The Enzymatic Nature of Phospholipid Synthesis in Normal Rabbit and Human Aorta

Results of In Vitro Studies

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SUMMARY

Evidence has been presented to show that phospholipids are synthesized by supernatants of homogenates of rabbit and human aorta when palmitate-1-C-14, or linoleate-1-C-14, are used. The synthetic process is enzymatic in nature; it depends on temperature, *p*H, and the presence of various cofactors. These requirements are not unlike those of cell-free liver preparations, which also synthesize phospholipids.

The origin of various lipid substances found in normal and atherosclerotic blood vessels has not been definitely determined. This study is concerned with some aspects of the source of phospholipids in normal rabbit and human arteries.

Chernick[1] first demonstrated in vitro that normal rat thoracic aorta incorporated P-32 into phospholipids. Zilversmit et al.,[2,3] utilizing in vivo technics, subsequently found the same to be true for normal rabbit aorta. In addition these investigators[4-6] found that the incorporation of P-32 into aortic phospholipids was accelerated in cholesterol-fed animals. Zilversmit[7] also administered P-32 to human subjects prior to vascular surgery and found the label was incorporated into intimal phospholipids. These experiments suggest that the phosphate moiety of arterial wall phospholipids is synthesized at least in part in situ.

Recently Newman[8] reported in vivo studies in rabbits utilizing acetate-1-C-14 and presented evidence to suggest that the carbon skeleton of phospholipids is also synthesized in the aorta.

Although these in vivo experiments are of great value, they do not provide direct information on specific enzymatic pathways of phospholipid synthesis. In the present study in vitro technics were utilized in an effort to elucidate these processes. A clear supernatant fraction of homogenates of aortic tissue was used so that any phospholipid increase could be due only to actual synthesis and various factors affecting this synthesis could then be scrutinized. Evidence will be presented to demonstrate that rabbit and human aorta synthesize phospholipids by enzymatic processes similar to those described for liver.

Recently Stein et al.[9] have used an in vitro system similar to the one reported in this communication. Many of their findings are confirmed in the present study.

METHODS

Female albino rabbits weighing approximately 2.5 kg. were anesthetized with 2 to 3 ml. of sodium pentobarbital (60 mg. per milliliter). The entire aorta was removed, chilled, and cleaned of adhering fat and as much adventitia as possible. Four to eight aortas were pooled and homogenized in one milliliter of Tris buffer (0.1 M, *p*H 7.4) per 200 mg. wet weight of tissue. The homogenate was centrifuged in the cold at 800 x G for twenty minutes and the slightly turbid supernatant fluid was drawn off through a No. 18 needle and filtered through a cotton wad to remove the thin layer of fat at the surface.[10]

One milliliter aliquots of the supernatant fluid were added to 10 ml. Erlenmeyer flasks containing 0.25 to 0.5 μc. of potassium palmitate-1-C-14 or potassium linoleate-1-C-14,* and 5 μmoles ATP, 1 μmole $MgCl_2$, 0.2 μmoles CoA, 25 μmoles DL-α-glycerophosphate, and 300 μmoles NaF in 0.5 ml. of Tris buffer, *p*H 7.4. The flasks were incubated at 37° C. in a Dubnoff incubator for

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*Radioactive fatty acids were purified by silicic acid chromatography and were then solubilized by adding concentrated KOH to the volume desired and then warming under tap water until the solution appeared clear.

varying periods of time, with air as the gas phase. At the end of the incubation period the reaction was stopped by adding 4 ml. of methyl alcohol. Two or more controls were run with each experiment. In the control flasks the reaction was stopped with methyl alcohol immediately after the addition of cofactors and radioactive substrates. The contents of the control and incubated specimens were extracted for lipids by the method of Freeman[11] and the lipids separated by silicic acid column chromatography into four fractions.[12] The major components of these fractions were: cholesterol esters in fraction I, triglycerides and free fatty acids in fraction II, cholesterol and mono- and diglycerides in fraction III, and phospholipids in fraction IV. The radioactivity of each lipid fraction was determined by counting in toluene[13] in a Packard Tricarb automatic liquid scintillation counter. Results are expressed as the per cent of radioactivity recovered that was incorporated into phospholipids over controls.

Phosphorus determinations were done on the phospholipid fractions by a modification of the method of Bartlett,[14] with 70 per cent perchloric acid used in place of sulfuric acid. Results are expressed as the increase in lipid-soluble phosphorus during incubation over control values.

Phospholipids were further separated by paper chromatography[15] and the papers scanned for radioactivity under a strip counter. In each paper run, known phospholipid standards were used.

Human aorta obtained immediately after vascular surgery and from autopsy specimens was stripped of all adventitia and treated in a manner identical to that described for rabbit aorta.

RESULTS

Rabbit aorta. Radioactive palmitic acid and linoleic acid were incorporated into phospholipids during two hours' incubation. As seen in table 1, the change in percentage of total counts recovered that was found in the phospholipids was quite variable from one experiment to the next but averaged 12 per cent for palmitate-1-C-14 and 23 per cent for linoleate-1-C-14. On infrequent occasions no radioactivity was incorporated into the phospholipids. The cause for these apparent "failures" was never adequately explained. The two fatty acids were not incorporated into the major neutral lipid fractions. Although in a few experiments mono- and diglycerides were found to contain radioactivity, this finding was not consistent nor large enough for any conclusions to be drawn.

Table 2 summarizes a number of experiments in which the phospholipid phosphorus was determined before and after two hours' incubation. There was a significant increase in phosphorus after the incubation interval. As can be seen from table 2 there was appreciable variation in the phospholipid phosphorus content in the control as well as incubated samples. On four occasions no rise in phosphorus was observed after incubation. These experiments have not been included in the figures in table 2.

In an effort to determine which phospholipids were being labeled the phosphatides were separated by silicic acid paper chromatography and the patterns of radioactivity determined on a strip counter. Figure 1 shows that these patterns were similar for the two radioactive substances used. The major labeled component was chromatographically identical with lecithin, with lesser amounts in phosphatidylinositol and/or lysolecithin, phosphatidylserine, phosphatidylethanolamine, and perhaps sphingomyelin. Two unidentified peaks ran near the solvent front.

Experiments were performed to investigate the effect that deletion or addition of various cofactors would have

TABLE 1

Per cent radioactivity incorporated into phospholipids after two hours' incubation of rabbit aorta

Labeled substance used	No. of experiments	Controls	Two hours' incubation	Average increase over control	Significance (Standard deviation)
Palmitate-1-C-14	14	3.5±1.7	15.6±4.5	12.1	<.001
Linoleate-1-C-14	10	4.4±1.7	27.3±6.5	22.9	<.001

A summary of all experiments done with supernatant fluid from homogenates of rabbit aorta using 1 gm. of aorta/5 ml. of Tris, and the various cofactors mentioned in the text. The values given in columns 3 and 4 are the per cent of radioactivity recovered which was incorporated into phospholipids and their standard deviations.

TABLE 2

Increase in lipid soluble phosphorus after two hours' incubation of rabbit aorta

Labeled substance used	No. of experiments	Controls	Two hours' incubation	Average increase over control	Significance (Standard deviation)
Palmitate-1-C-14	8	15.5±2.6	28.8±6.7	13.3	<.005
Linoleate-1-C-14	5	13.6±3.4	21.1±1.4	7.5	<.01

All experiments were done with supernatants from homogenates of rabbit aorta using 1 gm. of aorta/5 ml. of Tris, and the various cofactors mentioned in the text. The figures in the columns labeled "control," "two hours' incubation," and "average increase over control" are in μg. These figures represent μg. of lipid soluble phosphorus in the supernatant of homogenate from the equivalent of 200 mg. wet weight of tissue. In addition, the standard deviations are also listed.

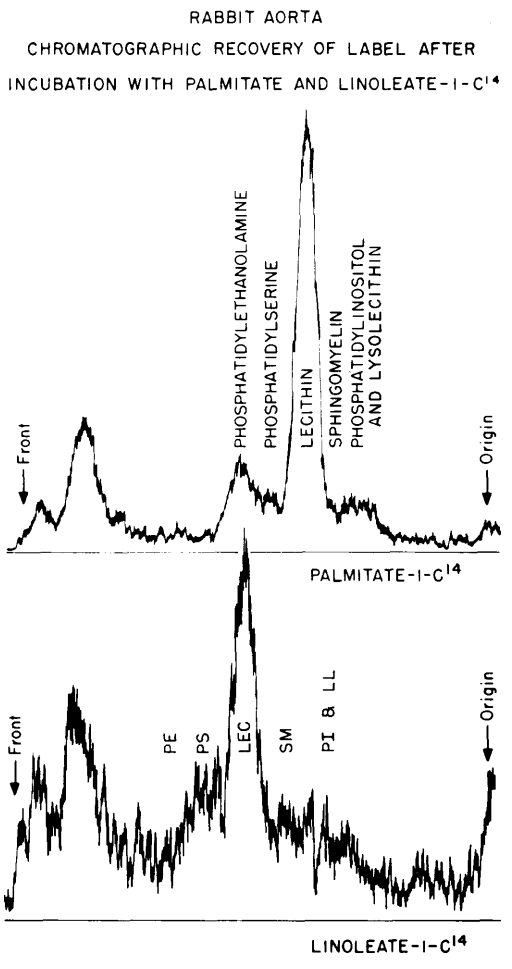

FIG. 1. The patterns of radioactivity of phospholipids separated by silicic acid paper chromatography are shown. One-fourth μc of either linoleate-1-C-14 or palmitate-1-C-14 was incubated with the supernatants from rabbit aortic homogenates and the cofactors mentioned in the text for two hours. This represents the supernatant of homogenate from 200 mg. wet weight rabbit aorta. The two peaks near the solvent front are unidentified. The positions of the various phospholipids were determined by simultaneously chromatographing known pure phospholipid standards.

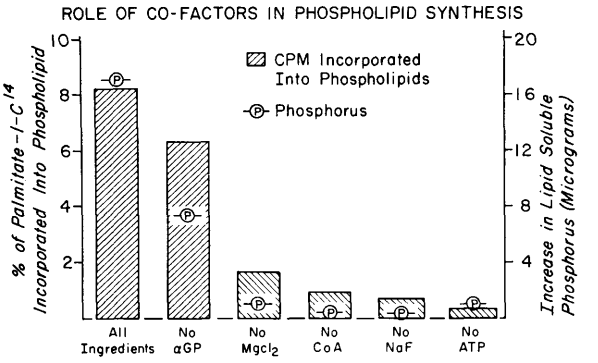

FIG. 2. Results are shown as per cent incorporation of palmitate-1-C-14 into rabbit aortic phospholipids over control values and as increase in lipid soluble (phospholipid) phosphorus after two hours' incubation. Each sample contains 5 μmoles ATP, 1 μmole $MgCl_2$, 0.2 μmoles CoA, 25 μmoles DL α-GP, and 300 μmoles NaF unless otherwise stated. Each sample has the supernatant of a homogenate from 200 mg. wet weight aorta.

on phospholipid synthesis. Figure 2 summarizes some of these findings when palmitate-1-C-14 was used. Maximal incorporation of radioactivity and increase in lipid-soluble phosphorus occurred when all cofactors were added to the supernatant fluids of aortic tissue homogenates. CoA and ATP were absolute requirements. The deletion of other cofactors depressed the reaction to varying degrees. The addition of cytidine triphosphate, cysteine, or unlabeled glucose did not enhance the reaction. Creatinine phosphate could be substituted for ATP as an energy source.

The rate of this reaction was studied over a six-hour period, and the results are depicted in figure 3. The per cent of radioactivity incorporated into phospholipids increased maximally and almost linearly for one hour. Similar curves were obtained if the rise in lipid-soluble phosphorus was plotted against time as in figure 3. About 5 μg. of phospholipid phosphorus were formed per 200 mg. of aorta per hour.

Modified rate curves were determined at three different temperatures with palmitate-1-C-14 (figure 4); 37° C. was the most efficacious.

The reaction was found to be *p*H dependent. Maximal incorporation of palmitate and linoleate-1-C-14 and maximal increase in lipid-soluble phosphorus occurred at *p*H 7.4, as seen in figure 5.

Experiments were performed to determine if the reaction could be blocked by certain enzyme inhibitors; figure 6 summarizes these studies. The incorporation of palmitate-1-C-14 was prevented by N-ethylmaleimide, p-hydroxymercuribenzoate, and iodoacetamide. These compounds are believed to act on enzyme systems containing SH groups.

Human aorta. Human aorta obtained at the time of vascular surgery or autopsy (sixteen to twenty-four hours after death) was prepared in a manner similar to rabbit aorta. Wherever possible only areas that appeared relatively normal were used, but in the surgical specimens little, if any, normal material was available.

Of three samples of aorta obtained at surgery two showed incorporation of radioactivity into phospholipids; these results are summarized in figures 7 and 8. Figure 7 shows that, as in rabbit, both palmitate-1-C-14 and

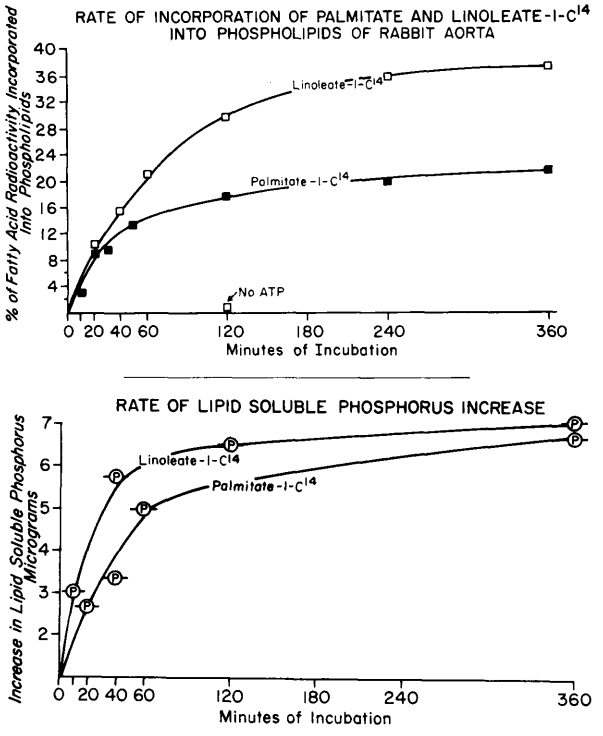

FIG. 3. Five gm. wet weight rabbit aorta was homogenized with 25 ml. Tris buffer. The supernatant obtained was incubated with 0.5 μc. of palmitate-1-C-14 or linoleate-1-C-14 and 12 ml. of Tris buffer containing all the cofactors discussed in the text. Duplicate samples were taken at zero time and at varying periods thereafter. One sample containing linoleate-1-C-14 was incubated two hours without ATP.

Top. Per cent incorporation of radioactivity into phospholipids. The rate is almost linear for the first hour. If ATP is deleted no incorporation results.

Bottom. The increase in phospholipid phosphorus over the zero time controls. Approximately 5 μg. of phosphorus is synthesized per 200 mg. of aorta per hour. Each point on this figure is an average of duplicate runs. In all instances the points given represent the phosphorus in the supernatant of homogenates from the equivalent of 200 mg. wet weight of tissue.

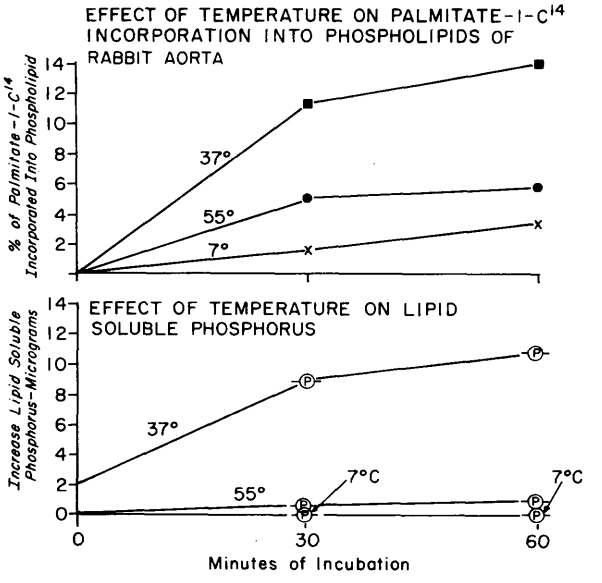

FIG. 4. A similar experimental procedure was used on this study as that shown in figure 3 except that only palmitate-1-C-14 was studied and the specimens of rabbit aorta were incubated at three different temperatures.

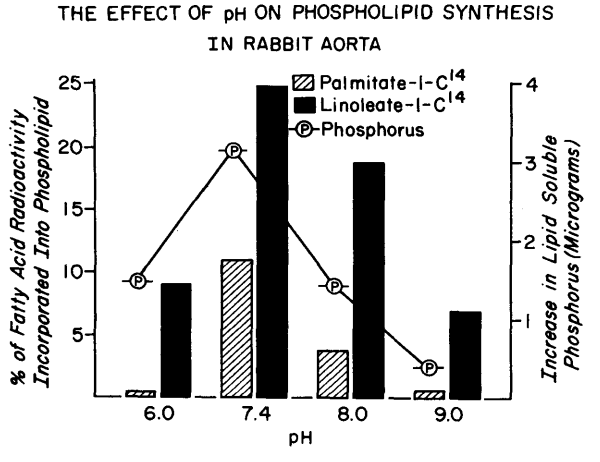

FIG. 5. Rabbit aorta was homogenized in 2.5 ml. of distilled water per gram of aorta. Then 0.5 ml. of the supernatant from the homogenate was added to 1 ml. of either 0.2 M. sodium acetate-acetic acid buffer, pH 5.8 (to attain a final pH of 6.0) or to 1 ml. of 0.1 M. Tris buffer made up to pH 7.4, 8.05, or 8.95 to give final pH's of 7.4, 8.0, and 9.0, respectively. The usual concentrations of cofactors were used.

linoleate-1-C-14 are incorporated into phospholipid. In addition, lipid-soluble phosphorus increases with incubation. If ATP or CoA are not added, the reaction does not proceed. Figure 8 shows the distribution of radioactivity in the various phosphatides. Again lecithin contains most of the radioactivity, with large amounts also found near the solvent front. The one surgical specimen which failed to incorporate these fatty acids into phospholipid was an abdominal aortic aneurysm with severe atherosclerosis. It is doubtful whether much viable tissue was present.

Four human aortas obtained at autopsy were studied, and the findings are summarized in figure 9. Again both fatty acids were incorporated into phospholipids, but to a much lesser extent than fresh human aorta. Phospholipid phosphorus values did not increase with incubation in any of these specimens. The radioactivity patterns on the paper chromatograms were similar to that of fresh human aorta and rabbit aorta.

DISCUSSION

The results presented in his paper support the contention of Zilversmit, Chernick, and Stein that phospho-

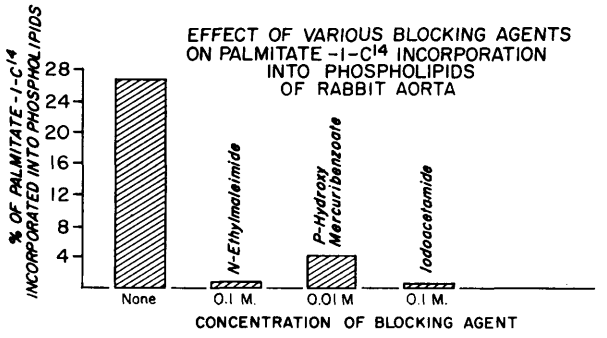

FIG. 6. Supernatants obtained from homogenates of 1 gm. rabbit aorta/5 ml. Tris were incubated for two hours, with all cofactors added with or without the various blocking agents. Each bar represents the average of three samples incubated.

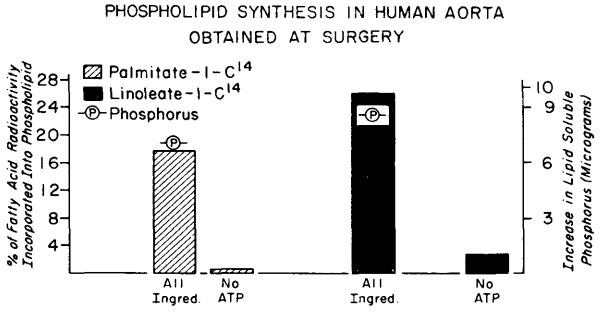

FIG. 7. Per cent of palmitate-1-C-14 and linoleate-1-C-14 incorporated into phospholipids and increase in lipid-soluble phosphorus with two hours' incubation of human surgical specimens. The following cofactors were added to each 1 ml. of supernatant (200 mg./ml. Tris) except where otherwise noted: 5 μmoles ATP, 1 μmole $MgCl_2$, 0.2 μmole CoA, 25 μmoles DL α-GP, and 300 μmoles NaF. This is the average of the results of two surgical specimens.

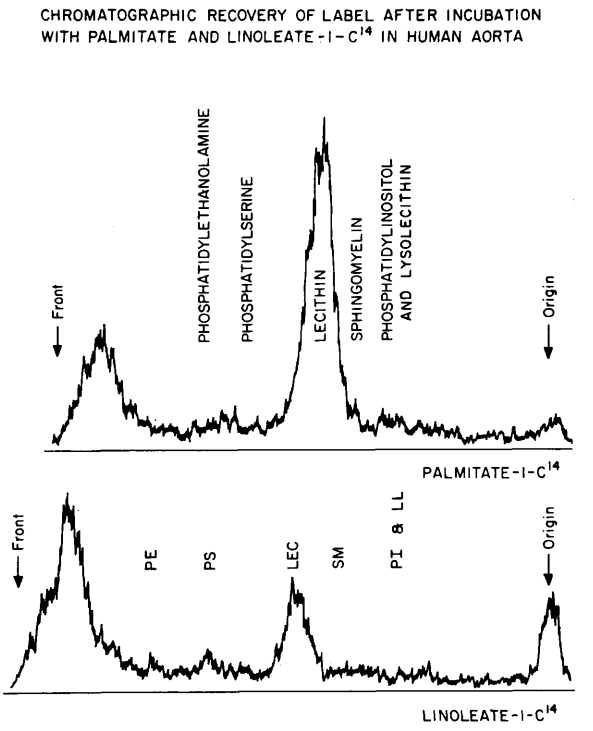

FIG. 8. Radioactivity patterns obtained from silicic acid paper chromatograms of phospholipids from human surgical specimens after two hours' incubation with palmitate-1-C-14 and linoleate-1-C-14.

lipids are synthesized in the arterial wall. Considered alone, the incorporation of fatty acids into phospholipids could represent a transfer phenomenon, but the fact that phospholipid phosphorus increased is evidence in favor of synthesis. This study adds to the present knowledge by demonstrating that fatty acids can be utilized for phospholipid synthesis. As noted in table 1 considerable amounts of radioactivity were found in the phospholipid fractions in the pre-incubated or control samples. This radioactivity represents labeled fatty acids which is either nonspecifically adsorbed to phospholipids or nonspecific spreading on the silicic acid column.

The in vitro technic employed in this study allows some quantitation of the synthetic reaction. Between 15 and 20 μg. of phospholipid phosphorus or between 400 and 500 μg. of phospholipids are synthesized per gram of aorta per hour in rabbits. Not enough data are available on human aorta to permit similar calculations. Whether this rate of synthesis occurs in vivo is not clear.

The requirements shown for phosphatide synthesis in rabbit and to a limited extent in human aorta are remarkably similar to those in liver. Figure 10 is a summary of the enzymatic processes involved in phospholipid synthesis according to studies done by Kennedy,[16,17] Kornberg and Pricer,[18-20] and Lehninger.[21] These reactions have been demonstrated in cell-free enzyme preparations. Palmitic, linoleic, and oleic acids have been shown to be incorporated into phosphatidic acid when added to α-glycerophosphate in these liver preparations; this requires CoA and ATP. The present study on aorta has also demonstrated the incorporation of fatty acids into phospholipids and has further shown that CoA and ATP are absolute requirements. These findings are very similar to those recently reported by Stein, et al.[9] This group of investigators also used the supernatants of homogenates of rabbit aorta and found linoleate-1-C-14 was incorporated into phospholipids in the presence of CoA and ATP. In addition, magnesium and fluoride ions, which were necessary in liver to incorporate P-32 labeled α-glycerophosphate into phospholipids, were

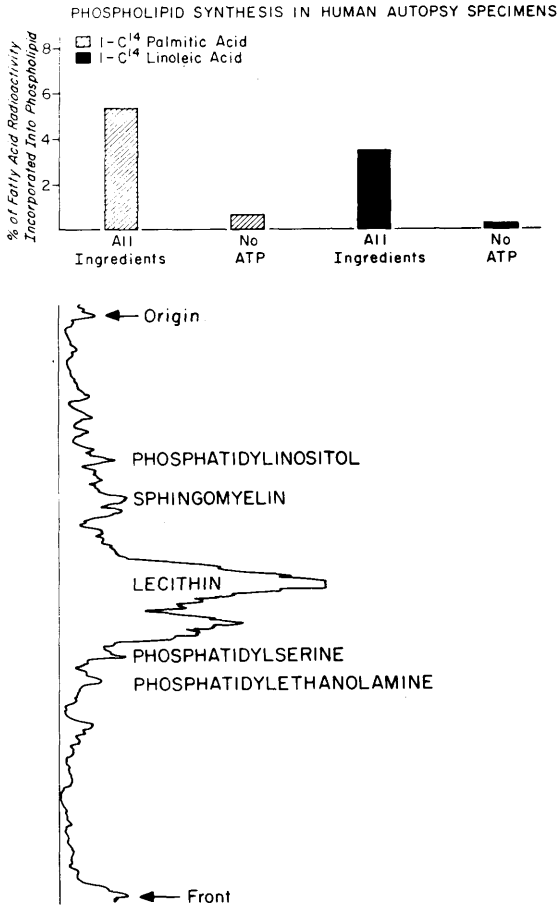

FIG. 9. Per cent radioactivity incorporated into the phospholipids of human aorta obtained at autopsy and incubated for two hours. Concentration of the homogenate 1 g. aorta/5 ml. Tris buffer. Below is the palmitate-1-C-14 pattern of the phosphatides separated by paper chromatography. This is the average of the results of four autopsy specimens.

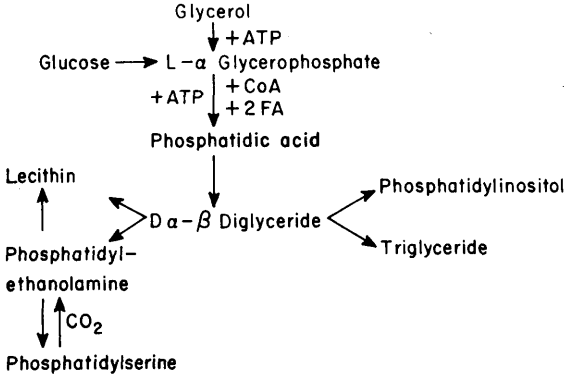

FIG. 10. Summary of the enzymatic processes involved in the biosynthesis of glycerides and glycerophosphatides in liver.

found necessary in the present system. The phospholipids synthesized by the enzymatic processes in the liver are identical to the phospholipids found containing radioactivity in the aorta. If a similar enzymatic system is operating in arterial wall one might expect to find the labeled precursors—phosphatidic acid and diglycerides. These were not definitely identified, although the unidentified radioactive peaks near the solvent front of the paper chromatograms have a chromatographic mobility similar to that of phosphatidic acid.[15]

The significance of this reaction in the arterial wall is not known. Zilversmit has postulated that the surface active properties of phospholipids might aid in the solubilization and therefore the removal of cholesterol from the arterial wall. There is evidence to suggest that most of the cholesterol found in atherosclerotic plaques is deposited from the plasma,[22] and Zilversmit[4-6] has presented evidence to suggest that there is an acceleration of the production of arterial phospholipids with cholesterol feeding in rabbits.

SUMMARIO IN INTERLINGUA

Le Natura Enzymatic del Synthese de Phospholipidos in Normal Aortas Conilian e Human. Resultatos de Studios in Vitro

Es presentate observationes que indica que phospholipidos es synthetisate per supernatantes de homogenatos de aorta conilian e human quando palmitato-1-C-14 o lineoleato-1-C-14 es usate. Le processo synthetic es de natura enzymatic. Illo depende del temperatura, del *p*H, e del presentia de varie cofactores. Iste requirimentos es comparabile a acellular preparatos hepatic le quales etiam synthetisa phospholipidos.

ACKNOWLEDGMENT

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Dr. Parker is Postdoctoral Fellow of the National Institute of Arthritis and Metabolic Diseases.

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Withering and the Quarterly Journal of Negative Results

(*Continued from page 176*)

So much for the comments of the learned journal. When the writer of this editorial discovered these documents, he felt that they were provocative and deserving of comments. It occurred to this author that the correspondence between Withering and the *Quarterly Journal of Negative Results* illustrates the growing schism between the two modern types of medicine. One is exemplified by Withering's article, as rejected by the *Quarterly Journal of Negative Results*. Withering's article and the work underlying it is the outgrowth of pure clinical observation, not watered down or enriched (according to the point of view) by quantitative analytical examination. This type of thinking has served medicine well, and no attempts should be made to consider it of less quality than any other type of medical research. The physician in academic medicine considers the busy but observing clinician often less than his equal; his attitude has introduced a dangerous schism in modern medicine. The academically inclined physician, usually working at a University hospital, whose papers are acceptable to scientific journals, often lacks the "feel" for the patient as well as the natural gift for simple observation which so often characterizes the practicing physician.

This division which is opening in modern medicine can only be overcome by a sincere effort of the man in academic medicine to learn from his purely clinical colleague. He must look at the whole patient, observe him, and understand his personality. He still can pursue his researches. Love for the patient is not incompatible with love for science. On the contrary! Love for the patient inspires interest to help, and therefore interest in the science of medicine. If the academic teacher of medicine does not make these adjustments, his ivory tower will become his prison. Let us learn from Withering!

RICHARD J. BING, M.D., *Professor and Chairman, Department of Medicine, Wayne State University College of Medicine, Detroit*

From *Arch. Int. Med.*—Bing—February, 1963.