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us statistically satisfactory changes. We are continuing to pursue experiments in both species of animals. No difficulty has been encountered in maintaining virulence in fertile hen's eggs.

Results of Experiments

Concept of Membrane Permeability Defect

The discussion of our concept of the effect of infections and immune processes on alterations in membrane permeability has been published: Harrell, G.T. and Aikawa, J.K.: Alterations in the Permeability of Membranes During Infections, J.A.M.A. 147:232-237 (Sept. 15) 1951.

Standardization of Methods

Studies on the normal values for the radiosodium space in rabbits have been completed and published as reported last year.

The values for radiosodium space and the comparison with the extravascular fluid space as measured by thiocyanate technique in normal individuals and in some disease processes has been published: Aikawa, J.K.: The Significance of Radiosodium Space in Human Disease, South. Med. J. 44:654-661 (July) 1951.

Data on radiopotassium space and tissue radiopotassium content in normal rabbits has been completed and published: Aikawa, J.K.: The Effect of Starvation on the Exchangeable Potassium in Tissue K^{42} Content in Rabbits, Proc. Soc. Exper. Biol. & Med. 78:524-528 (Nov.) 1951.

A study on the exchangeable radiopotassium space in twenty normal young women has been completed and the results compared with the previously known data in normal young men: Aikawa, J.K., Harrell, G.T. and Eisenberg, L.: The Exchangeable Potassium Content of Normal Women, J. Clin. Invest. Accepted for publication and scheduled for April, 1952. In these normal women, the mean K^{42} space was 31 milliequivalents per kilogram with a range of 25 to 35. This figure compares with a mean of 41 in normal men with a range of 35 to 50. No variation was detected with different stages of menstrual cycle, though serial determinations in the same individual have not been done. The lower value in women was interpreted as reflecting the relatively smaller proportion of muscle and greater proportion of fat in females as compared with males.

In order to study the effect of stress different from that to be induced later in definitive experiments on the breakdown of cells with the liberation of potassium, normal rabbits were starved and the effect on the exchangeable radiopotassium space and tissue content of radiopotassium were determined: Aikawa, J.K.: The Effect of Starvation on the Exchangeable Potassium in Tissue K^{42} Content in Rabbits, Proc. Soc. Exper. Biol. & Med. 78:524-528 (Nov.) 1951.

No significant correlation between the radiopotassium content and urinary creatinine excretion was detected in starved, normal male rabbits: reference cited.

Comparison of the blood volume by the human serum albumin technique tagged with radioactive iodine 131 and the non-isotopic Evans blue dye (T 1 - 1824) technique has been completed in normal human beings and the values found to run parallel.

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Time did not permit this year comparison of normal values for blood volume in rabbits by the tagged serum protein technique utilizing I^{131} as a marker.

Autoradiography of organs of normal rats following the injection of I^{131} and iodinated human serum albumin. A suitable technique for fixation of tissues after the injection of soluble ions of potassium and sodium has not yet been developed. An attempt to localize iodinated albumin molecules in the liver of rats was unsuccessful. These data were desired to serve as a basis for localization of antigen-antibody reactions. The lantern slide technique is proving satisfactory.

Intracellular Infections

Uniform experimental infections with Rocky Mountain spotted fever strain of rickettsias has been established in guinea pigs. Experimental infections in rabbits have been established but the disease is mild and the fatality rate so far has been low. Preliminary experiments in the initial groups have just been completed but are not yet analyzed.

Preliminary experiments on rickettsial spotted fever infection in guinea pigs treated with cortisone have shown no significant alteration in mortality. This experiment was performed to determine whether cortisone would increase the spread of an intracellular infection as it has been reported to do with extracellular infections such as those with staphylococci, streptococci and tubercle bacilli. Alterations in the height and duration of fever in guinea pigs treated with cortisone are not as striking as have been reported in human beings with naturally acquired Rocky Mountain spotted fever simultaneously treated with antibiotics. The comparable experiment combining cortisone and antibiotic therapy in guinea pigs has not been done.

Since we have not yet been able to step up the virulence of the rickettsias for rabbits, a preliminary group was run at the present virulence to detect alterations in the sodium 24 and potassium 42 spaces, but the data have not been analyzed. The tissue values have not been completed. The immunologic response as determined by complement fixation reaction has not been entirely dependable in rabbits and the titers have been low, so that data comparing alterations in permeability with immune responses are so far felt to be not entirely reliable.

No experiments were attempted this year tagging rickettsias with P^{32}.

Extracellular Infections

Time did not permit this year experiments on fluid changes in Trichinosis, an extracellular infection, applying the techniques for radioisotopic studies.

Immune Reactions Without Infections

The initial studies on active sensitization of rabbits with human plasma have been published: Aikawa, J.K. and Harrell, G.T.: The Immunophysiology of Serum Sickness. Alterations in the Blood Volume and Thiocyanate Space in Relation to the Development of Humoral Antibodies in the Rabbit, J. Clin. Invest. 30:360-368 (April) 1951. The continuation experiments utilizing different fractions of plasma have been published: Aikawa, J.K., Yount, E.H., Harrell, G.T. and Jennings, T.M.: Immunophysiology of Serum Sickness. Alterations in the Blood Volume and Thiocyanate Space in Relation to Humoral Antibodies in Rabbits Sensitized with Human Albumin or Globulin, J. Allergy, 22:508-517 (Nov.) 1951.

Experiments with a passive-transfer method for induction of serum sickness in rabbits have been published: Aikawa, J.K. and Rhoades, E.L.: Immunophysiology of Immune Reactions. Alterations in the Thiocyanate Space in Quantitative Passive-Transfer Experiments in Rabbits, J. Allergy 23:123-129 (March) 1952.

An attempt to localize the site at which fluid accumulates in the extravascular spaces as a result of immune reactions has been completed and published: Aikawa, J.K. and Rhoades, E.L.: Immunophysiology Increase in Radiosodium Space of Adrenal Glands in Rabbits Sensitized with Human Plasma, Proc. Soc. Exper. Biol. & Med. 79:233-235 (Feb.) 1952. The findings in this study are compatible with the thesis that the adrenal gland plays a role in immune reaction and is compatible with the clinical experience that cortisone is efficacious in the treatment of diseases of allergic etiology.

Rabbits have been actively sensitized by the injection of human plasma and the effect on external potassium balance and radiopotassium exchangeable space determined. Preliminary examination of the data do not reveal much alteration but the analysis is yet incomplete. This experiment is at present being repeated by hyperimmunizing a group of rabbits in an attempt to obtain more striking results.

Autoantibody Studies

The limitations of time and available technical assistance have not permitted inauguration of autoantibody studies.

Clinical Studies

The results of a preliminary survey of alterations in permeability to radiosodium in various disease states has been reported: Aikawa, J.K.: The Significance of Radiosodium Space in Human Disease, South. Med. J. 44:654-661 (July) 1951. Additional data collected in the past year have, in general, followed the results previously obtained.

The usefulness of the radiosodium technique as a tool in the study of the abnormal physiology of rheumatic fever was reported briefly at the meeting of the Southern Society for Clinical Research, Atlanta, Georgia, January. 1952. An abstract of these studies is in press in the AMERICAN JOURNAL OF MEDICINE. The data indicate that the radiosodium space is increased in untreated acute rheumatic fever and that therapy with salicylates will decrease the available exchangeable radiosodium space without greatly altering the body weight of the individual. These data have been interpreted as indicating an increased permeability of cells to sodium which is reversible with salicylates and which recurs on withdrawal of the drugs. We would like to extend these studies to a larger series of cases, but admission of acute instances of the disease have been rare during the past year.

An initial preliminary survey of the exchangeable radiopotassium content in human beings with various disease states has been submitted for publication. The results were interpreted as indicating that the radiopotassium method is more accurate and more sensitive than the external balance method for assaying changes in body potassium content. The radiopotassium method is particularly applicable to the study of such disease states as diabetes, myxedema, cirrhosis, congestive heart failure, and individuals with any disease under treatment with ACTH and cortisone.

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The magnitude of the deficiency of the body content of potassium as measured by the exchange of radiopotassium induced by the administration of adrenocortropic hormone (ACTH) or cortisone has been reported: Aikawa, J.K. and Felts, J.H.: Body Potassium Loss During Therapy with ACTH and Cortisone, N.E. Med. J. 13:[illegible] (Jan.) 1952. These data seem also to indicate that the renal tubules do not handle sodium and potassium entirely independently, at least under the influence of these hormones.

The radiopotassium space has been determined in a group of 50 diabetic patients. Diabetes is known to affect integrity of arterioles and capillaries producing hemorrhages in the eye grounds, albuminuria and hematuria in poorly regulated cases. The magnitude of the body deficiency of potassium and the ease of replacement, as measured by serial determinations while supplementary potassium salts were being given, has been studied. An abstract of this study will appear in the program of the American Society for Clinical Investigation, Atlantic City, New Jersey, May 5, 1952, and will be subsequently published in the JOURNAL OF CLINICAL INVESTIGATION.

The exchangeable potassium space has been determined in patients with cirrhosis of the liver, a disease in which the permeability of capillaries in the portal system is altered. These studies are being analyzed and the results correlated with studies on protein metabolism using human serum albumin tagged with I^{131}.

Studies have been done in patients with cirrhosis of the liver following the injection of human serum albumin tagged with I^{131} and the results reported: Tyor, M.P. and Sayer, R.: Studies with Iodine Tagged Albumin in Cirrhotic Patients, South. Med. J. 45:141-145 (Feb.) 1952 and Tyor, M.P., Aikawa, J.K. and Sayer, R.: The Plasma Disappearance and Urinary Excretion Rate of I^{131} Albumin in Patients with Cirrhosis, Gastroenterology, accepted for publication. In the course of these studies the rate of appearance of iodinated albumin in ascitic fluid and its rate of metabolism by the liver as determined by the appearance of inorganic iodine in the urine have been determined.

Studies utilizing I^{131} tagged human serum albumin and [illegible] are continuing as clinical material becomes available on the wards of the hospital. So far most patients studied have been instances of the chronic form of the disease process where the pathologic physiologic situation is reasonably stabilized. The number of instances of acute disease suitable for our studies has been limited this year.

Studies of Digitalis

The study previously completed has now been published: Aikawa, J.K., Knight, V.H. and Tyor, M.P.: Effective Intravenous Digitoxin on Fluid Distribution in Hospitalized Males Without Cardiovascular Disease, Proc. Soc. Exper. Biol. & Med. 76:250-252 (Feb.) 1951. No new experiments in this field have been undertaken this year.

ALTERATIONS IN THE BODY POTASSIUM CONTENT IN CIRRHOSIS OF THE LIVER

JERRY K. AIKAWA, M.D., JOHN H. FELTS, JR., M.D., AND GEORGE T. HARRELL, JR., M.D.
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Retention of sodium and water and depletion of the body store of protein are among the more striking features of decompensated hepatic cirrhosis. Hypoproteinemia has been thought to be one, but not the sole, factor responsible for fluid retention. Although the role of sodium in the pathogenesis of ascites has been extensively investigated, very little attention has been given to the possibility that there might also be an associated derangement in the metabolism of potassium. Such a possibility is suggested by the observation that the synthesis of protein is influenced by the availability of intracellular potassium.[1] Since the fluid and electrolyte pattern in cirrhosis resembles that found in subjects given the adrenocorticotrophic hormone, it appears probable that the metabolism of both sodium and potassium is altered.[2]

The introduction of the radioisotopic dilution technique has provided a method for measuring the exchangeable potassium content in human subjects.[3] The purpose of the present study was to determine the exchangeable potassium content in patients with cirrhosis of the liver, and the effect which the usually accepted dietary therapy would have upon this measurement.

MATERIALS

Subjects. Thirteen hospitalized subjects, 11 males and 2 females, with a diagnosis of cirrhosis of the liver were studied; their ages ranged from 23 to 67 years. Liver aspiration biopsy confirmed the diagnosis of Laennec's cirrhosis in 9 cases and of biliary cirrhosis in one. In one patient Laennec's cirrhosis was demonstrated at necropsy. In 2 cases no pathologic studies were done. All the patients showed clinical evidences of cirrhosis and had abnormal liver function tests. Four had ascites; the others had no apparent peripheral edema. None of the subjects has any evidence of renal disease.

A high protein, high carbohydrate, low fat diet with vitamin supplements was the basic therapy employed. Strict sodium restriction was avoided during

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the period of study, so that the pre-admission status of the patients could be more nearly simulated.

Isotopes. Isotopic potassium (K^{42}) was prepared for injection in the manner described by Corsa and his co-workers[3]. The subjects were given 100 microcuries of K^{42} contained in 1.5 or 3 milliequivalents of potassium chloride solution, depending on the age of the solution.

METHODS

Exchangeable potassium content (Ke). The initial determination of the exchangeable potassium content (Ke-1) was performed as soon as possible after the subject's admission to the hospital. When possible, two or more determinations were done.

The subjects received an intravenous injection of radioactive potassium by calibrated syringe between 7:30 and 9 a.m. They were then asked to void and to discard this specimen; all urine voided thereafter until 7 a.m. the following day was collected and the K^{42} content of the pooled specimen was determined. Determinations of the specific activity were made on two spot samples of urine obtained at 8 and 9 a.m. the day after injection.

The following formula was used to calculate the value for the exchangeable potassium content of the body:

$$Ke = \frac{Ki^{42} - Ko^{42}}{Ku^{42}/Ku^{39}}$$

Ke = quantity of exchangeable potassium in milliequivalents.
Ki^{42} = quantity of radiopotassium administered (arbitrary units).
Ko^{42} = quantity of radiopotassium excreted in the pooled specimen of urine.
Ku^{42} = concentration of radiopotassium in the spot samples.
Ku^{39} = concentration of nonisotopic potassium in the spot samples.
Ku^{42}/Ku^{39} = mean specific activity of the two spot samples.

Preliminary studies confirmed the observation of Corsa and his associates[3] that the specific activity of K^{42} in the urine reached an equilibrium in diseased as well as normal subjects within 24 hours. The mean difference in specific activity between the two spot specimens, when expressed as percentage of the mean Ke, was 6.52 ± 6.65 per cent.[4] In this laboratory Ke values are reproducible within 20.2 per cent in normal individuals and in hospitalized subjects who are in a steady state.[4]

Measurement of radioactivity. The activity of the urine and serum specimens was determined with a dipping tube and a scaling circuit. A total of 10,240 counts was made on each sample. All counting rates were at least ten times

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background, and were usually in the range of 500 to 3,000 per minute. At these counting rates, no correction for coincidence loss was necessary. All determinations were corrected for decay.

Chemical determinations. The total inorganic sodium and potassium concentrations in urine and serum were determined by flame spectrophotometry,

TABLE 1

Exchangeable potassium content and serum electrolyte concentration in patients with cirrhosis of the liver

SUBJECT	SEX	AGE (YRS.)	WEIGHT (KG.)	EXCHANGEABLE POTASSIUM CONTENT			SERUM SODIUM (MEQ/L.)	SERUM POTASSIUM (MEQ/L.)
				(meq.)	Change in Ke (%)	(meq/Kg.)		
1	M	49	82.4	1378		16.7	142.0	3.9
2	M	42	68.6	1640		23.9		
3	M	51	83.7	2436		29.1		
4	M	57	61.3	2053		33.5		
5	M	23	39.5	1360		34.4		
6	M	31	62.6	1687		26.9	144.0	4.6
			62.0	2165[7]*	+28.3	34.9	130.0	4.8
7	M	26	64.1	2492		38.8	143.0	3.7
			64.5	3275[16]*	+31.4	50.8	131.0	3.7
			64.0	3060[25]*	+22.8	47.9	152.0	4.8
8	F	56	73.4	1750		23.8		
9	F	67	44.1	1003		22.8	135.0	4.5
			45.6	1602[6]*	+59.0	35.1	128.0	3.9
			45.9	1976[12]*	+97.0	43.0	127.0	3.3
10†	M	60	71.4	2341		32.8	136.0	4.0
11†	M	34	111.0	2266		20.4		
			86.8	2183[21]*	−3.7	25.2	122.0	4.5
			83.2	2403[29]*	+6.0	28.9	111.0	6.1
12†	M	55	75.5	2084		27.7		
			68.6	2556[7]*	+22.6	37.2		
			68.2	2423[15]*	+16.2	35.5		
13†	M	45	64.1	2663		41.6	137.0	4.4
			61.1	2390[19]*	−10.3	39.1	150.0	4.9

* Interval in days after first Ke determination.

† Subjects with ascites.

using the method of Mosher and his co-workers.[5] The total serum protein, albumin and globulin concentrations were determined by the method of Howe.[6]

RESULTS

A total of 23 Ke determinations were made. Single determinations were obtained on 7 individuals. In the remaining 6 patients two or more determinations were made at the intervals specified in table 1.

The reported range for Ke in normal males is 2770–4470 meq. with a mean of 3402 meq., or 35.6–53.6 meq/kg. with a mean of 46.3 meq/kg. (3). For

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normal females the range in one series[7] was 1374–2164 meq. with a mean of 1776 meq., or 25.1–35.9 meq/kg. with a mean of 31.5 meq/kg.; in another series,[8] it was 28.0 to 47.2 meq/kg., with a mean of 40.7 meq/kg.

In the present study, all initial values for Ke were below the normal range, with the sole exception of a female subject (case 8) in whom the initial absolute value was normal (fig. 1). In terms of Ke/wt., however, this measurement was also below the normal range. When the subjects' body weights were taken into consideration, all initial values (Ke-1) fell below the normal means for their respective sexes. Both female subjects had initial Ke/wt. values below the range found for normal women in either series. Three of the males (cases 7, 12 and 13), including one with ascites (case 13), showed values in the lower normal range, while the remainder had values below the normal range. The lowest value for Ke/wt. (16.7 meq/kg.) was found in the subject with the

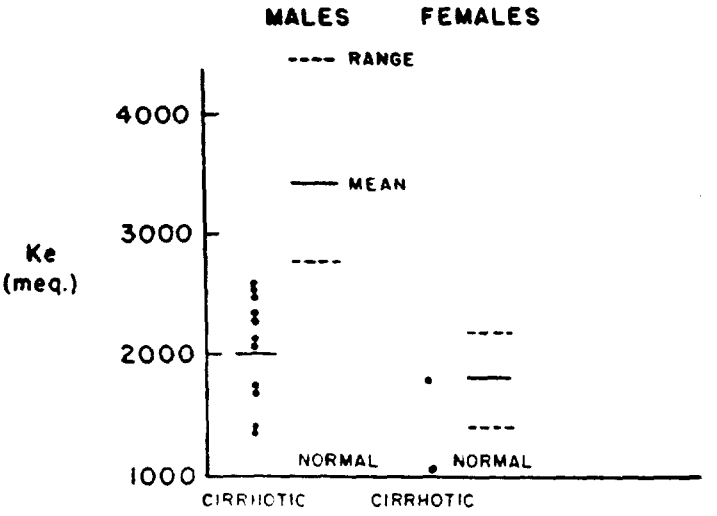

FIG. 1. Comparison of exchangeable potassium content of cirrhotic and normal subjects.

greatest amount of fat in the liver biopsy specimen; this patient (case 1) had no clinical edema, and his total serum protein concentration was normal.

The values for Ke/wt. could not be correlated with the serum sodium or potassium concentration, the total serum protein or globulin concentration, the duration of symptoms, the magnitude of weight change, or the degree of hepatic enlargement. In all instances, however, the serum albumin concentration was abnormally low (less than 4.5 gm. per 100 cc.).

The results of the serial determinations of Ke have been plotted in figure 2. In the three subjects without ascites, the exchangeable potassium content rose significantly after treatment (more than 25 per cent above the baseline values); the three subjects with ascites showed no significant change. In all of the patients who had repeat determinations, the body weight either decreased or did not change significantly during the period of study.

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Significance of the exchangeable potassium content and its response to therapy

These findings strongly suggest that an intracellular deficiency of potassium is a common occurrence in cirrhosis. While the low initial values for Ke in those individuals with ascites might be attributed to fluid retention, this explanation alone would not account for the low values in those without this manifestation. A low Ke might be attributed to depletion of the body store of protein, which is reflected in the clinical manifestations of weakness, weight loss, emaciation and hypoproteinemia. If, however, the low Ke is due solely to a reduction in lean muscle mass, this value should not increase out of proportion to changes in body weight when an adequate diet is administered. The data indicate that in some instances (cases 6 and 7), a significant increase in Ke occurred before any change in the body weight was noted. In another

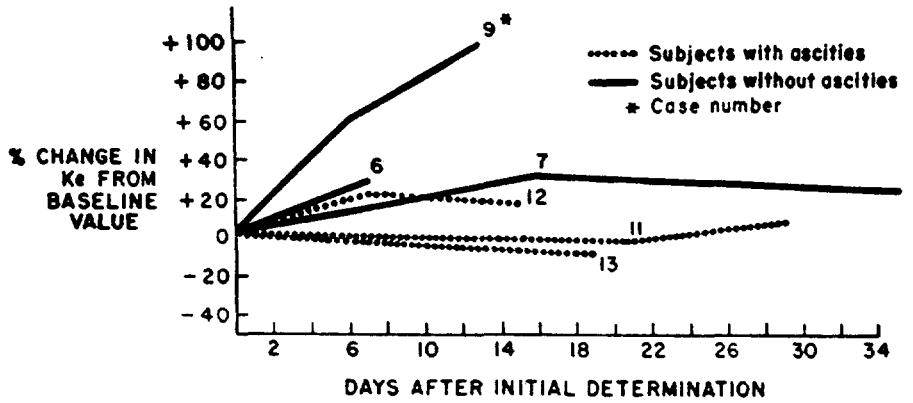

FIG. 2. Results of repeat determinations of the exchangeable potassium content in six subjects with cirrhosis of the liver.

subject (case 9), the rise in Ke was grossly out of proportion to a slight increase in body weight. None of these subjects had ascites.

In the absence of renal disease, an increase in the exchangeable potassium content during therapy suggests the existence of a prior deficiency state. Tarail and Elkinton[9] have shown that potassium is retained in the intracellular phase only when there has been a pre-existing deficit. Since none of the subjects studied had evidence of renal disease, the rise in Ke may be ascribed to the correction of a prior deficiency. This was accomplished primarily by an increase in the dietary potassium intake as hepatic function improved. The various other factors responsible for the increase in Ke are not too clear. One patient (case 6) had had an associated amebic colitis which responded dramatically to specific antiamebic chemotherapy; loss of potassium from the intestine was decreased as the dysentery subsided and his intake of food improved.

In the three subjects with ascites (cases 11, 12 and 13), diuresis and a decrease in the extent of the ascites caused the body weight to drop; however, no significant change occurred in the exchangeable potassium content. Two of

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these subjects (cases 11 and 12) were given 3 gm. of supplemental potassium daily for six weeks without any effect on the Ke; both subsequently expired in hepatic coma. In the other subject (case 13) the initial Ke value was in the lower normal range. Despite the presence of ascites, his appetite had not been impaired and his weight had remained constant.

Cirrhotic patients as a group have a lower serum protein concentration than normal subjects. The rate of turnover of iodinated human albumin is slower in cirrhotic patients with ascites than in those without this complication; this observation suggests that the presence of ascites is dependent to some degree upon the rate of synthesis of albumin (10, 11). This metabolic process may be dependent in part upon the availability of potassium, since it has been shown experimentally that in animals with a potassium deficiency the body weight does not increase, despite the administration of an otherwise adequate diet, unless potassium is also supplied.[1] In cirrhotic patients who lack a minimal amount of functional hepatic tissue, however, the correction of a potassium deficiency and even the intravenous administration of human albumin may not result in improvement. Such apparently was the case in patient 11. The limiting factor appears to be the quantity and the functional capacity of the surviving liver parenchyma. It appears that in cases 11 and 12, the amount of residual functional hepatic tissue was insufficient for the maintenance of life.

The possibility that there might be a relationship between the relatively excessive sodium intake in patients with cirrhosis and the development of overt signs and symptoms of potassium deficiency has not been previously suggested. The studies of Cannon and his co-workers on animals indicate that such a reciprocal relationship might exist. In the rat the effects of an experimentally induced potassium deficiency can be modified by altering the intake of sodium; when the sodium intake is lowered, the effects of the potassium deficit are minimized; and when the sodium intake is increased, the manifestations of potassium deficiency become more severe.[1] It is possible that the clinical improvement produced by a low sodium diet in patients with ascites may be due, at least partially, to suppression of the effects of a prior deficiency of potassium. Although none of the subjects in the present series had hypokaliemia, it is known that a decrease in the serum potassium concentration and electrocardiographic changes suggesting hypokaliemia may occur in cirrhotic patients with decompensation.[12] Artman and Wise[13] have reported such findings in 25 of 30 patients with hepatic decompensation. Studies in this laboratory have suggested that hypokaliemia does not develop unless there is a deficit of at least 25 per cent in the exchangeable potassium content.[14]

Previous studies [15,16,17] have demonstrated disturbances in sodium, water and protein metabolism in patients with cirrhosis of the liver. The results of the present study suggest, in addition, the existence of a potassium deficiency of a magnitude which may be of clinical significance.

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SUMMARY

Determinations of the exchangeable potassium content (Ke) by the radioisotopic method were performed on 13 subjects with cirrhosis of the liver, four of whom had ascites. Twelve of the 13 initial values were below the range found in normal subjects, and the remaining value was in the lower normal range. Repeat determinations were performed on 6 subjects. The 3 subjects without ascites showed a significant increase in Ke following a week or more of treatment with a high protein, high carbohydrate, low fat diet and supplementary vitamins. In the 3 subjects with ascites no significant change in Ke occurred, even after the administration of an oral supplement of potassium. The results suggest that an intracellular deficiency of potassium is commonly present in cirrhosis of the liver, and that this deficit is not correctible once hepatic decompensation has occurred.

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BODY POTASSIUM LOSS DURING THERAPY WITH ACTH AND CORTISONE

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ACTH is a hormone produced by the pituitary gland. Its administration stimulates the adrenal cortex to produce hormones which affect diverse physiologic functions. Cortisone appears to be but one of the hormones produced by the adrenal cortex. Its systemic effects are similar to those produced by ACTH.

When cortisone is administered therapeutically, this steroid acts as a substitute for the natural hormone. Both ACTH and cortisone alter electrolyte metabolism, causing retention of sodium and excessive urinary excretion of potassium. For this reason, a low sodium diet and the administration of supplementary potassium have been employed as adjuvants to therapy with either of these drugs[1].

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*Research fellow of the American Heart Association sponsored by the 20-30 International Rheumatic Fever Foundation.

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Quantitative data on this effect are based on external studies of the electrolyte balance, which are indirect measures. Such studies have indicated that the loss of potassium during therapy with ACTH and cortisone exceeds the amount of nitrogen which would be lost as a result of the destruction of tissue cells[1a] Hence the potassium loss cannot be explained solely on this basis, and it appears, therefore, that an absolute deficit of this cation is induced. The systemic effects of potassium depletion, such as muscular weakness, respiratory difficulties, and electrocardigraphic changes, are well known[2].

The availability of the radioactive isotope of potassium, K^{42}, has made possible direct measurements of the exchangeable potassium content of the body[3]. For this purpose, a tracer dose of K^{42} is injected intravenously. After sufficient time has elapsed for the tagged atoms to achieve equilibrium with the native atoms in the body, the specific activity of the urine specimens is used to calculate the exchangeable potassium content of the body[3]. By such a method, it has been found that the mean exchangeable potassium content (Ke) in 30 healthy young adult males is 46.3 milli-equivalents per kilogram, with a range of 35.6 to 57.1 milli-equivalents per kilogram[3]. In 20 normal young females, the mean value is 31.5 milli-equivalents per kilogram, with a range of 25.1 to 35.9 milli-equivalents per kilogram[4].

The purpose of this report is to point out the magnitude of the depletion in body potassium which can be caused by the administration of ACTH or cortisone. Four cases

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studied serially by the radioisotope technique are cited.

Case Reports

Case 1

A 65 year old woman was hospitalized for treatment of mild rheumatoid arthritis of four years' duration. During the seven day period of study, she received ACTH intravenously, 20 mg. daily, and potassium chloride orally, 3 Gm. daily. Exchangeable potassium (Ke) determinations were done on the first and last days of the study. The results were as follows:

Day of Study	Weight (Kg.)	Ke (m-eq.)	Ke/Wt. (m-eq./Kg.)
1	51.1	1777	34.8
7	49.0	1266	25.8

Comments: This case reveals a striking decrease in the total body potassium (29 per cent of the initial value), despite supplementary doses of this mineral. No signs or symptoms of potassium depletion were noted.

Case 2

Seven months prior to hospitalization, this 25 year old man had a sore throat, followed in two weeks by fever and polyarthritis. He was given cortisone, which he continued to take in varying dosages, depending upon the severity of his symptoms. Dietary sodium was not restricted, and no supplementary potassium was prescribed. Within six months the body weight increased 45 pounds. Three weeks prior to hospitalization, he noted hand pigmentation of the nails.

Physical examination on admission revealed acne, "moon" facies, a "buffalo hump," and the fat distribution typical of Cushing's syndrome. The temperature was 101 F. Marked improvement in the arthritic symptoms was observed when the patient was given salicylates. Hormone therapy was stopped. No supplemental potassium was administered, but the patient was placed on a liberal diet which contained approximately 3 Gm. of potassium per day. He was hospitalized for 15 days. Two months after discharge, he was symptom-free, and the signs of cortisone intoxication had disappeared.

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Day of Study	Weight (Kg.)	Ke (m-eq.)	Ke/Wt. (m-eq./Kg.)
1 (Second hospital day)	87.7	2340	26.7
6	85.4	2256	27.6
14	85.0	2966	34.9

Case 3

A 33 year old man began to have chills, fever, polyarthritis, cardiomegaly, and anemia in February, 1951, three weeks after a streptococcal pharyngitis. While hospitalized elsewhere, he received both ACTH and cortisone. In the early part of March, 1951, he was given oral cortisone, 100 mg. daily, which he continued without sodium restriction or potassium replacement until he was admitted to the North Carolina Baptist Hospital late in August.

Physical examination revealed acne, "moon" facies, and a cervical fat pad. The blood pressure was 150 over 94. Both knee joints showed effusion. The arthritic symptoms responded promptly to salicylates and aminopyrine. Hormone therapy was discontinued, and he was given a liberal diet. No added potassium was prescribed.

Day of Study	Weight (Kg.)	Ke (m-eq.)	Ke/Wt. (m-eq./Kg.)
1 (Second hospital day)	69.6	2099	30.2
7	70.2	2302	32.8

Comment: In these two patients, marked potassium depletion was present following unregulated cortisone therapy. In neither case had it progressed to the point where clinical signs of potassium deficiency were recognizable. Following withdrawal of hormone therapy, the body promptly began to make up the deficiency from the diet.

Case 4

A male painter, aged 56, was first admitted to the North Carolina Baptist Hospital in 1948 because of exfoliative dermatitis of undetermined etiology. At that time local and supportive therapy produced a remission. He was admitted for the second time in July, 1950, in a moribund state following a recurrence. Treatment with ACTH, 40 mg. daily, pro-

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duced marked improvement. Whenever the drug was temporarily withheld, evidences of recurrence appeared, and it was necessary to maintain him on ACTH during the next five months. Additional treatment included potassium chloride, 1 Gm. daily, and moderate sodium restriction. When he was discharged in December, 1950, his dermatitis was adequately controlled by cortisone, 50 mg. daily. He was told to continue his potassium supplement, and was given directions for a low sodium diet (less than 2 Gm. daily). He was seen monthly in the outpatient department, and aside from "moon" facies showed no visible sign of cortisone intoxication; no dermatologic symptoms or signs developed.

After eight months on cortisone, he was readmitted for a course of ACTH in preparation for the withdrawal of hormone therapy. During the period of study, he received ACTH intravenously on the following schedule: Day 1, 20 mg.; days 2-8, 40 mg.; days 9-14, 20 mg. During days 1-5, he also received oral cortisone, 50 mg. daily.

Ke studies were as follows:

Day of Study	Weight (Kg.)	Ke (m-eq.)	Ke/Wt. (m-eq./Kg.)
1	77.3	3127	40.5
7	79.1	3439	43.5
14	77.3	2856	36.9

Comment: In this instance, it appeared that moderate dietary sodium restriction and the administration of supplementary potassium over a long period of time provided adequate substitution therapy. The increase noted in the second Ke level, followed by a decrease on the third determination, suggests suppression of adrenal cortical activity due to the administration of cortisone. This effect became evident when cortisone was withdrawn and was counteracted when the full effect of ACTH on the gland was realized.

Discussion

Although data pertaining to electrolyte metabolism during long-term therapy with ACTH or cortisone are limited, external balance studies have shown that the urinary

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excretion of potassium is increased. The magnitude of the depletion of body potassium in case 1, as measured by the K^{42} method, was greater than had been anticipated from previous external balance studies. A deficit of 511 milli-equivalents (29 per cent of the initial value) developed in six days, despite the administraton of a total of 200 milli-equivalents of supplementary potassium. These figures illustrate the potential magnitude of the deficit which may be induced even when a patient is receiving what is assumed to be adequate replacement of potassium. Case 4 illustrates that an individual may be maintained in potassium balance during long-term therapy with ACTH or cortisone if the intake of sodium is adequately restricted (to less than 2 Gm. daily) and potassium supplements are given.

Similar studies in other subjects with potassium depletion indicate that a deficit of slightly more than 29 per cent of the normal body content may result in the sudden development of clinical symptoms and signs of potassium deficiency. The loss of body potassium of the magnitude demonstrated in these 4 cases, although not productive of symptoms, may potentially be very harmful. Alarming symptoms and signs may appear if the depletion is permitted to progress untreated.

Summary and Conclusions

Serial determinations of the exchangeable potassium content, as measured with a radioactive isotope, were performed in 4 patients during and after therapy with ACTH and cortisone. The data reveal a striking depletion in the body's store of potassium,

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even during replacement therapy with as much as 3 Gm. of potassium chloride daily. The results emphasize the importance of replacing this loss and of recognizing this potential hazard.

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STUDIES WITH IODINE-TAGGED ALBUMIN IN CIRRHOTIC PATIENTS*

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Patients with cirrhosis of the liver show alterations in many metabolic functions. In those patients who develop ascites one of the chief factors is believed to be the lowered concentration of serum albumin, since the albumin fraction is known to be the one which exerts the greatest intravascular osmotic attraction.

Many investigators have demonstrated a correlation between the presence of ascites and a lowered serum albumin level in cirrhotic patients.[1,2] It has been suggested that the prognosis in patients with cirrhosis is directly related to levels of albumin and other protein fractions.[1,3,4] Several observers have administered large amounts of human serum albumin up to 1,000 grams over a six-month period to patients with portal cirrhosis and ascites, and have studied the long-range effects.[5,6,7] None of these investigations allowed observations on the rate at which protein molecules are utilized or move about.

The body proteins are in a dynamic state.[8] Urinary nitrogen determinations furnish an index to the day-to-day utilization of dietary protein. Serum protein levels, when followed over long periods, may serve as an indication of the over all protein metabolism. These studies reflect the balance between the synthesis and breakdown of protein. It now appears that within the total protein content of the body there is an exchange between the cellular protein, that being synthesized, utilized and in the circulation. The plasma protein represents the labile exchange medium of the body's total protein pool.

Extensive experimental study has indicated that the liver is the chief source for the formation of plasma proteins.[9,10,11] This deduction has been supported by clinical studies in patients with liver disease.[12] Evidence has been produced to show that, in dogs, the mass of protein in the extravascular, extracellular fluid is approximately equal to the proteins in circulation.[13] In human patients with cirrhosis of the liver clinical recovery may be correlated with the ability to maintain a normal total circulating albumin.[14] The total circulating albumin seems to be a reflection of the body's total store of labile albumin.

Human serum albumin may be "tagged" with radioactive iodine (I^{131}) and injected into the body in very small amounts to serve as a tracer.[15] This permits dilution of negligible, yet detectable quantities of albumin with the body pool without altering homeostasis, and is a method by which the metabolism of native albumin can be measured and labile stores estimated.

MATERIALS AND METHODS

In the present study, a tracer dose of tagged human serum albumin* was injected into 11 control patients† without liver disease (Group 1), 7 cirrhotic patients (Group 2) without ascites, and 5 patients with portal cirrhosis and ascites (Group 3) (*Table 1*). Diuretics and parenteral fluids were not administered before or during the study period. Patients with ascites were studied before paracentesis.

The degree of radioactivity in the serum was determined at 24, 48 and 72 hours after injection of the tagged albumin.

The plasma volume was determined from the degree of radioactivity after 10 to 15 minutes was allowed for intravascular mixing. This

*Read in Section on Gastroenterology, Southern Medical Association, Forty-Fifth Annual Meeting, Dallas, Texas, November 5-8, 1951.

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†The opportunity to study eight of the patients in the control group was made possible through the cooperation of Dr. E. C. Harper, Chief Medical Officer, and his staff at the Veterans Administration Center, Mountain Home, Tennessee.

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period has been found to be sufficient for equilibration within the vascular compartment.[16] The serum albumin concentration was measured by a standard chemical method (Kjeldahl). The total circulating albumin was calculated from the plasma volume and the serum albumin level.

In a previous study in human patients it was found that significant radioactivity without albuminuria was detectable within three hours following the intravenous injection of tagged albumin.[16] This finding was interpreted as evidence that demonstrable catabolism of the injected albumin molecule had occurred. In order to use the concentration of tagged albumin in the serum as an index to the rate of metabolism of albumin, it was necessary to allow tagged molecules to reach a state of equilibrium with the body pool. It was found that a period of 24 to 48 hours was required for equilibration between the serum and the ascitic fluid in the patients with cirrhosis and ascites. The decrease in the serum level of tagged albumin between the 48 and 72 hour determinations was therefore used as an index to the rate of metabolism.

RESULTS (*Table 2*)

There was no significant difference in the mean plasma volumes of the three groups.

The serum albumin concentration in both groups of cirrhotic patients was significantly reduced as compared with the controls. The albumin level in the group with ascites was also significantly lower than that in the group of cirrhotic patients without ascites.

In the patients with cirrhosis and ascites the total circulating albumin was significantly less than the values found in cirrhotic patients without ascites. In Groups 1 and 2 the mean values for circulating albumin were comparable.

During the first 48 hours the rate of disappearance of the tagged albumin from the vascular compartment was similar in the three groups. At 48 to 72 hours, however, the rate of disappearance in the patients with cirrhosis and ascites (Group 3) was significantly less than that in the other two groups. A typical curve from each group is seen in *Fig. 1.*

COMMENT

The reduced rate of disappearance of the injected tagged albumin from the vascular compartment in the patients with portal cirrhosis and ascites is interpreted as indicating a slower rate of albumin metabolism in these patients.

The reduction in the serum albumin concentration of cirrhotic patients confirms the observations of other investigators.[17,18] Of further significance is the decrease in the serum albumin

CLINICAL DATA

Group	No. of Pts.	No. of Det.	Males	Females	Age	Follow up
I Controls	11	11	10	1	21–44	All living and well.
II. Cirrhosis without Ascites	7	8	4	3	23–56	One dead with hemorrhage; others living and improved.
III. Cirrhosis with Ascites	5	6	4	1	35–69	4 dead 2–4 weeks after study. One unknown.

TABLE 1

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level of patients with cirrhosis and ascites, as compared with the cirrhotic patients who did not have ascites.

The data also indicate that those individuals with cirrhosis who show no evidence of ascites have an essentially normal total circulating al-

COMPARISON OF RESULTS

	Plasma Volume	Serum Albumin Concentration	Total Circulating Albumin	Albumin Metabolism
Controls				
Cirrhotics $\bar{s}$ Ascites	↔	↓	↔	↔
Cirrhotics $\bar{c}$ Ascites	↔	↓	↓	↓
Cirrhotics $\bar{s}$ Ascites				
Cirrhotics $\bar{c}$ Ascites	↔	↓	↓	↓

↔ Similar Values
↓ Significant Reduction

TABLE I

Rate of Disappearance of I^{131} Albumin from the Vascular Compartment
Illustrative Cases

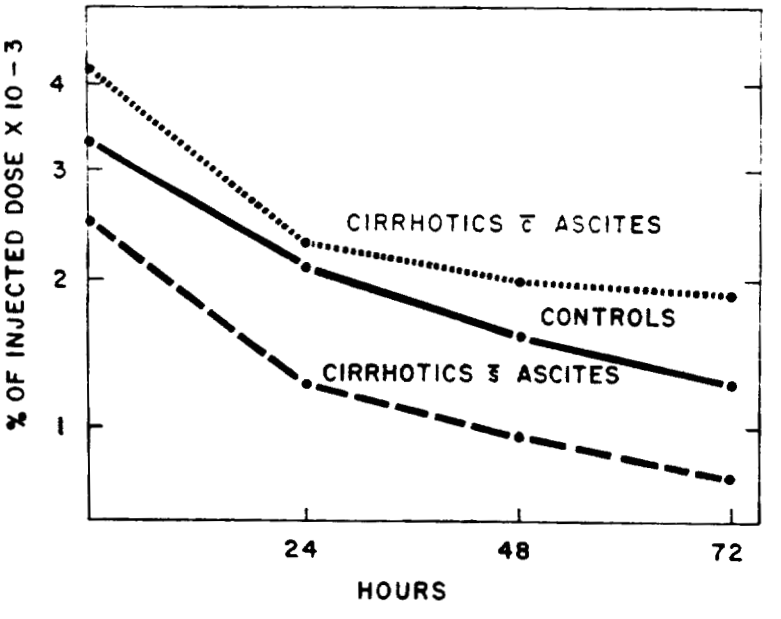

FIG. 1

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bumin. In the patients with cirrhosis and ascites, however, this value was significantly reduced indicating depletion of albumin stores.

During the 72 hours in which the patients were studied, there was no detectable alteration in body homeostasis. The reduction in the serum albumin concentration, the total circulating albumin, and the rate of albumin metabolism in the patients with ascites would suggest a decrease in the synthesis of albumin.

The data presented are consistent with the concept of a labile protein reserve, which has been depleted in patients with portal cirrhosis and ascites. It is felt that the reason for the depletion of this store is a reduction in the rate of albumin synthesis. When the labile stores of protein are depleted, the homeostatic mechanisms regulating the distribution of body fluids are altered, and ascites develops. Our data suggest that the defect in albumin metabolism is of primary importance in the pathogenesis of ascites, although factors such as portal hypertension, alterations in electrolyte metabolism and the possible influences of endocrines and lymphatics are significant and contributory.

The patients with ascites had a uniformly poor prognosis. Four died within six weeks after the study. The lower rate of albumin metabolism in ascitic patients, as compared to that of cirrhotic patients without ascites would appear to indicate that albumin metabolism proceeds at a constant, relatively normal rate, despite impairment of other less vital liver functions, until the terminal stages of liver disease.

SUMMARY

Following the injection of tracer amounts of albumin tagged with I^{131}, the plasma volume, serum albumin concentration, total circulating albumin, and rate of disappearance of albumin from the vascular compartment (albumin metabolism) were determined in patients without liver disease and in cirrhotic patients with and without ascites.

The serum albumin concentration of cirrhotic patients, with and without ascites, is significantly reduced.

In patients with cirrhosis and ascites the levels of serum albumin and total circulating albumin, as well as the rate of albumin metabolism were significantly less than those of patients without liver disease or cirrhotic patients without ascites.

These data demonstrate that depletion of labile albumin stores can be correlated with the presence of ascites, and suggest that albumin metabolism proceeds at a constant rate and is demonstrably altered only in the terminal stages of liver disease.

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THE SIGNIFICANCE OF THE RADIO-SODIUM SPACE IN HUMAN DISEASE*

A COMPARISON WITH THE THIOCYANATE SPACE

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Clinicians have long recognized the occurrence of edema during the course of such varied and seemingly unrelated disease processes as congestive heart failure, nephritis, chronic malnutrition, cirrhosis, toxemia of pregnancy, myxedema, infectious mononucleosis, serum sickness, trichinosis, pneumonia, and Rocky Mountain spotted fever. The presence of edema is obviously an indication that the distribution of body fluids has been altered. Although various substances have been used in the past to determine the magnitude of the edema, no single substance has been found which is thought to give a true index of the extracellular fluid volume. The purpose of this paper is to discuss briefly the clinical significance of quantitative determinations of the extracellular fluid space, and to present the results of a comparative study of two methods for measuring this space, one employing a positively charged ion, radiosodium, and the other, a negatively charged ion, thiocyanate.

NORMAL ANATOMY

By weight, approximately 70 per cent of the adult human body is composed of water. For example, an average man weighing 155 pounds or 70 kilograms will contain about 49 liters of water. Roughly, two-thirds of this volume, or 34 liters, is intracellular. The remaining 15 liters is extracellular, being distributed between the vascular and interstitial fluid spaces. The interstitial fluid space contains three times as much fluid as the vascular compartment (*Fig. 1*). The composition of the blood plasma differs from that of the interstitial fluid primarily in its greater content of protein (*Fig. 2*).

Some of the characteristics of the internal environment are well known. The sodium ion is the predominant cation in the extracellular fluid, and is normally maintained at a concentration of about 142 milliequivalents per liter. The potassium ion is located principally in cells, only 5 milliequivalents per liter being present in the extracellular fluid. One of the greatest unsolved mysteries in biology today is the mechanism whereby the differential concentration of sodium and potassium is maintained by the tissue cell membrane.

Sodium is such a ubiquitous ion, and the solubility of its salts is so great and their ionization so rapid that, until recently, it was difficult technically to determine its concentration in biologic fluids. By the use of flame spectrophotometry, this obstacle has been largely overcome. It is now possible to measure with relative ease the concentration of sodium and potassium in a solution.

If based on measurements of concentrations alone, however, conclusions as to the nature of the physiologic processes involved may be erroneous. The volume of a space, as well as the concentration of a substance within that space, must be known in order to calculate the total quantity of the substance present, and to determine whether an absolute insufficiency or excess exists.

Methods of Determining the Extracellular Fluid Volume.—In principle, the method of determining the extracellular fluid volume is a simple one. A known quantity of a substance is injected intravenously and its concentration in the blood is measured after sufficient time has elapsed for the substance to reach equilibrium.

Any substance which is employed to measure extracellular fluid volume should meet the fol-

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*The Na^{24} used was supplied by the National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

†AEC Fellow in the Medical Sciences of the National Research Council, 1948-1950.

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lowing requirements: (1) it must not cross the cell membrane barrier, or must do so very slowly; (2) it must be nontoxic when injected intravenously in the necessary amounts; (3) it must diffuse rapidly in the extracellular fluid space and must quickly attain a state of equilibrium with the native ions; (4) there must be an accurate and simple method for its quantitative measurement.

In the past, the following agents have been used for this purpose: chloride, bromide, thiocyanate, thiosulfate, sucrose, inulin, and mannitol. With the exception of chloride, none of these substances is normally present in the human body. The substance which has been most frequently used in clinical investigation has been the thiocyanate ion. However, it is stated that in certain pathologic conditions the thiocyanate space approaches the calculated total amount of body water;[1] consequently it has not been thought to be a true index of extracellular fluid volume.

Use of Radioactive Sodium.—With the introduction of artificial radioactive isotopes, an invaluable and physiologic substance has been made available for measurement of the extracellular fluid space. Isotopes are atoms of the same atomic number and containing the same number of valence electrons, but differing in atomic mass from ordinary atoms.

One method for producing radioactive sodium is to bombard Na^{23} with neutrons, as is done in the nuclear reactor at Oak Ridge (*Fig. 3*). An additional neutron is crowed into some of the Na^{23} nuclei, and an unstable radioactive isotope (Na^{24}) is thereby formed. Na^{24} changes to a stable state by transmutation into an atom of $Mg.^{24}$ In the process a beta ray is emitted and the residual excess nuclear energy is dissipated as gamma rays. The Geiger-Mueller tube is used to measure the ionizations produced by these radiations and to determine thereby the amount of isotope present in a sample.

It should be emphasized that the difference between Na^{23} and Na^{24} is a purely physical one, an extra neutron in the latter. The chemical characteristics of an atom are determined by the orbital electrons; the chemical properties of Na^{23} and Na^{24} are therefore identical. Na^{24} fulfills all the criteria necessary for the measurement of

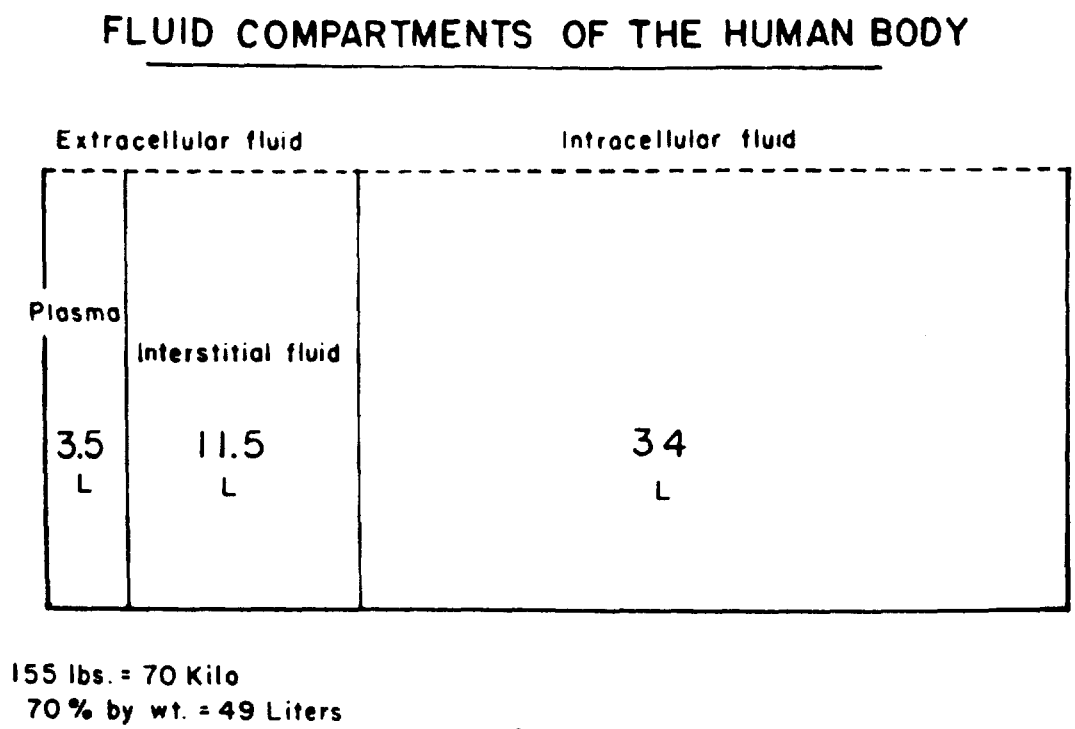

Fig. 1

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a volume occupied by the ordinary sodium atoms. Since it is chemically indistinguishable from ordinary sodium atoms, its metabolism in the organism will be the same as that of Na^{23} and it will be uniformly diluted among the nonradioactive ions. Another longer lived radioactive isotope, $Na,^{22}$ which is produced in limited quantities in the cyclotron, has been used by Reaser and Burch[5] in investigations on congestive failure.

In measuring the extracellular fluid space with $Na,^{24}$ a tracer amount of a sterile solution of sodium carbonate is given intravenously. After an interval of 3 hours, a sample of blood serum is drawn and a Geiger-Mueller counter is used to determine its content of the isotope.

There are certain disadvantages and hazards which preclude the routine use of this method in the office or in most hospitals:

(1) Na^{24} decays very rapidly, having a half-life of 14.8 hours. (That is, in a solution containing a certain number of radioactive atoms, one-half of this number would have disintegrated into stable magnesium in 14.8 hours; in the next 14.8 hours, one-half of the remaining quantity would have decayed, and so on.) It is then obvious that geographic proximity to the source of supply and a dependable and rapid method of transport of the isotope to the laboratory are essential.

(2) Na^{24} emits high energy beta and gamma rays; these create potential and unavoidable radiation hazards to the laboratory personnel and to the patient unless proper precautions are used.

(3) The shielding and protective equipment necessary for the safe use of even tracer doses of high energy isotopes is expensive.

IONIC COMPOSITION OF THE FLUID COMPARTMENTS

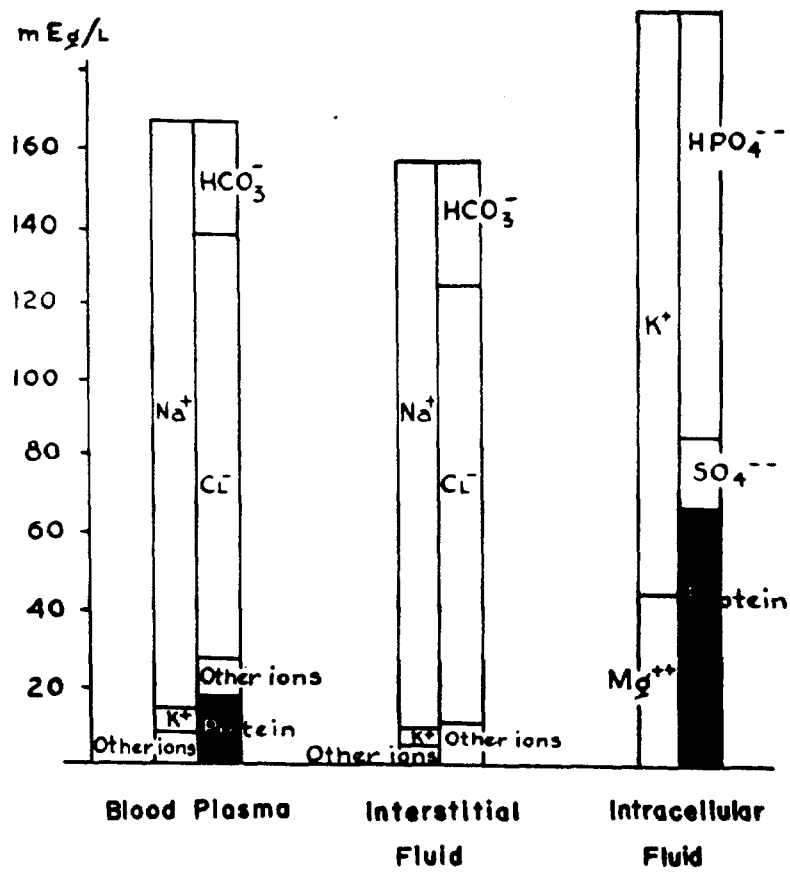

Fig 2

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The hazards of external radiation to those handling tracer doses of radiosodium are minimal, provided proper technics are employed. The harmful effects of radiation to the patients can be minimized by using the smallest dose necessary to obtain statistically significant counting rates. It has been calculated that the injection of 1.3 microcuries of radioactive Na^{24} per kilogram of body weight delivers a total body radiation of about 0.1 r. over an effective period of about two days, when both the beta and the gamma rays are assumed to be entirely absorbed within the body.[2] The accepted limit of tolerance for continuous irradiation is 0.3 r. per week; hence, from the standpoint of radiation hazard to the patient, it is safe to use tracer doses of 2 to 3 microcuries of radioactive sodium per kilogram of body weight and to repeat them a few times at reasonable intervals (two weeks to two months).

It is then apparent that it would be impractical to use radiosodium in routine determinations, or even for prolonged clinical investigations. However, by making simultaneous determinations of the thiocyanate space and the radiosodium space in normal and abnormal states, and comparing the result, it should be possible to determine whether the thiocyanate method is a valid measure of the distribution of the sodium ion. If so, the technically simpler thiocyanate method can be used for routine determinations of the extracellular fluid space and the isotopic technic reserved for special studies.

COMPARATIVE CLINICAL STUDIES

Simultaneous determinations of the radiosodium and thiocyanate spaces have shown that, following their intravenous injection, Na^{24} and the thiocyanate ion disappear from the blood serum rapidly, and at practically the same rate, up to the third hour (*Fig. 4*). After the third hour, the rate of disappearance of radiosodium from the blood stream is considerably slower than before. Kaltreider, Meneely, Allen and

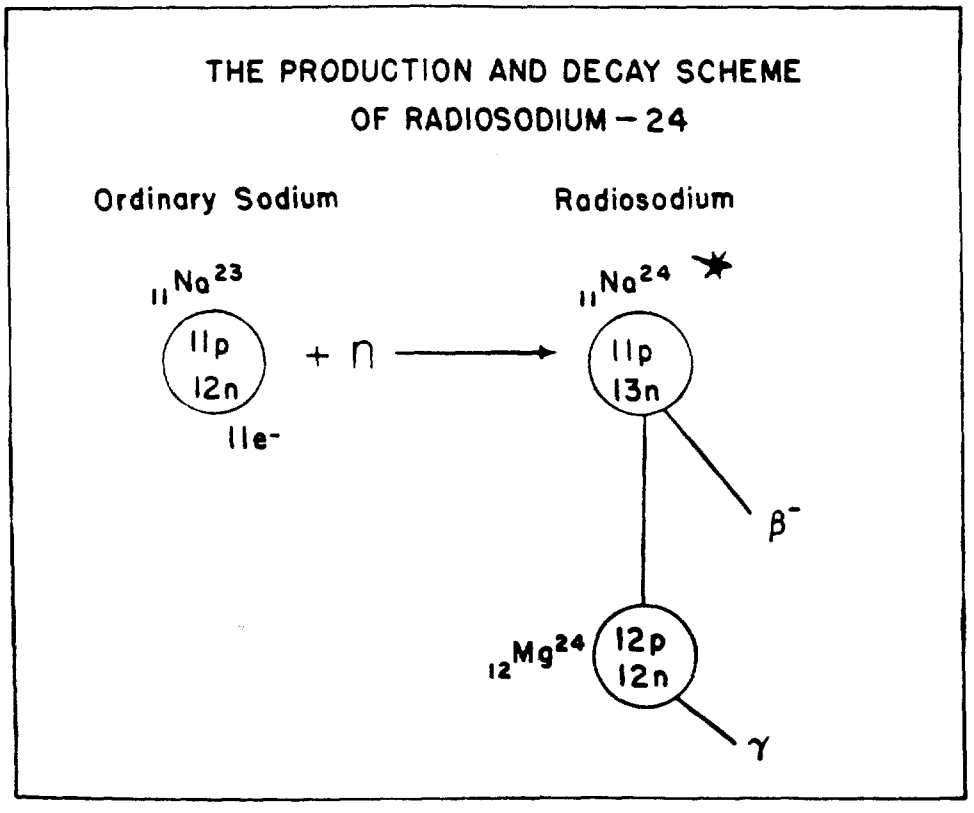

Fig 3

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Bale[3] have shown that in normal individuals the comparative values for the thiocyanate and radiosodium spaces are practically identical, when the calculations are based on the three-hour samples. Although it is not established that either the radiosodium or the thiocyanate ion in the extracellular fluid has reached complete equilibrium at three hours, the data show that the two substances measure an identical volume of fluid at that time. The question of whether there is subsequent diffusion of sodium into a larger space after the first three hours is beyond the scope of the present discussion.

It was not known whether the two values would be comparable in individuals in pathologic states. In an effort to clear up this point, we have to date performed a total of 32 simultaneous determinations of the thiocyanate and radiosodium spaces in 20 patients with such varied clinical conditions as hypertension, diabetes, myxedema, toxemia of pregnancy, serum sickness, rheumatoid arthritis, disseminated lupus erythematosus, typhoid fever, arteriovenous fistulas, and exfoliative dermatitis (*Table 1*).

In the three-hour samples the mean thiocyanate space was found to be 17.3 liters; the mean sodium space was 17.4 liters. The mean difference between the simultaneous determinations of the thiocyanate and radiosodium spaces in each individual was 0.069 liter, and the standard error of the difference was 1.46. In normal individuals, the standard error was considerably smaller. It is apparent from these experiments that, although the mean difference between the thiocyanate and the radiosodium space is slight in both normal and abnormal states, there is considerably greater variability in diseased states. The factors which may be responsible for the variability in the different disease processes are being investigated.

SIMULTANEOUS DETERMINATIONS OF RADIOSODIUM AND THIOCYANATE SPACES IN NORMAL AND DISEASED STATES

	Total Number of Comparative Determinations	Mean Radiosodium Space (Na^{24}) (Liters)	Mean Thiocyanate Space (Liters)	Mean Difference	Standard Error
Normal*	9	19.1	19.0	.044	0.16
Diseased states	32	17.4	17.3	.069	1.46

*From Kaltreider, McNeely, Allen, and Bale.

Table 1

COMPARATIVE DISAPPEARANCE CURVES OF RADIOSODIUM AND THIOCYANATE IN DISEASED STATES

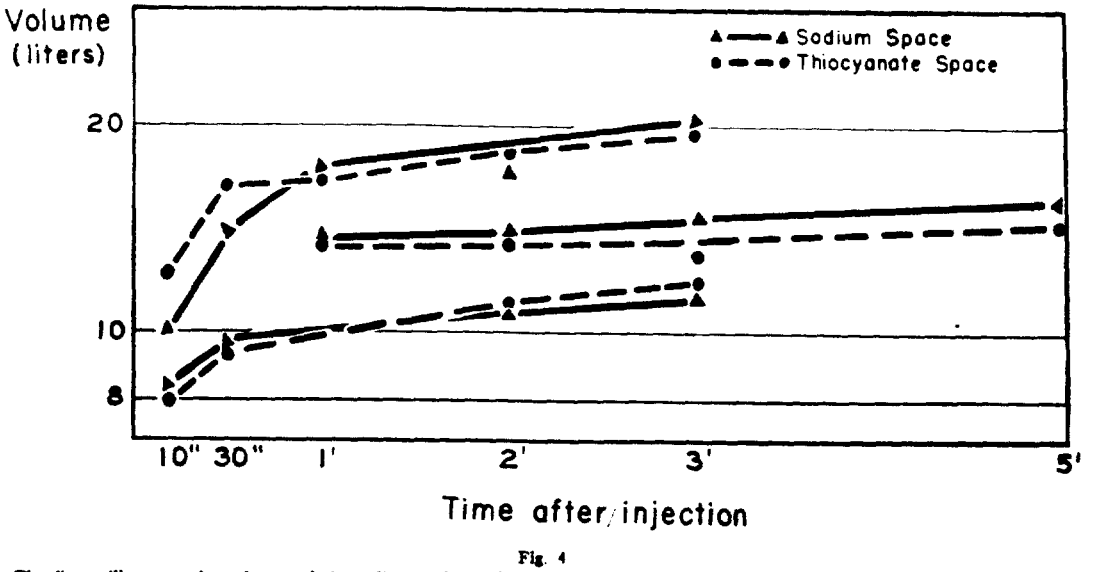

Fig. 4

The figure illustrates the volumes of the sodium and the thiocyanate spaces determined simultaneously from serial serum samples in three representative cases.

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It therefore appears, from the available data, that the thiocyanate ion measures a volume of fluid which for practical purposes is comparable to that measured by the radiosodium ion. The radiosodium method is the more physiologic of the two for the measurement of the extracellular fluid space. Because the results obtained by the two methods vary more widely in diseased than in normal states, both methods should probably be employed in critical investigations.

THE VALUE OF STUDIES ON THE DISTRIBUTION OF BODY FLUIDS

The methods now available for studying alterations in the distribution of body fluids may not be directly applicable to practice at present, except in a few instances. Direct measurement of the blood volume may be of value in the therapy of congestive heart failure and in states of circulatory collapse and shock. In patients with the low salt syndrome, determinations of the serum sodium concentration, which is at present estimated indirectly by the serum chloride determinations would be of value. The problem of potassium deficiency in diabetic acidosis and in diarrhea was presented in this Section two years ago by Nicholson and Spaeth.[6]

The fundamental physiologic mechanisms which may be clarified by studies on the distribution of body fluids may have a direct bearing upon the understanding of disease processes and on therapy in the future. To date, no satisfactory method has been devised for measuring directly the volume of the intracellular compartment, the largest pool of water in the body. In all probability, the state of hydration of the cells themselves is of much greater significance than the extracellular fluid volume, or the concentration of ions in it. An indirect method for measuring this volume can be employed[4] by determining the difference between the tritium, deuterium and

THE EFFECT OF THYROID HORMONE ON THE DISTRIBUTION OF BODY FLUID IN MYXEDEMA

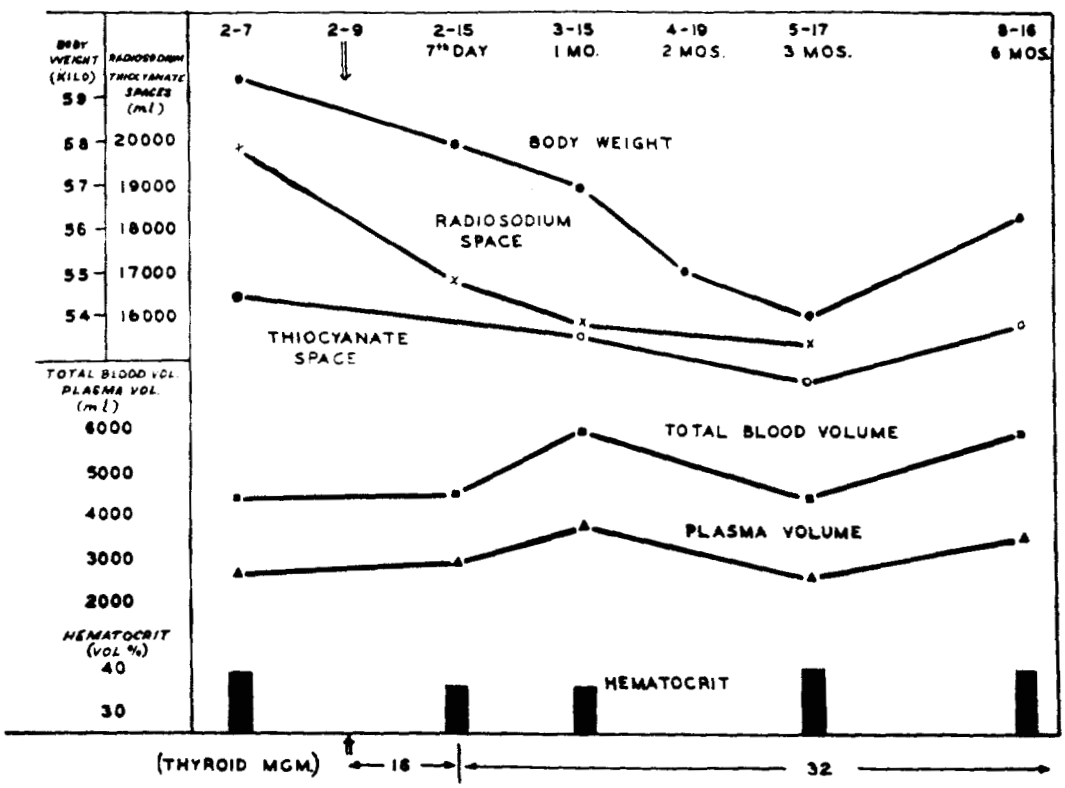

Fig 5

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antipyrine space and the volume of the extracellular fluid space.

The following case of myxedema included in our series illustrates the type of information which may be derived from studies on the distribution of body fluids.

In myxedema, the thickening of the skin is thought to be due not to true edema, but to the deposition of a semifluid albuminous substance. Serial determinations of the plasma volume and of the thiocyanate and radiosodium spaces were made during the course of thyroid therapy in a woman with frank untreated myxedema (*Fig. 5*). During the first two months of therapy a progressive decrease in body weight was paralleled by a decline in the radiosodium space. The correlation was so good that it is hard to escape the conclusion that the initial elevation of the radiosodium space was due to edema fluid. The correlation between the changes in weight and thiocyanate space was not so good. As is usually the case in this disease, the total blood volume and the plasma volume were low prior to therapy, and rose to normal levels during the course of treatment.

SUMMARY

Most of the substances which have been used in the past to measure the extracellular fluid space are foreign to the body. Since radioactive sodium, Na^{24} is chemically identical with ordinary sodium, Na^{23} the metabolism and distribution of the two substances in the body should therefore be the same.

Simultaneous determinations of the thiocyanate and radiosodium spaces were performed in individuals with various abnormal physiologic states. Although there was considerable variability in the individual cases, it was found that the mean volume of fluid measured by the two ions in the three-hour samples agreed very closely.

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DISCUSSION (*Abstract*)

Dr. George T. Harrell, Winston-Salem, N. C.—The studies done by Dr. Aikawa employ an isotope dilution technic. By injecting a known amount of the radioactive substance and then determining its concentration in a sample taken after mixing, the total quantity of that particular substance can be estimated. Under certain conditions, where diffusibility of the isotope is free, the distribution and total body content of the material in the body can also be measured. Since the salt is injected intravenously and subsequent measurements are made on samples of blood, the fluid space estimated by the degree of dilution and rate of disappearance from the blood must be extravascular. This space is assumed, on the basis of other investigations, to be predominantly extracellular in the normal individual.

Other isotopes which might be used in an attempt to check the size of the fluid spaces estimated by Dr. Aikawa are the isotopes of potassium, phosphorus and bromine. Chlorine would be a highly desirable isotope to use, since it is freely diffusible and is normally found both within and without cells, but the half-life is so very short that its use is impractical. Alteration in the extracellular (interstitial) fluid space in pathologic processes may also be estimated by measuring the rate of disappearance of various sized protein molecules tagged with radioactive iodine. As the defect in capillary permeability increases with the severity and type of the disease under investigation, larger and larger molecules would be lost from the circulation. A great technical difficulty involved in experiments of this type is the accurate measurement of the small amounts of radiation which can be tolerated by the human body. Further development of the scintillation counter, which is now being shown by a California group in the portion of the scientific exhibit devoted to medicine, gives promise of solving this technical problem.

Dr. Aikawa did not have time to discuss separately each disease process he has studied. The variations noted are apparently not due to differences in individuals; it should be stressed that the alterations seem to be the result of the disease processes. When ordinary chemical technics are used, it has been found that in pneumococcal lobar pneumonia the blood chlorides are low while the blood volume is increased. In Rocky Mountain spotted fever, as has been reported before this Section in the past, the blood chlorides are also low but the blood volume is decreased rather than increased. In congestive heart failure sodium is retained, though it is not yet completely clear whether the total amount of potassium in the body is also increased. In diabetes both sodium and potassium are lost from the tissues. It is of interest that this afternoon in the Section on Pathology a group from Oak Ridge is discussing the effect of irradiation on the permeability of capillary walls and hence on the plasma volume.

In spite of the intensive investigations of this general

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problem, the knowledge of what goes on within cells is still fragmentary. No technic is available by which direct measurements of the intracellular environment can be made. The radio-autographic technic, when it can be applied, gives a qualitative or semi-quantitative approach to the problem. Exact information on the hydration and ionic concentration of salts within cells is a very vital matter, since these factors are known to affect the function of enzyme systems on which life itself depends.

The basic studies of research groups such as Dr. Aikawa's are thankless, laborious and often dangerous. Such studies, however, are necessary if we are to obtain basic fundamental scientific knowledge. Only on such a foundation can our ultimate objective be reached, the improvement in therapy of human disease through the understanding of the mechanisms by which illness is produced.

Question.—Did you determine the activity of the edema fluid in the patient with myxedema?

Dr. Aikawa (closing).—In reply to question, we made no attempt to collect edema fluid. This preliminary study was intended to be merely a survey of the comparative values for the radiosodium and thiocyanate spaces in individuals with various pathologic physiologic states. A more comprehensive study is now in progress.

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WAKE FOREST COLLEGE
THE BOWMAN GRAY SCHOOL OF MEDICINE
WINSTON-SALEM, NORTH CAROLINA

DEPARTMENT OF
INTERNAL MEDICINE

March 21, 1951

Atomic Energy Commission
Office of Research and Medicine
Post Office Box E
Oak Ridge, Tennessee

Gentlemen:

You will find enclosed the progress reports on Titles I, II, and III of the contract number AT-(40-1) held by the Bowman Gray School of Medicine of Wake Forest College. These reports have been delayed several days because of the necessity of giving final examinations and generally winding up the work of the school trimester which ended March 17.

We are enclosing two reprints of the papers which have come out so far this year. As you will note from the progress reports additional manuscripts have been accepted for publication but have not yet come out and hence reprints are not available at present. Should you desire them, we can furnish copies of the manuscripts as they have been accepted.

We are very pleased this year with the amount of work that has been accomplished under Title I. We are still encountering technical difficulties in the design of experiments, some of which are extremely difficult to work out. We are also still encountering considerable difficulty in arranging satisfactorily for transportation of isotopes from Oak Ridge to Winston-Salem. As an example of the first four shipments of radiopotassium we were able to utilize only one. The specific activity is such that any delay in receipt of the material puts the required dose up to the point that chemical toxicity is encountered from the amount of potassium administered. We are all pleased with the progress made under Title II and with the recognition of the work by others. We are again pleased with the progress that has been made under Title III, considering the amount of the grant and the time available for the project.

Please let me express to you on behalf of all of those working here with us on the project our deepest appreciation for your continued help. We would especially like to thank you for the spirit of helpfulness and cooperation from an administrative point of view. This atmosphere has made our work very pleasant.

Respectfully,

George T. Harrell

George T. Harrell, M.D.

GTH:mfc

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PROGRESS REPORT

Work performed under AEC Contract No. AT-(40-1)-288 - Title I.
The Bowman Gray School of Medicine of Wake Forest College.

Distribution and turnover of sodium and potassium in acute infections.

Dr. George T. Harrell, Jr., Project leader July 1, 1950 to March 15, 1951

Facilities: The laboratory as previously constructed has proved in use to be very satisfactory. No changes have been made in the design or construction of the laboratory. The major items of equipment previously purchased have proved quite satisfactory. No different types of equipment have been needed this year. Additional counters, shields, and other items for expansion of the existing equipment have been purchased.

Personnel: Dr. Jerry K. Aikawa completed his AEC Fellowship. We have been able to obtain for him a fellowship from the American Heart Association which will enable him to continue work with us for at least another year. Financial support for him may be required by 1952.

Miss Eleise Rhoades, technician, has now been completely trained in our techniques and is proving quite satisfactory.

Claude Taylor, the negro technician, is proving satisfactory with the animal experiments.

Dr. Ernest Yount continues to assist in the design of experiments affecting human beings. He also is directing the experiments with use of iodine-131 which are not directly related to the primary research project.

Early in the year we were able to utilize house officers in the performance of certain groups of experiments. The majority of our house staff has now been called to military service and the shortage of personnel at this level is critical. Our human work may suffer during the next few years unless personnel not eligible for military service can be found.

Isotopes: Considerable time is still lost due to the vagaries of air transport. We have attempted to re-design experiments so that sodium-24 can be used even if it is late in arriving. In the case of potassium-42, however, it has proved impossible to design experiments which would give valid results should the isotope be late in arriving. The specific activity of the material received from Oak Ridge is such that if the shipment is as little as 12 hours late it would require the administration of a chemically toxic dose in order to achieve a satisfactory counting rate. This difficulty has made it impossible to use three of the first four shipments of potassium which started February 5. We have tried surface transportation by truck and air transportation by several different routes with about equal lack of success.

Animals: The animal breeding program mentioned in the previous progress report has been started. Very few animals were raised during the winter, but a few animals are now becoming available. It is not yet possible to estimate whether we can raise all the animals we need or not.

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Results of Experiments

Concept of Membrane Permeability Defect

A discussion of the concept of alterations in membrane permeability with various infectious and immune processes was presented at the June, 1950, meeting of the American Medical Association in the Section on Experimental Medicine and Therapeutics. This report is to be published in the JOURNAL OF THE AMERICAN MEDICAL ASSOCIATION. In it selected examples of our data on fluid volume changes in Rocky Mountain spotted fever, trichinosis, serum sickness and drug reactions in human beings are presented. The effect on intracellular fluid of heavy metal poisoning of enzyme systems by arsenic and the reversal after administration of BAL gives a clue as to possible mechanisms in infection.

Studies in Immunophysiology

As an extension of the original work on induced hypersensitivity in animals, the conditions of the experiments were slightly modified in order to decrease random variations. The physiologic effects of the injection of human plasma into rabbits were correlated with immunologic changes. Evidence for alterations in capillary and cell membrane permeability were found, which were thought to be related to the humoral antibody response. This work entitled, "The Immunophysiology of Serum Sickness: Alterations in the Blood Volume and Thiocyanate Space in Relation to the Development of Humoral Antibodies in the Rabbit," J. A. Aikawa and G.T. Harrell, has been accepted for publication in the Journal of Clinical Investigation and is scheduled for the April, 1951, issue.

A similar study was performed with purified human plasma fractions, specifically, albumin and globulin. Although the results varied quantitatively, the changes were qualitatively similar to those observed in the studies with human plasma. Here again, it was thought possible to attribute changes in blood volume, thiocyanate space and hematocrit as due to defects in membrane permeability as a result of immunologic phenomena. This work has been summarized and has been submitted for publication.

The current opinion is that in vivo antigen-antibody reactions are mediated by certain substances resident in the body in an inactive state, and which are released during the antigen-antibody reaction and thus cause the systemic effects. Histamine has often been implicated. It also is not established whether the increase in capillary permeability which is known to occur with various hypersensitive states, is due to damage to the endothelial cells or to an alteration in the intercellular cement substance. Hyaluronic acid is a constituent of the intercellular cement substance and is depolymerized by the enzyme hyaluronidase. In an attempt to determine whether these two substances contribute to the biochemical defect in hypersensitivity reactions, normal rabbits were injected with large doses of histamine and hyaluronidase. It was found that neither the plasma volume nor the thiocyanate space was altered in either group of animals. It therefore was concluded that neither of these substances was solely responsible for the changes previously observed in in vivo antigen-antibody reactions. This study is in the final manuscript stage.

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Preliminary studies with the passive transfer method are still in progress in order to determine the optimum dose of antigen and antibody required consistently to reproduce serum sickness and certain physiologic changes in rabbits. The difficulty to date has been in obtaining sufficiently high-titered sera for injection. After repeated sensitization of several animals, it now appears that such sera can be produced.

Comparative Studies with Thiocyanate and Radiosodium

In the previous progress report, it was mentioned briefly that a comparative study of the thiocyanate and radiosodium methods as indices of the volume of the extracellular space was in progress. These determinations were made in individuals with various pathologic physiologic states. To date, no data are available in the literature on the reliability of these two methods under abnormal conditions, although a report of a limited study in normal individuals is available. To date, we have performed studies with radiosodium-24 in more than 50 individuals and compared them with values for thiocyanate done simultaneously. In the group as a whole the two spaces checked as closely as in normal individuals. In specific diseases, however, wide divergence between the two methods was encountered which was thought to reflect differences in the pathologic-physiologic responses between different diseases. Much more work needs to be done before any definitive conclusions can be drawn.

A brief preliminary report of this work was presented at the November, 1950, meeting of the Southern Medical Association, in the Section on Medicine. This report is to be published soon in the SOUTHERN MEDICAL JOURNAL.

A discussion of the applicability of such methods in medical research and a demonstration of the actual methods used in this laboratory for such studies was given at the Oak Ridge Institute of Nuclear Studies at the Advanced Training Course in February, 1951.

A comprehensive analysis of the results is planned in the near future, after more data have been collected in individuals with various diseased states. At the present time, insufficient data are available for any single disease to warrant conclusions.

Studies with Radiopotassium-42

Until recently, there was not available a single valid method for measuring quantitatively any constituent of the intracellular compartment. The introduction of radioactive potassium-42 suggested that a tool was finally available for such a study. There is available in the literature a study of findings of the "exchangeable potassium" content in a series of normal young individuals. Such a study has not been reported in individuals with various pathologic physiologic states. For the past two months, study has been in progress on the determination of the total potassium content of hospitalized subjects with various abnormal states. A total of 35 individuals have been studied to date. In most of these individuals, a significantly lower potassium content has been obtained than has been previously suspected in these diseases. More data will be accumulated in normal and abnormal states before any publication is prepared.

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Studies in Normal Rabbits

Since the previous annual report was submitted, the data on fluid volumes and electrolyte concentrations in 45 normal rabbits have been summarized and published in the AMERICAN JOURNAL OF PHYSIOLOGY, 162:695, Sept., 1950. In this study, the results of the analyses of tissues and organs for their sodium-24 and water content, as well as a comparison of the thiocyanate and radiosodium spaces are given. This represents the first comprehensive study of this nature in normal rabbits, using artificial isotopes. It serves as a baseline for evaluation of the studies on pathologic states and establishes the validity of the methods.

Studies with Digitalis

It has not generally been appreciated that the digitalis glycosides which have been used for decades in the therapy of congestive heart failure are steroid compounds, structually related to the adrenal cortical hormone and the sex hormones. The latter substances are known to affect salt and water metabolism. It is generally stated that digitalis has a specific cardiotonic effect; there has been some speculation as to the existence of an extracardiac or peripheral mechanism of action as well. In the treatment of circulatory failure in the course of acute infections, digitalis is sometimes used. The use in such diseases as pneumonia and Rocky Mountain spotted fever would be placed on a firmer basis if a peripheral action of its mechanism could be established.

In order to study this problem, hospitalized subjects without cardiovascular disease were given a therapeutic dose of digitoxin intravenously. Serial determinations were then made of fluid volumes. The data indicated that there was an increase in the water and protein content of the vascular compartment within 24 hours after the administration of 1.2 mg. of digitoxin. This alteration is difficult to explain solely on the basis of a direct myocardial effect. A summary of the findings has been accepted for publication in the PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE.

In a more detailed study of the same problem, a group of 7 normal male medical students were next given the same dose of digitoxin intravenously. In this series, urinary excretion of sodium and potassium and the alterations in the body electrolyte concentrations and contents were also followed; sodium-24 was used as a check on thiocyanate space. This experiment has been completed and the data are being analyzed.

Since there is some experimental evidence that toxic doses of digitalis cause a reversal of the effects of the usual therapeutic dose, rabbits were given a sublethal toxic dose of digitoxin and the changes in fluid distribution and electrolyte concentration and content, including sodium-24, were followed. The changes found could not be explained solely on the basis of a direct myocardial effect of digitoxin. It was concluded that large doses of digitoxin had caused cell damage and alterations in cell membrane permeability to sodium ion and water. The effects resembled those produced in animals by adrenalectomy or the administration of large doses of desoxycorticosterone. This work has been submitted for publication.

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Comparative Study of Methods for the Determination of Plasma Volume

In previous studies on changes in capillary membrane permeability, the T-1824 (Evans Blue) dye method was used exclusively. Recently the iodinated serum fraction method has been introduced. From theoretic considerations, it would appear that this would be the ideal method for the clinical determination of the plasma volume or to detect alterations in capillary membrane permeability. Arrangements have been completed with Dr. D. L. Tabern of Abbott Laboratories to secure the iodinated plasma fractions from him. Preliminary comparative studies have been made in rabbits. Permission is awaited from AEC to use the material for clinical investigations in human beings.

George T. Harrell

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Reprinted from THE AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 162, No. 3, September, 1950

FLUID VOLUMES AND ELECTROLYTE CONCENTRATIONS IN NORMAL RABBITS[1]

JERRY K. AIKAWA[2]

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WINSTON-SALEM, NORTH CAROLINA

DURING the course of some studies in immunophysiology, it was necessary to determine the normal values and their random variations for the extracellular fluid compartments in normal rabbits. The plasma volume, total blood volume and the thiocyanate and radiosodium spaces were, therefore, measured in a total of 45 rabbits. Serial determinations of the plasma volume and the thiocyanate space were made in 6 animals. The reproducibility in any individual animal of values for the thiocyanate and the radiosodium spaces was determined. Serum sodium and potassium concentrations were measured. The radiosodium space of various tissues and organs was determined in 6 animals, as well as their total water content.

MATERIAL AND METHODS

A total of 45 normal domestic adult rabbits of both sexes, with initial body weights between 2 and 4 kg., were kept in individual cages and were fed a stock diet. Water was given without restriction.

Plasma Volume and Thiocyanate Space. The plasma volume and thiocyanate space were determined by the T-1824 dye (Evans Blue) and sodium thiocyanate techniques (1). The dye and the thiocyanate ion were injected as a sterile solution containing a mixture of one mg. cc. of Evans Blue and 50 mg cc. of sodium thiocyanate. Syringes were calibrated to deliver approximately 2 ml. of the mixture. No correction was made in dosage for variation in weights of the rabbits. The plasma volume was determined on a single 10-minute sample; 30-minute samples were drawn in some cases for comparison with the radiosodium space. The *hematocrit* was determined in Wintrobe tubes centrifuged at 3000 rpm for 30 minutes, at a radius of 15 cm.

Radiosodium Space. The radiosodium space was determined as follows: A known volume (2 ml.) of a sodium carbonate solution containing approximately 20μc ml. of Na^{24} was injected intravenously into a marginal vein of the ear immediately follow-

Received for publication February 27, 1950.

[1] This investigation was supported (in part) by the U. S. Atomic Energy Commission under a contract with the Bowman Gray School of Medicine and (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

The Na^{24} used was supplied by the National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

[2] AEC Fellow in the Medical Sciences of the National Research Council, 1948–1950.

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ing the injection of the mixture of Evans Blue and sodium thiocyanate. In a preliminary experiment, serial samples were obtained in order to determine the disappearance curve of radiosodium. Subsequently, a single blood sample was drawn to coincide with the equilibration point (one hour) for radiosodium. The activity was determined with a dipping tube and a scaling circuit. Counts were made to one per cent accuracy. The radiosodium space was calculated by the following formula: Volume of Na^{24} space in cc. = (Total counts/min. injected)/(Counts/min/ml. plasma). All values were corrected for decay. The standard solution which was used for injection was appropriately diluted with distilled water to approximate the counting rate of the plasma. No correction was made for urinary excretion of the radiosodium during the period of observation since the amount excreted was negligible.

Plasma Sodium and Potassium Concentrations. Plasma sodium and potassium concentrations were determined by flame spectrophotometry on the Beckman Model DU instrument. The following modification of the method of Mosher, Boyle, Bird, Jacobson, Batchelor, Iseri and Myers (2) was used: The slit width was set at 0.1 mm. in both sodium and potassium determinations. With the standard solution set on the transmission dial at 70 and 25, respectively, for sodium and potassium, the galvanometer needle was set at zero by altering the sensitivity setting.

Tissue Analyses. The 6 animals which were to be killed for tissue analyses were injected intravenously with 50μc/ml., with a total of 100 μc/animal. One hour after injection, these animals were exsanguinated by severing the jugular veins and the carotid arteries; the animals died within 4 to 5 minutes. Approximately one gm. of tissue was weighed on a torsion balance. Each sample was then completely digested with 5 to 10 ml. of fuming sulfuric acid; the digest was made to a volume of 20 ml. with distilled water. In order to maintain a constant geometry, 25-ml. graduated cylinders were used as containers for the digests. Because of the marked photosensitivity of the dipping tubes, all counting was done in a dark room. The radiosodium space of the various tissues and organs was calculated by the formula: Percentage radiosodium space = (Counts/min/gm. of tissue)/(Counts/min/ml. plasma). Aliquots were dried to constant weight in a drying oven at 110° to 120° C. for determinations of the total water content of the tissues and organs.

EXPERIMENTAL RESULTS

Serial Determinations of Plasma Volume, Hematocrit, and Thiocyanate Space. In the initial experiment, a total of 71 determinations of the plasma volume and the thiocyanate space were performed in 6 rabbits during an aggregate period of 206 days. The determinations were made every second or third day and occasionally at less frequent intervals. The calculations were based on single 10-minute samples. The results of this study are summarized in table 1.

Mixing Time for Radiosodium. Serial venous blood samples were obtained from 8 rabbits following the administration of radiosodium. Figure 1 shows graphically in cc/kg. the mean values for radiosodium space at each time interval. After the first 30 minutes, the disappearance curve became exponential. On the basis of the above findings, subsequent calculations of the sodium space were made on a single blood sample obtained one hour following the injection of Na^{24}. The rate of removal of

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radiosodium from the plasma was roughly 10 per cent hour. If the exponential portion of the curve was extrapolated to zero time, the radiosodium space was approximately 25 per cent of the body weight.

Mixing Time for Thiocyanate. In previous studies on immunophysiology (3, 4), for technical reasons, the thiocyanate space was calculated from the 10-minute sample. Upon serial determinations on any single rabbit, the results of this measurement were reproducible (table 1). However, it was recognized that equilibration had not occurred at that time. In figure 1 is shown the curve for thiocyanate space calculated from the mean values obtained at various time intervals in 2 normal rabbits. Equilibration was attained at 30 minutes. The disappearance slope was steeper than for the radiosodium space, so that the rate of removal of thiocyanate from the plasma was roughly 20 per cent/hour. Since, in the routine determination of the thiocyanate space, values were not extrapolated to zero time, and because of the rapid rate of removal of the ion from the circulation, 30-minute samples were drawn to compare with the one-hour radiosodium samples.

TABLE 1. MEAN VALUES OF SERIAL DETERMINATIONS OF FLUID SPACES IN NORMAL RABBITS

RABBIT NO.	DAYS OF OBSERVATION	TOTAL NO. DETERMIN.	PLASMA VOLUME	RED CELL VOLUME	TOTAL BLOOD VOLUME	HEMATOCRIT	THIOCYANATE SPACE
			cc/kg.	*cc/kg.*	*cc/kg.*	*Vol. %*	*cc/kg.*
1	46	16	45±4.5[1]	36±4.4	73±7.0	38±3.1	205±17
2	30	11	50±3.9	27±4.4	78±8.2	35±3.2	213±7
3	36	13	49±5.4	25±3.6	73±6.7	33±4.2	191±6
4	31	9	43±4.6	20±2.8	62±6.0	31±3.6	177±11
5	31	11	46±7.1	27±5.8	73±11.1	36±3.4	197±17
6	32	11	46±4.9	21±3.5	67±6.3	34±4.8	173±18
Total.	206	71	46±5.5[2]	27±7.6	72±8.0	35±4.3	194±21

[1] Standard deviation. [2] Standard deviation of the series.

Plasma Volume. The mean plasma volume, based on a total of 67 determinations on 39 rabbits, was found to be 42.3 cc kg. (table 2). Repeat determinations were made on a total of 28 animals (table 2). In 27 of these, the measurements were made within an interval of 2 to 14 days. In one animal, the interval between the 2 determinations was 50 days. The mean difference between such duplicate determinations was 4.14 cc. kg. There was no correlation of the interval between the determinations and the magnitude of the difference.

Hematocrit. The mean hematocrit, based on a total of 65 determinations on 39 animals, was 38.8 volumes per cent. The mean difference between duplicate determinations, based on 26 such pairs, was 4.46 volumes per cent. There appeared to be a rough correlation between the decrease in the second hematocrit as compared with the initial measurement and the time interval between the 2 readings, the decrease in the second hematocrit being greater, the shorter the interval between the determinations. Of the total of 26 duplicate determinations, in all cases except 4, the second hematocrit was equal to or less than the first. In these 4 instances, at least 14 days had elapsed between the 2 determinations. It appeared that with the volume of

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blood withdrawn for each study, at least 10 to 14 days were required for the hematocrit to return to the original value.

Total Blood Volume. The mean total blood volume, based on 65 determinations in 39 animals, was found to be 69.8 cc kg. (table 1). Repeat determinations in 26 animals varied by a mean of 6.34 cc kg. No correlation was noted of the interval between the repeat determinations and the magnitude of the difference between the 2 values.

Thiocyanate Space. The mean of 68 determinations of the thiocyanate space on a total of 39 rabbits, based on the 10-minute samples, was 217.8 cc/kg. (table 1). The mean of the 30-minute samples, based on a total of 50 determinations in 32 rabbits, was 237.6 cc/kg. The mean of the 30-minute samples, therefore, was 20 cc/kg. greater than the mean of the 10-minute samples. Thirty-minute samples revealed slightly less scatter than the 10-minute samples. The upper limit of the values for both the 10- and the 30-minute determinations was practically identical. Duplicate determinations of the 30-minute readings were more reproducible than the 10-minute

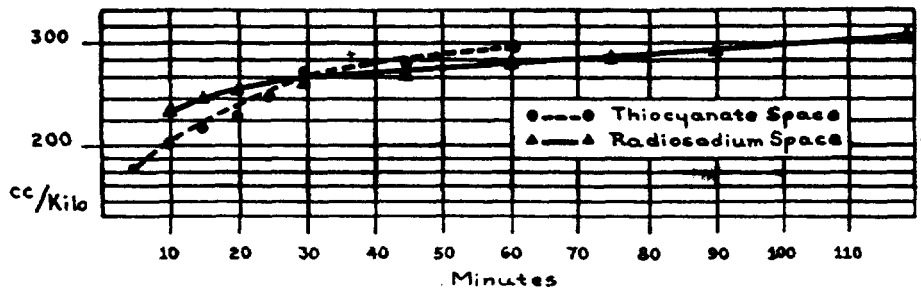

Fig. 1. THIOCYANATE AND RADIOSODIUM SPACES calculated from mean values at various time intervals.

readings, the mean difference in the former being 15.03 cc kg. and in the latter, 8.66 cc/kg. There was no correlation of the interval between duplicate determinations at either 10 or 30 minutes and the magnitude of the difference between the 2 determinations.

Radiosodium Space. The mean radiosodium space, based on the one-hour samples on 68 determinations in 39 rabbits, was found to be 263 cc kg. This value was 45 cc/kg. greater than the mean thiocyanate space based on the 10-minute samples, and 25 cc/kg. greater than the thiocyanate space based on the 30-minute samples. The variability of the 3 measurements was roughly equal, that of the 30-minute thiocyanate space being the least. The mean difference and the scatter of duplicate determinations of the radiosodium space was greater than those for the thiocyanate space at either 10 or 30 minutes.

Serum Sodium and Potassium Concentrations. The mean serum sodium concentration, based on a total of 55 determinations in 33 rabbits, was 131.9 mEq/l. with a standard error of 0.97. The mean serum potassium concentration, based on the same sera, was 4.58 mEq/l. with a standard error of 0.075. Serum sodium levels showed less random variation than serum potassium levels. There was no correlation

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of the interval between duplicate determinations of either serum sodium or potassium levels and the magnitude of the difference.

Tissue Radiosodium Space. The results of the analyses of the tissue radiosodium spaces in 6 normal rabbits are summarized in table 3. Of the tissues analyzed, the cartilage, skin and kidney contained the largest amount of radiosodium per unit weight. Skeletal muscle contained the least. Considerable variation was noted in the radiosodium space of some organs, e.g. subcutaneous connective tissue, liver, appendix and cartilage.

Total Water Content of Organs and Tissues. Total water content of the various tissues and organs are given in table 3. The appendix and the lung contained the largest amount of water per unit of weight of tissue and the tendon the least. The

TABLE 2. SUMMARY OF DATA ON EXTRACELLULAR FLUID VOLUME AND ELECTROLYTE CONCENTRATION IN NORMAL RABBITS

	RESULTS OF DETERMINATIONS IN 39 NORMAL ANIMALS			COMPARISON OF TWO DETERMINATIONS IN 29 ANIMALS		
	Total No. Determin.	Mean ± Stand. Deviation	Range	Total No. Duplicate Determin.	Mean Diff. ± Stand. Deviation	Range
Body weight (kg.)	68	3.17±.538	1.97–4.50			
Plasma volume (cc/kg.)	67	42.3±7.38	29–58	28	4.14±3.25	0–11
Hematocrit (vol. %)	65	38.8±4.76	27–48	26	4.46±3.73	0–14
Total blood vol., (cc/kg)	65	69.8±9.12	50–93	26	6.34±5.25	0–20
Thiocyanate space (cc/kg.) 10 min.	68	217.8±17.8	177–270	29	15.03±10.53	0–50
Thiocyanate space (cc/kg.) 30 min.	50	237.0±15.1	210–271	18	8.66±8.42	0–26
Radiosodium space (cc/kg.)	68	263.2±22.1	223–308	29	16.55±15.61	0–82
Serum sodium (mEq/l.)	55	131.9±7.2	110–147	22	6.72±4.16	1–14
Serum potassium (mEq/l.)	55	4.58±0.56	3.4–6.0	22	0.36±0.29	0–1.0

coefficients of variation for the determinations of the total water content of any tissue or organ were usually smaller than those for the determinations of the radiosodium spaces.

DISCUSSION

In the initial experiment (table 1), it was found that when a series of 9 to 13 determinations of the plasma volume, hematocrit, total blood volume and the 10-minute thiocyanate space were made every second to third day in normal rabbit, the respective means and standard deviations were 46±5.5 cc/kg., 35±4.3 volumes per cent, 72±8.9 cc/kg. and 194±21 cc/kg. The values obtained in the subsequent studies involving a single or 2 repeated determinations on normal rabbits agreed fairly closely with the previous serial determinations. In the only other similar study

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of normal rabbits which has been found in the literature (5), the mean of the determinations of the total blood volume in 60 rabbits was 70 cc/kg., based on samples obtained 6 minutes after injection of the Evans Blue. The mean value of the plasma volume was 50 cc/kg. The blood volume was found to be proportional to the body weight and not to the surface area. These results are consonant with the findings in the present investigation. No comparative data have been found regarding the thiocyanate space or the rate of disappearance of the ion from the blood stream in rabbits.

The results of the present study indicate that regardless of whether the thiocyanate determinations based on the 10- or 30-minute samples or the radiosodium space based on the one-hour sample are employed as an index of the extracellular fluid volume, consistently reproducible results can be obtained, at least in normal rabbits. There is valid argument against the use of the 10-minute readings, since equilibration had not occurred at that time. However, for technical reasons, this

TABLE 3. SUMMARY OF DATA ON TISSUE RADIOSODIUM SPACE AND TOTAL WATER CONTENT IN 6 NORMAL RABBITS

	TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION		TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION
Body weight (kg.)	3.66±0.15		Tissues—*Cont.*		
Radiosodium space (% body weight)	24.9±1.10		Liver	15.20±5.36	70.34±1.73
Tissues[1]			Abdominal muscle	16.20±3.56	69.05±4.44
Skin	44.05±6.85	63.99±3.78	Heart	23.67±2.16	77.97±3.16
Subcutaneous connective tissue	31.17±9.04	62.97±7.45	Kidney	42.24±6.53	76.38±0.09
			Appendix	23.56±6.59	80.21±1.08
Diaphragm	15.02±2.20	65.76±6.16	Stomach	24.41±5.68	77.95±1.08
Spleen	10.79±2.58	70.48[2]±1.58	Ear cartilage	51.91±10.1	65.09±2.20
Lung	37.10±3.31	79.63±0.09	Skeletal muscle	10.07±1.41	75.26±0.06
Adrenal	14.98[2]±1.08	65.75±8.32	Achilles tendon	30.06±4.61	60.01±3.58

[1] All values expressed in percentage of weight. [2] Based on 5 animals.

has proved to be the most practical method when serial determinations are made on a single animal. Actually, the 30-minute thiocyanate determinations revealed less scatter than either the 10-minute thiocyanate determinations or the radiosodium determinations. The exact significance of the thiocyanate space, however, remains in doubt. Determinations of the thiocyanate space in various tissues and organs and their comparison with the sodium space might clarify this problem. The present data indicate that in normal rabbits the thiocyanate ion at equilibration consistently measures a space roughly 10 per cent less than the space measured by radiosodium.

The mean radiosodium space of 26.3 per cent of the body weight found in the present study agrees with the values obtained by previous investigators with a smaller number of rabbits (6–8). Manery and Bale (8) compared the radiosodium content of rabbit tissues with their content of chemically determined sodium and found that the specific activity of all tissues except the nervous tissue was constant. They concluded that "with the exception of the nervous tissue, in 1 hour 8 minutes, injected

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radioactive sodium becomes distributed between tissues and plasma in precisely the same ratio as normally occurring sodium." Most of the tissue sodium values reported by Manery and Hastings (9) were higher than those obtained in the present study. One possible explanation for this discrepancy may be the difference in the methods employed in sacrificing the animals. Decapitation of animals does not result in as thorough exsanguination as severing the neck vessels. The two-fold discrepancy in the values for cartilage is difficult to explain.

The total water content of tissues obtained in the present study are summarized in table 3. These values agree well with those obtained by Manery and Hastings (9) and by Skelton (10). Less discrepancy was noted than with the determinations of the radiosodium space.

SUMMARY AND CONCLUSIONS

The normal values and their random variations for the extracellular fluid compartments in normal rabbits were determined. Plasma volume was determined by the T-1824 dye method; the thiocyanate space, by the dilution of sodium thiocyanate; and the radiosodium space, by the dilution of Na^{24}. Serum sodium and potassium concentrations were measured by flame spectrophotometry. In a series of 39 animals, the plasma volume was found to be 42.3±7.38 cc/kg.; the total blood volume, 69.8±9.12 cc/kg.; the thiocyanate space, 217.8±17.8 cc/kg.; and the radiosodium space, 263.2±22.1 cc/kg. The serum sodium concentration was 131.9±7.2 mEq/l.; the serum potassium concentration, 4.58±0.56 mEq/l. The mean difference between 2 determinations in 29 rabbits was as follows: plasma volume, 4.14±3.25 cc/kg.; total blood volume, 6.34±5.25 cc/kg.; thiocyanate space, 15.03±10.53 cc/kg.; radiosodium space, 16.55±15.61 cc/kg. Seventy-one serial determinations were made on 6 rabbits over an aggregate period of 206 days. The results were as follows: plasma volume, 46±5.5 cc/kg.; total blood volume, 72±8.9 cc/kg.; thiocyanate space, 194±21 cc/kg. The values obtained upon tissue analyses for total water content and radiosodium space are given.

The total blood volume, calculated from the plasma volume and the hematocrit, was 69.8 cc/kg. This value was constant in normal rabbits and appeared to be directly related to the body weight. A previous investigator (6) had obtained practically the identical value, so that the constancy of this value appears to have been established.

The exact significance of the thiocyanate space remains doubtful and will not be resolved until comparative studies of the concentration of the sodium ion and the thiocyanate ion in various tissues and organs are made. From the present study it can only be concluded that the thiocyanate ion at equilibrium measured a volume which was approximately 10 per cent less than that measured by the radiosodium ion. Whether this relationship would continue in abnormal physiologic states remains to be studied. Presumably the radiosodium ion at one hour measured in various organs and tissues the volume of fluid which was extracellular. The thiocyanate determinations were reproducible in normal rabbits, regardless of whether 10- or 30-minute samples were compared. The random variations in the values for the thiocyanate or the Na^{24} space were roughly equivalent. Tissue sodium spaces are probably not com-

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parable unless a uniform technique is utilized in sacrificing the animals, since it is obvious that variations in the amount of residual blood in the organs and tissues would alter their sodium content. Since serial determinations of the fluid volume spaces are more reproducible in individual animals than single determinations in separate animals, experiments should be so designed as to take this observation into consideration.

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FLUID VOLUMES AND ELECTROLYTE CONCENTRATIONS IN NORMAL RABBITS[1]

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DURING the course of some studies in immunophysiology, it was necessary to determine the normal values and their random variations for the extracellular fluid compartments in normal rabbits. The plasma volume, total blood volume and the thiocyanate and radiosodium spaces were, therefore, measured in a total of 45 rabbits. Serial determinations of the plasma volume and the thiocyanate space were made in 6 animals. The reproducibility in any individual animal of values for the thiocyanate and the radiosodium spaces was determined. Serum sodium and potassium concentrations were measured. The radiosodium space of various tissues and organs was determined in 6 animals, as well as their total water content.

MATERIAL AND METHODS

A total of 45 normal domestic adult rabbits of both sexes, with initial body weights between 2 and 4 kg., were kept in individual cages and were fed a stock diet. Water was given without restriction.

Plasma Volume and Thiocyanate Space. The plasma volume and thiocyanate space were determined by the T-1824 dye (Evans Blue) and sodium thiocyanate techniques (1). The dye and the thiocyanate ion were injected as a sterile solution containing a mixture of one mg cc. of Evans Blue and 50 mg cc. of sodium thiocyanate. Syringes were calibrated to deliver approximately 2 ml. of the mixture. No correction was made in dosage for variation in weights of the rabbits. The plasma volume was determined on a single 10-minute sample; 30-minute samples were drawn in some cases for comparison with the radiosodium space. The *hematocrit* was determined in Wintrobe tubes centrifuged at 3000 rpm for 30 minutes, at a radius of 15 cm.

Radiosodium Space. The radiosodium space was determined as follows: A known volume (2 ml.) of a sodium carbonate solution containing approximately 20μc./ml. of Na^{24} was injected intravenously into a marginal vein of the ear immediately follow-

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The Na^{24} used was supplied by the National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

[2] AEC Fellow in the Medical Sciences of the National Research Council, 1948–1950.

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ing the injection of the mixture of Evans Blue and sodium thiocyanate. In a preliminary experiment, serial samples were obtained in order to determine the disappearance curve of radiosodium. Subsequently, a single blood sample was drawn to coincide with the equilibration point (one hour) for radiosodium. The activity was determined with a dipping tube and a scaling circuit. Counts were made to one per cent accuracy. The radiosodium space was calculated by the following formula: Volume of Na^{24} space in cc. = (Total counts/min. injected)/(Counts/min/ml. plasma). All values were corrected for decay. The standard solution which was used for injection was appropriately diluted with distilled water to approximate the counting rate of the plasma. No correction was made for urinary excretion of the radiosodium during the period of observation since the amount excreted was negligible.

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Mixing Time for Radiosodium. Serial venous blood samples were obtained from 8 rabbits following the administration of radiosodium. Figure 1 shows graphically in cc/kg. the mean values for radiosodium space at each time interval. After the first 30 minutes, the disappearance curve became exponential. On the basis of the above findings, subsequent calculations of the sodium space were made on a single blood sample obtained one hour following the injection of Na^{24}. The rate of removal of

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4	31	9	43±4.6	20±2.8	62±6.0	31±3.6	177±11
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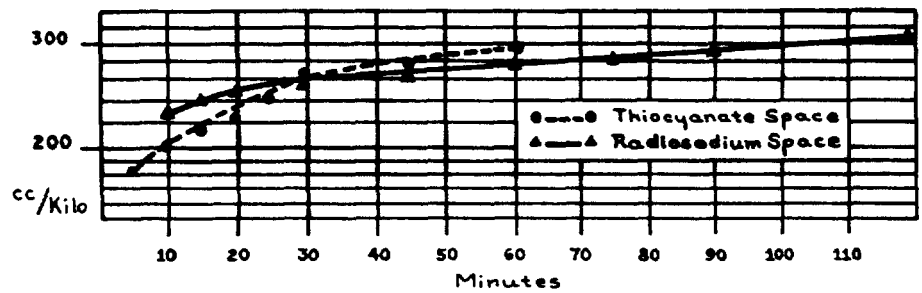

Fig. 1. THIOCYANATE AND RADIOSODIUM SPACES calculated from mean values at various time intervals.

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of the interval between duplicate determinations of either serum sodium or potassium levels and the magnitude of the difference.

Tissue Radiosodium Space. The results of the analyses of the tissue radiosodium spaces in 6 normal rabbits are summarized in table 3. Of the tissues analyzed, the cartilage, skin and kidney contained the largest amount of radiosodium per unit weight. Skeletal muscle contained the least. Considerable variation was noted in the radiosodium space of some organs, e.g. subcutaneous connective tissue, liver, appendix and cartilage.

Total Water Content of Organs and Tissues. Total water content of the various tissues and organs are given in table 3. The appendix and the lung contained the largest amount of water per unit of weight of tissue and the tendon the least. The

TABLE 2. SUMMARY OF DATA ON EXTRACELLULAR FLUID VOLUME AND ELECTROLYTE CONCENTRATION IN NORMAL RABBITS

	RESULTS OF DETERMINATIONS IN 39 NORMAL ANIMALS			COMPARISON OF TWO DETERMINATIONS IN 29 ANIMALS		
	Total No. Determin.	Mean ± Stand. Deviation	Range	Total No. Duplicate Determin.	Mean Diff. ± Stand. Deviation	Range
Body weight (kg.)	68	3.17±.538	1.97–4.50			
Plasma volume (cc/kg.)	67	42.3±7.38	29–58	28	4.14±3.25	0–11
Hematocrit (vol. %)	65	38.8±4.76	27–48	26	4.46±3.73	0–14
Total blood vol., (cc/kg)	65	69.8±9.12	50–93	26	6.34±5.25	0–20
Thiocyanate space (cc/kg.) 10 min.	68	217.8±17.8	177–270	29	15.03±10.53	0–50
Thiocyanate space (cc/kg.) 30 min.	50	237.6±15.1	210–271	18	8.66±8.42	0–26
Radiosodium space (cc/kg.)	68	263.2±22.1	223–308	29	16.55±15.61	0–82
Serum sodium (mEq/L)	55	131.9±7.2	110–147	22	6.72±4.16	1–14
Serum potassium (mEq/L)	55	4.58±0.56	3.4–6.0	22	0.36±0.29	0–1.0

coefficients of variation for the determinations of the total water content of any tissue or organ were usually smaller than those for the determinations of the radiosodium spaces.

DISCUSSION

In the initial experiment (table 1), it was found that when a series of 9 to 13 determinations of the plasma volume, hematocrit, total blood volume and the 10-minute thiocyanate space were made every second to third day in normal rabbit, the respective means and standard deviations were 46±5.5 cc/kg., 35±4.3 volumes per cent, 72±8.9 cc/kg. and 194±21 cc/kg. The values obtained in the subsequent studies involving a single or 2 repeated determinations on normal rabbits agreed fairly closely with the previous serial determinations. In the only other similar study

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of normal rabbits which has been found in the literature (5), the mean of the determinations of the total blood volume in 60 rabbits was 70 cc kg., based on samples obtained 6 minutes after injection of the Evans Blue. The mean value of the plasma volume was 50 cc kg. The blood volume was found to be proportional to the body weight and not to the surface area. These results are consonant with the findings in the present investigation. No comparative data have been found regarding the thiocyanate space or the rate of disappearance of the ion from the blood stream in rabbits.

The results of the present study indicate that regardless of whether the thiocyanate determinations based on the 10- or 30-minute samples or the radiosodium space based on the one-hour sample are employed as an index of the extracellular fluid volume, consistently reproducible results can be obtained, at least in normal rabbits. There is valid argument against the use of the 10-minute readings, since equilibration had not occurred at that time. However, for technical reasons, this

TABLE 3. SUMMARY OF DATA ON TISSUE RADIOSODIUM SPACE AND TOTAL WATER CONTENT IN 6 NORMAL RABBITS

	TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION		TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION
Body weight (kg.)	3.66±0.15		Tissues—*Cont.*		
Radiosodium space (% body weight)	24.9±1.10		Liver	15.20±5.36	70.34±1.73
Tissues[1]			Abdominal muscle	16.20±3.56	69.05±4.44
Skin	44.05±6.85	63.99±3.78	Heart	23.67±2.16	77.97±3.16
Subcutaneous connective tissue	31.17±9.04	62.97±7.45	Kidney	42.24±6.53	76.38±0.09
			Appendix	23.56±6.59	80.21±1.08
Diaphragm	15.02±2.20	65.76±6.16	Stomach	24.41±5.68	77.95±1.08
Spleen	19.79±2.58	76.48[2]±1.58	Ear cartilage	51.91±10.1	65.09±2.20
Lung	37.10±3.31	79.63±0.09	Skeletal muscle	10.07±1.41	75.26±0.06
Adrenal	14.98[2]±1.08	65.75±8.32	Achilles tendon	39.06±4.61	60.01±3.58

[1] All values expressed in percentage of weight. [2] Based on 5 animals.

has proved to be the most practical method when serial determinations are made on a single animal. Actually, the 30-minute thiocyanate determinations revealed less scatter than either the 10-minute thiocyanate determinations or the radiosodium determinations. The exact significance of the thiocyanate space, however, remains in doubt. Determinations of the thiocyanate space in various tissues and organs and their comparison with the sodium space might clarify this problem. The present data indicate that in normal rabbits the thiocyanate ion at equilibration consistently measures a space roughly 10 per cent less than the space measured by radiosodium.

The mean radiosodium space of 26.3 per cent of the body weight found in the present study agrees with the values obtained by previous investigators with a smaller number of rabbits (6–8). Manery and Bale (8) compared the radiosodium content of rabbit tissues with their content of chemically determined sodium and found that the specific activity of all tissues except the nervous tissue was constant. They concluded that "with the exception of the nervous tissue, in 1 hour 8 minutes, injected

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radioactive sodium becomes distributed between tissues and plasma in precisely the same ratio as normally occurring sodium." Most of the tissue sodium values reported by Manery and Hastings (9) were higher than those obtained in the present study. One possible explanation for this discrepancy may be the difference in the methods employed in sacrificing the animals. Decapitation of animals does not result in as thorough exsanguination as severing the neck vessels. The two-fold discrepancy in the values for cartilage is difficult to explain.

The total water content of tissues obtained in the present study are summarized in table 3. These values agree well with those obtained by Manery and Hastings (9) and by Skelton (10). Less discrepancy was noted than with the determinations of the radiosodium space.

SUMMARY AND CONCLUSIONS

The normal values and their random variations for the extracellular fluid compartments in normal rabbits were determined. Plasma volume was determined by the T-1824 dye method; the thiocyanate space, by the dilution of sodium thiocyanate; and the radiosodium space, by the dilution of Na^{24}. Serum sodium and potassium concentrations were measured by flame spectrophotometry. In a series of 39 animals, the plasma volume was found to be 42.3±7.38 cc/kg.; the total blood volume, 69.8±9.12 cc/kg.; the thiocyanate space, 217.8±17.8 cc/kg.; and the radiosodium space, 263.2±22.1 cc/kg. The serum sodium concentration was 131.9±7.2 mEq/l.; the serum potassium concentration, 4.58±0.56 mEq/l. The mean difference between 2 determinations in 29 rabbits was as follows: plasma volume, 4.14±3.25 cc/kg.; total blood volume, 6.34±5.25 cc/kg.; thiocyanate space, 15.03±10.53 cc/kg.; radiosodium space, 16.55±15.61 cc/kg. Seventy-one serial determinations were made on 6 rabbits over an aggregate period of 206 days. The results were as follows: plasma volume, 46±5.5 cc/kg.; total blood volume, 72±8.9 cc/kg.; thiocyanate space, 194±21 cc/kg. The values obtained upon tissue analyses for total water content and radiosodium space are given.

The total blood volume, calculated from the plasma volume and the hematocrit, was 69.8 cc/kg. This value was constant in normal rabbits and appeared to be directly related to the body weight. A previous investigator (6) had obtained practically the identical value, so that the constancy of this value appears to have been established.

The exact significance of the thiocyanate space remains doubtful and will not be resolved until comparative studies of the concentration of the sodium ion and the thiocyanate ion in various tissues and organs are made. From the present study it can only be concluded that the thiocyanate ion at equilibrium measured a volume which was approximately 10 per cent less than that measured by the radiosodium ion. Whether this relationship would continue in abnormal physiologic states remains to be studied. Presumably the radiosodium ion at one hour measured in various organs and tissues the volume of fluid which was extracellular. The thiocyanate determinations were reproducible in normal rabbits, regardless of whether 10- or 30-minute samples were compared. The random variations in the values for the thiocyanate or the Na^{24} space were roughly equivalent. Tissue sodium spaces are probably not com-

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parable unless a uniform technique is utilized in sacrificing the animals, since it is obvious that variations in the amount of residual blood in the organs and tissues would alter their sodium content. Since serial determinations of the fluid volume spaces are more reproducible in individual animals than single determinations in separate animals, experiments should be so designed as to take this observation into consideration.

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Progress report on the research project

FORMATION OF PHOSPHOLIPIDES IN TISSUES

sponsored by the Atomic Energy Commission, Division of Biology and Medicine under contract No. AT-(40-1)-288, Title II, with the Bowman Gray School of Medicine of Wake Forest College.

Project leader: Dr. Camillo Artom

The present report covers the work done from March 16, 1950 through March 15,195 This work is a direct continuation of that previously done under the sponsorship of the Atomic Energy Commission and summarized in my progress report of March 15, 1950. In addition to the writer, the following investigators participated to the research work: M. A. Swanson, Ph. D., M. Crowder, M. S., P. Mitchell, Graduate Student. Technical assistance was given by G. Terhune and for limited periods, by H. Wainer and J. Giles.

A. General outline of the work.

During the past year a few additional experiments have been made in order to complete the two series of investigations on the Lipide composition of mitochondria, and on the Incorporation of inorganic P^{32} into the phospholipides of isolated liver slices. Previous and present results on these subjects have been organized in two complete papers (one published, the other submitted for publication).

The largest part of the time and activity of the investigators has been devoted to a study of the Incorporation of C^{14}-labeled choline in the lipides of isolated liver tissue. The experimental part of this study is almost completed and the results will be presented shortly in a preliminary form. A simultaneous investigation has been carried out on the Metabolic pathways of choline in liver slices. Some of the results have been published; but much further work is needed before definitive conclusions can be drawn.

Since dimethylethanolamine is a likely precursor in the synthesis of choline, and since previous in vivo experiments in this laboratory indicated that it may be directly incorporated in the phospholipide molecule, the Synthesis of C^{14}-labeled dimethylethanolamine has been undertaken. The synthesized product will be added to the liver slices in experiments similar to those in which labeled choline has been used.

A number of recent data strongly suggests that formaldehyde (and/or formic acid) are important intermediates in the biological oxidation (perhaps also in the synthesis) of the methyl groups of several compounds, including choline. This has induced us to study the Disposal of formaldehyde in tissues. Some of the preliminary findings have been and others will be reported shortly.

The main results of each series of investigations are summarized below. Several of these results, even if they are considered separately, seem to be of a considerable interest. However, their significance is greatly enhanced by the fact that in our past and present investigations the problems have been approached simultaneously from various angles and with various experimental techniques (in vivo and in vitro experiments, isotopic labeling of different moieties of the phospholipide molecule, etc.). The comparison between the results obtained in the

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various series of experiments has made possible to draw certain deductions and interpretations of a more general interest. The writer was given the opportunity of developing and discussing these interpretations before two scientific groups of specialized workers (conferences on "Lipides" and on "Liver Injury.").

B. Lipide composition of liver mitochondria.

As reported previously, this study was suggested, first by the high content of mitochondria in total lipides; and, second, by the presence in these substructures of enzyme systems which are essential for many oxidation processes in the cells and which, more especially, under proper conditions actively synthesize phospholipides in vitro. Several complete analyses of the lipide mixtures in the "large granule" fraction, separated by differential centrifugation of liver homogenates, had been made last year and the results reported verbally in a summarized form. Additional analyses were made more recently, in order to compare directly the composition of the large granule fractions and that of the whole unfractionated liver. The results of such a comparison confirm our preliminary conclusion that mitochondria have a higher proportion of lipides, especially phospholipides, but that the composition of these lipides is not essentially different from that present in the whole liver. Unlike previous claims, no evidence was found for the presence of unusually large amounts of peculiar lipides, such as cerebrosides and lysophospholipides, in this fraction. A complete paper has been prepared and published on this subject.

C. Incorporation of inorganic P^{32} into the phospholipides of liver slices.

The experiments of this series have also been completed and most of our previous findings confirmed and extended. On the basis of these findings, it appears that a decrease in the incorporation of P^{32} into the lipides occurs in liver slices of rats on low protein diets. The decrease seems to be due to the decrease in cytoplasmic proteins and more especially in the enzyme proteins which are involved, directly or indirectly, in the synthesis of the phosphate bond of phospholipides. When these data are compared with those previously obtained on insect animals, it seems likely that in the liver of the latter animals, regulatory mechanisms (possibly hormonal) maintain the rate of the incorporation of P^{32} into phospholipides at a level adequate to the requirements of the animal. Presumably such a level is much below the maximum functional capacity of the liver, so that in the intact animal losses of enzymes in the liver can be easily compensated by the increased activity of the remaining cell structures. On the other hand, in the isolated tissue where hormonal mechanisms are absent, or much less efficient, the process does occur at the maximum speed compatible with the conditions, of the experiment. If the amounts of active enzymes present in the tissue are reduced, as in the liver of protein depleted animals, the speed of P^{32} incorporation into the lipides of the surviving slices will be correspondently decreased.

The effects of the in vitro addition of choline, ethanolamine and betaine to liver slices differ not only with the substance added but also with the previous dietary regime of the animal. The most unexpected finding was a marked inhibition of the incorporation of P^{32} in the lipides, when choline was added to liver slices

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from rats on adequate diets. It might be that such an inhibition is the result of a decrease in the endogenous respiration of the tissue caused by the added choline.

The manuscript of a complete paper reporting these results in detail has been prepared and submitted for publication.

D. Incorporation of labeled choline into the lipides of isolated liver tissue.

Choline labeled in the methyl group was prepared by methylation of dimethylethanolamine with C^{14}-labeled methyl iodide. The labeled choline was added to liver slices or liver homogenates and incubated for 3 and 1/2 hours in a Warburg bath. The tissues were then precipitated with colloidal ferric hydroxide and $MgSO_4$ and washed with $MgSO_4$ solution. The lipides were extracted from the precipitate, and phosphorus, choline and radioactivity determined on aliquots of the lipide extract. Much time and effort was spent in the elaboration of a technique which would allow a satisfactory elimination of the non-lipide choline, but could still be used as a routine procedure in experiments in which numerous parallel determinations had to be carried out simultaneously.

The results obtained consistently showed a well detectable incorporation of the added choline into the lipides. This finding, in conjunction with those previously obtained in this and other laboratories with P^{32}-labeled phosphate and C^{14}-labeled fatty acids complete the evidence for the ability of the isolated liver to synthesize the whole phospholipide molecule from its constituent parts.

In oxygen, the rate of the incorporation of choline into the lipides is generally lower with slices from rats on stock or high protein diets than with slices of rats from low protein diets. Substitution of nitrogen for air or oxygen does not considerably affect the process in surviving slices from rats on the stock diet. On the other hand, the absence of oxygen markedly decreases the incorporation of choline in the lipides of slices from rats previously maintained on deficient diets. Similar effects have been obtained with the in vitro addition of cyanide ions. Homogenization of the liver decreases but does not abolish the ability of the tissue to incorporate choline into the lipides.

The effects of the in vitro addition of a number of substances to liver slices (in air or oxygen) have also been tested. Ethanolamine, diethanolamine, triethylcholine strongly inhibit the incorporation of choline in the lipides. A less marked inhibition was observed by the addition of serine or glycine. No inhibition was detectable when thiocholine or trimethylamine were added to the liver slices. Experiments in which mixtures of isotopic choline and non-isotopic choline in various concentrations were added to the liver slices, suggest that the increase in the concentration of free choline may actually enhance its incorporation into the phospholipides.

The interpretation of the results mentioned above is still rather difficult, since obviously a number of factors are involved. It may be pointed out, however, that several of the findings concerning the incorporation of choline into the lipides are markedly different from those previously obtained by us and by others in experiments in which inorganic P^{32} was added to liver slices. Indeed, the

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incorporation of P^{32} is suppressed by homogenization of the liver and it is greatly decreased by a reduction in the oxygen supply. Furthermore, unlike the incorporation of choline, that of P^{32} is markedly decreased in slices from rats previously maintained on low protein diets. It seems therefore that the synthesis of the bond between the choline and the phosphate moieties of the phospholipides may, at least to a large extent, proceed independently from the synthesis of the other ester bonds in the phospholipide molecule. This also suggests that phosphatidic acids may represent an important intermediary product for the synthesis, the splitting and the interconversion of the various phospholipides in the liver. This hypothesis may also explain the effects of the addition *in vitro* of various substances which would compete with choline for the phosphatidic acid radicals in the system. Indeed, the substances which most markedly inhibited the incorporation of choline, are either components of the phospholipides or analogues of these components.

Two preliminary reports have been or will be presented at scientific meetings. It is believed that only a limited amount of additional experiments are required before these results can be organized in a complete paper.

E. Metabolic pathways of choline in liver slices.

In the experiments reported above the amounts of choline incorporated into the lipides represent only a small percentage of the labeled choline which actually disappears during the incubation with liver slices. Since choline was labeled in the methyl groups a study of other pathways for the metabolism of the methyl groups of choline under the conditions of our experiments became possible.

Little C^{14} was found as carbon dioxide, some occasionally as free formaldehyde, none as dimethylethanolamine. The last finding is in line with recent evidence, obtained in this and other laboratories, against the formation of dimethylethanolamine as a result of the transfer of the methyl groups of choline to other acceptors.

Quite detectable amounts of C^{14} were present as trimethylamine and especially as trimethylamine oxide. This would indicate that such compounds represent physiological products of the metabolism of choline in animal tissue, whereas the view most generally accepted is that the small amounts of trimethylamine oxide which are found in the urine originated from the activity of intestinal bacteria only.

The major portion of the choline which disappears during the incubation can be recovered from the aqueous extracts by a strong hydrolysis. These water-soluble combinations, in contrast to lecithin and glycerolphosphorylcholine, are quite resistant to a mild hydrolysis, thus resembling phosphorylcholine. Further work is necessary before the nature of these combinations can be established with certainty. At present, their formation appears to be the major pathway of choline metabolism in the isolated liver.

A preliminary report of these results has been published.

F. Synthesis of C^{14} methyl-labeled dimethylethanolamine, (a possible choline precursor).

As previously mentioned, dimethylethanolamine could be visualized as an intermediary product in the synthesis, or in the degradation of choline, or in both. At present, the formation of dimethylethanolamine by direct demethylation of choline is unlikely (see above). On the other hand, previous results from this laboratory indicated that dimethylethanolamine can be directly introduced in the molecule of phospholipides even before being completely methylated. It was thought that by the use of labeled dimethylethanolamine much information could be obtained on these points. Accordingly, the synthesis of a methyl labeled dimethylethanolamine has been undertaken. Mono-methylethanolamine has been converted into the dimethyl derivative by reaction with C^{14} labeled formaldehyde at an alkaline pH. The conditions for such a synthesis and for the separation of the product in a pure form have been studied carefully. The labeled product has been used in some preliminary experiments with surviving liver slices.

G. Disposal of formaldehyde in tissues.

The disappearance of formaldehyde in the presence of tissue homogenates has been studied. The most interesting finding was a considerable increase in the disappearance of the formaldehyde by the addition of pyruvic acid to the digestion mixture. The distribution of the enzyme responsible for this reaction, the conditions for its activity and the nature of the resulting products have been investigated. In several experiments C^{14}-labeled formaldehyde has been used and the isotope content of a fraction, obtained by precipitation with 2,4-dinitrophenylhydrazine was determined. While no definitive conclusions can still be made, it seems likely that a condensation product is later converted to the corresponding acylsin ("Pectol"). In view of the increasing evidence for the formation of one carbon compound in several processes of the intermediary metabolism, (including oxidation and synthesis of methyl groups), the significance of this reaction of formaldehyde with pyruvic acid may well be of a great importance in the general economy of living bodies. Two preliminary reports on these results have been prepared and presented at scientific meetings.

H. Bibliography.

Since March 16, 1950 the following papers based on the results obtained in the previous and present experiments of this project have been published:

1. "Studies on Dimethylethanolamine" by Camillo Artom and Marietta Crowder. Absts. of Commun. XVIII Intern. Physiol. Congr., Copenhagen, p.78 (1950).

2. "The Lipide Composition of the Large Granules (Mitochondria) From Rat Liver" by Marjorie A. Swanson and Camillo Artom, Journal of Biological Chemistry, Vol. 187, 281 (1950).

3. "Metabolism of Methyl-Labeled Choline in Surviving Liver Slices" by Camillo Artom and Marietta Crowder, Archives of Biochemistry, Vol. 29, 237 (1950).

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The following complete manuscript has been submitted for publication:

4. "Studies on the Formation of Phospholipides in Isolated Tissues. I. Incorporation of Inorganic P^{32} in Liver Slices From Rats on Various Diets" by Camillo Artom and Marjorie A. Swanson. (Submitted to the Journal of Biological Chemistry).

5. The results obtained from the investigation now in progress have also been the basis for a report on Phospholipide metabolism which was presented September 1950 at the Lipide Symposium of the Robert Gould Foundation, Rochester, N. Y. This report will be included in a monograph to be published by the Gould Foundation.

The following preliminary reports have been or will be on the program of scientific meetings:

6. "Enzymatic Disappearance of Formaldehyde in the Presence of Pyruvate" by Peggy Mitchell and Camillo Artom. N. C. Academy of Science, Salisbury, N. C. (May 1950); American Society of Biological Chemists, Cleveland, Ohio (April 1951).

7. "Incorporation of Labeled Choline in the Phospholipides of Isolated Liver Tissue" by Camillo Artom, Marietta Crowder, and Marjorie A. Swanson. Southeastern Section of the Society of Experimental Biology and Medicine, Winston-Salem, N. C., (January 1951); American Society Biological Chemists, Cleveland, Ohio (April 1951).

8. A paper on the "Mechanism of action of lipotropic factors" will also be presented at the Macy Conference on Liver Injury to be held May 1951 in New York City.

Camillo Artom

Camillo Artom, M. D.

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Summary of the work from March 16, 1950 through March 15, 1951 on the project described as

FORMATION OF PHOSPHOLIPIDES IN TISSUES

Choline is an essential dietary factor and a constituent part of certain types of phospholipides. The metabolism of choline labeled with radioactive carbon has been studied in isolated liver tissues. Part of the added choline is incorporated into the phospholipides. The influence exerted on this process by various factors such as the previous dietary regime of the animals, the supply of oxygen, the integrity of cell organization, etc., has been investigated. When the results of such a study are compared with those obtained in previous experiments on the incorporation of labeled phosphate into the phospholipides, it appears that the introduction of choline may occur, at least to a certain extent, independently from the synthesis of the remaining part of the phospholipide molecule.

In addition to its incorporation into the phospholipides, choline is metabolized by liver slices along various pathways. Among these, the production of trimethylamine and trimethylamine oxide and the synthesis of water-soluble combinations with a considerable degree of stability have been demonstrated.

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Progress Report of the work done on

TOXICITY OF P^{32} AS RELATED TO THE DIET AND TO THE FUNCTIONAL CAPACITY OF THE LIVER

sponsored by the Atomic Energy Commission under direct contract (AT-(40-1)-286, Title III with the Bowman Gray School of Medicine.

Principal Investigators: Dr. George T. Harrell, Jr., Dr. Camillo Artom, and Dr. David Cayer

The present report covers the work done from March 16, 1950 to March 15, 1951. This work is a direct continuation of that summarized in a previous report of March 15, 1950.

1. General plan of the experiments.

During the past years, the study of dietary factors which may protect the tissues against the injurious effects of the isotope has been continued actively. Since radioactive phosphate exchanges rapidly with the inorganic components of the bones, it seemed likely that the damage to the bone marrow and the consequent impairment of its blood cell forming capacity represents the chief factor for the toxicity of P^{32} (as well as of other radioisotopes which are also deposited preferentially in the skeleton). Accordingly, it was thought that a more or less marked protection against the toxic action of inorganic P^{32} could be obtained by either of the following mechanisms: a) an increase in the rate of mobilization of the isotopic phosphate from the bones; this mobilization should shorten the time of the exposure of the bone marrow to radiation and consequently decrease the severity of tissue damage; b) an increase in the recovery and regeneration of the tissue, initially damaged. In this respect, it seemed likely that the maintenance of an adequate supply of the factors involved in normal hemopoiesis may be of great importance.

2. Enrichment of the diets with phosphate or citrate.

In experiments which we have reported previously, the beneficial effects of an increase in the phosphate content of the diet have been noted. These results have been now extended and the interpretation that phosphate is mobilized and excreted further confirmed by the actual demonstration of an increased elimination of the isotope in the urine. Several other series of experiments have been made with the addition of considerable amounts of sodium citrate to the diets. It was hoped that by increasing the concentration of citrate in blood and tissue, (thus reducing Ca^{++}), the mobilization of both calcium and phosphorus from the bones would be enhanced. However, the data on both the mortality of the animals and the rate of excretion of the isotope have been quite inconsistent, and no statistically significant difference was observed between the experimental groups and the controls.

3. Effects of parathyroid hormone.

Since the administration of parathyroid hormone is said to mobilize calcium from the bone and increase the excretion of calcium and phosphorus in the urine, several groups of mice have been injected with parathyroid hormone shortly before

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and immediately after the introduction of P^{32}. The experiments are still in progress, and therefore no definite statement can be made on this point. It is our impression, however, that little or no protection is apparent against the effects of doses of P^{32} in the higher range of the LD_{50} dose. We are planning to continue this study on animals injected with smaller amounts of the isotope.

4. Effects of folic acid and vitamin B_{12} on the survival of mice injected with P^{32}.

Among the hemopoietic factors which might affect the recovery from radiation injury, folic acid and vitamin B_{12} seemed to be worth investigating. Some beneficial effects of folic acid on the survival of animals irradiated by x-rays have been described. However, neither folic acid, nor vitamin B_{12} appeared to modify the hematological changes following total body irradiation. In preliminary experiments (mentioned in our last year's report), the addition of large amounts of folic acid or vitamin B_{12} to the diets did not give any significant protection against the toxic effects of lethal doses of P^{32}. During the past months, the investigation was continued with the following changes in the disposition of the experiments: a) the dose of P^{32} injected has been decreased to the lower range of the LD_{50} dose, and the length of the experiments increased from 3 to 8 weeks, so that the effects of the vitamins on the recovery of the animals (rather than on the actual damage to the tissue) could be studied; b) since large amounts of vitamins are probably synthesized by the intestinal flora, sulfasuxidine was added to the diets of the animals in an attempt to reduce this source of vitamins. Thus, the effects of the isotope on vitamin-deficient animals could be directly compared with those observed in animals receiving adequate amounts of the preformed vitamins in the diet; c) since several observations point to a synergistic action of mixtures of various vitamins, especially of vitamin B_{12} and folic acid, the isolated and combined administration of these and other vitamins has been tested. A total of 525 mice has been used in these experiments. In the following table we are summarizing the results of experiments in which the action of folic (FA), vitamin B_{12}, biotin (Bio), and vitamin K have been tested in mice on sulfasuxidine-containing diets.

Table I

Effects of Certain Vitamins on the Survival of Mice Injected with P^{32} ($5 \mu c/g$) and Maintained on a Sulfasuxidine-Containing Diet*

Supplements	No. of Mice	Avg food Consumption g/day	Avg change in body wt. g	Time of 50% deaths days	% of survivors 21st day	% of survivors 56th day	Avg time of survival † days
None	73	3.5	- 1.6	27	58	16	29 ± 2.0
FA	39	4.1	+ 5.0	20	49	31	30 ± 3.3
B_{12}	24	3.7	- 3.8	34	58	46	36 ± 4.0
FA + B_{12}	34	4.1	- 0.1	56	76	56	42 ± 3.5
FA, B_{12}, K, Bio	67	4.0	+ 1.1	54	72	49	41 ± 2.5
None# (controls)	18	4.1	- 6.7	56	100	83	55 ± 0.8

Table I (continued)

* Diet No. 34 (1) containing 5% sulfasuxidine. A basal B vitamin mixture was incorporated daily in the diet.

† In the calculation of the avg. time of survival, a survival time of 56 days was ascribed to the mice still alive at the end of the experiments. Values preceded by ± are the standard errors of the means.

†† Not injected with P^{32}.

It should be pointed out that when the results of the control groups are compared with our previous data on the toxicity of P^{32} in mice on diets without sulfasuxidine, it is apparent that the inclusion of sulfasuxidine in the diet does not increase the susceptibility of the animals to the injurious action of the isotope. From the previous and present experiments, the following conclusions can be drawn: a) The addition of generous amounts of folic acid, or folic acid and vitamin B_{12} to diets without sulfasuxidine had no significant effect on the survival of mice injected with a dose of P^{32} in the higher range of the LD_{50} (21 days); b) Supplementation of the sulfasuxidine-containing diet with folic acid improved slightly the survival figures. Vitamin B_{12} alone was more effective. When, however, both vitamin B_{12} and folic acid were added to the diet, there was a significant improvement in the survival of the animals; c) The further addition of biotin and of a synthetic vitamin K-like product did not enhance the protection exerted by the mixture of folic acid and vitamin B_{12}.

The results of these experiments will be reported at the meeting of the Southeastern Section of the Society for Experimental Biology and Medicine to be held this coming April at Oak Ridge, Tenn. A complete manuscript has been submitted for publication in the Proceedings of the Society for Experimental Biology and Medicine.

5. Effects of folic acid and vitamin B_{12} on the hematological changes following the introduction of P^{32}.

These experiments were carried out with the purpose of studying if and to what extent the beneficial effects of vitamin B_{12} and folic acid on the survival of mice injected with P^{32} could be ascribed to a more rapid or complete recovery from the hematological changes caused by the isotope. In a number of experiments, several groups of animals were maintained on the same diets employed in the experiments of the preceding section. One animal in each group was sacrificed at regular intervals after the introduction of P^{32}, so that sufficient blood could be obtained for determinations of hemoglobin, red blood cells, white blood cells and hematocrit. After an initial fall, the hemoglobin, red cell, and white cell values rose progressively in all groups of animals. However, between the 12th and the 36th day after the administration of P^{32}, all these values in the blood of mice receiving the mixture of folic acid and vitamin B_{12} were consistently higher than in the blood of the other groups.

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While the differences are perhaps not quite statistically significant, the data may be taken as at least suggestive of a relationship between the improved hematological picture and the prolonged survival of mice receiving the mixture of folic acid and vitamin B_{12}.

6. Other experiments.

Preliminary series of experiments have also been carried out in order to explore the possible protection exerted by other factors. Some of these factors, such as the administration of cysteine, have been claimed to be effective in the protection against total body irradiation. To date, our results on mice injected with P^{32} and cysteine are inconclusive. The affects of adding to the diets sodium bicarbonate (to make the diet alkalosic), or ammonium chloride (to make the diet acidosic) are being tested, but much more work is required before definite statements on this point can be made.

Camillo Artom

Camillo Artom, M. D.

David Cayer

David Cayer, M. D.

George T. Harrell, Jr.

George T. Harrell, Jr., M.

Summary of the work from March 16, 1950 through March 15, 1951 on the project described as

TOXICITY OF P^{32} AS RELATED TO THE DIETS AND TO THE FUNCTIONAL CAPACITY OF THE LIVER

The influence of a number of dietary factors on the toxicity of P^{32} injected into mice as sodium phosphate has been studied. In an investigation of the importance of an adequate supply of B vitamins, sulfasuxidine has been included in the diet in order to reduce the synthesis of the vitamins by the microorganisms in the intestine. From a comparison between the survival of mice thus made vitamin-deficient and the survival of mice on the same diet supplemented with the vitamins, it appears that the combined administration of vitamin B_{12} and folic acid affords a quite significant protection against the injurious action of the radioisotope. It is suggested that such a protection is possibly due to the enhanced recovery and regeneration of the blood forming elements in the bone marrow.

1112314

Progress Report of the work done from July 1, 1949 to March 15, 1950 on the research project described as

TOXICITY OF P^{32} AS RELATED TO THE FUNCTIONAL CAPACITY OF THE LIVER

sponsored by the Atomic Energy Commission under direct contract AT-(40-1)-288, Title III, with the Bowman Gray School of Medicine.

Principal Investigators: Dr. Camillo Artom, Dr. G.T. Harrell, Jr., and Dr. David Cayer.

According to the original plan of the investigation, the acute toxicity of P^{32} has been studied in relation to the possibility that changes in the functional capacity of the liver may modify the susceptibility of the animals toward the radiation effects. In the first series of experiments the role of a dietary deficiency of lipotropic factors and of a partial damage of the liver by poisons such as CCl_4 has been studied. While the results seemed to miminize such a role in the acute toxic effects of the isotope, they indicated definite differences in relation to the composition of the diets. Accordingly, the effects of quantitative variations in several dietary components have been investigated with results which seem of a certain interest.

The general disposition of the experiments, the points investigated, the results obtained and the plans for future experiments are summarized below:

1. General plan of the experiments. Mice of approximately 20 grams were transferred from a stock diet to experimental diets containing various amounts of protein (casein), fat, minerals, and vitamins. After 5 days on the experimental diets, the mice were injected intraperitoneally with a single dose of Na_2HPO_4 containing P^{32} (mostly 6 microcuries per g. of body weight). Food consumption and changes in weight were recorded and the mice maintained on the diets for three weeks or more after the injection of P^{32}. As an indication of the effects of the isotopes, the following criteria were used: a) Time in days at which 50% deaths occurred; b) Percent of survivors at the 21st day; and c) Average time of survival. The results have been evaluated statistically using the χ^2 and the t tests of significance. A total of more than 650 mice have been used in the experiments summarized below.

2. Determination of the LD_{50} dose. The LD_{50} dose was determined in experiments of 21 days duration, using 90 mice maintained on a 10% protein, 32% fat diet. In these conditions the LD_{50} dose lies between 4 and 6 microcuries per gram of body weight.

3. Fatty infiltration of the liver, CCl_4 poisoning and lipotropic factors in the diet. The results obtained in 35 mice on a low protein, high fat diet (which is deficient in lipotropic factors and causes an extensive fatty infiltration of the liver) were compared with those obtained in 43 mice, maintained on the same diet supplemented with generous amounts of choline. In another series of 30 mice, prior to introduction of P^{32} the liver was partially damaged by 2 or 3 subcutaneous injections of CCl_4. Part of these animals were then maintained on the deficient diet and others on the same diet supplemented with choline. However, no significant difference was detected between the groups on either the choline deficient or the choline supplemented diet, whether or not the liver had been previously damaged by CCl_4.

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4. Variations in the level of dietary fat and protein. 280 mice were maintained on 4 different diets, respectively low in protein and fat, high in protein and low in fat, low in protein and high in fat, high in both protein and fat. The results clearly show that the susceptibility of the animals to the injurious action of P^{32} (6 microcuries per gram) increased distinctly with the increase in the level of protein, or fat, or both. Thus, with a dose of 6 microcuries of P^{32} per gram of body weight, the mortality of the mice on the high fat, high protein diet was 97% with 50% deaths at the 12th day, whereas in the mice on the low fat, low protein diet, the mortality at the 21st day was 60% with 50% deaths at the 16th day. It is tentatively suggested that the susceptibility to radiation injury parallels the rate of metabolism, and that the specific dynamic action of the dietary components (especially protein) may partly explain the results obtained.

5. Variations in the phosphate content of the diets. It was thought possible that by increasing the amount of phosphate in the diets, the mobilization of the P^{32}, initially deposited in the bone, would be enhanced and, consequently, the effective time of exposure to the isotope of the tissues (especially the bone marrow) would be shortened. Three series of experiments were made on a total of 167 mice, injected with 6 microcuries per gram of body weight. The results show a significantly decreased susceptibility in the mice on the phosphate enriched diets. Thus, in a group of mice on a diet high in phosphate (2.9% as H_3PO_4) a mortality of 39% at the 21st day was observed as compared with a mortality of 89% in the mice on a low phosphate diet (0.3% as H_3PO_4): the times of 50% deaths in these two groups were 23 and 14 days, respectively.

We have now in progress some additional experiments in which we are studying the rate of elimination of P^{32} in rats on diets with a low, medium and high phosphate content. The results obtained thus far seem to be in line with the data on the toxicity and therefore corroborate the interpretation we have suggested. Some of this data are reported here.

% Phosphate in Diet as H_3PO_4)	% of P^{32} eliminated in the urine 1st day	2 - 3 days	4 - 5 days
0.3	8.3	4.6	3.2
1.3	20.3	9.7	6.4
2.9	27.1	10.3	6.8

Additional experiments will soon be started, in which the action of other factors which might enhance the excretion of phosphate (such as parathyroid hormone, citrates, etc.) will be investigated.

6. Variations in the supply of certain vitamins. A protective action against external radiation by enriching the diet with folic acid has been described. We have reinvestigated this point on 160 mice injected with P^{32} and kept on various diets with or without additions of folic acid. The results thus far have been of doubtful significance. We are however, continuing our experiment with diets containing sulfasuxidine in an attempt to inhibit the bacterial flora in the intestine, which is an important source of several vitamins, including folic acid.

7. Future experiments. It is planned to use somewhat smaller doses of radioactivity and to keep the animals under experiment for longer periods. Indeed, it seems likely that in experiments of acute or subacute toxicity, the percentage and time of survival

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are primarily influenced by factors which might enhance or attenuate the actual damage to the tissues (as for instance, an increased phosphate content of the diet). Factors (such as the partial damage of the liver, or an increased supply of certain vitamins) may act, more probably, by impairing or favoring, directly or indirectly, the recovery of the damaged tissues. The action of such factors may not be detectable in experiments of short duration and with high doses of the isotope, while their role may become apparent in studies on the chronic effects of radiations.

The results summarized above have been reported or are on the programs of the following scientific meetings:

Southeastern Section of the Society of Experimental Biology and Medicine, Durham, N. C., January 1950.

Southern Society for Clinical Research, New Orleans, La., March 1950.

Federation of the American Biological Societies, Atlantic City, N. J., April 1950.

A more extended paper, "Subacute Toxicity of Radioactive Phosphorus as Related to the Composition of the Diet", by W. E. Cornatzer, G. T. Harrell, Jr., David Cayer, and Camillo Artom has been accepted for publication in the Proceedings of the Society for Experimental Biology and Medicine.

G. T. Harrell, Jr., M. D.

David Cayer

David Cayer, M. D.

Camillo Artom

Camillo Artom, M. D.

Summary of the work done from July 1, 1949 to March 1[illegible], 1950 on the project described as

THE EFFECT OF P^{32} AS RELATED TO THE NUTRITIONAL CAPACITY OF THE LIVER

sponsored by the Atomic Energy Commission under direct contract AT-(40-1)-288, Title III, with the Bowman Gray School of Medicine.

Principal Investigators: Dr. Camillo Artom, Dr. G.T. Harrell, Jr. and Dr. David Cayer.

In mice maintained on a 10% casein, 32% fat diet and injected intraperitoneally with a single dose of P^{32} as Na_2HPO_4, the LD_{50} dose at the 21st day lies between 4 and 6 microcuries per gram of body weight.

Neither the fatty infiltration of the liver (induced by a deficiency of lipotropic factors, with or without partial damage of the organ by poisoning with CCl_4), or its prevention (by supplementing the diet with choline) changes the survival figures.

The time of 50% deaths, the percentage of survival at the 21st day and the average survival time of mice injected with P^{32} are highest with a diet low in fat and in protein, and are significantly decreased when the level of the fat, or of the protein, or both, are increased in the diet.

Enrichment of the diets with inorganic phosphate seems to afford a significant degree of protection against the injurious action of P^{32}.

G. T. Harrell, Jr., M. D.

David Cayer

David Cayer, M. D.

Camillo Artom

Camillo Artom, M. D.

1112318

Progress report o. a. research project

FORMATION OF PHOSPHOLIPIDES IN TISSUES

sponsored by the Atomic Energy Commission, Division of Biology and Medicine under contract No. AT-(40-1)-288, Title II, with the Bowman Gray School of Medicine of Wake Forest College.

Project leader: Dr. Camillo Artom

The present report covers the work done from July 1, 1949 through March 15, 1950. This work is a continuation of previous work which was summarized in the final report to the Office of Naval Research. Copy of this report, dated September 1, 1949 was sent to the Division of Biology and Medicine of the Atomic Energy Commission.

A. Formation of phospholipides in the liver of intact animals.

As in previous experiments, the formation of phospholipides has been studied in the liver of animals killed at a given time after the administration of isotopic inorganic phosphate. Additional experiments were made in the past month using rats of various age groups and maintained on diets with various protein levels. To some of these diets guanidoacetic acid or diethanolamine was added, in an attempt to further inhibit the synthesis of certain phospholipide fractions. Analytical and isotopic determinations of both inorganic and lipide phosphorus were made in the liver. On the basis of the large number of data obtained in these and previous experiments, it became possible to compare the various methods of expressing the rates of formation of phospholipides in the liver ("percentage of the isotopic dose injected", specific activity", "relative specific activity","total radioactivity/specific activity of the inorganic phosphorus"). The statistical elaboration of the data has not yet been completed. However, it seems likely that the values calculated by the last method closely represent the amounts of phospholipi. synthesized in a given time by the liver of the animals. When such amounts are referred to the same weight of animal, the values are remarkably constant, in spite of considerable variations in the age and diet of the animals, and even when functional and probably also anatomical alterations are detectable in the liver. Accordingly, on the basis of previous and present findings the following general concepts might be suggested: a) The synthesis of phospholipides proceeds unimpaired, even when the dietary supply of their N-containing precursors is considerably reduced and other deficiency symptoms (such as fatty infiltration of the liver) are visible. Apparently the formation of phospholipides in the liver takes a high degree of priority in the processes of the intermediary metabolism, including those involving the transfer or synthesis of methyl groups; b) The rate of phospholipide synthesis in the liver is not considerably affected by conditions which presumably reduce the number of the active cells or their functional capacity; and c) Since in intact animals the synthesis of phospholipides proceeds to a rate not far from normal even under very adverse conditions, one should assume that the liver has a very extensive functional reserve for this process.

It is expected that more time will be required for the complete elaboration of the data, before a complete paper on the subject can be published.

B. Formation of phospholipides in surviving slices of the liver.

Previous experiments in which the synthesis of phospholipides has been studied in surviving slices of the liver of rats under various nutritional conditions have been continued. In some experiments the total phospholipides have been fractionated into choline-and non-choline-containing and the formation of these two groups of phospholipides followed comparatively. The amounts of isotopic phospholipides synthesized by one gram of tissue during a 4 hour period of incubation with radioactive phosphate

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has been taken as a basis for comparison. Results of the more recent experiments show that a fasting even of short duration, causes a detectable decrease in phospholipide formation. The finding of a much more marked decrease in this process, when the animals had been previously maintained on diets low in protein, has been confirmed. The recent data also further corroborate the concept that this decrease is due to the low intake of dietary proteins rather than to a deficient supply of choline or of its precursors. No difference was noted in the rate of formation of choline- or non-choline-containing phospholipides by the isolated tissue.

The results of the experiments with tissue slices may be compared with those of the experiments in intact animal. As mentioned above, the formation of phospholipides in the liver of intact animals is independent to a large extent from the composition of the diet, whereas phospholipide synthesis in the isolated tissue is greatly affected by the nutritional state of the animal. It is tentatively suggested that in the isolated tissue the phospholipide synthesis occurs at a maximum speed, whereas in the intact animal, regulatory mechanisms are operating to keep the rate of phospholipide formation at a level adequate to the needs of the body, such a level being in ordinary conditions much below the maximum functional capacity of the tissue.

The action of various substances (choline, ethanolamine, betaine) added in vitro to the surviving slices of liver has been also studied further and most of our previous findings have been confirmed. Further experiments are in progress, in which other substances (e.g. diethanolamine, thiourea, etc.) are added in vitro to the isolated tissue.

A preliminary report of the experiments on the isolated liver tissue will be presented next April at the meetings of the Federation of the American Biological Societies in Atlantic City, N. J.

C. Lipide composition of liver mitochondria.

Under proper conditions the synthesis of phospholipides does occur even with liver homogenates. When these homogenates are fractionated by differential centrifugation, the large granules fraction (resulting essentially of mitochondria) appears very active in this respect. Since a large proportion of lipides is present in the mitochondria it was thought worthwhile to make a detailed study of the lipide composition of these structures.

Six preparations of "large granules" separated from rat liver by differential centrifugation, have been analyzed. Complete extraction of the lipides seems to be difficult. Total lipides extracted from the trichloroacetic precipitate averaged 24% of the dry weight. The remaining part consisted essentially of proteins and nucleoprotides and a smaller proportion of substances soluble in trichloroacetic acid. The average composition of the lipides was as follows: 78% phospholipides, 4.5% cholesterol (half or more of it, as free cholesterol), 18% of neutral fat and other lipides. The tentative distribution of the individual phospholipides was: lecithin 43%, sphingomyelin 7%, non-choline-containing phospholipides 50%. Most of these were phosphatidyl ethanolamine, but a non-negligible fraction could not be justified as ethanolamine- or serine-containing phospholipides. With the exception of one preparation, very little excess of N over P was found in the lipide extracts. However, the possibility of an incomplete extraction of cerebrosides, if present, can not be dismissed. In conclusion, mitochondria have a characteristically high proportion of lipides, especially phospholipides, but their composition does not appear to be essentially different from that of the lipides extracted from the whole unfractionated liver.

The results of this investigation were reported at the January meeting of the Southeastern Section of the Society for Experimental Biology and Medicine, Durham, N. C.

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In the future experiments it is planned to study in more detail the *in vitro* synthesis of phospholipides under the action of mitochondria from the liver of rats in various nutritional states.

D. Incorporation of C^{14} labeled choline into the phospholipides.

These experiments have been hampered by difficulties in the synthesis of isotopic choline. Dr. Arthur Roe of the University of North Carolina has now developed for us a method of synthesis which seems both convenient and efficient. We are about to use the synthesized material in experiments on isolated liver slices. We are planning to run also parallel experiments with other labeled compounds which are, or may be intermediary terms in the formation or degradation of choline (such as betaine, dimethylglycine, and dimethylethanolamine). At our suggestion, Dr. Roe is studying methods for the synthesis of these compounds. It is hoped that in the course of our experiments some indications will also be obtained concerning the mechanism of other biological methylations, besides those directly involved in the synthesis of choline.

The following papers based on the results obtained in the previous and present experiments of this project have been published:

THE ACTION OF AN ANALOGUE OF ETHANOLAMINE (DIETHANOLAMINE) ON THE FORMATION OF LIVER PHOSPHOLIPIDES, Journal of Biological Chemistry, Vol. 180, No. 2, Page 495, September, 1949. (Complete report).

LIPIDE PHOSPHORYLATION IN THE LIVER AS RELATED TO THE DIETARY SUPPLY OF METHYL DONORS AND METHYL ACCEPTORS, Abstracts of the First International Congress of Biochemistry, Cambridge, England, August 1949, Page 22; Journal of the Elisha Mitchell Scientific Society, Vol. 65, No. 2, December 1949. (Preliminary report).

LIPIDE PHOSPHORYLATION IN THE LIVER OF RATS FED THIOUREA AND RELATED COMPOUNDS, Journal of the Elisha Mitchell Scientific Society, Vol. 65, No. 2, December 1949. (Preliminary report).

The following preliminary reports have been or are on the program of scientific meetings:

THE LIPIDE COMPOSITION OF THE MITOCHONDRIA FROM RAT LIVER, Society of Experimental Biology and Medicine, Southeastern Section, Durham, N. C., January 1950.

LIPIDE PHOSPHORYLATION BY SURVIVING SLICES OF THE LIVER OF RATS ON VARIOUS DIETS, Federation of the American Biological Societies, Atlantic City, N. J., April 1950.

Camillo Artom

Camillo Artom, M. D.

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Summary of the work from July 1, 1949 through March 15, 1950 on the project described as

FORMATION OF PHOSPHOLIPIDES IN TISSUES

sponsored by the Atomic Energy Commission, Division of Biology and Medicine under contract No. AT-(40-1)-288, Title II, with the Bowman Gray School of Medicine of Wake Forest College.

Project leader: Dr. Camillo Artom

In intact animals, the formation of phospholipides proceeds unimpaired even under very adverse conditions, such as a dietary deficiency of choline or choline precursors. This suggests that phospholipide formation in the liver takes a high priority in the processes of the intermediary metabolism. On the other hand, in isolated liver slices phospholipide synthesis is closely dependent upon the previous dietary regime and is reduced by a decreased intake of dietary proteins. It is suggested that in the isolated liver the formation of phospholipides proceeds at a maximum rate, whereas in the intact animals this process is kept at a level adequate to the needs of the animal, such a level being, in ordinary conditions, much below the functional capacity of the tissue.

A study of the synthesis of phospholipides by isolated liver mitochondria has been started. As a preparatory step for such a study, a detailed analysis of the lipide composition of mitochondria has been made.

Camillo Artom

Camillo Artom, M. D.

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PROGRESS REPORT

Work performed under AEC Contract No. AT-(40-1)- Title I.
The Bowman Gray School of Medicine of Wake Forest College.

Distribution and turnover of sodium and potassium in acute infections.

Dr. George E. Harrell, Jr., project leader July 1, 1949 to March 15, 1950

Facilities: The actual construction of the Isotope Laboratory on the fifth floor of the Medical School, built in part with funds from the preceding contract with ONR, was completed and the lab turned over to us June 20, 1949. It was discovered that the weight of the fume hood, delivered about June 30, was greater than the supporting girders of the old roof of the medical school could support and hence the floor plan of the laboratory had to be revised after construction was completed in order to relocate the hood over a major structural girder. This relocation involved moving of a wall and installation of additional shielding in the form of boiler plates. These alterations were completed and the remainder of the equipment purchased under this year's grant was installed by September 15, 1949. Shipments of sodium 24 were begun September 26, 1949.

Personnel: Dr. Jerry K. Aikawa, one of the AEC fellows assigned to us, has been working full time on the project since the completion of his training course at Duke.

Miss Eloise Rhoades, a technician, began work May 1, 1949, but had to be trained from scratch in techniques being utilized. She and Dr. Aikawa were sent to the Oak Ridge Institute of Nuclear Studies in March, 1950, for a short period of observation in refinement of techniques.

It has been impossible to find another satisfactory girl to add as a second technician to expedite the experiments. It was decided that much of the work now being done by Miss Rhoades could be better done by a man who would be primarily responsible for the handling of animals. Accordingly, Claude Taylor - a negro college graduate who has served as a farm agent, has been hired to begin work April 1, 1950. The school is underwriting from other funds any deficit in the salary of this negro technician which may be incurred in the three months remaining under the present grant.

Mr. Ernest Yount has assisted part time, chiefly in the design of experiments, monitoring, health physics, and in the few human experiments - which often have not been directly related to the primary research project - simultaneously performed.

Dr. Kingsley Stevens, our second AEC Fellow, transferred to Chicago in September 1949, just as he was becoming useful to us.

General plan of experiments

July to September 1949: In order to standardize techniques and to train the technician, determinations of plasma volume and thiocyanate space were done in rabbits in which serum sickness had been induced 1) by injection of human plasma fractions and 2) by passive transfer techniques utilizing immune serum previously prepared in other rabbits. During this interval, the flame photometer,

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spectrophotometer, and the counters were installed and standardized but no isotopes were employed.

September to November 1949: Similar experiments in rabbits with serum sickness and with passive transfer techniques were repeated and observations made on the sodium 24 space. Tissue analyses were done for sodium 24 and total sodium in an attempt to localize the area in which the fluid and electrolyte changes were taking place.

November 1949 to February 1950: The variation in the results in the experimental animals in the preceding groups necessitated determination of normal control values for rabbits. No such data were available in the literature.

March 1950: Preliminary experiments were begun utilizing the above techniques with a pneumococcal skin infection ("dermal pneumonia").

As suitable cases were available on the ward, the facilities and personnel of the isotope laboratory were utilized. No systematic attempt was made to study infectious disease in human beings at this time. Scattered observations have been made using I^{131} in thyroid disease and Na^{24} in Addison's and other problems in doses authorized for peripheral vascular studies here by Dr. Harold Green of the Department of Physiology.

Results

The results in repeat serum sickness in experimental rabbits were more variable than similar experiments performed the preceding year. In general an increase in the thiocyanate space at the time of the development of precipitins was observed. In the passive transfer experiments, simultaneous injection into previously unsensitized rabbits of antigen and antibody, prepared in another rabbit against that same antigen, was found six and 24 hours later to elevate significantly the thiocyanate space, which presumably measures extracellular fluid. Such changes were not found when either antigen or antibody alone was injected into control animals. The changes were interpreted as indicating an abnormal increase in membrane permeability directly or indirectly attributable to the immunologic reaction. No significant changes were found in the blood volume in either group .

Utilizing the radiosodium technique, rabbits were passively sensitized with anti-human gamma globulin and were actively sensitized with 10 cc. per kilogram of human plasma intravenously. The increase in the radiosodium space over the base line values was only about 1 to 5 per cent of the total body weight; the errors in the technique were in a comparable range, hence it was not possible to draw a definite conclusion from the study.

Statistically accurate data were obtained in over 50 normal rabbits for plasma volume, hematocrit, thiocyanate space, radiosodium space, serum sodium and serum potassium concentration.

Tissue analyses for radiosodium space were obtained on six control animals.

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In the preliminary pneumococcal experiments, satisfactory local edema has been produced, but in the present stage of the experiments bacteremia is developing too rapidly. It is hoped that a better experiment can be designed which will permit longer survival with slower invasion of the blood stream by the organism so that more marked edema and greater alteration in permeability will develop.

Critique of the experiments

Considerable time was lost due to the vagaries of air transport. In the animals with serum sickness it is necessary to start injections 10 days before the expected arrival of the shipment of radioisotope. Due to the weather, several shipments did not arrive on schedule. Because of the short half-life of the sodium and the fact that the animals had passed the peak of the immune response by the time the shipment could be retrieved by surface transportation, at least four weeks' work was completely futile. These very disheartening experiences led us to change to surface transportation entirely and to arrange with the Department of Physiology and the Isotopes Division for joint use of each shipment. The isotope is now arriving weekly rather than bi-weekly and is coming by truck with much greater regularity in the time of arrival.

We encountered great variability in the response of animals this year; in preceding years, the variability has not been so marked. Review of the data indicate that the age, weight, and breed of the animals are all factors which affect their response in our type of experiments. trip was made to Oak Ridge to consult with Dr. Jacob Furth of the Biology Division, Oak Ridge National Laboratories, in an attempt to devise means for reducing these variables. We discovered that Dr. Furth has encountered much the same problem and had arrived at the same ultimate solution as we. It would appear that we must plan in the future to raise our own animals from an inbred strain utilizing groups of the same age and weight. The negro animal technician [illegible] hired with this end in view, but it may be a year before the full benefits of the animal breeding program can be achieved. It is not clear how much additional expense this program may incur and how it can be borne.

Guy T. Hamilton

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SUMMARY OF PROJECT

Distribution and Turnover of Sodium and Potassium in Acute Infections

Contract No. AT-(40-1)-Title I

Patients recovering from infections or serum sickness have a diuresis during recovery; water and chloride are lost. In the acute phase of Rocky Mountain spotted fever and trichinosis, edema develops and the blood chlorides and proteins drop; no data are available on sodium and potassium.

It is not known from blood volume and thiocyanate studies whether the extravasated fluid is found between cells or within them. The increase in permeability can be visualized as a graded series of pores. In mild disease only crystalloids leave the blood stream; in more severe instances small protein molecules such as albumin, or larger ones such as gamma globulin will leak out. A small defect should be detectable by radioactive ions, a larger one by tagged protein molecules. An antigen-antibody reaction may be responsible for the defect.

If the mechanism by which increased amounts of electrolytes are passed across capillary and cell membranes can be established, it may be possible to correct the physiologic abnormality. Eventually it may be possible to induce an increase in permeability so that radioisotopes could enter certain tissues and exert a radiation effect.

Some tumors may be caused by filterable viruses; others can concentrate viruses such as vaccinia. If a virus could be introduced into a cancer and immune serum administered, an antigen-antibody reaction should take place only on parasitized cells and circulating radioisotope be concentrated in cancerous tissue. If, through application of the auto-antibody technique, an antiserum against cancerous tissue can be made, a similar reaction would take place and only on malignant cells.

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ALTERATIONS IN THE PERMEABILITY OF MEMBRANES DURING INFECTIONS

George T. Harrell Jr., M.D.
and
Jerry K. Aikawa, M.D., Winston-Salem, N. C.

The underlying physiological disturbances responsible for death during the course of acute infections remain a mystery. Thousands of years ago men began to record descriptions of febrile illnesses. A particular type of rash might be accompanied by a slow or fast pulse and by a steady or remitting type of fever, which would run its course within a reasonably constant period of time and would be followed by a period of convalescence. On the basis of clinical observations, various specific diseases were separated from one another—the small from the great pox, the typhuslike, or typhoid, fever from typhus. In the course of some acute infections the appearance of edema was noted; in others edema was not visible, but diuresis occurred during recovery—an observation which suggested that fluid was retained in the tissues, whether it was obvious or not. With the introduction of immunologic techniques, it was recognized that the patient would react to the infecting agent with demonstrable immune responses, which could be used for confirmation of the diagnosis or for prognosis.

In addition to the observations on acute generalized diseases, excellent clinical descriptions of different types

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Atomic Energy Commission Fellow in the Medical Sciences of the National Research Council, 1948 to 1950 (Dr. Aikawa).

From the Department of Internal Medicine, The Bowman Gray School of Medicine, Wake Forest College, and the North Carolina Baptist Hospital.

Read before the Section on Experimental Medicine and Therapeutics at the Ninety-Ninth Annual Session of the American Medical Association, San Francisco, June 29, 1950.

These studies were aided (in part) by research grants from the John and Mary R. Markle Foundation; the National Heart Institute of the National Institutes of Health, Public Health Service, and the Atomic Energy Commission under a contract with the Bowman Gray School of Medicine.

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of local infections in wounds or the skin were recorded. Lesions characterized by a thin serous exudate, or by uniform red swelling having a sharp and rapidly spreading raised edge in the skin (erysipelas), were contrasted with those having a thick purulent exudate or with more localized swelling which remained circumscribed (a boil or carbuncle). It was recognized that the redness and heat of the inflammation were the result of a local increase in blood flow. The swelling was attributed to a loss of fluid from the blood into the tissues. The pain was ascribed to stimulation of nerve endings—the result of an increase in the tension of tissues, produced by the accumulation of fluid. If the local infection were adjacent to a serous cavity, fluid might accumulate in that space; in tuberculosis, for instance, a pleural effusion might develop early. Because of its higher protein content, the exudate produced by infection was recognized to clot more readily than fluid accumulating as the result of mechanical factors, such as heart failure or venous and lymphatic obstruction.

On the supposition that the body's reaction to generalized infectious diseases might follow the same general principles involved in its response to localized infections, we began to make observations in various specific fevers. Any alteration in the permeability of membranes occurring during the course of an infection should affect the fluids in the entire vascular tree, as well as their local distribution in the affected tissues. Changes in the general circulation would explain the clinical observations that a weak pulse, faint heart sounds, and cyanosis frequently precede death during infectious diseases.[1] Direct toxic myocardial damage (central circulatory failure) might then be differentiated at the bedside from peripheral collapse (peripheral circulatory failure) resulting from toxemic alterations in the permeability of membranes.

1. Harrell, G. T.: Disturbances of the Circulation in Acute Infections, North Carolina M. J. 8: 559-563 (Sept.) 1947.

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ALTERATIONS IN PERMEABILITY ASSOCIATED WITH SPECIFIC DISEASES

Alterations Occurring Within Minutes to Hours.—Meningococcemia: In meningococcemia the integrity of membranes may be altered within a matter of minutes or hours. The characteristic hemorrhagic rash is due to the rupture of capillaries by groups of organisms. A loss of capillary tone may lead rapidly to a picture of peripheral circulatory collapse, without appreciable alteration in the permeability of membranes or without visible edema. Such a sequence of events occurred in the case of a girl, 17, who was admitted to the hospital with a typical rash (Fig. 1) and with circulatory failure. The

Fig. 1.—The hemorrhagic rash of meningococcemia on the third day of symptoms in a girl, 17. Note the edema of the fingers and back of the hand after the correction of dehydration with isotonic sodium chloride solution, which demonstrates an increase in the permeability of membranes due to toxemia. The course of the patient is shown in Figure 2.

simple administration of isotonic sodium chloride solution to correct dehydration and increase the circulating blood volume was all that was required to support the circulation (Fig. 2). The acute symptoms of infection were controlled with chemotherapy.

In these mild cases of meningococcemia, slight edema may be evident after several days; in this patient subcutaneous pitting edema developed, and the plasma proteins were found to be low. Plasma was then administered to correct the hypoproteinemia. This therapeutic measure apparently increased the circulating blood volume to such an extent (once the alteration in the permeability of membranes was corrected) that an esophageal

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varix (the result of a pre-existing Banti's syndrome) ruptured, and hemorrhagic shock occurred. Whole blood was given to replenish that lost from the circulation. This time the increase in blood volume produced central circulatory failure, which led to a fatal pulmonary infarction.

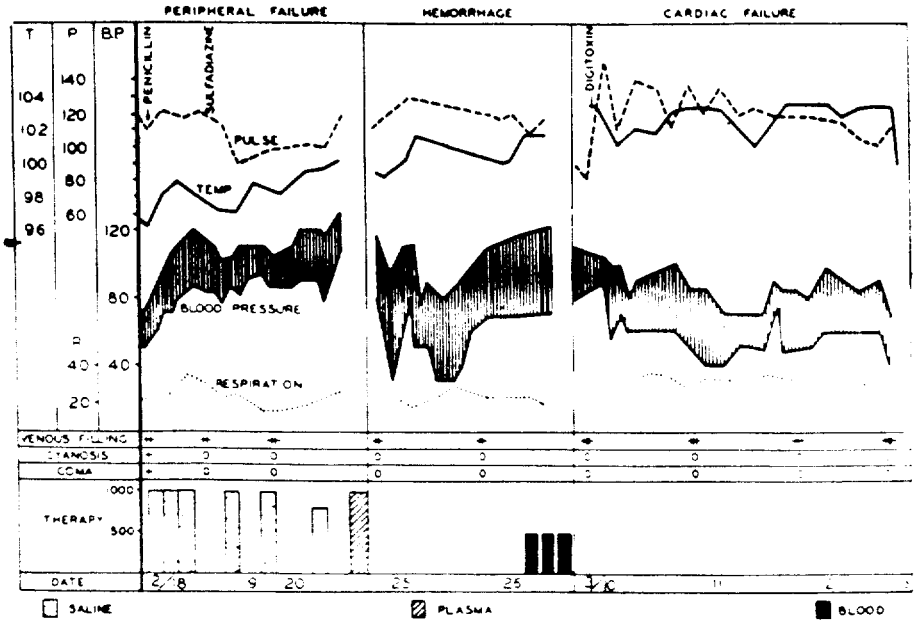

Fig. 2.—Three different types of circulatory failure occurring successively in a girl, 17, with meningococcemia with meningitis, circulatory failure, and pre-existing Banti's syndrome. Note the correction of the peripheral circulatory failure by therapy with isotonic sodium chloride solution alone and the prompt response to chemotherapy. The skin rash and edema visible in this patient on the second hospital day are shown in Figure 1. No appreciable change in the permeability of membranes occurred with the hemorrhage. Pulmonary and peripheral edema occupied the central myocardial failure. An adrenal gland removed at necropsy is shown in Figure 4.

In the hyperacute form of the disease, circulatory failure develops too rapidly for edema to become visible. Such was the case with an elderly shoemaker who entered the hospital with peripheral circulatory failure so severe that it had produced collapse of the neck and arm veins, as well as a fall in the arterial blood pressure. Because of the severity of the vascular collapse and the presence of deep cyanosis and coma, bilateral adrenal hemorrhage was suspected. Isotonic sodium chloride solution was given to correct the dehydration, but no clinical improvement resulted. Large amounts of plasma

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were then administered intravenously, but the blood pressure did not rise (Fig. 3). Since the failure of these initial therapeutic measures tended to confirm the clinical impression of adrenal failure, an aqueous solution containing 500 units of adrenal cortical hormone (50 dog units per cubic centimeter) was given intravenously. A dramatic change in the patient's clinical condition occurred after the administration of the first 150 units.

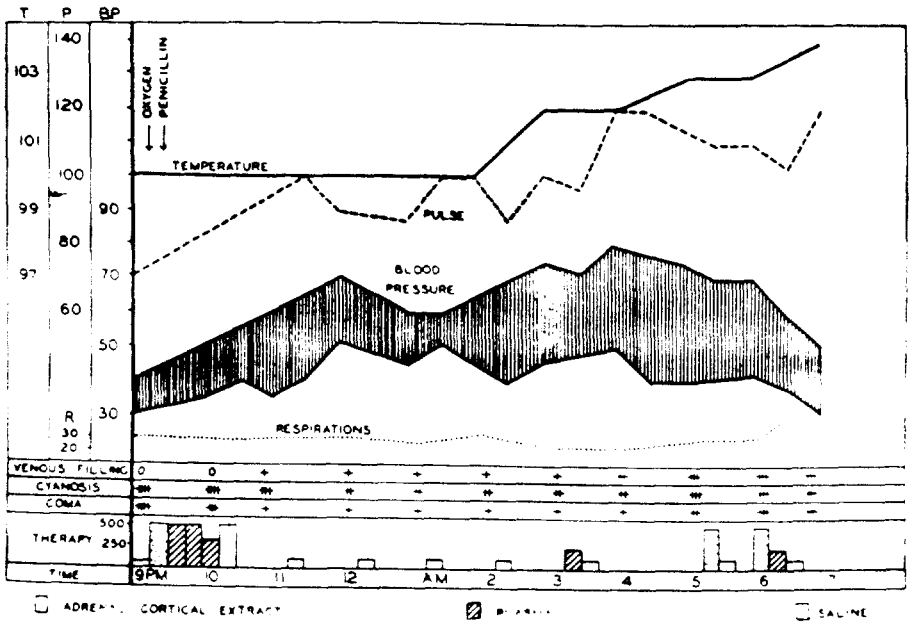

Fig. 3.—Clinical observations of circulatory changes occurring in a shoemaker, 66, with hyperacute meningococcemia of 17 hours' duration. The failure of therapy with fluid to support the circulation and the temporary response to adequate replacement therapy with an aqueous solution of adrenal cortical hormone support the clinical impression of bilateral adrenal cortical hemorrhage (Waterhouse-Friderichsen syndrome). Alteration in the permeability of membranes was minimal. The total duration of the illness was 27 hr.; an adrenal gland removed from this patient at necropsy is shown in Figure 4.

The blood pressure rose, the veins filled, and the patient roused from his coma. No edema appeared, but some hours later a gallop rhythm was noted and the neck veins rapidly became distended. Acute myocardial failure developed, and the patient died.[2]

The vascular changes occurring early in the course of meningococcemia are due to toxemia from products of the infecting organism and to dehydration. The differ-

2. Harrell, G. T.: Case Report, Clinico-Pathological Conference, North Carolina M. J. 7: 562-565 (Oct.) 1946.

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entiation between simple toxemic peripheral circulatory collapse and that due to adrenal destruction (Waterhouse-Friderichsen syndrome) can be made clinically at the bedside and confirmed at necropsy. The adrenals in the girl showed no evidence of hemorrhage; in the man both adrenals were completely destroyed by fresh hemorrhage (Fig. 4). It is evident that the clinical differentiation between peripheral vascular collapse due to infection alone and that due to adrenal cortical destruction was correctly made in both cases. In the latter case the distribution of fluids was not appreciably altered by the

Fig. 4.—The adrenals removed at necropsy from the patients with meningococcemia, whose courses are summarized in Figures 2 and 3. The gland on the upper right side is from the girl (Figure 1 and 2). The gland on the left, shown in cross section on the lower right side, was removed from the patient whose course is summarized in Figure 3. The structure is completely destroyed by extravasation of blood (Waterhouse-Friderichsen syndrome).

adrenal cortical deficiency. The severe peripheral collapse caused the blood pressure to fall below the level necessary for adequate glomerular filtration. The defect in the reabsorption of sodium by the renal tubules, which leads to the excessive loss of water and salt as well as to retention of potassium, did not have time to develop, as it does in the chronic deficiency of the cortical hormone which we recognize as Addison's disease.

Alterations Occurring Within Hours or Days.—Pneumococcus Lobar Pneumonia: In pneumococcus lobar pneumonia alterations in the permeability of membranes

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occur over a period of hours or days. Many years ago it was discovered that chlorides were lost from the circulating blood stream but retained in tissues during the acute phase of the disease.[3] Later observations demonstrated that the extravascular space (measured by the dilution of injected thiocyanate ions) and the circulating blood volume (measured by the Evans blue technique) were increased.[4] Apparently the chloride and its companion ions take water with them as they leave the vascular tree. The defect in permeability is apparently not great enough to decrease the blood volume.

These observations offer a logical explanation of the mechanism responsible for the tendency of the right side of the heart to fail during pneumonia, particularly after the excessive administration of intravenous fluids. The usual increase in blood volume is exaggerated by the additional fluids, and the myocardium is overloaded. The favorable response to digitalis which has been noted by older clinicians is probably due to correction of the central (myocardial) circulatory failure. A direct peripheral action on the permeability of blood vessels which apparently mobilizes tissue fluids may also play a part.[5]

The additional factor of anoxia, which has long been recognized as a contributing cause for alterations in the permeability of membranes, can be illustrated more clearly in pneumococcal pneumonia than in some other infections. The presence of the pulmonary lesion decreases the size of the vascular bed available for oxygenation of the blood, and cyanosis develops.

Alterations Occurring Within Days or Weeks.—Trichinosis: In trichinosis the alteration in the permeability of membranes is pronounced and develops more

3. Peabody, F. W.: Studies of the Inorganic Metabolism in Pneumonia with Especial Reference to Calcium and Magnesium, J. Exper. Med. **17**: 71-82 (Jan.) 1913. Wilder, T. S., and Drake, T. G. H.: Metabolism of Chloride and Total Fixed Base in Pneumonia and the Relation to Salt and Water Retention, J. Clin. Invest. **7**: 353-364 (Aug.) 1929.

4. Rutstein, D. D.; Thomson, K. J.; Tolmach, D. M.; Walker, W. H., and Floody, R. J.: Plasma Volume and "Extravascular Thiocyanate Space" in Pneumococcus Pneumonia, J. Clin. Invest. **24**: 11-20 (Jan.) 1945.

5. Aikawa, J. K.; Knight, V. H., and Tyor, M. P.: Effect of Intravenous Digitoxin on Fluid Distribution in Hospitalized Males without Cardiovascular Disease, Proc. Soc. Exper. Biol. & Med. **76**: 250-252 (Feb.) 1951.

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gradually—over a period of days or weeks. The earliest diagnostic sign, splinter hemorrhages in the nail beds, is due to traumatic rupture of capillary loops, with extravasation of blood, as larvae migrate from the circulation into muscles (Fig. 5).[6] The accumulation of edema fluid, especially around the eyes, has been recognized as one of the most frequent early clinical manifestations of the disease. In severe trichinosis the accumulation of fluid during the stage of edema is associated with oliguria, which lasts from one to two weeks and is followed by diuresis. Serial determinations of the blood proteins made in clinically severe cases of the disease have re-

Fig. 5.—Splinter hemorrhages in the nail bed early in the course of mild trichinosis in a business executive. ×2. The capillary loops are ruptured by trauma as the Trichinella larvae migrate from the circulation to the muscles. The alteration in the permeability of membranes with edema formation, which is so characteristic of the later stage of the disease, has not yet developed.

vealed hypoproteinemia.[7] The reversible psychic disturbances occasionally seen during the stage of edema suggest an excessive hydration of nerve cells.

By the time edema is recognizable, evidence of immunity can be detected in the blood by a positive precipitin reaction or in the cells by a positive skin test. This sequence of events suggests the possibility that immunologic mechanisms may play some part in the physiological disturbances associated with the disease. This thesis has been tested by the experimental production of

6. McNaught, J. B.: The Diagnosis of Trichinosis, Am. J. Trop. Med. **19**: 181-192 (March) 1939.

7. Hanes, F. M.: Trichinosis Complicated by Hypoproteinemia, Internat. Clin. **4**: 67-73 (Dec.) 1936.

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the disease in rabbits.[8] The physiological changes associated with alterations in the permeability of membranes were maximal just before humoral antibodies (precipitins) appeared (Fig. 6).

Rickettsial Spotted Fever: During the acute phase of Rocky Mountain spotted fever, the anatomic lesion is clearly reflected in the visible rash. The histological picture is essentially one of arteritis, with necrosis and

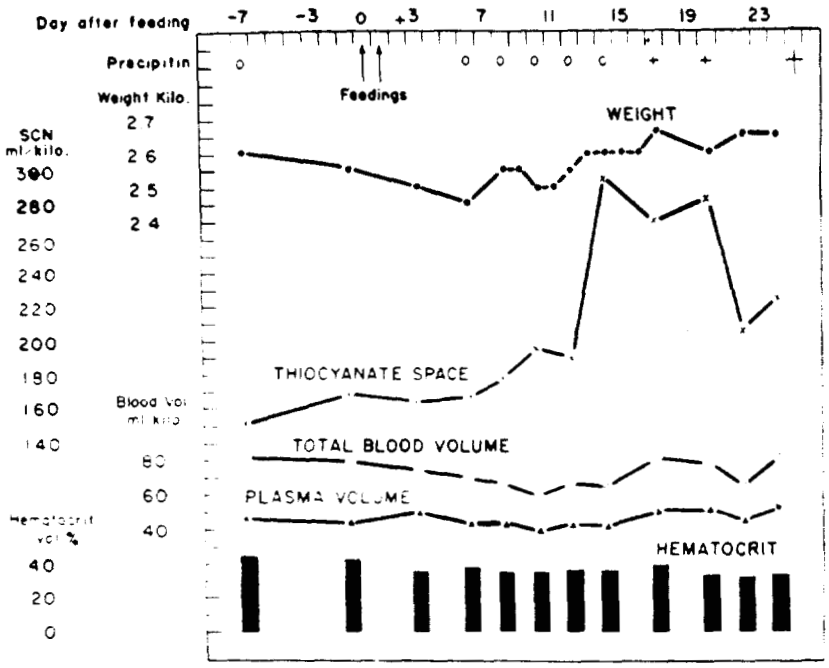

Fig. 6.—Fluid changes in a rabbit fed 6 gm. of trichinous rat meat. Note the increase in the thiocyanate space on the 14th day after the first feeding without a relative increase in body weight. The blood volume was decreased slightly during the 10th to 14th days. The physiological changes were beginning to reverse when the precipitin test became positive in the serum on the 17th day. This rabbit was severely ill, but died as the result of hematopericardium on the 25th day.

thrombosis of the walls of arterioles beginning at the capillary junction. The physiological disturbance, however, may be out of all proportion to the number of pathological lesions. The permeability of the membranes gradually increases during the first two weeks of the disease, and evidences of this increase are detectable

8. Aikawa, J. K.; Harrell, G. T., and Miller, T. B.: The Immunophysiology of Trichinosis: Alterations in the Blood Volume and the Thiocyanate Space in Relation to the Development of Humoral Antibodies in the Rabbit. J. Clin. Invest. **30**: 575-581 (June) 1951.

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generally throughout the body, often at some distance from the arteriolar lesions.[9]

Early in the course of the disease dehydration, accompanied by a rise in the nonprotein nitrogen and a decrease in blood chlorides, may be noted.[10] The administration of solutions of crystalloids, dextrose, and isotonic sodium chloride would seem to be indicated in such a situation. When crystalloids are given, however, the blood pressure may fall to shock levels and edema which has been minimal may become more pronounced

Fig. 7.—Marked edema in a girl, 4 yr., severely ill with Rocky Mountain spotted fever. The photograph on the left was made on the 16th day of rash. The photograph on the right, taken three weeks later during convalescence, illustrates the subsidence of edema with recovery. The clinical course of the patient is summarized in Figure 8.

(Fig. 7). At the time that circulatory failure or edema is detected, the level of the blood proteins are found to be low; the administration of preformed human protein in the form of albumin, plasma, or whole blood is then indicated.

Studies on the mechanism responsible for the circulatory changes have shown that the thiocyanate space is

9. Harrell, G. T., Jr.: Rocky Mountain Spotted Fever, Medicine **28**: 333-370 (Dec.) 1949.

10. Harrell, G. T.; Venning, W., and Wolff, W. A.: The Treatment of Rocky Mountain Spotted Fever, with Particular Reference to Intravenous Fluids: A New Approach to Basic Supportive Therapy, J. A. M. A. **136**: [illegible] (Dec. 9) 194[illegible]

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increased and the blood volume decreased during this critical period (Fig. 8).[11] When the intravascular osmotic pressure is maintained with plasma or blood, fluid is retained in the circulation, the blood pressure is increased, and the kidneys' powers of filtration are improved. Edema is decreased, and the effects of the physiological disturbances resulting from changes in permeability are lessened. If fluids are properly selected, large amounts may safely be given parenterally. We have, for instance, previously reported the case of a small

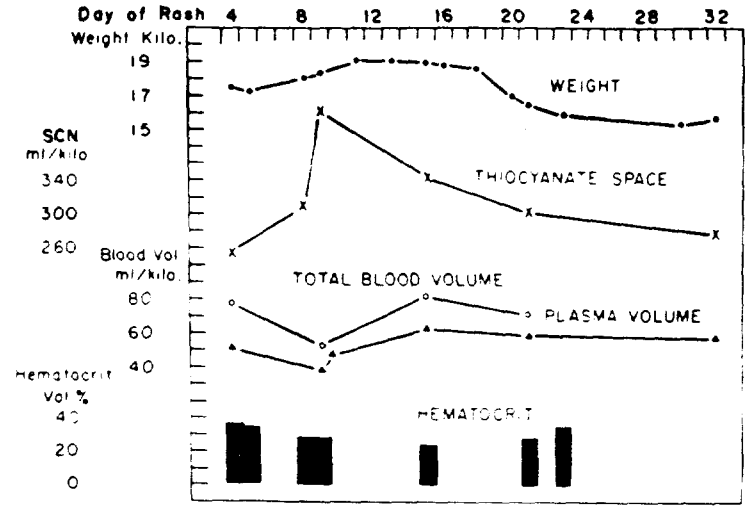

Fig. 8.—Alterations in the fluid spaces in a girl, 4 yr., severely ill with Rocky Mountain spotted fever, which was treated with supportive therapy alone. Note the rise in thiocyanate space with a drop in the blood volume on the 10th day of rash. These changes were accompanied by peripheral circulatory collapse, which was successfully treated by intravenous administration of human serum albumin. The photograph of this patient, on the left of Figure 7, was taken after the clinical peak of the disease as the symptoms were beginning to subside.

child whose entire circulating blood volume was twice completely replaced, first with an equivalent amount of proteins and then with an even larger amount of crystalloids.[12]

The early administration of effective chemotherapeutic agents which directly attack the rickettsiae will rapidly

11. Harrell, G. T., and Aikawa, J. K.: Pathogenesis of Circulatory Failure in Rocky Mountain Spotted Fever: Alterations in the Blood Volume and the Thiocyanate Space at Various Stages of the Disease, Arch. Int. Med. **83**: 331-347 (March) 1949.

12. Harrell, G. T.; Aikawa, J. K., and Kelsey, W. M.: Rocky Mountain Spotted Fever, Am. Pract. **1**: 425-435 (April) 1947.

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change the character of the rash (Fig. 9) and prevent the development of further edema (Fig. 10).[13]

Alterations Occurring in Immune Reactions.—Serum Sickness: When humoral antibodies produced by an immunized animal are used to assist the patient in overcoming his infection, another illness, serum sickness, often results. In many of its clinical aspects, such as fever,

Fig. 9.—The rash in a severe case of rickettsial spotted fever in a girl, 8 yr., treated with specific chemotherapy. The photograph at the top shows the hemorrhagic character of the eruption with edema of the hand and fingers at the time of admission on the ninth day of rash. The photograph at the bottom shows the marked fading of the rash with subsidence of edema and wrinkling of the skin on the hand four days later after treatment with aureomycin. The alterations in the fluid spaces are shown in Figure 10.

malaise, and skin eruption, serum sickness resembles an infectious disease. Indeed, edema, wheals in the skin, and swelling of the joints have long been recognized as prominent features of this illness; all these suggest that

13. Harrell, G. T.; Meads, M., and Stevens, K.: "Aureomycin" A New Orally Effective Antibiotic: Clinical Trial in Rocky Mountain Spotted Fever, Results of Susceptibility Tests and Blood Assays Using a Turbidimetric Method, South M. J. 42: 4-13 (Jan.) 1949.

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immune reactions can produce alterations in the permeability of membranes. In serum sickness resulting from the administration of Rocky Mountain spotted fever antiserum prepared in rabbits, measurements of the extravascular space (by the thiocyanate technique) show the same alterations which are seen in the original infection (Fig. 11). Similar changes have been observed in patients who have received diphtheria antitoxin prepared in horses (Fig. 12). After the administration of anti-

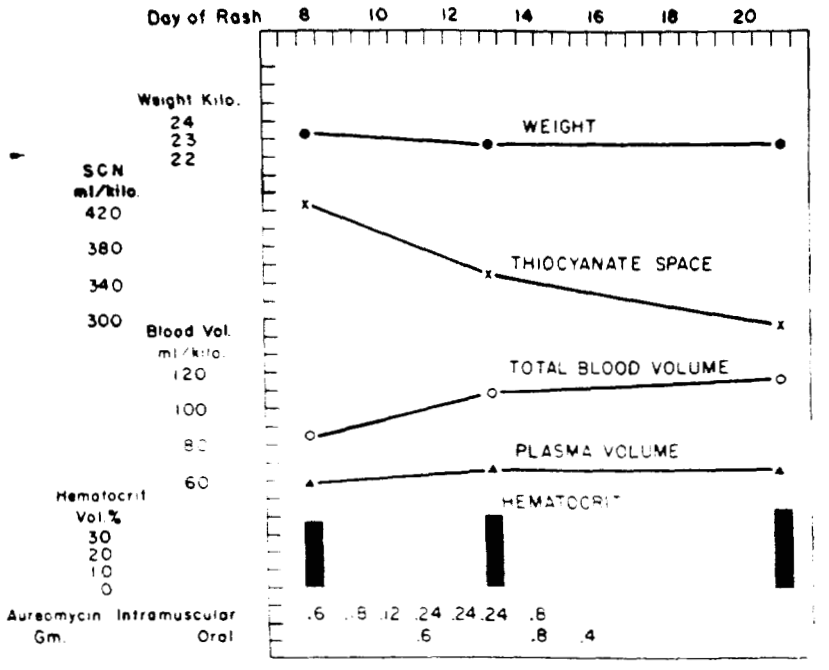

Fig. 10.—The fluid changes in a girl, 8 yr., severely ill with Rocky Mountain spotted fever treated with specific chemotherapy. Note the decrease in thiocyanate space and increase in blood volume as the clinical symptoms were controlled with aureomycin therapy. Photographs illustrating fading of the rash and subsidence of edema in this patient are shown in Figure 9.

reticular cytotoxic serum (ACS) prepared in rabbits, alterations in permeability have been demonstrated in the absence of any infection. An increase in the thiocyanate space and a decrease in the blood volume have been found to occur in serum sickness experimentally produced in rabbits by the injection of fractions of human plasma.[14]

14. Aikawa, J. K., and Harrell, G. T.: The Immunophysiology of Serum Sickness: Alterations in the Blood Volume and Thiocyanate Space in Relation to the Development of Humoral Antibodies in the Rabbit, J. Clin. Invest. **30**: 360-368 (April) 1951.

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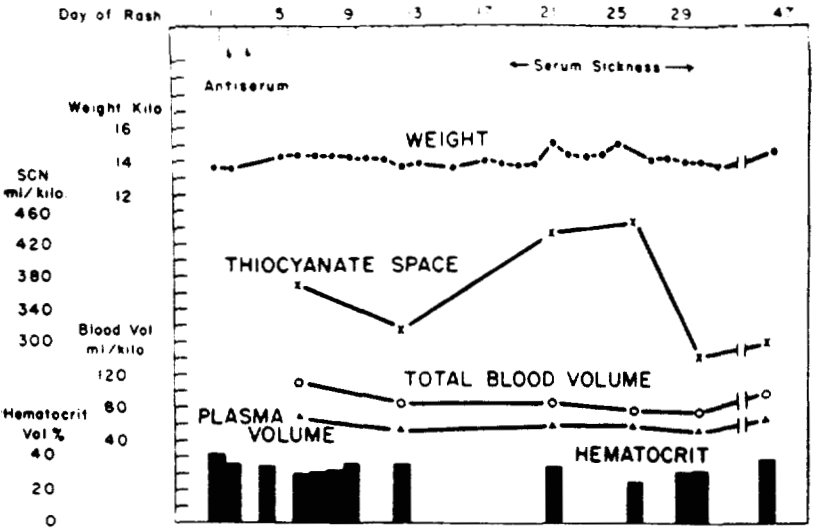

Fig. 11.—Alterations in the fluid spaces in a girl, 3 yr., with moderately severe rickettsial spotted fever treated with specific rabbit antiserum. Note the decrease in thiocyanate space on the 11th day as the clinical symptoms began to subside. The development of serum sickness on the 18th day after injection of Rocky Mountain spotted fever antiserum reproduced symptoms of infection, with fever and edema accompanied by a second rise in the thiocyanate space. The symptoms of serum sickness were controlled by the oral administration of diphenhydramine (benadryl®) hydrochloride with return of the thiocyanate space to baseline values.

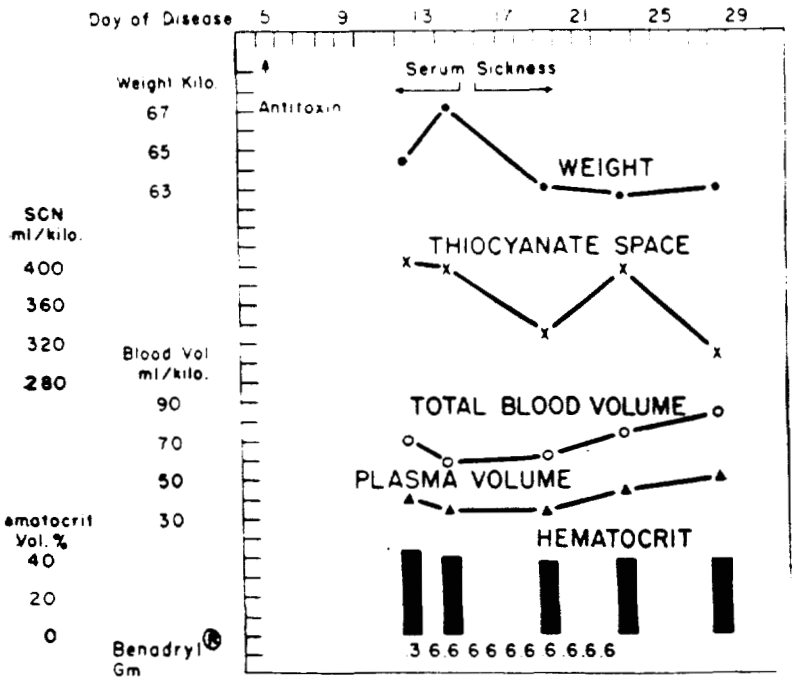

Fig. 12.—Fluid changes during serum sickness in a merchant seaman, 42, ill with diphtheria. The clinical symptoms of serum sickness appeared on the 12th day of diphtheria, seven days after the administration of antitoxin prepared in horses. Symptoms of serum sickness subsided with the oral administration of benadryl®. As the defect in permeability was repaired, edema disappeared, the thiocyanate space and weight decreased, and the blood volume increased.

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The clinical picture of serum sickness may follow the injection of antibiotics, such as penicillin (Fig. 13). When this reaction occurs, circulating antibodies to penicillin (precipitins) are usually detectable. In other instances the immune (or sensitivity) reaction appears to take place predominantly on cells and is manifested by dermatitis, often with exfoliation.

Prolonged serum sickness developed in one of our patients who received penicillin suspended in oil. Since the patient was known to have had repeated episodes of stomatitis following the ingestion of peanuts, the sensi-

Fig. 13.—Serum sickness due to a penicillin reaction in an electrician. 33. The photograph on the left taken on the second day shows moderate edema of the eyelids; mild generalized edema was also present. The photograph on the right was taken nine months after recovery. The fluid changes in this patient are shown in Figure 14.

tivity was thought to be due to the peanut oil used as a menstruum and not to the penicillin. Apparently the injection of any material which is allergenic to a particular patient may produce alterations in the permeability of membranes (Fig. 14).

COMMENT

Alterations in Capillary Permeability.—The observations reported can be interpreted as indicating an increase in the permeability of the vascular tree, permitting the progressive loss of crystalloids, colloids, and red blood cells from the circulation as the magnitude of the

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capillary defect increased with the clinical severity of the disease. The alterations in the vascular tree can be visualized as a series of pores or openings in the capillary wall where the normal extravascular circulation of crystalloids and, to a lesser extent, of small protein molecules is known to occur. In mild cases, water and ions which normally traverse the membranes would leave the blood more readily. Larger molecules, such as

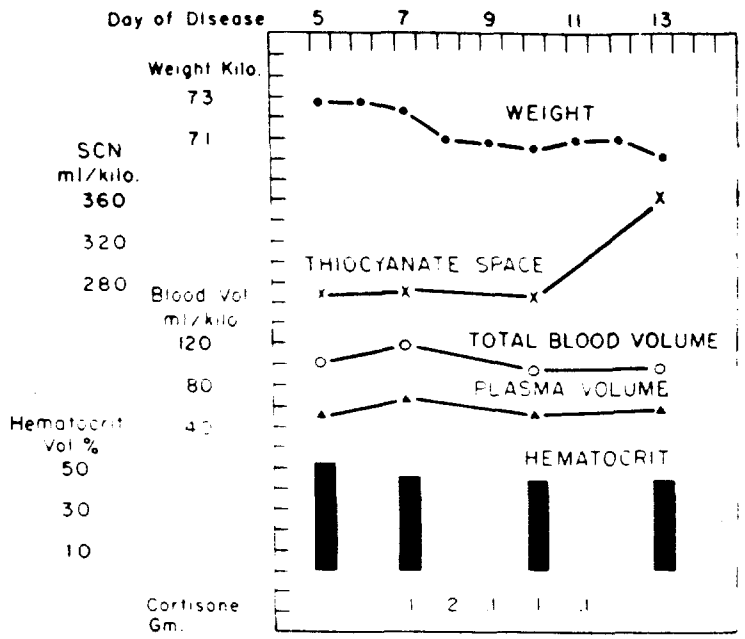

Fig. 14.—The fluid changes in an electrician, 33, with serum sickness due to a penicillin reaction treated with cortisone. The edema which appeared was less than that often seen because of the prompt suppression of symptoms by treatment. The fluid which did accumulate was mobilized, as shown by the temporary rise in blood volume, and was excreted with a drop in weight. The rise in thiocyanate space on the 13th day of the disease followed cessation of cortisone therapy. Clinical photographs of the patient are shown in Figure 13.

albumin and other proteins, would be lost in severer instances, while red blood cells would pass out of the vascular tree only in the extremest reactions.

Alterations in Permeability of Cellular Membranes.—The permeability of cell membranes is probably altered too, though no techniques are available for direct measurement of cellular changes. Viruses and rickettsiae are obligate intracellular parasites. Many bacterial infections—particularly those with a chronic or prolonged course, such as tuberculosis and brucellosis — pass

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through a phase in which the organisms are found intracellularly. During this stage the diffusion of toxemic products of the organism must be through the cell wall, with the attendant danger of damage. Furthermore, any immune reaction would take place within the cell, so that substances released in the process would affect primarily the parasitized cells. Additional light on the problem of cellular permeability may be shed by experiments now in progress,[15] utilizing the radioactive isotopic dilution technique with Na^{24} and K^{42}.

In children acute infections accompanied by diarrhea are reported to produce a potassium deficiency. How much of this deficiency is due to a change in the permeability of cellular membranes as a result of the infection itself and how much is due to the disturbances in fluid balance associated with the diarrhea is unknown. In either event potassium moves out of the cell in which the membrane of the cell wall normally retains it and is lost in the urine or feces.

An analogous situation in which the permeability of cellular membranes is altered in the absence of infection is illustrated by the edema occurring in a young woman with drug intoxication which followed the therapeutic administration of an arsenical preparation (Fig. 15). Arsenic apparently poisons intracellular enzyme systems which contain sulfhydryl (SH) groups in the molecule. The administration of British anti-lewisite (BAL), a substance which has large numbers of accessible sulfhydryl groups, will rapidly correct the enzymatic defect by combining with the metal. With correction of the defect, the alteration in the permeability of membranes is reversed and edema disappears (Fig. 16). Observations of this type may give further clues to the mechanism responsible for production of cell defects. Such questions are of more than academic interest, and are important in the design of therapy for acute infections. Intracellular enzyme systems are greatly affected by the

15. Aikawa, J. K.: The Significance of the Radiosodium Space in Human Disease: A Comparison with the Thiocyanate Space, South. M. J. 44: 654-661 (July) 1951.

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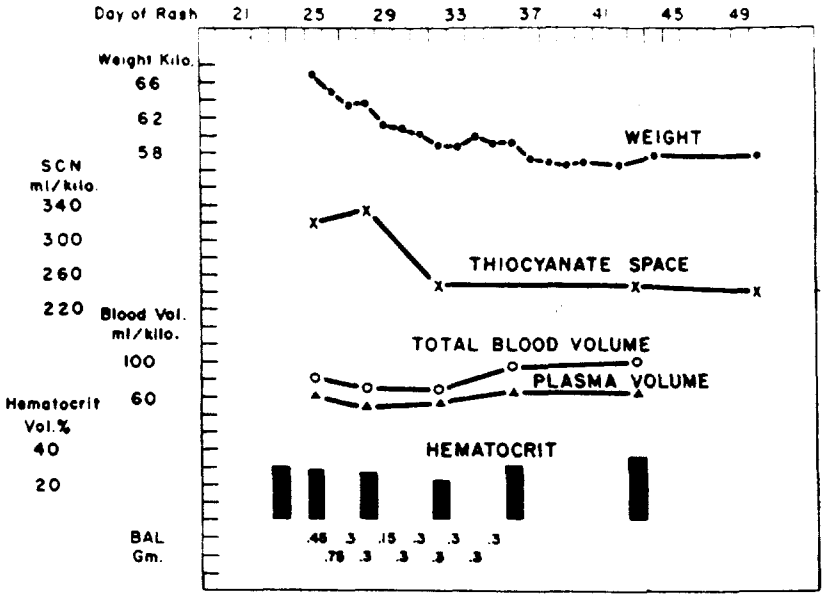

Fig. 15.—Fluid changes during arsenic intoxication (treated by inactivation) in a pregnant woman, 39, with early syphilis treated by intravenous injections of oxophenarsine hydrochloride (mapharsen®). The dermatitis appeared after the seventh injection, and the patient rapidly became ill. Note the decrease in thiocyanate space and weight following the administration of BAL. Clinical photographs of the patient are shown in Figure 16.

Fig. 16.—Edema and exfoliative dermatitis due to arsenic intoxication in a pregnant woman, 39. The photograph on the left was taken on the 27th day of rash, two days after the institution of therapy with British anti-lewisite. Note the generalized character of the edema and the scaling of dead skin. The photograph on the right was taken three months later showing complete subsidence of signs. The patient presented essentially the same appearance two weeks after the institution of therapy, illustrating how rapidly changes in permeability may revert with repair of the cellular defect.

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hydration of cells and by the intracellular concentrations of ions from salts.

Therapeutic Possibilities. — When infections produce an alteration in the permeability of membranes, treatment with maximum therapeutic doses of effective chemotherapeutic agents should be begun promptly in order to reduce the number of organisms and to prevent further damage. In diseases which frequently produce severe alterations in permeability—the rickettsial diseases, for example—every attempt should be made to prevent, in so far as possible, the development of the full-blown clinical picture seen in the natural course of the disease.

Salt, dextrose, and water must be administered with care. The drop in circulating proteins may be prevented or limited by the administration of a high-protein diet from the beginning of therapy.[16] When the patient is seen late in the course of the disease, after the proteins in the blood and tissues have been depleted, or after acute circulatory collapse has developed, preformed proteins in the form of concentrated human serum albumin, plasma, or whole blood must be administered. Fluid replacement therapy in specific diseases and under specific circumstances is discussed further in the illustrative cases presented in the section entitled "Alterations in Permeability Associated with Specific Diseases."

The fact that, in patients with serum sickness, administration of antihistaminic drugs relieves the joint pain and swelling and causes the wheals to disappear suggests that these drugs may find a useful place in the treatment of acute infections which have a large element of hypersensitivity. Salicylates and the closely related chemical compounds para-aminobenzoic acid (PABA) and procaine have long been successfully employed in the treatment of disease associated with immune reactions.

Metabolic factors which affect the permeability of membranes during the course of infections are as yet poorly understood. It is not known whether the increase

16. Harrell, G. T.; Wolff, W. A.; Venning, W. L., and Reinhart, J. B.: The Prevention and Control of Disturbances of Protein Metabolism in Rocky Mountain Spotted Fever: The Value of Forced Feedings of a High-Protein Diet and of the Administration of Specific Antiserum, South. M. J. 39: 551-558 (July) 1946.

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in metabolic rate observed during infections is an attempt by the body to compensate for the defect in permeability by increasing thyroid function. It is well recognized, however, that when the thyroid function is lowered, as in myxedema, the membranes become more permeable and fluid is retained in the tissues.[15] The salicylates apparently help to correct the defect in permeability in serum sickness; they may, through their antipyretic effect, also depress the increased level of metabolic activity.

Many ingenious theories have been proposed concerning the role of the adrenal in acute infections or in diseases which are indirectly related to the stress of illness.[17] In instances of adrenal destruction (Waterhouse-Friderichsen syndrome) occurring during meningococcemia, the indications for hormone-replacement therapy are clear. The decision to use adrenal cortical hormones must be made on clinical grounds. The necessary observations on the pulse, respirations, blood pressure, venous filling, and the degree of cyanosis and edema can be made at the bedside with the equipment usually carried in a physician's bag.

Corticotrophic hormone (ACTH) or cortisone often will dramatically relieve the symptoms of serum sickness and quickly restore the physiological integrity of the membranes (Fig. 14). In patients with pneumonia a reduction in the degree of toxemia and an increase in the sense of well-being following the administration of these hormones have been reported.[18] The possible usefulness of these hormones in the treatment of diseases which characteristically are associated with high degrees of allergy, such as tuberculosis, is still under investigation. It should be emphasized that the infecting agent is not attacked by the hormones and that they have no effect on bacteremia, if this is present; their action is solely on the tissues of the host. The suppression of toxemic reactions in the host may actually be accompanied by an increase in the number of organisms and an

17. Selye, Hans: The Physiology and Pathology of Exposure to Stress. Montreal, Acta, Inc., 1950.

18. Kass, E. H.; Ingbar, S. H., and Finland, M.: Effects of Adrenocorticotropic Hormone in Pneumonia: Clinical, Bacteriological and Serological Studies, Ann. Int. Med. 33: 1081-1098 (Nov.) 1950.

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exacerbation of the disease.[19] This sequence of events has long been recognized by clinicians in the recrudescence of clinical tuberculosis following the termination of pregnancy.

The sudden release of large amounts of antigen by the rapid destruction of organisms (such as the Herxheimer reaction which occurs in syphilis after the initial injection of penicillin) or by the dissolution of a granuloma (as occurs in some fungus infections after the administration of iodides) may not prove so detrimental to the patient if the harmful effects of the antigens on sensitized cells can be ameliorated by hormone therapy. Conversely, the apparent stimulating effect which hormone therapy has on the growth of the etiological agent may possibly be counteracted by the simultaneous or prior administration of specific chemotherapeutic agents.

In the final analysis, however, the patient must still heal himself. In spite of the most vigorous and carefully planned therapy, failures will occur. Patients will die of the toxemia of overwhelming infections and as the result of extensive vascular or cellular damage. However, intelligent management, with attention to alterations in the permeability of membranes, will allow the salvage of many cases now considered hopeless, will lessen the severity of the patient's reaction to his infection, and will shorten convalescence.

CONCLUSIONS

Our observations indicate that the permeability of membranes is altered during the course of acute infections. Early in the course of the disease the defect is probably induced by the toxemic products of the infecting organisms. Later in the course of the illness the defect may result from an immune reaction. In rickettsial diseases, for instance, the greatest alteration in permeability appears to come at the time when immunity is rising most rapidly; the balance between the antigens

19. Lurie, M. B.; Zappasodi, P.; Dannenberg, A. B., Jr., and Swartz, I. B.: Constitutional Factors in Resistance to Infection: The Effect of Cortisone on the Pathogenesis of Tuberculosis, Science **113**: 234-237 (March 2) 1951. ACTH and Cortisone in Active Infections, editorial, Arch. Int. Med. **87**: 1-3 (Jan.) 1951. Cortisone in Tuberculosis, editorial, J. A. M. A. **145**: 907 (March 24) 1951.

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liberated from or secreted by the organisms and the antibody produced by the host is being rapidly destroyed.[11] The point at which immunity overcomes the infection and the antibodies begin to appear in excess often is accompanied by a dramatic clinical change which is recognized as a crisis.

Antigen-antibody reactions are known to liberate a histamine-like substance. That histamine alters the permeability of membranes locally can be readily demonstrated by the intracutaneous injection of a small amount of the material; within a few minutes a wheal appears at the site of injection (Fig. 17). Petechial hemorrhages have

Fig. 17.—Wheal in the skin of a laboratory technician, 25, 15 min. following the intracutaneous injection of 0.1 mg. of histamine. Note the accumulation of fluid as the result of local alteration in the permeability of capillaries.

been recognized for years as a manifestation of anaphylactic reactions, a further clue to the mechanism of production of lesions in infections.

Since certain fundamental principles are common to all infectious diseases, alterations in the permeability of membranes can be detected in many infections occurring naturally in human beings or produced experimentally in animals. The observations reported indicate that the reactions are qualitatively similar, whether produced by a bacterial infection (meningococcemia), and extracellular infection due to a nematode (trichinosis), an intracellular infection caused by rickettsiae (Rocky Mountain spotted fever), or even an immune reaction

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occurring in the absence of actual infection (serum sickness).

SUMMARY

The permeability of membranes is altered during the course of acute infections. Edema develops during the acute phase, associated with an increase in extravascular fluid (thiocyanate space), and disappears with recovery. The alterations may occur within minutes to hours (meningococcemia), within hours to days (pneumococcus pneumonia), or within days to weeks (trichinosis and rickettsial spotted fever). The alterations in permeability which occur during immune reactions in the absence of infection (serum sickness) resemble the changes seen during an acute infectious disease. The observations are interpreted as indicating an alteration in the permeability of the capillary wall, permitting the progressive loss of crytalloids (water, ions, and dextrose), colloids (albumin and other proteins), and red blood cells as the clinical severity of the disease increases. These changes are often associated with alterations in blood volume and offer an explanation of the mechanism by which peripheral circulatory failure develops. The permeability of cell membranes is probably also altered, whether the cells are directly parasitized or not. The alterations during arsenic intoxication, which are reversible by therapy with British anti-lewisite (BAL), offer a clue as to a possible intracellular mechanism through interference with enzyme groups. The therapeutic possibilities are discussed, with emphasis on the correct use of crystalloids and proteins and the possible application of antihistamines and hormones in conjunction with specific chemotherapeutic agents in treatment. The early changes are due to toxemic products of the infecting organism; the later changes may result from humoral or cellular immune reactions. The alterations in permeability are qualitatively similar, whether produced by a bacterial infection (meningococcemia), an extracellular infection due to a nematode (trichinosis), an intracellular infection caused by rickettsiae (Rocky Mountain spotted fever), or an immune reaction in the absence of infection (serum sickness).

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Bowman Gray School of Medicine.

PROGRESS REPORT

Contract No. AT-(40-1)-288 - Title I
The Bowman Gray School of Medicine of
Wake Forest College.

Distribution and turnover of sodium and potassium in acute infections.

[redacted] Harrell, Jr., Project leader July 1, 1951 to March 15, 1952

Facilities: The laboratory as originally constructed has proved to be very [redacted]factory. No major changes in the design or arrangement of the facilities have been [redacted] No difficulty has been experienced with the major items of equipment with the [redacted] of a drying oven which has worn out and is being replaced. Additional shields [redacted]nets, and other relatively minor items of equipment have been purchased [redacted] the year.

Personnel: Dr. Jerry K. Aikawa has continued to work almost full time on the project with the principal investigator. His fellowship with the American Heart Asso- [redacted] will terminate July 1, 1952. [redacted] for part of his time from funds donated by the American Heart Association, which we hope will permit him to continue on the project for five more years. It will be necessary to continue supplementation of these funds from the grant in order to retain him at the present level of activity.

Miss Eloise Rhoades, technician, has proved entirely satisfactory and is continuing enthusiastically in her work.

Our former negro technician, Claude Taylor, has resigned and has been replaced by William Brooks, another negro, who has completed a technician's course at the Kate Bitting Reynolds Memorial Hospital in Winston-Salem. Though he is yet incompletely trained in our techniques, he is proving to be thoroughly reliable and dependable. He has worked seven days weekly throughout the spring without complaint in order to assure that the experiments run smoothly.

We have been extremely fortunate this year in being able to utilize, intermittently throughout the year, two excellent house officers, Drs. Malcolm Iyor and John H. Felts. As you can see from the bibliography, these men have been invaluable in pursuing the experiments with patients on the wards of the hospital. We are still losing men to military service and the shortage of personnel at this level is critical for the coming year. Our human work may suffer during the next years unless personnel are released from military service or returned to civilian training, or unless we can obtain financial support for one of these two men, both of whom have seen military service.

Isotopes: After much consultation with the airlines, including a conference with the presidents of Capital and Piedmont Airlines, we are obtaining our isotopes more regularly. We have redesigned our experiments so that we utilize sodium 24 and potassium 42 starting early Tuesday mornings. We are not encountering as much chemical [redacted] with potassium 42 as in the past year.

Animals: We have discontinued trying to breed the strain of animals obtained from Oak Ridge. Animals are in better supply from commerical breeders in the vicinity. We have been able to establish our rickettsial strain in guinea pigs without difficulty, but have not yet been able to adapt the technical methods to guinea pigs. We have been unable to establish the rickettsial strain in rabbits at sufficient virulence to give

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Effect of Intravenous Digitoxin on Fluid Distribution in Hospitalized Males without Cardiovascular Disease.* (18451)

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(Introduced by G. T. Harrell)

From the Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest College, and the Baptist Hospital, Winston-Salem.

Although the actions of digitalis on the heart are well known, its effect on the distribution of body fluids in normal individuals have not been reported. Because the cardiac glycosides are similar in chemical structure to other cyclopentenophenanthrene derivatives which are known to affect salt and water metabolism—for example, the sex hormones and the adrenal cortical hormones—they might be expected to produce some alteration in the distribution of fluids in the body.

The present study was conducted in order to determine the effect of intravenous digitoxin on the plasma and extracellular fluid spaces in normal individuals.

Material and Methods. Subjects. Serial studies were performed on 5 male patients who had been hospitalized for non-cardiovascular disorders and who had no obvious disturbances of salt and water metabolism. These individuals were ambulatory and were on a house diet. No attempt was made to control their intake of salt and water. *Material.* Commercial crystalline digitoxin prepared from Digitaline nativelle and dissolved in a 40% solution of alcohol, was used in all cases. The solution contained 0.1 mg of digitoxin per ml of fluid. *Method.* The methods which were used to determine the *hematocrit*, the *plasma volume* and the *thiocyanate space* have been described previously (1). In preliminary experiments it was demonstrated that the thiocyanate ion apparently reached a state of equilibrium in the extracellular fluid space 3 hours after injection (2); the 3-hour values, therefore, have been used for calculations and comparisons. Since the total volume of blood withdrawn for each complete study did not exceed 40 ml, the blood loss would not have affected the circulating blood volume appreciably. The *serum protein concentration* was determined by the Kingsley biuret method (3). This value and the plasma volume were used in calculating the *total circulating protein* content.

Plan of the experiment. A baseline deter-

* This work was supported in part by the U. S. Atomic Energy Commission through a contract with the Bowman Gray School of Medicine, and in part by a grant-in-aid from the American Heart Association.

† Research Fellow of the American Heart Association.

1. Aikawa, J. K., *Am. J. Physiol.*, 1950, v162, 695.
2. Aikawa, J. K., submitted for publication.
3. Kingsley, G. R., *J. Lab. and Clin. Med.*, 1942, v27, 840.

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mination of all the quantitative measurements described above was made on each individual at least a day prior to the administration of digitoxin, and the subject was weighed at this time. After completion of the baseline studies, a total of 1.2 mg of digitoxin was administered intravenously to each patient. This amount was divided into 2 equal doses of 0.6 mg each, given one hour apart. Five hours after the first injection of digitoxin, venous blood was withdrawn and the solution containing T-1824 (Evans Blue) and sodium thiocyanate was injected intravenously. The plasma and total blood volumes were measured on samples drawn 10 minutes later. The thiocyanate space was calculated from a sample drawn 3 hours later (8 hours after the initial injection of digitoxin). The total serum protein concentration was determined on samples drawn 5, 7, and 8 hours after the first dose of glycoside; and the hematocrit, on the samples drawn at 5 and 8 hours. In order to determine whether the alterations in fluid distribution were reversible, the entire battery of tests was repeated 72 hours after the injection of digitoxin.

Method of statistical analysis. Each patient served as his own control. In order to test the significance of the results, the differences between the baseline value and the values observed at the specified time intervals after the administration of digitoxin were determined for each individual. The mean difference and the standard error were then calculated; from these values, "t" was obtained.

Results. (Table I). A progressive decrease in the mean serum protein concentration during the first 8 hours was found. The decrease at 8 hours (0.75 g per 100 cc) was statistically significant ($t = 8.4$). The mean values for the total circulating protein content before the administration of digitoxin, and 5 and 72 hours after its administration were 215, 235, and 200 g, respectively. The increase of 20 g at 5 hours was significant ($t = 4.95$, $P = < 0.01$).

A mean increase of 381 ml in the plasma volume and of 348 ml in the total blood volume (each roughly 10% of the baseline value) was found at 5 hours; the values at 72 hours were lower than the baseline values in 4 of the 5 subjects. The mean increase in plasma volume at 5 hours was at least suggestive ($t = 2.98$).

TABLE I. Comparison of the Baseline and 5' Plasma Volumes

	Baseline	5'	d
Ro	3272	3841	+569
Lo	3492	3966	+474
Ga	3217	3841	+624
Br	2683	3004	+321
Be	2683	2596	— 87
		Sum of d	+1901
		Mean diff.	—380.2

$$\frac{S\,d^2 - \frac{(Sd^2)}{n}}{n(n-1)} \qquad S\,d^2 = 1{,}048{,}420 \qquad \frac{(Sd^2)}{n} = -722{,}760 \qquad Sd^2 - \frac{(Sd^2)}{n} = 325{,}660$$

$$\frac{325{,}660}{20} = 16{,}280.$$

Square root $= S_d = 128.0$.

$$t = \frac{380.2}{128.0} = 2.98.$$

The decrease in the mean hematocrit value was not significant at either time interval. The decrease in thiocyanate space at 72 hours, although of considerable magnitude, was not significant. No change was observed at 5 hours. No significant alterations in body weight were noted during the period of observation, although there was a slight decrease at 72 hours.

Comment. The results of the present study suggest that changes occur in fluid distribution when 1.2 mg of digitoxin is given intravenously to individuals without cardiovascular disorders. A significant decrease in the concentration of serum proteins and an increase in the mean total circulating protein content, associated with a suggestive increase in the mean plasma volume and a decrease in the mean hematocrit, indicate that there is either (1) a primary shift of water and a secondary mobilization of labile protein into the vascular compartment or (2) a primary shift of protein containing fluid into the vascular compartment. The maximum dilution

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of the blood proteins appears to have occurred at 8 hours. Unfortunately, the experiment was so designed that the plasma volume was not measured at that time.

It is known that in congestive heart failure the onset of diuresis is preceded by a fall in the specific gravity of the plasma, presumably due to an increased hydration of the blood plasma(4). The resultant increase in blood volume is thought to be responsible for the diuresis. A similar mechanism may have been in operation in the present study: this would explain the decrease in the thiocyanate space and in the body weight observed at 72 hours.

The changes which were observed in the fluid content of the vascular compartment may have been due to a primary decrease in cardiac output, with a consequent decrease in capillary pressure. However, a decrease in the venomotor tone, an increase in the interstitial fluid pressure, or both, may also cause the changes noted. Whether the results were due entirely to the glycoside's effect on the heart or whether there was also an extracardiac effect is not known. A more detailed study of the problem is now in progress.

Summary. The intravenous administration of 1.2 mg of digitoxin to 5 male subjects with no cardiovascular disorder was found to cause a significant decrease in the serum protein concentration and an increase in the total circulating protein content within 8 hours. The results were interpreted as indicating an increase in the water and protein content of the vascular compartment.

4. Stewart, H. J., *J. Clin. Invest.*, 1941, v20, 1.

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FLUID VOLUMES AND ELECTROLYTE CONCENTRATIONS IN NORMAL RABBITS

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FLUID VOLUMES AND ELECTROLYTE CONCENTRATIONS IN NORMAL RABBITS[1]

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DURING the course of some studies in immunophysiology, it was necessary to determine the normal values and their random variations for the extracellular fluid compartments in normal rabbits. The plasma volume, total blood volume and the thiocyanate and radiosodium spaces were, therefore, measured in a total of 45 rabbits. Serial determinations of the plasma volume and the thiocyanate space were made in 6 animals. The reproducibility in any individual animal of values for the thiocyanate and the radiosodium spaces was determined. Serum sodium and potassium concentrations were measured. The radiosodium space of various tissues and organs was determined in 6 animals, as well as their total water content.

MATERIAL AND METHODS

A total of 45 normal domestic adult rabbits of both sexes, with initial body weights between 2 and 4 kg., were kept in individual cages and were fed a stock diet. Water was given without restriction.

Plasma Volume and Thiocyanate Space. The plasma volume and thiocyanate space were determined by the T-1824 dye (Evans Blue) and sodium thiocyanate techniques (11). The dye and the thiocyanate ion were injected as a sterile solution containing a mixture of one mg/cc. of Evans Blue and 50 mg/cc. of sodium thiocyanate. Syringes were calibrated to deliver approximately 2 ml. of the mixture. No correction was made in dosage for variation in weights of the rabbits. The plasma volume was determined on a single 10-minute sample; 30-minute samples were drawn in some cases for comparison with the radiosodium space. The *hematocrit* was determined in Wintrobe tubes centrifuged at 3000 rpm for 30 minutes, at a radius of 15 cm.

Radiosodium Space. The radiosodium space was determined as follows: A known volume (2 ml.) of a sodium carbonate solution containing approximately 20μc ml. of Na^{24} was injected intravenously into a marginal vein of the ear immediately follow-

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The Na^{24} used was supplied by the National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

[2] AEC Fellow in the Medical Sciences of the National Research Council, 1948–1950.

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ing the injection of the mixture of Evans Blue and sodium thiocyanate. In a preliminary experiment, serial samples were obtained in order to determine the disappearance curve of radiosodium. Subsequently, a single blood sample was drawn to coincide with the equilibration point (one hour) for radiosodium. The activity was determined with a dipping tube and a scaling circuit. Counts were made to one per cent accuracy. The radiosodium space was calculated by the following formula: Volume of Na^{24} space in cc. = (Total counts/min. injected)/(Counts/min./ml. plasma). All values were corrected for decay. The standard solution which was used for injection was appropriately diluted with distilled water to approximate the counting rate of the plasma. No correction was made for urinary excretion of the radiosodium during the period of observation since the amount excreted was negligible.

Plasma Sodium and Potassium Concentrations. Plasma sodium and potassium concentrations were determined by flame spectrophotometry on the Beckman Model DU instrument. The following modification of the method of Mosher, Boyle, Bird, Jacobson, Batchelor, Iseri and Myers (2) was used: The slit width was set at 0.1 mm. in both sodium and potassium determinations. With the standard solution set on the transmission dial at 70 and 25, respectively, for sodium and potassium, the galvanometer needle was set at zero by altering the sensitivity setting.

Tissue Analyses. The 6 animals which were to be killed for tissue analyses were injected intravenously with 50μc/ml., with a total of 100 μc/animal. One hour after injection, these animals were exsanguinated by severing the jugular veins and the carotid arteries; the animals died within 4 to 5 minutes. Approximately one gm. of tissue was weighed on a torsion balance. Each sample was then completely digested with 5 to 10 ml. of fuming sulfuric acid; the digest was made to a volume of 25 ml. with distilled water. In order to maintain a constant geometry, 25-ml. graduated cylinders were used as containers for the digests. Because of the marked photosensitivity of the dipping tubes, all counting was done in a dark room. The radiosodium space of the various tissues and organs was calculated by the formula: Percentage radiosodium space = (Counts/min./gm. of tissue)/(Counts/min./ml. plasma). Aliquots were dried to constant weight in a drying oven at 110° to 120° C. for determinations of the total water content of the tissues and organs.

EXPERIMENTAL RESULTS

Serial Determinations of Plasma Volume, Hematocrit, and Thiocyanate Space. In the initial experiment, a total of 71 determinations of the plasma volume and the thiocyanate space were performed in 6 rabbits during an aggregate period of 200 days. The determinations were made every second or third day and occasionally at less frequent intervals. The calculations were based on single 10-minute samples. The results of this study are summarized in table 1.

Mixing Time for Radiosodium. Serial venous blood samples were obtained from 8 rabbits following the administration of radiosodium. Figure 1 shows graphically in cc./kg. the mean values for radiosodium space at each time interval. After the first 30 minutes, the disappearance curve became exponential. On the basis of the above findings, subsequent calculations of the sodium space were made on a single blood sample obtained one hour following the injection of Na^{24}. The rate of removal of

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radiosodium from the plasma was roughly 10 per cent hour. If the exponential portion of the curve was extrapolated to zero time, the radiosodium space was approximately 25 per cent of the body weight.

Mixing Time for Thiocyanate. In previous studies on immunophysiology [illegible], for technical reasons, the thiocyanate space was calculated from the 10-minute sample. Upon serial determinations on any single rabbit, the results of this measurement were reproducible (table 1). However, it was recognized that equilibration had not occurred at that time. In figure 1 is shown the curve for thiocyanate space calculated from the mean values obtained at various time intervals in 2 normal rabbits. Equilibration was attained at 30 minutes. The disappearance slope was steeper than for the radiosodium space, so that the rate of removal of thiocyanate from the plasma was roughly 20 per cent hour. Since, in the routine determination of the thiocyanate space, values were not extrapolated to zero time, and because of the rapid rate of removal of the ion from the circulation, 30-minute samples were drawn to compare with the one-hour radiosodium samples.

TABLE 1. MEAN VALUES OF SERIAL DETERMINATIONS OF FLUID SPACES IN NORMAL RABBITS

RABBIT NO.	DAYS OF OBSERVATION	TOTAL NO. DETERMIN.	PLASMA VOLUME	RED CELL VOLUME	TOTAL BLOOD VOLUME	HEMATOCRIT	THIOCYANATE SPACE
			cc/kg.	*cc/kg.*	*cc/kg.*	*Vol. %*	*cc/kg.*
1	46	16	45±4.5[1]	36±4.4	73±7.0	38±3.1	205±17
2	30	11	50±5.0	27±4.4	78±8.2	35±3.2	213±[illegible]
3	30	13	40±5.4	25±3.6	73±9.7	33±4.2	191±[illegible]
4	31	9	43±4.6	20±2.8	62±6.0	31±3.6	177±[illegible]
5	31	11	40±7.1	27±5.8	73±11.1	36±3.4	197±17
6	32	11	46±4.9	21±3.5	67±6.3	34±4.8	173±18
Total	206	71	4[illegible]±5.5[2]	27±7.6	72±8.6	35±4.3	191±21

[1] Standard deviation. [2] Standard deviation of the series.

Plasma Volume. The mean plasma volume, based on a total of 67 determinations on 30 rabbits, was found to be 42.3 cc/kg. (table 2). Repeat determinations were made on a total of 28 animals (table 2). In 27 of these, the measurements were made within an interval of 2 to 14 days. In one animal, the interval between the 2 determinations was 50 days. The mean difference between such duplicate determinations was 4.14 cc/kg. There was no correlation of the interval between the determinations and the magnitude of the difference.

Hematocrit. The mean hematocrit, based on a total of 65 determinations on 30 animals, was 38.8 volumes per cent. The mean difference between duplicate determinations, based on 26 such pairs, was 4.46 volumes per cent. There appeared to be a rough correlation between the decrease in the second hematocrit as compared with the initial measurement and the time interval between the 2 readings, the decrease in the second hematocrit being greater, the shorter the interval between the determinations. Of the total of 20 duplicate determinations, in all cases except 4, the second hematocrit was equal to or less than the first. In these 4 instances, at least 14 days had elapsed between the 2 determinations. It appeared that with the volume of

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blood withdrawn for each study, at least 10 to 14 days were required for the hematocrit to return to the original value.

Total Blood Volume. The mean total blood volume, based on 95 determinations in 39 animals, was found to be 69.8 cc/kg. (table 1). Repeat determinations in 26 animals varied by a mean of 6.34 cc/kg. No correlation was noted of the interval between the repeat determinations and the magnitude of the difference between the 2 values.

Thiocyanate Space. The mean of 68 determinations of the thiocyanate space on a total of 39 rabbits, based on the 10-minute samples, was 217.8 cc/kg. (table 1). The mean of the 30-minute samples, based on a total of 50 determinations in 32 rabbits, was 237.6 cc/kg. The mean of the 30-minute samples, therefore, was 20 cc/kg. greater than the mean of the 10-minute samples. Thirty-minute samples revealed slightly less scatter than the 10-minute samples. The upper limit of the values for both the 10- and the 30-minute determinations was practically identical. Duplicate determinations of the 30-minute readings were more reproducible than the 10-minute

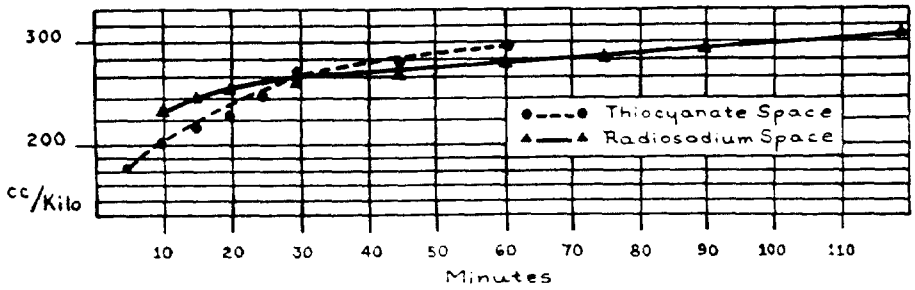

Fig. 1. THIOCYANATE AND RADIOSODIUM SPACES calculated from mean values at various time intervals.

readings, the mean difference in the former being 15.03 cc/kg. and in the latter, 8.66 cc/kg. There was no correlation of the interval between duplicate determinations at either 10 or 30 minutes and the magnitude of the difference between the 2 determinations.

Radiosodium Space. The mean radiosodium space, based on the one-hour samples on 68 determinations in 39 rabbits, was found to be 293 cc/kg. This value was 45 cc/kg. greater than the mean thiocyanate space based on the 10-minute samples, and 25 cc/kg. greater than the thiocyanate space based on the 30-minute samples. The variability of the 3 measurements was roughly equal, that of the 10-minute thiocyanate space being the least. The mean difference and the scatter of duplicate determinations of the radiosodium space was greater than those for the thiocyanate space at either 10 or 30 minutes.

Serum Sodium and Potassium Concentrations. The mean serum sodium concentration, based on a total of 55 determinations in 33 rabbits, was 131.9 mEq/l. with a standard error of 0.97. The mean serum potassium concentration, based on the same sera, was 4.58 mEq/l. with a standard error of 0.075. Serum sodium levels showed less random variation than serum potassium levels. There was no correlation

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of the interval between duplicate determinations of either serum sodium or potassium levels and the magnitude of the difference.

Tissue Radiosodium Space. The results of the analyses of the tissue radiosodium spaces in 6 normal rabbits are summarized in table 3. Of the tissues analyzed, the cartilage, skin and kidney contained the largest amount of radiosodium per unit weight. Skeletal muscle contained the least. Considerable variation was noted in the radiosodium space of some organs, e.g. subcutaneous connective tissue, liver, appendix and cartilage.

Total Water Content of Organs and Tissues. Total water content of the various tissues and organs are given in table 3. The appendix and the lung contained the largest amount of water per unit of weight of tissue and the tendon the least. The

TABLE 2. SUMMARY OF DATA ON EXTRACELLULAR FLUID VOLUME AND ELECTROLYTE CONCENTRATION IN NORMAL RABBITS

	RESULTS OF DETERMINATIONS IN 39 NORMAL ANIMALS			COMPARISON OF TWO DETERMINATIONS IN 29 ANIMALS		
	Total No. Determin.	Mean ± Stand. Deviation	Range	Total No. Duplicate Determin.	Mean Diff. ± Stand. Deviation	Range
Body weight (kg.)	68	3.17±.538	1.07–4.50			
Plasma volume (cc/kg)	57	42.5±7.38	20–58	25	4.14±3.25	0–11
Hematocrit (vol. %)	65	38.8±4.70	27–48	26	4.40±3.73	0–14
Total blood vol. (cc/kg)	65	69.8±9.12	50–93	26	6.34±5.25	0–20
Thiocyanate space (cc/kg., 10 min.)	8	217.8±17.8	177–270	20	15.03±10.53	0–50
Thiocyanate space (cc/kg., 30 min.)	50	237.0±17.3	210–271	18	8.[illegible]±8.42	0–26
Radiosodium space (cc/kg.)	8	[illegible]±22.1	223–308	20	[illegible]±[illegible]	0–82
Serum sodium (mEq/l.)	55	131.6±7.2	110–147	22	0.72±4.16	1–14
Serum potassium (mEq/l.)	55	4.50±0.57	[illegible]	22	0.30±0.20	0–1.0

coefficients of variation for the determinations of the total water content of any tissue or organ were usually smaller than those for the determinations of the radiosodium spaces.

DISCUSSION

In the initial experiment (table 1), it was found that when a series of 9 to 13 determinations of the plasma volume, hematocrit, total blood volume and the 10-minute thiocyanate space were made every second to third day in normal rabbit, the respective means and standard deviations were 46±5.5 cc/kg., 35±4.3 volumes per cent, 72±8.9 cc/kg. and 194±21 cc/kg. The values obtained in the subsequent studies involving a single or 2 repeated determinations on normal rabbits agreed fairly closely with the previous serial determinations. In the only other similar study

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of normal rabbits [illegible] found in the literature [illegible]. The mean of the determinations of the total blood volume in 96 rabbits was 70.[illegible] cc./kg., based on samples obtained 6 minutes after injection of the Evans Blue. The mean value of the plasma volume was 50.[illegible] cc./kg. The blood volume was found to be proportional to the body weight and not to the surface area. These results are consonant with the findings in the present investigation. No comparative data have been found regarding the thiocyanate space or the rate of disappearance of the ion from the blood stream in rabbits.

The results of the present study indicate that regardless of whether the thiocyanate determinations based on the 10- or 30-minute samples or the radiosodium space based on the one-hour sample are employed as an index of the extracellular fluid volume, consistently reproducible results can be obtained, at least in normal rabbits. There is valid argument against the use of the 10-minute readings, since equilibration had not occurred at that time. However, for technical reasons, this

TABLE 3. SUMMARY OF DATA ON TISSUE RADIOSODIUM SPACE AND TOTAL WATER CONTENT IN 6 NORMAL RABBITS

	TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION		TISSUE RADIOSODIUM SPACE MEAN ± STAND. DEVIATION	TISSUE WATER CONTENT MEAN ± STAND. DEVIATION
Body weight, kg.	3.[illegible]0±0.15		Tissues (*Cont.*)		
Radiosodium space (% body weight)	24.[illegible]9±1.10		Liver	15.20±5.36	70.34±1.73
Tissues			Abdominal muscle	1[illegible].20±3.5[illegible]	6[illegible].2[illegible]±1.44
Skin	44.05±6.85	63.00±3.78	Heart	23.07±2.1[illegible]	77.07±3.1[illegible]
Subcutaneous connective tissue	31.17±6.04	62.97±7.45	Kidney	42.24±0.53	76.3[illegible]±0.99
Diaphragm	15.02±2.20	75.70±6.1[illegible]	Appendix	23.56±0.59	80.21±1.05
Stomach	16.79±2.58	76.28[illegible]±1.58	[illegible]	24.[illegible]±[illegible]	77.[illegible]±1.06
Lung	17.[illegible]±3.[illegible]	7[illegible]±0.96	Eye (aqueous)	52.01±10.[illegible]	7[illegible].[illegible]±2.22
Adrenal	14.98[1]±1.08	65.75±8.[illegible]	Skeletal muscle	10.07±1.41	75.[illegible]±3.[illegible]
			Achilles tendon	30.00±4.[illegible]	60.21±3.58

[1] All values expressed in percentage [illegible]. [2] [illegible]

has proved to be the most practical method when serial determinations are made on a single animal. Actually, the 30-minute thiocyanate determinations revealed less scatter than either the 10-minute thiocyanate determinations or the radiosodium determinations. The exact significance of the thiocyanate space, however, remains in doubt. Determinations of the thiocyanate space in various tissues and organs and their comparison with the sodium space might clarify this problem. The present data indicate that in normal rabbits the thiocyanate for an equilibration consistently measures a space roughly 10 per cent less than the space measured by radiosodium.

The mean radiosodium space of 26.3 per cent of the body weight found in the present study agrees with the values obtained by previous investigators with a smaller number of rabbits (6–8). Manery and Bale (8) compared the radiosodium content of rabbit tissues with their content of chemically determined sodium and found that the specific activity of all tissues except the nervous tissue was constant. They concluded that "with the exception of the nervous tissue, in 1 hour 8 minutes, injected

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radioactive sodium becomes distributed between tissues and plasma in precisely the same ratio as normally occurring sodium. Most of the tissue sodium values reported by Manery and Hastings (9) were higher than those obtained in the present study. One possible explanation for this discrepancy may be the difference in the methods employed in sacrificing the animals. Decapitation of animals does not result in as thorough exsanguination as severing the neck vessels. The two-fold discrepancy in the values for cartilage is difficult to explain.

The total water content of tissues obtained in the present study are summarized in table 3. These values agree well with those obtained by Manery and Hastings (9) and by Skelton (10). Less discrepancy was noted than with the determinations of the radiosodium space.

SUMMARY AND CONCLUSIONS

The normal values and their random variations for the extracellular fluid compartments in normal rabbits were determined. Plasma volume was determined by the T-1824 dye method; the thiocyanate space, by the dilution of sodium thiocyanate; and the radiosodium space, by the dilution of Na^{24}. Serum sodium and potassium concentrations were measured by flame spectrophotometry. In a series of 39 animals, the plasma volume was found to be 42.3±7.38 cc/kg.; the total blood volume, 69.8±0.12 cc/kg.; the thiocyanate space, 217.8±17.8 cc/kg.; and the radiosodium space, 203.2±22.1 cc/kg. The serum sodium concentration was 131.0±7.2 mEq/l.; the serum potassium concentration, 4.58±0.56 mEq/l. The mean difference between 2 determinations in 20 rabbits was as follows: plasma volume, 4.14±3.25 cc/kg.; total blood volume, 6.34±5.25 cc/kg.; thiocyanate space, 15.03±10.53 cc/kg.; radiosodium space, 10.55±15.61 cc/kg. Seventy-one serial determinations were made on 6 rabbits over an aggregate period of 206 days. The results were as follows: plasma volume, 40±5.5 cc/kg.; total blood volume, 72±8.9 cc/kg.; thiocyanate space, 164±21 cc/kg. Data based upon tissue analyses for total water content and radiosodium space are given.

The total blood volume, calculated from the plasma volume and the hematocrit, was 69.8 cc/kg. This value was constant in normal rabbits and appeared to be directly related to the body weight. A previous investigator (10) had obtained practically the identical value, so that the constancy of this value appears to have been established.

The exact significance of the thiocyanate space remains doubtful and will not be resolved until comparative studies of the concentration of the sodium ion and the thiocyanate ion in various tissues and organs are made. From the present study it can only be concluded that the thiocyanate ion at equilibrium measured a volume which was approximately 10 per cent less than that measured by the radiosodium ion. Whether this relationship would continue in abnormal physiologic states remains to be studied. Presumably the radiosodium ion at one hour measured in various organs and tissues the volume of fluid which was extracellular. The thiocyanate determinations were reproducible in normal rabbits, regardless of whether 10- or 30-minute samples were compared. The random variations in the values for the thiocyanate or the Na^{24} space were roughly equivalent. Tissue sodium spaces are probably not com-

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parable unless a uniform technique is utilized in sacrificing the animals. Since it is obvious that variations in the amount of residual blood in the organs and tissues would alter their sodium content. Since serial determinations of the fluid volume spaces are more reproducible in individual animals than single determinations in separate animals, experiments should be so designed as to take this observation into consideration.

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Progress report on the research project

FORMATION OF PHOSPHOLIPIDS IN TISSUES

sponsored by the Atomic Energy Commission, Division of Biology and Medicine under contract No. AT-(40-1)-[illegible], Title II, with the Bowman Gray School of Medicine of Wake Forest College.

Project leader: Dr. Camillo Artom

The present report covers the work done from March 16, 1951, through March 15, 1952. This work is a direct continuation of that previously done with the aid of the Atomic Energy Commission and summarized in my progress report of March 15, 1951. In addition to the writer, the following investigators participated in the research work for various periods of time: [illegible] Ph.D., M. Crowder, M.S., F. Mitchell, M.S. Technical assistance was given by G. Fortuna, and H. Pollard.

A. General outline of the work.

The experiments on the Incorporation of P^{32} labeled choline in the lipides of isolated liver tissue have been completed and our previous findings [illegible] in a preliminary form) have been confirmed and extended. Both groups of results are now being organized for presentation in the complete papers.

The study on the Incorporation of labeled dimethylethanolamine (a likely precursor of choline) into the lipides of the isolated liver has been carried out actively. The data will be reported shortly in a summarized form and it is believed that only a limited number of additional experiments will be required for completion of this study. The labeled compound has been synthesized by a method which presents some unusual features. These have been described in a note which is now in press.

Some progress has been made in the investigation of the metabolic pathways of choline in the isolated liver. It is felt, however, that on several points definite conclusion can not yet be drawn and that the present data represent merely an indication for future work.

Studies on the metabolism of formaldehyde have been continued. Previous findings on the occurrence in most tissues of an enzyme condensing formaldehyde with pyruvic acid have been confirmed. While the products of this reaction have been only partially characterized, it appears that the reaction might be of considerable importance in [illegible] metabolism (including the formation and transfer of methyl groups). One preliminary report has been published and another will appear shortly.

Further details on each group of investigations are given below.

B. Incorporation of labeled choline into the phospholipides of isolated liver slices.

Definitive evidence has been obtained for the concept that the whole molecule of choline is incorporated as a unit into the phospholipides of the isolated liver. Some of the recent experiments made for such a purpose were (a) incubation of slices from the same liver with choline labeled either in the methyl, or in the ethylene moiety; (b) hydrolysis of the isolated lipides

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synthesized in vitro and determination of the radioactivity in various [illegible] of the lipide hydrolyzates; (c) degradation of the choline separated from these hydrolyzates and localization of the isotope in the same position as in the choline added to the tissue slices.

In other experiments a direct comparison has been made between the incorporation of labeled phosphate and labeled choline into the lipides of slices or homogenates prepared from the same liver. The results have confirmed our previous statement that the two processes are affected in quite different manners by a number of factors, such as homogenization of tissue, substitution of nitrogen for oxygen of air, addition of [illegible] inhibitors, previous dietary history of the animals. It appears definitely that the synthesis of the ester bond between choline and the remaining part of the lecithin molecule has a relatively low requirement of energy and can proceed independently from the formation of the phosphate ester bonds presumably. This would make possible the incorporation of choline and of the molecule [illegible] the whole compound. The findings also suggest that the [illegible] into another with a different nitrogen substituent, possibly as a result of a change in the concentrations of the [illegible] components within the cells. The results of the experiments [illegible] DME (or betaine) [illegible] further indications in the same direction.

C. Incorporation of labeled dimethylethanolamine (DME) into the phospholipides of liver tissue.

The labeled compound (synthesized by our method) was added to slices or homogenates of liver from rats maintained on various diets. A [illegible] part of the added compound was incorporated directly into the phospholipides. The isotopic values in slices from rats on adequate stock diet were smaller than in those from animals on choline-deficient diets. Under both conditions, DME incorporation in the lipides was greater in nitrogen than in oxygen or air. Homogenization of the tissue reduced but did not suppress the process. Addition of non-isotopic choline inhibited the incorporation of DME, whereas addition of non-isotopic DME inhibited the incorporation of isotopic choline into the phospholipides.

Only a minor proportion of the [illegible] in the lipides extracted after incubation. Furthermore, the amounts of labeled choline in the lipides of the incubated tissue are not significantly increased by the addition of methionine or betaine when [illegible] rats (although the formation of DME containing lipides is depressed under [illegible]). When [illegible] deficient animals and methionine was added to the [illegible] C^{14} in the lipide choline was definitely increased at the expense of the [illegible] of the [illegible] present in the lipides.

These results indicate that choline and DME are [illegible] phospholipides under conditions which are essentially similar, but probably by the activity of the same enzymatic system for which the two compounds compete. Accordingly the relative properties of DME and choline [illegible] phospholipides will depend upon their respective concentrations in the cells. DME is methylated to choline, but since the choline thus formed is rapidly oxidized to betaine aldehyde and betaine by liver slices of normal rats, [illegible] amounts of labeled choline formed from DME are found only [illegible]

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conditions in which the methylation of DME is enhanced and the activity of choline oxidase is depressed (slices from rat liver incubated in the presence of methionine and labeled DME).

b. Metabolic Pathways of choline in isolated liver.

The data obtained thus far gives definite information on the following points: (a) labeled trimethylamine (TMA) and TMA oxide were found after incubation of liver slices with labeled choline. It can now be stated that the formation of such compounds is due to [illegible] activity of liver and not to a bacterial contamination since the results were not affected by the addition of antibiotics ([illegible]). (b) a [illegible] portion of the labeled choline [illegible] could be recovered at the end of the incubation by a [illegible] extract. These findings [illegible] to us the [illegible] phosphate ester as one of the major intermediates [illegible] phoryl-choline [illegible] analysis. Analysis of the fractions separated by [illegible] ties. Before these possibilities can be [illegible] that the separation [illegible] used thus far should be [illegible] to liver slices causes a moderate increase in the oxygen consumption, but no such increase is found when DME is added to liver homogenates which oxidize added choline very actively. It seems therefore, that DME is [illegible] likely precursor of choline, and that methylation to choline is the main metabolic pathway of DME in the liver, little or no DME being oxidized [illegible] dimethylglycine. (d) On the other hand, it was further confirmed that partial demethylation of choline with formation of DME does not occur in the liver, the major products of choline degradation in the isolated liver being betaine aldehyde and betaine.

. Metabolism of Formaldehyde in Tissues

The widespread occurrence in animal tissue of a formaldehyde-pyruvate condensing enzyme was described by us in a previous report. Most of our preliminary data have been already confirmed in investigations carried out independently in other laboratories. We have now studied further the properties of the enzyme in a partially purified preparation (mitochondria, of rat liver). Additional experiments with [illegible] labeled formaldehyde have confirmed and extended our previous [illegible] characteristics of the addition product. The more recent information indicates that the enzyme is not a carboxylase [illegible] and is [illegible] part of the added formaldehyde [illegible] recovered by oxidation with HIO[illegible]. It appears now that the initial product of the reaction is a [illegible] adjacent [illegible] product, either [illegible] enzymatically.

[illegible]

It is believed that the results summarized above represent definite advancement in the development of our investigations. This has been accomplished in spite of adverse circumstances which have reduced considerably the efficiency of our [illegible] the full members of the Department of Biochemistry [illegible] the working personnel workers. The [illegible]

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on this project during 1950-1951, also left in July, and could be replaced only by a less skillful person who had to be trained for our specialized type of work. It is hoped that we will be able in the near future to make up for the present deficiencies in both professional and technical workers and thus speed up the investigative effort on this project.

6. Bibliography

From March 16, 1951, through March 15, 1952, the following papers based on the results of the investigations of this project have been published:

1. "Incorporation of Labeled [illegible] into the Lipides of Liver [illegible]", By C. Artom and M.A. Swanson, [illegible] Vol. 189, [illegible]

2. "The Synthesis of Choline (Methyl-C^{14}) [illegible]", By [illegible] Vol. 87, [illegible]

3. "Mechanism of Action of Lipotropic Factors in Animals", By [illegible] Transaction of the Fourth Conference on Liver Injury, J. May, [illegible] Pp. 32-40 (1951)

4. "Incorporation of Labeled Choline in Phospholipides of Isolated Liver Tissue", By C. Artom, E. Crowder, and M.A. Swanson, Federation Proc., Vol. 10, pp. 167-168 (1951)

5. "Enzymatic Disappearance of Formaldehyde in the Presence of Pyruvate", By F. Mitchell and C. Artom, Federation Proc., Vol. 10, pp. 224-225 (1951)

The following paper is presently in press:

6. "Preparation of C^{14} Methyl Labeled Dimethylaminoethanol", By C. Artom and E. Crowder, J. of Amer. Chem. Soc., Vol. 74

The following preliminary reports are on the program of scientific meetings:

7. "Incorporation of Labeled Monoethanolamine in Phospholipides of Isolated Liver Tissue", By E. Crowder and C. Artom, Am. Soc. of Biol. Chemists, New York, April 1952.

8. "Formaldehyde-pyruvate Condensing Enzyme", By F.C. Mitchell and C. Artom, Am. Soc. of Biol. Chemists, New York, April 1952.

Comprehensive surveys of the results obtained in the [illegible] recent investigation on this subject will be presented by C. Artom at a Symposium on Phosphorus Metabolism (Johns Hopkins University, Baltimore, Maryland, June 1952) and at the Second International Congress of Biochemistry (Paris, France, July 1952).

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Emilio Artom, M.D.

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Enzymatic disappearance of formaldehyde in the presence of pyruvate. PEGGY MITCHELL* AND CAMILLO ARTOM. *Dept. of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, N. C.*

Formaldehyde, incubated with homogenates of most animal tissues, is partly converted into compounds not detectable with chromotopic acid. The addition of pyruvate greatly enhances the rate of disappearance of formaldehyde. A similar, but less marked effect is obtained with alpha-ketoglutarate and oxaloacetate. From various lines of evidence (formation of the 2,4 dinitrophenylhydrazine derivative, oxidation with periodate, isotope determinations after incubation with $HC^{14}HO$) it appears that the reaction might be: $CH_3{\cdot}CO{\cdot}COOH + H{\cdot}CHO \rightarrow CH_3{\cdot}CO{\cdot}CH_2OH + CO_2$. The enzyme responsible for this reaction is stable at 37° and is completely inactivated only at relatively high temperatures (one hour at 60°-65°). It is resistant to concentrations of iodoacetate which inhibit the disappearance of formaldehyde in the absence of pyruvate. The enzyme is present in the large granule fraction ('mitochondria') of rat liver, little, or no formaldehyde being removed by the granules without added pyruvate. Our preparations were not activated by Mg^{++}, thiamine or cocarboxylase. However, the differentiation of the formaldehyde-pyruvate condensing enzyme from the diphosphothiamine-metal-proteins catalyzing the formation of acyloins from other aldehydes and pyruvate (GREEN *et al. J. Biol. Chem.* 145: 69, 1942) remains uncertain. (Work performed under Contract AT-(40-1)-288, Title II, with the U. S. Atomic Energy Commission).

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March, 1951, Vol. 10, No. 1, Part I
Printed in U. S. A.

Incorporation of labeled choline in phospholipides of isolated liver tissue. CAMILLO ARTOM, MARTHA CROWDER* AND MARJORIE A. SWANSON. *Dept. of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, N. C.*

C^{14} labeled choline chloride was added to slices or homogenates of liver from rats previously maintained on various diets. After $3\frac{1}{2}$ hours of incubation in Krebs Ringer phosphate (pH 7.4), the lipides were extracted and their contents of C^{14}, choline and phosphorus were determined. In oxygen the percentage of added choline incorporated into the phospholipides is much smaller with slices of liver from rats on stock diet than with slices of liver from rats maintained on low protein diets, with or without added guanidoacetic acid. Substitution of nitrogen for oxygen decreased the incorporation of choline in the lipides of the liver from rats on the low protein diets, but not from rats on stock diet. In both groups of animals homogenization of the liver reduced marked, but did not suppress completely, the uptake of labeled choline by the lipides. *In vitro* addition of ethanolamine and diethanolamine (0.003 M; rats on stock diet; slices in O_2) inhibited, whereas addition of betaine *in vitro* enhanced, the process. A comparison between the incorporation of labeled phosphate and labeled choline in the phospholipides of isolated tissues suggests that formation of the linkage between choline and the phosphoric acid moiety may occur to a certain extent independently of the formation of the other ester bonds in lecithins. (Work performed under Contract AT-40-1-288, Title II, with the U. S. Atomic Energy Commission.)

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INCORPORATION [illegible] LIPIDS OF LIVER SLICES

By CAMILLO ARTOM and MARJORIE A. SWANSON

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INCORPORATION OF LABELED PHOSPHATE INTO THE LIPIDES OF LIVER SLICES*

By CAMILLO ARTOM AND MARJORIE A. SWANSON

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(Received for publication, March 12, 1951)

Animals on diets with a low protein content develop a fatty infiltration of the liver, which is easily prevented or relieved by choline administration. These effects are generally interpreted by assuming that phospholipides play a major rôle in the transport of fatty acids out of the liver (or in their metabolism within the tissue). Since choline is an essential component of lecithins and sphingomyelins, a dietary deficiency of choline (or of its precursors) would lead to an accumulation of fat in the liver because of an impairment in the formation of phospholipides. Actually, in the livers of rats on low protein diets the increase in neutral fat is accompanied by a decrease in the level of total phospholipide (2). However, this level is not restored to normal by the addition of choline alone to the diet (2, 3). Moreover, the rate of phospholipide synthesis, as measured by the incorporation of P^{32} into the phospholipides, is not appreciably decreased in the livers of intact rats on "alipotropic" diets (4–6).

In view of the greater simplicity of the conditions of experiments *in vitro*, it was hoped that some useful information on this and other aspects of the biological synthesis of phospholipides could be obtained by studying the incorporation of inorganic phosphate into the lipides of liver slices from rats previously maintained on various diets. The effects of a number of factors (such as level of protein in the diet, amount of choline available to the animals, addition of choline and related compounds to the liver slices *in vitro*, etc.) were tested. The most definite indication which could be obtained is that the ability of the isolated tissue to synthesize phospholipides from inorganic phosphate is markedly reduced by a low intake of proteins. Furthermore, it appears that the response of the liver slices to the addition *in vitro* of choline is also dependent upon the previous dietary history of the animals.

* With the technical assistance of M. L. Haigler. A preliminary report was presented before the annual meeting of the American Society of Biological Chemists, Atlantic City, April, 1950 (1). This work was performed under a contract (No. AT-(40-1)-288, Title II) between the United States Atomic Energy Commission and the Bowman Gray School of Medicine. The P^{32} used in the experiments was obtained from the Clinton National Laboratories, Oak Ridge, Tennessee, on allocation from the United States Atomic Energy Commission.

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EXPERIMENTAL

Male albino rats raised on a stock diet (Rockland Farms rat diet, complete) were sacrificed or transferred to an experimental diet for various lengths of time (6 to 35 days). One of these (Diet 26 (7)) contained 5 per cent casein; the other (Diet 29 (8)) contained 25 per cent casein. In several experiments, the animals were further depleted of transferable methyl groups (9) by the addition of 0.5 per cent guanidoacetic acid (GAA) to Diet 26. In other experiments the choline deficiency was corrected by adding 0.4 per cent of choline chloride to Diet 26. A mixture of pure B vitamins (8) was incorporated daily into the experimental diets.

The livers of two rats, maintained on different diets, were often used in one experiment. The animals were killed by decapitation, and the livers rapidly removed, chilled in 0.9 per cent NaCl, and sliced free hand. Between 500 and 800 mg. of slices were added to 6 ml. of Krebs-Ringer phosphate solution, pH 7.3, containing 50 mg. of glucose, any other desired addition, and approximately 2 μc. of P^{32} (as sodium phosphate). The flasks were shaken 4 hours in a Warburg bath at 37°, in the presence of air or of a 5 per cent CO_2-95 per cent O_2 mixture. At the end of the incubation period, 12 ml. of 10 per cent trichloroacetic acid (TCA) were added. The precipitate was centrifuged, washed with three 6 ml. portions of 5 per cent TCA, and dehydrated overnight with acetone. It was then extracted three times each with boiling ethanol and ethanol-ether (2:1). The combined acetone-ethanol-ether extract was evaporated on a steam bath, and dried 3 hours *in vacuo* at 45°. The residue was dissolved in chloroform and the solution filtered. Aliquots of the filtrate were taken for the determination of total lipides (by weight, after evaporation of the solvent) and lipide P (after incineration in HNO_3-H_2SO_4 (10)). "Neutral fat" was estimated from the difference between the total lipides and the total phospholipides (lipide P × 25). In order to eliminate the last traces of inorganic P^{32}, the remainder of the chloroform solution was shaken with 1 gm. of Na_2HPO_4 and a few drops of water for 5 minutes. The solid was sedimented by centrifugation, and an aliquot of the supernatant solution taken for determination of radioactivity (11). In all experiments, at least one blank was prepared from each liver. This was treated exactly like the experimental samples, except that TCA was added immediately after the P^{32}. The counts in the lipides extracted from the blanks were usually 1 to 2 per cent, and never more than 5 per cent, of the counts in the lipides of the experimental samples. It seems therefore that our technique for removal of inorganic phosphate, although simpler than that used by other investigators, is adequate for our purpose.

The inorganic P was precipitated with magnesia mixture from an aliquot of the TCA extract. This precipitate was taken up in a few drops

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of 1 N HCl, diluted with H_2O, and used for the determination of P^{31} (10) and P^{32} (11). In preparing the samples for radioactivity measurement, addition of a few ml. of acetone facilitated the evaporation of H_2O and the deposition of an even film of salts. The samples were brought to a uniform weight by addition of a solution of non-radioactive lipides. Because of the presence of a comparatively large amount of phosphate in the buffer, the specific activity of the phosphate decreased by not more than 10 per cent during the incubation. In preliminary experiments in which other buffers were used (citrate, bicarbonate, glycerophosphate), the specific activity of the inorganic P was initially much higher, but declined to about one-fourth of its original value during the incubation. Variations of this magnitude would obviously increase the uncertainty in the calculation of the amounts of labeled phospholipides synthesized by the liver slices.

Calculations—Since the amounts of tissue, inorganic phosphate, and radioactivity in the samples varied somewhat from experiment to experiment, the results, corrected for the blanks, were calculated for 1 gm. of moist tissue, and divided by the specific activity of the inorganic phosphate. The formula used was as follows:

$$\left(\frac{\text{Counts per sec. in } E}{\text{Gm. tissue}} - \frac{\text{counts per sec. in } B}{\text{gm. tissue}}\right) \Big/ \frac{\text{Counts per sec. in } I}{\gamma\, I}$$

(where E = lipides from the experimental flask; B = lipides from the blank flask; I = inorganic P from the blank flask). The values obtained express the micrograms of inorganic P incorporated into the phospholipides, or, multiplied by 25, the micrograms of labeled phospholipides formed by 1 gm. of tissue. These are minimal values, since the calculation does not take into account several factors which tend to lower the concentration of P^{32} in the lipides, such as the increase in the amount of inorganic P during the incubation, the synthesis of phospholipides from preformed (hence unlabeled) precursors, or the breakdown of newly formed phospholipides before termination of the experiment.

Results

Effects of Diet—In Table I we are reporting representative data from experiments in which slices were prepared simultaneously from two rats maintained for the same length of time on different diets. The rats were of the same age, but of different weights because the animals on deficient diets grew poorly. In the livers of these rats, neutral fat was increased and total phospholipides were decreased, as expected (2). Slices of these livers incorporated inorganic P^{32} to a much lesser extent than liver slices from rats on the stock diet (Experiment 21). This difference is apparent

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in both the absolute and relative amounts of newly formed phospholipides. Addition of choline to the deficient diet neither prevented (Experiment 25) nor corrected (Experiment 23) this decrease, although the amount of neutral fat in the liver was reduced nearly to normal. Therefore the decreased synthesis of phospholipides in the isolated tissue cannot be ascribed to an interference by the excess fat with the penetration of P^{32} from the medium or with the enzymatic activities involved in phospholipide formation. When the rats were maintained on a purified diet containing 25 per cent casein (corresponding to the protein level of the stock diet), their livers synthesized phospholipides at a high rate (Experiment 26).

TABLE I

Incorporation of Inorganic P^{32} into Lipides of Liver Slices from Rats on Various Diets

Experiment No.	Diet No.	Days on diet	Per gm. moist tissue			Labeled phospholipides in total phospholipides
			Neutral fat	Total phospholipides	Inorganic P incorporated in lipides	
			mg.	*mg.*	*γ*	*per cent*
21	Stock		15	15.6	15.3	2.44
	26 + GAA*	15	81	9.0	3.3	0.91
25	26 + choline†	6	15	11.5	6.4	1.42
	26 + GAA*	6	60	9.6	6.9	1.78
23	26 then 26 + choline*	16 then 12	31	9.0	3.0	0.83
	26	28	176	7.9	2.3	0.73
26	29	16	16	14.9	13.7	2.29
	26	16	59	11.3	1.7	0.38

* Guanidoacetic acid, 0.5 gm. in 100 gm. of diet.

† Choline chloride, 0.4 gm. in 100 gm. of diet.

These findings indicate that the differences observed in the rate of phospholipide synthesis depend upon the protein level of the diet rather than upon the supply of choline or choline precursors, or upon some undetermined factors which may be present in the mixture of crude animal and vegetable products of the stock diet.

A statistical analysis of our data is presented in Table II. The figures have been calculated for both the moist weight and the dry, fat-free weight of the tissue, since the amounts of neutral fat in the liver differed considerably between the two groups of animals. By both types of calculations the mean differences between the groups on the high and low protein diets have a high degree of statistical significance.

Effects of Addition in Vitro of Choline and Related Substances—*In vivo* the administration of a single large dose of choline (13) or betaine (14)

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to rats on low protein diets increases the rate of phospholipide turnover as measured with P^{32}. Similar effects have been noted after administration of ethanolamine (15, 16), which is not clearly lipotropic. When the animals had been on adequate diets, choline caused little or no stimulation

TABLE II

Statistical Analysis of Results of Eleven Directly Comparable Experiments

	Inorganic P incorporated into lipides			t*
	High protein diets†	Low protein diets‡	Mean difference	
	γ	γ	γ	
Per gm. moist tissue	11.86	2.91	8.95 ± 1.38§	6.46
" " dry, fat-free tissue	45.5	10.9	34.6 ± 5.24§	6.61

* For $N - 1 = 10$ (degrees of freedom) and $P = 0.01$ (probability for a chance occurrence), $t = 3.169$ (12).

† Stock diet or Diet 29.

‡ Diet 26 or Diet 26 + GAA.

§ Standard errors of the mean differences.

TABLE III

Effects of Addition in Vitro of Choline Chloride to Liver Slices of Rats on Various Diets

Choline added (final concentration)	Rats on high protein diets*			Rats on low protein diets†		
	Degrees of freedom	Average change‡	t	Degrees of freedom	Average change‡	t
M		*per cent*			*per cent*	
8×10^{-3}	9	−19	4.0	8	+45	3.0§
3.3×10^{-3}	13	−29	6.4	11	+31	5.1
1.7×10^{-3}	16	−45	12.2	9	+51	3.8

* Stock diet or Diet 29.

† Diet 26 or Diet 26 + GAA.

‡ Increases (+) or decreases (−) in per cent of the values from the control flasks to which no choline was added.

§ $P < 0.05$. All other t values indicate a probability for a chance occurrence less than 0.02 (12).

(7). In the present experiments various concentrations of choline and of a few related substances were added to the liver slices *in vitro*. Under these conditions, the action of choline depends upon the previous dietary régime in qualitatively the same way as had already been observed *in vivo*. When choline was added to liver slices from rats on the low casein diet, the incorporation of P^{32} into the lipides was somewhat increased, but not nearly enough to reach the values found in the livers of rats on the

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high protein diets. There was no difference in the effect of choline *in vitro*, whether the rats had been fed Diet 26 alone or Diet 26 supplemented with GAA. On the other hand, choline definitely depressed the rate of phospholipide synthesis when the liver slices had been obtained from rats on the stock or high protein diet. The changes caused by the addition *in vitro* of choline to the liver slices of rats on either high or low protein diets appear to be statistically significant (Table III). In one experiment with liver slices from a rat on the stock diet, the phospholipides were fractionated according to Taurog *et al.* (17). It was found that the formation of both choline-containing and non-choline-containing phospholipides was depressed to approximately the same extent.

The effects of ethanolamine and betaine added *in vitro* will be mentioned only briefly, since these were less marked and less consistent than the effects of choline *in vitro*. In the livers of rats on the stock diet, usually ethanolamine inhibited phospholipide formation, while betaine stimulated the process. Increases as high as 50 per cent were found after addition of betaine in concentrations of 1.7×10^{-2} M or 3.3×10^{-5} M, but no such effect was observed when the substance was present at lower concentrations.

DISCUSSION

The present findings indicate that in rats on low protein diets there is a definite reduction in the ability of the isolated liver to synthesize phospholipide from inorganic phosphate. The association of this finding with a fatty infiltration apparently supports the commonly accepted explanation of the lipotropic action of choline. However, since supplementation of the diet with choline relieves the fatty infiltration but does not restore the ability of the liver slices to synthesize phospholipides, there seems to be no simple relationship between the two processes. The lowered rate of phosphate incorporation probably is more directly related to the decreases in cytoplasmic proteins (18, 5), especially in some of the oxidative enzymes (19–23), which occur rapidly in livers of protein-depleted rats. A decrease in oxidative systems of the cells might well account for our findings, because oxidations are necessary for the incorporation of inorganic phosphate into the lipides of liver slices (24). It is also possible that the inhibition by choline added *in vitro* to liver slices from rats on the stock diet may be explained in a similar way, since oxidation of choline by liver slices *in vitro* is apparently accompanied by a reduction in the endogenous respiration (25).

In conclusion, we would like to point out that the present findings concern only the incorporation of inorganic phosphate into the phospholipides.

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and therefore do not necessarily apply to the synthesis of the other ester bonds present in these compounds.[1]

SUMMARY

Slices from the livers of rats previously maintained on a 5 per cent casein diet incorporated inorganic P^{32} into the lipides at a lower rate than liver slices from rats on a stock diet or on a 25 per cent casein diet.

Supplementation *in vivo* of the low protein diet with choline prevented or reversed the fatty infiltration but did not raise the ability of the isolated liver to synthesize phospholipides from inorganic phosphate.

The addition of choline *in vitro* inhibited the formation of phospholipides in the liver slices from rats on the high protein diets. When the rats had been previously maintained on low protein diets, choline added *in vitro* to the liver slices stimulated the process to a certain extent.

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THE SYNTHESIS OF CHOLINE (METHYL-C^{14}) CHLORIDE
~~CONTAINING METHYL-C^{14}~~

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THE SYNTHESIS OF CHOLINE (METHYL-C^{14}) CHLORIDE ~~CONTAINING METHYL-C^{14}~~

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The preparation of choline (methyl-C^{14}) chloride was undertaken at the request of Dr. Camillo Artom of the Bowman Gray School of Medicine, Winston-Salem, N. C. The compound had not at that time been reported in the literature, although Du Vigneaud and Verly (1) reported the *in vivo* preparation of it after this work was completed.

An attempt was made to prepare the compound following the directions used by Du Vigneaud (2) in preparing choline chloride containing deuterium; the method consists of condensing methyl iodide with ethanolamine in alkaline solution, followed by precipitation of choline as the reineckate and decomposition of the reineckate; yields using this method proved to be unsatisfactory. The method finally developed is shown in the accompanying equations; *3*-dimethylaminoethanol (I) was condensed with labeled methyl iodide (II) in benzene solution at room temperature; choline iodide (III) precipitated in substantially quantitative yield. Choline iodide was changed into choline chloride by shaking with an aqueous suspension of freshly precipitated silver chloride; the yield was over 95%.

$$\underset{\text{I}}{CH_3\ N(CH_3)CH_2CH_2OH} + \underset{\text{II}}{C^{14}H_3I} \xrightarrow{\text{benzene}} \underset{\text{III}}{(CH_3)_3\overset{C^{14}H_3}{N}CH_2CH_2OH\ I}$$

Anhydrous choline chloride could be obtained by evaporation *in vacuo*; this was done for analytical purposes, but the main portion of the labeled choline chloride was kept in solution made 0.1 N with hydrochloric acid.

Several runs using inactive materials indicated the over-all yield to be better than 95% based on methyl iodide; the choline chloride and choline iodide were both isolated and analyzed for nitrogen. Analysis for *3*-dimethylaminoethanol by the method of Artom and Crowder (3) showed it to be absent from the choline chloride solution.

The active material was prepared using one millicurie of methyl iodide supplied by Tracerlab, Inc.; analysis of the choline chloride showed it to contain an activity of one millicurie per millimole.

Experimental

Twenty runs were made with inactive materials to find the optimum conditions; the final run made with the "hot" methyl iodide is described below.

The tube containing one millimole of methyl-C^{14} iodide (fitted with a break-off

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seal) obtained from Tracerlab was sealed to the vacuum line and the line evacuated. A dry-ice acetone bath was placed around the methyl iodide; it was noted that the liquid did not solidify, as pure methyl iodide does at that temperature. The seal was broken, and the methyl iodide allowed to distill into a conically shaped reaction tube containing 20 ml. of benzene, the tube and contents being cooled with liquid air while the tube containing the methyl iodide was warmed to room temperature. When the transfer was complete the reaction tube was allowed to come to room temperature and 1.5 millimoles (0.133 g.) of β-dimethylaminoethanol (b. p. 133–135°) was added; a drying tube was attached and after shaking to insure mixing the solution was set aside for 48 hours. It was noted that a cloudiness developed within five minutes, and a copious precipitate formed within an hour.

After 48 hours the solution was cooled to 5°C. and the benzene removed with the aid of a filter stick; the reaction tube was placed on the vacuum line and the last traces of benzene removed *in vacuo*. The trace of choline iodide adhering to the filter stick was rinsed into the reaction tube with 5 ml. of water. The carefully washed silver chloride formed from 2 g. of silver nitrate, was added to the tube, and the tube shaken on the shaker overnight. The aqueous solution was filtered from the yellow silver iodide with a clean filter stick; the precipitate was washed with several small amounts of water, each being added to the original filtrate until 10 ml. had been collected. A small quantity (0.083) ml. of conc. hydrochloric acid was added to the solution. After stirring, an aliquot was removed for carbon 14 analysis; this was done by evaporating the sample to dryness, burning to carbon dioxide, and measuring the activity in an ionization chamber on a Vibrating Reed Electrometer.

With the method above using inactive materials, yields of 95–98% of choline chloride based on methyl iodide were realized. A sample of inactive choline iodide was analyzed. Calcd. for $C_5H_{14}ONI$: N, 6.06. Found: N, 5.94, 6.05. A sample of inactive choline chloride was also analyzed. Calcd. for $C_5H_{14}ONCl$; N, 10.0. Found: N, 9.95, 10.1.

Acknowledgment: The aid of Dr. Paul H. Cheek in some of the analytical work and of Dr. R. L. McKee in discussing the problem and supplying the dimethylaminoethanol is gratefully acknowledged. This work was done under a contract between the Atomic Energy Commission and the Bowman Gray School of Medicine, Winston-Salem, N. C.; Contract No. AT-(40-1)-288, Title II; Principal Investigator, Dr. Camillo Artom.

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Immunophysiology. Increase in Radiosodium Space of Adrenal Glands in Rabbits Sensitized with Human Plasma.* (19335)

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Since von Pirquet and Schick published their description of serum sickness(1), many studies concerning the relationship of certain histologic findings to immunologic phenomena have been reported(2-5). Such anatomic changes have been well delineated, but the physiologic alterations which are induced by antigen-antibody reactions are still poorly understood. It has been found previously that in rabbits sensitized with human plasma shifts in the distribution of body fluids occur at the time humoral antibody is first detected. The data were interpreted as indicating that a severe immune reaction may be associated with changes in the permeability of membranes (6). The methods employed in these studies did not permit localization of the observed changes to any specific tissues or organs.

The present experiment was undertaken in order to determine the site of the maximum alteration in fluid distribution following the injection of a large dose of human plasma into rabbits. Radioactive sodium (Na^{24}) was used as a tracer.

Materials and methods. Domestic rabbits of mixed breeds, weighing from 2 to 4 kg each, were placed in individual cages and fed a stock diet. Water was given without restriction. The same pool of human plasma was used as the sensitizing antigen and as antigen for the precipitin test. Another pool of human plasma was denatured by being heated to 100°C for 10 minutes. The filtrate of heat-denatured plasma was diluted with physiologic saline solution to the original volume. The methods for the determination of the plasma volume, thiocyanate space, radiosodium space, tissue radiosodium content, and tissue water content have been described previously(7). The appearance of humoral antibody was detected by a qualitative ring precipitin test. The human plasma, diluted 1:100 in physiologic saline solution, was used as antigen to overlay an equal volume of undiluted rabbit serum in 2 x 20 mm test tubes.

Plan of the experiment. Eighteen rabbits were divided into 3 groups of 6 each. *Group 1* (test) and *Group 2* (control) consisted of 6 rabbits each. The animals in the 2 groups were paired as to sex and body weight. On each rabbit, two baseline determinations of the plasma volume, the hematocrit, and the thiocyanate and radiosodium spaces were made 6 to 7 days apart. Each of the animals in Group 1 then received intravenously 10 ml of human plasma per kg of body weight, 5 ml/kg being administered on each of 2 successive days. The animals in Group 2 did not receive injections of human plasma, but were otherwise treated identically. Beginning on the fourth day after the injection of the initial dose of antigen, precipitin tests were performed daily on all rabbits in both groups. On the day when humoral antibody first appeared in an animal in Group 1, the volume determinations were repeated on the animal in Group 2 which had been paired with it, and the 2 rabbits were then sacrificed by exsanguination. For each animal the tissue radiosodium space was determined on 2 portions (roughly 1-2 g each) of the following: cartilage, kidney, skin, tendon, lung, subcutaneous connective tissue, heart, appendix, stomach, adrenal, liver, spleen, abdominal muscle, diaphragm, and gastrocnemius muscle. The total water content was determined on one portion of each tissue. *Group 3*, which served as an additional control, consisted of 6 rabbits whose sex and weight distribution was comparable to that of the rabbits in the preceding two groups. Each animal received by intra-

* This investigation was supported in part by the U. S. Atomic Energy Commission under a contract with the Bowman Gray School of Medicine and in part by a grant-in-aid from the American Heart Association. Na^{24} was supplied by the National Laboratory, Oak Ridge, Tenn., on allocation from the U. S. Atomic Energy Commission.

† Research Fellow of the American Heart Association.

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venous injection 10 ml of denatured human plasma per kg of body weight, 5 ml/kg being administered on each of 2 successive days. No volume determinations were made in this group, since no changes had been detected in Groups 1 and 2. Six and a half days after completion of the injection of denatured plasma—the average interval at which precipitins were first detected in Group 1—the animals were sacrificed by exsanguination for tissue analyses. At that time the precipitin tests were negative to both denatured and undenatured plasma. The following organs were studied: adrenal, kidney, stomach, and skeletal muscle.

Statistical analysis. The total blood volume, plasma volume, thiocyanate and radiosodium spaces were recorded in milliliters per kg of body weight. For each animal the difference between the mean of the two baseline determinations and the single value obtained on the day the precipitin test first became positive was calculated. The mean difference between the baseline and response values was then obtained, and the significance of the mean difference was tested by the following formulae in which $\bar{d}$ = mean difference, $s\bar{d}$ = standard error of the mean difference, S = summation of, n = number of animals:

$$t = \frac{\bar{d}}{s\bar{d}} \qquad s\bar{d} = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n(n-1)}}$$

The calculated value for "t" was compared with the value at (n—1) degrees of freedom, as obtained from standard tables. "t" was considered significant if $P < 0.01$. The results of the tissue analyses were analyzed by groups. The mean values for tissue water content and radiosodium content were obtained for each group and their respective standard errors were then calculated. The significance of the difference between the means was tested by the following formulae:

$$\bar{d} = \bar{x}_1 - \bar{x}_2 \qquad s\bar{d} = \sqrt{s\bar{x}_1^2 + s\bar{x}_2^2}$$

$\bar{x}$ = group mean
$s\bar{x}$ = stand. error of mean

Results. Precipitins to undenatured human plasma developed in the animals in Group 1

TABLE I. Fluid Spaces in Rabbits Given Injections of Human Plasma and in Paired Uninjected Control Animals.

Group			Wt (kg)	Radiosodium space (ml/kg)	Thiocyanate space (ml/kg)	Plasma vol. (ml/kg)	Hematocrit (vol. %)	Total blood vol. (ml/kg)
1	Test animals inj. with human plasma, 10 ml/kg	Baseline	3.07 ± .60	256 ± 15.7	216.5 ± 19.6	41 ± 4.2	40.8 ± 2.4	69.8 ± [illegible]
		Response*	3.39 ± .45	261 ± 14.2	221.3 ± 28.8	43.7 ± 8.9	37.8 ± 2.8	70.5 ± 16
2	Controls not inj. with plasma	Baseline	3.57 ± .42	249 ± 11	209.2 ± 10.7	39.8 ± 6.7	40.3 ± 4.1	66.5 ± 9.5
		Response†	3.66 ± .47	249 ± 10.9	207.2 ± 18.9	37 ± 4.05	39.5 ± 3.3	61.5 ± 7.2

All values are expressed as mean ± stand. dev.
* Values obtained on day when humoral antibody first appeared (mean of 6.5 days after initial inj. of human plasma).
† Values obtained at a time interval corresponding with appearance of humoral antibody in Group 1.

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(test) at a mean of 6.5 days after the initial injection of antigen. No precipitins to undenatured or denatured plasma developed in the animals in Groups 2 or 3. When the average of the mean baseline values at the time of sacrifice were compared with the average of the mean baseline values, no significant difference was found in the weight, plasma volume, hematocrit, total blood volume, thiocyanate space, or total radiosodium space in any group (Table I).

Comparison of the results of the tissue analyses on the animals in Groups 1 and 2 shows the radiosodium space in the adrenal glands of the sensitized animals to be significantly increased, without an accompanying increase in total water content (Table II). No significant difference in the radiosodium space or the water content of any of the other tissues or organs was found when Group 1 is compared with Group 2.

The results of the tissue analyses in Groups 1 and 3 likewise reveal the radiosodium space to be significantly increased only in the adrenal glands of the animals injected with undenatured plasma as compared with those given denatured plasma. No significant difference in the total water content of the gland was found. The kidney, stomach, and skeletal muscle revealed no change in radiosodium space or total water content.

Comment. The development of humoral antibody in all animals injected with undenatured plasma (Group 1) indicates that a certain degree of sensitization was induced. Since no generalized physiologic alterations developed it must be assumed that this sensitization was minimal. The fact that changes in the tissue radiosodium space were limited to the adrenal glands of those animals injected with untreated human plasma and were not present in those given denatured plasma or no antigen at all suggests that this gland may play some vital role in the maintenance of homeostasis. It may be suggested that the change in the adrenal gland may be a compensatory response to the harmful effects of an antigen-antibody reaction. From the data available, it is not possible to determine whether this increase in the radiosodium space of the adrenal gland was due to interstitial edema, to an increase in the isotope content within the parenchymal cells, to a relative decrease in parenchymal tissue, or to a combination of these factors.

TABLE II. Radiosodium Space and Total Water Content of the Adrenal Gland in Sensitized and Control Rabbits. Six animals in each series.

Group		Mean values: Radiosodium space (% of wet wt)	Mean values: Water content (% of wet wt)
1	Inj. with undenatured plasma	19.06	63.03
2	Uninj. controls	14.98	65.75
3	Inj. with denatured plasma	11.26	56.80

Summary of statistical analyses

Groups	Radiosodium space $\bar{d}$	Radiosodium space $S\bar{d}$	Radiosodium space t	Water content $\bar{d}$	Water content $S\bar{d}$	Water content t
1 vs. 2	4.08	.63	6.48*	2.72	4.74	.57
1 vs. 3	7.80	1.32	5.91*	3.72	1.20	3.10
2 vs. 3	3.72	1.20	3.10	8.95	3.94	2.27

* Statistically significant. P = <.01.

Summary. Rabbits sensitized with human plasma (10 ml/kg) developed humoral antibody following a mean interval of 6.5 days. No changes were noted in the blood volume or in the thiocyanate or radiosodium space at that time. Tissue analyses revealed the radiosodium space to be significantly increased in the adrenal gland of those animals given injections of untreated plasma, as compared with animals given denatured plasma or no antigen at all. No such increase was noted in any of the 14 other tissues or organs studied. No significant differences in the water content of any of the tissues or organs were found.

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IMMUNOPHYSIOLOGY OF SERUM SICKNESS

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IMMUNOPHYSIOLOGY OF SERUM SICKNESS

Alterations in the Blood Volume and Thiocyanate Space in Relation to Humoral Antibodies in Rabbits Sensitized With Human Albumin or Globulin

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THE physiologic disturbances resulting from immune reactions which follow the introduction of closely related purified antigens have received scant attention. Many years ago disturbances in the distribution of body fluids were observed clinically and alterations in renal function were detected during the course of serum disease.[1-4] With the development of chemical methods for separation of serum into fairly homogeneous fractions of reasonable purity, it has been possible to approach the problem of experimental hypersensitivity to foreign proteins in animals more definitively.

The histological and serological sequences in rabbits sensitized with albumin and globulin fractions of bovine serum have been described.[5] The localization of protein antigens of various molecular sizes in the tissues of a heterologous animal by homologous antibody labeled with fluorescein has been reported.[6]

In a previous study it was found that rabbits sensitized by the intravenous injection of human plasma showed a decrease in the blood volume and an increase in the thiocyanate space which could be related to the time at which humoral antibody was first detected.[7] In preliminary studies with whole plasma, serial determinations of the fluid volumes detected two separate alterations in the space available for dilution of thiocyanate ion. These observations suggested that the two peaks were the result of physiologic defects attributable to reactions to two separate antigens from the plasma. These results were analogous to the appearance of anatomic lesions at different times following the injection of two separate serum fractions.[5]

Accordingly, the present experiments were conducted to study the changes in distribution of fluids induced by antigen-antibody reactions resulting from purified fractions of human serum of different molecular size.

MATERIALS AND METHODS

Twenty-nine domestic rabbits of mixed breeds, weighing roughly two kilograms each, were used. Animals were placed in individual cages and fed

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a stock diet* supplemented once weekly by fresh green vegetables. Water was given without restriction.

Crystalline Human Serum Albumin.†—The very limited quantity of crystalline human serum albumin available was dissolved in sterile physiologic saline solution (2 Gm. in 10 ml.). No preservative was added and the solution was injected immediately without other sterilization. This solution was further diluted to a final concentration of 1:1000 in physiologic saline for use as antigen in the precipitin tests.

Human Serum Albumin.†—Concentrated salt-poor human normal serum albumin (25 Gm. in 100 ml.) was injected undiluted. The material was diluted 1:100 in physiologic saline for use as antigen in the precipitin tests.

Human Serum Globulin.†—Human immune serum gamma globulin (12.3 Gm. in 100 ml.) was injected undiluted. The material was diluted 1:100 in physiologic saline for use as antigen in the precipitin tests.

Only one lot of each of the antigenic materials was used to complete all of the experiments.

Chemical Techniques.—The plasma volume was determined by the T-1824 (Evans Blue) dye method. Syringes holding 2 milliliters were calibrated to deliver 0.5 ml. of a solution containing 3 mg. of the dye per milliliter. Two different lots of dye were used for the albumin and globulin experiments. The thiocyanate space was determined simultaneously. Syringes holding 10 ml. were calibrated and 2.0 ml. of a solution containing 50 mg. of sodium thiocyanate per milliliter were injected.

The concentrations of the dye in rabbit serum were determined by the Evelyn photoelectric microcolorimeter and the thiocyanate concentrations with the Evelyn photoelectric macrocolorimeter.[7]

Hematocrit readings were made on oxalated blood placed in Wintrobe tubes, centrifuged at 3,000 revolutions per minute at a radius of 15 cm. for 30 minutes.

The total blood volume was calculated from the plasma volume and the hematocrit.

Immunologic Techniques.—Undiluted rabbit serum was placed in 2 by 20 mm. tubes to a height of 1 cm. and overlayed with 0.5 milliliter of the diluted antigen. The tubes were left at room temperature and the presence or absence of precipitation at the interface was detected by observation in reflected artificial light against a dark background after an interval of one hour. Each tube was compared with a control.

Skin tests were not done in order to avoid additional injections of antigen. The dye T-1824 has previously been shown not to induce precipitins or positive skin tests in rabbits.[7]

Experimental Procedure.—In the morning before feeding, an ear vein was nicked with a knife and 2 ml. of blood collected in a dry test tube and allowed to clot; 1 ml. was then collected in another dry tube containing 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate. The dye was injected into a

*Kasco Complete Rabbit Ration, Kasco Mills, Incorporated, Toledo, Ohio.
†Furnished by the American Red Cross.

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TABLE I. MEAN OF TWO BASELINE AND TWO RESPONSE VALUES IN RABBITS SENSITIZED BY THE INTRAVENOUS INJECTION OF 0.5 GM. PER KG. OF CONCENTRATED HUMAN ALBUMIN (GROUP 2)

	BASELINE					RESPONSE*					
RABBIT NUMBER	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %	PRECIPITIN POSITIVE DAY	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %
1	2.50	157.6	44.4	76.4	42.0	8	2.50	172.0	40.0	63.6	37.5
2	2.27	179.3	39.2	63.0	37.5	7	2.61	226.8	29.9	46.0	36.0
3	2.50	163.0	34.8	58.0	40.0	7	2.64	206.8	29.9	48.5	38.5
4	2.52	146.8	40.8	69.0	41.0	8	2.75	143.6	33.8	55.3	38.5
5	2.27	170.5	45.8	72.2	37.0	8	2.52	165.1	35.7	54.4	34.5
6	2.33	165.2	36.1	60.9	41.0	9	2.60	185.0	28.5	46.5	39.0
7	2.50	155.2	47.2	77.2	40.0	5	2.66	178.9	37.9	61.3	39.0
8	2.64	158.7	42.4	75.0	44.0	6	2.81	177.2	31.3	50.5	40.0
9	2.35	160.4	40.0	65.5	39.5	7	2.66	186.5	30.8	48.9	36.5
10	2.16	184.3	37.5	63.9	41.5	6	2.50	203.6	28.8	45.2	36.5
11	2.70	146.3	35.6	58.9	39.5	7	2.84	168.0	29.9	46.1	35.5
12	1.93	189.6	42.5	66.8	36.5	7	2.39	200.8	32.2	49.0	34.0
Mean	2.39	164.7	40.5	67.2	40.0	7.1	2.62	184.6	32.4	51.3	37.1

*The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same rabbit done on the day precipitins to concentrated human albumin appeared and on the next day.

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vein in the opposite ear, the syringe changed, and the sodium thiocyanate injected through the same needle within one minute. Ten minutes following completion of the injection, 2 ml. of blood were collected from the first ear. When serial determinations were previously done, this arbitrary mixing time was found to give reproducible results for both blood volume and thiocyanate space.[8]

Preliminary Experiments.—In order to establish the minimal sensitizing dose, 3 rabbits were given a single subcutaneous injection in the interscapular region of 0.5 Gm. per kilogram of concentrated human albumin. Two rabbits developed positive precipitin tests on the twenty-ninth day, but the third animal was still negative on the thirty-second.

A single subcutaneous injection of 0.75 Gm. per kilogram of concentrated human albumin was then given four other rabbits. Two animals developed positive precipitin tests at 13 days and 2 at 14 days following injection.

Definitive Experiments.—Two baseline determinations were made on successive days on each of 5 rabbits (Group 1). After the second determination, each animal was injected subcutaneously with a single dose of 0.75 Gm. per kilogram of crystalline albumin. Precipitin tests were performed daily on each rabbit, but no further fluid volume determinations were done until the day that a positive precipitin reaction was first obtained. The measurements were repeated on that day and the following day.

Twelve rabbits (Group 2) were treated in the same fashion except that a single intravenous injection of concentrated albumin in a dose of 0.5 Gm. per kilogram was given.

Six rabbits (Group 3) were studied in an identical manner except that 0.25 Gm. per kilogram of serum globulin was given intravenously.

Statistical Analysis.—The total blood volume, plasma volume, hematocrit, thiocyanate space and weight were recorded in absolute values. The mean of the two baseline determinations on each animal was compared with the mean of the two response determinations made on the day that precipitins appeared and on the following day. The mean differences between the baseline and response values were calculated. The calculated value for "t" was compared with the value for n-1 degrees of freedom as obtained from standard tables. The value was considered significant if P was less than 0.01.

The data obtained in absolute values were then calculated in terms of milliliters per kilogram of body weight and the statistical significance was determined. Each animal served as its own control. No attempt was made to compare one experimental group with another.

RESULTS

General Observations.—Clinical serum sickness, manifested by erythema or edema, was not observed in any of the groups with the doses used.

Crystalline Human Serum Albumin (Group 1).—Positive precipitin tests appeared in all animals at a mean of 22.5 days. No significant changes were found in any of the response values when they were compared with the baseline values.

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Human Serum Albumin (Group 2).—Positive precipitin tests developed in all animals at a mean of 7.1 days. The alterations in thiocyanate space, plasma volume, total blood volume, hematocrit and body weight are summarized in Table I, the statistical analyses in Table II.

TABLE II. STATISTICAL SIGNIFICANCE OF ALTERATIONS IN FLUID SPACES IN RABBITS SENSITIZED WITH CONCENTRATED HUMAN ALBUMIN

	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML.	PLASMA VOLUME ML.	TOTAL BLOOD VOLUME ML.	HEMATOCRIT VOLS. %
$\bar{d}$	+ .230	+91.92	−11.40	−26.40	−2.83
$s_{\bar{d}}$	.035	13.50	1.44	3.12	.37
t	6.570	6.81	7.92	8.46	7.65
		ML./KG.*	ML./KG.*	ML./KG.*	
$\bar{d}$		+19.82	−8.08	−16.04	
$s_{\bar{d}}$		4.50	.20	1.10	
t		4.40	40.40	14.58	

*Calculated in terms of weight on the day of determination.

$\bar{d}$ = mean difference

$s_{\bar{d}}$ = standard error of the mean difference

S = summation of, n = number of animals.

$$t = \frac{\bar{d}}{s_{\bar{d}}} \qquad s_{\bar{d}} = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n(n-1)}}$$

For 11 degrees of freedom t = 3.106 ; P = <.01

The data all show statistically significant differences between the baseline values and those obtained at the time of the humoral antibody response.

A significant increase in the mean thiocyanate space was noted at the time the humoral antibody was first detected. A significant increase in body weight occurred during the period of alteration in thiocyanate space. In contrast to the increase in thiocyanate space and weight, significant decreases in plasma volume, total blood volume, and hematocrit occurred in all animals.

All the data are statistically significant whether the calculations are based on the comparison of absolute values or of values based on milliliters per kilogram of body weight.

Human Serum Globulin (Group 3).—All animals developed positive precipitin tests at a mean of 9.0 days. The alterations in thiocyanate space, plasma volume, total blood volume, hematocrit and body weight are summarized in Table III, the statistical analyses in Table IV.

The increase in thiocyanate space was significant only when the data were analyzed in absolute values. The increase in body weight was significant. The decreases in plasma volume, total blood volume and hematocrit were significant whether the data were calculated in absolute values or in terms of milliliters per kilogram of weight.

DISCUSSION

The observations reported demonstrate that immune reactions to albumin and globulin fractions of human serum are associated with alterations in distribution of fluids within the body. The changes were detected earlier and were quantitatively greater after the injection of albumin than after globulin, but qualitatively they were the same. The absence of clinical manifestations of serum disease in our animals suggests that the disease was mild. The variation in the time of appearance of precipitins in relation to the experiments of others

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TABLE III. MEAN OF TWO BASELINE AND TWO RESPONSE VALUES IN RABBITS SENSITIZED BY THE INTRAVENOUS INJECTION OF 0.25 GM. PER KG. OF HUMAN GAMMA GLOBULIN (GROUP 3)

	BASELINE					RESPONSE*					
RABBIT NUMBER	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %	PRECIPITIN POSITIVE DAY	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %
13	1.96	250.0	56.1	93.4	39.0	9	2.10	261.9	46.2	74.3	36.5
14	2.09	234.0	58.4	101.9	41.0	7	2.33	215.5	46.4	79.8	39.5
15	1.91	266.0	67.5	103.4	35.0	9	1.93	302.6	57.0	85.0	33.5
16	1.56	313.5	69.2	112.8	36.5	10	1.70	329.4	52.9	89.4	34.5
17	1.93	273.6	64.8	106.2	39.0	8	2.05	275.6	55.1	81.5	34.0
18	2.10	262.8	70.0	103.8	42.0	11	2.18	278.8	56.0	79.4	39.0
Mean	1.93	233.3	64.3	103.4	38.8	9.0	2.05	277.3	52.3	81.6	36.2

*The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same rabbit done on the day precipitins to human gamma globulin appeared and on the next day.

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TABLE IV. STATISTICAL SIGNIFICANCE OF ALTERATIONS IN FLUID SPACES IN RABBITS SENSITIZED WITH HUMAN GAMMA GLOBULIN

	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML.	PLASMA VOLUME ML.	TOTAL BLOOD VOLUME ML.	HEMATOCRIT VOLS. %
$\bar{d}$	+0.122	+52.20	−16.80	−32.00	−2.58
$s_{\bar{d}}$	0.028	9.60	1.99	3.40	0.54
t	4.370	5.43	8.44	9.40	4.78
		ML./KG.*	ML./KG.*	ML./KG.*	
$\bar{d}$		+10.70	−12.23	−21.80	
$s_{\bar{d}}$		7.40	1.37	1.14	
t		1.45	8.93	19.10	

*Calculated in terms of weight on the day of determination.
For 5 degrees of freedom, t = 4.032; P = <.01.
With the exception of the thiocyanate space in ml. per kg. the data all show statistically significant differences between the baseline values and those obtained at the time of the humoral antibody response.

may be due to differences in the species of serum utilized as antigen. Other investigators have used chiefly horse or bovine serum.[4, 5, 9] The preliminary experiments indicate that the size of the dose affects the incubation period, but not the severity of the disease.

The crystalline serum albumin used may well have been antigenically altered in the process of crystallization. The appreciably longer period before precipitin tests became positive, as compared with concentrated serum albumin, is suggestive of impaired antigenicity. Another factor contributing to the longer incubation period may have been the difference in the route of administration of the antigen. The fact that significant physiologic alterations were not detected may be due to either of these factors, or to both.

The increase in space available for dilution of the thiocyanate ion at the time circulating humoral antibodies appeared, when compared with the control period in the same animal, indicates that this small molecule diffused into some extravascular space to a greater extent. The loss of water and ions from the vascular tree into tissues would readily explain the observations of retention by the body of water, salt and chlorides during serum disease and pneumonia.[2, 11] The decrease in circulating blood volume confirms the suggestion that particles had left the circulation.[7] The colloids lost to the interstitial spaces would tend to retain water extravascularly until they had been reabsorbed or broken down in the tissues. It is well known that in severe immune reactions of the anaphylactoid type hemorrhages, due to the extravasation of the even larger red blood cell, appear in various organs.

The observations can be interpreted as indicating an increase in permeability of the vascular tree, so that crystalloids, colloids, and red blood cells are progressively lost from the circulation as the magnitude of the defect increases with increase in the clinical severity of the disease. The alterations in the vascular tree can be visualized as a series of pores, probably in the capillary wall where the normal extravascular circulation of crystalloids, and to a lesser extent small protein molecules such as albumin, is known to occur. In mild instances of serum disease water and ions which normally traverse the membranes would

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leave the blood more readily. Larger molecules such as protein would be lost in more severe instances and red blood cells would pass out of the vascular tree only in the most severe reactions.

A uniform, significant reduction in the hematocrit occurred in all animals in Groups 2 and 3. Since the plasma volume had decreased, the total circulating red cell mass must have decreased also. There was no clinical evidence of increased capillary permeability to red cells as evidenced by hemorrhage or petechiae. The clinical disease induced appeared to be too mild to account for the decrease in hematocrit on the basis of extravasation of red cells out of the vascular tree. The possibility of hemolysis must be considered; jaundice has been previously observed in another group of animals sensitized with human plasma. No such sign was detected in the present study. It is also possible that a shunting and pooling of red cells in some inactive tissue or organs had occurred. No adequate explanation can be given at the present time for this reduction in hematocrit.

The proportionately smaller increase in thiocyanate space in the animals injected with globulin, as compared with those injected with albumin, may have been due to the fact that the antigenic stimulus was quantitatively less. A smaller dose was administered and the larger size of the molecule would mean that fewer antigenic particles were injected in a given weight of serum. Furthermore, the globulin molecules may be less antigenic than the smaller albumin molecules. The reduction in the incidence of clinical serum disease noted since the introduction of therapeutic antisera composed chiefly of the gamma globulin fraction lends further support to this hypothesis. The cells to which antigens of various size attach, as well as the speed of attachment, vary with the type of antigen injected.[5] The globulin molecule may attach to cells which have less capacity for the production of antibodies.

The increase in extravascular fluid space as measured by the thiocyanate ion could not always be correlated with an increase in weight. The alterations cannot therefore be attributed solely to a defect in capillary permeability with retention of extra fluid in the interstitial spaces. Apparently the normal distribution of fluid in the body was altered, probably as the result of an increase in permeability of cellular membranes as well. It is not known whether the thiocyanate ion normally diffuses into cells or not. In any event these observations indicate that at the time humoral antibodies were detected the permeability of membranes to these ions was increased, so that they diffused from blood into interstitial spaces and probably from interstitial fluid spaces into cells.

Histological techniques have demonstrated that after the injection of antigens cellular reactions can be detected by anatomic methods before humoral antibodies are present.[5, 9] It has been presumed that these histological changes are the result of antigen-antibody reactions taking place on the cell membrane or within the cell, but the speed with which specific antibodies are formed within cells or attached to them is not known.

The role of antigen-antibody reactions in the production of anatomic glomerular lesions following the injection of bovine globulin into rabbits has

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recently been questioned.[9] The fact that anatomic lesions appear before humoral immunity is detectable suggests the alternative possibility that physiochemical relationships concerned with the hydration of intraglomerular proteins may effect changes without an immune reaction taking place.

Correlation of these physiologic experiments with previous histologic studies could not always be made. The injection of albumin fractions of bovine or horse serum results in generalized arterial lesions; the globulin fractions produce glomerular lesions more frequently.[5, 9] It is likely that the physiologic defect which we have studied occurs not in the artery, but in the capillary for reasons given earlier. The physiologic defect reaches its maximum after the anatomic lesion appears, is completely reversible if the animal lives, and disappears at a time when the anatomic lesion may persist.[5, 7] In rickettsial diseases reversible physiologic changes may be present which are out of all proportion in magnitude to the number of anatomic vascular lesions present.[11] The similarity of the histological picture in rickettsial diseases and serum sickness suggest that some common denominator may be present. An immune reaction is such a factor.

SUMMARY

1. Alterations in the distribution of fluids associated with the development of humoral immunity were detected in rabbits injected intravenously with human serum albumin or globulin.

2. Increase in the space available for dilution of thiocyanate ions and decreases in plasma volume, total blood volume and hematocrit occurred at the time circulating antibody (precipitins) appeared.

3. The changes appeared earlier (at a mean of 7.1 days) following the injection of albumin than after the injection of globulin (9.0 days). Crystalline human serum albumin, injected subcutaneously, did not produce significant physiologic disturbances and the appearance of precipitins was delayed for a mean of 22.5 days. These findings were interpreted as indicating an alteration in antigenicity of the protein in the process of crystallization, but may have been related to the subcutaneous route of injection.

4. The redistribution of fluid was interpreted as indicating a defect in permeability of capillaries and of cell walls as a result of antigen-antibody reactions.

5. The correlation with anatomic findings by histologic techniques is discussed.

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EXTRACELLULAR FLUID SPACES IN NORMAL RABBITS

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In the course of some studies in immunophysiology, it was necessary to determine the normal values and their random variations for the extracellular fluid compartments of normal rabbits. The plasma volume, total blood volume and the thiocyanate and radiosodium spaces were, therefore, measured in a total of 45 rabbits. Serial determinations of the plasma volume and the thiocyanate spaces were made in six animals. The reproducibility in any individual animal of values for the thiocyanate and the radiosodium spaces were determined. Serum sodium and potassium concentrations were measured. The radiosodium space of various tissues and organs was determined in six animals, as well as their total water content. Since no comparable data were available in the published literature, the following report was prepared.

MATERIAL AND METHODS

A total of 45 normal domestic adult rabbits of both sexes, with initial body weights between two to four kg., were kept in individual cages and were fed a stock diet. Water was given without restriction.

The plasma volume and the thiocyanate space were determined by the T-1824 dye (Evans Blue) and sodium thiocyanate techniques (1). The dye and the thiocyanate ion were injected as a sterile solution containing a mixture of 1 mg. per cc. of Evans Blue and 50 mg. per cc. of sodium thiocyanate. Syringes were calibrated to deliver approximately 2 ml. of the mixture. No correction was made in dosage for variation in weights of the rabbits. The plasma volume was determined on a single 10 minute sample; 30 minute samples were drawn in some cases for comparison with the radiosodium space. Hematocrits were determined in Wintrobe tubes centrifuged at 3,000 rpm for 30 minutes.

The radiosodium space was determined as follows: A known volume (2 ml.) of a sodium carbonate solution containing approximately 20 microcuries per ml. of Na^{24} was injected intravenously into a marginal vein of the ear

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immediately following the injection of the mixture of Evans Blue and sodium thiocyanate. In a preliminary experiment, serial samples were obtained in order to determine the disappearance curve of radiosodium. Subsequently, a single blood sample was drawn to coincide with the equilibration point (1 hour) for radiosodium. The activity was determined with a dipping tube and a scaling circuit. Counts were made to 1 per cent accuracy. The radiosodium space was calculated by the following formula:

$$\text{Volume of } Na^{24} \text{ space in cc.} = \frac{\text{Total counts/ min. injected}}{\text{Counts / min. / ml. plasma}}$$

All values were corrected for decay. The standard solution which was used for injection was appropriately diluted with distilled water to approximate the counting rate of the plasma. No correction was made for urinary excretion of the radiosodium during the period of observation since the amount excreted was negligible.

Plasma sodium and potassium concentrations were determined by flame spectrophotometry on the Beckman Model DU instrument. The following modification of the method of Mosher, Boyle, Bird, Jacobson, Batchelor, Iseri and Myers (2) was used: The slit width was set at 0.1 mm. in both sodium and potassium determinations. With the standard solution set on the transmission dial at 70 and 25 respectively for sodium and potassium, the galvanometer needle was set at zero by altering the sensitivity setting.

The six animals which were to be sacrificed for tissue analyses were injected intravenously with 50 microcuries per ml., for a total of 100 microcuries per animal. One hour following injection, these animals were exsanguinated by severing the jugular veins and the carotid arteries; the animals died within four to five minutes. Approximately one gram of tissue was weighed on a torsion balance. Each sample was then completely digested with five to ten ml. of fuming sulfuric acid; the digest was made to a

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volume of 20 ml. with distilled water. In order to maintain a constant geometry, 25 ml. graduated cylinders were used as containers for the digest. Because of the marked photosensitivity of the dipping tubes, all counting was done in a dark room. The radiosodium space of the various tissues and organs was calculated by the formula:

$$\text{Per cent radiosodium space} = \frac{\text{Counts / min./ gm. of tissue}}{\text{Counts / min./ ml. plasma}}$$

Aliquots were dried to constant weight in a drying oven at 110 to 120 C for determination of the total water content of the tissues and organs.

EXPERIMENTAL RESULTS

Serial determinations of the plasma Volume, hematocrit and the thiocyanate space

In the initial experiment, a total of 71 determinations of the plasma volume and the thiocyanate space were performed in 6 rabbits during an aggregate period of 206 days. The determinations were made every second or third day and occasionally at less frequent intervals. The calculations were based on single 10 minute samples. The results of this study are summarized in Table I.

Mixing time for radiosodium

Serial venous blood samples were obtained from eight rabbits following the administration of radiosodium. Figures I shows graphically in cc/Kg. the mean values for radiosodium space at each time interval. After the first 30 minutes, the disappearance curve became exponential. On the basis of the above findings, subsequent calculations of the sodium space were made on a single blood sample obtained one hour following the injection of Na^{24}. The rate of removal of radiosodium from the plasma was roughly 10 per cent per hour. If the exponential portion of the curve was extrapolated to zero time, the radiosodium space was approximately 25 per

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cent of the body weight.

Mixing time for thiocyanate

In previous studies on immunophysiology (3, 4), for technical reasons, the thiocyanate space was calculated from the 10 minute sample. Upon serial determinations on any single rabbit, the results of this measurement were reproducible (Table I). However, it was recognized that equilibration had not occurred at that time. In Figure I is shown the curve for thiocyanate space calculated from the mean values obtained at various time intervals in two normal rabbits. Equilibration was attained at 30 minutes. The disappearance slope was steeper than for the radiosodium space, so that the rate of removal of thiocyanate from the plasma was roughly 20 per cent per hour. Since, in the routine determination of the thiocyanate space, values were not extrapolated to zero time, and because of the rapid rate of removal of the ion from the circulation, 30 minute samples were drawn to compare with the one hour radiosodium samples.

Plasma volume

The mean plasma volume, based on a total of 67 determinations on 39 rabbits, was found to be 42.3 cc./Kg. (Table I). Repeat determinations were made on a total of 28 animals (Table III). In 27 of these, the measurements were made within an interval of 2 to 14 days. In one animal, the interval between the two determinations was 50 days. The mean difference between such duplicate determinations was 4.14 cc./Kg. There was no correlation between the interval between the determinations and the magnitude of the difference.

Hematocrit

The mean hematocrit, based on a total of 65 determinations on 39 animals, was 38.8 volumes per cent. The mean difference between duplicate

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determinations, based on 26 such pairs, was 4.46 volumes per cent. There appeared to be a rough correlation between the decrease in the second hematocrit as compared with the initial measurement and the time interval between the two readings, the decrease in the second hematocrit being greater, the shorter the interval between the determinations. Of the total of 26 duplicate determinations, in all cases except 4, the second hematocrit was equal to or less than the first. In these 4 instances, at least 14 days had elapsed between the two determinations. It appeared that with the volume of blood withdrawn for each study, at least 10 to 14 days were required for the hematocrits to return to the original values.

Total blood volume

The mean total blood volume, based on 65 determinations in 39 animals, was found to be 69.8 cc./Kg. (6.98 per cent of the body weight) (Table I). Repeat determinations in 26 animals varied by a mean of 6.34 cc./Kg. No correlation was noted between the interval between the duplicate determinations and the magnitude of the difference between the two values.

Thiocyanate space

The mean of 68 determinations of the thiocyanate space on a total of 39 rabbits, based on the 10 minute sample, was 217.8 cc./Kg (Table I). The mean of the 30 minute samples, based on a total of 50 determinations in 32 rabbits, was 237.6 cc./Kg. The mean of the 30 minute sample, therefore, was 20 cc./Kg. greater than the mean of the 10 minute samples. Thirty minute samples revealed slightly less scatter than the 10 minute samples. The upper limit of the values for both the 10 and the 30 minute determinations was practically identical. Duplicate determinations of the 30 minute readings were more reproducible than the 10 minute readings, the mean difference in the former being 15.03 cc./Kg. and in the later, 8.66 cc./Kg. There was no

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correlation between the interval between duplicate determinations at either 10 or 30 minutes and the magnitude of the difference between the two determinations.

Radiosodium space

The mean radiosodium space, based on the one hour samples on 68 determinations in 39 rabbits, was found to be 263 cc./Kg (26.3 per cent of the body weight). This value was 45 cc./Kg. greater than the mean thiocyanate space based on the 10 minute samples of both, and 28 cc./Kg. greater than the thiocyanate space based on the 30 minute samples. The variability of the three measurements was roughly equal, that of the 30 minute thiocyanate space being the least. The mean difference and the scatter of duplicate determinations of the radiosodium space was greater than those for the thiocyanate spaces at either 10 or 30 minutes.

Serum sodium and potassium concentrations

The mean serum sodium concentration, based on a total of 55 determinations in 33 rabbits, was 131.9 mEq./L. The mean serum potassium concentration, based on the same sera, was 4.58 mEq./L. Serum sodium levels showed less random variation than serum potassium levels. There was no correlation between the interval between duplicate determinations of either serum sodium or potassium levels and the magnitude of the difference.

Tissue radiosodium spaces

The results of the analyses of the tissue radiosodium spaces in six normal rabbits are summarized in Table III. Of the tissues analyzed, the cartilage, skin and kidney contained the largest amount of radiosodium per unit weight. Skeletal muscle contained the least. Considerable variation was noted in the radiosodium spaces of some organs e.q. subcutaneous connective tissue, liver, appendix and cartilage.

Total water content of organs and tissues

Total water contents of the various tissues and organs are given in

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Table IV. The appendix and the lung contained the largest amount of water per unit of weight of tissue and the tendon the least. Less variation was noted in the determinations for any tissue or organ than was observed in the radiosodium spaces.

DISCUSSION

In the initial experiment (Table I), it was found that when a series of 9 to 13 determinations of the plasma volume, hematocrit, total blood volume and the ten minute thiocyanate spaces were made every second to third day in normal rabbits the respective means and standard deviations were 46 - 5.5 cc./Kg., 35 - 4.3 volumes per cent, 72 - 8.9 cc.;Kg. and 194 - 21 cc./Kg. The values obtained in the subsequent studies involving a single and/or a 2 repeated determinations on normal rabbits agreed fairly closely with the previous serial determinations. In the only other similar study of normal rabbits which has been uncovered in the literature (6), the mean of the determinations of the total blood volume in 60 rabbits was 70 cc./Kg., based on samples obtained 6 minutes after injection of the Evans Blue. The mean value of the plasma volume was 50 cc./Kg. The blood volume was found to be proportional to the body weight and not to the surface area. These results are consonant with the findings in the present investigation. No comparable data have been found regarding the thiocyanate space or the rate of disappearance of the ion from the blood stream in rabbits.

The results of the present study indicate that regardless of whether the thiocyanate determinations based on the 10 or 30 minute samples or the radiosodium space based on the one hour sample are employed as an index of the extracellular fluid volume, consistently reproducible results can be obtained, at least in normal rabbits. There is valid argument against the use of the 10 minute reading, since equilibration had not occurred at that time. However, for [illegible]

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time. However, for technical reasons, this has proved to be the most practical method when serial determinations are made on a single animal. Actually, the 30 minute thiocyanate determinations revealed less scatter than either the 10 minute thiocyanate determinations or the radiosodium determinations. The exact significance of the thiocyanate space, however, remains in doubt. Determinations of the thiocyanate space in various tissues and organs and their comparison with the sodium space might clarify this problem. The present data indicate that in normal rabbits the thiocyanate ion at equilibration consistently measures a space roughly 10 per cent less than the space measured by radiosodium.

The mean radiosodium space of 26.3 per cent of the body weight found in the present study agrees with the values obtained by previous investigators with a smaller number of rabbits (7, 8, 9). Manery and Bale (9) compared the radiosodium content of rabbit tissues with their content of chemically determined sodium and found that the specific activity of all tissues except the nerveus tissue was constant. They concluded that "with the exception of the nervous tissue, in 1 hour 8 minutes, injected radioactive sodium becomes distributed between tissues and plasma in precisely the same ratio as normally occurring sodium." Most of the tissue sodium values reported by Manery and Hastings (11) were higher than those obtained in the present study. One possible explanation for this discrepancy may be the difference in the methods employed in sacrificing the animals. Decapitation of animals does not result in as thorough exsanguination as severing the neck vessels. The two-fold discrepancy in the values for cartilage is difficult to explain.

The total water content of tissues obtained in the present study are summarized in colume V of Table V. These values agree well with those obtained by Manery and Hastings (11) and by Skelton (12). Less discrepancy

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was noted that with the [illegible]tions of the radiosodium space.

CONCLUSIONS

The total [illegible] calculated from the plasma volume and the hematocrit, was [illegible] This value was constant in normal rabbits and appeared to be [illegible] to the body weight. A previous investigator (6) had obtained [illegible] the identical value, so that its reliability cannot be questioned.

The exact significance of the thiocyanate space remains doubtful and will not be resolved until comparative studies of the concentration of the sodium ion and the thiocyanate ion in various tissues and organs are made. From the present study it can only be concluded that the thiocyanate ion at equilibrium [illegible] volume which was approximately 10 per cent less than that [illegible] the radiosodium ion. Whether this relationship would continue in abnormal physiologic states remains to be studied. Presumably the radiosodium [illegible] one hour measured in various organs and tissues the volume [illegible] intracellular. The thiocyanate determinations were reproducible in [illegible] rabbits, regardless of whether 10 or 30 minute samples were [illegible] random variations in the values for the thiocyanate or the Na^{24} [illegible] equivalent. Tissue sodium spaces are probably not comparable [illegible] technique is utilized in sacrificing the animals, since it is [illegible] that variations in the amount of residual blood in the organs and tissues would alter their sodium content. Since serial determinations of the fluid volume spaces are more reproducible in individual animals than single determinations in separate animals, experiments should be so designed [illegible] this observation into consideration.

SUMMARY

The plasma volume, total blood volume, the thiocyanate and the radiosodium

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spaces, and the serum concentrations of sodium and potassium. Repeat determinations were also made at various time intervals in a larger group of rabbits in order to determine the degree of random variation in any single animal. Serial determinations were made to check the constancy of the values. Tissue analyses for total water content and radiosodium space were also done.

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1951, v78, 524-528

Effect of Starvation on Exchangeable Potassium and Tissue K^{42} Content in Rabbits.*† (19127)

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In the course of a clinical survey of individuals with various diseased states, low values for exchangeable potassium, as measured by the radioactive isotopic technic, were encountered(1). In general, these low values appeared to be due to: (1) a decrease in body mass secondary to increased catabolism produced by the disease process; (2) a diminished intake of food from anorexia induced by the illness; (3) an intracellular deficiency of this cation from some inherent metabolic abnormality; or to a combination of these factors. The role of human disease and of metabolic abnormalities would be best studied in patients who present such problems. The effect on potassium metabolism of a decrease in food intake in the absence of disease can be approached experimentally. Since such a study was not feasible on human beings in our laboratory, rabbits were starved and the changes produced were followed serially.

The purpose of the present study is to determine the effect of starvation for a short period of time on the total potassium content of the body, as well as its concentration in various tissues. Because of the greater correlation of weight and urinary creatinine with exchangeable potassium content in human males as compared with females, the following experiments were performed in male animals (2,3).

Material. Nineteen normal male adult rabbits of mixed breeds were used. They were placed in individual metabolism cages and were fed a stock diet of compressed pellets. Tap water was given without restriction.

Methods. Experimental procedure. The experiment was planned to study the alterations in potassium metabolism which would be produced by a reduction in body weight of ap-

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†The technical assistance of Miss Eloise L. Rhoades and Mr. Claude Taylor is gratefully acknowledged.

‡ Research Fellow of the American Heart Assn.

1. Aikawa, J. K., Felts, J. H., Tyor, M. P., and Harrell, G. T., submitted for publication.

2. Corsa, L., Olney, J. M., Steenburg, R. W., Ball, M. R., and Moore, F. D., *J. Clin. Invest.*, 1950, v29, 1280.

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proximately 15%. A loss in weight of this extent is not uncommonly encountered clinically and would be comparable to a decrease of 12.5 kg (22.5 lb) in a 70 kg (150 lb) man. Because of the technical difficulties involved in the use of an isotope with such a short half-life (12.4 hours), an interval of one week was found to be convenient between isotopic determinations. Group A consisted of 7 animals which were observed for a control period of one week before starvation and subsequently during the deprivation of food. External potassium balance studies were conducted for the baseline period and the first week of starvation. At the conclusion of this period the animals were still in good condition, so that the observations with radiopotassium were continued for an additional week, but balance studies were not done. Each morning before feeding, the body weight, total food consumption and the daily urine volume were recorded. Creatinine determinations were performed in the morning on an aliquot of 24 hour daily sample. Baseline exchangeable potassium determinations were completed in the morning just before food was withdrawn. This determination was repeated after 1 and 2 weeks of starvation. Six rabbits (Group B) were fed for a period of one week to establish equilibrium on the diet. Food was then withdrawn completely, though water was allowed *ad libitum*. After one week the animals were sacrificed by exsanguination and analyses for radiopotassium and total water content were performed on 17 different tissues. Another group of 6 animals (Group C) served as a control for Group B. These rabbits were continued on food and water *ad libitum* but otherwise were treated identically with Group B.

Radioisotope methods. The preparation of the potassium solution for injection has been previously described(2). A calibrated volume of 5 ml of the K^{39} solution containing 30 μc of K^{42} in 0.5 milliequivalent was injected intravenously. The urine was collected for 24 hours; a spot specimen was then obtained by catheterization and its specific activity was determined. The following formula was used to calculate the value for the *exchangeable potassium content* of the body:

$$K_e = \frac{K_1^{42} - K_0^{42}}{\frac{Ku^{42}}{Ku^{39}}}.$$

K_e = quantity of exchangeable K in milliequivalents.

K_1^{42} = quantity of radiopotassium administered (arbitrary units).

K_0^{42} = quantity of radiopotassium excreted in urine until spot specimen was obtained.

Ku^{42}/Ku^{39} = specific activity of spot specimen.

Tissue analyses. Three aliquots (1 to 3 g) of each tissue were obtained. They were kept in covered Petri dishes and were weighed as rapidly as possible on a Gramatic balance to 0.1 mg. Two portions were digested in concentrated sulfuric acid for determination of *radiopotassium concentration.* The digest was made up to a volume of 20 ml [redacted] counted with a dipping tube and a scaling circuit. The tissue concentration of radiopotassium was expressed as per cent of the injected dose per g of wet tissue. *Tissue water content* was determined on the third aliquot, which was dried to constant weight at 110°C for 18-24 hours. The *potassium* concentration in the urine was determined by flame spectrophotometry by a method which has been previously described (4). Urinary *creatinine* concentration was determined by a modification of the method of Bonsnes and Taussky(5). *External balance of potassium.* Each g of feed was found to contain 0.159 meq. of potassium. The tap water contained a negligible amount. The potassium content of the ingested feed was found to be completely absorbed through the gastrointestinal tract. The content of potassium in the stool was too low to be detected by flame photometry. The loss of potassium from the body was, therefore, calculated on the basis of its urinary excretion. *Statistical method.* The significance of the difference between the means was derived by the use of the "t" test(6).

4. Aikawa, J. K., *Am. J. Physiol.*, 1950, v162, 695.
5. Bonsnes, R. W., and Taussky, H. H., *J. Biol. Chem.*, 1945, v158, 581.

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Results. The 7 animals followed by external balance and K_e determinations (Group A) were in potassium balance and gained weight during the baseline period (Table I, Fig. 1). During the period of starvation a progressive loss of body weight occurred. The mean weight loss for the group after the first week was 0.35 kg (15.6% of the mean initial weight) (Table I, Fig. 1); this amount, however, was not statistically significant. After 2 weeks, the mean decrease in weight amounted to 0.71 kg, which is significant. At the end of the first week the mean negative potassium balance amounted to 23 meq. The mean K_e decreased 41 meq. during the first week and an additional 34 meq. during the second week of starvation. By the end of the second week of starvation, a significant reduction in K_e/wt had occurred.

Although the mean creatinine excretion decreased 146 mg during the first week and 124 mg during the second week, as compared with the baseline values, these changes, as well as the decreases in the creatinine coefficient, were not significant. The urine volume increased the first week of starvation.

Discussion. The present results emphasize the importance of the concept that the body potassium is in dynamic equilibrium, and that the changes in the rate of exchange may be the first indication of a disturbance in its metabolism. Such an abnormality may occur before any alteration in concentration of potassium in blood or tissue is detectable.

During starvation, as tissue is catabolized, the weight decreases progressively and the total body content of exchangeable potassium decreases. The continued loss of potassium in the urine during starvation confirms the endogenous source of this cation. With tissue catabolism, the K_e to weight ratio decreases at a relatively constant rate, at least for the first two weeks of starvation.

The exchangeable potassium loss after a week of starvation exceeds by 18 meq. the potassium loss as measured by the external urinary balance method. According to the method of calculation of K_e, the more in-

6. Snedecor, G. W., *Statistical Methods*, Iowa State College Press, 1946.

TABLE I. Effects of Starvation on Potassium and Creatinine Metabolism in Normal Male Rabbits (Group A).

Starvation	Wt, kg	Exchangeable K (meq)	Ke/wt (mg/kg)	Urinary creatinine (mg/wk)	Creatinine coef. (mg/kg)	Urine vol (ml/wk)	Oral intake K (meq/wk)	Urinary K39 excretion (meq/wk)
Before	2.24 ± .33*	137.4 ± 15.4	62 ± 5.43	694 ± 207	45.8 ± 4.85	901 ± 356	77.8	76.6
1st wk	1.89 ± .33	96.4 ± 23	51.8 ± 14.80	548 ± 155	38.9 ± 7.95	1456 ± 165	0	23
2nd wk	1.53 ± .35	62.4 ± 11.7	41.4 ± 4.45	424 ± 160	34.8 ± 7.92	1149 ± 355	0	

* Standard deviation.

Results of Balance Studies

Starvation	wt	Exchangeable K (meq)	Ke/wt (meq/kg)	Urinary creatinine (mg/wk)	Creatinine coef. (mg/kg)	Urine vol (ml/wk)	Urinary K39 excretion (meq/wk)
1st wk	−.35	−41*	−10.2	−146	−6.9	+555*	+23
2nd wk	−.71*	−75*	−20.6*	−270	−11	+248	

* Statistically significant difference when compared with baseline values. $P = < .01$.

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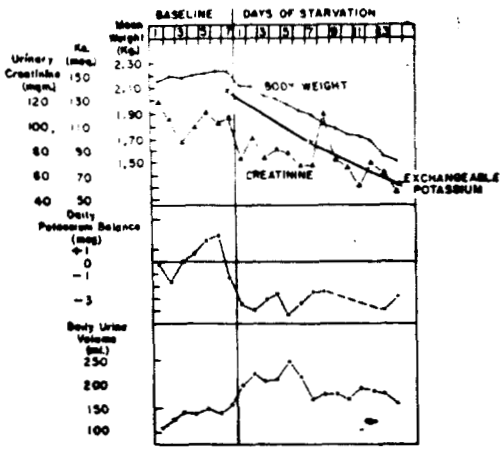

FIG. 1. The effect of starvation on potassium and creatinine metabolism in normal male rabbits.

complete the exchange of radioactive atoms with the normal intracellular untagged potassium ions, the smaller would be the calculated exchangeable potassium content. In normal rabbits approximately 90% of the body potassium would have exchanged in 24 hours(2). It appears that with starvation and the consequent reduction in metabolic rate, which is known to set in quickly during fasting, the rate of turnover of intracellular potassium is decreased, or the relative amount of "bound" potassium is increased.

Comparison of the values in the normal animals (Group C) and the rabbits starved for one week (Group B) revealed no significant difference in the concentrations of tissue water or tissue radiopotassium for the following tissues: skin, subcutaneous connective tissue, abdominal muscle, gastrocnemius muscle, quadriceps muscle, diaphragm, tendon, cartilage, heart ventricle, heart auricle, lung, liver, spleen, stomach, appendix, adrenal and kidney. The mean weight loss of the starved animals after one week of starvation was 0.43 kg (13.8% of the initial mean body weight). Since the tissue analyses show no significant difference, it is possible, therefore, to account for the total decrease in body potassium on the basis of tissue breakdown without postulating an intracellular deficiency in the remaining tissues.

The creatinine coefficient serves as an index of relative muscle mass. Although the decrease in this ratio observed with starvation may imply 1) a decrease in the rate of metabolism of muscle tissue; 2) a decrease in the total muscle mass, or both, the differences at one and 2 weeks were not significant when compared with the baseline value. With diminished intake of food, a relatively greater utilization of tissue fat and carbohydrate in preference to protein would be expected initially. The utilization of fat was evident grossly at the time the samples for analyses were obtained; the amount of perirenal fat in the starved animals, for instance, was considerably less than in the control rabbits, though the muscles appeared similar.

Since no significant change in radiopotassium concentration was found in any specific tissue or organ in starved animals, the decrease in rate probably was generalized over the body. The possibility that such an alteration might be limited to the skeletal or nervous system must be considered, since these two tissues were not included in the present analyses. The radioactive potassium method, therefore, appears to be a more sensitive index of the functional defect produced by starvation than the external balance method.

The possibility that the loss of potassium in the feces during starvation was increased over the normal amount must also be considered. In such an event, the urinary balance study alone would underestimate the total loss. However, since, during the prestarvation period, there was no loss in the stool, it would be highly unlikely that the discrepancy of 18 meq. between the external urinary balance and the K_e could be explained on this basis alone.

These studies complement our clinical observations and lend support to the hypothesis that whenever catabolic processes exceed the anabolic, the loss of body potassium, weight, and muscle tissue parallel each other(1).

Summary. 1. Male rabbits were starved in order to study the effect of a decrease in food intake on potassium metabolism. 2. Serial determinations of the exchangeable potassium content and urinary potassium and creatinine excretion were made in one group of animals. The mean decrease in exchangeable potassium

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content was 29.8 and 54.6% of the baseline value after one and two weeks respectively. The corresponding mean decrease was 15.6 and 31.7% in body weight. No significant changes in urinary creatinine excretion occurred. 3. In a separate group of rabbits, tissue analyses for radiopotassium and water concentration after one week of starvation revealed no significant changes when compared with control animals, continued on a constant diet. 4. The decrease in total body potassium content, as measured by the exchange of isotopic potassium, as well as the loss in the urine during starvation could be accounted for on the basis of tissue catabolism without postulating an intracellular deficiency of this cation in the remaining tissues. A functional abnormality in potassium metabolism may be detectable by the radioisotope method prior to its manifestation by the external balance method.

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THE IMMUNOPHYSIOLOGY OF SERUM SICKNESS. ALTERATIONS IN THE BLOOD VOLUME AND THIOCYANATE SPACE IN RELATION TO THE DEVELOPMENT OF HUMORAL ANTIBODIES IN THE RABBIT[1]

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(Submitted for publication November 22, 1950; accepted, January 22, 1951)

The development of edema during the course of acute infectious diseases has been observed in patients for many years. The retention of water and salt during the course of untreated pneumococcal pneumonia was described early in the century (1). The loss of chlorides from the blood and their subsequent excretion in the urine during recovery was shown to be associated with retention by the body of sodium and calcium while potassium and magnesium were excreted normally or in excess.

After antipneumococcal horse serum became available for treatment of pneumonia, serum sickness developed frequently in patients during convalescence. The clinical picture of serum disease with edema and fever was shown to be accompanied by the retention of chlorides and water in the body (2). Further investigation of serum disease in human beings revealed that the clinical symptoms are associated with the appearance in the blood of precipitins against the species of animal in which the therapeutic serum was prepared (3). The appearance of precipitins precedes recovery from the disease by a short interval and is coincident with disappearance of the antigen. These studies indicated that antigen-antibody reactions were of clinical significance in the production of disease in human beings, and that the mechanism was in some way related to that of experimental anaphylaxis in animals.

During the course of studies on patients with Rocky Mountain spotted fever, it was observed that alterations in the distribution of body fluids with the development of marked clinical edema reached a maximum just before clinical recovery begins. The alterations in distribution of fluid appear to predispose to peripheral circulatory collapse. In such crises the blood chlorides, blood volume and extra-vascular thiocyanate space are altered transiently but return to normal with recovery (4). Observations in one patient who had been given hyperimmune rabbit antiserum for Rocky Mountain spotted fever showed that the alterations in the blood volume and thiocyanate space, which occurred during classic serum sickness following recovery from the initial disease, resembled strikingly those seen in the same patient during the acute phase of the rickettsial infection. These observations suggested the possibility that an immune reaction may be directly or indirectly responsible for the changes in the permeability of membranes which permit alterations in fluid distribution in rickettsial spotted fever.

The present experiments were undertaken to determine if changes in the blood volume and thiocyanate space occur in an antigen-antibody reaction induced *without infection* and to relate the physiologic alterations to the development of immunity. The anatomic changes occurring with serum sickness have been well studied, but the physiologic alterations induced by the disease are very poorly understood (5). Accordingly, rabbits were injected with human plasma and the changes in distribution of body fluids were followed serially.

MATERIAL

Domestic rabbits of mixed breeds, weighing 2 to 4 kilograms each, were placed in individual cages and fed a stock diet, supplemented once weekly by fresh green vegetables. Water was given without restriction.

The same sample of pooled human plasma was used as the sensitizing antigen and as antigen for the precipitin tests in each individual experiment.

[1] This study was supported (in part) by research grants from the John and Mary R. Markle Foundation, the National Heart Institute of the National Institutes of Health, Public Health Service, and the Atomic Energy Commission.

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METHODS

Chemical techniques: The *plasma volume* was determined by the T-1824 (Evans Blue) dye method. Syringes holding 2 ml. were calibrated to deliver 0.5 ml. of a solution containing 3 mg. of the dye per milliliter. The *thiocyanate space* was determined simultaneously. Syringes holding 10 ml. were calibrated, and 2.0 ml. of a solution containing 50 mg. of sodium thiocyanate per milliliter were injected.

The concentrations of the dye in rabbit serum were determined by the Evelyn photoelectric microcolorimeter and the thiocyanate concentrations with the Evelyn photoelectric macrocolorimeter (6). *Hematocrit* readings were made on oxalated blood placed in Wintrobe tubes, centrifuged at 3,000 revolutions per minute for 30 minutes.

The *total blood volume* was calculated from the plasma volume and the hematocrit.

Immunologic techniques: The humoral antibody titer was followed by ring *precipitin tests.* The human plasma was diluted 1 : 100 in water and used as antigen to overlay rabbit serum serially diluted in 2 × 20 mm. tubes to a maximum of 1 : 32. In the definitive experiment human plasma was diluted in physiologic saline, but the rabbit serum was undiluted. In the preliminary experiments dilution of human plasma (antigen) as high as 1 : 100,000 gave positive tests with undiluted rabbit serum (antibody) as definite as those obtained with 1 : 100 dilution of antigen and 1 : 32 dilution of antibody.

The dye was diluted as much as 1 : 50,000 for precipitin tests in group 1; no precipitins were encountered, so the test was not performed in later groups.

Skin tests to the dye were never positive in group 1; so they were not performed in later groups. No skin tests were done with plasma in any group.

Experimental procedure: In the morning before feeding the animals were weighed and strapped on their backs to wooden boards. In groups 1 and 2 blood was withdrawn without anesthesia in an oiled syringe by cardiac puncture.

Comparison of data indicated that reliable results could be obtained without deaths from hemopericardium by nicking an ear vein with a knife and collecting the blood in dry test tubes. Accordingly in group 3, 2 ml. were collected and allowed to clot; 1 ml. was then collected in another dry tube containing 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate. The dye was injected into a vein in the opposite ear, the syringe changed, and the sodium thiocyanate injected through the same needle within one minute. Ten minutes following completion of the injection, 2 ml. of blood were collected from the first ear.

Preliminary experiments: No data were available on the reliability of the technical methods in small animals, on the effect of repeated bleedings at short intervals, or on the normal values for plasma volume and thiocyanate space in rabbits. It was not known whether rabbits might become sensitized after repeated injections of the dye and hence develop a picture analogous to serum sickness.

Accordingly, six animals (group 1) were used as a control group on the technical procedures. Determinations of the blood volume, thiocyanate space and precipitin titers, as well as a skin test, were done simultaneously every second or third day. The animals received no plasma. They were kept under observation for a minimum of 30 and a maximum of 46 days. A minimum of nine and a maximum of 16 determinations were done on each individual animal.

A second experiment was performed in order to study the variability of the physiologic changes in individual animals ill with serum sickness. It was also necessary to determine the optimum route of sensitization, the required dose of plasma and the time relationship of any observed alterations to the development of humoral immunity.

Accordingly, 11 animals (group 2) were injected with plasma. The two groups were treated in the same fashion except for the sensitizing injections of plasma. After a baseline for the individual animal had been established, the rabbits in group 2 received injections of plasma ranging from 2.2 to 5.0 ml. per kilogram of body weight. Eight animals received the sensitizing dose intravenously and three were injected subcutaneously in the intrascapular region. Three of the former animals received a second injection intravenously on the day following the initial injection.

During the clinical peak of the disease, determinations of the blood volume and thiocyanate space were done on two successive days. All animals in group 2 were followed at least 24 days following the injection of plasma, unless accidental death from hemopericardium supervened. A minimum of seven determinations were done on each animal in group 2 after the baseline was established.

In order to interpret the data critically a satisfactory statistical method of analysis had to be devised. It was necessary to determine whether groups could be compared or whether each individual animal must serve as its own control. The data from groups 1 and 2 were analyzed by several techniques and the results obtained were submitted to an impartial arbiter for criticism.[3] A satisfactory statistical method was devised and the definitive experiment designed to improve the statistical significance of the data obtained in the preliminary experiments.

Definitive experiment: Twelve rabbits (group 3) of more uniform size, weighing approximately 2 kilograms each, were injected with human plasma. The chemical and immunologic techniques and experimental procedure as described above were followed. At least two baseline determinations were made every second day on each animal. After the second determination, each animal was injected intravenously with a single dose of 3 ml. of plasma per kilogram of weight. Precipitin tests were performed daily on each rabbit, but no further fluid vol-

[3] We are deeply indebted to the Institute of Statistics of the University of North Carolina, Raleigh, N. C., for critical review of the data and for suggesting the method subsequently used.

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ume determinations were done until the day that a positive precipitin reaction was first obtained. The measurements were repeated on that day, on two consecutive days thereafter, and at varying intervals subsequently until the chemical values had returned approximately to the baseline.

Statistical analysis: The total blood volume, plasma volume, hematocrit and thiocyanate space were recorded in absolute values. The mean of two successive baseline determinations on each animal was compared with the mean of the two successive determinations made on the day the precipitins became positive and on the following day. The mean differences between the baseline values and those obtained after the precipitin test became positive were calculated. The significance of the mean difference was tested by the following formulae in which $\bar{d}$ = mean difference, $s_{\bar{d}}$ = standard error of the mean difference, S = summation of, n = number of animals:

$$t = \frac{\bar{d}}{s_{\bar{d}}} \qquad s_{\bar{d}} = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n(n-1)}}.$$

The calculated value for t was compared with the value at n − 1 degrees of freedom as obtained from standard tables.

The data as obtained in absolute values were then calculated in terms of milliliters per kilogram of body weight on the day the measurement was made. The statistical significance was determined by the same formulae.

In view of the fact that the experiment was planned to evaluate changes in a febrile illness occurring in a growing animal the difference in the amount of food and water ingested by the animals unavoidably introduced variables which were difficult to control. Accordingly, the data were re-calculated in terms of milliliters per kilogram of *initial* body weight (the day of the first baseline determination) in order to avoid consideration of differences in food and water intake and in order to permit ready comparison with the data in group 1. The statistical significance was then determined by the same method.

It should be noted that in the definitive experiment no attempt was made statistically to compare the experimental group as a whole with the control group. Each animal served as its own control.

RESULTS

Control on methods: None of the animals in group 1 showed any symptoms or signs suggesting serum sickness. No reaction was noted in any rabbit to the repeated intravenous injection of dye and no evidence of sensitization was detected by skin or precipitin tests. The fatal dose of T-1824 dye was found to be 3 mg. per kilogram of body weight given intravenously; death occurred within 15 minutes.

Moderate variations in *plasma volume, thiocyanate space* and *total blood volume* per kilogram of body weight and in the *hematocrit* were noted among the different animals. In serial determinations on a single rabbit, however, the results were much more uniform. When the data obtained near the end of the experiment were examined, a slight decrease in the thiocyanate space in relation to

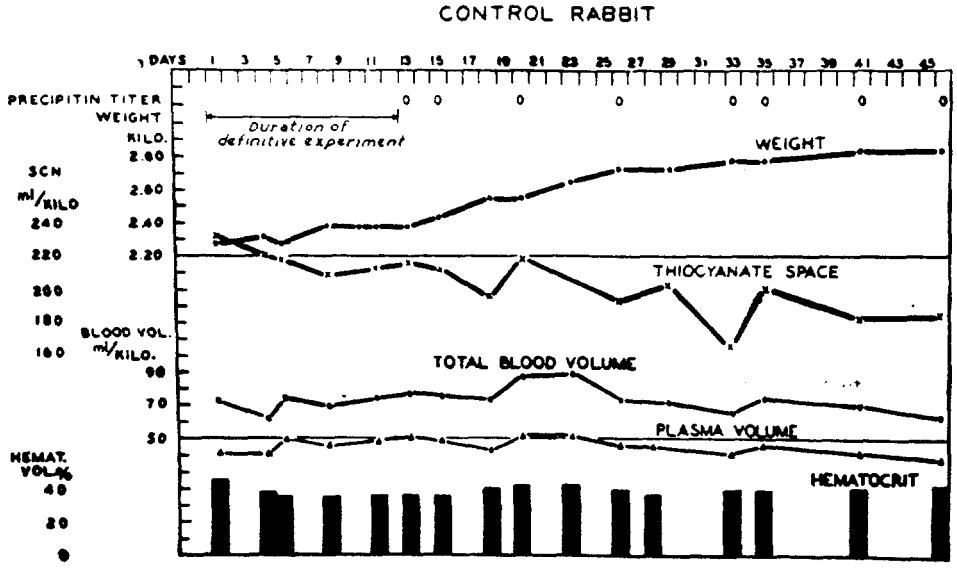

FIG. 1. FLUID SPACES IN A CONTROL RABBIT (GROUP 1) NOT INJECTED WITH PLASMA

All of the serial determinations are plotted in this graph; only four values which fall within the indicated duration of the definitive experiment are used in the calculations in Tables I and II.

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TABLE I

Control of technical methods (group 1)

Rabbit number	Baseline					Time of expected response				
	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit
	Kilo.	*ml./Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*Vol. %*	*Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*Vol. %*
1	2.30	219.6	44.2	68.8	36.0	2.39	216.1	49.4	75.5	34.5
2	3.04	197.8	47.0	68.8	31.3	3.04	197.0	55.8	87.2	35.7
3	2.53	210.3	51.3	73.5	30.0	2.78	193.8	49.8	72.8	31.5
4	3.27	177.5	43.0	63.5	33.3	3.24	162.5	40.0	62.1	35.5
5	2.76	207.7	49.7	77.3	35.8	2.93	198.1	47.9	73.3	34.5
6	2.73	155.3	42.7	67.0	36.3	2.92	162.2	45.0	62.7	28.0
Mean for group	2.77	194.7	46.3	69.8	33.8	2.88	188.3	48.0	72.3	33.3
s	3.05	24.00	3.62	4.90	2.68	0.29	21.50	5.27	9.27	2.99
s_x	0.14	4.69	1.47	2.00	1.08	0.12	8.80	1.02	3.78	1.22

The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same animal six to eight days after the baseline was established. No injections of plasma were made.

s = Standard deviation

s_x = Standard error of the mean

weight was observed; this finding would be expected as the animals increased in size from normal growth. Figure 1 illustrates the serial values in a representative control rabbit.

The mean of two consecutive baseline determinations is recorded in Table I and compared with the mean for two consecutive determinations done between six and eight days after the second determination, the time interval at which a positive precipitin test would be expected were the animals injected with plasma. The statistical analysis is summarized in Table II and indicates that, in absolute values or in milliliters per kilogram of body weight on the day of the determination, no significant difference was found. This statement is true for plasma volume, thiocyanate space, total blood volume, hematocrit, and weight.

These data have subsequently been extended in a series of 45 animals in conjunction with other studies on normal values in rabbits (7).

General observations: Clinical serum sickness, manifested by erythema, edema, or both at the base of the ears, generalized subcutaneous edema and hyperirritability developed in 10 of the 11 animals in group 2, and in 11 of the 12 animals in group 3. Clinical signs usually began on the fifth or sixth day following the initial injection of plasma and usually lasted two or three days. The edema was most striking in the skin over the abdomen, but was not evident unless the fur had been clipped; the skin appeared pale and felt soggy, hot, and moist. Fever (rectal temperature over 40 C.) was not a constant feature of clinical serum sickness. The clinical severity of serum sickness bore no relation to the size of the sensitizing dose. Multiple injections produced no more severe reactions than did single injections. The subcutaneous route of sensitization was as effective as the intravenous route, but variations in absorption could not be detected or controlled.

TABLE II

Statistical significance of controls on methods (group 1)

	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit
	Kilo.	*ml.*	*ml.*	*ml.*	*Vol. %*
$\bar{d}$	+.11	+4.33	+10.00	+15.33	+.50
$S\bar{d}$	.05	13.60	5.30	9.35	1.81
t	2.20	.32	1.89	1.64	.28
		ml./Kilo.	*ml./Kilo.*	*ml./Kilo.*	
$\bar{d}$		−6.42	+1.67	+2.45	
$S\bar{d}$		3.67	1.90	3.58	
t		1.75	.88	.68	

No significant difference is noted between the baseline values and those obtained at the time of the expected response. The data are calculated in absolute values and in terms of weight on the day of the determination. The value of t was not considered significant if P was greater than .01.

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Preliminary experiments: The alterations in plasma volume, thiocyanate space, total blood volume, and hematocrit which were observed in a representative rabbit sensitized with human plasma are illustrated in Figure 2. The changes in fluid distribution bore no direct relationship to the sensitizing dose of plasma, but paralleled the clinical severity of the disease.

In 10 of the 11 animals in group 2 increases in the *thiocyanate space* developed at some time during the period of observation. In eight animals two rises occurred, the first usually between the third and sixth days, and the second between the 11th and 20th days. The first rise usually coincided with the onset of the clinical manifestations; the second was usually marked by an increase in the subcutaneous edema. The changes in the thiocyanate space usually paralleled the weight changes, especially with the second reaction. Certain discrepancies were noted, however.

The most profound drop in *plasma volume* was noted in those animals which developed the severest clinical signs of serum sickness and the greatest and most prolonged increases in thiocyanate space. In seven animals there was no significant decrease in plasma volume, however.

In the four animals which showed a definite drop in plasma volume an appreciable decrease in the *total blood volume* also occurred. In four animals no changes in the total blood volume occurred during the experimental period.

In all the animals with severe early clinical manifestations of serum sickness a drop in *hematocrit* was associated with a rise in thiocyanate space and a decrease in plasma volume. In six animals no striking variations in the hematocrit occurred

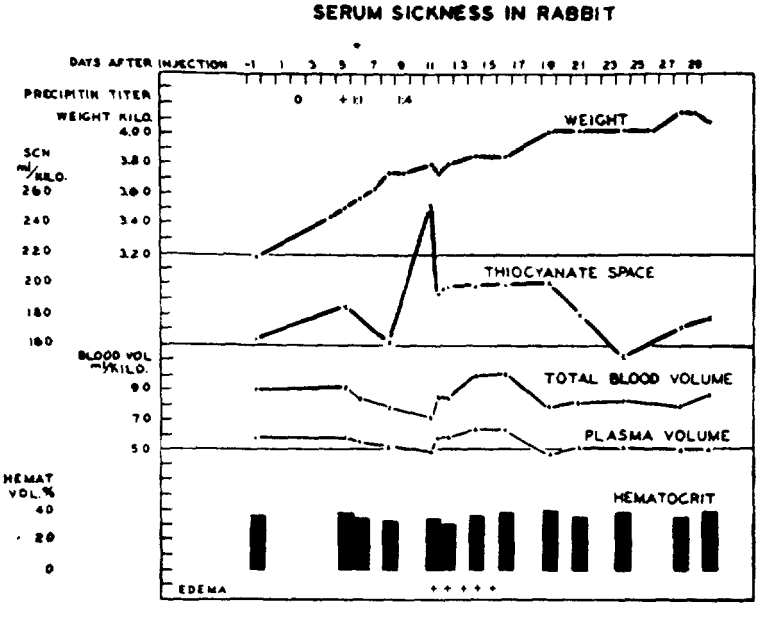

FIG. 2. FLUID SPACES IN A RABBIT (GROUP 2) GIVEN AN INTRAVENOUS INJECTION OF HUMAN PLASMA

Note the increase in thiocyanate space, which is out of proportion to the slight increase in weight, on the 11th day and the associated decrease in the plasma and total blood volume. On the following day, there was a marked decrease in the thiocyanate space, which was out of proportion to the decrease in weight. All the thiocyanate values from the 11th to the 21st day were elevated. After the 21st day, the variations in the thiocyanate space paralleled the weight changes and were not significant.

In the statistical calculations in the definitive experiment (group 3), Tables III and IV, two baseline values, two values obtained at the time precipitins appeared, and two values obtained at the termination of the experiment were used.

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TABLE III

Alterations in fluid spaces in rabbits ill with serum sickness after an injection of human plasma (group 3)

Rabbit number	Baseline					Response*				
	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit
	Kilo.	*ml./Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*Vol. %*	*Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*ml./Kilo.*	*Vol. %*
18	2.10	228.5	46.0	75.5	39.0	2.10	322.5	48.0	74.0	35.0
19	2.14	235.5	48.5	80.0	40.0	2.14	242.5	47.0	75.5	37.5
20	1.99	246.5	48.5	79.5	39.0	2.14	282.5	39.5	61.5	35.7
21	1.87	245.5	45.0	75.5	40.5	1.86	296.0	39.0	62.5	37.5
22	1.74	289.5	48.5	78.5	37.5	1.96	273.0	44.0	68.5	35.2
23	1.87	266.0	45.5	74.0	38.5	2.04	313.5	40.0	62.0	36.0
24	1.76	229.5	50.0	80.0	37.0	2.05	281.5	35.0	54.5	36.0
25	2.10	212.5	51.0	84.0	39.5	2.30	265.0	39.0	62.0	36.7
26	2.22	183.0	54.0	88.5	39.0	2.24	225.5	40.5	62.5	35.7
27	1.76	215.0	63.0	108.0	42.0	1.93	280.0	47.0	78.0	40.0
28	1.96	202.5	52.0	85.5	39.5	2.07	307.5	40.5	63.5	36.0
29	1.85	220.5	54.0	92.0	41.0	1.90	311.0	48.5	78.0	38.5
Mean for group	1.95	231.2	50.5	83.4	39.4	2.06	283.4	42.3	66.9	36.7
s	0.17	28.60	4.95	9.47	1.38	0.13	28.00	4.40	8.44	1.48
s_x	0.05	8.21	1.45	2.73	0.40	0.04	8.42	1.27	2.54	0.42

* The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same rabbit done on the day precipitins to human plasma appeared and on the next day.
s = Standard deviation
s_x = Standard error of the mean

with the initial increase in thiocyanate space. None of the animals revealed a decrease in hematocrit in association with the second rise in thiocyanate space.

TABLE IV

Statistical significance of alterations in fluid spaces in rabbits ill with serum sickness (group 3)

	Weight	Thiocyanate space	Plasma volume	Total blood volume	Hematocrit
	Kilo.	*ml.*	*ml.*	*ml.*	*Vol. %*
$\bar{d}$	+.11	+126.00	−11.08	−24.67	−2.73
$S\bar{d}$	.03	21.40	2.81	4.57	.23
t	3.70	5.89	3.94	5.40	11.87
		*ml./Kilo.**	*ml./Kilo.*	*ml./Kilo.*	
$\bar{d}$		+51.30	−8.50	−16.50	
$S\bar{d}$		9.85	1.49	2.60	
t		5.21	5.70	6.35	
		ml./Kilo.†	*ml./Kilo.*	*ml./Kilo.*	
$\bar{d}$		+74.16	−6.25	−12.25	
$S\bar{d}$		9.98	1.23	2.35	
t		7.43	5.08	5.21	

* Calculated in terms of weight on the day of determination.
† Calculated in terms of weight on the day of the first baseline.
The data all show statistically significant differences between the baseline values and those obtained at the time of the humoral antibody response. The value of t was considered significant if P was <.01.

In all 11 animals positive *precipitin tests* to human plasma developed between the third and the 12th days following the initial injection of plasma. In eight animals the thiocyanate space decreased and the clinical symptoms and signs subsided as the precipitin tests became positive. In all animals except one the precipitin tests remained positive throughout the period of observation. In this animal, which was followed longer than the others, the precipitin test was negative after the 28th day.

Definitive experiment: The alterations in plasma volume, thiocyanate space, total blood volume, hematocrit and body weight are summarized in Table III.

The statistical analyses are summarized in Table IV.

A statistically significant increase in *thiocyanate space* was noted in each animal at the time the humoral antibody was first detected. In all instances a return to baseline values had occurred by the 20th day after injection of the sensitizing dose. A significant increase in body *weight* oc-

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curred during the period of alteration in thiocyanate space. The body weight continued to increase from normal growth after the animal recovered from serum disease.

In contrast to the increase in thiocyanate space and weight, a statistically significant decrease in *plasma volume, total blood volume* and *hematocrit* occurred in all animals.

All of the data are statistically significant, whether the calculations are based on the comparison of *1*) absolute values; *2*) values based on milliliters per kilogram of weight on the day of the determination; or *3*) values based on milliliters per kilogram of weight on the day of the first baseline measurement.

In order to determine whether these changes were reversible, the mean of two consecutive determinations at the termination of the experiment was compared with the mean baseline values in each animal. The mean differences for the body weight, plasma volume, total blood volume, and the thiocyanate space were not significant. The decrease in the hematocrit at this time was significant. These data are not included in the tables.

In all 12 animals positive *precipitin tests* to human plasma developed between the fourth to the eighth day after injection. The distribution was as follows:

	Onset of positive precipitin tests				
Days after injection	4	5	6	7	8
Number of rabbits	2	1	2	4	3

DISCUSSION

The experiments indicate that immune reactions may be associated with alterations in the blood volume and the fluid space which is available for the diffusion of thiocyanate ion. The maximum alterations in rabbits sensitized with pooled human plasma occurred at the time circulating humoral antibodies (precipitins) appeared in the serum. This finding correlates well with the previous clinical observation that the symptoms of serum sickness begin to disappear with the appearance of precipitins (3).

The greatest change was observed in the thiocyanate space. Alterations in the plasma volume and the hematocrit were less pronounced in magnitude and were more transient, lasting only one to three days as compared with the changes in thiocyanate space which occasionally persisted into the second week. The weight of the animals tended to increase with the increase in thiocyanate space, though the changes could not always be correlated closely. Apparently the normal distribution of fluid in the body was altered as the result of an increase in permeability of membranes which was completely reversible.

Methods: The chemical methods were reliable since the results in the control animals (group 1) were consistently reproducible. The possibility that the dye might act as an antigen, producing serum sickness, or as a haptene, forming a new compound which might induce an auto-antibody response, was ruled out by the preliminary experiments.

The variability between individual animals in the experimental group (group 3) was controlled by determining baseline values on each rabbit and comparing these values with the response in the same animal for the statistical analysis. The effect of growth and of variations in food and water intake was apparently of little significance in this experiment, since the data were found to be statistically significant whether calculated in terms of absolute values or in relation to weight at the time of the first baseline determination or on the day of the immunologic and clinical response.

Though whole plasma is a complex of many protein fractions, each of which might induce its own slightly different immunologic response, it was felt that a mixed antigen might more closely simulate a natural infection in which multiple antigens are liberated by the infecting organism. Anatomic studies have indicated that the body responds at different times after the injection of albumin or globulin fractions derived from the same plasma (5). The two peaks in the thiocyanate space observed in the preliminary experiments may be due to this difference.

Permeability of membranes: The present experiment offers a logical explanation for the phenomenon of retention of salt and water; if the permeability of membranes to ions is increased, these ions could diffuse from blood into interstitial spaces and from interstitial fluid spaces into cells. The

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resultant hypochloremia would decrease the renal excretion of salt and water. The administration of saline would increase the edema, until restoration of the integrity of membranes corrected the alterations in the internal environment. It is not clear whether the physiologic alteration is confined solely to vascular walls, as pathologic experiments might suggest, or whether cellular membranes are also affected. Von Pirquet suggested that the edema was interstitial, but the data in the present experiments may indicate that some of the change occurs within cells (8).

Capillary permeability: The alterations in the vascular tree can be visualized as a series of pores in the capillary wall whose size is related to the clinical severity of the disease. In mild instances of the disease water and ions which normally traverse the membrane would leave the blood more readily. Larger molecules such as protein would be lost in more severe instances and red blood cells would pass out of the vascular tree only in the most severe reactions. Petechial hemorrhages in organs have been recognized for years as a manifestation of anaphylactic reactions.

The loss of water and ions from the vascular tree into tissues is compatible with the observations on retention of water, sodium and chloride during pneumonia and serum disease (1, 2). The permeability of the vascular tree to some colloids must have been increased because the decrease in plasma volume was concomitant with the increase in thiocyanate space. The colloids lost from the circulation to the interstitial spaces would tend to retain water extravascularly until they had been reabsorbed, presumably through the lymphatics, broken down in the tissues, or until other homeostatic mechanisms had been operative.

It is probable that the amount of protein lost from the vascular tree during serum disease is not great. The dye is known to be bound to albumin, one of the smaller protein molecules of the blood. Any great loss of albumin into interstitial spaces would reduce the amount of circulating dye and hence lead to a larger value for plasma volume by the chemical method; a loss in blood volume of any magnitude would thus be obscured. If protein was lost from the vascular tree, the defect was detected only with the first rise in thiocyanate space and was transient.

The drop in hematocrit though slight may indicate a loss of red blood cells from the vascular compartment. The drop was apparently not due to repeated bleeding since the hematocrit remained constant in group 1 in which more determinations were done in the same interval of time. No jaundice was noted clinically and the serum was never icteric, hence it seems unlikely that the red cells were hemolyzed. It is possible that the red cells were reduced in size or that they were pooled in some portion of the vascular tree which was not measured by the techniques used.

Cellular permeability: It is not known whether the thiocyanate ion normally diffuses into cells or not. The increase in thiocyanate space therefore could be due to *1*) interstitial edema, *2*) increase in permeability of cells to the thiocyanate ion, *3*) destruction of tissue cells with liberation of cellular water, or *4*) dehydration of cells with a shift of intracellular fluid into the interstitial spaces. The fact that some animals showed a sudden increase in thiocyanate space without an increase in body weight would favor the thesis that some redistribution of fluid had occurred whether or not additional fluid ingested in food or water was retained. The hypothesis that tissue cells were destroyed, liberating additional water and making it available for dilution of thiocyanate ion, does not seem likely. The rapidity of the increase in the thiocyanate space and its reversibility makes this explanation appear highly improbable. Such a sudden increase in thiocyanate space without an increase in body weight would have to mean that the associated tissue destruction exceeded the amount of interstitial edema. Furthermore, preliminary nitrogen balance studies in other rabbits have disclosed no marked increase in the excretion of urinary nitrogen, and presumably in cell destruction, during clinical serum sickness.

Mechanisms: It is not known whether the antigen in serum disease attaches solely to the vascular wall or to tissue cells outside the vascular system as well. As far as the effect on the circulation is concerned, it would be immaterial whether the loss of substances from the vascular tree is attributable to an abnormal passage of fluid between the endothelial cells, as would occur if the permeability of the intercellular cement substance were decreased, or whether the permeability of the

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cells themselves is abnormally increased, so that the fluids pass through the cells into the interstitial spaces.

The participation of cells in immune reactions is recognized clinically in allergic states; the deleterious antigen-antibody reaction is assumed to occur on the cell surface or perhaps within the cell. It has been postulated that histamine or some similar substance is released during the interaction of antigens with antibodies. Histamine will alter the local permeability of capillary walls. The liberation at the cell wall of some substance which would increase the permeability of the cell membrane would enhance the transmission of molecules, including the thiocyanate ion, across the surface. Such a hypothesis is of more than academic interest, since it is known that intracellular enzyme systems are affected by the extent of hydration of cells and by the concentration of ions. An increased cellular content of ions could be proved by isotopic techniques which could measure not only the diffusion of labelled thiocyanate but the relative movement of radioactive sodium, potassium, and chloride ions across cell membranes.

Relation to infections: The present findings indicate that the physiologic alterations which may result from antigen-antibody reactions are more profound than has been recognized in the past. The physiologic changes deserve more attention in the therapy of infectious diseases. In infections, the invading micro-organisms multiply within the host and release or secrete multiple antigens. With organisms known to locate intracellularly during some phase of the disease, as is true with the viruses and rickettsias, antigens are produced within the cell. Those antigens which could readily diffuse out would produce predominantly humoral antigen-antibody reactions; those antigens which would be retained within cells would be more likely to induce intracellular antigen-antibody reactions and hence increase the extent of the permeability of cellular walls. A most profound alteration in functions of the body could thus be produced. The possible application of antihistaminic drugs or adrenal cortical hormones to therapy of infections with an allergic element in the host response is based on such alterations in function.

SUMMARY

1. Rabbits sensitized by the intravenous injection of pooled human plasma developed clinical edema with significant increases in weight and in the fluid space available for dilution of thiocyanate ions.

2. Significant decreases in the plasma volume, total blood volume and hematocrit were observed transiently during the most marked alteration of thiocyanate space.

3. The changes were interpreted as indicating an increase in permeability of the vascular tree, probably in the capillary wall, so that crystalloids, colloids, and red blood cells are progressively lost from the circulation as the magnitude of the defect increases with increase in the clinical severity of the disease.

4. Occasional discrepancies observed between the changes in thiocyanate space and in weight suggest that cellular permeability is probably also increased to water and ions.

5. The physiologic changes occurred at the time humoral antibodies (precipitins) appeared, suggesting that the alterations may have been due directly or indirectly to an antigen-antibody reaction.

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[illegible] as report on the research project.

TOXICITY OF P^{32} AS [illegible]

[illegible] by the Atomic Energy Commission under direct contract [illegible], with the Bowman Gray School of Medicine of Wake Forest [illegible]

Principal Investigators: Drs. S. Warren and L. T. Harrell, Jr.

The present report covers the work done from March 16, 1951 to March 15, 1952. In addition to the principal investigators, Dr. W. S. Cornatzer and Dr. M. M. Stevens participated for short periods in the research work. Technical assistance was given by J. Giles and C. Downs.

A. General outline of the work:

During the past year the experiments have been carried along two main parallel lines: (a) continuation of our systematic study of the dietary factors which might protect against, or favor the recovery from, the effects of internal radiation by P^{32}, (b) continuation of our attempts to modify by various means the deposition of the isotope in the bones or its mobilization from the bones.

Until now, the latter group of investigations did not yield satisfactory results. On the contrary, the experiments on the role of pyridoxin in the protection against the injury by P^{32} were quite conclusive. Our present findings in this respect parallel those previously obtained by us with the combined administration of vitamin B_{12} and folic acid. In both cases a significant protection against the effects of internal radiation could be demonstrated only in animals in which the vitamin deficiency was already developed. Since damage to the blood-forming tissues probably plays a dominant role in the toxicity by radioactive phosphate, it seems possible that the protective action of B_{12}+ folic acid and of pyridoxin is related to the high requirement for these vitamins of rapidly regenerating structures, such as the erythro- and lympho-poietic tissues. Future work on this aspect of the problem is contemplated and in this respect, the results of a collateral investigation on the uptake of P^{32} by the nucleic acids of developing chick embryo, may perhaps be of some interest.

B. Protective Role of Pyridoxin against P^{32}

Two series of experiments were made on a total of about [illegible] rats. In preliminary experiments the animals were maintained on a low protein diet without pyridoxin and injected with mid-lethal doses of P^{32}. Administration of large [illegible] of pyridoxin [illegible] animals [illegible] the survival figures.

Since in these experiments the rats did not show symptoms of vitamin deficiency, the experiments were repeated on animals in which the deficiency of pyridoxin was enhanced by the administration of a pyridoxin analog [illegible] (desoxypyridoxin (DOP)). Symptoms of a severe deficiency developed rapidly and many deaths occurred even in the controls not injected with the isotope. By reducing [illegible] the first few days of the experiment the period in which DOP was added to the diet, it was possible to slow down the progress of the deficiency and permit the survival of most of the controls not receiving the P^{32}. Under these conditions a marked reduction in the number of deaths

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and a definite prolongation of the survival time of the mice injected with [illegible] was observed, when pyridoxin was added to the diet, which has metabolic antagonist. For instance, in one of the series (35% casein diet with [illegible] g. of diet - mice injected: 5 microcuries p. g. of body weight), supplementation of the diet with pyridoxin (2 mg / 100 g.) shifted the day of 50% deaths from the 14th to beyond the 42nd, the average time of survival from 19 to 33 days and the proportion of survivors (at the 21st day after the introduction of the isotope) from 19% to 77%.

In subsequent experiments a relative deficiency of pyridoxin was induced by increasing the metabolic demands for this vitamin. Mice placed on a high protein diet to which either cystine or methionine was added, developed obvious symptoms of pyridoxin deficiency. In these animals also pyridoxin administration exerted a quite significant protection against the injurious action of P^{32}. For instance, in animals maintained on a 32% casein diet supplemented with 0.6% cystine and injected with 5 microcuries of P^{32} per g. of body weight, the administration of pyridoxin (1 mg / 100 g. of diet) shifted the day of 50% deaths from the 18th to beyond the 42nd day, the average time of survival from 21 to 36 days and the proportion of survivors (at the 21st day) from 22% to 63%.

A statistical analysis of the results obtained in the various series of experiments indicates that the differences observed have a high degree of significance and are much larger than those which might be ascribed to a simple additive effect of internal radiation and vitamin deficiency.

The results of these experiments have been reported at the winter meeting of the Southeastern Section of the Society for Experimental Biology and Medicine. A complete report is now in press in the Proceedings of the Society for Experimental Biology and Medicine.

C. Effects of the administration of "chelating" agents.

A fairly large number of experiments have been made on the effects of the introduction of "Versene", a preparation which is known to bind calcium ions in blood plasma. It was thought that this effect would be followed by a mobilization of both calcium and phosphorus from the bones and thus decrease the amount of ionizing radiation received by the bone marrow. In various series of experiments mice injected with P^{32} received versene (1-2 injections daily), and the amounts of the isotope in the urine were determined. Differences in the P^{32} excretion between the experimental and the control groups were observed, but the effects on the survival figures of the animals were often slight and not consistent. The experiments are now being continued on mice maintained on a phosphate-rich diet. If these experiments will not be clearly successful, it is planned to make some further attempt, using larger animals in which versene will be introduced by continuous instillations in the veins.

D. Experiments with pyrophosphate of P^{32}.

In various series of experiments the effects of the same radioactive dose introduced in the form of either pyrophosphate, or ordinary phosphate were compared. Here again differences in the rate of elimination of the isotope were observed, but up to now these differences did not cause [illegible] or

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[illegible] pyrop[illegible] to shortly after the [illegible] recently [illegible].

[illegible] by nucleic acids of developing chick embryo.

[illegible]rtile chicken eggs at various [illegible] of incubation (9 to 16) were [illegible] radioactiv[illegible] phosphorus. [illegible] hours later the embryos were dissected, deoxyribose - and ribose-nucleic acids were isolated, and their content in [illegible] and P^{31} was determined. In both nucleic acids the uptake of [illegible] increased rapidly to a peak on the 13th day, then fell abruptly. At its peak [illegible] the turnover rate of phosphorus in the nucleic acids of the embryo [illegible] much [illegible]ractor (1[illegible] to 2[illegible] times) than the corresponding values obtained by [illegible] on the bone marrow (which shows the highest turnover among the tissues [illegible] adult animals.)

[illegible] ra[illegible]by

[illegible] the period covered by the present report, the following complete [illegible].

1. "[illegible] Folic Acid on Recovery from Internal [illegible]adiation by P^{32}." by [illegible] Cornatzer, [illegible] Artom, G. T. Harrell, Jr., [illegible], [illegible] Vol. 70, pgs. 552-554 (19[illegible])

2. "[illegible] of U[illegible] Nucleic Acids to [illegible] of Developing [illegible] embryos." by [illegible] Cancer [illegible], Vol. 12, pgs. [illegible] (19[illegible]).

The following complete [illegible] are currently in press:

[illegible] Protective [illegible] Against the Toxic [illegible] to [illegible]." by [illegible] Artom, [illegible] Cornatzer and [illegible] Harrell, Jr., Proc. Soc. Exp. Biol. [illegible] (1952).

E. Camille Artom
Camille Artom, M.D.

George T. Harrell Jr.
George T. Harrell, Jr., M.D.

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Beneficial Effects of Vitamin B_{12} and Folic Acid on Recovery from [illegible]

W. E. CORNA[illegible] [illegible] [illegible] T. H. [illegible], JR. [illegible] [illegible]

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1951, v76, 552-554

Beneficial Effects of Vitamin B_{12} and Folic Acid on Recovery from Internal Radiation by P^{32}.* 18553

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In a previous paper the toxicity of P^{32} has been investigated in relation to the content of lipotropic factors, protein, fat and phosphate in the diets(1). In a continuation of such a study, the effect of varying the supply of certain vitamins has now been tested. Damage to the bone marrow is a constant occurrence in radiation injury and should be of even greater importance after administration of radioactive compounds, such as isotopic phosphate which is deposited preferentially in the skeleton. Under this condition, one might expect that the recovery of the animals will be affected markedly by a deficiency of hematopoietic factors such as folic acid and vit B_{12}. Some beneficial effects of folic acid on the anemia and leukopenia following radiation therapy in human beings have been reported(2,3). An increase in the % of survivors and in the survival time of x-ray irradiated mice receiving generous amounts of either pyridoxine or folic acid has also been described(4). However, no significant effect of these vitamins on the hematological changes of the animals was detected. Folic acid also failed to prevent such changes after total body irradiation with x-rays of rats(5), cats(6), and swine(7), and after introduction of P^{32} in human beings, or Sr^{89} in rats(8). Likewise, vit B_{12} failed to modify the anemia and leukopenia of rats exposed to x-rays(9).

To our knowledge, no study has yet been reported concerning the effectiveness of the combined administration of folic acid and vit B_{12} on the recovery from radiation injury: a point which is directly suggested by the close relationships in the biological actions of these two compounds. Moreover, since large amounts of these and other vitamins are synthesized by the intestinal flora, an attempt to reduce such a synthesis by the ingestion of poorly absorbed antibacterial agents, would tend to give a more accurate estimation of the value of the vitamins.

Experimental. Four series of experiments were carried out on a total of 525 mice under conditions identical to those of our previous experiments(1), except as indicated below. In series I and II, 172 mice were maintained on experimental diets without added sulfasuxidine and injected with 6 μc/g of P^{32}. Large doses of folic acid (20 mg/100 g of diet) or of a mixture of folic acid (20 mg/100 g) and vit B_{12} (0.75 mg) were added to the diet of part of these animals. In Series III and IV the low fat, low protein diet (Diet No. 34) (1) contained 5% sulfasuxidine and a basal mixture of B vitamins (Thiamine HCl 0.5 mg, riboflavin 0.5 mg, pyridoxine HCl 0.5 mg, Ca

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TABLE I. Effects of Certain Vitamins on Survival of Mice Injected with P^{32} (5 $\mu c/g$) and Maintained on a Sulfasuxidine Containing Diet.*

Supplements	No. of mice	Avg food consumption, g/day	Avg change in body weight	Time of 50% deaths	% of survivors		Avg time of survival† days
					21st day	56th day	
0	53	3.5	[illegible]	27	75	15	[illegible] ± 2.0
FA	39	4.1	[illegible]	29	49	21	[illegible] ± 3.3
B_{12}	24	3.7	[illegible]	34	58	46	[illegible] ± 4.0
FA + B_{12}	34	4.1	−0.1	>56	76	56	42 ± 3.5
FA, B_{12}, K, Bio.	67	4.0	+1.1	54	72	49	41 ± 2.5
0‡ (controls)	18	4.1	−6.7	>56	100	83	55 ± 0.8

* Diet No. 34(1) containing 5% sulfasuxidine. A basal B vit. mixture (see text) was incorporated daily in the diet.

† In the calculation of the avg time of survival, a survival time of 56 days was ascribed to the mice still alive at the end of the experiments. Values preceded by ± are the standard errors of the means.

‡ Not injected with P^{32}.

TABLE II. Statistical Comparison of the Survival of Mice Injected with P^{32} (5 $\mu c/g$) and Maintained on a Sulfasuxidine-containing Diet.

Supplements	Avg time of survival			No. of survivors at 56th day	
	n	t	P	χ^2	P
FA	110	0.23	>.05	2.32	>.05
B_{12}	95	1.67	>.05	7.56	<.01
FA + B_{12}	105	3.19	<.01	15.70	<.01
FA, B_{12}, K, Bio.	138	3.85	<.01	15.92	<.01

* n = Degrees of freedom; t = According to Fisher(10); P = Probability for a chance occurrence.

pantothenate 2 mg, inositol 2 mg). In addition, some groups received also, as "supplements" to each 100 g of the diet: folic acid alone (FA = 1.5 mg), or vit B_{12} alone (B_{12} = 0.45 μg), or both folic acid and B_{12} (FA + B_{12}; 1.5 mg and 0.45 μg, respectively), or a mixture (FA,B_{12},K,Bio) of folic acid (1.5 mg), vit B_{12} (0.45 μg), 2,methyl-1,4-naphthoquinone (Menadione, 15 μg), and biotin (1.2 μg). Three groups of 6 mice each were maintained for the whole period of the experiments on the sulfasuxidine-containing diet without supplements and were not injected with the isotope. All other animals were maintained on the diets, unsupplemented or supplemented, for 10 days before the introduction of P^{32}, and for 56 more days thereafter. Each of these mice received a single injection of P^{32} (5 $\mu c/g$): from our previous data this dose was estimated to be slightly below the LD_{50} (at the 21st day and for a low-protein, low-fat diet)(1). In the experiments of Series IV, one animal in each group was sacrificed by decapitation immediately before and at regular intervals after the introduction of P^{32}, so that blood could be obtained for determinations of the hemoglobin content, red blood cell and white blood cell counts, and hematocrit.

Results. In the experiments of Series I and II the diets did not contain sulfasuxidine, and therefore notable amounts of vitamins were probably still available to the animals through synthesis in the intestine. Supplementation of such diets with folic acid, or folic acid and vit B_{12}, did not appear to cause any significant effect on the survival of mice injected with a dose of P^{32} in the higher range of the LD_{50} (at the 21st day)(1). Data of the experiments of these series (and of Series IV also) are omitted for brevity. Only the data on the survival of mice on the diets containing sulfasuxidine and the results of a statistical treatment of these data are recorded in Tables I and II, respectively. A few of

10. Fisher, R. A., *Statistical Methods for Research Workers*, London, 1936, 6th Edition.

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the mice not injected with P^{32} died after several weeks on the low protein diet with added sulfasuxidine. On the other hand, in the groups of mice injected with 5 μc/g of P^{32}, the % of survivors at the 21st day and the time of 50% deaths were the same as, or greater than the values previously observed in mice injected with a smaller dose of P^{32} (4 μc/g) and maintained on a diet without added sulfasuxidine (Diet 31, which differed from Diet 34 only in a higher fat content) (1). It seems therefore that the inclusion of sulfasuxidine in the diet did not enhance the susceptibility of the animals to the injurious action of the isotope.† Supplementation of the sulfasuxidine-containing diet with folic acid alone increased the % of survivors at the 56th day, but the increase was not statistically significant ($P>0.05$). Vit B_{12} seemed more effective, however only the increase in the % of survivors was significant. On the other hand, the combined administration of folic acid and vit B_{12} offered a marked protection with a high degree of significance for both % of survivors and survival time. The further addition of menadione and biotin did not enhance the beneficial action of the mixture of folic acid and vit B_{12}. Altogether our results suggest that none of the vitamins tested modify appreciably the damage caused by the introduction of P^{32}, but that an adequate supply of both vit B_{12} and folic acid may be an important factor for the recovery of the damaged tissues.

The hematological changes following the introduction of P^{32} in mice on the sulfasuxidine-containing diet showed approximately the same general behavior in all groups of Series IV. After an initial fall, the values rose progressively and often declined again at the longest intervals. However, between the 12th and the 36th day, that is, during the recovery period after the initial fall, the % hemoglobin and the red and white cell counts in the blood of mice on the diet supplemented with both folic acid and vit B_{12} were all higher than in the other groups. Because of the limited number of determinations, the differences observed can only be taken as a suggestive indication for a relationship between the improved hematological picture and the prolonged survival of mice receiving the mixture of the two vitamins.

Summary. The addition of generous amounts of folic acid, or folic acid and vit B_{12}, to experimental diets had no significant effect on the survival of mice injected with a dose of P^{32} in the higher range of the LD_{50} (21st day). On the other hand, when sulfasuxidine was added to the diet and the mice were injected with a dose of P^{32} slightly below the LD_{50} (21st day), the administration of vit B_{12} and folic acid increased significantly the time of 50% deaths, the average time of survival and the % of survivors (at both the 21st and the 56th day).

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† Several recent reports suggest that antibiotics may even be beneficial in the treatment of radiation injury by preventing general or localized infections. For example, streptomycin and penicillin effectively reduced the mortality or prolonged the survival of rats injected with P^{32} (11).

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RELATION OF UPTAKE OF RADIOPHOSPHORUS INTO NUCLEIC ACIDS TO AGE OF DEVELOPING CHICK EMBRYOS

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Relation of Uptake of Radiophosphorus into Nucleic Acids to Age of Developing Chick Embryos*

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While much work has been done on the turnover rates of nucleic acids in young and adult animals, especially the rat (5), little is to be found on their turnover in embryonic tissue. Since the relative rate of growth of tissue is much greater during embryonic development than during adult life, it was felt that a study of the changes in turnover rates of desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) in relation to age would be of interest. When mature animals are used, separate organ analyses can be made, but such analyses are not generally feasible on embryonic material. With the use of radiophosphorus,[1] we have demonstrated high turnover rates with large differences in these rates at different ages.

MATERIALS AND METHODS

Fertile chicken eggs were incubated for from 9 to 16 days at 38° C. In each experiment from four to eleven eggs were used, the larger number being used for the younger embryos. Five-tenths ml. of a solution containing .5 μc. of carrier-free $Na_2HP^{32}O_4$, 25 units of penicillin, and 250 μg. of streptomycin was injected into the yolk sac of each egg. After 6 hours' further incubation at 38°, the embryos were dissected free, observed for viability, pooled, weighed, and blended. The nucleic acids were then extracted by the method of Hammarsten (4), with the modification that the PNA was precipitated by the neutralization of the acid lanthanum after the DNA was removed. Although a great deal of PNA is lost by this technic, the loss does not alter the specific activity. Phosphorus determinations on aliquots of the DNA and PNA were made by the method of Allen (2) and DNA determinations by the method of Stumpf (9). When a value of DNA phosphorus of 9.1 per cent was assumed, the DNA values as determined by the two methods checked within 10 per cent. DNA analyses were made on the PNA to detect possible contamination. Only one experiment showed contamination of over 6 per cent for which appropriate corrections were made. Counts were made on the entire dried sample brought to constant mass with sodium chloride. Over 10,000 counts in duplicate were taken in all cases.

Since the administered $Na_2HP^{32}O_4$ equilibrated with the total inorganic P of the egg, it was necessary to multiply the "raw" specific activity values by the quantity of inorganic P per egg. These data for eggs of varying ages were secured from Kugler (6). The term "relative specific activity" as used here refers to the product of counts minute milligram nucleic acid P × the milligrams of inorganic P per egg. Since the same quantity of P^{32} was injected in all experiments, this term is proportional, though not equal, to the more conventional definition of "relative specific activity."

RESULTS

The results are shown in Chart 1.

The "relative specific activities" of both PNA and DNA are seen to rise rapidly from the ninth day, reach a peak on the thirteenth day, and then drop precipitously. The DNA remains low through the sixteenth day, while the PNA begins to rise moderately. The ratio of PNA/DNA "relative specific activities" remains essentially constant until the fourteenth day, and then rises.

DISCUSSION

Novikoff and Potter (8) studied the changes in PNA and DNA concentrations in the developing chick embryo. Their data are shown in Chart 2, along with data from Needham (7), showing changes in protein concentrations.

In our study only 9–16-day embryos were in-

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[1] It has been demonstrated that inorganic P^{32}- and C^{14}-labeled purines give similar nucleic acid turnover rates (1).

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vestigated. Younger embryos required a larger number of eggs than were available, while older ones could not be extracted satisfactorily by this technic.

The similarity of the position of the peaks in Charts 1 and 2 is apparent. Chart 2 represents concentrations of these substances, while Chart 1

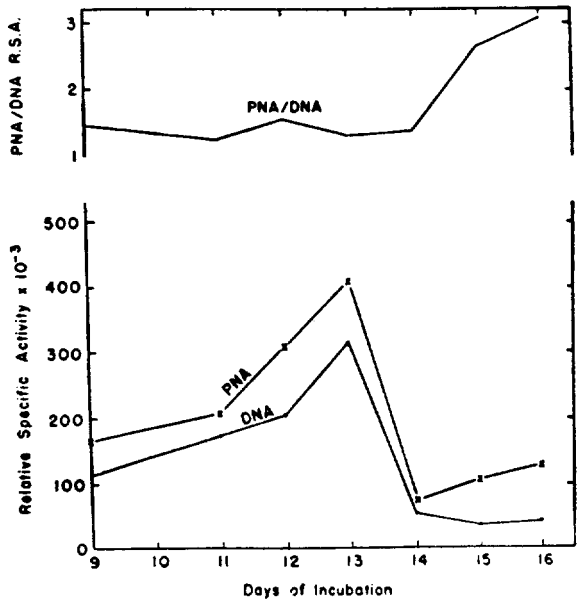

CHART 1.—Changes in PNA and DNA relative specific activities (counts/min./mg nucleic acid P×mg inorganic P/egg) with increasing age. No values were obtained for the tenth day. Days 9 through 13 represent single experiments. Day 14, three experiments, standard deviation PNA, 24,300; DNA, 18,000. Day 15, four experiments, s.d. PNA, 19,600; DNA, 11,200. Day 16, two experiments, s.d. PNA, 1230; DNA, 1520.

presents the pattern of change in turnover rates. Hence, not only do the concentrations but also the turnover rates increase greatly about the thirteenth day. Since it is difficult to determine accurately just how long an egg has been incubating, the difference of 1 day in the peak is probably not significant. If both the turnover rates and the amounts of nucleic acids are important factors in the rate of protein synthesis, then around the thirteenth or fourteenth day the product of the two is at a level several times higher than at earlier or later periods between 9 and 16 days.

A rough estimation of the absolute turnover rates can be calculated.[2] For the peak value of the thirteenth day, a rate of 148 per cent per 24 hours is found for PNA and 116 per cent for DNA. This contrasts strongly with the rate for adult animals, since the highest nucleic acid turnover rates in rabbits, found in bone marrow, were only 13 per cent for PNA and 4 per cent for DNA per day.[3] Even the lowest rates in the embryo, found on the fourteenth day, are 28 per cent and 20 per cent per day for PNA and DNA, respectively.

After the thirteenth day the DNA activity drops and then remains constant, while the PNA activity drops but quickly begins rising. This may reflect a continued high rate of cytoplasmic protein synthesis in contrast to a relative decrease in cell division.

Since these studies were made on whole embryos, the particular organs responsible for the increase remain unknown. Davidson and Leslie (3) could demonstrate no marked increase in the amounts of nucleic acids in the heart or liver of chick embryos in the twelfth- to sixteenth-day period. However, in adult animals, the nucleic acid turnover rates are much higher in lymphoid tissue, spleen, bone marrow, and intestinal mucosa (5).[3] Study of these tissues in embryos would be preferable if sufficient quantities of tissues could be secured.

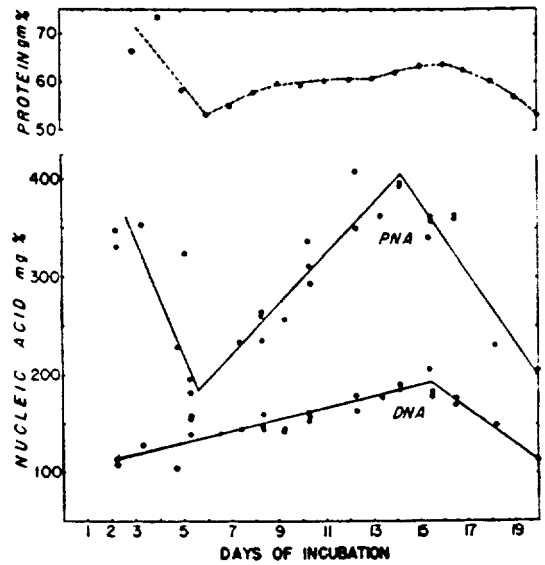

CHART 2.—Changes in PNA and DNA concentrations with age, from Novikoff and Potter (8). Top curve: changes in protein concentration, from data of Needham (7). Dry weight basis.

It is clear that, about the thirteenth or fourteenth day, the chick embryo undergoes drastic changes in its nucleic acid metabolism. Further study of this period may reveal important information concerning normal growth.

[2] Method of calculation: 5 μc. P^{32} injected which produce 11×10^6 disintegrations per minute. The counter efficiency for P^{32} was 9.9 per cent. Hence, 5 μc. would produce 10.9×10^5 counts per minute. There are 17.2 mg. of inorganic P in a 13-day egg. This will give a specific activity of 6.3×10^4 counts/min./mg P. The PNA-P specific activity was 23.6×10^3 counts/min/mg PNA-P. Hence, the turnover rate would be 37 per cent for 6 hours, or 148 per cent per day.

[3] K. M. Stevens, unpublished data.

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SUMMARY

1. The uptake of radiophosphorus into desoxyribonucleic acid (DNA) and pentosenucleic acid (PNA) in 9–16-day chick embryos has been determined.

2. The "relative specific activities" of both DNA and PNA rise rapidly to a peak on the thirteenth day, then fall abruptly to about 20 per cent of this peak value. The DNA activity then continues constant, while the PNA begins to rise again.

3. The turnover rates of both nucleic acids, but especially DNA, appear to be much higher in embryos than in adult tissues.

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Effect of Cortisone Acetate on Experimental Rocky Mountain Spotted Fever in the Guinea Pig. (20223)

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In certain diseases, the administration of cortisone may relieve many of the symptoms and suppress the signs of illness. In spite of this apparent beneficial effect, cortisone therapy may be harmful in diseases such as pneumonia or tuberculosis, since it often accelerates the spread of the infection(1,2). It has been suggested, however, that in the therapy of Rocky Mountain spotted fever and typhoid fever, this hormone may be a valuable adjunct to chloramphenicol(3,4). Because of the intracellular position of rickettsiae as opposed to the extracellular location of most bacteria, one might expect a different response to cortisone in animals or subjects infected with the former micro-organisms. The hormone might alter the rate of spread from the site of inoculation, the rate of growth within cells, or the response of the host cells.

The present study was undertaken in an effort to determine the effects of two different dosage schedules of cortisone acetate administered to guinea pigs with experimental spotted fever.

Material and methods. A total of 91 guinea pigs were used. The Bitter Root strain of *R. rickettsii* was passed serially by intraperitoneal injections. Whole bacteriologically sterile, citrated blood obtained by cardiac puncture, or a splenic emulsion in sterile physiologic saline solution was used as passage material. The animals from which passage material was obtained were sacrificed on either the fourth or fifth day of fever. Daily determinations of the body weight and the rectal temperature were made on all animals. Complement fixation tests were performed by a standard method(5). The significance of the difference in the mortality rate between the test and control groups was tested by the chi-square method(6).

Plan of the Experiment. Exp. 1 was designed to determine whether the daily administration of a dose of cortisone acetate comparable to a clinically therapeutic dose in human beings would influence the course of the infection. Five sets of observations were made upon 51 male animals. Each set of animals was given a different preparation of rickettsiae. In each set, the animals were divided into test and control groups of comparable body weight. All animals in the 2 groups were inoculated with the same volume of material containing the rickettsiae. Starting on the day of infection, each animal in the *test group* was given daily subcutaneous injections of 1 mg of cortisone acetate (a dose which is comparable to 150 mg for a 75 Kg man) for 17 days. The *control group* was not given cortisone. All observations were continued for 17 days after infection. *Exp.* 2, performed several months after completion of Exp. 1, was designed to determine whether the administration of a large dose of cortisone acetate for 5 consecutive days at the peak of the clinical illness would alter the mortality rate or the degree of fever. Forty guinea pigs were divided equally into 2 sets, which were studied one month apart. The 20 guinea pigs in each set were observed simultaneously and were given aliquots of a single inoculum of rickettsiae. Ten animals in each set were not given cortisone; the remaining 10 received 5 daily intramuscular injections of

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TABLE I. Effect of Cortisone Acetate, 1 mg Daily, Mortality Rate in Guinea Pigs with Spotted Fever (Exp. 1)

Severity of Infection	Total No. of Animals	Survivals	Deaths
Control group (*R. rickettsii* only)			
Mild, no fatality	3	3	0
Moderate	15	4	11
Severe, fatality rate over 90%	8	1	7
Total	26	8	18
Test group (*R. rickettsii* and cortisone)			
Mild, no fatality	3	3	0
Moderate	14	8	6
Severe, fatality rate over 90%	8	0	8
Total	25	11	14

cortisone acetate, 25 mg, starting on the morning of the fourth day of infection.

Results. Exp. 1. Sixty-nine per cent of the control animals infected with spotted fever and 56% of the test animals also given cortisone acetate died; the difference in the overall fatality rate was not significant. However, when the 5 sets of animals were arbitrarily divided into 3 groups, depending upon the severity of the infection, there was a suggestion that the administration of cortisone acetate did have a beneficial effect (Table I). All of the six animals with "mild" infections survived. None developed fever (rectal temperature of 104° F or greater). Complement-fixing antibody to spotted fever antigen was found in all animals on the twenty-second day of infection. This set of animals was given the original lyophilized material, which subsequently increased in virulence upon serial passage in guinea pigs. All but one of the 16 animals with infections classified as "severe" succumbed. There was no significant difference between the test and control groups as to the duration or height of fever or the day of death. In the 29 animals classified as having "moderately severe" infections, 43% of those given cortisone acetate and 73% of the control guinea pigs succumbed. The difference in the mortality rates in the two groups was statistically significant ($P = 0.01$ to 0.02). The mean duration of fever in the test group was 5.5 days, as compared with a mean of 4.1 days in the control group. In the test group, the average day of death was 11.5 days after inoculation; in the control group, 10.4 days. In general, the changes in body weight in the two groups paralleled each other.

Exp. 2. Eighty per cent of the animals in each group died during the period of observation. Only 6 of the 16 deaths in the test group occurred while the animals were receiving cortisone acetate, whereas 14 deaths occurred during the same period in the control group (Table II). Thus, most of the fatalities in the test group occurred after cortisone was discontinued. The mortality rate in the test group during the first 8 days of infection was significantly lower than that in the control ($P =$ less than 0.01). The mean day of death in the infected animals not given cortisone acetate was 8.1 days; that in the test group which received the hormone was 9.9 days. A striking difference was noted clinically in the physical appearance of the test and control animals within 48 hours after the institution of cortisone therapy in the test group. Those given the drug were much livelier and showed less ruffling of the coat than those not receiving cortisone.

The mean daily decrease in body weight in the 2 groups was practically the same during the first 8 days. An abrupt fall in the mean daily rectal temperature occurred in the control group between the sixth and seventh days of infection, and all except 2 of the animals were dead by the ninth day. In the group given cortisone acetate, the highest mean temperature of 104.7° F was found on the fourth day of infection; it subsequently decreased gradually until the thirteenth day of infection.

Comment. Although the daily administration of 1 mg of cortisone acetate did not significantly alter the overall mortality rate in guinea pigs infected with *R. rickettsii*, analysis of the results according to the severity of the disease suggested that the hormone may be of value if the infection is of moderate severity. The justification for such an arbitrary classification lies in the fact that the efficacy of a therapeutic agent which does not directly inhibit the growth of an infecting organism may not be apparent, either in the presence of a minimal infection without systemic reactions or in the presence of an over-

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TABLE II. Effect of Cortisone Acetate, 25 mg Daily, on Period of Survival in Guinea Pigs Infected with *R. rickettsii*. Experiment 2. [illegible] in each group.

	Total deaths	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Test (Given *R. rickettsii* and cortisone)	16	1	2		2	1	1	2	2	1
Control (Given *R. rickettsii*)	16	7	7	1					1	

whelming infection. In the presence of a moderately severe infection, when there is sufficient time for the body's defense mechanisms to be mobilized, any beneficial effect which the drug possesses may become evident. It has been observed that in guinea pigs with fatal infections of *R. rickettsii*, the temperature drops rather precipitously to subnormal values about 12 hours prior to death. The longer mean duration of fever in the group given cortisone acetate and classified as having moderately severe infections has therefore been attributed to a reduction in toxemia resulting from the administration of cortisone. The longer period of survival in the cortisone-treated animals is also compatible with this interpretation.

The observations in Exp. 2 appear to confirm the impression that cortisone acetate has a beneficial effect on the course of moderately severe cases of Rocky Mountain spotted fever. No deaths occurred in either group prior to the fourth day of infection, at which time the administration of large doses of cortisone acetate was begun in the test group. During the period of drug therapy, the number of deaths in the treated group was significantly smaller than that in the control group. In addition, the clinical appearance and behavior of the treated animals was less in keeping with a severe illness. Following discontinuation of cortisone, the number of deaths in the test group was greater than that in the control group, so that the overall mortality rate was identical. It appears, therefore, that the beneficial effects of cortisone acetate were transitory.

It is not possible from the present study to determine the exact mechanism of the protective effect apparently afforded by cortisone acetate in guinea pigs infected with *R. rickettsii*. There is no evidence to suggest that it may be due to a suppression of the growth of rickettsiae in the infected animals, and it has been previously observed that cortisone acetate does not affect the rate of growth of *R. rickettsii* in the yolk sac of embryonated chick eggs(7).

Summary. 1. The effect of 2 different dosage schedules of cortisone acetate on guinea pigs infected with Rocky Mountain spotted fever of varying degrees of severity has been investigated. The overall mortality rate in 25 animals given daily intramuscular injections of 1 mg of the drug, starting on the day each received, by intraperitoneal injection, an inoculum of rickettsiae, did not differ significantly from the mortality rate in infected guinea pigs which received no treatment. The severity of the infection induced by different preparations of rickettsiae varied considerably; when this factor was taken into consideration, it appeared that cortisone acetate in doses comparable to those employed in human beings reduced the mortality rate in those animals with moderately severe infections. 2. In another group of 22 animals which received daily intramuscular injections of 25 mg of cortisone acetate for 5 days at the peak of the illness (fourth through the eighth day of infection) the overall mortality rate was identical with that in 20 infected animals not given the hormone. During the period of administration of this relatively huge dose of cortisone acetate, however, the fatality rate was significantly lower than that in the untreated animals.

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WAKE FOREST COLLEGE
THE BOWMAN GRAY SCHOOL OF MEDICINE
WINSTON-SALEM NORTH CAROLINA

DEPARTMENT OF
INTERNAL MEDICINE

March 20, 1953

Atomic Energy Commission
Office of Research and Medicine
Post Office Box E
Oak Ridge, Tennessee

Gentlemen:

You will find enclosed the progress report on Title I of the contract number AT-(40-1)-288 made with the Bowman Gray School of Medicine of Wake Forest College. This report has been delayed several days because of the necessity of giving final examinations and generally winding up the work of the winter trimester in the school which has just ended.

We have already forwarded to you two reprints of each of the papers which have appeared so far this year. As you will note from the progress report, additional manuscripts have been accepted or submitted for publication but have not yet come out. Should you desire copies of the manuscripts which have been accepted, we would be most happy to furnish them. Reprints will be forwarded when they are available after the studies have been published.

We are again very pleased with the amount of work which has been accomplished this year, primarily by the intensive endeavors of Dr. Gerry K. Aikawa. We are pleased with the recognition of the work by the American Medical Association; an exhibit summarizing the past four years endeavor will be presented at their New Orleans meeting in May, 1953.

Please let me express to you on the behalf of all the people working here with us on these projects our deepest appreciation for your continued help. We would like again especially to express our appreciation for the complete cooperation which we have had in the administration of these grants. This cordial atmosphere has made our work very pleasant. Without this support, particularly of Dr. Aikawa and the house officers, it would have been impossible to have completed the experiments.

The bibliography of this year's work and available reprints have already gone forward to you, under separate cover, March 20, 1953.

Respectfully,

George T. Harrell

George T. Harrell, M.D.
Research Professor of Medicine

GTH:vs
Encl:

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CONTRACTS
Correspondence
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PROGRESS REPORT

Contract No. AT-(40-1)-288 - Title I
The Bowman Gray School of Medicine of
Wake Forest College

DISTRIBUTION AND TURNOVER OF SODIUM AND POTASSIUM IN ACUTE INFECTION

Dr. George T. Harrell, Jr., Project leader — July 1, 1952 to March 15, 1953

Facilities: No changes have been made in the laboratory. Minor items of equipment have been added.

Personnel: Dr. Jerry K. Aikawa has continued to work almost full time on the project. He probably will be called for two year's military duty about July 1, 1953.

Miss Eloise Rhoades has continued to work enthusiastically. She has resigned and will leave the state by July 1, 1953. No replacement is in sight at present.

William Brooks has continued to work well. He will remain with the project.

The academic position of the project leader was changed during the current year. He no longer has charge of the house staff or beds in the hospital. It has not been possible to utilize house officers for experiments with patients to the same extent as in the previous years. A former house officer participant, Dr. Malcolm P. Tyor, is now on the staff of the Medical Division, Oak Ridge Institute of Nuclear Studies; another, Dr. Jack Felts, enters military service this year.

Isotopes: Transportation was much improved this year. Only two shipments failed to arrive as [illegible] because of the weather.

Animals: An epidemic during the course of the year wiped out several groups of rabbits on which [illegible] already been collected, particularly on myxedema. More guinea pigs than rabbits have been used this year.

[illegible] and Plan of Approach

Concept of Membrane Permeability Defect

Experiments this year have investigated single factors which might alter the permeability of either capillary or cell membranes. The distribution and turnover of sodium and potassium may be affected in the course of acute infections by alterations in absorption, distribution, excretion, or utilization of ions. Alterations were detected and the magnitude of the changes determined by isotope dilution techniques. The studies on starvation in rabbits, with a [illegible] in the incidence of cancer, were directed toward the problem of absorption. The studies on myxedema in patients and animals and on rheumatic fever have been directed toward alterations in distribution. The mechanism for alterations in distribution has been investigated by the studies on immune reactions in animals. The effects of hormones on distribution and have upon excretion by the kidney been investigated by the studies on cortisone and ACTH in patients under treatment, as well as in normal and infected animals.

Results of Experiments

1. Standardization of Methods

Normal values for Na_2 space and Na content of selected tissues in guinea pigs have been established. The data are included in the paper on myxedema [illegible] for publication.

[illegible]

In spite of the extensive literature on the effects of cortisone and ACTH, detailed analysis of the effects of the hormones on normal animals has been lacking. Before the results of treatment with these hormones in animals could be interpreted it was necessary to determine the effect of the hormones on normal animals. Two groups of rabbits were given cortisone for two or three weeks in two different dosages and the effects on sodium and potassium metabolism were observed. The results of this study have been published and reprints are enclosed.

The data on K_{42} space in normal young women have been published and reprints are enclosed. The space is appreciably less in women than in men. It has not been possible to extend the observations to older age groups.

It has not been possible because of lack of beds and clinical help to determine in human beings the effect of bed rest and starvation without immobilization on the K_{42} space of normal human beings.

The group of rabbits with myxedema was lost in an epidemic so that the experiment could not be completed this year.

2. Intracellular Infections

It was not possible to increase the virulence of our strains of spotted fever rickettsias for rabbits. Observations were completed on mild infections, however. Serial determinations of the fluid volume and electrolyte concentrations were made. Physiologic changes were minimal although comparable in every way to those previously described in human patients with Rocky Mountain spotted fever. The results of these experiments are being prepared for publication.

The strains of rickettsias [illegible] were severe infection in guinea pigs. The paucity of veins in this species precluded any serial studies of fluid spaces. Groups of guinea pigs were infected and studies of the [illegible] of radiosodium at the peak of the infection were completed. The data are now being analyzed and prepared for publication.

Observations on human beings indicate that cortisone or adrenocorticotrophic hormone (ACTH) may be harmful in some infections, particularly during the extracellular phase of those due to the pneumococcus, tubercle bacillus or brucella organism. The host defense mechanism is suppressed, but it has not been clear whether the organisms actually are able to spread through the body. The effect on the intracellular phase of these infections is not clear. Accordingly, a study was completed in guinea pigs infected with spotted fever rickettsias to determine whether the administration of cortisone would increase the spread of the rickettsias and alter the mortality rate. The hormone apparently does not effect either extremely severe or very mild infections; in those of moderate severity it may be beneficial as judged by the lowering of the rate in animals with this degree of severity of infection. These results are comparable to those observed previously in human beings.

No further autoradiographic studies have been done.

3. Intracellular Infections

No extracellular infections were studied.

4. Immune Reactions without Infection

A pilot experiment with rabbits hyperimmunized by repeated subcutaneous injec- [illegible]

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subside. Other groups of rabbits showed an increase in radiosodium space within twenty-four hours after reinjection of antigen; in some cases the changes persisted for as long as a week. The data are now being analyzed for correlation of changes in radiosodium space with humoral antibody titers. At the end of the experiments, animals were sacrificed and tissue analyses for radiosodium space were performed. These data are now being analyzed.

A second group of rabbits has been under observation for two months but the experiment is not completed.

5. Clinical Studies

A comparison of the thiocyanate and radiosodium spaces in various diseases has been published and reprints are enclosed.

The results of radiosodium studies in acute rheumatic fever have been published and reprints are enclosed. Very few cases of rheumatic fever suitable for study have been available this year and it has not been possible to collect data on K_{42} space.

Studies on the rate of disappearance of human albumin tagged with I_{131} from plasma of patients with cirrhosis have been made and the rate of excretion of I_{131} in the urine of these patients followed. The results have been published and reprints are enclosed.

In an attempt to evaluate the changes occurring during infections, it has been necessary to determine the extent of the alterations in K_{42} content produced by other types of disease processes. A survey of various disease states, including some infections, has been done and the results have been published; reprints are enclosed. Of particular interest in this study [illegible] the suggestion in a small number of patients that the total body potassium may be reduced in psychoneurosis. Loading of these patients with supplementary potassium has resulted [illegible] retention of administered potassium. It is hoped that others will attempt to confirm these results and to extend them in a much larger series of cases. Our series is definitely too small to draw any conclusions as to a possible role in the production of symptoms of psychoneurosis or of any beneficial results from treatment by the administration of potassium.

In an attempt to determine whether a defect in intracellular utilization of potassium occurs in diabetes mellitus, a series of poorly regulated diabetics was studied. These patients were not in acidosis and presumably the deficiency in total body potassium uncovered was not the result of prolonged upset in acid-base balance. The study has been published and reprints are enclosed.

Additional groups of patients have been studied by the K_{42} technique for defects in potassium utilization. In males with thyrotoxicosis the total body potassium is significantly reduced; in females the changes are less. It is believed these alterations are a result of the increase in rate of metabolism with thyrotoxicosis so that body stores of fat have been exhausted and muscle cells are being depleted. The reduction in potassium is comparable to that occurring in destruction of cells from starvation. These data have been analyzed and submitted for publication.

Similar studies in patients with cirrhosis of the liver have been done. The data have been accumulated chiefly in males in whom the disease is more frequent though a few data have been obtained in females. In the patients without ascites, the deficits in total body potassium may be due to impaired utilization; administration of supplements of potassium has resulted in retention of the ion and increase in radipotassium space. In patients with ascites the situation is more complicated.

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because of the alteration in fluid balance in addition to the defects in synthesis of protein; administration of a supplement has not appreciably altered the total body potassium. These experiments have been analyzed and accepted for publication.

A limited number of patients on prolonged treatment with cortisone or ACTH with or without sodium restriction or administration of potassium supplements have been studied. The magnitude of the defect in total body potassium was greater than had been anticipated. These results have been published and reprints are enclosed.

From these rather extensive studies on exchangeable potassium, we have concluded that total body deficiency of this ion occurs in many clinical states in which potassium deficiencies have not been previously recognized. Apparently a defect of greater than 25% of the total body store must be present before clinical symptoms of potassium deficiency occur and before depletion can be recognized by changes in the serum potassium or electrocardiogram. The results of our studies indicate that the radiopotassium method is more sensitive and more accurate than the conventional external balance method in the detection of mild abnormalities in potassium metabolism. Most of these defects could not have been detected by conventional methods. Of particular interest, were the results in patients under treatment with prolonged administration of hormones for arthritis which indicated that deficits of total body potassium can occur on usually accepted treatment regimens. The deficiencies resulting from alterations in excretion as a result of hormone therapy are reversible with alterations in the treatment regimen. It has not always been possible to correct the defects resulting from alteration in administration in other disease processes by administration of potassium supplements.

Reports of these studies have been made by various members of our group before medical societies throughout the south; these talks are reflected in the abstracts listed in the bibliography.

An [illegible] summarizing our work over [illegible] years has been accepted for presentation at the American [illegible] Association meeting in New York City, June 1,53.

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SUMMARY OF SCOPE AND PURPOSE

Contract No. AT-(40-1)-233- Title I
The Bowman Gray School of Medicine of
Wake Forest College

Distribution and Turnover of Sodium and Potassium in Acute Infections.

Dr. George T. Harrell, Jr., Project leader July 1, 1952 to March 15, 1953

Patients with infections retain fluids and salts during the acute phase and excrete them in convalescence. Edema often develops, indicating alteration in permeability of capillary and cell membranes. Sodium is the predominate ion in the extracellular space and potassium in the intracellular, but it is not known how or where their metabolism is upset. The concentration of both in body fluids can be measured by flame photometry. The total body content or exchangable ion space can be estimated indirectly by isotopic dilution techniques. Both methods are necessary to study changes during disease.

Alterations in the content and turnover of ions may result from defects in absorption, distribution, excretion or utilization. Recovery from [redacted] disease is a complicated process involving many mechanisms and sites of action. Experiments are being conducted to study the effect of a single defect on the behavior of solutions of ions in the body.

Before the data in disease can be interpreted, values must be determined for the range of normal physiologic variation in both sexes of the human being and in the various species of animals used in these experiments. The effects of reduction in food intake, increase in rate of metabolism as occurs with fever, alteration in renal excretion from increase adrenal function, and changes in permeability with immune reactions are examples of types of experiments under way.

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PROGRESS REPORT

Contract No. AT-(40-1)-288 - Title I
The Bowman Gray School of Medicine of
Wake Forest College

DISTRIBUTION AND TURNOVER OF SODIUM AND POTASSIUM IN ACUTE INFECTION

Dr. George T. Harrell, Jr., Project leader — July 1, 1952 to March 15, 1953

Facilities: No changes have been made in the laboratory. Minor items of equipment have been added.

Personnel: Dr. Jerry E. Aikens has continued to work almost full time on the project. He probably will be called for two years military duty about July 1, 1953.

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William Brooks has continued to work well. He will remain with the project.

The academic position of the project leader was changed during the current year. He no longer has charge of the house staff or beds in the hospital. It has not been possible to retain house officers for experiments with patients to the same extent as in the previous years. A former house officer participant, Dr. Malcolm [illegible], is now on the staff of the Medical Division, Oak Ridge Institute of Nuclear Studies; another, Dr. Jack [illegible], enters military service this year.

Isotopes: Transportation was much improved this year. Only two shipments failed to arrive as scheduled because of the weather.

Animals: An epidemic during the course of the year wiped out several groups of rabbits on which much data had already been collected, particularly on [illegible]. More Guinea pigs than rabbits have been used this year.

Scope and Plan of Approach

Impact of Sodium. Permeability Defect

Experiments this year have investigated single factors which might alter the permeability of either capillary or cell membranes. The distribution and turnover of sodium and potassium may be affected in the course of acute infections by alterations in absorption, distribution, excretion, or utilization of ions. Alterations were detected and the magnitude of the changes determined by isotope dilution techniques. The studies on starvation in rabbits, with a clinical counterpart in the anorexia of cancer, were directed toward the problem of absorption. The studies on myxedema in patients and animals and on rheumatic fever have been directed toward alterations in distribution. The mechanism for alterations in distribution has been investigated by the studies on immune reactions in animals. The effects of hormones on distribution and turnover and upon excretion by the kidney have been investigated by the studies on cortisone and ACTH in patients under treatment, as well as in normal and infected animals.

Results of Experiments

1. Standardization of Methods

Normal values for the space and turnover of injected Na[illegible] in Guinea pigs have been established. The data are included in the paper on spotted fever submitted for publication.

[illegible]

In spite of the extensive literature on the effects of cortisone and ACTH, detailed analysis of the effects of the hormones on normal animals has been lacking. Before the results of treatment with these hormones in animals could be interpreted it was necessary to determine the effect of the hormones on normal animals. Two groups of rabbits were given cortisone for one or three weeks in two different dosages and the effects on sodium and potassium metabolism were observed. The results of this study have been published and reprints are enclosed.

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The group of rabbits with myxedema was lost in an epidemic so that the experiment could not be completed this year.

2. Intracellular Infections

It was not possible to increase the virulence of our strains of spotted fever rickettsiae for rabbits. Observations were completed on mild infections, however, serial determinations of the fluid volume and electrolyte concentrations were made. Physiologic changes were minimal although comparable in every way to those previously described in human patients with Rocky Mountain spotted fever. The results of these experiments are being prepared for publication.

The strains of rickettsiae induced a much more severe infection in guinea pigs. The [illegible] of veins in this species precluded any serial studies of fluid space. [illegible] guinea pigs were infected and studies of the [illegible] distribution of radiosodium at the peak of the infection were completed. The data are now being analyzed [illegible] publication.

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No further autoradiographic studies have been done.

3. Extracellular Infections

No extracellular infections were studied.

4. Immune Reactions without Infection

A pilot experiment with rabbits hyperimmunized by repeated subcutaneous injections of [illegible]

cuticide. Other groups of rabbits showed an increase in radiosodium space within twenty-four hours after reduction of antigen; in some cases the changes persisted for as long as a week. The data are now being analyzed for correlation of changes in radiosodium space with humoral antibody titers. At the end of the experiments, animals were sacrificed and tissue analyses for radiosodium space were performed. These data are now being analyzed.

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In an attempt to determine whether a defect in intracellular utilization of potassium occurs in diabetes mellitus, a series of poorly regulated diabetics was studied. These patients were not in acidosis and presumably the [illegible] in their body potassium uncovered was not the result of prolonged upset in acid-base balance. The study has been published and reprints are enclosed.

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because of the alteration in fluid balance in addition to the defects in synthesis of protein; administration of a supplement has not appreciably altered the total body potassium. These [illegible] for publication.

A limited number of patients under prolonged treatment with cortisone or ACTH with or without sodium restriction or administration of potassium supplements have been studied. The magnitude of the defect in total body potassium was greater than had been anticipated. These results have been published and reprints are enclosed.

From these rather extensive studies on exchangeable potassium, we have concluded that total body deficiency of this ion occurs in many clinical states in which potassium deficiencies have not been previously recognized. Apparently a defect of greater than 25% of the total body store must be present before clinical symptoms of potassium deficiency occur and before depletion can be recognized by changes in the serum potassium or electrocardiogram. The results of our studies indicate that the radiopotassium method is more sensitive and more accurate than the conventional external balance method in the detection of mild abnormalities in potassium metabolism. Most of these defects could not have been detected by conventional methods. Of particular interest, were the results in patients under treatment with prolonged administration of hormones for arthritis which indicated that deficits of total body potassium can occur on usually accepted treatment regimens. The deficiencies resulting from alterations in excretion as a result of hormone therapy are [illegible] with alterations in the treatment regimen. It has not always been possible to correct the defects resulting from alterations in utilization in other disease processes by administration of potassium supplements.

Reports of these studies have been made by various members of our group before medical societies throughout the south; these talks are reflected in the abstracts listed in the bibliography.

An edited manuscript of our work over the past four years has been prepared for presentation at the American Medical [illegible] meeting in New Orleans, May June 1953.

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SUMMARY OF SCOPE AND PURPOSE

Contract No. AT-(40-1)-2[illegible]- Title I
The Bowman Gray School of Medicine of
Wake Forest College

Distribution and Turnover of Sodium and Potassium in Acute Infections.

Dr. George T. Harrell, Jr., Project leader July 1, 1952 to March 15, 1953

Patients with infections retain fluids and salts during the acute phase and excrete them in convalescence. Edema often develops, indicating alteration in permeability of capillary and cell membranes. Sodium is the predominate ion in the extracellular space and potassium in the intracellular, but it is not known how or where their metabolism is upset. The concentration of both in body fluids can be measured by flame photometry. The total body content or exchangable ion space can be estimated indirectly by isotopic dilution techniques. Both methods are necessary to study changes during disease.

Alterations in the content and turnover of ions may result from defects in absorption, distribution, excretion or utilization. Recovery from human disease is a complicated process involving many mechanisms and sites of action. Experiments are being conducted to study the effect of a single defect on the behavior of solutions of ions in the body.

Before the data in disease can be interpreted, values must be determined for the range of normal physiologic variation in both sexes of the human being and in the various species of animals used in these experiments. The effects of reduction in food intake, increase in rate of metabolism as occurs with fever, alteration in renal excretion from increase adrenal function, and changes in permeability with immune reactions are examples of types of experiments under way.

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Reprinted from the Journal of Infectious Diseases
November-December, 1958, Vol. 103, 222-225

ISOTOPIC STUDIES OF FLUID AND ELECTROLYTE CHANGES IN DOMESTIC RABBITS WITH ROCKY MOUNTAIN SPOTTED FEVER

JERRY K. AIKAWA* AND GEORGE T. HARRELL†

From the Department of Internal Medicine, The Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, North Carolina

Previous studies on patients with Rocky Mountain spotted fever[1] have suggested that the distribution of body fluids and electrolyte metabolism are altered during the course of this disease. Further studies of the disturbed physiology of spotted fever have been performed on experimental animals. Although the disease is induced most easily in guinea pigs, their small size and the paucity of veins make them unsuitable for the performance of serial determinations of fluid spaces. The rabbit is best suited for such a study, and Foot[2] has previously reported that clinical spotted fever can be induced in the domestic rabbit.

The present experiment was undertaken to study in rabbits the changes in fluid distribution and electrolyte concentrations resulting from infection with Rocky Mountain spotted fever. Radioactive isotopes of sodium (Na^{24}) and potassium (K^{42}) were used to measure directly the radiosodium space and the exchangeable body potassium content.

Received for publication March 12, 1958.

This work was supported in part by the U. S. Atomic Energy Commission under a contract with the Bowman Gray School of Medicine, and in part by a grant-in-aid from the American Heart Association.

Na^{24} and K^{42} were supplied by the National Laboratory, Oak Ridge, Tennessee, on allocation from the U. S. Atomic Energy Commission.

The Bitter Root strain of *Rickettsia rickettsii* and the rickettsialpox antigen were kindly furnished by Dr. Herald R. Cox, Lederle Laboratories, Pearl River, New York.

* This work was performed during a tenure as a Research Fellow of the American Heart Association sponsored by the 20-30 International Rheumatic Fever Foundation. Present address: Department of Medicine, University of Colorado School of Medicine, Denver, Colorado.

† With the technical assistance of Eloise L. Rhoades and William Brooks.

1. Harrell, G. T. and Aikawa, J. K. 1949, Arch. Int. Med. **83**: 331-347.
2. Foot, N. C. 1919, J. Med. Res. **39**: 495-507.

MATERIAL

Lyophilized yolk sac material which contained the Bitter Root strain of *Rickettsia rickettsii* was suspended in sterile physiologic saline solution and was then injected intraperitoneally into guinea pigs. [illegible] serial passages in guinea pigs were maintained by the intraperitoneal injection of physiologic saline solution, containing a mixture of whole blood and splenic emulsion which was obtained from animals in the preceding passage group. The animals from which passage material was obtained were sacrificed on either the 4th or 5th day of fever rectal temperature [illegible]. All passage materials were checked for bacteria [illegible] contaminated materials were used.

Fifteen adult domestic rabbits of mixed breeding [illegible] individual metabolism cages and were [illegible] compressed pellets which contained [illegible] 0.275 meq. of potassium and 0.09 meq. of sodium. Tap water which contained negligible [illegible] of sodium and potassium was given without restriction.

Since it was not technically feasible to use Na^{24} and K^{42} simultaneously in the same animals, the rabbits were divided into two groups. Each of the seven rabbits in group 1 received an intraperitoneal injection containing 3 ml of a pooled mixture of whole blood and splenic emulsion obtained from two guinea pigs of the third passage. Each of the eight animals in group 2 was inoculated with similar material from the sixth passage in guinea pigs.

METHODS

The methods for determining the plasma volume, hematocrit, thiocyanate and radiosodium spaces, and exchangeable body potassium con-

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tent have been described previously.[3,4] Serum sodium and potassium concentrations were determined with a Beckman Model DU flame spectrophotometer.

Complement fixation tests were performed on the rabbit serums by a standard method,[5] using as antigen a commercial rickettsialpox material.

Plan of the experiment.—On each rabbit in group 1, two baseline determinations of the plasma volume, the hematocrit, the 30-minute thiocyanate and 60-minute radiosodium spaces, and the serum sodium, potassium and protein concentrations were made 7 days apart. The body weight was recorded daily. Baseline complement fixation tests were performed on serums obtained a day before the animals were injected with *R. rickettsii*.

Eleven days after the second baseline determinations, each animal was given 3 ml of the spotted fever inoculum intraperitoneally. (One ml aliquots of this same inoculum, when injected intraperitoneally into guinea pigs, killed 10 of 11 animals.) Daily rectal temperature readings were made thereafter until the termination of the study. The measurements of fluid volume were repeated in each animal at intervals of 7, 13 and 20 days after injection of the rickettsias.

In group 2, two baseline determinations of the exchangeable potassium content (Ke) were made 7 days apart in each animal. On the day the second Ke determination was completed, each animal was given 3 ml of the spotted fever inoculum intraperitoneally. Rectal temperature readings were made thereafter until the termination of the study. The Ke determinations were repeated in each animal 7 and 13 days after injection of rickettsias.

Statistical analysis.—The total blood volume, plasma volume, and thiocyanate and radiosodium spaces are recorded in ml per kg of body weight. In the statistical analysis, each animal served as its own control. For each animal the difference between the mean of the two baseline determinations and each of the single values obtained on the 7th and 13th days (and also on the 20th day in group 1) was determined. The mean differences between the baseline and the response values at the stated intervals were then calculated for the entire group and their significance was tested by the following formulas, in which d = mean difference, s_d = standard error of the mean difference, Σ = summation of, and n = number of animals.

$$t = \frac{d}{s_d} \qquad s_d = \sqrt{\frac{\Sigma d^2 - (\Sigma d)^2/n}{n(n-1)}}$$

The calculated value for t was compared with the value at $(n-1)$ degrees of freedom, as obtained from standard tables; t was considered significant if P was 0.01 or less.[6]

RESULTS

All rabbits survived. Clinically, only a mild disease was induced. There was no evidence of peripheral edema or of circulatory collapse—signs which have been previously noted in severe cases occurring in human beings.[1] No scrotal lesions were observed. Two of the animals showed petechial hemorrhages on the ears at the end of the first week of infection; no other skin lesions were noticed.

The statistical results have been summarized in table 1 and the statistically significant changes have been indicated.

Group 1.—In 6 of the 7 animals fever developed on the 3rd day after inoculation, and the 7th had fever on the 5th day. The mean duration of fever was 5 days. Four of the seven rabbits showed a diagnostic increase in complement fixing antibody, the titer rising from 0 to 16 by the end of the second week after inoculation. The serums were anticomplementary in the remaining three animals.

The following statistically significant changes in fluid volumes and electrolyte concentrations were found: One week after inoculation, the plasma volume and thiocyanate space were elevated and the hematocrit was decreased. This interval corresponds to the peak of the clinical disease in human beings. On the 13th day, when the animals were afebrile, the total blood volume and

3. Aikawa, J. K. 1950, Am. J. Physiol. **162**: 695–702.
4. Aikawa, J. K. 1951, Proc. Soc. Exper. Biol. & Med. **78**: 524–528.
5. American Public Health Association. Diagnostic Procedures for Virus and Rickettsial Diseases, 1948. New York.
6. Snedecor, G. W. 1946, Statistical Methods. Ames, Iowa, The Iowa State College Press.

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TABLE 1. *Summary of data on fluid and electrolyte changes in 15 rabbits infected with* R. rickettsii.

	Mean baseline value	Days of infection: Mean value, 7th day	Mean value, 13th day	Mean value, 20th day
	Group 1 (7 animals)			
Weight (kg)	3.464	3.478	3.540	3.514
Plasma volume (ml/kg)	38.9	49.7*	53.5	46.7*
Hematocrit (vol. %)	39.6	30.2*	34.6	38.5
Total blood volume (ml/kg)	64.6	71.2	77.7*	75.8
Thiocyanate space (ml/kg)	209.9	243.1*	237.3*	241.4*
Radiosodium space (ml/kg)	254.0	269.1	241.3	254.8
Serum sodium (meq./L)	144.4	145.0	144.9	151.4
Serum potassium (meq./L)	4.87	4.96	3.81*	4.59
Total serum protein (gm %)	6.06	6.56	6.67	6.57
	Group 2 (8 animals)			
Weight (kg)	2.029	2.109	2.189	2.089
Exchangeable potassium				
(meq.)	166.7	148.4	163.2	
(meq./L)	81.5	70.0	74.7	

* Statistically significant difference when compared with the mean baseline value; $P = <0.01$.

thiocyanate space were increased and the serum potassium concentration was decreased. This time interval corresponds to early convalescence in patients. At 20 days, the plasma volume and the thiocyanate space were increased. No significant changes were noted in the radiosodium space or the serum sodium concentration at any time.

Group 2.—The febrile course in this group of animals was similar to that in group 1. The mean exchangeable potassium contents 7 and 13 days after infection showed no significant changes when compared with the mean baseline value.

COMMENT

The physiologic changes observed in the rabbits are comparable to those found in a typical mild case of Rocky Mountain spotted fever in a human being (see case 2[1]), inasmuch as no evidence of peripheral circulatory collapse or of edema was noted, and no significant changes in the concentration of serum protein occurred. While the plasma volume was increased, the hematocrit was decreased, so that very little change occurred in the total blood volume.

The thiocyanate space was increased in the rabbits, as it is even in mild cases occurring in human subjects. Significant increases, as compared with the individual control baseline values, were noted on the 7th, 13th and 20th days after the inoculation of the rabbits with spotted fever rickettsias. No significant change was found in the radiosodium space, however. The baseline values in this group of animals were lower than those reported in a previous study.[3] Since, however, serial determinations in any one animal were reproducible, the following interpretation of the experimental results is suggested. These discrepant findings between the thiocyanate and the radiosodium spaces suggest that mild spotted fever in the rabbit increases the rate of exchange of the thiocyanate ion between the extracellular and the intracellular phases, whereas it does not effect that of the radiosodium ion. Since these two volumes are practically identical in normal individuals[7] and normal rabbits,[3] this increase in the thiocyanate space without a corresponding increase in the radiosodium space suggests that some abnormal physiologic change of a rather subtle nature had occurred in the tissue cell membrane. The increase in thio-

7. Aikawa, J. K. 1952, Am. J. Med. Sc. **224**: 632–637.

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ion space persisted for at least 19 days after infection. It would be difficult to explain this finding as being a shift of water into the extracellular compartment, since such an event should also have increased the radiosodium space.

The decrease in serum potassium on the 13th day of infection in group 1 suggests that some disturbance in potassium metabolism occurred at this time. Measurement of the exchangeable potassium content in group 2, however, showed no significant changes 1 and 2 weeks after infection. In other disease states a decrease in the serum concentration of potassium does not usually occur unless the body store of this ion has been previously depleted. Hence the finding of a low serum potassium in group 1 and of an unchanged Ke in group 2 suggests that more severe physiologic changes were induced in group 1 than in group 2, despite the observation that the febrile course of the two groups was similar. A profound degree of protein catabolism has been previously demonstrated in severe cases of spotted fever in human patients.[8]

The results of the present study suggest that the physiologic changes which are induced in domestic rabbits by experimental inoculation with *R. rickettsii* are comparable to those produced by a mild natural infection in human patients. Foot's observations that only a mild infection with *R. rickettsii* can be induced in domestic rabbits is confirmed. Domestic rabbits of mixed breeds appear to be naturally more resistant to this disease than guinea pigs or human beings. The characteristic febrile response and the increase in complement fixation titers, however, indicate that the animals were indeed infected with the disease.

8. Harrell, G. T., Wolf, W. A., Venning, W. L. and Reinhart, J. B. 1940. South. M. J. **39**: 551–558.

SUMMARY

Experimental infection with *Rickettsia rickettsii* was induced in domestic rabbits by the intraperitoneal injection of splenic emulsion from infected guinea pigs. Serial determinations of the body weight, temperature, plasma volume, hematocrit, thiocyanate and radiosodium spaces, the exchangeable body potassium content and the serum concentrations of sodium, potassium, and protein were made. Significant increases in the thiocyanate space were found on the 7th, 13th and 20th days of infection; the radiosodium space was unchanged at these intervals. The serum potassium concentration was reduced on the 13th day of infection. However, subsequent determinations of the exchangeable potassium content revealed no significant changes. It is concluded that the physiologic changes found in experimental Rocky Mountain spotted fever in the domestic rabbit are qualitatively comparable to those observed in mild human cases.

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TABLE 1.—*Mean organ weights of infected and control guinea pigs*

	Group 1 (4 animals infected with *R. rickettsii*)	Group 2 (6 normal control animals)*
Kidney	A. 1.08 B. 3.56	A. 0.87 B. 3.24
Heart	A. 0.32 B. 1.05	A. 0.26 B. 0.98
Adrenal	A. 0.10 B. 0.33	A. 0.05 B. 0.20
Spleen	A. 0.35 B. 1.17	A. 0.13 B. 0.49
Liver	A. 4.60 B. 15.55	A. 3.17 B. 11.70
Total body weight	337	369

A. Mean values expressed as percent of total body weight at the time of sacrifice.
B. Mean absolute values in grams at the time of sacrifice.

animals of group 1 and in all six animals of group 2 at the time of sacrifice.

Plan of the experiment.—Weights and rectal temperatures of all animals were recorded daily. On the sixth day after inoculation, three animals in each group were put to death with ether. The remaining six guinea pigs were sacrificed on the following day.

Previous observations on guinea pigs infected with the Bitter Root strain of *R. rickettsii* have shown that the clinical peak of the illness occurs on the sixth or seventh day after infection.

RESULTS

None of the six animals in the control group had fever (rectal temperature of 104 F or higher). In all of the infected animals fever developed on the fourth day after inoculation; thus, three of these animals were sacrificed on the third and three on the fourth day of fever. None of the control animals lost weight during the period of observation. The infected animals lost an average of 13.8% of their original body weight.

The spleens of the infected animals were grossly enlarged and engorged, and in spite of the loss in total body weight the mean weights of all the organs were greater in the infected than in the noninfected guinea pigs (table 1). Because of the small number of observations in the test group, a statistical analysis of these data was not attempted.

There was no difference between the mean total body radiosodium spaces in the test and control animals (table 2). The serum sodium concentration was significantly decreased in the test animals, but no change was noted in their serum potassium concentration. The radiosodium spaces of the adrenal, liver and kidney were significantly increased in the infected animals, whereas those of the spleen and heart were not.

COMMENT

The results of the present study indicate that in guinea pigs infection with spotted fever results in a redistribution of the sodium ion in the body. The decrease in the serum sodium concentration suggests a diminution in the tonicity of the extracellular fluid space. Since the total radiosodium space was not altered, the total body content of

TABLE 2.—*Comparison of radiosodium spaces and serum electrolyte concentrations in guinea pigs with Rocky Mountain spotted fever and in noninfected animals.*

		Total radiosodium space (ml/kg)	Serum sodium (meq./l)	Serum potassium (meq./l)	Radiosodium spaces of body organs (percent of weight)				
					Spleen	Heart	Adrenal	Liver	Kidney
Group 1 (6 animals infected with *R. rickettsii*		428.0	134.0	5.38	16.56	25.30	22.03	17.02	43.40
Group 2 (6 normal control animals)		453.0	149.0	6.05	14.33	26.08	16.94	13.14	32.98
Statistical analysis	d	−25.0	−15.0	−0.67	+2.23	−0.78	−5.09	−3.88	+10.42
	s	34.3	2.97	0.58	0.91	2.40	1.56	0.67	2.61
	s_d	0.73	5.05*	1.16	2.46	0.33	3.26*	5.86*	3.99*

* Statistically significant difference. $P = <0.01$.

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sodium may have declined during the infection.

The characteristic pathologic picture in Rocky Mountain spotted fever is that of a generalized involvement of peripheral blood vessels. The degenerative changes found in the endothelial cells and in the smooth-muscle cells of the media are thought to result from a direct injury caused by the rickettsiae. In guinea pigs with experimental infections, the spleens were grossly enlarged, but the relative radiosodium spaces were not significantly altered. The latter finding suggests that the splenic enlargement was not due solely to extracellular edema, and that it might be attributed to tissue proliferation. In contrast, the increase in the relative radiosodium spaces of the adrenal, kidney and liver suggests the presence of extracellular fluid edema, or perhaps an abnormal increase in the permeability of tissue cell membrane to the radiosodium ion. Such changes may depress the functional capacity of these organs, and, indeed, it has been previously demonstrated in human subjects that disorders of renal, adrenal and hepatic function are common in Rocky Mountain spotted fever.[1] The radiosodium method serves as a sensitive index of physiologic aberrations in organs and tissues—aberrations which may not be demonstrable by conventional histopathologic or chemical techniques.

SUMMARY

In guinea pigs infected with *Rickettsia rickettsii* the radiosodium spaces of tissues and organs were determined by the use of Na^{24}. When the animals were sacrificed at the clinical peak of the illness, significant increases were found in the radiosodium spaces of the adrenal, liver and kidney, but not in those of the spleen or heart. The serum sodium concentration was decreased. The moist weights of all the organs were greater in the infected animals than in normal control animals. The data suggest that infection with Rocky Mountain spotted fever results in redistribution of the sodium ion in the organs and tissues of the body.

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Progress report on the research project

TOXICITY OF P^{32} AS RELATED TO THE DIET

1112451

Winston-Salem, N. C.,
March 15th, 1953.

Progress report on the research project

TOXICITY OF P^{32} AS RELATED TO THE DIET

sponsored by the Atomic Energy Commission under direct contract AT-(40-1)-288 Title III, with the Bowman Gray School of Medicine of Wake Forest College.

Principal Investigators: Drs. Camillo Artom and George T. Harrell, Jr.

The present report covers the work done from March 16, 1952 to March 15, 1953. Technical assistance was given by C. Downs, and, for a short period, by W. Sears.

A. General outline of the work: As indicated in our original proposal, during the past year our experiments have been carried along several parallel lines of investigations. In continuation of our studies on the importance of vitamins for the protection against internal radiation by P^{32}, the roles of vitamin E and pantothenic acid (PA) have been investigated. No significant protection could be detected, when a vitamin E deficient diet was supplemented with tocopherol. On the other hand, a quite definite improvement in the percent of survivors and in the survival time was obtained by administration of PA to mice which were deficient in this vitamin and had been injected with P^{32}. The unexpected finding than an analog of PA, pantoyltaurine, also has a significant protective action, led us to extend our investigation to the action of certain other S-containing substances.

Several series of experiments have been made also on various agents which might be expected to increase the mobilization of the radioactive material from the bones by a direct, or indirect protective mechanism. Pyrophosphate, Dihydrotachysterol and Versene were tested. However in none of these experiments was any definite beneficial effect observed in respect to the survival of the mice injected with the isotope. Likewise exploratory experiments on the effects of repeated exposure to internal radiation failed to show any increase in the resistance of the animals as a result of the previous treatment with a sublethal dose of P^{32}.

B. Protective Role of Pantothenic Acid Against P^{32}. In several series of experiments, one group (a) of mice was maintained on a high fat diet containing sulfasuxidine. The diet was supplemented with all the known vitamins, except PA. After a depletion period, a dose of P^{32} in the midlethal range was injected, and the animals were maintained on the deficient diet for 3 more weeks. Hereafter, PA was given in order to reduce the fatalities due to the prolonged vitamin deficiency. Three groups of controls were run simultaneously with the experimental groups, that is: (b) mice on the same diet, supplemented with PA and injected with P^{32}; (c) PA-deficient mice, not injected with P^{32}; and (d) mice on the PA supplemented diet, not injected with P^{32}. The occurence of a severe epidemic (probably infectious, ectromelia) obscured the significance of the results obtained in the experiments then in progress and compelled us to discontinue our work for several weeks. However the data obtained from five series of experiments on about 300 animals are believed to be reliable. These data indicate a very definite increase in the survival of the groups receiving PA as compared with the survival of the vitamin deficient mice injected with P^{32}. Additional experiments are now in progress, in which the effects of administration of PA to animals on

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a milder vitamin deficiency are being studied. When these experiments will be completed, we believe that we shall be able to report the main results in a scientific journal.

C. Action of Certain S-Containing Substances. Pantoyltaurine is an analog of PA which has been claimed to act on bacteria as an antagonist of the vitamin. However conflicting reports have been published on its effectiveness as an antivitamin for higher animals. Under the conditions of our experiments the substance exerted a significant protection against BAL, and a protection was almost as marked as that exerted by its natural analog. The question arises whether this unexpected effect can be ascribed to a vitamin-like action, or, more specifically, to the S-containing moiety of pantoyltaurine. Experiments are now in progress, in which taurine is given to the animals. Preliminary results indicate that this compound also is active. This finding is of obvious interest since the S in taurine is in a completely oxidized form ($-SO_3$). On the contrary, an -SH group is present in BAL. Preliminary [illegible] show to exert a definite protection against [illegible]. In other experiments which are also under way, we are testing the possibility of a protective action against radiation by certain other proprietary compounds which contain S and are chemically related to BAL.

D. Role of Vitamin E. Tocopherol added in relatively large amounts to a low fat, low protein diet caused a definite decrease in the percent of survivors and in the survival time of mice injected with P^{32}. This decrease is partly due to the toxic effects of the vitamin itself, since a considerable mortality occured in the controls receiving the same doses of tocopherol, but not injected with P^{32}. Some of our results are exemplified in the table below.

No. of mice	Tocopherol added to diet	P^{32} injected $\mu c/g$.	Percent Survivors 21 d.	42 d.	Average time of survival days
36	+	5	44	18	22.7
18	0	5	62	48	29.6
18	+	[illegible]	[illegible]	[illegible]	[illegible]
18	0	[illegible]	100	[illegible]	[illegible]

In future experiments we shall attempt to produce a more severe vitamin E deficiency, either by adding oxidized fat to the diet, or by administration of vitamin E analogs. Experiments similar to those summarized above will be carried out on such more severely deficient mice.

E. Experiments With AT-10 and with Pyrophosphate. Dihydrotachysterol (AT-10) partly simulates the action of parathyroid hormone, but it has a much longer lasting effect. This drug is believed to lower the inorganic phosphate in blood by increasing phosphate excretion through the kidneys. The possibility was tested that such a mechanism may be used in order to increase the mobilization of the radioactive phosphate initially deposited in the bones. However, in our experiments treatment with AT-10 increased (instead of decreasing) the mortality of the mice injected with the P^{32}, suggesting that the substance in the doses which we used was definitely toxic. Moreover the elimination of P^{32} in the urine was quite irregular and no clear differences could be detected in this respect between the mice treated with AT-10 and the untreated controls.

Inactive pyrophosphate was injected before, together with, or after introduction of radioactive phosphate, with the idea that under the action of bone pyrophosphatase a high concentration of inactive phosphate could be obtained at the surface of the bone, and thus favor the mobilization of P^{32}. Actually a somewhat higher elimination of P^{32} in the urine was observed in the mice receiving inactive pyrophosphate. However the increases were less marked than those observed previously with the ingestion of large amounts of phosphate and the differences in the survival of the experimental and the control mice were not statistically significant.

F. Action of Chelating Agents. Versene was injected daily in mice receiving P^{32}. In the various series of experiments the number and the timing of the injections was varied. The mortality was somewhat lower in certain of the groups treated with Versene, but there was a great variability in the data and the mean differences between the experimental and the control groups in the complete series were not statistically significant. Likewise no significant differences were detected in the elimination of P^{32} in the urine of animals receiving or not receiving the chelating agent.

G. Effects of Repeated Exposure to Internal Radiation. In two series of experiments mice (33 and 36, respectively) were injected with a sublethal dose of P^{32} (3, and 1.5 μc, respectively). A second injection of P^{32} in the midlethal range was done after several weeks (3, and 4, respectively). In the two series of experiments 55% and 51% of the mice were surviving after 42 days. The average survival time was 34.6 days in the first series and 33 days in the second. These figures should be compared with a 62% survival at the 42nd day and with an average survival time of 32.5 days in the 72 control mice which received the higher dose of P^{32} only.

H. Remarks. Since the results of the experiments with vitamin E, AT-10 and Versene were negative, we believe that publication of these results is not warranted. On the other hand, it is felt that the experiments on the protective action of pantothenic acid have given clearcut results and that a relatively short time only is needed for the completion and the statistical evaluation of these data, which we shall then publish in a scientific journal. Of the other groups

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of investigations, those concerning the action of S-containing substances look promising and will be continued actively during the next few months.

In the period covered by the present report the following complete paper has been published: "A Protective Role of Pyridoxin Against the Toxic Effects of P^{32}, by C. Artom, W. E. Cornatzer and G. T. Harrell, Jr., Proc. Soc. Exp. Biol. and Med. 79: 494-497 (March 1952).

Dr. Camillo Artom

Camillo Artom, M. D.

George T. Harrell, Jr.

George T. Harrell, Jr.

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Summary of the work from March 16, 1952 through March 15, 1953 on the project described below

TOXICITY OF P^{32} AS RELATED TO THE DIET

sponsored by The Atomic Energy Commission under direct contract AT-(40-1)-288, Title III, with the Bowman Gray School of Medicine.

Principal Investigators: Drs. Camillo Artom and George T. Harrell, Jr.

In a continuation of previous studies the protective role of pantothenic acid (PA) toward the effects of internal radiation by P^{32} has been investigated. Mice were placed on a synthetic diet containing sulfasuxidine and supplemented with all known vitamins except PA. The animals were then injected with a dose of radioactive phosphate in the midlethal range. A marked increase in the percent of survivals and in the average t ime of survival was observed, when calcium pantothenate was added to the deficient diet.

In other experiments, the effects of various agents which might be expected to enhance the mobilization of the isotope, initially deposited in the bones, were tested. However, after introduction of dihydrotachysterol (AT-10), of inactive pyrophosphate, or of a chelating compound (versene), no significant protection against the injurious action of P^{32} could be detected. Likewise, no improvement was observed in the survival of the animals which had received a small dose of P^{32}, 3 or 4 weeks before the lethal dose of the radioisotope.

Dr. Camillo Artom

Camillo Artom, M. D.

George T. Harrell, Jr.

George T. Harrell, Jr., M. D.

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Dr. Camillo Artom

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George T. Harrell, Jr.

George T. Harrell, Jr., M. D.

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A Protective Role of Pyridoxin Against the Toxic Effects of P^{32}

CAMILLO ARTOM, W. E. CORNATZER, AND GEORGE T. HARRELL, JR.

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A Protective Role of Pyridoxin Against the Toxic Effects of P^{32}.* (19422)

CAMILLO ARTOM, W. E. CORNATZER,† AND GEORGE T. HARRELL, JR.

From the Departments of Biochemistry and Internal Medicine, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

In a continuation of our study on the effects of internal radiation by P^{32} as related to various dietary factors(1,2), the role of pyridoxin has now been investigated. After

* Technical assistance was given by J. Giles and C. Downs. This work was performed under a contract between U. S. Atomic Energy Commission and the Bowman Gray School of Medicine. The P^{32} was obtained from the Oak Ridge National Laboratories. Desoxypyridoxin was graciously supplied by Merck and Co., Rahway, N. J. A preliminary report was presented before the Southeastern Section of the Soc. Exp. Biol. and Med., Atlanta, Ga., Jan., 1952.

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whole body irradiation of mice with mid-lethal doses of X-rays, large amounts of pyridoxin prolonged the survival, but did not improve the anemia and leukopenia(3). In the present experiments when pyridoxin was added to a pyridoxin deficient diet with a low protein content, no change in the survival of mice injected with P^{32} was detectable. On the other hand, a marked degree of protection by the vitamin became apparent when the deficiency was made more severe by the administration of a pyridoxin analog (desoxypyridoxin)(4), or by diets which presumably increase the requirements for pyridoxin (high protein diets(5) with added cystine or methionine(6)).

Experimental. Two basal diets were employed, one with a 5% casein content (Diet 34(1)), the other with a higher protein level (Diet 41, containing: Casein 30 p.p. 100, dextrin 29.5, sucrose 29.5, Crisco 4, cod liver oil 1, Ruffex 2, salt mixture (U.S.P. No. 2) 4). A mixture of B vitamins(2) from which pyridoxin had been omitted, was incorporated in the diets. Six series of experiments were made on a total of 558 male albino mice, placed under experimental conditions which have been described(1,2). In each series of experiments the animals were distributed in 4 groups: *a*, *b*, *c*, and *d*. All 4 groups received the same diet and the same supplements, except that pyridoxin HCl (1 mg 100 g of diet unless otherwise indicated) was added to the diet of groups *b* and *d* only. After 3 to 10 days on the experimental diet, the mice of groups *a* and *b* were injected intraperitoneally with a solution of radioactive $Na_2HP^{32}O_4$ (5 or 6 $\mu c/g$) and kept under observation for 42 more days. Groups *c* and *d* were used as controls and observed for the same length of time. The criteria previously used as an indication of the toxic effects of the isotope(1,2) were applied also to the results of the present experiments, except that in computing the average length of survival, a survival of 42 days was ascribed to the mice still alive at the end of the experiment.

Results. In the experiments of Series I (Table I) supplementation of the low protein diet with pyridoxin did not alter the survival of the mice injected with P^{32}. The controls maintained on the pyridoxin-deficient diet and not injected with the isotope grew fairly well and did not show skin lesions, thus confirming previous indications(7) that, unlike the rat, the mouse on a moderately low protein diet does not develop typical symptoms of pyridoxin deficiency. In the experiments of Series II, III and IV (Table II) in which the low protein diet contained desoxypyridoxin, survival figures were higher in the groups injected with P^{32} and receiving pyridoxin, in addition to the anti-vitamin. Similar differences between the groups receiving or not re-

TABLE I. Survival of Mice Injected with P^{32} (6 $\mu c/g$) and Maintained on a Low Protein Diet (Diet 34).*

Groups of mice	Pyridoxin in diet	No. of mice	Survivors 21st day, %	Avg length of survival, days†
I a	—	24	67	32 ± 2.8
b	+	15	67	30 ± 2.5
c‡	—	12	100	42
d‡	+	12	100	42

* Time of 50% deaths was >42 days for all 4 groups.

† Values preceded by ± are the stand. errors of the means.

‡ Control groups not injected with P^{32}.

TABLE II. Survival of Mice Injected with P^{32} (5 $\mu c/g$) and Maintained on a Low Protein Diet (Diet 34) with Added Desoxypyridoxin.

Groups of mice		Pyridoxin in diet*	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival,† days
II§	a	—	46	13	15	16 ± 1.4
	b	+	46	>42	74	34 ± 1.9
	c‡	—	39	>42	87	38 ± 1.5
	d‡	+	13	>42	100	42
III	a	—	30	14	29	20 ± 2
	b	+	27	>42	79	30 ± 1.8
	c‡	—	26	41	93	36 ± 1.5
VI¶	a	—	18	15	22	22 ± 2.6
	b	+	18	>42	78	36 ± 2.5
	c‡	—	24	>42	92	39 ± 1.4
	d‡	—	12	>42	100	42

* 2 mg/100 g of diet, then 1 mg/100 g when desoxypyridoxin was omitted from diet.

† Values preceded by ± are the stand. errors of the means.

‡ Control groups not inj. with P^{32}.

§ Desoxypyridoxin (5 mg/100 g diet) for 9 days.

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TABLE III. Survival of Mice Injected with P^{32} (5 μc/g) and Maintained on a High Protein Diet (Diet 41) with Added Cystine (Series V) or Methionine (Series VI).

Series	Group	Pyridoxin in diet	No. of mice	Time of 50% deaths, days	Survivors, % 21st day	Avg. length of survival, days
V‡	a	—	23	18	22	21 ± 2.6
	b	+	23	>42	83	36 ± 2.4
	c†	—	24	41	96	37 ± 1.4
	d†	+	19	>42	95	39 ± 1.3
VI§	a	—	28	15	28	21 ± 2
	b	+	27	28	67	27 ± 2.4
	c†	—	24	>42	100	39 ± 1
	d†	+	23	>42	91	40 ± 1

* Values preceded by ± are the stand. errors of the means.
† Control groups not inj. with P^{32}.
‡ Cystine (.3 g/100 g diet) for 52 days.
§ Methionine (.5 g/100 g diet) for 47 days.

TABLE IV. Statistical Appraisal of the Protection by Pyridoxin Against P^{32} Toxicity.

		10% casein + desoxypyridoxin	30% casein + S-containing amino acids
No. of mice inj. with P^{32}		185	101
No. of survivors 21st day	Chi^2	41	24
	P*	< .01	< .01
	Diff. between adjusted means	11 ± 1.12†	10 ± .98†
Avg. length of survival	t	9.9	10.2
	P*	< .01	< .01

* Probability for such a chance occurrence.
† Stand. errors of the diff. between adjusted means.

ceiving pyridoxin are apparent (Table III) in the experiments with a high protein diet supplemented with cystine (Series V), or methionine (Series VI). In the pyridoxin dedeficient groups of all 5 Series of experiments acrodynia appeared early and often progressed to the almost complete loss of hair. Growth of these mice was also definitely impaired. For a statistical evaluation, the results of the experiments of Series II-IV, or Series V and VI, respectively, have been grouped together. The average values from the groups *a* and *b* were then adjusted on the basis of the data from the corresponding controls which did not receive the isotope (groups *c* and *d*). The significance of the differences between these adjusted means was then estimated, using the Chi^2 method for the number of survivors at the 21st day and the *t* test of significance for the average length of survival (Table IV).

Discussion. It is apparent that the administration of pyridoxin to mice which are actually deficient in pyridoxin exerts a significant protection against the injurious effect of P^{32}. This protection is of about the same degree as that observed previously with the combined administration of vitamin B_{12} and folic acid to mice, placed on sulfasuxidine-containing diets and injected with P^{32}(2). It is possible that both groups of findings may be due primarily to a beneficial effect on the recovery of the bone marrow, although evidence for a specific role of pyridoxin in erythropoiesis is somewhat equivocal and restricted to certain animal species (see, for instance(8)). On the other hand, atrophy of the thymus and lymphoid tissue accompanied by changes in the white blood cells is a striking and constant finding in acute pyridoxin deficiency(9). It is tempting to compare our present observations with others(10), suggesting that the integrity of large sections of the lymphatic system (as by lead-shielding the spleen during exposure to X radiation) increases the survival of the animals and hastens the regeneration of hemopoietic tissues.

Summary. Mice placed on pyridoxin-free diets were injected with a single dose of radioactive phosphate (5-6 μc/g). In the animals maintained on a low protein diet (in which symptoms of vitamin deficiency did not appear), the administration of pyridoxin did not affect the survival. On the contrary a marked protection by pyridoxin against the injurious effects of P^{32} was observed in experiments in which the vitamin deficiency had been made more severe by adding a pyridoxin analog (desoxypyridoxin) to the low protein diet, or by increasing the requirements for the vitamin (high protein diets with added cystine or methionine). In these experiments the differences between the groups receiving and those

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not receiving pyridoxin were larger than the differences which might result from a simple additive effect of the internal radiation and vitamin deficiency.

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Received February 18, 1952. P.S.E.B.M., 1952, v79.

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Progress report on the research project

"FORMATION OF PHOSPHOLIPIDES IN TISSUES"

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's report on the research project

FORMATION OF PHOSPHOLIPIDES IN TISSUES

ed by the Atomic Energy Commission, Division of Biology and Medicine,
ontract No. AT-(40-1)-288, Title II, with the Bowman Gray School of
e of Wake Forest College.

leader: Dr. Camillo Artom

e present report covers the work done from March 16, 1952 through
5, 1953. This work is a direct continuation of that previously done
e aid of the Atomic Energy Commission and summarized in my progress
of March 16, 1952. In addition to the writer, the following investigators
pated in the research work for various periods of time:
ofland, Ph. D., and M. Crowder, M. S.,. Technical assistance was
y H. Pollard, B. A., B. Blue, B. A., and J. Girard.

eral outline of the work.

cause of the importance of the results and of their bearing on
erpretation of the lipotropic action of choline and related
factors, the activity of the investigators has been concentrated on the
choline in the oxidation of fatty acids in the liver. The data
definite evidence for the concept that oxidation of fatty acids
essed in the liver of choline deficient animals and that choline
tration restores the ability of the tissue to oxidize fatty acids
rmal rate.
me definite progresses have been made in the investigations on the
diate stages in the synthesis of the phospholipide molecule, and on
abolic pathways of choline in the isolated liver. As anticipated,
e and effort has been expended in the elaboration of appropriate
ies. While the results are not conclusive, it is felt that the data
d give useful indications for the future development of these investigations.
was not possible to obtain a preparation of labeled ethanolamine
k of time did not permit to study the synthesis of this compound in
oratory. Only in a few experiments in which liver preparations were incubated
beled glycine, some indications were obtained concerning the
ation of ethanolamine into phospholipides.

details on each group of investigation are given below.

e of choline in the oxidation of fatty acids in the isolated liver.

indicated in the proposed plan of investigation, the action of choline
as precursors in preventing and curing fatty livers (lipotropic action)
generally interpreted by assuming that the fatty acids are carried

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dation of the fatty acids in the liver itself.
n each experiment slices or homogenates were prepared simultaneously
e liver of 2 to 4 rats, maintained for the same length of time on
ent diets, that is: (a)- a low protein, choline-deficient diet; or
low protein diet, supplemented with choline; or (c)- an adequate diet
ed vegetable and animal origin (stock diet). In most experiments
the animals was also injected with choline shortly before death.
ices and homogenates were then incubated with stearic or palmitic
abeled with C^{14}, the $C^{14}O_2$ produced during the incubation was collected
s radioactivity was determined.
n a first group of experiments, the results showed that slices of the
of rats on the low protein, choline-deficient diet produced much less
from added stearate than slices of rats on the stock diet. On the other
higher values of isotopic CO_2 were obtained with the livers of rats,
he low protein diet was supplemented with choline. Even more marked
ses in the formation of $C^{14}O_2$ were obtained with the liver of choline-
ent rats which had been injected with a large dose of choline shortly
death. The effects of choline, injected or added to the diet, were
ed after short (9 days) as well as after prolonged periods (up to 6 months)
low protein diet. In all experiments, differences between the values
ed from rats receiving or not receiving choline were quite significant,
ective of the method used for expressing the results (per one gm of tissue,
e mg of N, in the whole liver of a 100gm rat).
n a second series of experiments, similar results were obtained, by using
nates of the liver (instead of slices), a finding which indicates that the
ed differences in the production of $C^{14}O_2$ cannot be ascribed to differences
permeability of cell membranes.
n a third series of experiments palmitate-1-C^{14} was used as substrate.
ntially identical effects were obtained, although in most of the experiments
rats were used, and in these animals apparently higher doses of choline
equired in order to enhance fatty acid oxidation.
he lack of an inverse parallelism between the rate of production of
and the amount of liver fat excludes the possibility that the results
erely due to isotopic dilution of the added fatty acid. More direct evidence
is point was obtained in an additional series of experiments with washed
les or mitochondria, prepared form the liver of choline deficient rats,
ing, or not receiving choline respectively. Much less $C^{14}O_2$ was produced
parations from the latter animals, although the previous washing ot the
les resulted in the almost complete elimination of the excess fat accumulated
tissue.
rom the results of other experiments it appears that the production
topic acetoacetate was affected by choline deficiency, or choline
stration, respectively, in the same manner as the production of $C^{14}O_2$.
n vitro addition of choline to liver slices or homogenates did not enhance
acid oxidation. This finding strongly suggests that the active substance
choline, but some other compound formed in vivo from choline. Identification
s compound, or compounds, should be the aim of future investigations.

termediate Stages of Phospholipides in the Isolated Liver

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scheme represented the major pathway for the incorporation of choline
.ospholipides of the isolated liver. However the possibility of an
e pathway which may include phosphorylcholine as an intermediate stage is
d by our experiments, and is more directly suggested by recent data
g and Pricer (Federation Proc., 11, 242 (1952)).

lic Pathways of the Metabolism of Choline in the Isolated Liver.

ous experiments with methyl-labeled choline indicated that the following
are among the probable metabolic products in liver slices and homogenates:
line, trimethylamine oxide, betaine aldehyde, betaine, and, in addition,
arge amounts of an unknown compound, or compounds, from which choline
erated by a relatively strong hydrolysis. Attempts to separate these
by various means have been thus far only partly successful. We are
systematically paper chromatography and other adsorption and partition
an effort to identify the unknown compounds, and to estimate the relative
of these individual metabolic pathways under varied experimental conditions.

ration of Nitrogenous Constituents (other than choline) into
ospholipides.

otope dilution method was also used in a few experiments in which we
hypothesis that dimethylethanolamine (DME) is the natural precursor
as suggested by the following hypothetical scheme:
ethanolamine $\rightarrow$ DME $\rightarrow$ choline. In these experiments, glycine-2-C^{14} was
with liver slices in the presence or absence of non-isotopic DME.
cant activity was found in the DME fractions. Doubtful or negative
re also obtained in experiments in which the isotopic glycine was
d in vivo together with large amounts of non-isotopic DME. Because
uity with which glycine is metabolized through numerous pathways, it
at similar experiments should be repeated, using labeled ethanolamine
the labeled glycine.
incubation of liver slices with glycine-2-C^{14} as much as 30% of the
ity in the lipide extract was present in the ethanolamine moiety.
ound in this fraction after incubation with glycine-1-C^{14}, suggesting
irst reaction in the above scheme should be actually written as follows:
serine $\rightarrow$ ethanolamine + CO_2.

arch 16, 1952 to March 15, 1953, the following papers based totally
n the results of the investigations of this project have been published:

poration of Dimethylethanolamine into Phospholipides of Isolated
Tissue"
Crowder and C. Artom, Federation Proc., 11, 199 (March 1952)

ldehyde-Pyruvate Condensing Enzyme"
C. Mitchell and C. Artom, Federation Proc., 11, 261 (March 1952)
Commun. 2nd Intern. Congr. Biochem., P.38 (Paris, July 1952)

ration of C^{14}-Labeled Dimethylethanolamine"
Artom and M. Crowder, J. Amer. Chem. Soc., 74, 2412 (May 1952)

olipide Formation in Isolated Liver Tissue"
Artom, M. A. Swanson and M. Crowder, Abst. Commun. 2nd Intern. Congr. Biochem.,
(Paris, July 1952)

tion of Phospholipides in Animal Tissues"
Artom, Phosphorus Metabolism, 2, 203 (The Johns Hopkins Press, Baltimore,
er 1952)

of Choline in the Oxidation of Fatty Acids in the Isolated Liver"
. Artom, Nature, 171, 347 (February 1953)

onal reports on the subject mentioned under 6) are also on the programs
ings of the Federation of the American Soc. for Exper. Biology
pril 1953) and of the 19th Intern. Congress of Physiology
eptember 1953).

K. Camilla Artom

C. Artom, M. D.

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om, N. C. March 16, 1953

ary of the work done from March 18, 1952 through March 18, 1953
he project described below.

FORMATION OF PHOSPHOLIPIDS I. THIS [illegible]

sored by The Atomic Energy Commission under direct contract AT-(40-1)-288,
e II, with the Bowman Gray School of Medicine.

cipal investigator: Dr. Camillo Artom

g the various lines of investigation carried out in this period,
cially important were the results obtained by studying the production
sotopic CO_2 from C^{14}-labeled stearate or palmitate, added to slices
omogenates of the liver of rats which had been previously maintained
arious diets. It appears that the oxidation of fatty acids is markedly
essed in the liver of choline deficient animals, and that choline added
e deficient diet or injected into the rat shortly before removal of the
r, raises fatty acid oxidation to the normal level or above this level.
e in vitro addition of choline was not effective, the active factors is
ably not choline, but some substance formed from choline in vivo.
e the nature of this substance is still undetermined, the present
ings strongly suggest that the well known action of choline in
venting and curing the fatty infiltration of the (lipotropic action)
ue to an enhancement of fatty acid oxidation in the liver rather
t an increased mobilization from this organ in the form of
ns phospholipides.

D. Camillo Artom

Camillo Artom, M. D.

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Reprinted from FEDERATION PROCEEDINGS
Vol. 11, No. 1, March, 1952
Printed in U.S.A.

Incorporation of labeled dimethylethanolamine in phospholipides of isolated liver tissue.

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Slices or homogenates of rat liver were incubated with C^{14}-methyl labeled dimethylethanolamine (DME). Considerable amounts of the compound were incorporated directly into the phospholipides without previous methylation to choline. This finding parallels those of previous experiments *in vivo* (*Federation Proc.* 8: 180, 1949). In oxygen the apparent uptake of DME by the lipides is greater with liver slices from rats on methyl-deficient diets than from rats on a stock diet. Homogenization reduces but does not suppress this uptake. With both slices or homogenates the amounts of DME incorporated in the lipides are greater in nitrogen than in oxygen. The process is markedly inhibited by betaine added *in vitro*, less markedly by methionine. The above findings will be compared with those previously reported for the incorporation of labeled phosphate (*J. Biol. Chem.* 193: 473, 1951) and labeled choline (*Federation Proc.* 10: 157, 1951) into the phospholipides of isolated liver tissues.

(Aided by the Markle Foundation and by the U. S. Atomic Energy Commission, Contract No. AT-(40-1)-288, Title II).

1112469

Reprinted from FEDERATION PROCEEDINGS
Vol. 11, No. 1, March, 1952
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Formaldehyde-pyruvate condensing enzyme.

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The widespread occurrence in animal tissue of a formaldehyde-pyruvate condensing enzyme has been reported (*Federation Proc.* 10: 224, 1951). We have studied further the properties of this enzyme in partially purified preparations ('large granules' of rat liver). Our previous data on some characteristics of the addition product(s) have been confirmed and extended by the use of C^{14}-labeled formaldehyde. It appears now that the enzyme is different from the diphosphothiamine-metal-proteins catalyzing the formation of acyloins from other aldehydes and pyruvate. Since in the reaction between formaldehyde and pyruvate, no CO_2 is evolved, it seems likely that the initial product is a four carbon compound from which other compounds can be formed enzymatically or non-enzymatically. (Aided by the U. S. Atomic Energy Commission, Contract No. AT-(40-1)-288, Title II).

1112470

FORMATION OF PHOSPHOLIPIDS IN ANIMAL TISSUE

CAMILLO ARTOM

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The Johns Hopkins Press, Baltimore, 1952.

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Reprinted from *Phosphorus Metabolism*, Vol. II, edited by William D. McElroy and Bentley Glass. The Johns Hopkins Press, Baltimore, 1952.

FORMATION OF PHOSPHOLIPIDES IN ANIMAL TISSUE

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BECAUSE OF LIMITATIONS in time and space, only certain aspects of our subject will be considered, namely: (a) the pathways and mechanism of phospholipide formation, (b) the differences in the rate of synthesis in various tissues, and (c) the biological purpose of phospholipide formation (in other words, the functions which may be ascribed to phospholipides in animal tissues). Even within these limits, it will be possible to discuss only a part of the many recent contributions, and I hope to be forgiven if, in making such a necessary selection, the results obtained in our own laboratory are given special attention. On the other hand, I am confident that several of the points which I have omitted or mentioned only too briefly, will be discussed more adequately by other participants in this symposium.

BUILDING BLOCKS

The experiments with various labeled compounds introduced into intact animals clearly indicate that phospholipides are rapidly synthesized from their constituent parts, but they do not exclude the possibility of a partial synthesis in certain locations, completion of the whole molecule occurring in other tissues. Direct evidence for the complete synthesis of phospholipides in one site could be supplied only by experiments on isolated tissues. The incorporation of P^{32}-labeled phosphate into the lipides of liver slices was first described by Robinson et al. (121), and similar findings were reported by the same group of workers for several other tissues (69, 139, 81, 126, 53). In the experiments of Pihl and Bloch (112)

[1] Most of our recent work has been done with the aid of the J. and M. R. Markle Foundation and of the U. S. Atomic Energy Commission. Part of this material has been presented also at a Symposium on Lipides (R. Gould Research Foundation, Rochester, N. Y., Sept. 1950) and at the 10th Conference on Liver Injury (Josiah Macy, Jr., Foundation, New York, May 1951).

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on liver slices incubated with C^{14}-acetate, a notable proportion of the newly formed long-chain fatty acids was found to be present in the phospholipides. The introduction of labeled choline into the phospholipides of liver slices and homogenates has recently been demonstrated in our laboratory (20). The hypothesis that in these experiments the presence of the isotope is due to a physical exchange between the labeled compound and the corresponding radical in the phospholipide molecule is disproved by the results of control experiments in which the labeled compound is incubated with boiled tissue, or added at the end of the incubation period. Moreover, the incorporation of phosphate into the phospholipides is reduced or suppressed by conditions, such as homogenization of the tissue, anaerobiosis, or addition of respiratory inhibitors, which do not impair the hydrolysis of phospholipides. As pointed out by Chaikoff (50), this would also rule out the possibility that the results can be ascribed to the reversibility of hydrolytic reactions, a factor which is certainly small from the quantitative viewpoint but which cannot be dismissed a priori, because of the high degree of sensitivity of the isotopic technique. There is, therefore, little doubt that the liver and probably many or all other tissues are capable of carrying out the complete synthesis of a phospholipide molecule provided that adequate amounts of its building stones are supplied by the diet or by synthesis in the body.

Highly unsaturated fatty acids. In this respect it seems unlikely that a deficiency of phosphate, glycerol, or fatty acids may represent a limiting factor in the formation of phospholipides in intact animals. The only reservation on this point is suggested by the presence in the liver phospholipides of relatively large amounts of highly unsaturated fatty acids with 20 or 22 carbon atoms. These fatty acids are apparently not synthesized by the animals and must therefore be provided in the diet. The avidity with which such fatty acids, when present in the diet, are taken up by the tissue phospholipides, and the tenacity with which they are retained when the animals are transferred to a diet poor in unsaturated fatty acids, have been clearly shown, many years ago, by R. G. Sinclair (129).

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Ethanolamine. As for the availability of the nitrogenous components of phospholipides, nothing is known about the synthesis of sphingosine. The formation of ethanolamine from glycine and serine was clearly proved in the earlier experiments of Stetten with N^{15}-labeled compounds (133). The more recent evidence indicates that glycine is an immediate precursor of serine (Sakami, 122), but that ethanolamine is formed only or chiefly by decarboxylation of serine. In some unpublished experiments from our laboratory α-C^{14}-labeled glycine was incubated with liver slices, and the lipides were extracted, fractionated, and hydrolyzed. While the isotope was distributed widely among the various fractions, including cholesterol and the fatty acids of both glycerides and phospholipides, the ethanolamine contained as much as 30 per cent of the counts present in the lipide extract. In intact animals receiving serine labeled in the β-carbon, the ethanolamine isolated from the tissue phospholipides contains appreciable amounts of the isotope (Levine and Tarver, 99). On the contrary, none was found after glycine labeled in the carboxyl was given (Greenberg and Harris, 83). Likewise in rats injected with α-C^{14}-glycine, N^{15}-glycine, or β-C^{14}-serine the isotopes were present in both the methyl and the ethylene moieties of choline. After administration of carboxyl-labeled glycine, choline was totally inactive (Arnstein, 4).

Weissbach (154) has recently reported that after administration of ethanolamine doubly labeled with C^{13} and C^{14} these atoms appear in the glycine moiety of hippuric acid and in carbons 4 and 5 of uric acid, which are known to be derived from glycine (Buchanan et al., 44). Since in the earlier experiments of Stetten (133) the nitrogen of ethanolamine proved not to be a precursor of the N of glycine, it seems logical to postulate a sequence of reactions, starting with the formation of glycolaldehyde by oxidative deamination of ethanolamine. Glycolic and glyoxylic acids have been shown to be converted very rapidly to glycine (Weinhouse and Friedman, 152). These views are illustrated in Fig. 1.

Choline. Current hypotheses regarding the biochemical relationships between ethanolamine, choline, and betaine are summarized in

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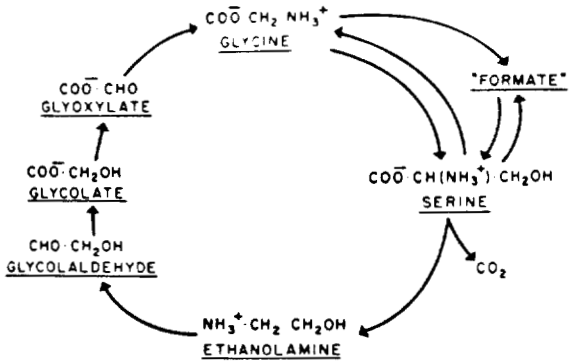
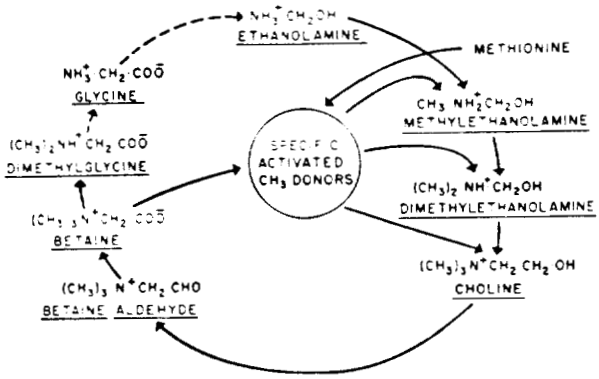
Fig. 2. The present scheme is similar to the one drawn five years ago by Jukes, who has also discussed in some detail the evidence for the various steps included in his scheme (89). I mention here



BIOCHEMICAL RELATIONSHIPS BETWEEN GLYCINE, SERINE AND ETHANOLAMINE

FIG. 1.



BIOCHEMICAL RELATIONSHIPS BETWEEN ETHANOLAMINE, CHOLINE AND BETAINE

FIG. 2.

only the few changes which are suggested by more recent investigations.

The concept of a pool of labile methyls to which a number of different methyl donors may contribute, and for which various methyl acceptors would compete, appears now to be questionable. The results of Cantoni (48) and Vignos and Cantoni (148) strongly

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suggest a transfer of methyl from a given activated donor to a given acceptor, each reaction being catalyzed by a specific enzyme, or enzyme system. As for the stepwise methylation of ethanolamine to choline, it should be pointed out that evidence for such a process is still quite indirect and perhaps susceptible of somewhat different interpretations. If the scheme of Fig. 2 is accepted provisionally, the data of Noland and Baumann (108) on the choline requirements of roaches may be of interest. These insects fail to grow or survive on a diet without choline. Choline may be substituted by betaine, but not by ethanolamine, methionine, or dimethyl ethanolamine (DME). However, the last two compounds given together are partially effective. It would appear, therefore, that at least in these insects the transfer of methyl groups to ethanolamine and methyl ethanolamine may occur from betaine but not from methionine. It may be noted in this respect that previous results on the synthesis of choline by liver tissue incubated with ethanolamine and methionine (Steensholt, 132; Barrenscheen et al., 37) have been questioned recently (Veitch and Zweig, 145).

For some time dimethyl ethanolamine was regarded both as a precursor of choline and as a product of its demethylation. In the present diagram instead, dimethyl glycine is indicated as the product of the demethylation of betaine, and as an initial step in the conversion of betaine to glycine. Evidence for the latter process has been given by Stetten in rats fed betaine labeled with N^{15} (133).

The concept that betaine rather than choline acts as a direct methyl donor was first suggested by Dubnoff (62), who showed that no methionine was formed from choline and homocysteine, incubated with the liver of rabbit, guinea pig, or chick, which contains little or no choline dehydrogenase. More direct evidence was supplied by Muntz (107) in experiments on rat liver homogenates incubated with homocysteine and N^{15}-labeled choline. A high concentration of N^{15} was present in the dimethyl glycine fraction, whereas none was found in the dimethyl ethanolamine. We have also failed to detect any activity in this compound after incubation of liver slices with C^{14}-methyl labeled choline in the presence of DME as a carrier (Artom and Crowder, 18).

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Dimethyl ethanolamine. The results of some of our experiments on intact rats may be considered in line with the interpretation mentioned above (Artom and Crowder, 17). After the administration of a large dose of DME, notable amounts of the compound were present in the lipides as well as in the aqueous extracts of liver and muscle. This was taken as an indication that the disposal of DME in vivo is sufficiently slow to allow its detection, if at any one time large quantities were produced in the tissues. On the other hand, after similar doses of choline were given, neither free nor combined DME could be detected in the tissues (Table 1).

TABLE 1

DME IN AQUEOUS AND LIPIDE EXTRACTS OF RAT LIVER*

Substances Administered (1-2 mM)	Hours After Administered	DME (micro M) in Liver	
		Aqueous extract	Lipide extract
H_2O	6	0	0
DME	3	47	20
"	6	35	70
" **	3	110	29
" **	6	25	90
Choline	3	0	0
"	6	0	0
" **	6	0	0

* Rats on choline-deficient diets
** By intraperitoneal injection

Attempts to demonstrate the presence of DME in the hydrolyzate of the phospholipides from the tissues of rats under various dietary conditions were unsuccessful, a result indicating that DME is not a quantitatively important constituent of phospholipides, even when the dietary supply of methyl donors is restricted. Levine and Chargaff also failed to detect DME in their analyses of the nitrogenous components of phospholipides by paper chromatography (98).

DME added to liver slices causes increases in the oxygen consumption which are smaller than those found after the addition of choline. No increases occur when DME is added to liver homogenates which still oxidize added choline very actively (Table 2). Part of

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the DME added to liver homogenates disappears in oxygen but not in nitrogen (Artom and Crowder, 19). In another series of experiments (Crowder and Artom, 59), in which liver slices were incubated with DME labeled with C^{14} in the methyls, only minimal amounts of the isotope were found in a fraction which should include dimethyl glycine. At the end of the incubation most of the C^{14} was still present as DME, whereas after incubation with either methyl- or ethylene-labeled choline only a small fraction of the isotope is recovered as unchanged choline. These data suggest that in the

TABLE 2

EFFECT OF DME OR CHOLINE ON O_2 CONSUMPTION BY LIVER TISSUE*

	Substrate (0.01 M)	
	DME	Choline
Slices	52	119
Slices + L-Methionine	155	312
Homogenate	4	71
Homogenate + L-Methionine	0	91
Homogenate + Betaine	−1	101

* Microl O_2/hour/100 mg tissue in excess over controls without added substrate

isolated liver no oxidation of DME to dimethyl glycine by reactions similar to the oxidation of choline to betaine aldehyde and betaine occurs, or that it represents only a metabolically unimportant pathway.

It seems reasonable to assume that under physiological conditions DME is formed in the tissues but that this compound is methylated to choline as rapidly as it is formed. Direct incorporation into the phospholipides would occur only if and when the amounts of DME present exceed the ability of the tissue to convert it to choline.

INTERMEDIATES

Presence in tissues. Consideration of the structural formulas of the phospholipides suggests a number of possible intermediates, many

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of which have been actually isolated from animal tissues. Glycerol phosphate is probably present in all tissues, although the fraction generally indicated as "glycerol phosphate" probably includes several different compounds. Phosphoryl ethanolamine, found first by Outhausen in tumors (110) and later by Colowick and Cori in pig and rabbit intestine (54), has been shown now to be present in most tissues of rats and in several human tumors (Awapara et al., 34). Similar findings were also reported recently in rat brain (Ansell and Dawson, 3) and in pig liver (Campbell and Work, 47).

Phosphoryl choline, first isolated in small amounts from the liver (Inukai and Nakahara, 88), is apparently present in large amounts in seminal fluid (Lundquist, 101). Lignoceryl sphingosine (Fränkel and Bielschowsky, 76; Tropp and Widersheim, 144) and sphingosine choline phosphate (Booth, 43; King and Small, 93) were characterized in several tissues. Glyceryl phosphoryl ethanolamine was recently isolated in many tissues by Campbell and Work (47) and by Walker (150). Paper chromatography has often been used by the recent workers, and it may be expected that a wider application of this method will show that these and other intermediates are constantly present in all tissues. I should like to mention, however, that according to Campbell and Work (47) glyceryl phosphoryl ethanolamine runs together with citrulline in two-dimensional chromatograms with the solvents most commonly used, and could be separated only by treatment with additional reagents. The chromatographic similarity between two substances with such different structures suggests a note of caution against too ready an acceptance of chromatographic evidence in the identification of unknown substances.

Hydrolytic Enzymes. Obviously the presence in the tissues of such intermediates as the above can be ascribed to hydrolytic as well as to synthetic processes. Indeed, evidence for the existence in many biological materials of a variety of enzymes which may split the phospholipide molecule in various ways has increased rapidly. A schematic representation of the action of these enzymes is outlined in Table 3. Except for the substitution of the more general term of "phospholipases" for "lecithinase," I have retained the no-

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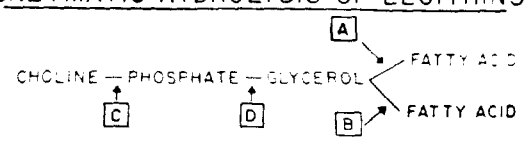
menclature of Contardi and Ercoli, who postulated the existence of specific enzymes acting on each of the four ester linkages in the lecithins (55). Since the literature on this subject is adequately covered in recent reviews (Zeller, 156; Wittcoff, 155), only a few of the latest developments will be mentioned here.

Phospholipase A was first described in cobra venom, but it is now known to be present in the gastrointestinal tract and in most animal tissues together with phospholipase B. Separation from the latter is easily attained because of its high stability against inactivating agents, such as heat and organic solvents. The recent data of

TABLE 3

ENZYMATIC HYDROLYSIS OF LECITHINS



ENZYME	SUBSTRATE	PRODUCTS
Phospholipase A	Lecithin	Lysolecithin + Fatty Acid
» B	Lysolecithin	Glyceryl phosphoryl choline + Fatty Acid
» C	Lecithin	Phosphatidic Acid + Choline
» D	Lecithin	Phosphoryl choline + Diglyceride

Hanahan (84) on the phospholipase A of pancreatic extracts suggest the formation of an enzyme-substrate complex soluble in ethyl ether, where the reaction proceeds unimpaired. In agreement with the findings of the earlier workers, only unsaturated fatty acids seem to be released. However, several years ago King (92) showed that totally hydrogenated lecithin is digested by extracts of intestinal mucosa as fast as the natural lecithin. Very recently, Zeller (157) has reported the ready formation of lysolecithin by the action of snake venoms on synthetic L-α-dimyristoyl lecithin, and has postulated that the decisive factor is the position α or β of the linkage between glycerol and fatty acid, a point which of course implies that in the natural lecithins the unsaturated acid is located preferentially in one of these two positions. A fairly pure prepara-

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tion of a phospholipase B, very active on lysophospholipides, but not on intact phospholipides, was described by Fairbairn in extracts of *Penicillium notatum* (67). Splitting of both fatty acids by the combined action of phospholipases A and B with formation of glyceryl phosphoryl choline and glyceryl phosphoryl ethanolamine is possibly the major pathway for the breakdown of lecithins and cephalins in tissues, if the same pattern is followed in vivo as in autolyzing tissues (Artom, 2; Schmidt et al., 125). In these, liberation of choline is somewhat slower, and it remains uncertain whether the actual substrate for such a reaction is glyceryl phosphoryl choline, or the intact phospholipide molecule (Fairbairn, 66).

Unequivocal evidence for the existence of a true phospholipase C that forms phosphatidic acids has been obtained only in plant tissues (Hanahan and Chaikoff, 85). Likewise, a phospholipase D was demonstrated with certainty only in the toxin elaborated by *Clostridium welchii* (Macfarlane and Knight, 103), by *Clostridium oedematiens* (Macfarlane, 102), and more recently in the venom of a South American snake (Vidal Breard and Elias, 147). However, liver extracts liberate lignoceryl sphingosine from sphingomyelins (Thannhauser and Reichel, 142). It is probable that a more careful search for such enzymes will reveal their presence in many animal tissues and thus indicate that phosphatidic acids, phosphoryl choline, phosphoryl ethanolamine, sphingosine-fatty acid amides, and diglycerides may all be formed by direct splitting of phospholipides. It should be pointed out also that several of the phospholipases exhibit marked differences in their ability to attack the various phospholipides. Thus the phospholipase D of *Clostridium welchii* splits lecithins and sphingomyelins, but not cephalins (Macfarlane, 102).

Role in the synthesis of phospholipides: a) *Experiments in vivo.* In the experiments of Chargaff and Keston (52) and of Riley (120), phosphoryl ethanolamine or phosphoryl choline, respectively, was labeled in the phosphate moiety and injected. Both esters were extensively split, and the concentrations of P^{32} in the lipides were no greater than those found in parallel experiments in which the isotope had been introduced as inorganic phosphate. Moreover,

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when the radioactivity was determined separately in the lecithins and cephalins, the differences in the specific activities of these fractions were just opposite to those which should have been expected if the ester had been utilized as a unit. However, the hypothesis of a role of phosphoryl ethanolamine in the synthesis of brain cephalins is compatible with the data of Ansell and Dawson (3). These authors showed that minced tissue incubated with radioactive phosphate synthesize phosphoryl ethanolamine. When isotopic phosphate was injected intracysternally into the brain of intact rats, the specific activity of the phosphoryl ethanolamine P (isolated by paper chromatography) was many times greater than the specific activity of the lipide P.

Evidence that formation of glycerol phosphate in vivo is an initial step in the synthesis of phospholipides is more abundant, but still somewhat presumptive.

The concentrations of P^{32} in the tissue lipides are essentially the same, whether the isotope has been injected as phosphate or as glycerol phosphate (Zilversmit et al., 160) and whether the labeled phosphate was injected alone or together with large amounts of non-isotopic glycerol phosphate (Artom and Swanson, 27). On the other hand, after introduction of isotopic phosphate into birds (Flock and Bollman, 71) or into rats (Artom and Swanson, 30), the gradients of the specific activity of the inorganic P, glycerol phosphate P, and lipide P of the liver were compatible with the assumption that glycerol phosphate is an intermediate in the synthesis of phospholipides. In more extensive experiments, Zilversmit et al. (160) and Popják and Muir (115) have compared the specific activity time curves of various phosphorus fractions in the liver of rats which had received isotopic phosphate. The behavior of the lipide and glycerol phosphate P curves corresponded to what one might theoretically ascribe to the specific activity time curves of a compound and of its immediate precursor (Zilversmit et al., 161). Actually, this relationship merely indicates that glycerol phosphate *can be* the immediate precursor of the phospholipides, but does not necessarily prove that *it is* such a precursor. Moreover, as mentioned above, the reliability of the specific activity values of the glycerol

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phosphate fraction may be questioned. Two recent papers reemphasize the heterogeneity of fractions separated from the extract of the liver and believed to be relatively pure glycerol phosphate (Olley and Blewett, 109) or ATP (Ennor and Rosenberg, 64). It should be pointed out that phosphoryl ethanolamine and phosphoryl choline would probably be included in the fractions which, in the experiments mentioned above, were regarded as glycerol phosphate.

b) *Experiments in vitro.* In spite of their apparently greater simplicity, the experiments in which glycerol phosphate (Taurog, 138; Popják and Muir, 115), or phosphoryl choline (Taurog, 138) labeled with P^{32} were added in vitro to tissue slices, have given only inconclusive results. In a few experiments in which we incubated liver slices with isotopic phosphate and variable amounts of inactive glycerol phosphate, the specific activity of the phospholipide P varied to a much less extent than would have been expected had glycerol phosphate been an intermediate in the synthesis (Artom and Swanson, 31). The significance of results of this type is obviously restricted by the possibility that glycerol phosphate did not permeate the cell membrane and therefore could not reach the cell structures where the synthesis of phospholipides probably occurs (Popják and Muir, 115). Liver slices were used by us and by others, because the earlier data of Chaikoff's group had indicated that homogenization of the tissue suppresses (in liver) or markedly reduces (in kidney or brain) the incorporation of P^{32} into the phospholipides (69, 81, 139).

Later, however, Friedkin and Lehninger (82) showed that cell-free preparations of liver mitochondria actively synthesize phospholipides from inorganic phosphate, provided that ATP and malate are added. Such fortified systems were used in the experiments reported recently by Kennedy (90). Addition of glycerol markedly enhanced the incorporation of P^{32}-phosphate into the phospholipides. The effects of the addition of inactive glycerol phosphate corresponded closely to those expected from the isotope dilution of the synthesized glycerol phosphate (or of a compound in equilibrium with glycerol phosphate). In spite of somewhat conflicting evidence, it appears

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tion. Thus in the experiments of Pihl and Bloch (112), when liver slices were incubated with C^{14} acetate, the concentration of the isotope was higher in the triglycerides than in the phospholipides. A similar relationship was found in the livers of intact rats receiving C^{14} palmitic acid (Volk et al., 149) (but not in rabbits fed C^{14} acetate (Popják and Beeckmans, 114)).

Formation and Interconversion of Individual Phospholipides

The widespread occurrence of enzymes which attack specifically one or another of the various linkages of phospholipides and the demonstration that at least in vitro some of the products of partial hydrolysis can be used as units in the synthesis of phospholipides suggest some interesting possibilities. For instance, if glycerol phosphate or phosphatidic acids are first formed, then choline and ethanolamine might compete with each other for these common precursors in the final stages of the synthesis of lecithins and cephalins. This would explain those rapid changes in the relative amounts and in the rates of formation of these phospholipides which have been observed in the liver after administration of single large doses of choline (Entenman et al., 65), of ethanolamine (Artom and Cornatzer, 12; Platt and Porter, 113), or of probable precursors (Artom and Cornatzer, 16) and analogs of these nitrogenous components (Artom, Crowder, and Cornatzer, 15). Some of our data are exemplified in Fig. 3.

Combination of partial hydrolysis and resynthesis could also represent a mechanism for the conversion of individual phospholipides into each other. The results obtained by Popják and Muir (115) after intravenous injection of lecithins or of cephalins, labeled with P^{32}, are of interest in this respect. In the livers of these animals the specific activities of both cephalins and lecithins were always higher than the specific activities of the inorganic and of the glycerol phosphate P. These findings obviously suggest a formation of lecithins from the injected cephalins, and vice versa, with only a partial breakdown of the phospholipides injected.

By a similar mechanism one might even visualize the interconver-

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that mitochondria are osmotically active systems and that their membrane-like barrier is impermeable to glycerol phosphate (Berthet et al., 38). To explain the discrepancy in the results of isotope dilution experiments on liver slices (Artom and Swanson, 31) or mitochondria (80), one may postulate either that the synthesis of phospholipides occurs at the interface between the granules and the surrounding medium, or that the mitochondrial membrane is easily damaged during incubation, with a consequent increase in its permeability.

In another very recent report Kornberg and Pricer (94) used aqueous extracts of liver, fractionated with methanol at low temperature. Glycerol phosphate labeled with P^{32} is rapidly converted by these extracts into a compound which was not completely characterized. However, a tentative identification with a phosphatidic acid is suggested by its solubility in ether and acetone and by the increased rate of its synthesis when long-chain fatty acids were added to the system. The same preparations, when incubated with phosphoryl choline labeled with C^{14}, synthesized products which were soluble in organic solvents and which contained 10 times more isotope than after incubation with C^{14}-labeled choline. When phosphoryl choline labeled in both moieties was added, the ratio of P^{32} to C^{14} in the reaction product approximated the ratio of these isotopes in the substrate. Coenzyme A and especially ATP markedly enhanced the process.

These two recent investigations supply the first unequivocal evidence for a role of glycerol phosphate and phosphoryl choline as intermediates in the synthesis of phospholipides. Moreover, from the data of Kornberg and Pricer, it would appear that the sequence of the reactions is not necessarily the same, and that, even in the same tissue, alternate pathways are open. One of these pathways would start with the formation of glycerol phosphate, the other with the formation of phosphoryl choline and perhaps phosphoryl ethanolamine also. Combination of the two last compounds with a diglyceride might lead directly to the complete molecule of lecithins and cephalins. While on this point no direct evidence is yet available, some data in the recent literature are compatible with this assump-

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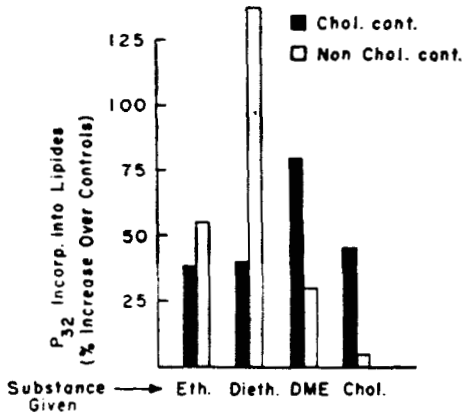
sion of lipides with different structures, such as sphingomyelins and cerebrosides (Reichel and Thannhauser, 117), or cholesterol esters and phospholipides. Somewhat questionable evidence for the occurrence of the latter conversion during the incubation of blood plasma has been presented recently (Le Breton and Pantaleon, 99).

These views necessarily imply that the breakdown as well as the formation of one ester linkage in the phospholipide molecule may occur independently of the breakdown and formation of the re-



Effects of a single dose of ethanolamine (Eth.), diethanolamine (Dieth.), dimethylethanolamine (DME) and choline (Chol.) — Rats on a 5% casein diet.

FIG. 3. Incorporation of $H_3P^{32}O_4$ into the choline-containing and non-choline-containing phospholipides of the liver.

maining linkages. Weinman et al. (153) have injected the plasma of a donor dog, containing phospholipides doubly labeled with P^{32} in the phosphate and with C^{14} in the fatty acids, and have then determined the rate of their disappearance from the circulation of the recipient dog. This rate was identical for both the isotopic labels. Tolbert and Okey (143) compared the specific activity time curves of the lipide phosphorus and choline carbon in the liver of rats simultaneously injected with P^{32}-labeled phosphate and C^{14}-methyl-labeled choline, and from the similarity of these curves they inferred that the turnover rate of the two phospholipide moieties was approximately the same. Because of the complications and uncertainties in such calculations the significance of this conclusion may be questioned. However, it seems unlikely to me that, under physio-

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logical conditions, the various parts of the phospholipide molecule are renewed at markedly different rates. A relative independence in the turnover of these moieties should probably be detectable only under conditions which may accelerate (or inhibit) the formation or the breakdown of only one of the linkages.

Our experiments on the synthesis in vitro of phospholipides have given some suggestive indications on this point. Liver slices from rats on high protein diets incorporate labeled phosphate into the phospholipides much more actively than liver slices of rats on low protein diets (Artom and Swanson, 32). On the contrary, the incorporation of labeled choline into the phospholipides by liver slices of rats on a high protein diet was lower than that observed with

TABLE 4

Incorporation of Labeled Phosphate and Choline into the Lipides
of Slices from Liver of Rats on Various Diet +

Labeled Compound	No of exp	High Protein Diets γ	Low Protein Diets γ
H_3PO_4	11	11 9	2 9
Choline	17	3 6	15 3

+ 3½ h incubation in air – Average values for 1 g tissue

slices from rats on low protein diets (Artom, Crowder, and Swanson, 20) (Table 4). Confirming Taurog et al. (139), we also found that the incorporation of phosphate into the lipides of liver slices was inhibited as much as 90 per cent by the absence of oxygen. When in our experiments, nitrogen was substituted for oxygen or air, the incorporation of choline into the phospholipides was never decreased and often was greater than in aerobic conditions. Table 5 illustrates the results of one experiment in which some slices from the same liver were incubated with labeled phosphate and others with labeled choline.

Homogenization of the liver completely suppresses the incorporation of labeled phosphate, whereas the incorporation of choline is less extensive but still quite appreciable in liver homogenates (Table 6). The addition of cyanide or azide to liver slices causes a very

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considerable inhibition in the incorporation of phosphate (159). Choline incorporation into the phospholipides is also depressed by these substances (as well as by dinitrophenol), but apparently to a much lesser extent (Artom, Crowder, and Swanson, 21).

The results of experiments in which C^{14} methyl-labeled dimethyl-

TABLE 5

Incorporation of Labeled Phosphate and Choline into the Lipides of Liver Slices in O_2 or N_2 *

Labeled Compound	Oxygen % X 10^2	Nitrogen % X 10^2
H_3PO	13	0.5
Choline	78	270

* 3½ Incubation – Values for 1 g. tissue
Rat on stock diet

TABLE 6

Incorporation of Labeled Phosphate and Choline into the Lipides of Liver Slices or Homogenates *

Labeled Compound	Slices % X 10^2	Homogenates % X 10^2
H_3PO_4	36	0
Choline	157	45

* 3½ h. incubation in air – Values for 1 g. tissue.

ethanolamine was incubated with liver slices (Crowder and Artom, 58) parallel those obtained with labeled choline. The apparent uptake of DME by the lipides is also greater with liver slices from rats on a low protein diet than from rats on a stock diet (Table 7). Homogenization of the tissues reduces but does not suppress this uptake. With either slices or homogenates the amounts of DME incorporated into the lipides are greater in nitrogen than in oxygen (Table 8). It was mentioned previously that unlike choline, DME is not readily oxidized to dimethyl glycine by the isolated tissue.

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Accordingly, the similarity in the results obtained in the experiments with labeled choline and labeled DME strongly suggests that variations in the activity of choline dehydrogenase are not, or are only partly, responsible for the observed differences in the incorporation

TABLE 7

INCORPORATION OF DME IN THE LIPIDES
EFFECT OF PREVIOUS DIET[1]

Previous Diet	Days on Diet	Neutral Fat in Liver (mg /g)	Radioactivity in Lipides[2]	
			Before Hydrolysis	DME
STOCK		12 16	64 37	45 26
Low Protein	10	40	79	68
Low Protein + GAA[3]	7 7 17	38 41 95	133 138 139	102 116 88

1 Liver slices – 3½ hours incubation in air
2 % of counts added x 10^2, per g of tissue
3 Guanido acetic acid, 0.5 %

TABLE 8

INCORPORATION OF DME IN THE LIPIDES OF RAT LIVER
SLICES OR HOMOGENATES IN AIR OR N_2[1]

	Gas Phase	Radioactivity in Lipides[2]			
		Before Hydrolysis (a)	After Hydrolysis		
			DME (b)	Choline (c)	Recovery $\frac{b+c}{a}$ x 100 %
SLICES	Air N_2	50 310	35 233	4 26	78 84
HOMOGENATES	Air N_2	16 26	13 18	4 2	106 77

1 Rats on stock diet – 3½ hours incubation
2 % of counts added x 10^2, per g of tissue

of choline into the lipides under various conditions. It seems reasonable to conclude that the rate of synthesis of the various linkages in the lecithin molecule is modified in a different manner by a number of factors. While the incorporation of choline and DME probably also requires energy, the requirements are apparently much lower than those needed for the synthesis of the glycerol phosphate linkage. If this assumption is correct, one might expect that the reactions

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involved in the combination between the nitrogenous components and the phosphatidic acid radical would be easily reversed, and that therefore the composition of the phospholipide mixture at equilibrium would be markedly affected by changes in the concentration of the reacting substances within the cell.

TABLE 9

INCORPORATION OF DME OR CHOLINE IN THE LIPIDES
EFFECTS OF ADDING INACTIVE CHOLINE OR DME [1]

Previous Diet	C^{14} added as	MicroM of Substance		Radioactivity in Lipides [2]
		DME	Choline	
STOCK	DME	15	None	66
	"	15	20	33
5% Casein + GAA[3] (7 Days)	Choline	None	2.5	134
	"	30	2.5	52
	DME	30	2.5	46
	Choline	None	32.5	24

1 Liver slices – 3½ hours incubation in air

2 % of counts added X 10^2, per g of tissue

3 Guanido acetic acid, 0.5 %

TABLE 10

Effects of Substances added "in vitro" on the Incorporation of Choline in the Lipides of Rat Liver Slices *

Substance Added [illegible]	Relative amounts of C^{14}-labeled choline incorporated in the lipides
None	100
Ethanolamine	47
Diethanolamine	32
Methylethanolamine	30
Dimethylethanolamine	36
Triethylcholine	56
Thiocholine	106
Betaine	134 (?)
Serine	74
Glycine	83
Trimethylamine	101

* 3½ hour incubation in O_2 – Rats on stock diet.

Indeed, the addition in vitro of DME inhibits the incorporation of labeled choline into the phospholipides, and conversely the addition of inactive choline inhibits the incorporation of labeled DME (Table 9). The effects of a number of other compounds on the incorporation of C^{14}-choline in vitro are summarized in Table 10.

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It is apparent that this process is markedly inhibited by substances which are either natural components of the phospholipides, such as ethanolamine, or by precursors of these components, such as methyl ethanolamine or DME, or by chemical analogs, such as triethylcholine or diethanolamine, which may substitute for the natural components in the phospholipide molecule. The only discrepant results in this respect are those obtained with the addition of thiocholine, which in our experiments was totally ineffective. No inhibition was observed by substances, such as betaine or trimethylamine, which are not utilized directly in the synthesis of phospholipides.

Rate of Phospholipide Formation in Various Tissues

The earlier work in which radioactive phosphate was introduced into intact animals showed characteristic differences in the amounts and concentrations of the isotope present in the phospholipides of the various tissues. Obviously a number of factors may have been responsible for these differences, such as, for instance, differences in the rate of transfer of inorganic phosphate from the plasma to the intercellular fluids and from these fluids to the cells. Indeed, when the specific activities of the lipide P were expressed as relative specific activities, that is, as a function of the specific activity of the inorganic phosphate, some of the differences became less marked. This is especially true for the brain, because of the low permeability of the so-called blood-brain barrier. However, the general arrangement of the values remained essentially unchanged. Moreover, when instead of phosphate the fatty acid or the nitrogenous moiety of the phospholipides was labeled, the various tissues arranged themselves in the same order (Table 11). There is, therefore, little doubt that the observed differences are due primarily to differences in the rate of phospholipide formation. No relationship is clearly apparent between these differences and the differences in the amounts of total phospholipides or in the oxygen consumption (as determined in the isolated tissues). On the other hand, our early data showed a rough parallelism between the relative specific activi-

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ties of the lipide P and those of the acid-insoluble P (Artom et al., 27) (Table 12). It would be of some interest to reinvestigate the possibility of a correlation between the rate of phospholipide formation and that of the synthesis of ribonucleic acid in the various tissues.

TABLE 11

ISOTOPE CONCENTRATIONS IN THE TISSUE PHOSPHOLIPIDES OF RATS RECEIVING LABELED PHOSPHATE, FATTY ACIDS, OR CHOLINE

TISSUE	Compound and Isotope Given		
	H_3PO_4 (a) (P_{32})	Palmitic acid (b) (C^{14})	Choline (c) (C^{14})
Liver	100	100	100
Small Intestine	96	38	88
Kidney	57	22	73
Other Internal Organs	32	(Lung) 46	(Spleen) 59
Heart	27	18	19
Skel. Muscle	9	11	
Brain	3		5

(a) Artom et al. (1938)
(b) Goldman et al. (1950)
(c) Tolbert and Okey (1952)

TABLE 12

COMPARISON OF SOME VALUES DETERMINED IN VARIOUS RAT TISSUES

TISSUE	Rel. Spec. Act.		Total Phospholipides (micro M per g.)	Q_{O_2}
	Lipide P	Acid-ins. P		
	(a)	(b)	(c)	(d)
Liver	100	100	100	17
Small Intestine	97	83	91	
Kidney	77	52	84	38
Other Int. Organs	61	60	56	9 – 13
Skel. Muscle	19	53	33	
Brain	13	43	165	26

(a) (b) From Artom, C. et al. (1937)
(c) From Artom, C. (1945)
(d) From Krebs, H. A. C. (1950)

It seems likely that the differences in the rate of phospholipide formation observed in vivo are due not to differences in the amounts of specific enzymes present in each tissue but rather to regulatory mechanisms (perhaps of a hormonic nature) which maintain the rate of the process at a level more or less characteristic of each tissue.

On the other hand, in a given tissue changes will presumably occur in the amounts of phospholipides or in the rate of their synthe-

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sis when the metabolic needs of the tissue are increased or decreased, or when the supply of materials needed for the synthesis of phospholipides becomes insufficient, or when the efficiency of the regulatory mechanism is impaired. Many years ago we observed a decrease in the phospholipide level of the liver after removal of the thyroid or of the ovaries (Artom, 5; Artom and Marziani, 24). More recently, Flock et al. (73) have found that in rats the turnover of liver phospholipides is increased by thyroxine administration and decreased by thiouracil. Similar findings were reported by us (Cornatzer and Artom, 56). Variations in the phospholipide content of the corpus luteum were noted, which seemed to correlate with the changes in the physiological activity of that organ (Bloor et al., 41; Weinhouse and Brewer, 151). However, much of the available evidence on this subject has been obtained in investigations aiming to test the hypothesis that choline is lipotropic because it promotes the formation of phospholipides in the liver. I have recently had the opportunity of reviewing this topic (Artom, 10), and some of these findings will be mentioned later in connection with the postulated role of phospholipides in fat metabolism. Here I shall merely point out that choline deficiency is only one, and probably not the most important, factor for the effects of low protein diets. In the liver of rats on such diets the following changes have been noted: (a) a considerable increase in neutral fats; (b) a moderate decrease in total phospholipides; (c) a more marked decrease in the lecithins (Artom and Fishman, 22; Fishman and Artom, 70). The fatty infiltration is always easily prevented or reversed by supplementation of the diet with choline. The decreases in the liver lecithins are prevented by choline, but only under certain experimental conditions and even under these conditions total phospholipides remain definitely below the level found in animals on adequate diets. As an example, the results of our experiments in which choline was added to low protein diets with a variable fat content are reported in Table 13. Actually the decreases in the total phospholipides in the liver parallel the decreases in total proteins, as indicated by the constancy of the ratio of lipide P to protein N (Artom, 9) (Table 14). The same conclusion has been reached by Campbell and Kosterlitz, who have used

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as a reference standard the values of desoxyribonucleic acid in the liver (46).

Attempts to demonstrate changes in the rate of phospholipide formation in the livers of animals fed low protein diets were made

TABLE 13

EFFECT OF DIETARY FAT AND CHOLINE ON LIVER LIPIDES

DIET			LIVER LIPIDES*			
	% FAT	CHOLINE ADDED	TOTAL PH LIP mg	CHOLINE PH LIP mg	CHOLINE PH LIP % of TOTAL	NEUTRAL FAT mg
10 % CASEIN	0-10	—	23.5	12.1	52	135
	"	+	23.7	14.8	62	13
	20-40	—	23.7	12.7	54	199
	"	+	26.0	20.0	77	34
STOCK	5	—	31.5	19.5	62	9

* Values per g. of lipide-free tissue

(Fishman and Artom, 1946)

TABLE 14

PHOSPHOLIPIDE AND PROTEIN CONTENT OF RAT LIVER

DIET	Total Phospholipides mg/g	Ratio $\frac{\text{Lipide P}}{\text{Protein N}} \times 100$
5 % Casein + GAA*	20.0	18.7 (14.7 — 24.7)
5 % Casein + Choline	22.3	19.7 (15.1 — 24.4)
Stock	31.8	19.0 (15.1 — 24.2)

* GAA = 1% guanidoacetic acid added to the diet.

by several groups of workers, with results which are in substantial agreement (Bollman and Flock, 42; Artom and Cornatzer, 14; Campbell and Kosterlitz, 45). In the livers of our rats on low protein diets the specific activities were higher than in the controls on the stock or on a high protein diet, but the total radioactivity calculated for the whole liver of a 100 g. rat was approximately the same in all groups. This is also true if the values are expressed as relative specific activities and relative radioactivities (that is, if the

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data are corrected for the slight variations in the specific activity of the inorganic P). Moreover, these results, obtained in protein-depleted animals, closely resemble those of Flock and Bollman on rats partially hepatectomized or poisoned with CCl_4 (72).

It seems reasonable to conclude that in the livers of intact animals the amount of total phospholipides newly formed in a given time interval is not markedly affected by a reduction in the size of the organ, in the amount of cytoplasmic materials, or in the functional capacity of the cells, such a reduction being probably compensated by an increased rate of phospholipide synthesis in the remaining tissue. The higher specific activity of the lipide P in the damaged or protein-depleted liver is probably the expression of this compensatory hyper-activity. According to the recent data of Singal et al. (130), in the liver of protein-depleted animals these increases in the specific activity occur in both the large and small granules, but are more marked in the latter fraction.

On the other hand, in our experiments with liver slices the incorporation of phosphate into the phospholipides was considerably depressed when the animals had been previously maintained on a low protein diet (Artom and Swanson, 32). This apparent discrepancy between experiments in vivo or in vitro can be reconciled by the assumption mentioned earlier that in the livers of intact animals the rate of phospholipide synthesis is maintained at a level adequate to the metabolic needs of the tissue. Because of the large functional reserve of the liver, in intact animals such a level remains unchanged even under very adverse conditions. On the other hand, in isolated tissues where regulating mechanisms are absent or less effective, phospholipides are probably formed at the highest speed compatible with the condition of the experiment. The decreased formation of phospholipides in liver slices from rats on low protein diets would then be the direct result of the decrease in cytoplasmic materials (especially enzyme proteins) which develops rapidly under these conditions.

At any rate, it appears that in the livers of animals on diets which contain little protein and no preformed choline, choline and ethanolamine are still available in amounts sufficient for the synthesis of

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phospholipides at a rate about normal. This statement appears today less surprising than at the time those observations were made, since much direct evidence is now available for the ability of the rat to synthesize methyl groups from various sources.

Furthermore, separate determinations of the concentrations of P^{32} in the choline-containing and non-choline-containing fractions of the liver of protein-depleted animals did not show any preferential

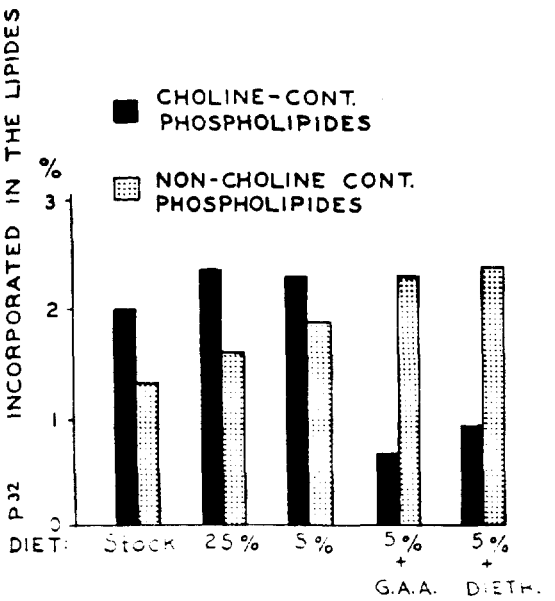

FIG. 4. Incorporation of $H_3P^{32}O_4$ in the choline-containing and non-choline-containing phospholipides of the liver of rats on various diets.
25% = 25% casein diet.
5% = 5% casein diet.
5% + GAA = 5% casein diet with added guanidoacetic acid 0.5%.
5% + Dieth = 5% casein diet with added diethanolamine 0.5%.

change in the rate of formation of either fraction. The only experiments in which the specific activity of the lecithin P was markedly decreased were those in which guanidoacetic acid or diethanolamine had been added to the low protein diet (Artom and Cornatzer, 14) (Fig. 4). The first of these two substances probably drains the available methyls for creatine synthesis (Stetten and Grail, 104), the second possibly acts as a metabolic antagonist of ethanolamine and thus may impair its role of methyl acceptor in the synthesis of choline (Artom, Cornatzer, and Crowder, 15). It seems therefore

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that the synthesis of phospholipides has a high priority among the various metabolic processes in which choline is involved, including the lipotropic effect. To obtain a well detectable reduction in lecithin formation one has to superimpose upon the dietary restriction of choline or choline precursors, the administration of substances which interfere specifically with the synthesis or availability of choline. The above considerations also suggest that the increased phospholipide turnover after administration of a large dose of choline, ethanolamine, or related substances (Cornatzer and Artom, 57) cannot be interpreted merely as the replacement of a deficiency. Rather, one is tempted to ascribe this effect to a stimulation of phospholipide synthesis by the high concentration of the substance administered in the liver cells.

Functions of Phospholipides

The large amounts of phospholipides present in certain formations, such as seeds of leguminous plants and egg yolk, almost certainly represent reserve materials from which the growing organism draws phosphate and fatty acids. It might be pointed out in this respect that in rats and mice the total body content of phospholipides is highest at birth and decreases to about one-half during the first three months of postnatal development (Sinclair, 128). It has been speculated also that the lecithins and sphingomyelins which are so abundant in the nervous tissue might represent a readily available source of the choline needed for the synthesis of acetylcholine in that tissue. Finally, the importance of certain phospholipides as the components of the lipoprotein involved in the coagulation of blood is generally recognized.

Aside from these few peculiar examples, three main functions have been proposed for phospholipides: first, that these compounds are structural materials upon which the characteristic properties of the living cell (including permeability phenomena) are dependent; second, that they are involved in the biological oxidations; and third, that they represent intermediate compounds in the absorption, transport, and metabolism of fatty acids. I will discuss here in some detail only the evidence pertaining to the last mentioned theory.

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Fat Absorption

O. Franck first suggested in 1898 that the glycerides of the intestinal lymph may originate by interaction of the soaps absorbed and the lecithin of the intestinal mucosa (75). In 1929, R. G. Sinclair found that in the intestines of rats receiving highly unsaturated fats, while the amounts of phospholipides remained constant, their iodine number markedly increased (127). Our experiments with iodized fats provided even more direct evidence for the rapid incorporation of ingested fatty acids into the phospholipides of the intestinal mucosa (Artom and Peretti, 25). These results and those of later investigations from several laboratories in which variously labeled fatty acids, radioactive phosphate, and N^{15}- or C^{14}-labeled choline were used, definitely indicate that phospholipides are actively formed from their building stones in the intestinal mucosa. Actually, as mentioned earlier, the small intestine and the liver are the most active tissues in this process, but this is true in both animals absorbing fats and in animals not absorbing fats.

A more suggestive indication for a role of phospholipides in the absorption of fats was supplied by the finding that the incorporation of P^{32} into the intestinal lipides was greater in animals fed diets rich in fats than in those fed fat-free diets (Artom et al., 29). Similar results were obtained more recently by Schmidt-Nielsen, who compared the uptake of P^{32} by the phospholipides of alternate segments of rat intestine absorbing or not absorbing fat, respectively (126). On the other hand, Zilversmit et al. (159) failed to find significant differences in the synthesis of phospholipides by the small intestine of fat-fed and control dogs. It may be pointed out, however, that the results obtained by these authors in similar experiments on rats seem to be in general agreement with the findings of previous workers. Recently, Favarger (68) has stated that during the absorption of fat the turnover of phospholipides, especially of the choline-containing fraction, is more active in the epithelial layer, which represents but a small fraction of the total mucosa—a consideration which might perhaps account for some of the discrepancies noted above.

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A few years ago we showed that in rats fed low protein diets choline stimulated the incorporation of P^{32} into the phospholipides of the intestinal mucosa and that this effect was enhanced when fat was simultaneously administered (Table 15). Since little or no stimulation was observed in such animals when fat alone was given, we suggested that the supply of choline (or choline precursors) may represent a limiting factor in the formation of phospholipides during fat absorption (Artom and Cornatzer, 13). However, it should be pointed out that the increases in phospholipide turnover of the intes-

TABLE 15

PHOSPHATE INCORPORATION INTO THE PHOSPHOLIPIDES OF RAT SMALL INTESTINE *

SUBSTANCES GIVEN**	TOTAL RADIOACTIVITY	SPECIFIC ACTIVITY
H_2O	100	00
Choline	136	125
Choline + Oil	187	46
Oil	147	104

*Rats on a 5% casein diet for 7 days, killed 6 hours after injection of $H_3P^{32}O_4$

**By stomach tube, 5 mins before injecting $H_3P^{32}O_4$

(Artom and Cornatzer, 1947)

tine of animals receiving choline and fat, while quite significant, are still much below the range which one should expect if all of the absorbed fatty acids were converted into phospholipides.

Several authors have described increases in the lipide P of the lymph of the intestine or of the thoracic duct during the absorption of fats. In rabbits these increases are apparently quite marked and consistent (Süllman and Wilbrandt, 136), whereas in dogs the changes are irregular and often minimal (Eckstein, 63; Freeman and Friedmann, 80). Our analyses of the lipides in the thoracic lymph of dogs absorbing fats showed that the figures for the lipide P included a large proportion of compounds other than true phospholipides. Moreover, even on the basis of the lipide P values, it was apparent that the increases over the corresponding values in fasting animals accounted for only a very small fraction of the increases in the total fatty acids. Thus while the total fatty acids increased 10 or 20 times during fat absorption, the lipide P was only doubled

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(Artom and Peretti, 26). Recently, Bloom et al. (39) have found that after feeding labeled palmitic acids to rats as much as 96 per cent of the labeled compound was present in the lymph in forms other than phospholipides. The results obtained by Flock et al. on the intestinal lymph of rats receiving P^{32} definitely indicate that much of the newly formed phospholipide actually originated in the intestine (120). However, a quantitative estimation of this portion seems difficult, since a large proportion of the labeled phospholipides injected into the circulation can be recovered in the lymph of the thoracic duct (Reinhardt et al., 118).

As for the increases in plasma phospholipides which occur after a fatty meal, it would appear from the calculations of Hevesy and Lundsgaard (87), that only a small fraction of the newly formed phospholipides could have been derived from the intestinal mucosa. The possibility remains, of course, that phospholipides synthesized in the intestine are added to the blood of the portal systems and are taken up by the liver before they reach the general circulation. Without attempting to discuss here the much debated problem of the respective roles of the lymphatic and venous pathways for the absorbed fatty acids, I mention only that the latest experiments of Chaikoff's group with labeled palmitic acid or tripalmitin strongly suggest that only very small amounts of the fatty acid are absorbed through the portal route (Bloom et al., 40). Years ago, Verzàr and Laszt showed that fat absorption was inhibited by the administration of monoiodoacetate or phlorhizin or by removal of the adrenals (146). Since in all these conditions a number of reactions involving the synthesis of phosphate esters was also inhibited, it was inferred that phospholipide formation is a necessary step in fat absorption. From later reports, it would appear that fat absorption is inhibited by phlorhizin or by adrenalectomy only to a slight or questionable degree (Sarzana, 123; Barnes et al., 36; Stillman et al., 135; Schmidt-Nielsen, 126). On the other hand, the concentrations of iodoacetate used by Verzàr and Laszt were such that the metabolism of many compounds other than phospholipides would also be inhibited. A marked impairment in the functional capacity of the cells and therefore probably also in fat absorption would thus result, whether

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or not phospholipides are directly involved in the absorption of fat. It seems that evidence of this type could be acceptable only if and when some conditions are found in which fat absorption and phospholipide turnover are inhibited to exactly the same degree, and no other important metabolic reaction is affected (Artom, 8).

Perhaps of greater significance is the other observation of Verzàr and Laszt (146) that fat absorption was increased when glycerol phosphate, or a mixture of glycerol and phosphate, was administered together with the fat. These findings, have been confirmed by Cera and Bellini (49), and more recently by Frazer (77), who has stated that choline also increases by as much as 50 per cent the absorption of fat. Likewise, Tidwell (140) and Tidwell and Nagler (141) found a higher rate of absorption of the fat given with choline and ascribed the negative results of Shoshkes et al. (131) to the use of too large amounts of choline which may depress fat absorption. In the experiments of Tidwell and Nagler fat absorption was decreased in rats previously maintained on low protein diets, but the values were raised to normal when choline was given. These data are obviously suggestive, when compared with those mentioned above on the increased phospholipide turnover in the intestines of choline-deficient rats receiving choline and fat (Artom and Cornatzer, 134). It has been reported also that administration of unsplit lecithin increases the rate of fat absorption in humans (Adlersberg and Sobotka, 2) and animals (Augur et al., 33; Tidwell, 140).

In summary, there is at present no acceptable evidence for the concept that phospholipides are obligatory intermediates in fat absorption. On the other hand, certain findings are at least suggestive of a quantitatively less important role of phospholipides. While we can only speculate on the nature of this role, it may be pointed out that several recent reports strongly support the hypothesis that complete hydrolysis of neutral fats is not a prerequisite for their absorption.

Many years ago we have shown that in the digestion in vitro of neutral fats by the lipase of the pancreas monoglycerides and diglycerides are formed, and we suggested that, because of their greater affinity for water, these compounds could be absorbed before total

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hydrolysis (Artom and Reale, 28; Peretti, 111). Our findings were later confirmed (Frazer and Sammons, 78) and extended (Desnuelle et al., 60). In a recent study by Reiser et al. (119), synthetic triglycerides, labeled with C^{14} in the glycerol and with conjugated double bonds in the fatty acids, were fed, and the lipides of the lymph were then analyzed. From the results, it is inferred that between 25 and 45 per cent of the ingested glycerides had been completely hydrolyzed and from 55 to 75 per cent had been split to the stage of monoglycerides. Half of the phospholipides in the lymph had been synthesized from absorbed monoglycerides and the remaining half from free fatty acids. If, during fat absorption, large proportions of partially hydrolyzed glycerides are formed, one might speculate that these products combine easily with (and perhaps are later also easily split from) a relatively stable phosphoryl-choline (or phosphoryl-ethanolamine) radical. Such a mechanism (for which no evidence is yet available) could account for a very active incorporation of fatty acids into the phospholipides along with a comparatively slow turnover of the phosphate moiety. Or, possibly, the phospholipides exert a physical rather than a chemical role by favoring the passage of emulsified products across the mucosa of the small intestine.

Frazer et al. (79) have shown that extremely fine emulsions, stable between pH 6 and pH 9, are formed in mixtures of fatty acids, bile salts, and monoglycerides. In the presence of these emulsions, even paraffines are absorbed, and it seems likely that unsplit triglycerides could also cross the intestinal wall. Parenthetically, I may mention here that we have also obtained definite evidence for the direct absorption of intact phospholipide molecules (Artom and Swanson, 30).

According to either of these speculative interpretations, phospholipide formation in the intestinal mucosa would represent only an accessory factor in the absorption of fats. It is obvious, however, that in this as in other metabolic phenomena, accessory factors may become important when large amounts of materials have to be rapidly disposed of, or when the principal mechanisms are less efficient: just as secondary roads become important when the traffic is very heavy or when the main highway is blocked.

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Fatty Acid Transport

The hypothesis that phospholipides represent a more suitable form than neutral fats for the transport of fatty acids in plasma and for their transfer to the tissues is suggested by the greater affinity of the former components for water. However, phospholipides are present in plasma essentially as lipo-protein complexes. From the parallelism in the protein and phospholipide content of transudates (Mann and Peters, 104), as well as from the results of experiments with artificial membranes of graded permeability (Artom and Freeman, 23), it appears unlikely that phospholipides diffuse into the tissues more readily than the proteins to which they are linked in the plasma. But the most convincing evidence against the role of plasma phospholipides in fatty acid transport has come from the numerous results of Chaikoff's group. These results (which I have discussed elsewhere, 10) clearly indicate that there is a continuous exchange of phospholipides between liver and plasma, but not between plasma and other tissues. Moreover, choline administration appears to increase the turnover of lecithins in the liver, but not in the plasma. The conclusion seems justified that, under physiological conditions, plasma phospholipides do not represent a major chemical form for the transport of fatty acids. As it is worded, this statement allows for some reservations or exceptions. Thus, in laying birds considerable amounts of phospholipides are synthesized in the liver and transported in the plasma to the ovaries (Hevesy and Hahn, 86). From the evidence discussed in the preceding section a limited contribution of phospholipides synthesized by, or directly absorbed from, the small intestine seems probable. But even more likely is the assumption that, because of the simultaneous presence of polar and non-polar groups in their molecules, phospholipides may have an important physical role not only in the absorption but also in the transport of lipides such as neutral fats or cholesterol esters, which lack centers of attachment. Indeed, these highly hydrophobic lipides can possibly form solid solutions in the phospholipide moiety of the lipoproteins present in blood plasma (Chargaff, 51).

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Fat Metabolism

The hypothesis that phospholipides represent intermediate compounds in the metabolism of fatty acids was first suggested a long time ago by Loew, who stated that "lecithin was a machine for burning fats" (100). Later Leathes (96) suggested that incorporation of fatty acids into liver phospholipides could be a preliminary stage in their catabolism.

As mentioned above, the finding of a comparatively high turnover of phospholipides in the liver or in the intestinal mucosa may not be evidence for a participation of these compounds in fatty acid metabolism or absorption. Only the actual demonstration that in a given tissue the turnover of phospholipides, their amount, or both are increased or decreased in conditions in which the metabolism of fatty acids is also specifically increased or decreased should be regarded as significant in that respect. Some evidence of this type is listed here. The administration to dogs of very large amounts of fats causes a temporary but readily detectable increase in the liver phospholipides (Artom, 7). In our early experiments with radioactive phosphorus, incorporation of the isotope into the phospholipides of the liver, intestine, and possibly also of the kidney was greater in rats fed a diet rich in fat than in rats on a glucidic diet. No such difference was detectable in other tissues (Fig. 5). We interpreted these results by assuming that in the liver and intestinal mucosa, and perhaps also in the kidney, a high proportion of "lipometabolic phospholipides" specifically involved in fat metabolism were present, while in the other tissues the phospholipides represented chiefly essential constituents of the cells, i. e. "cytoplasmic phospholipides" (Artom et al., 29).

Zilversmit and DiLuzio (158, 61) have also shown recently that in depancreatized or phlorizinized dogs phospholipide turnover is markedly increased in the liver, small intestine, blood plasma, and to a lesser extent also in the kidney, but not in muscle and brain. These results are of obvious interest, in view of the extensive catabolism of fats in diabetic animals.

Since fats are largely used in the defense against cold, one might also interpret in a similar way the older data of Mayer and Schaeffer

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(106), who described a very rapid increase in the liver phospholipide of rabbits exposed to low temperatures. Interestingly enough, the increases in the amounts of phospholipides, determined by chemical methods, were accompanied by increases in the number and size of the mitochondria visible under the microscope (Mayer et al., 107).

Finally, many of the observations on the increased phospholipide turnover after administration of choline or related substances are

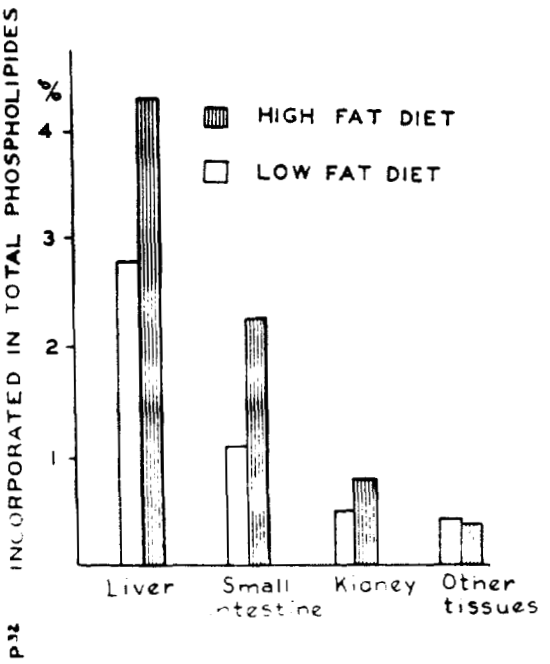

FIG. 5. Incorporation of $H_3P^{32}O_4$ in the tissue phospholipides of rats on high or low fat diet.

suggestive of a relationship between the formation of phospholipides and disposal of the fatty acid present in the liver. Without discussing again these observations (10), I shall merely point out here that if, as mentioned above, choline does not enhance the mobilization of fatty acids in the form of plasma phospholipides, one is inclined to ascribe the lipotropic effect of this substance to an increased catabolism of fatty acids in the liver itself. In a series of experiments now in progress in our laboratory, we are attempting to obtain a more direct evidence for this possibility (11).

Slices of the liver of rats maintained on various diets were incu-

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bated with C^{14}-labeled stearate, and the radioactivity of the CO_2 evolved was determined. As shown in Table 16, liver slices from rats on a low protein diet with added guanidoacetate produced less isotopic CO_2 than slices from rats on a stock diet. Supplementation of the deficient diet with choline consistently prevented the fatty infiltration of the liver. Production of isotopic CO_2 by these livers was increased in most experiments (but not in all). When the rats on the low protein diet, supplemented or not supplemented with

TABLE 16

$C^{14}O_2$ PRODUCTION FROM I-C^{14} STEARATE IN LIVER SLICES

Exper No.	DIET	Liver Fat mg./g	$C^{14}O_2$ (counts/min.)	
			1 g Tissue	Whole Liver of 100 g Rat
8	Choline def	220	394	1650
	Choline suppl.	32	573	2440
	Stock	13	1820	5830
12	Choline def	92	443	2[illegible]00
	Choline suppl.*	30	2910	13400
	Stock	22	1594	6410
9	Choline def.	260	539	2480
	Choline " *	135	1440	10250
	Choline suppl*	28	1062	4650

* Injected with 0.5 mM choline HCl [illegible] 5 h and 0.5 h before death

* 0.5 — 0.7 g. liver slices. Additions: 1 — C^{14} Stearate (1 — 1.5 micromoles, 2 — 3 × 10^5 c.p.m.), penicillin and streptomycin (3 mg. each). Total Vol.: [illegible] ml. Incubation: 3 hrs. at 37° C., in air (pH. 7.4).

choline, were injected with choline shortly before the removal of the liver, high values of radioactive CO_2 were always obtained. It is suggested that these high values are dependent upon the existence, *at the beginning of the experiment*, of a certain concentration of choline in the tissue, and that ingestion of a diet containing preformed choline does not always maintain such a concentration at the required level. At any rate, the occasional finding of a decreased $C^{14}O_2$ production in non-fatty livers indicates that the decrease is not merely due to isotopic dilution of the added stearate by the fat accumulating in the tissue. There may be still some question about the interpretation of our data, since the specific activity of the stearate

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at the site of its oxidation in the cells is not known. If our future experiments support the assumption that the isotopic CO_2 is a valid measurement of fatty acid oxidation in the isolated liver, it would appear that in this process choline is important. Moreover, since addition of choline in vitro (at least in the concentrations we have tested; 0.003 M to 0.015 M) was not effective, the active substance is probably not choline, but some other compound formed in vivo from choline.

It does not follow necessarily that this compound is a phospholipide.[2] Still one might well speculate about certain attractive possibilities which may be in line with some recent observations. Cytoplasmic particles such as the mitochondria, which are capable to carry out the oxidation of many substances, including fatty acids, are rich in phospholipides. The inhibition of succinodehydrogenase by substances which dissolve the phospholipides or may interact with these compounds (Ball and Cooper, 35) could be an indication of the importance of phospholipides for the enzymatic activities of cytoplasmic granules. The recent experiments of Quastel and Braganca on the action of heated snake venom are also extremely interesting in this respect (116). The heat-stable lecithinase A present in such material does not affect a large number of " soluble " enzymes, but inactivates all the enzyme systems which are located chiefly or only in the granular fractions of the cytoplasm. The functional activities of these substructures seem to be dependent upon the presence of intact phospholipide molecules. Some time ago Kielley and Meyerhof (91) described a preparation of muscle ATPase with a high content of phospholipides and showed that the enzyme was rapidly inactivated by the phospholipase D of *Clostridium welchii* which splits only choline-containing phospholipides. Recently, in our laboratory, Swanson and Mitchell also found very considerable amounts of phospholipides in a highly purified preparation of ATPase, separated from the nucleoproteins of liver nuclei (137).

[2] The hypothesis that choline is a component of enzymes involved in the respiration of muscle tissue has been suggested (Abdon and Börglin, 1). It has been claimed recently that choline pyrophosphate is present in both acid and alkaline phosphatases (Kutscher and Sieg, 95).

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It seems likely that future investigations will extend these observations and that phospholipides will be found to be an integral part of many other important enzymes. If this assumption is correct, it will perhaps become possible to reconcile into a single interpretation the various roles which have been suggested for the phospholipides. For instance, the large amounts of these compounds in the mitochondria undoubtedly represent structural materials and at the same time their presence seems to be essential for the occurrence of oxidation processes in the cell. Furthermore, one may also think that the so-called "lipometabolic phospholipides" actually are lecithin component of enzyme systems, directly or indirectly involved in some stages of the catabolism of fatty acids. Thus the evidence for a close relationship between phospholipide turnover and fatty acid metabolism could be explained without postulating that phospholipide formation represents an intermediary step in the metabolism of fatty acids.

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Preparation of C^{14}-Methyl Labeled Dimethylaminoethanol

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, BOWMAN GRAY SCHOOL OF MEDICINE, WAKE FOREST COLLEGE]

Preparation of C^{14}-Methyl Labeled Dimethylaminoethanol

BY CAMILLO ARTOM AND MARIETTA CROWDER

2-Methylaminoethanol (1 mM./ml.) is refluxed for 6 hours with C^{14}-formaldehyde (1 mM./ml.). Unreacted 2-methylaminoethanol and formaldehyde are destroyed with nitrous acid and alkaline iodine, respectively. C^{14}-Dimethylaminoethanol is recovered by two successive steam distillations (yield 40% based on the isotopic formaldehyde). The solution is of isotopic purity sufficient for biological experiments. No dimethylaminoethanol is formed at $pH < 7$, unless formic acid is also added.

In the course of our studies on the role of dimethylaminoethanol (DMA) as a likely intermediate in choline metabolism,[1-3] C^{14}-methyl-DMA was prepared by refluxing 2-methylaminoethanol (MMA) with C^{14}-formaldehyde (FA) in alkaline solution.

$$CH_3NHCH_2CH_2OH + 2HC^{14}HO \longrightarrow C^{14}H_3CH_2NCH_2CH_2OH + HC^{14}OOH$$

The highest yield, based on the FA, was obtained with about 1 mM. each of FA and MMA per ml. of solution, and was but very slightly increased by addition of formate. No DMA was formed at $pH < 7$, unless formic acid was also added. A comparison between these conditions and those of other methods for the reductive methylation of amines[4-7] may be of some theoretical interest. From the practical viewpoint, the present procedure, in spite of its lower yield, is extremely simple and the product is of a sufficient isotopic purity to be used directly for most biological experiments.

Experimental

For the radioactive measurements (thin mica window or Q-gas flow counter), both standard and unknown samples were brought to a uniform weight by adding the proper amounts of inactive materials (egg lipides), and were spread as thin films onto aluminum dishes covered by lens paper.

2-Dimethylaminoethanol.—Into a small flask fitted with a ground glass condenser, 2 ml. of a 2.5 *M* solution of 2-methylaminoethanol, b.p. 158-160° (5 mM.), 1.8 ml. of a solution of C^{14}-labeled formaldehyde[8] (300 microcuries, 4.6 mM.) and 0.7 ml. of alkaline borate, *p*H 10.0 (6.2 g. of boric acid in 100 ml. of *N* NaOH) are added. The mixture is refluxed for 6 hours, cooled and neutralized with 2 *N* HCl. The unreacted 2-methylaminoethanol is destroyed by adding 10 ml. of 30% sodium nitrite and 5 ml. of glacial acetic acid. After 10 minutes in ice, the mixture is made alkaline with saturated NaOH, aerated vigorously for 0.75 hour (to remove the oxides of nitrogen), then steam distilled (Parnas-Wagner apparatus[9]) for 0.5 hour into 4 ml. of *N* HCl. To eliminate traces of C^{14}-formaldehyde which may be carried over in the distillate, 1 ml. of 1% inactive FA, 1 ml. of *N* iodine and a slight excess of *N* NaOH are added. After acidification, the excess iodine is reduced with 5% $NaHSO_3$. The solution is concentrated to a few ml., made alkaline and steam distilled again into 4 ml. of *N* HCl. The distillate contained 1.87 mM. of DMA (by titration: a 40.6% yield, based on the added FA) and 41.0% of the counts introduced as C^{14}-FA.

The extent to which other isotopic products, introduced or formed during the reaction, had been eliminated was determined. Inactive formaldehyde, formic acid, or methanol was added to separate aliquots of the solution of isotopic DMA. The mixtures were acidified and distilled. In the distillates the per cent. recoveries of C^{13} and C^{14} (measured as $BaCO_3$ after oxidation with cold alkaline $KMnO_4$) were, respectively, formaldehyde, 97 and 0.08; formic acid, 99 and 0; methanol, 99 and 0.09.

From both active and inactive materials the picrolonic acid derivative was prepared[10] with variable yields (60-95%, depending on the amounts of DMA present or added as a carrier). The m.p. was 196° (unchanged by admixture with an authentic sample of DMA picrolonate; reported,[11] 197°). Within the limits of error of our measurements, the specific activity of the picrolonate was the same as that calculated for the DMA in solution and was not changed after several recrystallizations from water and alcohol.

Anal. Calcd. for $C_{14}H_{19}N_5O_8$: C, 47.59; H, 5.42; N, 19.82. Found: C, 47.48, 47.69; H, 5.35, 5.58; N, 19.45, 19.71.

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