

FORMATION OF PHOSPHOLIPIDES IN TISSUES

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Project leader: Dr. Camillo Artom

The present report covers the work done from March 16, 1950 through March 15, 1951. This work is a direct continuation of that previously done under the sponsorship of the Atomic Energy Commission and summarized in my progress report of March 15, 1950. In addition to the writer, the following investigators participated in the research work: M. A. Swanson, Ph. D.; M. Crowder, M. S., P. Mitchell, Graduate Student. Technical assistance was given by C. Terhune and for limited periods, by H. Wainer and J. Giles.

A. General outline of the work.

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During the past year a few additional experiments have been made in order to complete the two series of investigations on the Lipide composition of mitochondria, and on the Incorporation of inorganic P³² into the phospholipides of isolated liver slices. Previous and present results on these subjects have been organized in two complete papers (one published, the other submitted for publication).

The largest part of the time and activity of the investigators has been devoted to a study of the Incorporation of C¹⁴-labeled choline in the lipides of isolated liver tissue. The experimental part of this study is almost completed and the results will be presented shortly in a preliminary form. A simultaneous investigation has been carried out on the Metabolic pathways of choline in liver slices. Some of the results have been published; but much further work is needed before definitive conclusions can be drawn.

Since dimethylethanolamine is a likely precursor in the synthesis of choline, and since previous in vivo experiments in this laboratory indicated that it may be directly incorporated in the phospholipide molecule, the Synthesis of C¹⁴-labeled dimethylethanolamine has been undertaken. The synthesized product will be added to the liver slices in experiments similar to those in which labeled choline has been used.

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

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

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

B. Lipide composition of liver mitochondria.

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

C. Incorporation of inorganic P³² into the phospholipides of liver slices.

The experiments of this series have also been completed and most of our previous findings confirmed and extended. On the basis of these findings, it appears that a decrease in the incorporation of P³² into the lipides occurs in liver slices of rats on low protein diets. The decrease seems to be due to the decrease in cytoplasmic proteins and more especially in the enzyme proteins which are involved, directly or indirectly, in the synthesis of the phosphate bond of phospholipides. When these data are compared with those previously obtained on intact animals, it seems likely that in the liver of the latter animals, regulatory mechanisms (possibly hormonal) maintain the rate of the incorporation of P³² into phospholipides at a level adequate to the requirements of the animal. Presumably such a level is much below the maximum functional capacity of the liver, so that in the intact animal losses of enzymes in the liver can be easily compensated by the increased activity of the remaining cell structures. On the other hand, in the isolated tissue where hormonal mechanisms are absent, or much less efficient, the process does occur at the maximum speed compatible with the conditions of the experiment. If the amounts of active enzymes present in the tissue are reduced, as in the liver of protein depleted animals, the speed of P³² incorporation into the lipides of the surviving slices will be correspondently decreased.

The effects of the in vitro addition of choline, ethanolamine and betaine to liver slices differ not only with the substance added but also with the previous dietary regime of the animal. The most unexpected finding was a marked inhibition of the incorporation of P³² in the lipides, when choline was added to liver slices

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

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

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

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

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

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

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

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

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

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

E. Metabolic pathways of choline in liver slices.

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

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

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

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

A preliminary report of these results has been published.

F. Synthesis of C¹⁴ methyl-labeled dimethylethanolamine, (a possible choline precursor).

As previously mentioned, dimethylethanolamine could be visualized as an intermediary product in the synthesis, or in the degradation of choline, or in both. At present, the formation of dimethylethanolamine by direct demethylation of choline is unlikely (see above). On the other hand, previous results from this laboratory indicated that dimethylethanolamine can be directly introduced in the molecule of phospholipides even before being completely methylated. It was thought that by the use of labeled dimethylethanolamine much information could be obtained on these points. Accordingly, the synthesis of a methyl labeled dimethylethanolamine has been undertaken. Mono-methylethanolamine has been converted into the dimethyl derivative by reaction with C¹⁴ labeled formaldehyde at an alkaline pH. The conditions for such a synthesis and for the separation of the product in a pure form have been studied carefully. The labeled product has been used in some preliminary experiments with surviving liver slices.

G. Disposal of formaldehyde in tissues.

The disappearance of formaldehyde in the presence of tissue homogenates has been studied. The most interesting finding was a considerable increase in the disappearance of the formaldehyde by the addition of pyruvic acid to the digestion mixture. The distribution of the enzyme responsible for this reaction, the conditions for its activity and the nature of the resulting products have been investigated. In several experiments C¹⁴-labeled formaldehyde has been used and the isotope content of a fraction, obtained by precipitation with 2,4-dinitrophenylhydrazine was determined. While no definitive conclusions can still be made, it seems likely that a condensation product is later converted to the corresponding acyloin ("acetol"). In view of the increasing evidence for the formation of one carbon compound in several processes of the intermediary metabolism, (including oxidation and synthesis of methyl groups), the significance of this reaction of formaldehyde with pyruvic acid may well be of a great importance in the general economy of living bodies. Two preliminary reports on these results have been prepared and presented at scientific meetings.

H. Bibliography.

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

1. "Studies on Dimethylethanolamine" by Camillo Artom and Marietta Crowder. Absts. of Commun. XVIII Intern. Physiol. Congr., Copenhagen, p.78 (1950).
2. "The Lipide Composition of the Large Granules (Mitochondria) From Rat Liver" by Marjorie A. Swanson and Camillo Artom, Journal of Biological Chemistry, Vol. 187, 281 (1950).
3. "Metabolism of Methyl-Labeled Choline in Surviving Liver Slices" by Camillo Artom and Marietta Crowder, Archives of Biochemistry, Vol. 29, 237 (1950).

The following complete manuscript has been submitted for publication:

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

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

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

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

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

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

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

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

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