The Role of Choline in the Turnover of Phospholipids

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LET US FIRST consider the nature of the question, "What is the role of choline in the turnover of tissue phospholipids?" If our interest derived solely from the fact that choline is a constituent of a large portion of body phosphatides, one might equally enquire about the role of the phosphate or glycerol moiety in the turnover of phospholipids. Clearly then, the implication is that choline may influence the metabolism of phosphatides in a manner that might explain a more intriguing problem, namely, that of lipotropism and it is this question that we shall consider at the present time.

The recent emphasis on choline, methionine, vitamin B_{12}, and folic acid as lipotropic agents might tend to obscure the fact that lipotropism was discovered by Best and co-workers[1] as an activity of fed phosphatide. It was only after painstaking investigation that it was established that the whole phosphatide molecule was not essential for lipotropic action, but that choline alone could prevent or cure fatty infiltration of the liver.[2] Later, it was discovered that other agents, one or more steps removed from the phosphatide molecule also exhibited lipotropic activity. Nonetheless, the question remained whether these lipotropes acted on fat oxidation or fat transport directly, and whether their lipotropic activity was mediated through the phosphatide molecule. Although no direct evidence has yet been obtained on this problem, there are several reasons why one might wish to adhere to the previously proposed concept that choline removes excessive liver fat by increasing fat oxidation via its action on liver phosphatides. The relation of choline to fat oxidation has been reviewed by Artom in the previous paper.[3] We shall now consider some data relevant to the second part of the hypothesis: "Is the lipotropic action of choline mediated by stimulating the turnover of liver phosphatides?"

One of the earliest investigations in which tracer P^{32} was used, showed that choline increased the incorporation of this label into liver phosphatides of rats maintained on a high-fat, low-protein, choline-deficient diet.[4] At that time phosphatides were considered to be important vehicles for the transport of fatty acids and it was thus logical to study the incorporation of P^{32} into the plasma phosphatides. Figure 1 shows a typical result obtained from a study by Friedlander *et al.*[5] in choline-deficient dogs. There can be no doubt that a single administration of choline to a choline-deficient dog markedly increased the incorporation of inorganic P^{32} into plasma phosphatides. One might be tempted to conclude that in this animal, plasma phosphatide turnover was markedly increased, but a careful study of specific activity relations of liver and plasma phosphatides[6] revealed that the turnover of plasma phosphatides was not increased by choline and that the increased plasma phospholipid specific activity resulted from an increased synthesis of the precursor, namely, liver lecithin (Fig. 2). Here then, was direct proof that if choline diminished liver fat by acting on phosphatide

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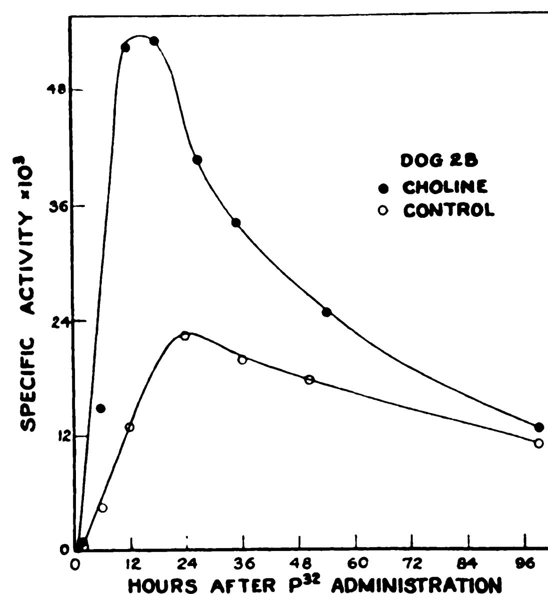

Fig. 1. Specific activity-time relations of plasma phospholipids of the dog after a single injection of P^{32}. The same animal was studied after 14 days on a choline-deficient diet with and without oral choline supplement prior to injection of P^{32}. (Reproduced from Friedlander, H. D., Chaikoff, I. L., and Entenman, C.: *J. Biol. Chem.* 158: 231, 1945; permission of the authors and publishers.)

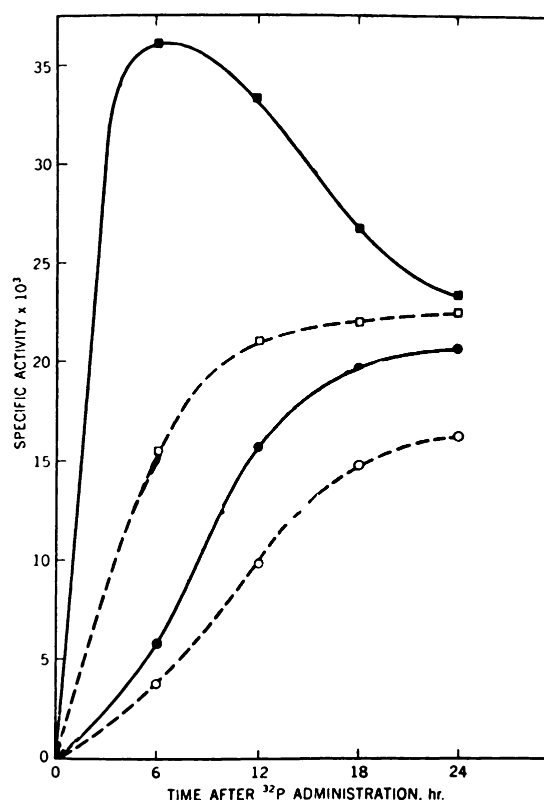

Fig. 2. Specific activity-time relations of plasma and liver choline containing phospholipids in choline-deficient dogs. O plasma, □ liver phospholipid specific activity after P^{32}. ● plasma, ■ liver phospholipid specific activity after P^{32} and a single dose of choline chloride. (Reproduced from Entenman, C., Chaikoff, I. L., and Friedlander, H. D.: *J. Biol. Chem.* 162: 111, 1946; permission of the authors and publishe.s.)

metabolism, it did *not* do so by increasing the transport of fatty acids from liver to extrahepatic tissues as an integral part of the plasma phosphatide molecule.

This conclusion was further supported by evidence obtained from hepatectomized animals[7] which demonstrated that plasma phosphatides played little or no role in the transport of fatty acids from one organ to another. If however, choline effects the removal of liver fat through its action on phosphatides, the possibility had to be studied that this action occurred through stimulation of fat oxidation. There is no direct evidence that liver phosphatides participate in fat oxidation but Table I summarizes some of our data on liver phosphatide synthesis in depancreatized[8] and phlorizinized[9] dogs during periods when severe ketosis might be taken to indicate increased utilization in body fat. In both these preparations, the incorporation of P^{32} into liver phosphatides ap-

TABLE I

Liver Phospholipid Synthesis in Diabetic and Phlorizinized Dogs

	Control	Depancreatized	Control	Phlorizinized
Number of dogs	15	10	7	8
Lipid P, mg/g	1.08 ± 0.04	1.13 ± 0.05	1.30 ± 0.10	1.32 ± 0.12
Specific activity*	8.21 ± 0.99	14.9 ± 1.5	4.98 ± 1.07	18.3 ± 1.3

Dogs were injected with P^{32}-phosphate and sacrificed 6 hours later.

* Per cent of the injected P^{32} per g of phospholipid P.

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peared to be increased parallel to the increase in fat oxidation. This furnishes at least circumstantial evidence that liver phosphatides may be involved in the catabolism of body fat.

Thus, we may summarize the available evidence as follows: (1) liver phosphatides may well participate in the oxidation of liver fat; (2) a single dose of choline stimulates liver phosphatide synthesis as well as the oxidation of fatty acids; and (3) choline does not increase the transport of liver fat by way of plasma phosphatides. Thus far the evidence supports the view that choline increases liver fat oxidation by stimulating liver phosphatide synthesis but we shall now consider some discordant facts.

First of all there are the experiments with liver slices derived from choline-deficient rats[10] and dogs.[11] As shown in Table II, phosphatide synthesis in the slices of dog liver incubated with P^{32} is depressed as might be expected if choline were essential for the normal turnover of liver phosphatides. Similar results were obtained in rat-liver slices.[10] The addition of choline to the incubation medium had no effect on phosphatide synthesis of normal liver slices or on slices from animals supplemented with dietary choline but greatly accelerated the incorporation of P^{32} into the choline-containing phosphatides of the choline-deficient liver slice. Thus, if choline were to act on fat catabolism merely by increasing liver phosphatide synthesis one might reasonably expect that whenever choline stimulated liver phosphatide turnover it would also increase the rate of fat oxidation. This is clearly not the case. Artom[12] showed that although injected choline stimulates fat oxidation by liver slices, the addition of choline to the incubating medium itself had no such effect. It is possible, of course, that fat oxidation in the liver slice is limited by factors other than the availability of phosphatides, but whatever the explanation, it is clear that stimulation of liver phosphatide synthesis is not the only prerequisite for an increase in fat oxidation.

Another disturbing aspect of the situation appeared when the effect of a single dose of choline on the synthesis of liver phosphatides was compared with the effects of chronic supplements of this lipotropic agent. In one experiment, rabbits weighing 1.5 to 3 kg were placed on four different diets: (1) Purina chow; (2) high-fat, low-protein, choline-deficient diet;* (3) the same diet with daily choline supplements, and (4) the same diet with a single intravenous injection of choline at the end of the 14-day experimental period. Radioactive phosphate was administered to all animals to measure liver phosphatide synthesis. The rabbit was originally chosen for this experiment to study the relationship between choline deficiency, phosphatide metabolism, and atherosclerosis.[13] However, it appears that the rabbit may be particularly suited for the study of choline deficiency since this animal converts the methyl of methionine to choline very poorly.[14] The results in Table III indicate that although the experimental animals were maintained on a low-protein, high-fat, choline-supplemented diet, their liver phosphatide concentration or synthesis did not differ from the chow-fed con-

TABLE II
Effect of Choline on Phospholipid Metabolism of Dog Liver Slices

Diet	Choline-containing phospholipids *mg/g*	P converted choline-containing phospholipid/hr/g	
		Without choline	With choline
Purina chow	0.67 ± 0.02	7.6 ± 0.67	8.3 ± 0.91
High-fat, low-protein, plus 1% choline (21 days)	0.44 ± 0.02	3.7 ± 0.32	4.7 ± 0.46
High-fat, low-protein, choline-deficient (21 days)	0.35 ± 0.01	4.0 ± 0.32	18.4 ± 1.17

Dog liver slices were incubated for 1 hour with P^{32}-phosphate in the presence and absence of choline chloride, 100 mg per 100 ml.

* The diet consisted of 38 g lard, 8 g casein, 44 g sucrose, 3 g of brewer's yeast, 5 g of Cowgill's salt mixture, and 2 ml of cod liver oil.

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TABLE III

Liver Phosphatide Metabolism of Rabbits

		High-fat-low-protein diet 14 days		
	Purina chow (1)	Daily oral choline (2)	No treatment (3)	Single dose intravenous choline (4)
Number of animals	9	6	5	6
Lipid P, mg P/g	1.06 ± 0.10	1.17 ± 0.04	1.25 ± 0.09	1.16 ± 0.12
Specific activity*	12.4 ± 1.2	13:5 ± 1.9	22.6 ± 2.1	38.7 ± 5.1

Rabbits were sacrificed 6 hours after administration of P^{32}-phosphate.

* Per cent of the injected P^{32} per g of phospholipid P.

trols as long as choline was present in the diet (compare columns 1 and 2). Apparently the presence of large amounts of fat or little protein *per se* did not affect liver phosphatide metabolism. However, when choline was *withdrawn* from the diet, liver phosphatide synthesis, as measured by the incorporation of P^{32}, nearly doubled. A further marked increase in liver phosphatide P^{32} took place (columns 3 and 4) when a single dose of choline was given to the choline-deficient animals simultaneous with the injection of P^{32}. Thus, the discussion of choline action requires a sharp differentiation between the effects of a single dose of choline and that of daily choline supplements. A somewhat similar result was obtained by Cayer and Cornatzer[15] in patients with liver disease who showed an above normal incorporation of P^{32} into plasma phosphatides the first time they received choline but failed to show any increase after a period of choline therapy.

To further evaluate these findings similar studies were undertaken in the dog (Table IV). Although liver phospholipid concentrations in the deficient animals decreased significantly, the P^{32} data are essentially similar to the data previously obtained in the rabbit. They show that incorporation of inorganic phosphate into liver phosphatides in the choline-deficient animals is greater than that of the choline-supplemented controls. While a single dose of choline in the dog resulted in enhanced liver phosphatide P^{32} formation, the daily administration of choline decreased this process below that of the choline-deficient animals. At the present time we cannot reach a satisfactory explanation for the results which we have obtained. It appears that the stimulation of liver phosphatide P^{32} synthesis by choline occurs only when the supply of dietary choline is the limiting factor in the series of reactions leading to phosphatide formation. In the animal which has been treated for some time with choline, and in which liver fat concentrations have decreased to normal levels, one might assume that factors such as the supply of triglycerides become limiting and keep the rate of phosphatide synthesis down at the level observed in animals fed low-fat commercial chow.

An alternative explanation might be based on the observation that free choline normally is removed from the bloodstream very rapidly.[16] The two processes which presumably are most important in this removal are the oxidation of choline by the aid of choline oxidase and the incorporation of choline into tissue lecithin.

TABLE IV

Liver Phospholipid Metabolism in Dogs

		High-fat-low-protein diet 21 days		
	Purina chow	Daily oral choline	No treatment	Single oral dose choline
Number of animals	12	6	8	6
Lipid P, mg/g	1.19 ± 0.04	0.97 ± 0.05	0.88 ± 0.05	1.09 ± 0.10
Specific activity*	10.2 ± 0.87	12.2 ± 1.55	19.4 ± 1.98	25.4 ± 3.0

Dogs were sacrificed 6 hours after administration of P^{32}-phosphate.

* Per cent of the injected P^{32} per g of phospholipid P.

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Since in the choline-deficient animal choline oxidase activity in the liver is sharply reduced[17] one might assume that in the deficient animal more choline would remain available for incorporation into phosphatides.

It is understood, of course, that one must be careful in concluding that the increased incorporation of P^{32} into a liver phosphatide fraction necessarily represents increased synthesis or increased turnover of liver phosphatide. While no differences in the specific activities of liver inorganic and organic acid-soluble phosphates were noted in the various experimental groups, it is quite possible that a single dose of choline administered to a choline-deficient animal might stimulate the formation of a specific phosphatide precursor or by some other means increase the specific activity of a phosphorylated intermediate such as cytidine diphosphate choline. To differentiate between the stimulation of phosphatide synthesis proper and the increased labeling of some phosphatide precursor one must measure the specific activity-time relations of the phosphatide fraction and its immediate precursor. Such an analysis may well clarify the differences between single and continued choline administrations and supply the missing links in our present knowledge about the effect of dietary choline on liver phosphatide synthesis.

It is obvious, however, from the results which we have presented, that the relationship of phosphatides to the lipotropic activity of choline is still obscure. The available evidence indicates that there is no simple relation between the rate of lipid phosphorylation in liver and the amount of lipotropic factors in the diet, or the extent of lipotropic activity from a single dose of choline or methionine (see also Horning and Eckstein[18]). The observed stimulation of liver phosphatide metabolism by a single dose of choline may turn out to be unrelated to lipotropic activity. Yet the fact that choline is an integral part of the liver lecithin molecule, and that under some conditions choline stimulates liver lecithin synthesis, presents enough circumstantial evidence to discourage the investigator from rejecting a hypothetical causal relationship between lipotropism and phosphatide metabolism.

SUMMARY

The effect of choline and choline deficiency on liver phosphatide metabolism has been studied in various species. Evidence to date indicates that a single dose of choline administered to choline-deficient animals increases the oxidation of liver fat as well as the synthesis of liver phosphatide P^{32}. On the other hand, the addition of choline to liver slices derived from choline-deficient animals promotes the incorporation of P^{32} into the phosphatide molecules without stimulating fat oxidation. In addition, it was observed that in the rabbit and dog the rate of incorporation of P^{32} into liver phosphatides is greater during periods of choline deficiency than during daily supplementation of the diet with 1 per cent choline. The implications of these findings are discussed in reference to the mechanism whereby choline prevents or cures fatty livers.

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DISCUSSION

Dr. W. Wells (University of Pittsburgh, Pittsburgh, Pennsylvania): Dr. Zilversmit in his excellent presentation has pointed out again the confusing nature of the role of choline in the phospholipid turnover. The importance of the administration of choline was pointed out as well as the importance of the level of fat in the diet. I would like Dr. Zilversmit to comment (1) on the effect of age. That is, as I understand it, in the adult animal there is little effect of choline on the phospholipid turnover, whereas in a weanling animal the effects of dietary choline toward increasing the phospholipid concentration and turnover have been shown and (2) on an earlier finding of his that the turnover of plasma cephalin, lecithin, and sphingomyelin were significantly increased in concentration in animals that had been fed cholesterol. I believe these were rabbits. In other words, is there any connection between cholesterol absorption and the phospholipids? It has been shown that choline has very little effect on the absorption of cholesterol. The work of Mann and the Harvard group suggests that atherosclerosis cannot be induced in monkeys which have been fed a low-methionine diet or a low sulfur-containing diet unless choline is added to that diet.

Dr. Kennedy: I should like to make one very brief comment, and that is strongly to reinforce the suggestion that Dr. Zilversmit made that in a P^{32} experiment of this kind one should compare the specific activity of the lecithin with its immediate precursor rather than with the total acid-soluble phosphorus fraction, because the cytidine diphosphate choline content of liver is so low that it is a very minute fraction of the total acid-soluble phosphorus fraction. Perhaps the otherwise very puzzling results that Dr. Zilversmit reports could be explained on the basis of the higher specific activity of the CDP-choline in some experiments.

Dr. W. Hartroft (Washington University, St. Louis, Missouri): I would like to ask if from the experiments with dogs and rabbits you have any data on the effect of a single injection of choline into control animals receiving the basal diet supplemented with choline during the entire experimental period? Results might clarify what part (if any) of the effects observed from this procedure in choline-deficient animals was the result of a pharmacologic or physiologic action of the injected choline.

Dr. Artom: I was interested in the difference in the results obtained by Dr. Zilversmit, when choline was given in one dose, or when it was added to the diet of his rabbits. We have observed a similar difference in our study of the action of diethanolamine on the incorporation of P^{32} into the lipids of rat liver. Given in one single dose, this analogue of ethanolamine stimulated the synthesis of cephalins and also of lecithins. On the other hand, as I mentioned in my paper, when diethanolamine was mixed with the diet, and therefore absorbed in small repeated doses over a period of several days, it acted as an inhibitor of the synthesis of lecithins.

Dr. Herndon: Isn't the effect of giving diethanolamine pharmacologic rather than physiologic?

Dr. Olson: During the last several years we have been engaged in a study of the lipoproteins of liver (*Chemistry of Lipides as Related to Atherosclerosis*, pp. 108–111, Thomas, Springfield, Illinois, 1958). As has already been mentioned the lipids of liver and the lipoproteins of which they are a part are contained largely in the particulates (nuclei, mitochondria, microsomes, and membranes). The cytoplasm is essentially free of these entities. These lipoproteins (and I speak of the group collectively since our studies thus far have been confined to whole homogenates made with a Waring blendor) appear to be quite different from those in the serum. They are more unstable, of high density (flotate only at 1.21 with a $-S$ value of 40 to 60), of apparently different frictional ratio than the high density alpha's of serum, and contain nitrogen, total lipid, phospholipid, and cholesterol in the approximate ratio of 4:40:20:2. Of course, these are rough approximations of the properties of this heterogeneous group of compounds and much more work must be done with the fractions from individual particulates before our knowledge of this interesting group of lipoproteins is complete. We must remember, however, that when we speak of lipid metabolism in the liver we are talking

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about the biochemical activity of the lipids in the particulates under normal conditions. The accumulation of neutral fat in the cytoplasm in choline deficiency probably results from relative "overproduction" of triglyceride by the mitochondria under conditions which prevent it from being moved into the serum at the proper rates. The subsequent effects of this triglyceride upon the integrity of the particulates may be related to a biophysical rather than a biochemical effect by which some of the lipids of the particulates are "solubilized" in the sea of neutral fat. Dianzani and Viti (*Biochem. J.* 59: 141, 1955) have reported the transfer of cytochrome C, from mitochondria to cytoplasm in the fatty liver produced by carbon tetrachloride. Finally, although the role of the lipids in the lipoprotein micelle which is found in the organized mitochondrion has been thought to represent merely a "glue" to hold various hydrogen transport and other enzymes in proper apposition, it is apparent from a recent paper by Marinetti *et al.* (*J. Biol. Chem.* 229: 1027, 1957) that the lipids of cytochrome b-cytochrome c_1 complex may participate in electron transport.

Dr. Zilversmit (closing remarks): We have no data ourselves on the effect of age on phospholipid turnover and the effect of choline thereon. There are numerous studies on the phospholipid level of choline-treated animals at different ages and I would be interested in hearing of studies on the turnover of phospholipid.

As far as the relation of choline to cholesterol is concerned, I am afraid I have very little to offer except to say that we were interested in this question. This was one reason why we performed the rabbit experiment since, as you know, in the rabbit cholesterol feeding produces a pronounced fatty liver and lesions in the aorta. We thought that since a single dose of choline has a stimulating effect on liver phospholipid formation, it might have an effect on other organs, such as the aorta as well. We were highly disappointed to learn that a single dose of choline in either a choline-deficient animal or in a cholesterol fed rabbit has no effect whatsoever on the synthesis of phospholipids (*Proc. Soc. Exper. Biol. & Med.* 92: 454, 1956).

There is some disagreement between our laboratory and Chaikoff's laboratory (*J. Biol. Chem.* 128: 735, 1939) and the group of Clement in France (*Compt. Rend.* 236: 412, 1953) on the effect of cholesterol on liver phospholipid synthesis. Both Clement and Chaikoff claim that in the rat liver phospholipid synthesis is greatly inhibited by cholesterol feeding. We have never exactly reproduced this experiment but we have fed some cholesterol to rats and found very little, if any, effect. On the other hand in the rabbit, cholesterol feeding causes a marked increase in phospholipid synthesis.

With regard to Dr. Hartroft's question, we have done the experiment he suggested, and the single dose of choline had no effect in animals which had been supplemented; neither had it any effect in animals which were on a chow diet. I think it has been also shown by Cornatzer and others in patients who have been treated with choline or methionine for a while that a single dose of choline has no effect on phospholipid formation. This comment also would be pertinent to the drug action. Even though there might still be some nonspecific type of effect, say upon oxygen consumption, which we have not measured, the fact remains that these nonspecific effects do not occur in these other preparations and this possibility is eliminated.

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