The Labeling of Phospholipid with Orthophosphate ^{32}P as Influenced by Procedures Which Alter Plasma Cholesterol Concentration (35611)

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Previous studies (1–4) from this laboratory have indicated that a primary increase in plasma phospholipid concentration induces a secondary increase in plasma free cholesterol concentration. Since an elevation in plasma phospholipid is observed in almost all varieties of clinical hypercholesterolemia, it seems possible that in certain varieties of hypercholesterolemia, an initial dysfunction occurs in phospholipid metabolism, which then in turn effects a sequential rise in the plasma cholesterol. Indeed, from our own studies (5), it would appear that the primary cause of at least one variety of hypercholesterolemia, namely that observed after biliary obstruction, is the initial rise in plasma phospholipid occurring after such obstruction.

In view of this experimentally observed sequential relationship of hyperphospholipidemia and hypercholesterolemia, and in view of the observation (6) that dietary cholesterol depressed ^{32}P uptake by liver phospholipids in rats, we thought it of interest to study labeling of hepatic phospholipid in various states associated with alterations in the plasma level of cholesterol, namely following experimental elevation of plasma phosphatide, biliary obstruction, administration of clofibrate (7, 8) and induction of a hypothyroid state (9–13).

Methods and Materials. A. Infusion of Lecithin. Male rats of the Long-Evans strain, 8 weeks of age and weighing approximately 290 g, were divided into experimental groups, designated I, II, and III together with corresponding groups of control animals designated I-C, II-C, and III-C. Each Group consisted of 5 animals.

All rats were maintained on laboratory chow (Simonsen Farms "Green Diet") until 4:00 p.m. prior to day of use; food was then withdrawn. The morning after food withdrawal each rat was anesthetized with ether and a polyethylene catheter was inserted into the iliolumbar vein. The rat was then placed in a restraining cage (14) and the free end of the catheter was connected to a constant infusion pump. At the start of the infusion period each rat received 2 ml of infusion fluid within approximately 15 sec. The infusion was continued thereafter at the rate of 1 ml/hr until termination of the experiment. The experimental animals received an infusion of 5.2% crude brain lecithin[1] (15) in 0.85% NaCl, adjusted to pH 7.3 with NaOH. Control animals received the same volume of 0.85% NaCl. At indicated times after the start of infusion, each rat received a single injection of carrier-free ^{32}P as neutral orthophosphate in 0.85% NaCl administered through the intravenous catheter. The dose was identical for all rats in a group and their corresponding controls, however, some groups received a dose of 0.21 mCi/rat, others greater amounts up to 0.5 mCi/rat.

Group I rats were infused with lecithin and Group I-C control rats with 0.85% NaCl for 6 hr. Both groups received orthophosphate-^{32}P at the end of the fifth hour of infusion.

[1] Nutritional Biochemical Co., "Animal lecithin 90% pure." The hypercholesterolemic effect of this material is comparable to that of a variety of phospholipids (15). When chromatographed on a thin layer of silica gel H and developed with chloroform–methanol–acetic acid–water, 95:35:3:4, 3 spots were seen after sulfuric acid char. The largest, containing approximately 80% of the total phosphorus, was located where phosphatidyl choline was expected; the other two spots were comprised in about equal amounts of material located where sphingomyelin and lysolecithin would be expected. No spots corresponding to phosphatidyl ethanolamine or phosphatidyl serine were observed.

Group II experimental and Group II-C control rats received lecithin or NaCl for 12 hr and received orthophosphate-^{32}P at the end of the sixth hour of infusion. Groups III and III-C rats received lecithin or NaCl for 24 hr and received orthophosphate-^{32}P at the end of the 18th hour.

At the end of this infusion period, each animal was bled from the aorta into a syringe containing heparin. In order to flush residual blood from the liver, 0.85% NaCl solution was injected retrograde through the superior vena cava. The blanched liver was removed, sealed in a polyethylene bag, and preserved frozen until analyzed.

B. Biliary obstruction. Seventeen rats (Group IV) similar to those described above were subjected to double ligation of the common bile duct, ligation thus obstructing the flow of both bile and pancreatic juice. Sixteen control rats (Group IV-C) were subjected to sham operation during which the bile duct was manipulated with forceps. Eighteen hr after operation, the rats of both groups received carrier-free ^{32}P orthophosphate by injection into the penis vein. Six hr later, the rats were bled, killed, and the liver was removed and frozen until analyzed.

C. Oral administration of Clofibrate. Ten male rats (Group V) were fed *ad libitum* the chow diet mixed with clofibrate[2] in proportions of 250 mg of clofibrate for each 100 g of diet. Ten control rats (Group V-C) were fed the same diet without clofibrate. After consuming these respective diets for 4 weeks the experimental rats received 625 mg of clofibrate emulsified in 1 ml of water by stomach tube. The control rats were given 1 ml of 0.85% NaCl. Exactly 1 hr after the oral intubation, each rat received a mixture of carrier-free orthophosphate-^{32}P and 0.3 mCi of tritiated sodium acetate (0.26 mg)[3] by injection via the penis vein. Six hr following this injection, the rats were bled, killed, and the liver was obtained and frozen.

D. Administration of propylthiouracil. Ten male rats (Group VI) were fed *ad libitum* the chow diet containing 150 mg of propylthiouracil/100 g of diet. Ten control rats (Group VI-C) ingested the unsupplemented diet. Three weeks after the beginning of the diet, all rats received carrier-free orthophosphate-^{32}P and tritiated sodium acetate via the penis vein. Six hr after injection, the rats were bled, killed, and the liver was secured and frozen.

E. Chemical procedures. All plasma samples were analyzed quantitatively for cholesterol by the method of Saifer and Kammerer (16), for lipid phosphorus by the method of Zilversmit and Davis (17) and for phosphorus in the form of acid-soluble phosphate by the method of Bartlett (18). The plasma samples obtained from Groups VI and VI-C rats were also analyzed for their thyroxine content by a commercial laboratory according to the method of Pileggi and Kessler (19).

Each liver was weighed wet after light blotting. The livers of rats of Groups I–IV and their respective controls were analyzed only for their labeled and unlabeled phospholipid contents. This was accomplished as follows: A weighed portion of liver was ground in a Potter-Elvejhem homogenizer, suspended in H_2O, and a sample of the suspension was precipitated with 9 vol of 10% trichloroacetic acid. A portion of the supernatant from the trichloroacetic precipitation was analyzed for phosphorus by the method of Bartlett (18) and another portion was counted for ^{32}P activity in a Packard 3003 liquid scintillation counter. Efficiency was checked by counting a commercial standard of known activity.[5] The trichloroacetic acid precipitate was extracted with 20 vol of $CHCl_3$:MeOH (2:1; v/v), and the extract was partitioned with H_2O (20). A sample of the $CHCl_3$ layer was removed for phospholipid analysis by the method of Zilversmit and Davis (17). A factor of 25 was used in calculation to convert lipid P to phospholipid. The remainder of the $CHCl_3$ layer was evaporated to dryness and ^{32}P was counted in the Packard counter.

The livers of rats of Groups V and VI and their respective controls were analyzed for

[2] Atromid-S, Ayerst Laboratories, Inc.

[3] New England Nuclear Co.

[4] Bay Area Pathologists Central Laboratories, San Francisco.

[5] Amersham-Searle, Buckinghamshire, England.

both their labeled and unlabeled phospholipid and cholesterol. The phospholipid analyses and ^{32}P counts of these livers were done exactly as described above except that a window excluding tritium was used in the Packard counter.

For the cholesterol-^{3}H counts, a weighed portion of the liver was cut finely and extracted under reflux with 50 vol of ethanol:acetone(1:1;v/v). The extract was divided, a portion being used for quantitative analysis of cholesterol by the method of Saifer and Kammerer (16) while the remainder was dissolved in chloroform and treated with zeolite[6] to remove phospholipid (21). The phospholipid-free extract was evaporated, taken up in ethanol:acetone (1:1;v/v), saponified with alcoholic KOH, neutralized with acetic acid and precipitated with 1% digitonin in 50% ethanol. The digitonin precipitate was washed twice with ethanol–acetone and once with ether. It was dried and dissolved in glacial acetic acid. A sample of the acetic acid solution was removed for cholesterol determination while ^{3}H radioactivity in the remainder was counted in a Packard model 3003 scintillation counter. Quench was monitored by the channels ratio method and checked by addition of an internal standard of tritiated toluene.

Results. A. Infusion of Lecithin. No significant differences in concentration of liver phospholipid were observed between any group infused with lecithin and its corresponding control group (Table I, Groups I vs I-C, II vs II-C, and III vs III-C). Hence there was no evidence that lecithin infusion resulted in appreciable storage or retention of phospholipid in the liver. The specific activity of liver phospholipid in each experimental group, however, was significantly lower than that of its control group (Table I), showing that lecithin infusion may have depressed the incorporation of inorganic phosphorus into phospholipid. An alternative possibility that lecithin promoted the release of phospholipid-^{32}P into plasma, seems less likely since the total amount of radioactive P in plasma phospholipid did not differ significantly between experimental and control groups (Table I); this also indicates that infusion of lecithin did not suppress entrance of phospholipid from liver to plasma. Plasma cholesterol concentration increased up to 7-fold under the influence of lecithin infusion, the longer the duration of infusion the greater the plasma cholesterol concentration. The plasma ^{32}P radioactivity in Table I is expressed in terms of dpm/ml, rather than as specific activity, to avoid distortion by the large amounts of phospholipid infused.

B. Biliary obstruction. This procedure did not alter the concentration of phospholipid in liver, nor did it affect the incorporation of orthophosphate-^{32}P into liver phospholipid (see Table I, Groups IV and IV-C). Bile duct ligation brought about a 70% increase in plasma cholesterol level and an increase of 150% in plasma phospholipid level, as compared with control values. This plasma phospholipid increase was supplied disproportionately from radioactive phospholipid, since plasma from ligated animals contained over 4 times as much phospholipid-^{32}P as did control plasma.

C. Oral administration of clofibrate. As others (22) and we (23) have reported, the average weight (21.0 g) of the livers of the clofibrate-treated rats was significantly greater ($p<0.001$) than that (14.3 g) of the control rats. Despite this disparity in hepatic weight, the hepatic phospholipid concentration (see Table II) was closely similar in the 2 groups. Similarly the average amount of ^{32}P in the total livers also was approximately the same in the 2 groups. However, the specific activity of liver phospholipid (see Table II) was lower in the clofibrate-treated animals. As also found by others (24), clofibrate was seen to depress incorporation of acetate-^{3}H into cholesterol, whether measured by the specific activity of cholesterol or by radioactivity in the total cholesterol content of the whole liver. A similar depression of acetate-^{3}H incorporation was observed in plasma cholesterol. Clofibrate administration also effected (see Table II) a fall in plasma phospholipid concentration. However, it did not cause any change in the specific activity of plasma phospholipid.

D. Administration of propylthiouracil. The

[6] Taylor Chemicals, Inc., Baltimore, Md.

TABLE I. Effect of Lecithin Infusion and of Bile Duct Ligation on Incorporation of Orthophosphate-^{32}P into Phospholipid.

Duration of infusion (hr)	Elapsed time until ^{32}P injection (hr)	Substance infused or procedure performed	No. of rats used	Designation of group in text	Liver phospholipid (mg/g)	p value	Liver phospholipid (dpm/g of liver $\times 10^5$)	p value	Plasma cholesterol (mg/100 ml)	p value	Plasma phospholipid (mg/100 ml)	p value	Plasma phospholipid (dpm/ml $\times 10^2$)	p value
6	5	Lecithin	5	I	20.5[a] ±1.0	NS[b]	0.8 ±0.08	<0.02	110 ±10	<0.001	2000		8.7 ±1.1	NS
		Saline	5	I-C	21.7 ±0.9		1.3 ±0.08		41 ±3.5		95		12 ±1.0	
12	6	Lecithin	5	II	25.9 ±0.6	NS	6.0 ±0.3	<0.001	271 ±11	<0.001	3446		270 ±20	NS
		Saline	5	II-C	28.3 ±0.7		11 ±1		51 ±4		90		280 ±18	
24	18	Lecithin	5	III	28.8 ±1.4	NS	12 ±0.57	<0.01	340 ±49	<0.001	2215		54 ±3.8	NS
		Saline	5	III-C	25.3 ±1.2		17 ±0.48		47 ±8		96		48 ±3.7	
No infusion; bile duct ligation or sham operation	18	Ligation	17	IV	24.3 ±0.8	NS	9.6 ±1.0	NS	75 ±8	<0.01	216 ±31	<0.01	900 ±92	<0.001
		Sham operation	16	IV-C	25.1 ±1.0		8.3 ±0.9		44 ±3		86 ±8		210 ±16	

[a] Values are averages with standard error of the mean and corresponding values of p (probability of occurrence of observed difference from Table of Student's t).

[b] NS = not significant, or $p > 0.05$.

TABLE II. Effect of Treatment with Clofibrate or Propylthiouracil on Incorporation of Orthophosphate ^{32}P into Phospholipid and of C^3H_3ONa into Cholesterol.

Treatment	No. of rats used	Designation of group in text	Liver phospholipid (mg/g)	p value	(dpm/mg of phos. $\times 10^6$)	p value	(dpm/whole liver $\times 10^7$)	p value	Liver cholesterol (mg/g)	p value	(dpm/mg)	p value	(dpm/whole liver)	p value
Clofibrate	10	V	21.3[a] ±1.3	NS[b]	1.9 ±0.14	<0.05	2.9 ±0.24	NS	1.7 ±0.12	NS	1800 ±147	<0.01	47,000 ±4259	<0.02
Control	10	V-C	20.1 ±1.2		2.3 ±0.11		2.6 ±0.13		2.1 ±0.14		4700 ±887		106,000 ±19,553	
Hypothyroid	10	VI	22.6 ±1.4	NS	1.5 ±0.07	NS	4.6	NS	1.8 ±0.10	NS	2200 ±188	<0.01	35,000 ±3817	<0.02
Control	10	VI-C	20.1 ±1.3		1.7 ±0.15		5.1		2.0 ±0.28		6300 ±952		121,000 ±24,783	

Treatment	No. of rats used	Designation of group in text	Plasma phospholipid (mg/100 ml)	p value	(dpm/mg of phos. $\times 10^6$)	p value	Plasma cholesterol (mg/100 ml)	p value	(dpm/mg)	p value	T-4 (μg/100 ml)
Clofibrate	10	V	75 ±4.7	<0.001	1.2 ±0.7	NS	50 ±3.7	<0.001	2400 ±377	<0.01	
Control	10	V-C	105 ±4.9		1.2 ±0.5		73 ±3.5		4400 ±703		
Hypothyroid	10	VI	115 ±9.3	NS	2.2 ±0.53	NS	86 ±5.8	<0.001	2200 ±162	<0.001	0.5
Control	10	VI-C	94 ±11.2		2.9 ±0.58		51 ±5.0		6100 ±1258		2.8

[a] Values are averages with standard error of the mean and corresponding values of p (probability of occurrence from Table of Student's t).
[b] NS = not significant, or $p > 0.05$.

administration of propylthiouracil led to a hypothyroid state as indicated by the markedly reduced level (see Table II) of plasma thyroxine of the treated rat. However this induced hypothyroidism had no discernible effect upon the rate of incorporation of orthophosphate-^{32}P into liver phospholipid (see Table II) or upon the other aspects of phospholipid metabolism investigated. As expected from our earlier studies (9), the hepatic rate of incorporation of acetate-^{3}H into cholesterol was depressed significantly in the hypothyroid rats.

E. Inorganic phosphate. The concentration and specific activity of acid-soluble phosphorous in the liver and plasma did not differ significantly between any experimental group and its corresponding control. Therefore, the extent of incorporation of ^{32}P into phospholipid may be taken to reflect the rate of the incorporation process itself, rather than to depend upon differences of substrate concentration.

Discussion. The synthesis of liver phosphatide is multicompartmental, of sequential complexity, and yields products showing structural and metabolic heterogeneity (25). The analytical methods we have used have the virtue of simplicity but suffice for only a limited first approach to information about the heterogenous hepatic pool.

The present results indicate that the extent of incorporation of ^{32}P into liver phosphatide was not significantly altered in any of the experimental studies except that one in which the animals were rendered hyperphosphatidemic by the continuous infusion of a phosphatide itself. Thus experimental increase of plasma cholesterol by induced biliary obstruction or hypothyroidism or its decrease by clofibrate administration failed to alter significantly the extent of incorporation of ^{32}P into hepatic phosphatide, even though in the latter two experimental conditions, the extent of incorporation of labeled acetate into hepatic cholesterol was significantly depressed.

The changes in plasma phospholipid in these various conditions were of some interest. Thus the plasma content of labeled phosphatide rose considerably in rats after biliary obstruction despite the fact that no significant change was observed in their extent of incorporation of ^{32}P into hepatic phosphatide. Apparently then, following biliary obstruction not only does retention of phospholipid in plasma occur, but also a good fraction of the latter consists of newly synthesized materials. On the other hand, no change was observed in the plasma content of labeled phospholipid in the rats infused with lecithin despite the fact that a significant fall had occurred in their incorporation of ^{32}P into hepatic phospholipid.

Despite the expected (9) decreased hepatic rate of cholesterol synthesis found in the hypothyroid rats, we were unable to detect, as were also earlier observers (10, 12), any significant change in the plasma content of labeled or unlabeled phospholipid. On the other hand, clofibrate administration significantly depressed the plasma content of both unlabeled and labeled phospholipid as well as that of cholesterol, phenomena also observed (26, 27) in the clofibrate-treated human subject.

The presence of lysolecithin and sphingomyelin in the lecithin preparation was indicated by thin-layer chromatography. It is possible that the effects of this preparation may have been due in part, or entirely, to these substances. Lysolecithin is of particular concern since it has been considered to be toxic because of its hemolytic action (28). It is noteworthy that lysolecithin occurs physiologically in rat plasma, which has been reported to contain on the average 17.5% of its lipid phosphorus as lysolecithin (29). Lysolecithin phosphorus has been reported to constitute 7%, and sphingomyelin phosphorus 19% of the lipid phosphorus of human plasma (30).

Summary. Changes in the rat plasma concentration of cholesterol were effected by lecithin infusion, biliary obstruction, clofibrate administration, and by induction of hypothyroidism. The extent of incorporation of ^{32}P into hepatic phospholipid was altered (*i.e.*, decreased) in only those rats subjected to lecithin infusion. However, changes in the plasma content of labeled phospholipid were observed only in those animals subjected to

biliary obstruction (increase) or clofibrate (decrease) administration.

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1. Friedman, M., and Byers, S. O., Amer. J. Physiol. **186**, 13–18 (1956).

2. Byers, S. O., and Friedman, M., Proc. Soc. Exp. Biol. Med. **92**, 459-462 (1956).

3. Di Luzio, N. R., and Zilversmit, D. B., Amer. J. Physiol. **199**, 991-994 (1960).

4. Byers, S. O., Friedman, M., and Sugiyama, T., J. Biol. Chem. **237**, 3375–3380 (1962).

5. Friedman, M., and Byers, S., Amer. J. Physiol. **188**, 337 341 (1957).

6. Morin, R. J., and Alfin-Slater, R. B., Metabolism **13**, 69–74 (1964).

7. Cramer, K., J. Atheroscler. Res. **3**, 500–508 (1963).

8. Hayward, P. J., Davies, T. D., and McKendrick, C. S., J. Atheroscler. Res. **3**, 571–579 (1963).

9. Byers, S. O., Rosenman, R. H., Friedman, M., and Biggs, M. W., J. Exp. Med. **96**, 513–516 (1952).

10. Bayton, S., Dayton, J., Drimmer, F., and Kendall, F. E., Amer. J. Physiol. **199**, 71–76 (1960).

11. Flock, E. V., Bollman, J. L., and Berkson, J., Amer. J. Physiol. **155**, 402–408 (1948).

12. Cornatzer, W. E., Gallo, G., and Davison, J. P., Proc. Soc. Exp. Biol. Med. **84**, 103-105 (1953).

13. Nelson, D. R., and Cornatzer, W. E., Endocrinology **77**, 37-44 (1965).

14. Bollman, J. L., J. Lab. Clin. Med. **33**, 1348 (1948).

15. Byers, S. O., and Friedman, M., Amer. J. Physiol. **193**, 435-438 (1958).

16. Saifer, A., and Kammerer, O. F., J. Biol. Chem. **164**, 657-677 (1946).

17. Zilversmith, D. B., and Davis, A. K., J. Lab. Clin. Med. **35**, 155–160 (1950).

18. Bartlett, G. R., J. Biol. Chem. **234**, 466-468 (1959).

19. Pileggi, V., and Kessler, G., Clin. Chem. **14**, 339–347 (1968).

20. Folch, J., Lees, M., and Stanley, G. H. S., J. Biol. Chem. **226**, 497-509 (1957).

21. Cheng, A. L. S., and Zilversmit, D. B., J. Lipid Res. **1**, 190–191 (1960).

22. Best, M. M., and Duncan, C. H., J. Lab. Clin. Med. **64**, 634–642 (1964).

23. Byers, S. O., and Friedman, M., Atherosclerosis **11**, 373-382 (1970).

24. Thorp, J. M., and Waring, W. S., Nature (London) **194**, 948–949 (1962).

25. Balint, J. A., Beeler, D. A., Treble, D. H., and Spitzer, H. L., J. Lipid Res. **8**, 486–493 (1967).

26. Hellman, L. B., Zumoff, B., Kessler, G., Kara, E., Rubin, I. L., and Rosenfeld, R. S., J. Atheroscler. Res. **3**, 454-466 (1963).

27. Carlson, L. A., Hogsted, B., and Örö, L., J. Atheroscler. Res. **3**, 467-474 (1963).

28. Lovern, J. A., "The Chemistry of Lipids of Biochemical Significance," p. 17. Wiley, New York (1955).

29. Newman, H. A. I., Liu, C. T., and Zilversmit, D. B., J. Lipid Res. **2**, 403–411 (1961).

30. Phillips, G. B., Biochim. Biophys. Acta **29**, 594–602 (1958).

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