The Biosynthesis of Phospholipids

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A CURRENT theory of the mechanism of the lipotropic action of choline and inositol suggests that these substances exert such an effect because they are important constituents of phospholipids. It is not known, however, precisely how the metabolism of phospholipids may be related to lipotropic activity. The interesting and important experiments of Dr. Zilversmit, discussed in this Symposium may shed some light on this problem. In this paper I will discuss some current work on the enzymatic synthesis of phospholipids, with the hope that some clue as to the mechanism of the lipotropic action of choline may emerge. Three main topics are to be considered: the enzymatic synthesis of lecithin; the enzymatic synthesis of sphingomyelin; and some preliminary experiments on the level of CDP-choline† in the livers of rats on a choline-deficient diet.

THE ENZYMATIC SYNTHESIS OF LECITHIN

The enzymatic processes by which lecithin is built up from simple precursors in the living cell has now been worked out in considerable detail. Some of these reactions are summarized in Figure 1. Since this subject has been reviewed recently elsewhere[1,2] the present discussion will be limited to features particularly pertinent to the lipotropic action of choline and to the choline-containing phospholipids. It should be mentioned however that an essentially similar scheme of reactions leads to the formation of phosphatidyl ethanolamine.

PC-Cytidyl Transferase Reaction: It has now been firmly established that a coenzyme form of choline, cytidine diphosphate choline (CDP-choline), is an essential intermediate in the bio-synthesis of lecithin. The structure of CDP-choline, which is that of a doubly substituted pyrophosphate, is shown in Figure 2. The synthesis of CDP-choline and related compounds in labeled form by purely chemical procedures has been described.[3] The enzymatic synthesis of CDP-choline from CTP + P-choline [reaction (e) of Fig. 1] is catalyzed by PC-cytidyl transferase, an enzyme widely distributed throughout nature. A detailed study of this enzyme has recently been carried out by Borkenhagen and Kennedy.[4]

The PC-cytidyl transferase reaction may be viewed as an "activation" of phosphorylcholine by incorporation into the energy-rich pyrophosphate structure of CDP-choline, and is one of the many reactions for which metabolic energy is required to carry out the biosynthesis of phospholipid.

PC-Glyceride Transferase Reaction: CDP-choline reacts with a D-1,2-diglyceride [reaction (f) Fig. 1] to form lecithin and CMP (cytidine-5′-phosphate). The enzyme catalyzing this reaction has been named PC-glyceride transferase and its properties have been described by Smith, Weiss, and Kennedy.[5] The enzyme requires magnesium or manganese ion and is severely inhibited by very low concentrations of calcium ion.

The CMP which is formed in reaction (f) may be rephosphorylated to CTP at the expense of ATP. The CTP which is thus regenerated may combine with another mole of phosphorylcholine, participating in a catalytic

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Presented at the *Symposium on Mode of Action of Lipotropic Factors in Nutrition* at the University of Pittsburgh, October 22–23, 1957, with the cooperation of The National Vitamin Foundation, Inc., New York, New York.

This work has been supported by grants from the Nutrition Foundation, the Life Insurance Medical Research Fund, and the National Institute for Neurological Diseases and Blindness U.S.P.H.S. (B-1199).

† CDP-choline = cytidine diphosphate choline; CoA = coenzyme A; CTP = cytidine triphosphate; ATP = adenosine triphosphate; CMP = cytidine-5′-phosphate.

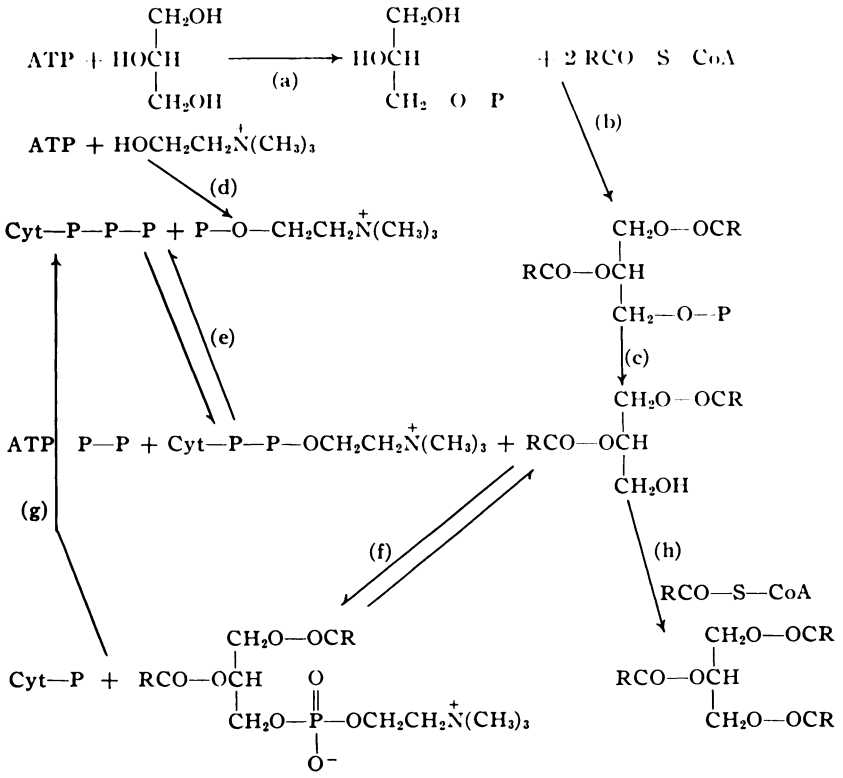

Fig. 1. Pathways for the enzymatic synthesis of phospholipids and triglycerides.

cycle, at every turn of which one mole of lecithin is formed from phosphorylcholine + diglyceride. During the course of this cycle, phosphorylcholine forms a part of the structure of the coenzyme itself, in a fashion somewhat analogous to the function of the uridine coenzymes, e.g., uridine diphosphate glucose.

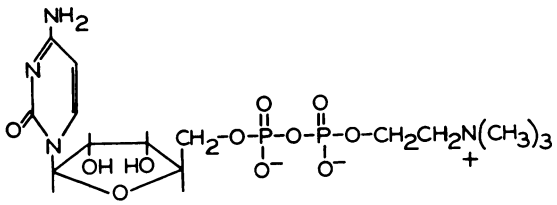

Fig. 2. The structure of cytidine diphosphate choline.

The PC-glyceride transferase is specific for CDP-choline; analogues in which adenosine, uridine, or guanosine replace the cytidine portion of the molecule have been prepared synthetically and are completely inactive. The enzyme is also highly specific for D-1,2-diglycerides; 1,3-diglycerides are only slightly active. The D-1,2-diglyceride must possess at least one unsaturated fatty acid, preferably in the alpha-prime position, and must be emulsified in a suitable nonionic detergent such as "Tween-20" to penetrate to the enzyme surface. The requirement for an unsaturated fatty acid may be related to the greater ease of emulsification of unsaturated glycerides.

A recent generous gift by Drs. E. Baer and D. Buchnea of samples of pure synthetic D- and L-1,2-diolein has made it possible to test the optical specificity of the enzyme, with the results shown in Table I. The D-1,2-diglyc-

TABLE I

The Optical Specificity of the PC-Glyceride Transferase

Diglyceride added	Lecithin synthesized $m\mu M$
1. L-1,2-diolein	13
2. D-1,2-diolein	126

The assay for lecithin synthesis was carried out as described by Weiss, Smith, and Kennedy.[5]

eride is seen to be much more active than the L-enantiomorph.

The PC-glyceride transferase reaction is freely reversible, which may be of some physiologic importance.

Interrelationships of Phospholipid and Triglyceride Synthesis: The scheme shown in Figure 1 indicates that L-α-phosphatidic acids and D-1,2-diglycerides may be intermediates of hitherto unsuspected importance in the biosynthesis of glycerophosphatides. [For references, see review.][2] This conclusion is supported by the discovery[6] that D-1,2-diglycerides may react with long-chain thioesters of coenzyme A to form triglycerides, and thus may be regarded as precursors of neutral fat as well as of phospholipides. Several laboratories have sought an alternative pathway for the formation of triglyceride, i.e., a direct reaction between free glycerol and long-chain thioesters of coenzyme A. To date no such reaction has been found, and the only known route for the incorporation of free glycerol into triglycerides is by way of L-α-glycerophosphate, phosphatidic acid, and D-1,2-diglyceride.

Is CDP-Choline a Lipotropically Active Form of Choline? One of the great advances in achieving an understanding of nutrition on a biochemical level has been the realization that water-soluble vitamins are of special importance in the diet because they are building blocks for coenzymes. It has further been learned that in general the vitamin does not function as a coenzyme as such, but is transformed in the body to a catalytically active form, often a nucleotide.

Although choline is not a vitamin in the strict sense of the word, its importance as a special dietary factor is well recognized. Since we now know that choline may be converted in the body to a catalytically active nucleotide form, the hypothesis that CDP-choline is a lipotropically active form of choline deserves careful consideration. The close interrelationship between triglyceride and phospholipid metabolism shown in Figure 1 makes this hypothesis all the more attractive, since the profound alteration in the triglyceride metabolism of liver, resulting in fatty liver, is one of the most dramatic consequences of a diet deficient in choline and other lipotropic agents. To consider but one obviously oversimplified interpretation, any condition which disturbs the balance between reactions (f) and (h) by blocking reactions (d), (e), or (f) (Fig. 1) might lead to a "spilling over" of 1,2-diglyceride into neutral fat rather than phospholipid. Reactions (d), (e), or (f) might be blocked or reduced either by an insufficient supply of choline to the cell or by metabolic poisons such as carbon tetrachloride.

THE ENZYMATIC SYNTHESIS OF SPHINGOMYELIN

The experimental results which have just been described suggested that the biosynthesis of another choline-containing phospholipid, sphingomyelin, might take place by a reaction essentially similar to the PC-glyceride transferase reaction:

(1) Cyt-P-P-choline + N-acylsphingosine $\rightleftarrows$ Sphingomyelin + CMP

It has recently been reported[7] that an enzyme from chicken liver catalyzes an extensive net synthesis of sphingomyelin according to reaction (1). The name phosphorylcholine-ceramide transferase has been proposed for this enzyme.

Phosphorylcholine-ceramide transferase is highly specific both for CDP-choline and for N-acylsphingosine (ceramide). Ceramides with fatty acids of short or intermediate chain length in amide linkage are much more active than the naturally occurring long chain compounds, presumably because the short chain compounds penetrate more readily to the enzyme surface. Much more surprising is the discovery[7] that the N-acylsphingosine must possess the *threo* rather than the *erythro* configuration in the sphingosine portion of the molecule. It has been generally accepted that naturally occurring sphingolipids have the *erythro* configuration. The physiologic significance of this puzzling observation merits further study.

The participation of CDP-choline in the biosynthesis of sphingomyelin makes it clear that the functions of this nucleotide form of choline are not limited to the realm of glycerophosphatides and strengthens the view that mechanisms controlling the synthesis and utili-

zation of CDP-choline may be of central importance in lipid metabolism generally.

MEASUREMENTS OF CDP-CHOLINE CONCENTRATION

It is now known that CDP-choline is widely distributed in nature[1] being found in the liver of various species including the rat and the hen, in brain and other tissues of the rat, and in yeast.

As a preliminary attempt to determine whether CDP-choline is a lipotropically active form of choline, it seemed of interest to determine the concentration of CDP-choline in the livers of rats under various conditions. Efforts to work out an enzymatic assay for CDP-choline have so far been unsuccessful, and it has been necessary to use an isotope dilution method. This procedure, although sensitive and accurate, is laborious and time-consuming and has therefore limited the number of experiments which can be done.

In one experiment, 18 male rats, about five weeks old, were divided into two groups of nine each. One group was fed *ad libitum* on a commercially available choline-deficient diet (Nutritional Biochemicals Co.). The control group was fed the same diet, supplemented with choline. After eight weeks, both groups of animals were killed. The livers of three animals were pooled for CDP-choline assay, resulting in three assays for each group. The results are shown in Table II. There was no significant difference between control and deficient animals.

TABLE II

CDP-Choline Content of Rat Liver in Choline-Deficient and Control Animals

$\mu M/100$ g

	I	II	III
Control	6.2	7.1	6.2
Deficient	5.9	6.9	5.9

These results, although very fragmentary, seem to suggest that the level of CDP-choline in liver may be relatively constant, even on a choline-deficient diet. However, other experiments, unfortunately incomplete, seem to indicate that the CDP-choline content of the livers of choline-deficient animals first falls, and then returns to normal, so that a period of eight weeks may be too long to detect such changes.

It is clear that much more extensive and better planned experiments will be needed to explore these points.

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DISCUSSION

Dr. Wilgram: Dr. Kennedy, I am wondering whether I am right in assuming that you think that the most important findings in this field in the last year or two are that 1-α-glycerophosphate is a very important precursor of D-1,2-diglycero-phosphatidic acid and that this compound, after fission of the phosphate group then reacts with cytidine diphosphate choline to form lecithin.

From the biochemical viewpoint there is nothing that I can add to your presentation at all, but as a physiologist there is some amazement about the fate of the phosphate group. After the formation of phosphatidic acid, phosphate is split off. I am wondering why Nature should take such a complicated and roundabout course, first, in putting phosphate on 1-α-glycerophosphate to yield the phosphatidic acid, and then removing it by a phosphatase, only to put another phosphate from CDP-choline back in the same position. From a physiologic viewpoint it seems to me that Nature, if it takes this course, is somewhat wasteful and I am wondering what your personal reactions are as to this complicated phenomena.

The second point I would like to ask you again arises from physiologic and not biochemical considerations. There are certain aspects of the problem which have been touched upon but not dealt with extensively by Dr. Kennedy. They are however, vital to the understanding of what goes on in the living

body. How does this biochemical finding of the mode of enzymatic synthesis of lecithin in the test tube lead to a better understanding of the mode of action of choline, in the intact organism, which is the main topic of the symposium today? You have mentioned several possible mechanisms by which neutral fat and phospholipids may be interrelated to each other in their metabolism. I wonder whether you would care to indicate which possibilities, if any, you think might account for the lipotropic action of choline.

Dr. Kennedy: With regard to the first point that Dr. Wilgram made, we are not responsible for how Nature chooses to make these compounds. Yet I think it would be unfortunate if the impression got around that Nature really would do well to study biochemistry rather than vice versa. I think there is some rhyme or reason to this merry-go-round in which the glycerol is phosphorylated, converted to phosphatidic acid and then dephosphorylated, because when the glycerol is phosphorylated it is phosphorylated stereospecifically, giving only L-α-glycerophosphate. Then when the phosphatidic acid is formed, it has the L configuration. It is a derivative of L-α-glycerophosphate but by a quirk in the arbitrary nomenclature of these compounds, the compound which is formed by splitting the phosphate from an L-phosphatidic acid is a D-1,2-diglyceride. That is simply because you change the end of the molecule you regard as the precursor of the aldehyde of D-glyceraldehyde. It gives specifically then the form of 1,2-diglyceride which is needed to react with cytidine diphosphate choline to given an L-lecithin. As far as we can tell, all of the glycerophosphatides in nature are of the L series.

This is exactly what Baer does when he makes lecithins synthetically. He takes the D-acetone-glycerol molecule and makes the benzyl ether to maintain stereospecificity. He puts the fatty acids on and then removes the benzyl ether and phosphorylates, using chemical methods.

Actually, there is quite a strict analogy between the chemical synthesis of lecithin and the biochemical synthesis of lecithin. In each case a protective group is put on in the alpha position while the fatty acids are introduced in order to preserve stereochemical purity, and then the protector is removed.

I realize this does not answer completely your question, which has real force to it. We see again and again that enzymatic reactions occur which seem at first to be wasteful, for example, splitting out of pyrophosphate rather than orthophosphate in certain reactions. I think there is a deeper physiologic significance, as you point out, and I cannot cope with it at that level at the moment.

As far as the second question is concerned, which of these interrelationships between triglyceride and phospholipid one might regard as a fruitful point of departure for investigating the lipotropic activity of choline, all I can say is that my instinct tells me it will not be as simple as blocking cytidine diphosphate choline utilization and switching the diglyceride over to triglyceride. It can't be that simple in view of the immense literature on the lipotropic activity of choline, almost unrivaled for its complexity. On the other hand, I think it would be extraordinary, really, if we found a cofactor form of choline which had no relationship at all to the lipotropic activity of choline because there are so many examples—nicotinamide, for example, in which the dietary nutrient is incorporated into a nucleotide or cofactor form and then exerts its biological activity. I have no evidence at all to cite that CDP-choline has lipotropic activity, but I still think it would be a good bet in the long run.

Dr. W. Cornatzer (University of North Dakota, Grand Forks, N. D.): The data on Dr. Kennedy's last slide where he found the cytidine diphosphate choline content in fatty livers identical to the control animals are similar to other data where no change in phospholipid turnover was observed in production of dietary cirrhosis (*Ann. New York Acad. Sc.* 57: 919, 1954) or liver necrosis (*J. Lab. & Clin. Med.* 31: 478, 1946). The composition of liver lipids can be altered by dietary intake of proteins. There is usually a slight decrease in the level of total phospholipids in the liver of animals maintained on a low-protein diet. The decrease is especially marked in the lecithin fraction with a consequent lower ratio of choline-containing to total phospholipids. The uptake of P^{32} or phospholipid turnover, however, is the same in the fatty livers as in the controls.

Dr. H. Segal (University of Pittsburgh, Pittsburgh, Pa.): I would like to comment on Dr. Kennedy's remark regarding the reversibility of the transfer of phosphoryl choline from CDP-choline to glyceride. I think this is perhaps not so surprising. An analogous situation is the reversibility of polynucleotide phosphorylase. It apparently means that we have to include phospho diesters as high energy phosphate compounds.

Dr. Kennedy (closing remarks): I would like to agree that the glyceride transferase reaction and polynucleotide synthesis are analogous. However, at least to my mind it was unexpected that the phospholipids present in considerable amount in the cell could represent an unsuspected reserve of "high energy" compounds. Whether this has any physiologic meaning at all I do not know.