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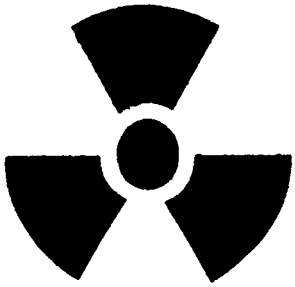

QUARTERLY PROGRESS REPORT

REPORT NO. 7. COPY NO. 58. ISSUED OCT 15 1950.

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(1)

METABOLISM AND TOXICITY OF RADIOACTIVE METALS (2)

V. COLUMBIUM 95, YTTRIUM 91, AND TANTALUM 182.

John Doull, M. F. Sullivan, and Kenneth P. DuBois

A study of the metabolism of certain fission products and other radioactive metals has been carried out in this laboratory during the past several months (1-5) to obtain information of value in the interpretation of toxicological data on these metals. For metabolism and excretion studies tracer doses of the radioactive metals were administered orally, intraperitoneally, and intravenously. The daily excretion of the isotopes was measured and the animals were usually sacrificed in six days for tissue distribution measurements. This procedure facilitated the acquisition of information on the extent of absorption and the duration of retention of the isotopes in animal tissues. Other investigators (6-8) have previously provided much of the desired information regarding the metabolism of fission products and our data were in agreement with previous findings.

We have previously reported (1-5) the results of metabolism studies with tantalum 182, yttrium 91, strontium 89, and zirconium 95. It was found that tantalum 182 as the oxide (Ta_2O_5) was not absorbed from the gastro-intestinal tract and was completely eliminated within two days after administration. When given intraperitoneally nearly all of the injected dose remained at the site of injection due to its insolubility and consequent poor absorption. Yttrium 91 was also poorly absorbed from the gastro-intestinal tract as evidenced by less than 0.01 per cent of the material being present in the tissues six days after administration. After intraperitoneal administration of yttrium 91 about 40 per cent of the injected dose was excreted in six days and after intravenous injection a similar rate of excretion was noted. Strontium 89 was well absorbed with 12 to 25 per cent of oral doses appearing in the tissues. After intraperitoneal and intravenous administration of strontium 89 the amount present in the tissues in six days was 50 to 65 per cent of the administered doses. Zirconium 95 was not absorbed to any significant degree when given orally and after parenteral administration 50 to 60 per cent of the material remained in the body six days later.

The present report contains information on the rate of excretion of columbium 95 administered intravenously and intraperitoneally to rats. Our investigations on this fission product were begun previously (5) with measurements of the absorption of orally administered columbium 95. In agreement with the findings of Hamilton (6) we observed that columbium 95 was absorbed to a greater extent when given orally than most of the other fission products.

With information on the metabolism of yttrium 91 and tantalum 182 at hand toxicity studies with these materials are being conducted. In preliminary tests we found (5) that doses of 800 uc/kgm. of yttrium 91 caused some decrease in the growth rate of rats but only one of three animals died in 180 days. The present report contains further information on the acute toxicity of yttrium 91 as well as information on the effects of intraperitoneally administered tantalum 182.

MATERIALS AND METHODS

For these experiments adult male Sprague-Dawley rats were employed. They were maintained in an air-conditioned laboratory (9) equipped for housing animals after treatment with isotopes and a special injection apparatus (10) was used for administering the isotopes. After injection of radioactive metals the animals were kept in metabolism cages for six days. This procedure was followed both for metabolism and for toxicity tests because it facilitated the disposal of the large fraction of the injected dose generally excreted during the first few days after administration of the radioactive metal. In metabolism studies with columbium 95 the urine and feces were collected daily for six days and the animals were then sacrificed for tissue distribution measurements.

Yttrium 91 and columbium 95 were obtained from the Oak Ridge National Laboratory. A solution of columbium 95 (12.6 mc./ml.) in 5 per cent oxalic acid was used after dilution to 50 uc/ml. with 0.9 per cent saline solution. A solution of yttrium 91 in 0.133 N HCl was neutralized to pH 6.5 with sodium hydroxide and the solution used for injection contained 750 uc of yttrium 91/ml.

For measurement of the distribution of columbium 95 tissues and feces were homogenized in distilledwater. Bone samples were ashed at 450°C. and the residue was dissolved in dilute HCl and neutralized. Samples were plated on copper discs, dried, and counted with an end-window mica counter. An aluminum absorber (13.5 mgm. Al/cm^2) was placed over the sample to decrease the beta counts.

EXPERIMENTAL

Rate of Excretion and Tissue Distribution of Columbium 95 Given Intraperitoneally.

In our previous experiments (5) with Cb 95 it was found that an average of 18.1 per cent of a 4-uc dose and 34.4 per cent of a 2-uc dose was present in the organs and carcass six days after oral administration. Most of the retained Cb 95 was present in the bones with 10.9 per cent of the 4-uc dose and 31.9 per cent of the 2-uc dose being found in the carcass.

To ascertain the distribution and rate of excretion of Cb 95 given intraperitoneally groups each containing three rats were given 2 uc and 4 uc of the isotope. Urinary and fecal excretion was measured daily for six days after which time the animals were sacrificed for tissue distribution measurements. The rate of excretion of Cb 95 given intraperitoneally is shown by the data in Table 1. The average total fecal excretion of Cb 95 was 26.4 per cent of the 4-uc dose and 31.4 per cent of the 2-uc dose. Urinary excretion was considerably less as evidenced by the average excretion of the 16.5 per cent of the 4-uc dose and 7.3 per cent of the 2-uc dose. Excretion occurred throughout the 6-day period in both the urine and feces suggesting that Cb 95 is not held by the tissues as tenaciously as some of the other fission products (8).

Analysis of the tissues from rats sacrificed six days after tracer doses of Cb 95 showed that most of the material was present in the carcass as shown

TABLE 1

RATE OF EXCRETION OF Cb 95 FOLLOWING INTRAPERITONEAL ADMINISTRATION TO RATS

Rat Number	7 Cb	8 Cb	9 Cb	10 Cb	11 Cb	12 Cb	Avg.	Avg.
Dose Administered	4 uc			2 uc			4 uc.	2 uc.
Weight of Animal	300	300	297	300	285	260	299	282
% of Injected Dose in Feces								
Days after Cb 95								
1	6.7	10.6	4.6	5.1	5.0	4.1	7.3	4.7
2	3.6	7.5	4.6	2.8	7.9	5.2	5.2	5.3
3	0.0	0.0	0.0	40.0	0.0	0.0	0.0	13.4
4	2.1	0.0	0.8	3.0	2.7	3.3	0.9	3.0
5	1.0	6.6	12.6	2.7	6.0	4.1	6.7	4.3
6	0.0	2.9	15.9	2.0	0.2	0.0	6.3	0.7
Total % in feces	13.4	27.6	38.5	55.6	21.8	16.7	26.4	31.4
% of Injected Dose in Urine								
1	6.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0
2	6.2	9.2	5.2	3.8	6.0	4.1	6.9	4.6
3	7.0	0.0	0.3	0.0	1.0	0.3	2.4	0.4
4	0.0	0.0	0.4	0.9	0.9	0.3	0.1	0.7
5	3.8	7.6	2.8	0.9	2.2	1.3	4.8	1.5
6	0.5	0.0	0.4	0.2	0.0	0.2	0.3	0.1
Total % in urine	23.5	16.8	9.1	5.8	10.1	6.2	16.5	7.3
Total % excreted in feces & urine	36.9	44.4	47.6	61.4	31.9	22.9	42.9	38.7
Total % in tissues	49.0	57.9	64.1	51.1	41.0	80.4	57.0	57.5
Total % recovered	85.9	102.3	111.7	112.5	72.9	103.3	99.9	96.2

by the data in Table 2. An average of 57 per cent of the 4-uc dose and 57.5 per cent of the 2-uc dose was retained in the tissues and over 90 per cent of the material was found in the carcass. Spleen and kidney each contained approximately 1 per cent of the administered dose while the other tissues contained smaller amounts of Cb 95.

A comparison of the radioactivity of tissues on a weight basis is given in Table 3. These data show that the specific activity of kidney was highest among the several tissues examined. Skeletal muscle and bone contained similar amounts on a weight basis and the value for carcass retention in Table 2 thus represents the Cb 95 in both the skeletal muscle and the bone. In this respect Cb 95 differed from other fission products which were deposited entirely in the bone. It is apparent from the data in Table 3 that Cb 95 is rather evenly distributed in several tissues and the large mass of the carcass accounts for the high percentage of the injected dose found in the carcass.

Excretion and Tissue Distribution of Columbium 95 Given Intravenously to Rats.

To determine the rate of excretion of Cb 95 after intravenous administration groups each containing three rats were given 10-uc and 20-uc of the radioactive metal. The daily excretion of Cb 95 was followed for six days and the results of these measurements are shown in Table 4. The average fecal excretion of Cb 95 was 8.7 per cent of the 10-uc dose and 3.8 per cent of the 20-uc dose. Urinary excretion of the isotope was considerably higher than fecal excretion as evidenced by an average of 32.3 per cent of the 10-uc dose and 20.4 per cent of the 20-uc dose in the urine during the 6-day period. The higher urinary excretion differed from the results obtained after intraperitoneal administration (Table 1) where the largest percentage of the Cb 95 appeared in the feces. However, the total percentage excreted in the urine and feces was nearly the same for the two routes of administration.

Measurements of the distribution of Cb 95 in rat tissues six days after intravenous administration showed that most of the material was present in the carcass as shown in Table 5. After 10 uc of Cb 95 53.4 per cent of the injected dose was present in the tissues of which 43.6 per cent was found in the carcass. Most of the remainder of the retained isotope appeared in the liver. Similar distribution occurred in the group of animals which received 20 uc of Cb 95. The average amount of the injected dose found in the tissues was 57.4 per cent of which 49.1 per cent occurred in the carcass, 6 per cent in the liver, and 1.6 per cent in the kidney. Other tissues contained small amounts of the isotope. When expressed in terms of activity per gram of tissue as shown in Table 5 it is evident that liver contained the highest specific activity and the higher total amount of Cb 95 in the carcass was due to the large mass of skeletal muscle and bone.

The Toxicity of Yttrium 91 and Tantalum 182 Given Intraperitoneally to Rats.

In preliminary toxicity tests with yttrium 91 we found(5) that a dose of 800 uc/kgm. given intraperitoneally caused a slight inhibition of growth of rats and one of a group of three animals died in 70 days. However, the other two animals in the group have survived for 270 days. Animals receiving 100 uc

TABLE 2

TISSUE DISTRIBUTION OF Cb 95 GIVEN INTRAPERITONEALLY TO RATS

Rat Number	7 Cb	8 Cb	9 Cb	10 Cb	11 Cb	12 Cb	Avg.	Avg.
Dose Administered	4 uc			2 uc			4 uc	2 uc
	% of Injected Dose							
Liver	1.2	1.1	3.1	1.4	1.9	0.4	1.8	1.2
Kidney	1.2	1.0	1.5	0.2	1.5	0.8	1.2	0.8
Spleen	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.1
Brain	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Heart	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lungs	0.7	0.8	0.0	0.0	0.0	0.1	0.5	0.1
Carcass	45.8	54.9	59.5	49.5	37.6	79.0	53.4	55.3
Total % in tissues	49.0	57.9	64.1	51.1	41.0	80.4	57.0	57.5

TABLE 3

COMPARATIVE RADIOACTIVITY OF TISSUES AFTER INTRAPERITONEAL ADMINISTRATION OF Cb 95

Rat Number	7 Cb	8 Cb	9 Cb	10 Cb	11 Cb	12 Cb	Avg.	Avg.
Dose Administered	4 uc			2 uc			4 uc	2 uc
% of Injected Dose/Gm. Wet Tissue								
Blood	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Lungs	0.5	0.1	0.0	0.0	0.0	0.0	0.2	0.0
Liver	0.2	0.1	0.2	0.1	0.2	0.1	0.5	0.2
Kidney	0.6	1.1	0.7	0.2	0.6	0.4	0.8	0.4
Spleen	0.2	0.1	0.1	0.0	0.0	0.1	0.1	0.4
Skeletal muscle	0.2	0.2	0.3	0.7	0.2	0.2	0.2	0.4
Bone	0.3	0.3	0.3	0.2	0.1	0.1	0.3	0.2
Brain	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Skin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Heart	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TABLE 4

RATE OF EXCRETION OF COLUMBIUM 95 ADMINISTERED INTRAVENOUSLY TO RATS

Rat Number	13 Cb	14 Cb	15 Cb	16 Cb	17 Cb	18 Cb	Avg.	Avg.
Dose of Cb 95	10 uc			20 uc			10 uc	20 uc
Fecal Excretion (% of Administered Dose)								
Days after Cb 95								
1	9.5	7.9	0.0	2.1	2.0	0.9	5.8	1.7
2	0.5	3.4	3.1	1.1	1.1	1.1	2.4	1.1
3	0.6	1.0	0.0	0.6	1.5	0.4	0.5	0.7
4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.9	0.0	0.0	0.3
6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total % in feces	10.6	12.3	3.1	3.8	5.5	2.4	8.7	3.8
Urinary Excretion (% of Administered Dose)								
Days after Cb 95								
1	13.4	10.8	12.4	9.0	8.5	9.0	12.2	8.8
2	8.5	9.9	8.8	4.4	6.0	4.3	9.1	5.0
3	4.2	3.0	3.2	1.5	1.7	1.7	3.5	1.6
4	3.2	2.5	4.3	1.3	1.8	4.8	3.3	2.7
5	2.5	2.2	3.0	1.3	1.7	1.6	2.6	1.5
6	1.0	1.4	2.5	0.5	0.6	1.2	1.6	0.8
Total % in urine	32.9	29.8	34.2	18.0	20.3	22.6	32.3	20.4
Total % excreted in urine and feces	43.5	42.1	37.3	21.8	25.8	25.0	41.0	24.2
Total % in tissues	47.4	57.9	55.0	55.8	67.5	48.8	53.4	57.4
Total % recovered	90.9	100.0	92.3	77.6	93.3	73.8	94.4	81.6

TABLE 5

TISSUE DISTRIBUTION OF Cb 95 GIVEN INTRAVENOUSLY TO RATS

Rat Number	13 Cb	14 Cb	15 Cb	16 Cb	17 Cb	18 Cb	Avg.	Avg.
Dose of Cb 95	10 uc			20 uc			10 uc	20 uc
% of Administered Dose in Organs								
Liver	3.6	11.6	5.6	11.7	3.4	2.8	7.0	6.0
Heart	0.3	0.3	0.2	0.1	0.1	0.2	0.3	0.1
Kidney	1.2	1.7	1.7	1.0	1.4	2.2	1.5	1.6
Spleen	0.2	0.1	0.9	0.5	0.2	0.5	0.4	0.4
Brain	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lungs	1.3	0.2	0.5	0.3	0.1	0.2	0.6	0.2
Carcass	40.8	44.0	46.1	42.2	62.3	42.9	43.6	49.1
Total % in tissues	47.4	57.9	55.0	55.8	67.5	48.8	53.4	57.4
% of Administered Dose per Gram of Tissue								
Liver	1.5	1.5	0.6	2.9	0.7	0.5	1.2	1.7
Kidney	0.6	0.8	0.8	1.0	1.5	2.0	0.7	1.5
Spleen	0.3	0.0	1.3	1.2	0.7	1.5	0.5	1.1
Lungs	0.5	0.2	0.3	0.3	0.1	0.4	0.3	0.3
Bone	1.0	1.2	1.1	1.1	1.4	1.3	1.1	1.3
Heart	0.4	0.4	0.2	0.1	0.2	0.3	0.3	0.2
Skin	0.1	0.1	0.3	0.2	0.3	0.3	0.0	0.3
Blood	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Brain	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Skeletal muscle	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

and 200 uc have survived for 40 weeks. Further toxicity tests have been conducted in which groups each containing four rats were given Y 91 intraperitoneally. The mortality among rats receiving from 1 to 4 mc/kgm. of Y 91 is shown in Table 6.

The results of these tests indicate that the 30-day LD-50 for Y 91 administered intraperitoneally is between 3 and 4 mc/kgm. Anthony previously found (11) that the 30-day LD-50 for Y 91 administered intracardially was between 1 and 3 mc/kgm. The results of our tests give further indication of the high toxicity of parenterally administered Y 91.

The animals which have survived for 30 days after administration of Y 91 are being observed and the growth rate is being recorded. The average growth rates for the groups of animals which received various doses of Y 91 are shown in Figure 1. After 4 mc/kgm. of Y 91 there occurred a rapid decrease in body weight immediately after injection of the isotope and the animals all succumbed within 10 days. A similar initial decrease in weight was observed after 3 mc/kgm. of Y 91 and in 40 days after injection 36 per cent inhibition of the growth rate of this group was observed. Animals receiving 1 and 2 mc/kgm. of the isotope showed only slight decreases in weight during the observation period. The surviving animals will be observed for longer periods of time to determine whether delayed mortality and weight changes occur.

Animals receiving various doses of tantalum 182 as Ta_2O_5 have been under observation for several months. Rats which received doses of 2 mc and 4 mc/kgm. intraperitoneally have survived for 39 weeks with no significant inhibition of the growth rate being observed. Animals receiving 8 and 16 mc of Ta 182/kgm. have survived for 30 weeks and exhibited only a slight inhibition of the growth rate. These results indicate that Ta 182 is much less toxic than Y 91.

DISCUSSION

In previous studies on the metabolism of orally administered Cb 95 we observed (5) that this material is absorbed to a greater extent than most of the other fission products in agreement with the findings of Hamilton (8). Further studies with this isotope have now been conducted using the intravenous and intraperitoneal routes of administration. These experiments showed that about 50 per cent of tracer doses were retained in the tissues six days after administration by both routes. Most of the retained Cb 95 was found in the carcass as was the case with the other fission products studied previously (2-5). However, a higher percentage of the injected dose was found in soft tissues and the liver and kidney contained almost 10 per cent of the injected dose.

Toxicity tests with Y 91 indicated that the 30-day LD-50 is close to 3 mc/kgm. when the material is given intraperitoneally to rats. This value is near the LD-50 value obtained by Anthony (11) after intracardial administration of Y 91 to rats. The inhibition of growth of animals which received 3 mc/kgm. indicates that delayed death of these animals may occur. Although Ta 182 as Ta_2O_5 is retained in the body for a much longer time than Y 91 the

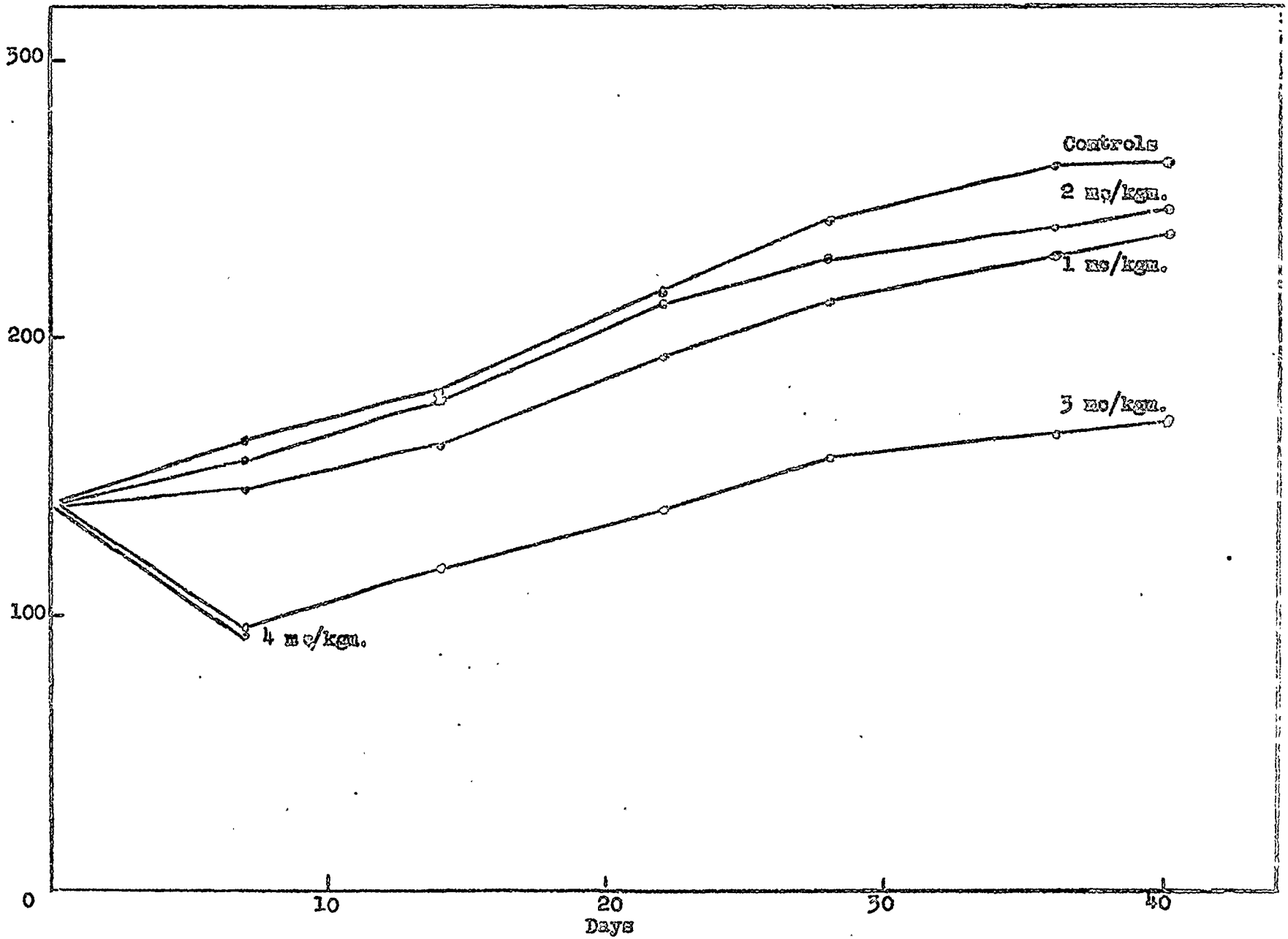
TABLE 6

THE TOXICITY OF YTTRIUM 91 GIVEN INTRAPERITONEALLY TO RATS

Dose of Y 91 mc./kgm.	10-Day Mortality	30-Day Mortality	% Mortality 30 Days
1	0/4	0/4	0
2	0/4	0/4	0
3	1/4	1/4	25
4	4/4	4/4	100

FIGURE 1

THE EFFECT OF YTTRIUM 91 GIVEN INTRAPERITONEALLY ON THE GROWTH RATE OF RATS



toxic effects are not as great as evidenced by survival of rats for 30 weeks after doses as high as 16 mc/kgm. Further toxicity tests with these and other radioactive metals are in progress.

SUMMARY

1. After intraperitoneal administration of Cb 95, 31.4 per cent of a 2-uc dose and 26.4 per cent of a 4-uc dose appeared in the feces in six days. Urinary excretion during this period amounted to 7.3 per cent of the 2-uc dose and 16.5 per cent of the 4-uc dose.

2. Tissue distribution studies showed that 57 per cent of the tracer doses of Cb 95 given intraperitoneally was retained in the tissues and over 90 per cent of the material was found in the carcass. Liver and kidney contained the highest activity on a weight basis.

3. After intravenous administration of tracer doses of Cb 95 to rats 32.3 per cent of a 10-uc dose and 20.4 per cent of a 20-uc dose was excreted in the urine during the first six days after injection while the feces contained 8.7 per cent and 3.8 per cent of the two doses respectively.

4. About 50 per cent of the Cb 95 given intravenously was found in the tissues and most of this amount was present in the carcass. The liver contained an average of 6 per cent and kidney 1.6 per cent of the injected doses.

5. In toxicity tests with Y 91 death of all of the rats receiving 4 mc/kgm. occurred within 10 days. The mortality of rats receiving 3 mc/kgm. was 25 per cent but a decrease in the growth rate of surviving animals was noted throughout a 40-day observation period.

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EFFECTS OF IONIZING RADIATIONS ON ENZYMES

IV. THE INFLUENCE OF GAMMA AND X-IRRADIATION ON PHOSPHATASES AND AEROBIC PHOSPHORYLATION.

Kenneth P. DuBois, Marcella Mazur, Kenneth W. Cochran, and John Doull

In recent studies on the effects of ionizing radiations on enzymatic reactions we observed (1) that the rate of hydrolysis of certain phosphate esters by alkaline phosphates was increased after exposure of mice to lethal doses of gamma radiation and X-rays. Stimulation of the alkaline phosphatase activity of serum has been reported previously by Ludewig (2) and by Ross and Ely (3) who found that the hydrolysis of several biologically important phosphate esters by the alkaline phosphatases of rat duodenum was increased after neutron irradiation.

In our experiments (1) no change in the adenosine triphosphatase activity of several tissues was noted after irradiation but the hydrolysis of β-glycerophosphate and 5-adenylic acid was significantly increased in some tissues. The 5-nucleotidase activity of mouse liver tissue gradually increased to twice the normal value in five days after 800 r of x-rays. After 2000 r of gamma radiation given continuously over a 48-hour period the nucleotidase activity was about twice the normal value at the end of the exposure period. Pretreatment of mice with para-amino propiophenone which was demonstrated by Storer and Coon (4) to exert a protective effect against x-ray mortality greatly reduced the activation of liver nucleotidase following radiation. The rate of hydrolysis of β-glycerophosphate by liver was approximately doubled immediately following 2000 r of continuous gamma radiation. Other evidence for an acceleration of phosphatase activity came from experiments on anaerobic glycolysis by liver and kidney tissue from irradiated mice in which lactic acid production and phosphate esterification were measured by the procedure of Stoesz and Le Page (5). Only a slight decrease in the ability of tissues to produce lactate was observed but a significant decrease occurred in the organic phosphorus of the medium accompanied by an increase in inorganic phosphorus.

To obtain further information on the nature and extent of the increase in phosphatase activity of tissues after irradiation we have continued investigations on alkaline phosphatases. Since an increased rate of hydrolysis of 5-adenylic acid was the most pronounced change observed (1) during our previous studies on phosphatases further experiments have been conducted on 5-nucleotidase. An assay method applicable to several tissues was devised and measurements of the 5-nucleotidase activity of several tissues from mice, rats, and guinea pigs were performed. Additional experiments on the effects of radiation on nucleotidase activity were performed.

The increased phosphatase activity of tissues after irradiation stimulated our interest in ascertaining whether the transfer of phosphate by the catalytic action of transphosphorylases was affected by radiation. The ability of tissues from irradiated animals to carry out oxidative phosphorylation was measured and results presented in this report indicate that phosphorylation is not significantly affected by radiation. However, the increased phosphatase

activity results in rapid breakdown of organic phosphates so that breakdown of phosphate esters approaches or outpaces the synthesis and the final result of this action is analagous to inhibition of phosphate esterification.

MATERIALS AND METHODS

Mice were exposed to gamma radiation using tantalum 182 as the source in the exposure chamber previously described (6). The animals were exposed continuously for 48 hours during which time they received 2000 r total body radiation. For experiments with x-rays animals were given 800 r total body irradiation in a single body exposure (250 kv, 15 ma, 1/4 mm Cu 50 cm. distance, and 40 r/min.).

5-Nucleotidase activity was determined by the procedure previously developed (7) in this laboratory for studies on beryllium poisoning. Aerobic phosphorylation was measured by the procedure of Potter (8) using isotonic KCl homogenates and creatine as the phosphate acceptor. Succinate was employed as the oxidizable substrate for most of the experiments; however, other intermediates of the Krebs cycle were also used as substrates at a final concentration of 0.004 M.

EXPERIMENTAL

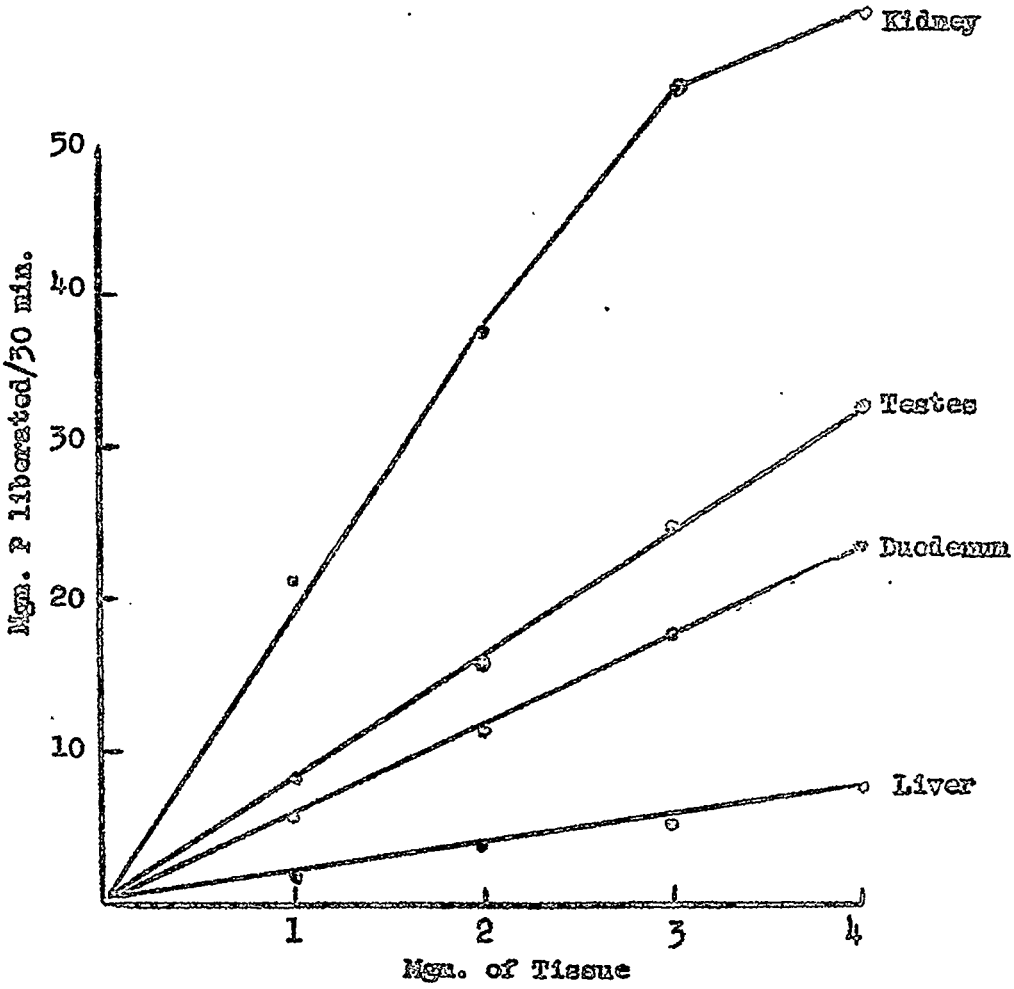
Measurement of the 5-Nucleotidase Activity of Animal Tissues

During studies on the mechanism of action of beryllium we developed (7) a procedure suitable for measuring the nucleotidase activity of animal tissues which was similar to the adenosine triphosphatase assay method of DuBois and Potter (9). The test system contained 0.15 ml. of 0.025 M barbital buffer, pH 8.9, 0.05 ml. of 0.04 M $MgCl_2$, 0.15 ml. of 0.013 M 5-adenylic acid, 1 or 2 mgm. of tissue as a 1%, 2%, or 5% aqueous homogenate and enough water to make a final volume of 0.65 ml. After incubation for 30 minutes at 38°C. the reaction was stopped by the addition of 0.1 ml. of 50% trichloracetic acid and the mixture was centrifuged. Inorganic phosphorus was determined on 0.3 ml. aliquots of the supernatant. The 5-nucleotidase activity was expressed as the mgm. P liberated from 5-adenylic acid by 1 gram of tissue in one hour. The applicability of this procedure for nucleotidase assays on several tissues is shown by the data in Figure 1.

Under the conditions described above the rate of hydrolysis of 5-adenylic acid by tissue homogenates is proportional to the tissue concentration when 1 to 4 mgm. of liver, testes, and duodenum were used. In the case of kidney tissue which has a high 5-nucleotidase activity the rate of the reaction was proportional to the tissue concentration when less than 3 mgm. of tissue was employed. Although there exists (10) evidence for the presence of a specific 5-nucleotidase in animal tissues it should be pointed out that the conditions of our assay permitted the estimation of the total capacity of various tissues to hydrolyze 5-adenylic acid rather than a single specific phosphatase. Thus, at a pH of 8.9 it is probable that at least part of the activity of some tissues is due to non-specific alkaline phosphatases.

FIGURE 1

5-NUCLEOTIDASE ACTIVITY OF MOUSE TISSUES



5-Nucleotidase-Activity of Animal Tissues

In previous studies (7) on rat tissues it was seen that the 5-nucleotidase activity of various tissues was markedly different from alkaline phosphatase activity as determined using β-glycerophosphate as the substrate. There was also a marked difference in the adenosine triphosphatase and 5-nucleotidase activity of tissues. These differences support the possibility that the hydrolysis of 5-adenylic acid is catalyzed by a specific phosphatase. In connection with studies on the effects of radiation on nucleotidase activity normal values have been obtained for several tissues from rats, mice and guinea pigs. The normal nucleotidase activity of tissues from these species is shown in Table 1 in which each value is the average for at least four animals.

As shown in Table 1 all of the tissues from the three species exhibited nucleotidase activity. However, there were striking species differences in the enzyme activity of some of the tissues. The activity of rat liver was about five times that of mouse liver and approximately 13 times higher than the activity of guinea pig liver. The values for rat and mouse kidney were much higher than the value obtained for guinea pig kidney. The activity of brain and skeletal muscle was similar for the three species but the activity of other tissues was generally highest in rat tissues and lowest in guinea pig tissues. There was no correlation between the distribution of 5-nucleotidase in animal tissues and the adenosine triphosphatase (9) activity of tissues. There were also noticeable differences between nucleotidase and alkaline phosphatase activity as measured using β-glycerophosphate as the substrate.

Effect of Citrate and Fluoride on 5-Nucleotidase Activity

Inhibitors of 5-nucleotidase activity were of particular interest in the present investigation in connection with attempts to decrease the elevated nucleotidase activity of tissues from irradiated animals. Since fluoride inhibits the activity of many phosphatases we measured the effect of fluoride on the nucleotidase activity of kidney, spleen and liver from normal and irradiated mice. Citrate has been employed by Pardee and Potter (11) to prevent the breakdown of adenylic acid during phosphorylation. It is presumed to act as an inhibitor by combining with magnesium thus decreasing nucleotidase activity by removing the activator. Since no direct measurements on the inhibitory action of citrate on nucleotidase activity had been reported previously we measured the inhibitory effect of citrate on liver nucleotidase of normal and irradiated animals in the presence and absence of added magnesium. The results of tests with fluoride and citrate are presented in Table 2.

The results of tests with fluoride indicated that this ion is not especially effective as an inhibitor of nucleotidase activity. A final concentration of 1.5×10^{-2} M fluoride inhibited kidney nucleotidase to the extent of 10 per cent in the absence of added magnesium and 17 per cent in the presence of magnesium. Liver was affected to a greater extent as evidenced by 48 per cent and 74 per cent inhibition in the absence and presence of magnesium respectively. In five days after 800 r a rise in the enzyme activity had occurred and fluoride produced about the same depression of enzyme

TABLE 1

THE 5-NUCLEOTIDASE ACTIVITY OF NORMAL TISSUES FROM RATS, MICE, AND GUINEA PIGS

Tissue	5-Nucleotidase Activity (mgm. P/gm. tissue/hr.)	
	No Mg	Mg
Rats		
Liver	23.3 (18.2-28.3)	24.4 (20.8-28.7)
Kidney	37.0 (27.3-42.5)	39.8 (30.9-43.3)
Skeletal muscle	1.6 (1.5- 1.7)	2.0 (1.9- 2.1)
Heart muscle	12.8 (10.5-14.2)	13.7 (10.5-15.7)
Lung	6.1 (5.8- 6.4)	7.1 (6.7- 7.5)
Ileum	18.3 (13.7-21.4)	17.6 (14.1-23.3)
Brain	6.0 (4.6- 7.1)	7.3 (5.6- 8.6)
Testes	20.3 (18.6-23.1)	24.3 (21.5-28.6)
Spleen	10.7 (7.6-13.3)	12.9 (8.4-11.9)
Thymus	9.0 (8.2-12.4)	10.4 (9.1-14.0)
Mice		
Liver	4.1 (3.4- 5.3)	4.9 (4.4- 5.4)
Kidney	48.3 (45.0-52.7)	49.6 (45.5-55.0)
Skeletal muscle	0.9 (0.6- 1.1)	1.0 (0.8- 1.4)
Heart muscle	3.0 (2.4- 3.4)	3.1 (2.6- 3.9)
Lung	8.5 (6.4-10.6)	6.0 (4.1- 8.0)
Ileum	16.2 (14.6-23.0)	17.9 (14.2-25.0)
Brain	3.7 (3.5- 4.1)	4.2 (3.9- 4.6)
Testes	16.3 (14.6-18.3)	18.8 (17.2-20.9)
Spleen	2.7 (2.2- 3.2)	3.4 (2.8- 3.7)
Guinea Pigs		
Liver	1.8 (1.7- 2.5)	2.6 (2.4- 2.6)
Kidney	8.3 (6.5-11.2)	10.7 (9.1-13.2)
Skeletal muscle	3.0 (2.1- 3.9)	3.8 (2.7- 5.0)
Heart muscle	4.2 (2.9- 5.4)	6.0 (4.3- 7.6)
Lung	14.4 (12.9-17.6)	17.5 (16.7-21.2)
Brain	4.9 (4.1- 5.6)	5.9 (5.0- 6.6)
Testes	9.6 (8.0-12.0)	12.1 (11.3-12.6)
Spleen	7.7 (5.6- 9.2)	9.7 (7.4-11.7)
Thymus	3.3 (3.0- 3.6)	4.7 (4.6- 4.8)

TABLE 2

EFFECT OF FLUORIDE AND CITRATE ON 5-NUCLEOTIDASE ACTIVITY

Tissue	Days after 800 r	5-Nucleotidase Activity mgm. P/gm. tissue/hr.			
		Without Inhibitor			
				1.5×10^{-2} M F	
		Mg	No Mg	Mg	No Mg
Kidney	Normal	43.3	43.5	38.9	35.9
Liver	Normal	4.3	4.8	2.3	1.3
	5	5.6	6.6	3.8	2.5
Spleen	Normal	2.6	3.3	1.7	1.4
	5	5.3	6.4	3.5	2.4
				1.5×10^{-2} M Citrate	
Liver	Normal	3.8	4.8	1.8	3.8
	7	4.8	5.8	2.1	4.2

activity as was noted with normal liver. Results with spleen were similar to those obtained with liver.

Citrate at a final molar concentration of 1.5×10^{-2} produced about 50 per cent inhibition of the activity of liver, but when magnesium was added to the system (0.003 M) the inhibition was reduced to about 25 per cent indicating that the combination of citrate with magnesium is responsible for the inhibition of the hydrolysis of 5-nucleotidase. These tests demonstrated that neither fluoride nor citrate are especially active as inhibitors of 5-nucleotidase.

Rate of Hydrolysis of Various Phosphorus-Containing Compounds by Tissues of Normal and Irradiated Mice.

To compare the rate of hydrolysis of 5-adenylic acid and other phosphorus-containing compounds we employed the nucleotidase test system described above and substituted other compounds for 5-adenylic acid as the substrate. The compounds employed for these tests were yeast adenylic acid (3-adenylic acid), phosphoglyceric acid, and diphosphopyridine nucleotide (DPN). A comparison of the rate of hydrolysis of these compounds by mouse liver, kidney and testes is given in Table 3.

The results of this comparison showed that 5-adenylic acid is hydrolyzed at a faster rate than any of the other substrates under identical experimental conditions. The results with 3-adenylic acid were of particular interest because of its close structural similarity to 5-adenylic acid. All of the substrates were hydrolyzed at a much slower rate than muscle adenylic acid by liver and kidney but the most pronounced difference was observed with testes. When yeast and muscle adenylic acid were mixed and employed together as substrates the same amount of phosphorus was liberated by liver as when 5-adenylic acid alone was used. This demonstrated that while 3-adenylic acid is not hydrolyzed as rapidly as 5-adenylic acid its presence does not inhibit the hydrolysis of the latter compound. Indications of substrate specificity were obtained in these tests particularly in the case of testes and liver where it appears that a specific 5-nucleotidase catalyzed most of the hydrolysis of 5-adenylic acid. In five days after 800 r of x-rays there was a 60 per cent increase in the rate of hydrolysis of 3-adenylic acid by liver tissue and the liberation of inorganic phosphorus from DPN was increased 36 per cent in liver and 15 per cent in kidney. The hydrolysis of phosphoglyceric acid by liver and testes was not appreciably increased after radiation but kidney showed a 20 per cent increase in ability to liberate phosphorus from this compound. The results of these tests are in agreement with our previous observations (1) on the increase in activity of various alkaline phosphatases in the tissues of irradiated animals.

Effect of pH on the Activation of 5-Nucleotidase after Radiation

It was observed by Ross and Ely (3) that the alkaline phosphatase activity of duodenum showed increased activity after irradiation when the reaction was carried out at pH 9 but not at pH 7. In order to determine whether the activation of nucleotidase after irradiation was pH dependent assays were carried out at pH 8.9 and at pH 7.4 on liver from normal and irradiated mice.

TABLE 3

RATE OF HYDROLYSIS OF PHOSPHORUS-CONTAINING COMPOUNDS BY MOUSE TISSUES

Tissue	Activity. (Mgm. P/gm. tissue/hr.)							
	5-Adenylic Acid		3-Adenylic Acid		DPN		Phosphoglyceric Acid	
	No Mg	Mg	No Mg	Mg	No Mg	Mg	No Mg	Mg
Liver	4.1	4.9	0.8	2.2	2.3	2.8	1.6	1.8
Kidney	48.3	49.6	28.0	30.5	9.1	10.4	16.0	16.7
Testes	16.3	18.8	2.0	2.8	---	----	2.8	3.0

There was no appreciable difference in the activity of normal liver at the two pH values. In 48 hours after 2000 r of gamma radiation there was an increase in activity from the normal of 4.1 mgm. P/gm. tissue/hr. to 7.4 when the reaction was carried out at pH 8.9. At pH 7.4 the value was 6.0 which represented a 46 per cent increase as compared with an 80 per cent increase at pH 8.9. This experiment indicated that the activation caused by radiation was somewhat greater as the alkalinity of the reaction medium increased.

Effects of X-Irradiation on Aerobic Phosphorylation by Mouse Kidney Homogenates.

The increase in phosphatase activity of tissues after irradiation stimulated our interest in ascertaining whether the synthesis of phosphate esters was affected by ionizing radiations. Our previous experiments (1) on phosphorylation and glycolysis showed a decrease in the ability of tissues from irradiated animals to maintain phosphorus in organic form. However, we attributed the breakdown of high energy phosphate to the increased phosphatase activity rather than a direct action of radiation on transphosphorylation. To obtain information on the activity of transphosphorylating enzymes in tissues taken from irradiated animals oxidative phosphorylation was studied. For these tests it was advisable to use a system in which the results would not be determined by factors such as the increased hydrolytic action of phosphatases or a decrease in oxygen consumption. The procedure of Potter (8) in which succinate is used as the oxidizable substrate and creatine as the phosphate acceptor seemed suitable because we had previously shown (12) that the oxidation of succinate by kidney homogenates is unaffected by ionizing radiations. The system of Potter (5) contains fluoride to block the action of alkaline phosphatases. Coupled oxidation and phosphorylation was measured using kidney tissue taken from mice at intervals after 800 r total body x-irradiation. The results of these assays are presented in Table 4 in which each value is the average for at least four animals.

As shown by the data in Table 4 there was a marked decrease in esterification of inorganic phosphorus after 800 of x-rays. The loss of phosphorylative ability of kidney occurred gradually following irradiation and in this respect resembled the gradual increase in phosphatase activity which was previously observed (1). No decrease in the ability of kidney tissue to oxidize succinate was observed throughout the 5-day period after irradiation and the failure of phosphocreatine synthesis was therefore not due to inhibition of the oxidization which provides the energy for phosphorylation.

To determine the effect of various doses of radiation on aerobic phosphorylation 400 r, 800 r, and 1200 r of x-rays were given to mice. Other animals received 2000 r of continuous gamma radiation administered over a 48-hour period. Aerobic phosphorylation was measured using kidney tissue and the results of these measurements are summarized in Table 5 in which each value represents the average for at least four animals.

Inhibition of the aerobic synthesis of phosphocreatine was only slightly inhibited by the sublethal dose of 400 r. After 1200 r and 800 r of x-rays similar inhibition was noted at four and five days respectively and in two days after 2000 r of continuous radiation 95 per cent inhibition was observed. Thus, the time of onset and extent of inhibition of phosphorylation was dependent upon the dose of radiation.

TABLE 4

THE EFFECT OF WHOLE BODY X-IRRADIATION ON AEROBIC PHOSPHORYLATION BY MOUSE KIDNEY

Days after 800 r	ugm. P esterified/ 30 mgm. tissue	cmm. O_2 consumed/10 min.	% Inhibition of P esterification
Control	83	41	
2	78	45	4
3	47	40	44
4	32	41	61
5	21	45	75

TABLE 5

THE EFFECT OF VARIOUS DOSES OF IONIZING RADIATIONS ON AEROBIC PHOSPHORYLATION BY MOUSE KIDNEY

Dose of Radiation	Days After Irradiation	ugm. P Esterified/ 30 ugm. Tissue	cmm. O_2 Consumed/ 10 min.	% Inhibition of P uptake
Control	-	83	41	
400 r X-rays	5	72	41	13
800 r X-rays	5	21	45	75
1200 r X-rays	4	27	40	68
2000 r gamma radiation	2	4	41	95

Effect of Radiation on Phosphorylation and the Coupled Oxidation of Various Substrates.

Different phosphorylases catalyze the uptake of inorganic phosphorus during oxidation of the various intermediates in the Krebs citric acid cycle. Thus, measurements of coupled oxidation and phosphorylation using various substrates would reveal whether the effects of ionizing radiations on phosphorus esterification are limited to certain phosphorylation reactions or whether the effect is independent of the substrate employed. To determine whether the decrease in phosphate esterification was influenced by the substrate used for coupled oxidation and phosphorylation we substituted several compounds for succinate in the test system of Potter (8). The final molar concentration of each substrate was 0.00^4 and when two substrates were added to the system each was present at a final concentration of 0.00^4 M. A comparison of phosphate esterification and oxidation of various substrates by kidney tissue from normal and irradiated mice is shown in Table 6. The assays were performed 24 hours after 2000 r of gamma radiation and each value is the average for three animals.

As shown in Table 6 a decrease in phosphorus esterification was observed with all of the substrates tested. The inhibition was greatest when pyruvate, glutamate, oxalacetate, and succinate were employed as substrates. Combinations of pyruvate with succinate, fumarate, and glutamate were also employed and inhibition of phosphorus esterification was observed. In this experiment DPN (0.1 ml. of 1%) was added to each flask since DPN increases esterification. The rate of oxidation of succinate plus pyruvate was much higher than that of either substrate alone; however, this combination of substrates should not be used for oxidative phosphorylation studies because the results may be confused by the fact that succinate can be oxidized at a rate which in independent of the other reactions in the cycle as has been pointed out by Potter (13). A small decrease in the oxidation of nearly all the substrates tested was observed in the kidney tissue from irradiated animals. In most cases this decrease in respiration was not great enough to account for the decrease in phosphocreatine production.

Phosphorylation by Liver and Kidney from Irradiated Mice in the Presence and Absence of Fluoride.

To determine whether inhibition of oxidative phosphorylation occurred in liver tissue after irradiation mice were given 800 r of x-rays and the phosphorylative ability of liver tissue was measured five days after irradiation. When succinate was used as the substrate the oxygen consumption was 37 cmm./30 mgm. of tissue for normal liver and 42 cmm. for liver from irradiated mice. There was likewise no difference between the normal and irradiated liver with respect to phosphorus esterification as evidenced by phosphate uptake of 67 ugm./30 mgm. of normal liver tissue and 69 ugm. by irradiated liver. When glutamate was used as the substrate esterification by normal and irradiated liver was also similar. In seeking an explanation for the differences in phosphorylative ability of liver and kidney from irradiated animals we gave consideration to the differences in the alkaline phosphatase activity of the two tissues. As described in a preceding paragraph of this report the 5-nucleotidase activity of mouse liver is much lower than that of kidney.

TABLE 6

THE EFFECT OF RADIATION ON THE OXIDATION OF VARIOUS SUBSTRATES AND PHOSPHORYLATION BY KIDNEY TISSUE FROM MICE

Substrate	ugm. P Esterified/ 30 mgm. Tissue		cmm. O_2 Consumed/10 min.		% Inhibition of P Esterification
	Normal	2000 r	Normal	2000 r	
Succinate	125	75	43	40	40
Fumarate	89	75	31	25	15
Pyruvate	87	22	23	18	74
Glutamate	94	36	31	27	61
α-Ketoglutarate	118	100	43	39	15
Oxalacetate	110	64	44	35	42
Fumarate + pyruvate	135	101	56	54	25
Succinate + pyruvate	117	80	73	67	32
Glutamate + pyruvate	127	180	39	37	37

Although fluoride is present in the test system used for aerobic phosphorylation this inhibitor does not effectively block the breakdown of adenylic acid by kidney tissue. However, fluoride is effective in inhibiting the low nucleotidase action of liver. It, therefore, seemed possible that the apparent inhibition of aerobic phosphorylation by kidney tissue from irradiated animals was due to increased phosphatase activity in spite of the presence of fluoride. This possibility was tested by measuring phosphorylation by kidney and liver tissue from normal and irradiated mice in the presence and absence of fluoride. The results of this experiment are given in Table 7.

The results of this experiment demonstrated that there was no decrease in phosphate esterification by liver from irradiated mice when fluoride was present in the medium. In the absence of added fluoride phosphocreatine formation still occurred with normal liver but not in the case of liver from irradiated animals. This difference would appear to be explainable on the basis of increased phosphatase activity which is effectively inhibited in liver by fluoride. Kidney tissue was unable to maintain phosphorylation in the absence of fluoride due to the high phosphatase activity in this tissue and it therefore appears that the apparent inhibition of transphosphorylation in kidney after irradiation is the result of increased phosphatase action. The results in Table 7 together with the data in Table 2 demonstrating the ineffectiveness of fluoride as an inhibitor of 5-nucleotidase support this conclusion.

DISCUSSION

In previous studies we found (1) that the alkaline phosphatase activity of several tissues was increased after lethal doses of ionizing radiations. This increase was particularly noticeable in the case of 5-nucleotidase but was also observed when substrates other than 5-adenylic acid were employed. In order to extend the observations on nucleotidase activity after irradiation to species other than mice it was necessary to obtain normal values for tissues from various species. The present report contains evidence that the assay method which was previously developed (7) in this laboratory for nucleotidase is generally applicable to many animal tissues. A comparison of the 5-nucleotidase activity of tissues from rats, mice, and guinea pigs revealed some rather striking species differences in enzyme activity. The activity of rat tissues was generally highest among the three species studied with the greatest difference being observed in the case of liver where the value for rats was five times higher than for mice and 13 times higher than the activity of guinea pig liver.

When equal quantities of liver and kidney were used fluoride at a final molar concentration of 1.5×10^{-2} M produced 10 per cent inhibition of the nucleotidase of kidney and 48 per cent inhibition of the activity of liver indicating the fluoride is not an effective inhibitor of kidney nucleotidase. Citrate was about equal to fluoride as an inhibitor of the enzyme.

The increased phosphatase activity after irradiation would be expected to interfere with the level of high energy phosphate compounds in the tissues by hydrolyzing phosphate esters at an abnormally high rate. Thus, the final result of phosphatase activation in the intact animal would be comparable to inhibition of the synthesis of phosphate esters because breakdown would ap-

TABLE 7

AEROBIC PHOSPHORYLATION BY NORMAL AND IRRADIATED LIVER AND KIDNEY IN THE PRESENCE AND ABSENCE OF FLUORIDE

Tissue	Fluoride	ugm. P Esterified/ 30 mgm. Tissue		cmm. O_2 Consumed/10 Min.	
		Normal	5 Days After 800 r	Normal	5 Days After 800 r
Liver	Present	84	87	37	39
	Absent	51	-7	50	53
Kidney	Present	101	60	41	41
	Absent	-62	-113	65	59

proach or exceed the rate of formation. In experiments on the phosphorylative ability of tissues from irradiated animals a marked inhibition of the formation of phosphocreatine by kidney was observed following irradiation even in the presence of fluoride. These results might be interpreted as indicating that ionizing radiations exerted a direct action on transphosphorylases. However, phosphorylation by liver was unaffected by radiation when fluoride was present in the system but was depressed in the absence of fluoride. This finding indicated that increased phosphatase activity was the main factor responsible for the apparent inhibition of phosphorus esterification. The results obtained with kidney would seem to be explainable on the basis that fluoride was unable to inhibit completely the stimulation of phosphatases after irradiation. The results of these tests indicate that in tissues from irradiated animals the rate of hydrolysis of phosphate esters may outpace the rate of synthesis. Transphosphorylases do not appear to be affected at least in the tissues examined and it would therefore appear that further studies on phosphatases offers the best approach to an explanation of the disturbances in phosphorus metabolism produced by ionizing radiation.

SUMMARY

1. A method for the determination of 5-nucleotidase activity of animal tissues was developed and shown to be applicable to a number of animal tissues.

2. The 5-nucleotidase activity in rat liver was five times higher than in mouse liver and 13 times higher than the value for guinea pig liver. The activity of brain and skeletal muscle was similar for all three species but the activity of other tissues was generally highest in rats and lowest in guinea pigs.

3. Citrate at a final molar concentration of 1.5×10^{-2} produced about 50 per cent inhibition of the nucleotidase of liver while fluoride at the same molar concentration produced 48 per cent inhibition of liver and 18 per cent inhibition of kidney nucleotidase.

4. The rate of liberation of phosphorus from 5-adenylic acid by liver, kidney, and testes was much higher than the rate of hydrolysis of 3-adenylic acid, DPN, and phosphoglyceric acid. These findings are indicative of specificity toward 5-adenylic acid.

5. Aerobic phosphorylation of creatine by kidney tissue from irradiated mice (800 r X-rays) was markedly decreased after irradiation. This decrease was progressive during a 5-day period. No decrease in ability to oxidize succinate accompanied the loss of phosphorylative ability. The time of onset and extent of inhibition of phosphorylation was dependent upon the dose of radiation.

6. No inhibition of phosphorylation was observed in liver tissue from mice five days after 800 r of x-rays when fluoride was added to the test system. However, when fluoride was omitted normal liver was able to phosphorylate creatine but no phosphate uptake occurred with liver from irradiated mice. It thus appeared that fluoride was better able to inhibit the low phosphatase activity of liver than the high activity of kidney and the apparent inhibition of transphosphorylation with the latter tissue was due to increased phosphatase activity caused by irradiation.

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FURTHER STUDIES ON SCREENING FOR USEFUL AGENTS TO PROLONG SURVIVAL OF MICE EXPOSED TO ACUTE WHOLE BODY X-IRRADIATION

Maxwell Dauer, Ann M. Budy and John H. Rust

A previous report (1) of the results of the screening program in progress gave in some detail the theories and therapies of x-radiation sickness. The methods of treatment and exposure, and the rationale for the statistical approach were also presented in detail. In the present report, a 250 kilovolt x-ray machine operated at 15 milliamperes with added filtration of 0.5 mm. Cu + 1.0 mm Al was used. In each case, except as indicated, the animals were divided into a pre-treatment, post-treatment, irradiated control and drug control groups. The animals used were CF-1 mice. The ages of the mice and the substances used are presented in the account of each of the results.

RESULTS

Adenylic Acid. Twelve male and 12 female mice, equally divided into a pre-treated and post-treated group, ages 6-1/2 weeks, and weighing 20 to 24 grams, were each injected intraperitoneally with 13.2 mgm. of adenylic acid in 0.2 ml. of N/10 NaOH solution. Twenty-four irradiated controls were used and six drug controls. The x-ray exposure time was 18 minutes and 42 seconds for a dose of 800 r.

So far as is known, adenylic acid has not been tried as an agent to alleviate the acute x-radiation syndrome. It appears that adenylic acid, as administered and under the conditions of the experiment, has no prophylactic or therapeutic value in acute whole body radiation (Table 2).

Vitamin B_{12}, Glutathione, Desoxyribonucleic Acid. Data presented in our previous report (1), gave some indication that vitamin B_{12} given after irradiation, or glutathione, or DNA given before irradiation, increased the survival time of mice exposed to 800 r. Using a larger number of mice, a second test of each substance was made in an attempt to repeat the results of the preliminary trials. For each compound 24 males and 24 females, equally divided into a pre-treated and post-treated group, ages 4-1/2 weeks, and weighing 20 grams, were exposed to 500 r over a period of 11 minutes and 12 seconds in each of the following tests. One group of non-treated irradiated mice was used as controls for all three tests. All surviving mice were sacrificed at 62 days.

Vitamin B_{12}. One and five-tenths mgm. of vitamin B_{12} (Rubramin, Squibb) was given intraperitoneally five minutes after irradiation. The dose used was twice the amount given in the first test (1). An analysis of the data using Fischer's Exact Method gives a P of 0.23 at 30 days. The values of the per cent survivals show that Vitamin B_{12}, administered under the conditions of these experiments, has no therapeutic value.

Glutathione. A dose of 15.5 mgm. of glutathione in 0.2 ml. of propylene glycol was given intraperitoneally five minutes before irradiation. The dose was 15.5 mgm. in 0.2 ml. of distilled water. The statistical analysis of the first test gave a P of 0.05. Using Fischer's Exact Method in the analysis of the second test, a P of 0.04 was obtained. The per cent survivals are listed in Table 2.

Desoxyribonucleic Acid. A dose of 30.0 mgm. of DNA in 0.2 ml. of solution (pH 7) was given intraperitoneally five minutes before irradiation. The dose was 30 mgm. in 0.2 ml. in distilled water. The statistical analysis of the first test gave a P of 0.05. The test was repeated using older mice as well as a larger number. Fischer's Exact Method gave a P of 0.08 at 30 days. The results of the third test gave a P of 0.2. The per cent survivals are listed in Table 1.

Adenosine. Twelve males and 12 females, equally divided into a pre-treatment and post-treatment group, ages 5-1/2 weeks, and weighing 20 to 26 grams, were injected intraperitoneally with a suspension of 15.5 mgm. of adenosine in 0.2 ml. of distilled water. Twenty-four irradiated controls and six drug controls were used. The exposure time was 17 minutes and 12 seconds for a dose of 800 r.

There are no known reports upon the use of this agent in acute whole body x-radiation sickness. Table 2 shows that this agent, when used before irradiation, has no therapeutic value. As shown by the P obtained, i.e. < 0.1, but > 0.05 in the post-treated animals, this bears repeating in the future in order to test the validity of the first trial.

Ribose Nucleic Acid. Two groups of investigators (2,3) have reported some success with the use of this agent in the treatment of the acute x-radiation syndrome.

In testing the agent, three trials were made because the preliminary test suggested that this substance might be of some value after irradiation. In each case 24 irradiated controls and 6 drug controls were used. In the first test, 12 males and 12 females, equally divided into pre-treated and post-treated groups, ages 13-1/2 weeks, and weighing 20 to 25 grams, were injected intraperitoneally with 10 mgm. of ribose nucleic acid in 0.2 ml. of distilled water. The solution was allowed to stand at room temperature over a period of six weeks. Exposure time was 16 minutes and 54 seconds for a dose of 800 r. Table 2 shows that pre-treatment with RNA had no effect; however, it had some value when given after irradiation.

The second trial was made using 24 males for post-treatment, ages 4-1/2 weeks, and weighing 18 to 20 grams. The same dose was given using a freshly prepared solution of ribose nucleic acid. The results were not comparable and even suggest that the agent when given after radiation, reduced the survival time in mice.

A third trial using 12 males and 12 females for post-treatment, ages 5-1/2 weeks and weighing 20 to 24 grams was made. The same dose was used as

TABLE 1

PER CENT SURVIVALS IN A GROUPED-EXPERIMENT USING THREE SUBSTANCES

Days	Non-Treated	Treated		
		Vitamin B	Glutathione	DNA
7	95.8%	79%	91.7%	95.8%
14	33.3%	41.7%	66.6%	37.6%
21	33.3%	29.2%	58.3%	29.2%
28	33.3%	29.2%	54.3%	29.2%
35	33.3%	25%	54.3%	29.2%
42	33.3%	25%	50%	29.2%
62	33.3%	25%	50%	29.2%

TABLE 2

TABULATED SUMMARY OF THE RESULTS OBTAINED IN SCREENING AGENTS GIVEN BEFORE OR AFTER ACUTE WHOLE BODY RADIATION WITH 800 r

Agent	Dose	Pre-Treatment			Post-Treatment		
		d.f.	t value	P	d.f.	t value	P
Desoxyribonucleic acid (1)	30.0 mg	11	2.25	0.05	11	-3.03	0.01
✲ " " (1)	30.0 "	--	----	0.08	--	----	----
Ribose nucleic acid	10.0 "	11	0.45	0.7	10	1.98	0.05
" " "	10.0 "	--	----	----	23	-6.91	0.01
" " "	10.0 "	--	----	----	23	-5.15	0.01
Xanthine (1)	10.0 "	11	-0.25	0.8	11	-0.29	0.8
" (1)	15.5 "	11	----	----	23	-1.73	0.1
Xanthosine (1)	15.5 "	11	0.88	0.4	11	-3.62	0.01
Hypoxanthine	15.5 "	11	1.26	0.2	11	1.07	0.3
Guanine (1)	15.5 "	11	-0.86	0.4	11	-1.12	0.3
Guanosine (1)	20.0 "	11	0.55	0.6	10	-0.28	0.8
Adenine sulfate (1)	10.0 "	11	1.07	0.3	11	-0.60	0.6
Adenylic acid	13.2 "	11	0.49	0.6	11	-0.07	0.9
Adenosine	15.5 "	11	0.19	0.8	11	1.98	0.1
Thymine	15.5 "	10	1.87	0.1	11	-1.32	0.2
Amino Acids or Polypeptids:							
Cysteine (1)	20.0 "	11	2.45	0.05	11	0.70	0.5
✲ " (1)	20.0 "	--	----	0.10	--	----	---
✲Methionine	10.0 "	--	----	0.52	--	----	---
Histidine	13.2 "	10	1.87	0.1	11	-1.32	0.2
Glutathione (1)	15.5 "	11	2.07	0.05	11	-0.31	0.8
Hormones:							
Insulin	1.25 U	11	-1.51	0.2	11	0.14	0.9
Insulin plus desoxy-corticosterone acetate	1.25 U 1.0 mg	21	-0.13	0.9	23	-0.16	0.9
Vitamins:							
Vitamin B (1)	0.75	11	0.71	0.5	11	2.63	0.02
Miscellaneous:							
Sodium thiosulfate	110.8 mg	11	0.00	0.9	9	-2.17	0.05
Sodium nitrite (1)	2.0 "	11	-0.73	0.5	11	0.55	0.6

✲ Fischer's Exact Method
(1) Presented in the previous report - corrected data
d.f. Degrees of freedom

The agents with a negative t value and a P of 0.05 or less are considered to be harmful under the conditions of the tests.

in the previous trials, but this solution was allowed to stand at room temperature as in the first test in an attempt to duplicate the experiment. The exposure time was 18 minutes and 24 seconds for 800 r. The results are similar to the second trial. These observations are presented because of the equivocal results.

Methionine. Occasionally, autopsy findings revealed fatty livers in mice exposed to acute whole body x-radiation. Although it has been shown that cystine does not influence the survival (4), it was felt that methionine, an SH-amino acid might be of some value in protecting the liver or in increasing the survival time. Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 12 to 14 weeks, and weighing 22 to 25 grams were given intraperitoneal injections of 10 mgm. of methionine in 0.2 ml. of distilled water. Twenty-four irradiated controls and six drug controls were used. The exposure time was 19 minutes and 30 seconds for 800 r. An analysis of the data by Fischer's Exact Method indicates that there is no significant difference in the treated and the untreated animals. Gross pathologic findings were essentially the same in all the animals.

Hypoxanthine. Because of the rather interesting results obtained with xanthosine (1), a trial was made using hypoxanthine. Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 5-1/2 weeks, and weighing 18 to 25 grams, were injected intraperitoneally with 15.5 mgm. of a suspension of hypoxanthine in 0.2 ml. of distilled water. Twenty-four irradiated controls and six drug controls were used. The exposure time was 17 minutes and 2 seconds for a dose of 800 r.

The results of the tests indicate that this agent does not increase or decrease survival time when used under the conditions described.

Insulin. This hormone was empirically selected for the following tests. Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 5-1/2 weeks, and weighing 20 to 24 grams, were injected with 1.25 units of insulin* (Iletin). Twenty-four irradiated controls and six drug controls were used. This dose was arbitrarily used since it produced hypoglycemia with typical convulsions within four hours, and recovery occurred in the drug controls within eight hours after injection. The x-ray exposure time was 17 minutes and 42 seconds for 800 r. The results indicate that insulin in this amount, and under the conditions of the experiment, did not influence the survival time.

Insulin and Desoxycorticosterone Acetate. This combination was also selected empirically. In the trial, 12 males and 12 females, equally divided into a pre-treated and post-treated group, were used in each test with 24 paired controls, a total of 98 animals. One mgm. of DCA in 0.2 ml. of oil was given four hours before irradiation to insure absorption of the hormone. The mice, 6-1/2 weeks old and weighing 21 to 25 grams, were injected with 1.25

* Courtesy of the Eli Lilly Company, Indianapolis, Indiana.

units of insulin. Exposure was given over a period of 17 minutes and 1 second for a dose of 800 r. There is no beneficial effect in mice treated as described in the above tests.

Thymine. Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 5-1/2 weeks, and weighing 19 to 24 grams were injected intraperitoneally with a suspension of thymine (5-methyl uracil). Twenty-four irradiated controls and six drug controls were used. The dose given was 15.5 mgm. in 0.2 ml. of N/10 NaOH solution. The exposure time was 17 minutes and 42 seconds for a dose of 800 r. Table 2 indicates that thymine given before irradiation had some effect; whereas it somewhat decreased the survival time when given after irradiation.

Histidine. Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 6-1/2 weeks and weighing 19 to 24 grams were injected intraperitoneally with 13.2 mgm. of DL-histidine monohydrochloride in 0.2 ml. of distilled water. Twenty-four irradiated controls and six drug controls were used. The exposure time was 18 minutes and 12 seconds for a dose of 800 r. Table 2 indicates that histidine given before irradiation may be of some value. Given after irradiation, histidine decreases the survival time.

Sodium Thiosulfate. This agent was selected in view of the fact that 20 per cent thiosulfate was effective when administered to rats with partial body irradiation (5). Twelve males and 12 females, equally divided into a pre-treated and post-treated group, ages 5-1/2 weeks, and weighing 18 to 24 grams, were injected intraperitoneally with 110.8 mgm. of sodium thiosulfate in 0.5 ml. of a 20 per cent solution in distilled water. Twenty-four irradiated controls and six drug controls were used. Exposure was given over a period of 20 minutes and 30 seconds for a dose of 800 r. Table 2 shows that sodium thiosulfate given before irradiation had no effect on the survival time, whereas when given following irradiation, it significantly decreased the survival time.

SUMMARY

The agents tested seemed to be of no great value in prolonging the survival time of mice exposed to a single acute dose of whole body irradiation. However, histidine, adenosine and thymine showed some promise. On the basis of t values, it will be of interest to repeat the pre-treatment with histidine and thymine, and the post-treatment with adenosine, using a larger number of animals. A systematic testing of the nucleoproteins and their derivatives will be continued.

Glutathione, when given before irradiation, is an effective agent in prolonging the survival time in mice exposed to 500 r under the conditions of these experiments.

The substances tested for which results are presented in this report are: listed.

END

800 r:		500 r:
Adenylic acid	Insulin + DCA	Vitamin B_{12}
Adenosine	Thymine	Glutathione
Ribose nucleic acid	Histidine	Desoxyribonucleic acid
Hypoxanthine	Sodium thiosulfate	
Insulin		

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EXPOSURE OF ANIMALS TO CONTINUOUS GAMMA IRRADIATION

John F. Thomson, Magdalene S. Carter, and
Robert S. Cox, Jr. p. 38 - 45 →

RATS EXPOSED TO 20 R/DAY

Seventeen Sprague-Dawley female albino rats, with an average weight of 208.7 gm., were placed in the chamber on December 6, 1949, and have been continuously exposed to 20 r/day since that time (1,2,3), for a total of 304 days.

There have been four deaths, all occurring between 165 and 184 days of exposure (3). Because of the loss of weight shown by the four rats that died, the average weight of the group decreased slightly during the period while those animals were sick. During the past 14 weeks, the average weight has risen to 254 gm. This value is still considerably below the 293 gm. average of control rats of the same age.

There has been an increasing incidence of breast tumors in this group. Six of the thirteen survivors now bear breast tumors, an increase of two since the last report (3). Two rats have multiple tumors. Aside from the tumors, twelve of the thirteen survivors appear to be healthy. The thirteenth rat is suffering from an ulcerated tumor and probably will be the next animal to die.[1]

MORTALITY AND INCIDENCE OF TUMORS IN RATS EXPOSED TO 120 R/DAY FOR 10 AND 20 DAYS

The rats which survived limited exposure to gamma radiation from the cobalt^{60} source (1), 120 r for 10 and 20 days, have been observed for 354 and 334 days, respectively.

Seventeen rats survived the 30 day observation period following the 10 day exposure. Seven of these rats have since died, five within the last 40 days. There have been four deaths, however, among the 10 control rats. Of the ten survivors of the exposed group, seven have tumors; since four of the rats that died had tumors, the incidence in the exposed group is 11/17. The incidence among the controls is 2/10.

There were 14 survivors among the 20 rats exposed for 20 days to 120 r after the 30 day observation period. Only four of those 14 are now alive. The median survival time of this group of 14 was 313 days. All four of the survivors have developed tumors, and six of the ten dead animals had tumors, for a total of 10/14. Three of the nine control rats are dead; two tumors have been noted in this group. Thus the effect of radiation in accelerating the rate of development of spontaneous tumors is borne out by these experiments.

[1] After this report was prepared, this rat died after 306 days of exposure.

GUINEA PIGS EXPOSED TO 30 R/DAY

On May 22, 1950, a group of 20 hybrid female guinea pigs, with an average weight of 302.0 gm., were introduced into an exposure chamber (4) and exposed continuously to 30 r/day (3). Fifteen animals, averaging 271.2 gm. in weight, were set aside as controls.

The guinea pigs received Purina rabbit checkers and water daily. Three times a week they were given fresh cabbage. The guinea pigs in the exposure chamber were transferred to a clean cage on alternate days. Both groups were weighed once a week.

The first guinea pig died on the 22nd day of exposure, having lost 76 gm. in the previous week. Whether radiation was responsible for this death is not certain; this animal was the smallest of the 20 used*. The next animal died on the 44th day; during the next 45 days, 18 guinea pigs died. The last animal died after 102 days of exposure. The median survival time was 60.5 days. The average was 62.8 days, with a standard deviation of 17.3 days. These times correspond to total doses of 1815 r for the median, with an average of 1884 ± 519 r. The survival times are shown in Figure 1.

The weights of both the exposed and control guinea pigs increased steadily during the course of the experiment. The initial weight loss seen in rats exposed in our chambers occurred in only one of the guinea pigs. In some cases, there was a sudden fall in weight immediately before death. In all of the animals autopsied there was evidence of severe hemorrhages; intraluminal hemorrhages were particularly noteworthy, as was the case in animals exposed to 60 r/day (5). The weight changes are presented in Figure 2.

Another group of 20 guinea pigs has been exposed to a dose of 30 r/day for 30 days, in order to study late effects of this limited exposure.

The data obtained to date on continuous exposure of guinea pigs are presented in Table 1.

LOSS OF WEIGHT IN EXPOSED RATS

Mention has been made previously of the immediate weight loss shown by rats exposed continuously to gamma irradiation under the conditions we have employed (3). Since both the rate at which the animals lost weight and also the duration of the period of weight decrease seemed to be proportional to the dose rate of gamma radiation, it appeared that this loss was attributable primarily to effects of radiation and not to other environmental factors. Nevertheless it was considered desirable to study the effects of modification of the environment on weight loss.

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* There is a slight positive correlation between initial weight and survival. The coefficient, +0.20, indicates that this correlation is not significant.

FIGURE 1

SURVIVAL OF GUINEA PIGS EXPOSED CONTINUOUSLY
TO 30 R/DAY

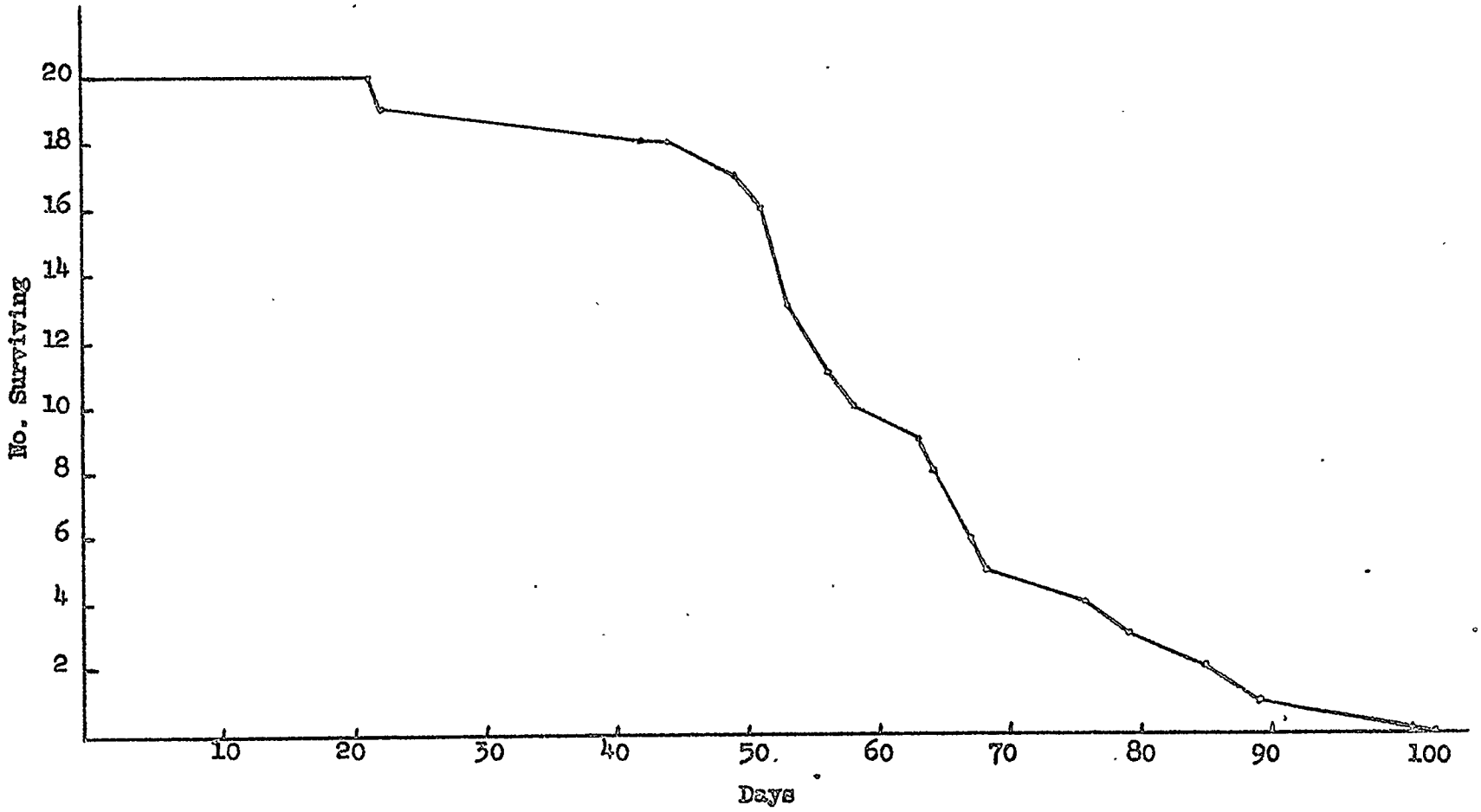


FIGURE 2

WEIGHT CHANGES IN GUINEA PIGS EXPOSED TO 30 R/DAY

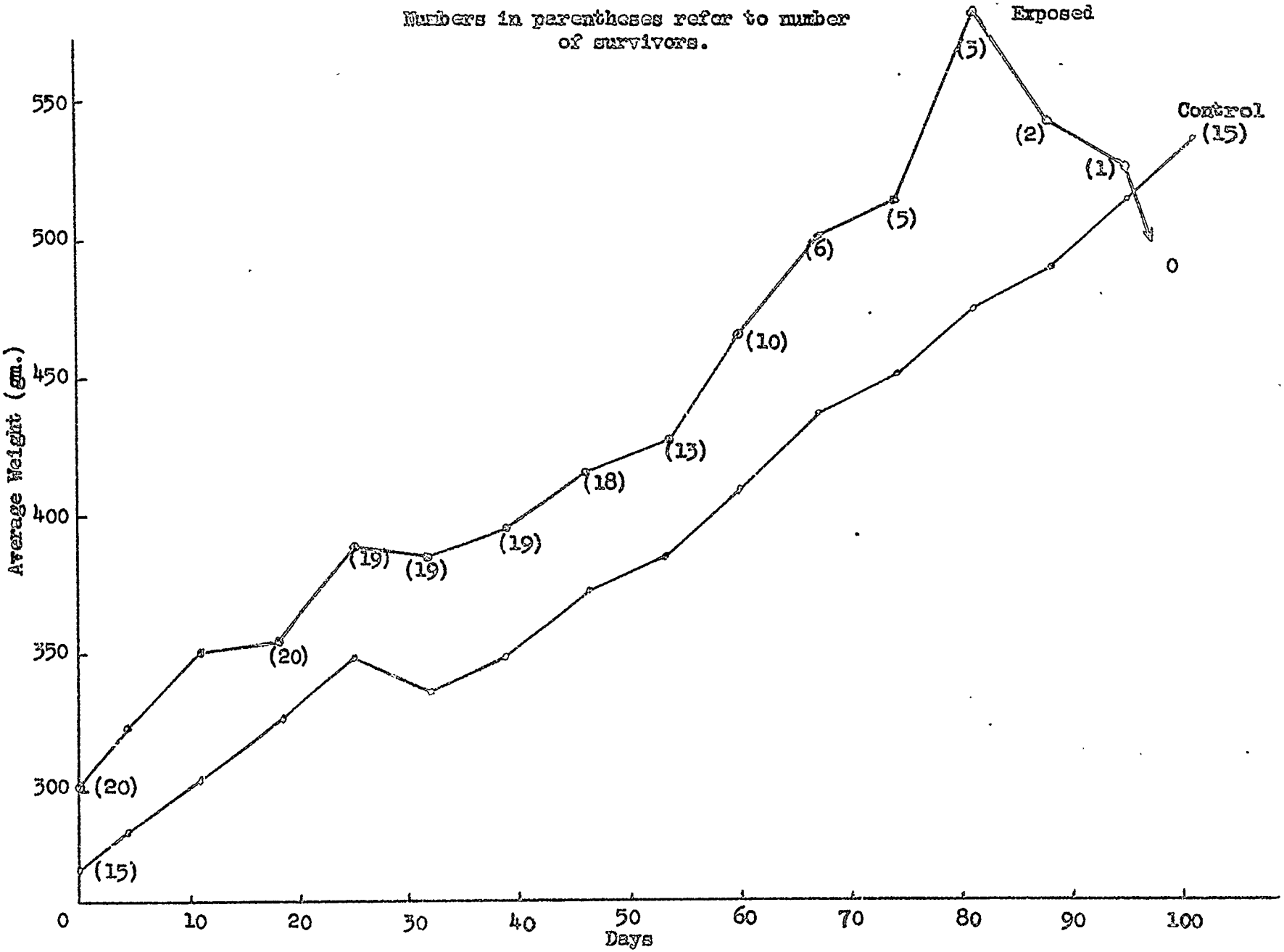


TABLE 1

EFFECT OF DOSE RATE ON SURVIVAL OF GUINEA PIGS EXPOSED TO CONTINUOUS GAMMA RADIATION

Dose (r/day)	No. Animals	Days					Mean Accumulated Dose (r)
		First Death	Median	Last Death	Average	σ	
30	20	22	60.5	102	62.8	17.3	1884
60	20	32	41	53	40.2	5.64	2412
90	25	12	18	28	17.8	3.80	1502
120	20	12	17	29	18.4	5.66	2208

Sprague-Dawley female rats have been used throughout. Their weights have varied between 210 and 250 gm., except in one study when 170 - 180 gm. rats were used. A fourteen day exposure period was used in most cases. All of the rats received Wayne Dog Blox and water ad libitum. Wayne Dog Blox are composed of meat meal, animal liver meal, riboflavin supplement, dried whey, corn flakes, wheat flakes, dried beet pulp, dried tomato pomace, soybean oil meal, coconut oil meal, bread meal, wheat germ meal, corn germ meal, brewers' yeast, cane molasses, wheat germ oil, irradiated yeast, Vitamin A and D feeding oil, 2 per cent steamed bone meal, 0.02 per cent iron oxide, 0.0043 per cent KI, 0.5 per cent $CaCO_3$, 0.0002 per cent $CuSO_4$, 0.002 per cent $MnSO_4$, and 0.5 per cent salt.

It had been noticed in all experiments carried out at a dose rate of 120 r/day that although the exposed rats ate very little of their regular ration of dog biscuits, they avidly consumed dead animals. Thus it was felt that they might more readily eat raw meat than the dog food.

"Perk" dog food was used as the meat supplement. According to the manufacturer, this product contains meat by-products, meat, liver, hearts, soya flour, bone broth, cracked wheat, wheat germ, cooked ground bone 3 per cent, 4000 international units of Vitamin D per pound, brewers' yeast 1 per cent, charcoal 0.05 per cent, iron oxide 0.02 per cent, carotene 0.004 per cent, sodium nitrite 0.002 per cent. There is about 26 per cent dry matter, 12 per cent protein, between 2 and 5 per cent fat, 3 per cent ash, 0.25 per cent Ca, 0.30 per cent P, 0.3 per cent NaCl, and not more than 1 per cent crude fiber.

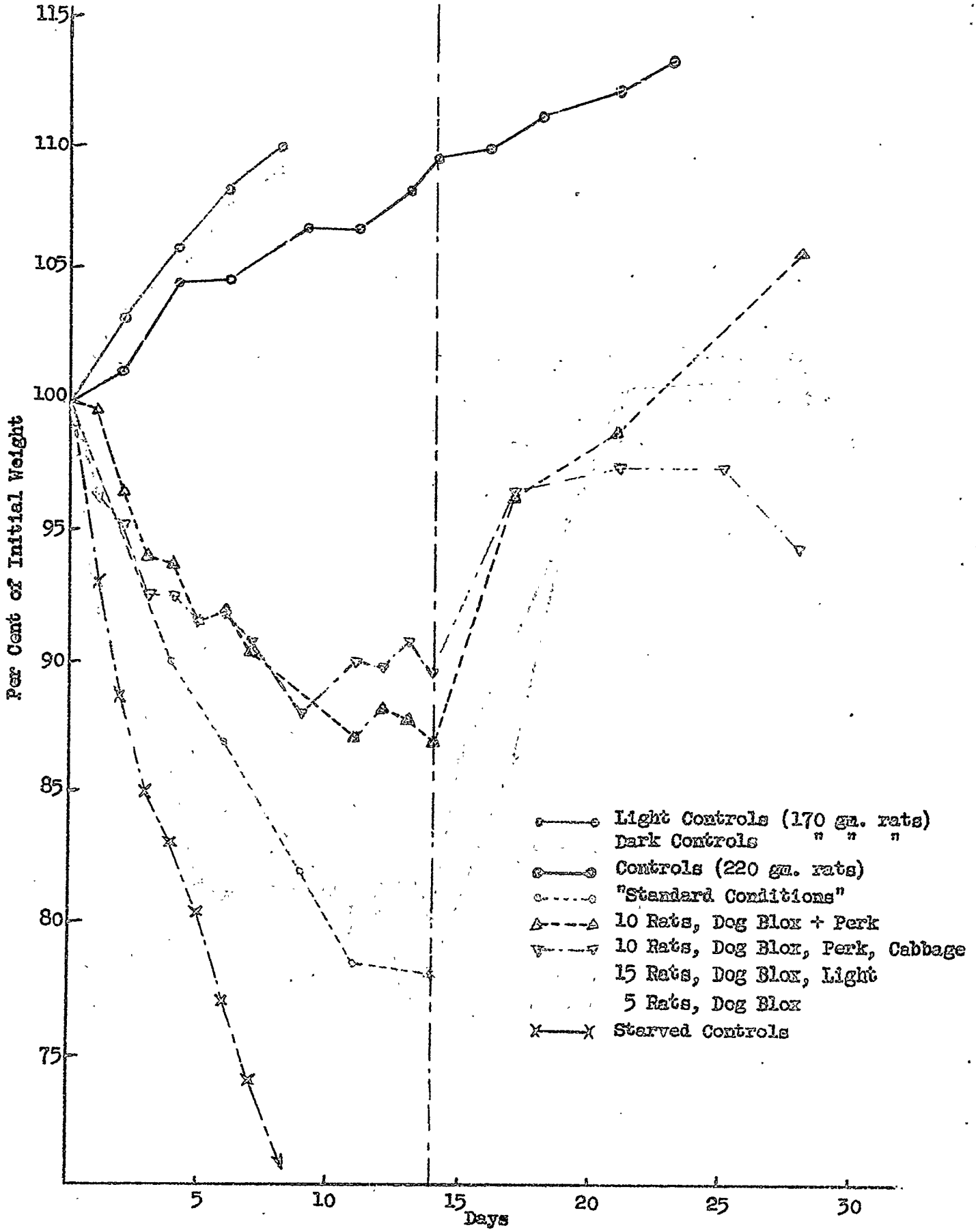
"Standard Conditions". A composite curve was constructed from the weight changes in rats exposed in groups of 20 to 120 r/day. These experiments have been reported previously (1,6). The curve (Figure 3) represents the average weight changes in 80 rats exposed to 120 r/day. These rats were kept in the dark, and were fed Wayne Dog Blox ad libitum. The temperature within the chamber was 24°C. It can be seen that their weights dropped to 78 per cent of the initial during 14 days of observation.

Effect of Crowding. It was possible that 20 rats were too many for the conditions employed, and that the weight loss may have been due at least in part to inadequate ventilation, excessive heat radiation, or physical inaccessability of food and water. However, the average weight loss seen when only five rats were exposed was as marked as when 20 were exposed; the initial rate of fall was even slightly faster, while at 14 days, the weight was 78 per cent of the initial weight, the same as with the standard conditions.

Effect of Light. The first experiment consisted of comparing over an eight day period the weight gain of unexposed rats stored in dark chambers with those exposed in the conventional stock cages. As mentioned in the last report, there was no difference. These rats weighed about 178 gm. at the start of the experiment.

A second experiment consisted of placing a 60-watt incandescent light in the chamber, and exposing 15 rats to 120 r/day with 15 hours of light and 9 hours of darkness per day. Again, the average weight loss was the same as under standard conditions. The presence of the light caused an increase in

UNDER VARYING CONDITIONS



the chamber temperature to 30.5°C. Whether any possible beneficial effects of the light may have been counterbalanced by the increased temperature has not been studied.

Effect of Meat. Ten rats were given daily supplements of about 5 cc. of Perk Dog food in addition to the Wayne Dog Blox. Their average weights fell to a value of 87 per cent of the initial weight after 14 days. Another group of rats received cabbage three times a day in addition to Perk. The cabbage did not appear beneficial in preventing the weight loss.

Controls. Data on some 120 rats used as weight controls in other experiments were combined to construct a curve for comparative purposes.

In addition, five rats were deprived of food but given water ad libitum. After nine days, the average weights had fallen to 69 per cent of the original level. The experiment was then stopped because one of the starved rats was eaten by the others.

The results of the aforementioned experiments are summarized in Figure 3. It would seem that dietary considerations, at least as far as weight loss is concerned, are more important than illumination or possible effects of crowding. Experiments are in progress to ascertain the effects of meat supplements on survival time.

SUMMARY

1/ The current status of several experiments concerned with the continuous exposure of rats and guinea pigs to gamma radiation is discussed. The results of certain modifications of the usual experimental conditions are outlined. /

END

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STUDIES ON RAT THYMUS. III. PHOSPHORYLATION

John F. Thomson, Magdalene S. Carttar, and John B. Heff

Because of the rapid decrease in concentration of ribonucleic (RNA) and desoxyribonucleic (DNA) acids in the thymus of rats following gamma irradiation from a Co^{60} source (1), it was of interest to investigate further this phenomenon. As a first consideration, we have studied the capacity of thymus homogenates to esterify inorganic phosphate, an imperative step in endergonic processes, such as nucleic acid synthesis (2), the energy for which is provided by high-energy phosphate bonds maintained by catabolic processes.

METHODS

The reaction mixture described by Potter (3) was first tried in these studies. It was found that under the conditions employed by Potter for studying esterification in kidney homogenates, the thymus of the rat showed a low oxygen uptake and little esterification. Increasing the succinate concentration increased the oxygen consumption but not the amount of phosphorylation.

After trying a number of combinations of substrates, we found that a mixture of succinate and pyruvate, both at a final concentration of 0.00^4 M, gave good oxygen uptake and phosphate esterification with 50 mg. of homogenate. Pyruvate alone failed to support esterification. The addition of coenzyme I did not materially increase either phosphorylation or oxygen consumption. These data are shown in Table 1.

Thus, except for the addition of sodium pyruvate, and increasing the amount of tissue to 50 mg. per flask, all conditions were the same as those given by Potter. In addition, in each experiment one flask was run without any substrate, since it was felt that any possible inhibition of phosphorylation might be due to an increased phosphatase activity. The differences between the esterification observed in the presence of succinate and pyruvate and the increase in inorganic phosphate seen in the absence of substrate may be taken as an index of the total capacity of the tissue to esterify inorganic phosphate against a dephosphorylation gradient.

Sprague-Dawley female rats weighing about 150 to 180 gm. were used in these studies. Their thymus weights ranged from 250 to 510 mg.

Exposures were carried out as described previously (4). In all experiments the rats were decapitated three hours after irradiation and the thymuses rapidly removed, chilled, weighed, and homogenized in nine volumes of 0.15^4 M potassium chloride. 0.5 ml. of the homogenate was used in all studies.

OBSERVATIONS

The results are tabulated in Table 2. It may be seen that there was not a significant alteration in the oxygen consumption of the thymus three hours following exposure of the animal to 800 r of gamma radiation.

TABLE 1

PHOSPHATE ESTERIFICATION IN RAT THYMUS

Each flask contained 50 mg. homogenized thymus in a final volume of 3.0 ml. Substrate concentrations were 0.004 M. Other additions according to Potter (3).

Substrate	µl O2/10'/50 mg.	µg P/20'/50 mg.*
Succinate	10.2	+ 4
Pyruvate	3.6	-24
Succinate and pyruvate	19.2	+16
None	1.0	-24

TABLE 2

PHOSPHORYLATION IN NORMAL AND IRRADIATED RAT THYMUS

	No. Rats	µg. P esterified per 20 min. per 50 mg.			µl O_2/10'/50 mg.	
		(1) Succinate + Pyruvate	(2) No Substrate	(3) Total Capacity (1)-(2)	(4) Succinate + Pyruvate	(5) No Substrate
Controls						
Average	10	+15.0	-21.0	+36.0	19.2	3.4
Std. error		2.52	2.25	3.24	1.08	0.89
Exposed						
Average	7	- 7.0	-28.0	+21.0	18.1	3.0
Std. error		5.71	3.10	3.73	0.92	0.63
"t" Values		3.52	1.84	3.04	0.8	0.3

There was, however, a marked decrease in the ability of the irradiated thymus to esterify inorganic phosphate. The esterification was negative (increased inorganic phosphate after incubation) in four of the seven animals, while only one of the three positive values fell within the broad normal range of +6 to +29 µg. esterified per 20 minutes per 50 mg. Thus despite the large deviations of both normal and exposed thymuses, the difference is large enough to be significant ($p < 0.01$).

Some of this difference may be explained by the larger amounts of inorganic phosphate found after incubation of the exposed thymus in the absence of substrate. This difference is not significant, however, and cannot account for the large difference between the total capacities of the irradiated and control rat thymuses to esterify inorganic phosphate.

DISCUSSION

The failure of the exposed tissue to show a decreased rate of oxygen utilization is in keeping with results previously reported (1,4). Barron (5) found that four hours after irradiation rat thymus slices showed no change in glycolytic or respiratory capacities.

The inhibition of phosphate esterification is of some interest because it indicates a possible explanation for the decreased concentration of nucleic acids, especially DNA, in the irradiated thymus.

The possibility that the diminution of nucleic acid concentration is referable to an accelerated phosphatase activity is not altogether ruled out, although the evidence presented here certainly militates against such a circumstance as the only mechanism of importance. Carter's findings with radiophosphorus indicate an interference with nucleic acid synthesis rather than an acceleration of breakdown (6). It may be remarked that the slight increase of inorganic phosphorus found after incubation of thymus from exposed rats, in comparison with control tissue, is in keeping with the work of several authors (7,8,9) who have reported that following X- or gamma irradiation there is an increased activity of a number of enzymes which hydrolyze various organic phosphatases.

On the other hand, further work must be done to establish a causality between the impaired phosphorylative mechanisms and the decreased DNA and RNA levels of the thymus following irradiation. It is possible, of course, that the decreased phosphorylation may be a sequel to, rather than a cause of the nucleic acid changes. It is also noteworthy that no change in the concentration of phospholipid phosphorus was seen in the thymus of animals irradiated and sacrificed three hours after exposure (unpublished results); tentatively, this would imply that the interference with DNA synthesis was independent of phospholipid synthesis. Since, moreover, the incorporation of P-32 into phospholipids requires the same coupled oxidative system as incorporation into RNA (2), there is some question as to the validity of the impedence of esterification as a primary metabolic defect induced by radiation.

SUMMARY

Three hours after exposure to 800 r, the thymuses of irradiated rats showed a decreased capacity to esterify inorganic phosphate. This impairment of phosphorylation was not accompanied by a decreased rate of oxygen consumption.

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CHARACTERIZATION OF RAT LIVER RIBONUCLEIC ACIDS

I. PRELIMINARY NUCLEOTIDE ANALYSES OF SUBCELLULAR FRACTIONS OF RESTING AND REGENERATING RAT LIVER

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INTRODUCTION

The problem of the composition of the ribonucleic acids of animal tissues, and even of yeast, is far from solved. Apparently the most native ribonucleic acids that have been prepared to date are those from plant viruses (1,2). The great sensitivity of yeast ribonucleic acid to the methods of preparation has been shown by Fairley (3), and these data are reasonably extensible to animal ribonucleic acids. The ribonucleic acid of beef pancreas has been shown to be extremely labile (4,5), and consequently different nucleotide ratios have been reported for this substance, based upon analyses of the isolated ribonucleic acid (6-8). It therefore seemed advisable to attempt to determine nucleotide ratios *in situ*, i.e. without prior isolation of the ribonucleic acid. For this purpose homogenization of the tissues in trichloracetic acid and extraction of the phospholipids with alcohol-ether by the method of Schneider (9) followed by the separation of the desoxyribonucleic acid from the ribonucleic acid by the method of Schmidt and Thannhauser (10) appeared satisfactory. Although this did not give a pure preparation of the ribonucleic acid it was possible to analyze for the constituent nucleotides by the methods described below.

It has been shown by Taylor, Greenstein, and Hollaender (11) that x-rays cause depolymerization of desoxyribonucleic acid and Errera (12) has studied the effect of x-rays on nucleoproteins *in situ* and *in vivo*. It was thought that it might be of interest to investigate the action of gamma radiation on the ribonucleic acid *in vivo*. For this purpose rat liver appeared to be a suitable tissue since it is easy to obtain in reasonably large quantities from a single animal, and is a relatively homogeneous tissue. In addition it was possible to remove part of the liver and obtain a preparation of rapidly growing (regenerating) liver tissue. Before proceeding with the radiation work, however, it was deemed advisable to characterize the ribonucleic acids present in normal and regenerating rat liver.

The ribonucleic acid of adult animal cells has been shown by Brachet et al (13) to be present largely in the form of sedimentable granules while that in rapidly growing cells is sedimentable to a lesser extent. Schneider (14) has separated homogenized tissue into four fractions by differential centrifugation: (1) nuclei and unbroken cells, (2) mitochondria, (3) particles, and (4) supernatant. All of these fractions were shown to contain ribonucleic acid. Chantremme (15) has shown that if the centrifugation is extended to 102,000 times gravity that all the ribonucleic acid is sedimented. He arbitrarily divided his preparations into six fractions instead of the four of Schneider. The division of the homogenatesinto four fractions, as done by Schneider is purely empirical; but a division of the cellular constituents is obtained which furnishes a standard basis for determining differences between different tissues or different forms of the same tissue. The mitochondria fraction contains the majority of the Janus Green B staining material, which has been termed mitochondria. Since so much difference has

been observed in the analyses of different preparations of nucleic acid from the same source it was decided to analyze the nucleic acid from each of the fractions of Schneider. Much evidence indicates that the preparation ribonucleic acid obtained from whole cells is not a single homogeneous compound, but rather a mixture of ribonucleic acids. If sufficient fractionation of cell constituents were carried out, however, a more nearly homogeneous preparation might be obtained, and more constant analytical results possible.

This paper deals with the preparation of rat liver homogenates from both normal and regenerating liver, their fractionation by the method of Schneider, and the nucleotide analyses of the ribonucleic acid fractions, recovered by the method of Schmidt and Thannhauser. The ribonucleic acid from the total cell homogenate was also isolated and analyzed as above.

METHODS

Sprague-Dawley rats were used for all of the following work. They were hepatectomized by the method of Brues, Drury, and Brues (16) and the removed portion of the liver weighed and used for the experiments on resting liver. The liver was allowed to regenerate for three days, and then the rat was killed and the regenerated portion of the liver removed, weighed, and used for the experiments on regenerating liver. The liver of each rat was kept separate and no experiments were done on pooled rat livers.

The livers were placed in ice cold 10 per cent trichloracetic acid immediately after removal, homogenized and fractionated into nuclei and intact cells, mitochondria, particles, and supernatant by the method of Schneider (14) previously described (17). The latter three fractions were then treated by a combination of the methods of Schneider (9) and Schmidt and Thannhauser (10) as previously described (17) to obtain a solution of the ribomononucleotides. The latter method was modified in that sulfuric acid was used for the precipitation of the desoxyribonucleic acid instead of hydrochloric acid; so silver precipitation of the purines could be carried out on the ribonucleic acid fraction without the interference of chloride ion. This, however, prevented the determination of phosphoprotein phosphorus since the presence of sulfate ion prevented either calcium or barium precipitation of inorganic phosphate. For another experiment whole liver homogenates were treated as above and a solution of the nucleotides obtained.

One tenth volume of 10 N sulfuric acid was added to these solutions of ribomononucleotides and they were hydrolyzed for two hours, freeing the purine bases and leaving the pyrimidine ribomononucleotides intact (18). This hydrolysis also effectively destroyed all the trichloracetic acid remaining in the solution (19). These solutions were then diluted to 25 ml. and analyzed for phosphorus by the method of King (20). The phosphorus content varied from 400 to 1500 micrograms per 25 ml. The free purine bases were then precipitated from 3 ml. aliquots of this acidic solution using a modification of the method of Schmidt and Levene (21). Two ml. of hot saturated silver sulfate solution were added to each of the 3 ml. aliquots and the tubes placed in a boiling water bath for 10 minutes. The tubes were then placed in ice in the refrigerator overnight, and centrifuged. The precipitate was then washed three times with 50 per cent methyl alcohol made 0.25 N with respect to sulfuric acid,

being kept ice cold all the time. The combined supernatant and washings were used for the analysis for the pyrimidine ribonucleotides, and the precipitate for the purine bases.

The analysis for the purine bases was carried out by taking the silver precipitate obtained above and extracting it three times with 2 ml. portions of 0.1 N hydrochloric acid. Each time the tubes were placed in a boiling water bath for 15 minutes, and the precipitate frequently stirred. The tubes were then cooled in ice and centrifuged. The combined extracts were then diluted to 25 ml. with 0.1 N hydrochloric acid. This solution was then placed in silica absorption cells 1 cm. wide with a light path of 1 cm., and the absorption spectrum taken between 220 and 300 mμ using a Beckman Model DU spectrophotometer with ultraviolet attachments. A blank was always run through the whole procedure and used in the first cell. This solution contained both the adenine and guanine and since the absorption curves of pure adenine and guanine in 0.1 N hydrochloric acid were accurately known (22) it was possible to calculate the concentration of both constituents by the following method.

At any wave length (λ_1) the density (D) equals the molar extinction coefficient of component 1 (ε_1) times its concentration (c_1) plus the molar extinction coefficient of component 2 (ε_2) times its concentration (c_2). If the expressions for the densities at two different wave lengths

$$D_{\lambda_1} = \varepsilon_{1\lambda_1} c_1 + \varepsilon_{2\lambda_2} c_2 \text{ and } D_{\lambda_2} = \varepsilon_{1\lambda_2} c_1 + \varepsilon_{2\lambda_2} c_2)$$

are solved simultaneously the following expressions are obtained:

$$c_1 = \frac{D_2 \varepsilon_{2\lambda_1} - D_{\lambda_1} \varepsilon_{2\lambda_2}}{\varepsilon_{1\lambda_2} \varepsilon_{2\lambda_1} - \varepsilon_{1\lambda_1} \varepsilon_{2\lambda_2}} \qquad c_2 = \frac{D_{\lambda_1} \varepsilon_{1\lambda_2} - D_{\lambda_2} \varepsilon_{1\lambda_1}}{\varepsilon_{1\lambda_2} \varepsilon_{2\lambda_1} - \varepsilon_{1\lambda_1} \varepsilon_{2\lambda_2}}$$

It will be noted that for any pair of wave lengths the denominator is the same regardless of the concentrations of the compounds. For adenine and guanine a careful examination of the curves of the two substances indicates that 262 and 285 mμ are a convenient pair of wave lengths to choose for this calculation. At these points both curves are a considerable distance apart and neither one is changing its slope with extreme rapidity, 262 mμ being the maximum for adenine and 285 mμ an approximately level region of the curve for guanine. The molar extinction coefficients at 262 mμ are adenine 13290 and guanine 7590, while those at 285 mμ are adenine 1820 and guanine 5380. The denominator is 5.77×10^7. In addition to calculating the concentrations of the individual purine components in this manner the total purine concentration may be calculated by taking a point where the adenine and guanine curves cross and using the common molar extinction coefficient to calculate the total concentration. One cross point is 276 mμ, and the molar extinction coefficient at that point is 7100.

In addition guanine was determined independently on many of the samples by the colorimetric method of Hitchings (19). For this purpose the purines were precipitated with silver as described above, the silver precipitate extracted with 0.1 N hydrochloric acid, and diluted to 25 ml. Three ml. aliquots were thus analyzed colorimetrically. Since adenine gives no color with the reagent this gives an independent check of the guanine concentration.

As has been stated the combined washings and supernatant from the silver precipitation were used in the analysis for the pyrimidine ribomononucleotides. It had been shown (22,24) that when using silver fractionation on a purified sample of nucleic acid this solution could be analyzed directly spectrophotometrically. The main question in this case was whether sufficient interfering substances were still present in the sample after the Schmidt-Thannhauser purification to interfere with this method. It has already been pointed out that the highly absorbing trichloracetic acid should be largely destroyed, and this was borne out in blanks that were run. It was decided to take these combined supernatants and determine the absorption curves under different pH conditions and see if the results could be satisfactorily proved. In addition the precipitation of the pyrimidine nucleotides with silver in basic solution after the addition of three volumes of isopropyl alcohol (18) was attempted unsuccessfully.

For the spectrophotometric determination the combined washings and supernatant were freed of silver by the addition of solid sodium chloride and centrifuged in the cold after having been heated in a boiling water bath. The silver-free solution was then diluted to 20 ml. and adjusted to pH 2.0 using a Beckman Model G pH Meter with external glass electrode. The solution was finally diluted to 25 ml. and the absorption curve taken using the Beckman spectrophotometer previously described. Again a blank was run through the whole procedure and used in the first cell. Since the solution was assumed to be a mixture of uridylic and cytidylic acids the calculations from the ultraviolet absorption curve were carried out in the same way as has been described under the purine determination. The calculations were carried out at 260 and 278 mμ. The molar extinctions coefficients (22) at 260 mμ are uridylic acid 9980 and cytidylic acid 6720, while those at 278 mμ are uridylic acid 4160 and cytidylic acid 12720. The denominator for the simultaneous equations is 9.90×10^7. The curves for uridylic acid and cytidylic acid at pH 2.0 cross at 266 mμ so it is possible to calculate the total pyrimidine nucleotide concentration at that wave length. The common molar extinction coefficient is 9390 (22). It would appear to be a fairly good check on the results obtained if the total moles of pyrimidine nucleotide calculated at 266 mμ equalled the total moles calculated from the sums of the uridylic and cytidylic acids determined at 260 and 278 mμ.

As an additional check, however, the silver free solutions were diluted to about 20 ml., sufficient disodium phosphate added (173 mg.) to make the final concentration 0.05 M, the solution adjusted to pH 7.0 using the pH meter, and finally diluted to 25 ml. The ultraviolet absorption spectrum of this solution was also taken and the results calculated as above using the densities at 260 and 280 mμ. Again a blank was used in the first cell. The molar extinction coefficients at 260 mμ (22) are uridylic acid 10090 and cytidylic acid 7670, while those at 280 mμ are uridylic acid 3470 and cytidylic acid 6860. The denominator for the simultaneous equations is 4.26×10^7. At pH 7.0 the curves of uridylic acid and cytidylic acid cross at 247.5 and 268 mμ with common molar extinction coefficients of 7100 and 8600 respectively (22). Again the total number of moles of pyrimidine nucleotide calculated at the cross points should check the total number of moles calculated from the sum of the uridylic acid plus the cytidylic acid.

A further additional check of the concentration of cytidylic acid is possible. The molar extinction coefficient of uridylic acid in 0.05 M pH 7.0

phosphate and in 0.01 N acid is approximately the same at 278 mμ (4430 and 4160), while the difference between the molar extinction coefficients of cytidylic acid at pH 2.0 and pH 7.0 at 278 mμ is 5170 (12720 at pH 2.0 and 7550 at pH 7.0)(22). If, therefore, the spectrum of the solution is taken at both pH's the approximate concentration of cytidylic acid may be calculated by using the change in the density at 278 mμ (i.e. $\Delta D_{278} = \Delta\varepsilon_{278}c$).

As will be shown below the agreement between these different methods was not good with respect to the ratio of cytidylic to uridylic acid, but the total moles of pyrimidine nucleotide checked well by the different methods. Further work, therefore, needs to be done to determine the relative amounts of uridylic and cytidylic acids. The paper chromatographic method of Carter (23) appears to offer the most satisfactory solution. In all the spectra run on pyrimidine nucleotide samples the amount of absorption in the shorter ultraviolet wave lengths (220 to 250 mμ) was much greater than could be predicted on the basis of a mixture of pyrimidine nucleotides. It must therefore be assumed that interfering substances were present. The nature of this interference will be discussed later in this paper. The fact that the total moles of pyrimidine nucleotide checks well by the different methods and that it also checks between the cross points of the curve and the total moles calculated from the simultaneous equations indicates that the errors are due to a warping of the curve and not to gross errors in the magnitude of the whole absorption spectrum.

These methods of analysis have been thoroughly checked and tested in previous work with regard to their reliability when used on pure nucleic acid samples (10,22,24).

RESULTS

Check Analyses of Yeast Nucleic Acid (Schwarz BN 4705)

As a check of the effect of the Schmidt-Thannhauser method of purification on the analytical method described above a pure sample of yeast nucleic acid was analyzed directly by the methods described and also after treatment according to the Schmidt-Thannhauser method (17). This experiment gives a direct check of the effect of the basic hydrolysis and the trichloracetic acid extraction on the subsequent acid hydrolysis and analysis by the ultraviolet absorption spectra of the silver insoluble and silver soluble fractions. It does not control the effect of other impurities that might be carried over into the "ribonucleic acid" fraction from the tissue homogenate.

For the experiment 100 mg. of nucleic acid (Schwarz BN 4705) was dissolved in 50 ml. of distilled water. Twenty-five ml. of the solution were put through the Schmidt-Thannhauser procedure and finally diluted to 50 ml., while the remaining 25 ml. were diluted to 50 ml. The resulting solutions were then acid hydrolyzed, 3 ml. aliquots fractionated with silver, and the resulting supernatants and washings adjusted to pH 2.0 and diluted to 100 ml. The acid extracts of the silver precipitate were also diluted to 100 ml. The results of these analyses are summarized in Table 1.

These data indicate that the Schmidt-Thannhauser treatment does not affect the results obtained by this method of analysis, and that after such

TABLE 1

NUCLEOTIDE ANALYSES OF YEAST NUCLEIC ACID
(SCHWARZ HN 4705)

Nucleotide	Mg./Mg. of Nucleic Acid	
	Direct Analysis	Schmidt-Thannhauser
Guanylic acid	0.296	0.292
Adenylic acid	0.222	0.215
Uridylic acid	0.218	0.219
Cytidylic acid	0.173	0.167

treatment this method is valid for use with tissue homogenates if additional impurities can be eliminated.

Analyses of the Ribonucleic Acids of Rat Liver Homogenates

As has been previously described rats were hepatectomized and then the livers allowed to regenerate for three days. Eight-hundred to 900 mg. of the normal and regenerating livers were homogenized in cold trichloracetic acid and analyzed for the constituent ribomononucleotides after purification by the methods of Schneider and of Schmidt and Thannhauser as previously detailed. This experiment was carried out to determine if any differences could be detected between the ribonucleic acids of normal and regenerating rat liver, before proceeding to the analyses of the nucleic acids of the subcellular components of the liver cells. The livers of three different rats were analyzed in this manner, and duplicate samples were run in parallel throughout. All the analyses for the pyrimidine nucleotides were carried out at pH 2.0, and were not checked in pH 7.0 phosphate. The results of these experiments are given in Table 2.

Examination of these results appears to indicate that the analyses are valid since a satisfactory check is found between calculated and observed phosphorus values. Although the ratios observed are very different from those reported for yeast nucleic acid (See Table 1, and 25), they are more in line with those reported for human liver and other animal tissues by Chargaff et al (26) using paper chromatograms. The great discrepancy between Chargaff's uridylic to cytidylic acid ratio and that observed here should be noted. As has been mentioned later work indicated that results more similar to those found by Chargaff are obtained if the pyrimidine nucleotide analyses are carried out in pH 7.0 phosphate instead of 0.01 N acid. Further work is in progress to clarify this point. The pyrimidine to purine ratio observed in this work is also considerably greater than that found by Chargaff, but on the basis of our work described later these data appear to be reliable. Further checks on the concentrations of the pyrimidine nucleotides should ascertain this more certainly, however. The trends of these results are discussed more fully in the discussion.

Analyses of the Ribonucleic Acids of Rat Liver Subcellular Fractions

Since appreciable differences appeared to exist in the compositions of the ribonucleic acid from resting and regenerating rat livers it was decided to determine if these differences could be localized in any of the subcellular fractions, or whether it was a general property of all the ribonucleic acid in the liver cell. In addition this procedure would show any differences normally existing in the ribonucleic acids of the different subcellular fractions arbitrarily chosen by Schneider (14). The differential centrifugation and the isolation of the ribonucleic acid from the mitochondria, particles, and supernatant fractions was carried out as described under Methods. All determinations were run in duplicate. The results of these experiments are not strictly comparable with those of the total liver homogenate since there is ribonucleic acid in the nuclei, and also there is considerable additional loss by adsorption to this fraction. The nucleic acid of the nuclei was not analyzed since the concentration of ribonucleic acid is very small compared to that of the

TABLE 2

NUCLEOTIDE ANALYSES OF WHOLE RAT LIVER HOMOGENATES

Rat Number	Resting Liver					Regenerating Liver				
	Molar Ratios			Mg. Phosphorus/25 ml.		Molar Ratios			Mg. Phosphorus/25 ml.	
	Guanine / Adenine	Uridylic / Cytidylic	Pyrimidine / Purine	Calculated	Found	Guanine / Adenine	Uridylic / Cytidylic	Pyrimidine / Purine	Calculated	Found
1	3.00	0.91	2.41	0.692	0.661	3.14	0.88	2.05	1.040	1.072
2	4.10	0.93	2.00	0.700	0.673	3.02	0.83	1.73	0.976	0.976
3	3.90	0.85	1.90	0.842	0.801	2.96	0.91	1.71	0.960	0.986

desoxyribonucleic acid, and for these preliminary experiments it was difficult to isolate sufficient material in order to carry out reasonably accurate analyses. In the initial experiments the analyses for the pyrimidine nucleotides were only carried out at pH 2.0, while later on check analyses were run on some samples in 0.05 M phosphate at pH 7.0. The results of these analyses are shown in Table 3, while the check analyses of the pyrimidine nucleotides in phosphate are shown in Table 4. In addition Table 4 shows the good checks obtained between the sum of the uridylic and cytidylic acids calculated at pH 2.0 at 260-278 mμ and the total calculated at the cross point at 266 mμ. Check analyses of guanine by the colorimetric method of Hitchings (19) and total purine nitrogen analyses are shown in Table 5.

The analyses in Table 3 show a good check between the calculated phosphorus values and those actually found by analysis. The single exception is that the calculated phosphorus values are consistently low in the mitochondria fractions, which would appear to indicate the presence of another phosphorus containing compound in this fraction. Further work is contemplated to attempt to explain this discrepancy more thoroughly. The general trend and significance of these results will be discussed more fully in the Discussion.

As has already been mentioned it was decided to check the concentrations of the pyrimidine nucleotides that had been found by ultraviolet absorption analysis of the supernatants and washings adjusted to pH 2.0 by adjusting the solutions to pH 7.0 after making them 0.05 M in phosphate. For this purpose an aliquot of the solution that was read at pH 2.0 was adjusted to pH 7.0 after the addition of phosphate (solid disodium phosphate). This was done so that individual differences between samples would be eliminated and as accurate a check procedure as possible would be carried out. The values obtained in phosphate were then corrected for difference in volume so that a check should be obtained with the pH 2.0 values. In addition cytidylic acid was calculated independently by the change in density at 278 mμ between pH 2.0 and pH 7.0 as previously described. The total number of moles of cytidylic plus uridylic acid obtained from the sum of the two constituents calculated at 260-278 mμ at pH 2.0 and at 260-280 mμ at pH 7.0 were also compared with the totals calculated from the common molar extinction coefficient at the points where the two curves cross (266 mμ at pH 2.0 and 247.5 and 268 mμ at pH 7.0). These results are summarized in Table 4.

The results in Table 4 indicate no correspondence between the results obtained at pH 2.0, pH 7.0, and cytidylic acid calculated by density difference at 278 mμ. The total moles calculated by the sum of the uridylic and cytidylic acids at pH 2.0 corresponds well with the total found at the cross point. The same holds true in the pH 7.0 determination. Thus the curves are internally self consistent. In addition the total moles at pH 2.0 and pH 7.0 are in fairly good agreement; so the agreement of the total phosphorus values reported in Table 3 appears to be still valid. The explanation for the differences in the results between pH 2.0 and pH 7.0 cannot be explained at this time, but further work is being undertaken by paper chromatography to resolve this point.

It will be noted, however, that the value for cytidylic acid by difference in density at 278 mμ is considerably lower than that found by simultaneous calculation at either pH 2.0 or pH 7.0. Since the total moles of pyrimidine nucleotide found appears to check well with the observed phosphorus values it

TABLE 3

NUCLEOTIDE ANALYSES OF SUBCELLULAR FRACTIONS OF RAT LIVER

Rat Number	Resting Liver					Regenerating Liver				
	Molar Ratios			Mg. Phosphorus/25 ml.		Molar Ratios			Mg. Phosphorus/25 ml.	
	Guanine / Adenine	Uridylic / Cytidylic	Pyrimidine / Purine	Calculated	Found	Guanine / Adenine	Uridylic / Cytidylic	Pyrimidine / Purine	Calculated	Found
MITOCHONDRIA										
1	4.44	0.99	2.48	0.631	0.740	4.86	0.94	2.30	0.713	0.867
2	2.64	0.82	1.86	0.892	1.060	3.89	0.89	2.09	0.510	0.605
3	3.22	0.84	2.02	0.881	1.060	3.56	0.85	1.83	0.873	1.020
4	3.02	0.84	1.92	0.924	1.130	3.51	0.92	1.96	0.811	0.914
PARTICLES										
2	3.81	0.99	1.82	0.429	0.565	3.87	0.93	1.61	0.678	0.660
3	4.62	0.92	1.73	0.535	0.564	3.60	0.87	1.48	0.920	0.875
4	3.74	0.86	1.60	0.761	0.740	3.76	0.92	1.57	0.818	0.796
SUPERNATANT										
1	4.21	0.86	2.21	0.816	0.780	3.76	0.82	1.74	1.747	1.680
2	3.07	0.80	2.03	1.127	1.090	3.35	0.85	1.68	1.156	1.080
3	3.48	0.85	2.06	1.031	1.060	2.55	0.74	1.48	1.760	1.590
4	3.03	0.82	1.85	1.453	1.380	2.93	0.82	1.46	1.560	1.490

TABLE 4

COMPARISON OF PYRIMIDINE NUCLEOTIDE ANALYSES AT pH 2.0 AND pH 7.0

Fraction[3]	Determination	Moles[1] at pH 2.0 260-278 mμ	Moles[1] at pH 7.0 260-280 mμ	Moles[1] 278[2] mμ	Total Moles[1] pH 2.0 266 mμ	Total Moles[1] pH 7.0 247.5 mμ	Total Moles[1] pH 7.0 268 mμ
Rat 1 Resting Mitochondria	Cytidylic acid	3.51	5.37	2.40	----	----	----
	Uridylic acid	3.46	1.74	----	----	----	----
	Total Pyrimidine	6.97	7.11	----	6.74	7.70	6.98
	Molar ratio U:C	0.99	0.32	----	----	----	----
Rat 1 Resting Particles	Cytidylic acid	0.86	1.28	0.46	----	----	----
	Uridylic acid	0.92	0.67	----	----	----	----
	Total Pyrimidine	1.78	1.95	----	1.78	1.85	1.98
	Molar ratio U:C	1.07	0.53	----	----	----	----
Rat 1 Resting Supernatant	Cytidylic acid	4.68	8.21	1.79	----	----	----
	Uridylic acid	4.02	1.82	----	----	----	----
	Total Pyrimidine	8.70	10.03	----	8.41	10.07	9.59
	Molar ratio U:C	0.86	0.22	----	----	----	----
Rat 1 Regenerating Mitochondria	Cytidylic acid	3.95	6.50	1.70	----	----	----
	Uridylic acid	3.73	2.13	----	----	----	----
	Total pyrimidine	7.68	8.63	----	7.51	8.79	8.43
	Molar ratio U:C	0.94	0.33	----	----	----	----
Rat 3 Regenerating Mitochondria	Cytidylic acid	4.71	7.38	2.21	----	----	----
	Uridylic acid	3.99	1.73	----	----	----	----
	Total pyrimidine	8.70	9.11	----	8.51	9.00	8.95
	Molar ratio U:C	0.85	0.23	----	----	----	----
Rat 3 Regenerating Supernatant	Cytidylic acid	9.33	14.65	4.68	----	----	----
	Uridylic acid	6.94	3.00	----	----	----	----
	Total pyrimidine	16.27	17.65	----	15.94	17.17	17.31
	Molar ratio U:C	0.74	0.21	----	----	----	----

[1] Values expressed as moles x 10^{-5} in the solution read in the spectrophotometer.
[2] Cytidylic acid calculated by the change in density between pH 2.0 and pH 7.0.
[3] Rat numbers and fractions correspond to those in Table 3.

would seem to eliminate the possibility of a large amount of non-nucleic acid adsorption at all wave lengths. If the low uridylic to cytidylic acid ratios found at pH 7.0 are assumed to be more correct (as this is in line with Chargaff's results on human liver (26)) then the high ratio found at pH 2.0 could be explained by excessive absorption at 260 mμ, due to impurities carried through the Schneider and Schmidt-Thannhauser procedures from the liver homogenate. The low cytidylic acid value by density difference at 278 mμ could well be caused by an impurity extinction coefficient of which at 278 mμ is greater at pH 7.0 than at pH 2.0. Thus, the apparent density difference would be much less than the actual change due to cytidylic acid. Such an increase in the absorption at pH 7.0 in the vicinity of 278 mμ would also raise the cytidylic acid value calculated simultaneously at 260 and 280 mμ. This effect would be especially apparent if the increase in absorption at pH 7.0 around 278 mμ were also accompanied by a corresponding decrease around 260 mμ. On the basis of these suppositions the true uridylic and cytidylic acid values probably lie somewhere between those found at pH 2.0 and pH 7.0. Examination of the simultaneous equations used for calculation shows that a small amount of impurity changing the spectrum as described above could account for the differences observed without radically altering the total pyrimidine nucleotide concentration. This is largely true because the molar extinction coefficients of nucleic acid and its derivatives are considerably higher than most other common biological materials.

As has previously been mentioned guanine was checked independently by the colorimetric method of Hitchings (19). In addition the total purine content of two samples was checked by total nitrogen analyses (semi-micro Nesslerization)(27) of the silver insoluble fraction. For the nitrogen analyses 3 ml. aliquots of the original 25 ml. dilution of the ribonucleic acid obtained from the Schmidt-Thannhauser purification were used. For the guanine determination the silver precipitate from a 3 ml. aliquot was extracted with 0.1 N hydrochloric acid in the same manner as was done previous to analysis by ultraviolet spectrophotometry. The colorimetric test of Hitchings was then applied directly to the extract. The copper precipitation method for isolation of guanine originally described in Hitching's paper (19) was also tried on the original solution, and gave comparable results. The results of these nitrogen and guanine analyses are summarized in Table 5.

It may be seen from Table 5 that the colorimetric guanine values check closely with those found by calculation from the ultraviolet data, and that the total purine nitrogen found agrees fairly well with that calculated from the results of the ultraviolet spectrophotometry.

DISCUSSION

The significance of the results has been briefly discussed at the end of each section, but a few additional comments on the trends shown are necessary. It will be noted that there is an extreme amount of individual variation between individual rats, although all the rats were of the same strain and approximately the same age and weight. Marrian et al (28) have also observed extreme variation between individual rats in the incorporation of labeled purines into nucleic acid. This variation makes interpretation of results difficult with the small number of cases available to date. Another difficulty is the unreliability of the pyrimidine

TABLE 5

GUANINE AND PURINE NITROGEN ANALYSES

Fraction[1]	Mg. Guanine per 25 ml.		Total Mg. Purine Nitrogen per 25 ml.	
	Colorimetric	Spectrophotometric	Nesslerization	Spectrophotometric
Rat 3 Resting Mitochondria	-----	-----	680	659
Rat 3 Resting Supernatant	-----	-----	804	765
Rat 1 Resting Mitochondria	0.718	0.722	---	---
Rat 1 Resting Supernatant	0.960	1.001	----	---
Rat 1 Regenerating Mitochondria	0.938	0.875	---	---
Rat 1 Regenerating Particles	1.336	1.202	---	---
Rat 1 Regenerating Supernatant	2.581	2.457	---	---

[1] Rat numbers and fractions correspond to those in Table 3

nucleotide ratios as shown in Table 4 As has been pointed out, however, the total pyrimidine nucleotide concentration appears to check satisfactorily, and hence the pyrimidine to purine ratio seems valid. With the analytical methods used in this work under the conditions of these experiments accuracy cannot be assumed to be greater than 5 to 7 per cent.

The most striking difference between resting and regenerating liver is a lower pyrimidine to purine ratio in the regenerating fractions. This is clearly shown in the total homogenate, and is especially prominent in the supernatant fraction. The particles show this effect, but it does not appear to be present in the mitochondria. The total liver homogenates appear to indicate a drop in the guanine to adenine ratio from the resting to regenerating state but this does not appear to be borne out in the portions of the ribonucleic acid analyzed in the subcellular fractions. The supernatant fractions show this tendency more than the others, but the trend is not clear-cut. The high guanine to adenine ratios (2.5-4.5) are in line with the results of Chargaff (26) on human liver, but the high pyrimidine to purine ratios (1.5-2.5) differ markedly from his values approximating 1.0.

The observation of Brachet et al (13) that more of the ribonucleic acid is present in the more difficultly sedimentable fractions is rapidly growing (regenerating) tissue is confirmed as can be seen from the phosphorus values in Table 3. In resting liver the phosphorus values for the mitochondria and supernatant are approximately equal, while in regenerating liver the value for the mitochondria is only 40 to 60 per cent that of the supernatant. The particles have less than either of the other fractions. As has already been mentioned the phosphorus value calculated from the results of the ultraviolet absorption analyses of the mitochondria fractions was generally about 20 per cent less than that found experimentally. In the particles and supernatants, however, the calculated value was generally slightly greater than that found by experiment. This is in accord with the observation that the phosphorus content of yeast nucleic acid is generally slightly lower than the theoretical. The nature of the extra phosphorus in the mitochondria is an interesting speculation. A large amount of phosphoprotein phosphorus could account for this discrepancy. It has already been pointed out it was not possible to analyze for phosphoprotein phosphorus on these samples due to the use of sulfuric acid to precipitate the desoxyribonucleic acid in the Schmidt-Thannhauser procedure. It could possibly be a metaphosphate type of substance as described by Wiame (29) in yeast, or it could be a polyphosphorylated nucleic acid enzyme as recently described by Binkley and Olson (30). Further work is necessary on this point.

SUMMARY

Total rat liver homogenates and the mitochondria, particle, and supernatant fractions have been analyzed for the nucleotide contents of their ribonucleic acids. Guanine to adenine rations from 2.5 to 4.5 and pyrimidine to purine ratios of 1.5 to 2.5 were found. The uridylic to cytidylic acid ratios were found to be unreliable. The predominant differences observed between resting and regenerating liver were a lower pyrimidine to purine ratio and the presence of a greater percentage of the phosphorus in the more difficultly sedimentable fractions in the regenerating liver. Suggestions for further work are also made.

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PREPARATION OF NUCLEOPROTEIN FROM INTESTINAL MUCOSA

Robert M. Feinstein and Carrie L. Butler

In an attempt to extract from intestinal mucosa that substance, expected to be mucoprotein, which imparted to the tissue its mucous, viscous nature, a highly viscous fraction was obtained which appears upon analysis to be nucleoprotein in nature.

A somewhat detailed description of this technique and of the resulting product will be given here, because of the simplicity of the method and because of the biochemical demonstration (succeeding report) of an effect of whole body irradiation upon this fraction of intestinal mucosa.

EXPERIMENTAL

Analytical Method. Protein was determined by the biuret method of Robinson and Hogden (1), as modified by Keyser and Vaughn (2). Total phosphorus was determined by the method of Fiske and Subbarrow (3) on an hydrogen peroxide-sulfuric acid digest modified (4) from the procedure of Koch and McMeekin (5). Phosphorus fractionation was performed by the method of Schneider (6).

Relative viscosities were determined by the use of Ostwald viscosimeters. In the calculation of relative viscosities, no allowance was made for density, since no material was handled which had a density differing from that of water by as much as one per cent. Tyrosine was determined by the method of Anson (7). Carbohydrate was determined by the carbazole method as outlined by Seibert and Atzo (8).

Preparation of Nucleoprotein. Nucleoprotein was prepared from whole rat intestine and from the intestinal mucosa of rats, rabbits, and sheep. Rats were killed by decapitation, and rabbits were killed by intravenous injection of air. Sheep intestines were obtained from the slaughterhouse. Rat and rabbit intestines were removed from the animal, placed on ice to cool, cut into segments of a foot or less, and flushed with cold 0.9 per cent NaCl solution to remove fecal matter where necessary. The flushing was done with a blunt-tipped 15 gauge hypodermic needle attached to a syringe or, in the case of rabbit intestine, simply with the tip of the syringe. For preparation from whole rat intestine, the cleaned segments were minced in a Latapie mincer, then blended in a Waring blendor for 30 seconds at room temperature with nine volumes of N/10 NaOH. The blend was then filtered through a nylon filter (National Filter Media Co., #1002) under suction.

Mucosa was obtained from rat intestine by squeezing through a device resembling a washing machine wringer. The rollers were made of stainless steel, lightly milled to afford traction. The segments were slit lengthwise and fed open through the rollers, which were maintained under slight, adjustable spring tension. By this method it was possible to obtain over 40 per cent of the original weight of intestine as mucosa.

Mucosa was obtained from the cleaned intestine of rabbits or sheep by pressing intact segments with bone spatulas. By judicious manipulation, over 60 per cent of rabbit intestine could be quickly expressed as mucosa, and probably a similar amount was obtained from sheep.

For preparation from rat, rabbit, or sheep separated mucosa, it was not necessary to mince the material. The mucosa was simply blended in the Waring blendor with nine volumes of N/10 NaOH. Filtration was not necessary.

The alkaline blend was then brought to pH 11 with vigorous stirring and placed in the cold room (2-5°C.). After a period of one to four days without disturbance, a separation takes place into two liquid layers. The upper layer, thin and watery, increases further in amount as the material remains in the cold. The lower layer is considerably more viscous and forms a cohesive mass very similar to the yolk of an egg, although with no apparent membrane. This cohesive mass shrinks with the passage of time, expressing more watery fluid, and itself becoming viscous to the point of almost complete gelation. In the earlier stages of this two-phase separation, it is possible to accelerate the separation by high speed centrifugation (ca. 18,000 x g) in the cold. Before the beginning of a separation is visible, however, such centrifuging will not bring it about. If the pH is not reduced to 11 before placing in the cold room, the two-phase separation will occur anyway, but it may take several weeks for it to be brought about. At the same time it will be found that the pH of these solutions has decreased to and below pH 11, presumably by absorption of CO_2 from the air and by reaction of the alkali with the glass vessel.

The heavier phase, the cohesive, viscous mass, will hereafter be referred to as the "tactoid", a not entirely accurate usage of the expression of Freundlich (9), Langmuir (10), and others for the microscopic or submicroscopic particles which, when properly oriented, form such a mass. This tactoid from animal intestine has been found to be highly concentrated nucleoprotein.

Factors Influencing Formation of the Nucleoprotein Tactoid. (a) pH. As noted above, the pH is of importance in determining the rate of tactoid formation. Not only will a sharp, decantable separation take place more rapidly in a preparation brought to pH 11 before chilling than in one left at pH 12 - 12.5, but also the amount of tactoid that can be obtained by high-speed centrifugation at any given time (up to final complete separation) is greater in the former. Thus, for example, in one case 14 per cent (by weight) of a preparation set in the cold room at pH 11 was obtained as tactoid after five days, while another portion of the same preparation set in the cold room at pH ca. 12.5 yielded only 3.4 per cent tactoid after five days. Upon remaining in the cold room for 20 days, however, the two aliquots yielded 9.7 per cent and 10.2 per cent tactoid, respectively. The reduction from 14.0 to 9.7 per cent is due to the compression of the tactoid, with resultant expression of fluid from it.

If the pH is reduced much below 11, a precipitate forms and no tactoid is visible. The precipitate undoubtedly contains a wide variety of other proteins as well as the nucleoprotein.

(b) Temperature. Tactoid formation is faster in the cold (2-5°C.) than at room temperature, and no tactoid has yet been observed to form in a bath

maintained at 37°C. In fact, at this higher temperature appreciable degradation was apparent; both the viscosity and the protein content were observed to decline, as shown in Table 1.

(c) Concentration. Formation of the tactoid appears to be critically dependent upon a high enough concentration of the material. In one experiment a 1:7 homogenate exhibited a very firm tactoid at a time when the usual 1:9 homogenate, simultaneously prepared from the same intestinal mince, had begun to exhibit only a slight, indefinite separation. In another case, one aliquot was kept at 2-5°C., while another aliquot was frozen, then partly thawed, and ca. 25 per cent of the material was discarded as ice. In this experiment, too, the more concentrated material showed tactoid separation long before the less concentrated.

(d) Other physical factors. Since the tactoid formation is considered to be due to orientation of asymetric particles* (presumably hydrated protein aggregates), it was felt that any treatment which helped to align the particles would accelerate the two-phase separation.

1. "Seeding". The addition to a freshly prepared homogenate of a small bit of a previously formed tactoid helped speed separation only slightly.

2. Shearing. A portion of fresh homogenate was forced through a 3 inch long, 27 gauge hypodermic needle. Tactoid formed no faster than in a control.

3. High-speed centrifuging. In Langmuir's (10) review, it is stated that tactoid particles have a higher density than the surrounding sol. Since it may be assumed that the small, denser particles are precursors to the separation of macrotactoid, it might be expected that prolonged, high speed centrifugation might at least concentrate the micro particles to the extent that eventual separation would be speeded up. This has not been found to be the case. Up to two hours of centrifuging at 18,000 xg were without effect on eventual rate of tactoid formation.

(e) Chemical factors. Two general principles from the papers of Freundlich (9) and Langmuir (10) were applied.

1. Salting out. It is possible that the addition of salts may reduce the effective concentration of water to the point where tactoid formation is facilitated. The addition of solid NaCl, Na_2SO_4, or $(NH_4)_2SO_4$, to a final concentration of 5 per cent, had no such effect.

2. Unmixing (coacervation). Langmuir (10) used these terms to refer to the addition of a chemical of opposite sign; as an example, he states that dilute gelatin and dilute gum Arabic form a two-phase liquid system, as do $SrCl_2$ and $(NH_4)_2MoO_4$. Not knowing the sign of our tactoid, we tested the effect of each of these four agents. They were added as solids, to a final concentration of 5 per cent. They had no effect on the rate of tactoid formation.

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* We wish to thank Dr. Frank W. Putnam for helpful discussion.

TABLE 1

EFFECT OF TEMPERATURE ON VISCOSITY, PROTEIN CONTENT, AND APPEARANCE OF TACTOID

Temperature and Time	Rel. Viscosity	Protein (mg/ml)	Visual Observation
Immediately tested	2.74	7.96	No tactoid
2-5°C. for 9 days	2.22	7.00	Slight tactoid
37°C. for 9 days	1.66	5.94	No tactoid

Materials were homogenized before determination of relative viscosity or protein content.

Physical Properties of the Tactoid. (a) Effect of dilution on viscosity. Although further dilution of a relatively dilute solution of the tactoid in N/10 NaOH has only the expected linear reduction in viscosity, dilution of a highly-viscous tactoid solution causes a much greater than linear reduction in viscosity. This is illustrated in Table 2.

(b) Separation beyond tactoid stage. Under certain circumstances, not yet well defined, it appears that a formed tactoid will develop a tendency to disintegrate into a second state, in which the viscosity is sharply reduced, and a granular precipitate slowly replaces the lower liquid layer. Neither the chemical nor physical nature of this change is understood as yet.

(c) Tactoid formation from other tissues. Tactoid formation has not been observed in identically prepared alkaline blends prepared from rabbit kidney, heart, lungs, liver, or testes. On the other hand, addition of an equal volume of N/10 NaOH to a preparation of thymus chromosomes (prepared according to Mirsky and Ris (11), but with a final suspension of chromosomes of such a concentration than each ml. of suspension represents one gram of original thymus) did very quickly yield a firm, clear, two-phase separation. When whole thymus was blended with nine volumes of N/10 NaOH, the resulting solution was extremely viscous (η = 74.8). Perhaps as a result of this, separation was sluggish, although a firm tactoid was formed after 2 to 3 weeks.

Data comparing the viscosity with the protein content of these various tissue blends are listed in Table 3.

Chemical Composition of the Tactoid. The chief difficulty in characterizing the tactoid lies in its irregular composition, dependent upon time, temperature, pH, speed and time of centrifuging, if any, and undoubtedly many biological factors. As the material stands in the cold room, fluid is continually expressed, and the chemical composition of the material is thus constantly changing. Methods of further purification have not yet been developed. Table 4 summarizes present information on the chemical composition of alkaline blends of intestine, before and after formation of tactoid.

Until methods of purification are devised, one fruitful approach seemed to be to follow chemical changes occurring as the tactoid expresses fluid. Thus it would be expected that with the progress of this procedure, certain continuing changes would become apparent, the asymptote of which would represent the approximate chemical composition of the tactoid free of excess fluids. Preliminary data are listed in Table 5. The chemical constancy with time of expressed fluid is indicated in Table 6.

Preliminary Identification of the Tactoid as Nucleoprotein. Identification of the tactoid as nucleoprotein is not yet solidly based. That the tactoid is probably nucleoprotein is indicated, however, by the following evidence.

(a) Phosphorus fractionation. The phosphorus fractionation shown in experiment 2, Table 5, indicates definitely that the bulk of the total phosphorus of the tactoid, indeed essentially all of the acid-insoluble phosphorus, is

TABLE 2

EFFECT OF DILUTION ON DILUTE AND ON CONCENTRATED TACTOID

Experiment Number	Degree of Dilution		Relative Viscosity (37°C.)
	Volume of Tactoid	Volume of N/10 NaOH	
1	all	0	91.3
	2	1	26.4
	1	1	14.1
	1	2	7.03
	1	4	3.57
2	all	0	81.1
	1	1	4.16
	0	all	1.02

The tactoid used in experiment 1 was prepared from a very firm one from which a considerable amount of fluid had been expressed. This tactoid was homogenized with slightly more than an equal volume of N/10 NaOH, and it is this diluted material which is referred to, above, as "tactoid".

The "tactoid" used in experiment 2 was a quite fresh alkaline blend of whole rat intestine, in which tactoid formation was just becoming apparent. The whole blend, well mixed, was used for the viscosity determinations.

TABLE 3

VISCOSITY AND PROTEIN CONTENT OF ALKALINE BLENDS OF VARIOUS TISSUES, AS RELATED TO ABILITY TO FORM TACTOID

Tissue	Relative Viscosity	Protein (mg/ml)	Tactoid Formation
Rabbit intestinal mucosa	4.64	7.90	rapid
kidney	2.12	10.62	none
lung	3.22	7.98	none
heart	1.92	8.84	none
liver	1.79	11.50	none
testis	1.48	5.20	none
Calf thymus chromosomes*	10.39	3.28	rapid
Whole calf thymus	74.8	7.84	slow

*See text for preparation. All other preparations are simple blends with nine volumes N/10 NaOH.

TABLE 4

CHEMICAL COMPOSITION OF TACTOID FROM INTESTINE

Sample		1	2	3	4	5
Total N (mg/ml)	original					1.10 (17.9% acid-soluble)
	tactoid		1.71		2.73 (21.6% acid-soluble	2.60
	supernatant		1.61		1.76 (24.8% acid-soluble	0.80
Total P (mg/ml)	original					0.19 (17.3% acid-soluble
	tactoid		0.52		1.05 (12.4% acid-soluble)	1.18
	supernatant		0.21		0.26 (46.2% acid-soluble)	0.14
% Dry weight	original					
	tactoid		3.37	2.48	3.61	3.96
	supernatant		2.09	2.02	1.78	1.12
Protein (mg/ml)	original	8.52		8.48		4.66
	tactoid	8.70	1.98	4.13	9.66	5.40
	supernatant	8.70	5.09	4.43	7.83	0.89
Polysaccharide (mg/ml)	original					0.15
	tactoid				1.14	7.40
	super.				0.59	0.25
Tyrosine (meqr10^{-4} per ml)	original					43.0 (35.8% acid-soluble
	tactoid		51.2 (49.7% acid-soluble	61.0 (18.7% acid-soluble	72.1 (17.7% acid-soluble	
	supernatant		52.2 (48.5% acid-soluble	58.2 (18.6% acid-soluble	59.4 (17.6% acid-soluble	
Relative viscosity	original	2.91		5.08		2.74
	tactoid	47.7	200-2000	16.40	not done (extremely viscous)	
	supernatant	1.50	1.43	4.17	1.47	

Samples 1 and 2 are from rat; samples 3 and 4 are from rabbit; and sample 5 is from sheep intestine.

Figures for acid-soluble fractions represent that per cent of the total constituent which is soluble in 5 per cent CCl_3COOH.

TABLE 5

CHEMICAL CHANGES IN TACTOID EXPRESSING FLUID

Experiment 1

	Tactoid, 2 Weeks After First Appearance	Tactoid 2 Weeks Later
% Dry weight	2.97	3.96
Total N (mg/ml)	2.04	2.60
Total P (mg/ml)	0.88	1.18

Experiment 2

	16 Day Old Tactoid	Same Tactoid, 28 Days Old	Same Tactoid, 32 Days Old
Phosphorus fractionation (Schneider (6) procedure)			
Acid-soluble P	28	24	20
Lipid P	6	3	9
Nucleic acid P	60	69	69
Phosphoprotein P	6	4	2

Figures are expressed as per cent of the total P present.

TABLE 6

CONSTANCY OF CHEMICAL COMPOSITION OF FLUID EXPRESSED FROM A SINGLE CONTRACTING TACTOID

Date fluid obtained	6/9/50	6/12/50	6/14/50	6/26/50
Dry weight (%)	1.31	1.42	1.34	1.12
Total N (mg/ml)	0.86	0.93	1.22	0.80
Total P (mg/ml)	0.15	0.12	0.15	0.14
Protein (mg/ml)	1.61	1.63		0.89
Total carbohydrate (mg/ml)	0.033	0.036		0.25
Relative viscosity	1.38	1.23		

Tactoid first appeared 5/30/50. To obtain data above, the tactoid was centrifuged on day shown, and the supernatant fluid was collected and analyzed.

found in the nucleic acid phosphorus fraction, as defined by Schneider (6). This evidence alone would seem conclusive were it not for a tentative challenge of Schneider's definition, as discussed below.

(b) Absorption spectrum*. A portion of sheep tactoid was extracted to remove acid-soluble and fat-soluble materials, then treated with perchloric acid to dissociate nucleic acids from protein combination. The absorption spectrum of the soluble portion was then determined. The absorption curve was found to be very similar to that of desoxyribose nucleic acid (DNA). It was slightly skewed, and the peak was shifted ca. 6 m μ toward the higher wave lengths, but otherwise the coincidence was close.

(c) Nucleotide analysis*. A portion of sheep tactoid was treated by a modification of the procedures of Schneider (6) and Schmidt and Thannhauser (12) in which ribose nucleic acid (RNA) nucleotides are solubilized, while DNA and its constituent parts remain insoluble and are discarded. Absorption analysis of the RNA nucleotide fraction further fractionated with Ag_2SO_4 demonstrated that ca. 10 per cent of the total phosphorus of the tactoid could be accounted for as attached to RNA nucleotides.

DISCUSSION

Identification of the tactoid as nucleoprotein must be considered still incomplete, although all chemical evidence listed above seems to point in the same direction. Some doubt has been thrown on the validity of Schneider's (6) implied definition of nucleoprotein phosphorus as that phosphorus which is insoluble in cold 10 per cent trichloracetic acid and in fat solvents, but solubilized by hot 5 per cent trichloracetic acid. This doubt is due to the isolation from intestinal mucosa (succeeding section of this report) of a protein fraction which, based only on its method of isolation, would seem to be a mucoprotein. This, too, has been found to release its phosphorus when treated with hot 5 per cent trichloracetic acid.

On the other hand, the tactoid resembles in its physical properties and behavior certain known nucleoproteins. Thus, an alkaline solution of a preparation of thymus chromosomes quickly forms a similar mass when chilled. Also it has been shown (13,14) that highly purified solutions of tobacco mosaic virus, a nucleoprotein, if stronger than 2 per cent, separate into two liquid layers upon standing.

SUMMARY

A highly viscous fraction has been obtained from the intestinal mucosa of rats, rabbits, and sheep. The fraction is considered, upon evidence not yet entirely complete, to be nucleoprotein in nature.

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* We wish to thank Drs. John F. Thomson and Robert J. Cox, Jr., for their assistance.

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ISOLATION OF A PRESUMED MUCOPROTEIN FRACTION FROM RAT INTESTINE

Robert N. Feinstein and Carrie L. Butler

The mucoproteins of intestinal mucosa are of interest to radiation biochemistry for a variety of reasons: (a) known sensitivity of intestine to x-radiation (1); (b) post-irradiation bacteremia characterized by the presence in the blood of intestinal microorganisms (2), which may indicate degradation of such a "ground substance" as mucoproteins are considered to be (3); (c) post-irradiation desquamation of intestinal epithelium (4); and (d) post-irradiation increase in serum polysaccharide (5), which may arise from degradation of x-ray sensitive glycoprotein (mucoprotein).

Considerable effort has therefore been put into an attempt to isolate an intestinal mucoprotein on which to test the effects of irradiation. A protein fraction has now been isolated which, on the basis only of its method of preparation, is suggested may be a mucoprotein. The material has not yet been fully characterized, nor is its degree of homogeneity known.

EXPERIMENTAL

Preparation of the (Muco)Protein Fraction. Rats are killed by decapitation, and the intestines are cleaned and flushed with cold saline from an hypodermic syringe fitted with a blunt-tipped 15-gauge needle. The cleaned segments are cooled on ice, then passed through a Latapie mincer. The mince is weighed and extracted with nine volumes of cold 0.05 M Na_2HPO_4 for one hour in the cold room, with continuous stirring. The phosphate extraction is based on a suggestion of Robertson et al (6). The suspension is then filtered through cheesecloth and the filtrate is centrifuged to free it of cellular debris. The milky fluid resulting is then treated in one of two ways.

(a) The bulk of the proteins is precipitated by the addition of four volumes of 0.75 M $HClO_4$ and is filtered off. To the filtrate is added 0.2 volume of 5 per cent phosphotungstic acid in 2 N HCl. The precipitated (muco)protein is centrifuged down and washed with phosphotungstic acid. This procedure is due to the suggestion of Winzler et al (7) and yields a product which is certainly highly heterogeneous.

(b) To one volume of the phosphate extract is added an equal volume of 0.1 N sodium acetate, then solid $(NH_4)_2SO_4$, with continual stirring, to bring to a final concentration of 2.73 M ammonium sulfate. This is kept in the cold room for at least 16 hours, then filtered through a double thickness of Whatman No. 1 filter paper. The pH of the filtrate is reduced to 4.9 with HCl, and the resulting suspension is kept in the cold room at least 16 hours, then filtered as before. The filtrate is then brought to pH 3.7, again kept in the cold room for at least 16 hours, and again filtered as before. Finally the (muco)protein is precipitated by saturating the filtrate with solid $(NH_4)_2SO_4$ at $4^{\circ}C$. This time the material is kept cold for at least 72 hours. The (muco)protein is then collected on a double thickness of Whatman No. 1 paper. It is dissolved in a little distilled water, exhaustively dialyzed against cold tap water, then

distilled water and finally dried by lyophilization. This procedure is adopted in toto from that of Weiner et al (8).

Although the yield from procedure (a) above has not been calculated, the yield from procedure (b) has been found to be approximately 40 mgm. of dried material per whole rat intestine.

Preliminary Characterization of the Mucoprotein. Table 1 lists some data taken from analyses of the initial phosphate extract of rat intestine, and of the protein fraction prepared from it by procedure (b) above.

DISCUSSION

It is obvious from the foregoing that the sole reason thus far for considering this to be a mucoprotein is that it appears in a fraction prepared from intestine extract by a procedure identical with that which yields a mucoprotein from blood plasma (8). The analytical data does not particularly bear out this interpretation; in particular, the low polysaccharide value militates against it. On the other hand, until the exact nature of the carbohydrate present is known, it is impossible accurately to measure it. The method here used, that of Seibert and Atno (9) using carbazole, is highly dependent upon the particular carbohydrate present, and probably also upon its particular conjugation, if any.

The low relative viscosity is not in accord with the usual concepts of mucoproteins, but Weiner et al (8) demonstrated, with their carefully characterized mucoprotein from plasma, that a 2 per cent solution of their mucoprotein had a relative viscosity of only 1.165.

The high percentage of acid-soluble phosphorus found in this product is not readily understandable. It is presumed that this would be reduced by repeated recrystallization, an impractical procedure at the time, because a total of only 40 mg. was obtained. The appearance of a high percentage of "nucleic acid" phosphorus may indicate nucleoprotein or, more likely, may constitute a challenge to the fractionation proposed by Schneider (10), which implies that the only protein-attached phosphorus which is solubilized by hot 5 per cent trichloracetic acid is nucleoprotein phosphorus.

A larger quantity of the material is now being prepared, and it is expected that a clearer understanding can be obtained of the chemical nature of this fraction.

SUMMARY

A protein fraction has been isolated from rat intestine which, on the basis only of its method of preparation, is suggested may be a mucoprotein.

TABLE 1

CHEMICAL COMPOSITION OF PHOSPHATE EXTRACT OF RAT INTESTINE AND OF (MUCO) PROTEIN FRACTION PREPARED FROM IT BY METHOD OF HEIMER ET AL (8)

Phosphate extract:	Dry weight	1.84%
	Relative viscosity	1.08
	Protein	3.55 mg./ml.
	Total tyrosine	41.6 meq. x 10^{-4}/ml.
	Polysaccharide	0.26 mg./ml. (as glucose)
(Muco) protein fraction:	Relative viscosity (0.5% in H_2O):	1.09
	Polysaccharide	1.7% (as glucose)
	Total N ca.	17.4%
	Total P ca.	4.4%
	Phosphorus fractionation (as % of total P):	
	acid soluble	58%
	lipide	2
	nucleic acid	37
	phosphoprotein	2

Analytical methods are listed in the preceding section of this report.

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EFFECT OF X-RADIATION ON SERUM POLYSACCHARIDE OF RABBITS

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We have previously reported (1) that 48 hours after a lethal dose of x-rays to rats, the serum polysaccharide is significantly increased over that preceding the irradiation. We have now performed a similar experiment on rabbits and have found a similar tendency, but with results not statistically significant.

EXPERIMENTAL

Methods are listed in the earlier report (1). Rabbits used in this experiment weighed from 1800-2900 gm. Six control rabbits and six to be irradiated were paired off according to weight. The test animals were given ca. 1000 r of x-rays (1/2 mm Cu, 22 cm. distance, 200 KV, 86 r per minute, for 11 min. 36 sec.). That this is well over the LD-50 is indicated by the fact that all six irradiated animals were dead within 17 days (average survival time 7.5 days). All animals, test and control, were fasted from the time the test animals were x-rayed until the 48-hour samples had been drawn. Blood samples were drawn from the ear vein by hypodermic needle, both before and 24 and 48 hours after irradiation, and also two weeks after irradiation.

RESULTS

The data of Table 1 indicate a tendency to a greater increase in serum polysaccharide 48 hours after irradiation than in the controls. This effect is not, however, statistically significant.

DISCUSSION

The effect of starvation alone on the serum polysaccharide level is so great as to tend to obscure superposed factors. The starvation effect, like most other factors (cancer, burns, tuberculosis) which increase serum polysaccharide (2-6), is probably due to tissue destruction. It is, of course, essential to starve all animals in an experiment of this sort because of the diminished appetite of x-irradiated animals. It seems probable that the tendency of irradiated rabbits towards a greater serum polysaccharide level would have been statistically significant had a greater number of animals been used.

SUMMARY

The serum polysaccharide level of fasted rabbits increased after a lethal dose (1000 r) of x-rays, but the increase was not statistically significant.

TABLE 1

EFFECT OF WHOLE BODY X-RADIATION ON THE SERUM POLYSACCHARIDE OF STARVED RABBITS

Treatment	Increase in Serum Polysaccharide, as mg./100 ml.		
	After 24 Hours	After 48 Hours	After 2 Weeks
None	47.7 ± 19.0	26.9 ± 30.6	4.9 ± 14.9
1000 r x-rays	44.3 ± 16.2 (t = 0.22)	57.0 ± 8.5 (t = 1.52)	6.0

Six rabbits used for all control points. Four rabbits used for 24 and 48 hour test points, and only one survivor available for 2-week test point.

Data presented are mean values, average deviation from the mean, and Student's t values, where a t of 2.5 or greater is considered to indicate a significant difference from the corresponding control point.

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EFFECT OF WHOLE BODY X-RADIATION AND OF INTRAPERITONEAL HYDROGEN PEROXIDE ON A NUCLEOPROTEIN FRACTION OF RAT INTESTINE AND ON WHOLE RAT INTESTINE

Robert M. Feinstein and Carrie L. Butler

In a preceding section of this report (page 67), a method was described for the isolation from intestinal mucosa of a fraction which has been identified, although not entirely conclusively, as nucleoprotein. The present report indicates that this fraction is changed -- whether quantitatively or qualitatively is not yet known -- after the animals received a lethal dose of x-rays to the whole body. In addition, certain changes in this fraction have been observed after the intraperitoneal injection of hydrogen peroxide, which has been shown (references in (1)) to be radiomimetic in certain respects.

EXPERIMENTAL

For ease in following the work here presented a short recapitulation of the method of preparation of the fraction in question, and the terminology employed are given.

Intestine is removed and cleaned by flushing with saline. It is blended with nine volumes of M/10 NaOH, filtered through nylon, and put away in the cold room after being brought to pH 11. After a variable period of time, ranging from one day to several weeks (ordinarily 4 to 5 days), a two-phase liquid separation occurs. The bottom layer is an extremely viscous, cohesive mass, the composition and quantity of which continually change as the mass contracts, expressing more of the non-viscous supernatant fluid. The viscous mass, considered essentially nucleoprotein, is referred to as the "tactoid", and the watery phase as the "supernatant". The two phases can be separated by decantation or, more effectively, by high-speed centrifugation.

It was found extremely difficult to put this phenomenon on a quantitative basis, chiefly because the amount and exact appearance of the tactoid is critically dependent upon the time and temperature and upon the speed and time of centrifugation if any. In addition, some evidence suggests that the rate of tactoid formation is probably somewhat dependent upon the exact nature and shape of the containing vessel as well. In order to obviate as many as possible of these factors, the following precautions were taken:

(1) All intestinal segments were frozen in a dry ice-acetone bath and kept in deep freeze until all samples had been collected. They were then all thawed as nearly simultaneously as possible, by blending, one after the other, with the proper amount of M/10 NaOH. All were placed in the cold room at the same time.

(2) All samples were placed in newly purchased and cleaned 400 ml. beakers.

(3) In centrifuging, one control, one x-rayed, and one H_2O_2-treated sample were spun down in the same centrifuge load.

(4) In addition, to minimize biological variations all animals were handled in groups of three. Thus each datum represents such a pool of the intestines of three animals.

It is particularly unfortunate that the only high-speed centrifuge available broke down after centrifugation of the 24-hour samples. For the 48-hour samples, it was impossible to centrifuge, and decantation was impractical, because of the incomplete state of formation of the tactoid. The device finally used was to remove, by pipet, a measured sample of the supernatant fluid; this was easily done without contamination by the tactoid. The tactoid and remaining supernatant were then thoroughly mixed together and sampled as such. It was argued that, since uncontaminated tactoid could not be obtained, at least it should be possible to detect any differences between supernatant fluids and hence to infer differences in the tactoid.

These data, however, proved extremely difficult to interpret, and hence the only data presented here for the 48-hour samples are the total values, i.e., tactoid plus supernatant.

Animals used in this experiment were Maguran male rats, weight 220 to 290 gm. The rats were matched in such a way that the total weight of all groups were approximately constant. X-rayed rats received 800 r of whole body x-radiation; H_2O_2-treated rats received 2.0 milliequivalents H_2O_2 per 100 gm. body weight, intraperitoneally. Both the x-ray and the H_2O_2 doses are somewhat higher than the LD-50.

RESULTS

In Table 1 are listed the weights of intestine obtained, and the relative viscosity and protein content of the alkaline blends prepared from these. These data are thus independent of tactoid formation. It is demonstrated that both x-rays and H_2O_2 significantly decrease the relative viscosity of these alkaline blends, while the protein content is not significantly affected. In addition, it is of interest to note that there is some decrease in total intestine weight after x-radiation, and an increase after intraperitoneal H_2O_2; both of these, however, are of dubious statistical significance.

In Table 2 are listed the percentages of tactoid centrifuged down from the 24-hour samples. As noted above, centrifuging of the 48-hour samples was impossible, and decantation was inadequate. Although a very considerable variation is to be noted in Table 2 (due chiefly to centrifuge difficulties and consequent variation in time and speed of centrifuging), in each individual case the control tactoid was very much larger in amount than were the x-rayed and H_2O_2-treated.

Tables 3, 4, 5, and 6 present, respectively, data describing changes in the dry weight, alcohol-precipitable carbohydrate (i.e., polysaccharide), total phosphorus, and total nitrogen. In these tables the data are treated differently for the 24 hour and 48 hour samples, for the reason discussed above. Thus it is possible to determine the quantity of a given constituent in the tactoid and in the supernatant, separately, of the 24 hour sample, but only the totals in the 48 hour samples.

TABLE 1

EFFECT OF X-RAYS AND OF H_2O_2 ON WEIGHT OF INTESTINE AND RELATIVE VISCOSITY AND PROTEIN CONTENT OF ALKALINE BLENDS OF THESE

Sample	Average Weight of Intestine Obtained per Pool of Three (gms. Wet Weight)	Relative Viscosity	Protein Content (mg./ml.)
24 hours			
Control	7.1 ± 0.5	3.31 ± 0.06	4.53 ± 0.38
X-ray	6.2 ± 0.4 (t=2.31)	2.56 ± 0.16 (t= 6.81)	4.40 ± 0.18 (t=1.08)
H_2O_2	8.7 ± 0.7 (t=2.96)	2.39 ± 0.14 (t=10.22)	4.63 ± 0.23 (t=0.67)
48 hours			
Control	6.2 ± 0.3	3.04 ± 0.04	4.49 ± 0.58
X-ray	5.7 ± 0.2 (t=2.17)	2.01 ± 0.02 (t=34.3)	4.48 ± 0.14 (t=0.27)
H_2O_2	7.8 ± 1.2 (t=2.19)	2.80 ± 0.21 (t= 1.85)	5.10 ± 0.20 (t=1.61)

Data presented are mean, average deviation from the mean, and Student's t value. Each datum represents the average of four pools of three rats each.

TABLE 2

EFFECT OF X-RAYS AND OF H_2O_2 ON AMOUNT OF TACTOID FORMING IN 24-HOUR SAMPLES

Sample	Per Cent of Total Blend Appearing as Tactoid
Control	29.3 ± 12.6
X-Rayed	5.4 ± 0.5 ($t=3.03$)
H_2O_2	5.6 ± 2.0 ($t=2.96$)

Data are mean, average deviation from the mean, and Student's t. Four pools of three rats each were averaged for each datum shown.

TABLE 3

EFFECT OF X-RAYS AND OF H_2O_2 ON THE DRY WEIGHT OF RAT INTESTINE TACTOID, SUPERNATANT, AND TOTAL HOMOGENATE

Sample	Total Grams Dry Matter			% in Tactoid
	In Tactoid	In Supernatant	Total Homogenate	
24-Hour				
Control	1.96 ± 0.67	2.23 ± 0.47	4.19 ± 0.53	46.8
X-Ray	0.72 ± 0.13	2.18 ± 0.85	2.90 ± 0.83 (t=2.05)	24.8
H_2O_2	0.91 ± 0.18	3.86 ± 0.39	4.77 ± 0.22 (t=1.59)	19.1
48-Hour				
Control			4.11 ± 0.29	
X-Ray			3.09 ± 0.10 (t=5.37)	
H_2O_2			4.75 ± 0.46 (t=1.89)	

TABLE 4

EFFECT OF X-RAYS AND OF H_2O_2 ON THE ALCOHOL-PRECIPITABLE CARBOHYDRATE (POLYSACCHARIDE) OF RAT INTESTINE TACTOID, SUPERNATANT, AND TOTAL HOMOGENATE

Sample	Total Milligrams Polysaccharide			% in Tactoid
	In Tactoid	In Supernatant	Total Homogenate	
24-hour				
Control	29.6 ± 13.0	52.4 ± 11.7	81.9 ± 5.5	36.2
X-Ray	18.7 ± 9.5	62.2 ± 3.9	80.9 ± 12.8 (t=0.12)	23.1
H_2O_2	54.2 ± 21.5	113.2 ± 17.1	167.4 ± 23.1 (t=5.78)	32.4
48-hour				
Control			65.3 ± 25.5	
X-Ray			63.5 ± 13.1 (t=0.10)	
H_2O_2			124.0 ± 52.4 (t=1.60)	

TABLE 5

EFFECT OF X-RAYS AND OF H_2O_2 ON THE TOTAL PHOSPHORUS OF RAT INTESTINE TACTOID, SUPERNATANT, AND TOTAL HOMOGENATE

Sample	Total Milligrams Phosphorus			% in Tactoid
	In Tactoid	In Supernatant	Total Homogenate	
24-Hour				
Control	21.0 ± 6.7	24.4 ± 1.7	45.4 ± 5.0	46.3
X-Ray	7.3 ± 0.8	25.8 ± 5.3	33.1 ± 6.1 (t=2.51)	22.1
H_2O_2	13.2 ± 2.2	39.1 ± 10.4	52.3 ± 8.4 (t=1.12)	25.3
48-Hour				
Control			41.9 ± 1.1	
X-Ray			25.0 ± 1.1 (t=17.1)	
H_2O_2			50.7 ± 11.0 (t=1.27)	

TABLE 6

EFFECT OF X-RAYS AND OF H_2O_2 ON THE TOTAL NITROGEN OF RAT INTESTINE

48 Hour Samples	Total Milligrams Nitrogen
Control	316.9 ± 26.8
X-Ray	207.9 ± 12.9 (t=5.86)
H_2O_2	340.2 ± 45.8 (t=0.52)

24 hour samples were not analyzed for nitrogen.

The following phenomena are apparent from a perusal of the data on these four tables:

(1) X radiation decreases the total dry matter of intestine; in addition, both x radiation and H_2O_2 injection decrease the percentage of the total dry matter which is found in the tactoid (Table 3).

(2) Although x-radiation has no effect on the total polysaccharide content of intestine, the intraperitoneal injection of H_2O_2 had a very considerable effect in increasing the total polysaccharides (Table 4).

(3) X-radiation causes the loss from the intestine of an appreciable portion of the total phosphorus ordinarily present. Hydrogen peroxide has no such effect. In both cases, however, there appears to be a shift in phosphorus from the tactoid to the supernatant (Table 5).

(4) X-radiation significantly reduces the total nitrogen of the intestine, while H_2O_2 is without effect (Table 6).

DISCUSSION

The phenomena described in this paper properly fall into two separate, yet related groups. The first group consists of those changes observed in the whole intestine, and the second group consists of those changes noted in the tactoid fraction. Since the tactoid appears to be a relatively large, hence at least quantitatively important, fraction of whole intestine, changes in it may be reflected in the overall picture.

Omitting momentarily a discussion of the effects of H_2O_2 (which in this work has not appeared to be entirely radiomimetic), it should be noted that perhaps the most striking effect of whole body x-radiation on these alkaline blends of intestine is the reduction in viscosity. Since it has been demonstrated (this report, page 67) that the major portion of the viscosity is due to the tactoid fraction, it is not surprising to find that the per cent of the total blend which appears as tactoid is greatly diminished after x-radiation of the animal. It is of great interest that, despite this major effect, the total protein and total polysaccharide are not decreased. Considerable phosphorus, however, is lost, all of it from the tactoid fraction. A similar percentage of whole intestine total nitrogen is also lost.

If we assume that the tactoid represents nucleoprotein, an assumption not yet entirely proven (this report, page 67), these results appear to indicate that whole body x-radiation affects intestinal nucleoprotein in some way which causes no loss of the protein moiety, but does result in destroying the particular asymetry which presumably is responsible both for tactoid formation and for the high viscosity of these materials. The nature of this effect is yet to be revealed, but it is presumably a dissociation or depolymerization of the nucleic acid moiety.

In vitro effects of x-radiation on viscosity of nucleic acid and nucleohistone have been described (2,3). Also, Ely and Ross (4) showed, by ultraviolet light absorption, the presence of decreased amounts of nucleoprotein.

in the crypts of Lieberkuhn of rats at various levels along the intestinal tract. The present work thus confirms these results and in addition presents a technique which may be quite helpful in further studies of this problem.

The effect of intraperitoneal hydrogen peroxide is also of interest. Peroxide, too, decreases the viscosity of alkaline solutions of intestine; this effect, however, is apparent only 24 hours after injection, and after another 24 hours has disappeared. Like x-rays, too, the H_2O_2 causes reduction in the amount of tactoid formable; this is probably parallel with the viscosity decrease.

In the effects on dry weight and carbohydrate content of intestine, H_2O_2 behaved differently than did x-rays. Whereas x-rays caused an appreciable decrease in the total dry weight of the intestine (less significantly observable also in the total (wet) weight of the intestine), H_2O_2 actually caused an apparent increase in wet and dry weight, although these effects are relatively low in statistical significance. The polysaccharide content of intestine after treatment with H_2O_2 is considerably increased, while no increase is apparent after x-radiation. The significance of this observation is unknown.

In the matter of phosphorus loss, H202 again does not follow the effect of x-rays, although it is of interest that the percentage of the total phosphorus which appears in the tactoid is decreased, as with x-rays. It might thus be a tenable hypothesis that H_2O_2, like x-rays, affects intestinal nucleoprotein, but in some sort of reversible manner, so that viscosity is soon restored, and phosphorus dissociated from the nucleoprotein is retained in some form within the intestine, presumably permitting re-association.

SUMMARY

Alkaline solutions of whole intestine, when chilled soon show a two-phase liquid separation. The heavier, viscous phase is designated as a "tactoid" and has been tentatively identified as nucleoprotein.

Either whole body x-radiations or intraperitoneal injection of H_2O_2, each in slightly greater than LD-50 dose, cause a reduction in the viscosity of the alkaline solution. At the same time, the percentage of total solution which appears as tactoid is decreased, an effect which is considered to parallel the viscosity decrease, since the tactoid is considered the chief viscogen in these solutions.

X-radiation causes the loss from the intestine of an appreciable portion of the total phosphorus ordinarily present. Although H_2O_2 does not cause such a loss, it does, like x-rays, cause a shift in the phosphorus from the tactoid to the non-tactoid fraction.

H202, but not x-radiation, causes an increase in the total polysaccharide of intestine.

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EFFECT OF WHOLE BODY X-RADIATION ON A PRESUMED MUCOPROTEIN OF RAT INTESTINE

Robert N. Feinstein and Carrie L. Butler

In a preceding section of this progress report (page 79), the separation was described of a protein fraction from rat intestine, this fraction being considered, solely on the basis of its method of preparation, to be mucoprotein. We wish to describe here an experiment indicating that whole body x-radiation of rats reduces the amount of this fraction to a statistically significant degree.

EXPERIMENTAL

The mucoprotein fraction (it being understood that the identification of this fraction as mucoprotein is highly tentative) was isolated by a combination of the phosphate extraction of Robertson et al (1) and the perchloric-phosphotungstic acid fractionation of Winzler et al (2).

Each rat was killed by decapitation, and the intestine was removed, chilled on cracked ice, flushed with cold saline from a hypodermic syringe fitted with a blunt-tipped 15-gauge needle, minced with scissors, then blended in the micro head of the Waring blendor with nine volumes of cold 0.05 M Na_2HPO_4 for 30 seconds. It was then stirred mechanically in the cold room for one hour. The cellular debris was strained off through cheesecloth, then centrifuged off. To 2 ml. of the supernatant were added 8 ml. of 0.75 M $HClO_4$. The mixture was shaken and permitted to stand 10 minutes, then filtered through 9 cm. Whatman No. 3 filter paper. To 5 ml. of filtrate was added 1 ml. of 5 per cent phosphotungstic acid in 2 N HCl. After letting stand 15 minutes, the mixture was centrifuged, and the precipitate was washed with 1 ml. of the phosphotungstic acid in HCl. The precipitate was then dissolved in NaOH, and its tyrosine content was determined by the method of Anson (3).

In a preliminary experiment to determine the consistency of this method of analysis, four rats were sacrificed and the intestines treated as above. The tyrosine contents of the precipitates were found to be 4.60, 4.48, 4.36, and 4.48 meq. x 10^{-4}, which is considered a satisfactory consistency.

Nine rats were then x-rayed (1/2 mm. Cu, 45 cm. distance, 250 KV, 40 r per minute, for 20 minutes, or a total of 800 r). At the same time, these and nine controls rats were fasted, although given water ad lib. After 24 hours four fasted control and four fasted x-rayed rats were sacrificed and treated as above. After 48 hours, five fasted controls and five fasted x-rayed rats were sacrificed and treated similarly, as were three fed, non-x-rayed rats. The data are presented in Table 1 and indicate a probably significant decrease in this mucoprotein fraction 24 hours, but not 48 hours, after x-radiation. It is of interest that three fed rats, not x-rayed, gave values similar to those of starved, x-rayed rats.

TABLE 1

EFFECT OF A WHOLE BODY X-RADIATION (800 r) ON A "MUCOPROTEIN" FRACTION FROM RAT INTESTINE

Group	No. of Rats	Tyrosine in Precipitate* (meq. x 10^{-4})	Student's t
Fed	3	4.61 ± 0.25**	
24 hour starved controls	4	5.39 ± 0.29	
24 hour starved x-rayed	4	4.50 ± 0.25	3.73
48 hour starved controls	5	5.22 ± 0.38	
48 hour starved x-rayed	5	4.72 ± 0.68	1.15

* See text for details of preparation and analysis.

** Data presented are mean values and average deviation from the mean.

DISCUSSION

In an earlier report (4), some reasons were presented for expecting a decrease in intestinal mucoprotein after x-radiation. The present report gives some support to this hypothesis, although it must be borne in mind that the identification of the presently discussed protein fractions as mucoprotein is highly tentative.

The anomalous effect of nutritional status is of great interest. Although the data in Table 1 represent such small populations that it is impossible to be entirely certain of the significance of the figures, it appears that the mucoprotein content of the intestines of fed rats more nearly resembles that of starved, x-rayed rats than starved non-x-rayed rats. Until this is confirmed by repetition with larger numbers of animals, no explanation is suggested.

SUMMARY

On the basis of limited data, it appears that 24 hours after whole body x-radiation of rats, an intestinal protein fraction, tentatively identified as mucoprotein, decreases in amount.

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SEASONAL VARIATION IN MOUSE LIVER CATALASE

Robert N. Feinstein and Carrie L. Butler

In the course of previous work (1) on the possible relation of hydrogen peroxide and catalase to the radiation syndrome, it was realized that the liver catalase level of the mice received by this laboratory was highly fluctuant, and it eventually became necessary to halt the work because of this fact. Specifically, it was found that with the arrival of the very hot summer of 1949, the liver catalase of our mice decreased appreciably. In order to determine the range of fluctuation of this enzyme and to determine the most propitious time to resume work on the problem, the liver catalase was determined of representatives of each shipment of mice received.

EXPERIMENTAL

Mice of the Carworth Farms CF-1 strain are received in this laboratory weekly and are maintained on a stock diet of Wayne Dog Food Blox. Three male mice, selected at random from each shipment, have been used for liver catalase assays, which have been done by the perborate method (2).

RESULTS

Unfortunately, no record was kept of shipment dates during the several months prior to appreciating the variance in liver catalase with different shipments. Over the period February 21, 1949, to June 13, 1949, a total of 19 normal mice, of unknown shipment date, were assayed for liver catalase as controls for various experiments. The liver of these normal animals assayed 0.73 ± 0.08 perborate units (2) per mg. wet weight. Since they were divided into five experiments, it may be assumed they represent five shipments. The date of use, which of course is not likely to be coincidental with the date of receipt of shipment, is listed in Table 1, together with the liver catalase assays. From Table 1 it is apparent that during the period February - June, 1949, mouse liver catalase assayed roughly 0.6 - 0.8 perborate units per mg. wet weight.

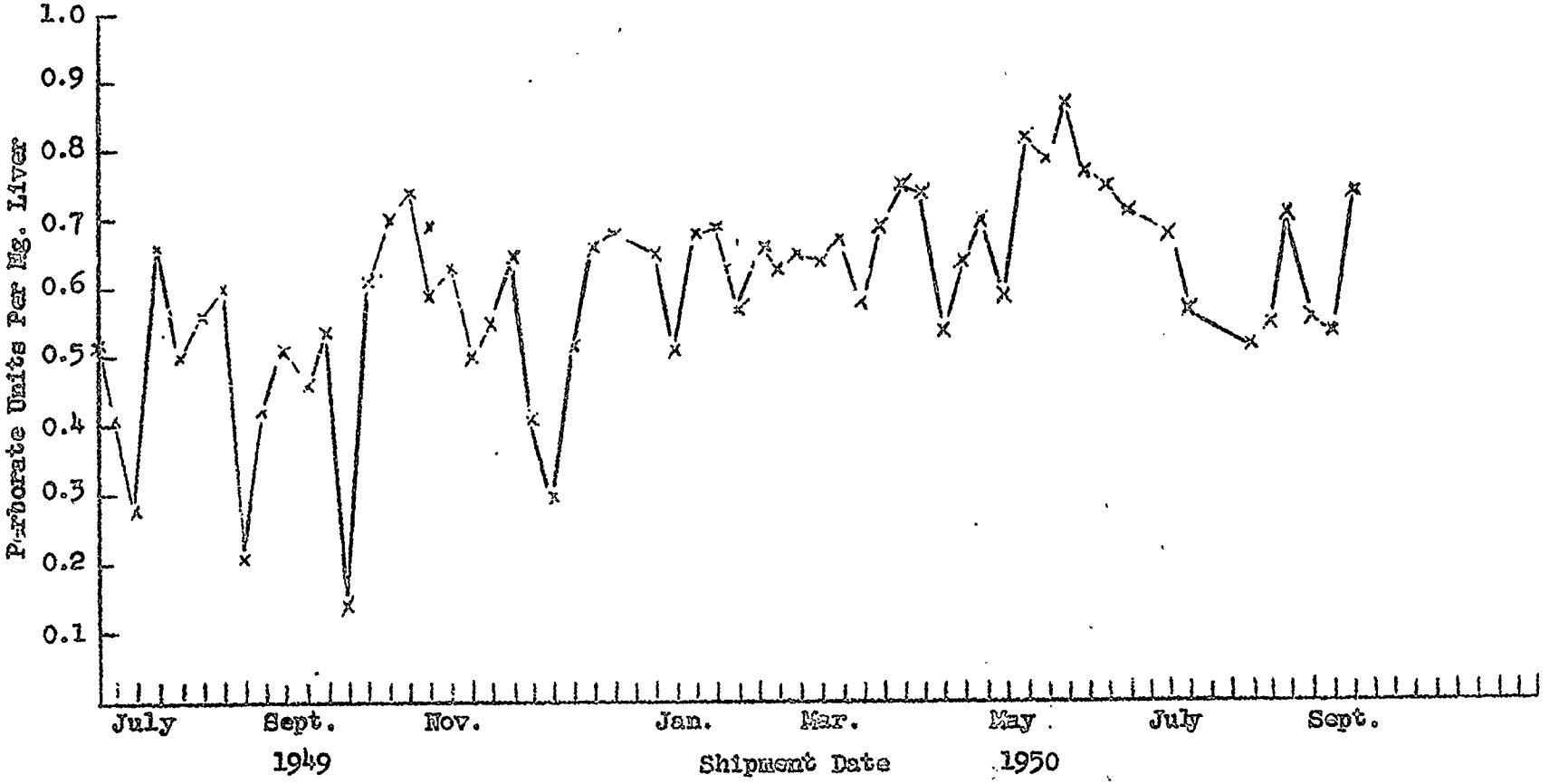
Figure 1 shows how this picture began to change in July 1949, and how the low, erratic level of liver catalase continued until December 1949. Thus in the months of July through November 1949, only seven shipments of mice were received with a liver catalase in the range 0.6 - 0.8 units per mg., while mice from 14 showed a catalase level below this range. Mice from three shipments showed less than 0.3 units/mg. Thus, the mice selected from 33 per cent of the shipments received during this period had liver catalase in the "normal" range of 0.6 - 0.8 units/mg. During the rest of the observations, stretching from December 1949 to September 1950, 22 out of a total of 37 shipments, or 60 per cent, were in the "normal" range, while two shipments were slightly above 0.8 units/mg., and only a single shipment showed a liver catalase level as low as 0.4 units/mg.

TABLE 1

SUMMARY OF NORMAL LIVER CATALASE IN MICE OF SHIPMENTS OF UNKNOWN DATE BUT PRECEDING KNOWN FALL IN CATALASE

Date of Use	No. of Mice	Liver Catalase (Perborate Units/Mg. Wet Weight)
2/21/49	6	0.77 ± 0.10
5/20/49	1	0.59
5/26/49	4	0.70 ± 0.04
6/1/49	4	0.62 ± 0.04
6/13/49	4	0.83 ± 0.02

FIGURE 1


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
P-Borate Units Per Mg. Liver
July
Sept.
Nov.
Jan.
Mar.
May
July
Sept.
1949
Shipment Date
1950

That random sampling of three mice from each weekly shipment of 100 to 300 mice gives a reliable summary of the entire shipment is indicated by the average deviations of the groups of three. The entire series of average deviations averaged 0.06 ± 0.03.

SUMMARY

The liver catalase of mice received by this laboratory suffered a precipitous and erratic drop for several months following July 1949. After December 1949, the liver catalase returned to levels considered normal and has remained approximately so for the subsequent ten months.

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STUDIES OF F_1 GENERATION HYBRID MICE FROM AN IRRADIATED PARENT: PRELIMINARY REPORT

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The object of these experiments is to ascertain whether a single dose of 200 r of x-radiation delivered to male or female mice would produce effects on (a) life span of offspring, and (b) litter size, when these irradiated animals were mated to untreated animals. For this purpose it was thought desirable to carry out the studies on hybrids between dba and C_{57} strains in order to minimize the spontaneous and radiation-induced lymphomas which are a major source of mortality in CF_1 mice. The ages of the mice at the initial pre-irradiation control mating ranged from 12 to 14 weeks for the C_{57}, and from 8 to 12 weeks for the dba. In each case the irradiated parent was from a C_{57} black line 1 strain. The x-ray treatments were administered with a G.E Maximar X-ray machine operating at 250 KV; 15 M.A. and 60 cm. T.S.D. The exposure time was three minutes and forty-two seconds.

RESULTS

The first post-irradiation matings were confined to the ten day period immediately following x-ray treatment. A second post-irradiation mating is planned after a four month interval has elapsed. While the main interest in this experiment is in the fitness of the progeny, certain other results presently available are summarized in Table 1.

As can be seen in the table, the proportion of pregnancies was slightly reduced in each case following irradiation. The reduction in litter size which was anticipated from previous work (1-5) was not observed. This may have been due to the small numbers of litters available in this series. However, the mortality of the weanlings was decreased in one series and increased in the other following irradiation.

SUMMARY

C_{57} mice irradiated with 200 r of x-rays produced approximately normal litter sizes in crosses with unirradiated animals. Life span data on these offspring will be reported later

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TABLE 1

	Irradiated Male Series						Irradiated Female Series					
	C_{57} Male X dba Female						C_{57} Female X dba Male					
	No. of Preg. / No. of Pairs	Total Number Born	Average Litter Size	Number Weaned M	Number Weaned F	Number Weaned Total	No. of Preg. / No. of Pairs	Total Number Born	Average Litter Size	Number Weaned M	Number Weaned F	Number Weaned Total
Pre-irradiated F_1 Controls	13/14	71	5.4	31	21	52	15/15	119	7.9	70	47	117
First post-irradiated F_1	9/14	47	5.2	23	22	45	13/15	106	8.1	41	37	78
Second post-irradiated F_1	(Data not available for this report--scheduled)											

Time intervals: 1st mating 65 days pre-irradiation
2nd mating immediately post-irradiation
3rd mating 120 days post-irradiation

TABLE 2

TABULATED SUMMARY OF THE RESULTS OBTAINED IN SCREENING AGENTS GIVEN BEFORE OR AFTER ACUTE WHOLE BODY RADIATION WITH 800 r

Agent	Dose	Pre-Treatment			Post-Treatment		
		d.f.	t value	P'	d.f.	t value	P
Desoxyribonucleic acid (1)	30.0 mg	11	2.25	< 0.05	11	-3.03	> 0.01
* " " (1)	30.0 "	--	----	0.08	--	----	----
Ribose nucleic acid	10.0 "	11	0.45	< 0.7	10	1.98	> 0.05
" " "	10.0 "	--	----	----	23	-6.91	< 0.01
" " "	10.0 "	--	----	----	23	-5.15	< 0.01
Xanthine (1)	10.0 "	11	-0.25	> 0.8	11	-0.29	< 0.8
" (1)	15.5 "	--	----	----	23	-1.73	< 0.1
Xanthosine (1)	15.5 "	11	0.88	< 0.4	11	-3.62	< 0.01
Hypoxanthine	15.5 "	11	1.26	> 0.2	11	1.07	> 0.3
Guanine (1)	15.5 "	11	-0.86	> 0.4	11	-1.12	< 0.3
Guanosine (1)	20.0 "	11	0.55	< 0.6	10	-0.28	< 0.8
Adenine sulfate (1)	10.0 "	11	1.07	> 0.3	11	-0.60	< 0.6
Adenylic acid	13.2 "	11	0.49	> 0.6	11	-0.07	> 0.9
Adenosine	15.5 "	11	0.19	> 0.8	11	1.98	< 0.1
Thymine	15.5 "	10	1.87	< 0.1	11	-1.32	> 0.2
Amino Acids or Polypeptids:							
Cysteine (1)	20.0 "	11	2.45	< 0.05	11	0.70	< 0.5
* " (1)	20.0 "	--	----	0.10	--	----	----
*Methionine	10.0 "	--	----	0.52	--	----	----
Histidine	13.2 "	10	1.87	< 0.1	11	-1.32	> 0.2
Glutathione (1)	15.5 "	11	2.07	> 0.05	11	-0.31	< 0.8
Hormones:							
Insulin	1.25 U	11	-1.51	< 0.2	11	0.14	< 0.9
Insulin plus desoxy-corticosterone acetate	1.25 U 1.0 mg	21	-0.18	< 0.9	23	-0.16	< 0.9
Vitamins:							
Vitamin B_{12} (1)	0.75 γ	11	0.71	< 0.5	11	2.63	> 0.02
Miscellaneous:							
Sodium thiosulfate	110.8 mg	11	0.00	> 0.9	9	-2.17	> 0.05
Sodium nitrite (1)	2.0 "	11	-0.73	< 0.5	11	0.55	< 0.6

* Fischer's Exact Method
(1) Presented in the previous report - corrected data
d.f. Degrees of freedom

The agents with a negative t value and a P of 0.05 or less are considered to be harmful under the conditions of the tests.