[7] A. Novick and L. Szilard, *Science*, **112**, 715, 1950; A. Novick, *Ann. Rev. Microbiol.*, **9**, 97, 1955; J. Monod, *Ann. Inst. Pasteur*, **79**, 390, 1950.

The chemostat maintains by continuous dilution a culture of bacteria growing indefinitely at constant density and under constant conditions. There is a growth tube which contains the growing population of bacteria, and there is a reservoir from which nutrient is fed into the growth tube in such a fashion that the contents of the tube are diluted at a rate equal to the bacterial growth rate. The bacterial growth rate is determined by the low concentration in the growth tube of some limiting growth factor. A similar apparatus, called the "bactogen," has been described by Monod. The chemostat is useful for kinetic studies because the concentrations of all chemical substances in the growth tube remain constant indefinitely, and, as a result, the bacteria remain in a constant physiological state.

[8] J. Lederberg, *J. Bacteriol.*, **60**, 381, 1950.

[9] The rate of induction, $K$ (to be described later), increases roughly linearly with $CO_2$ concentration up to as high as 10 per cent $CO_2$. This phenomenon will be described in a later publication.

[10] This fall is discussed in the section on "Intermediate Saturation."

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## *THE ISOLATION OF LYSOLECITHIN FROM HUMAN SERUM**

BY GERALD B. PHILLIPS

DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, AND PRESBYTERIAN HOSPITAL, NEW YORK, NEW YORK

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The phosphorus-containing fraction of lipid extracts of human serum has been reported to contain lecithin, sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine, and plasmalogen. The techniques employed for the isolation of the individual phospholipid components have usually been laborious and have often been unreliable. An improved method of separation using adsorption chromatography on silicic acid columns or on silicic acid–impregnated filter paper has been recently reported by Lea, Rhodes, and Stoll.[1] With a modification of this procedure, an additional phosphorus-containing component of a lipid extract of human serum has been isolated, which, by staining properties, chromatographic mobility, chemical analysis, and hemolytic activity, appears to be lysolecithin.

### MATERIALS AND METHODS

The serum used in these studies was either pooled hospital patients' sera refrigerated for 1 day prior to use or normal sera processed within 1 or 2 hours after withdrawal.

*Extraction.*—The serum was added dropwise with shaking to 15 times its volume of a 1:1 (v/v) mixture of methanol and chloroform. After standing for 1 hour, the mixture was filtered and the filtrate emulsified with an equal volume of distilled water. Following centrifugation, the upper layer was discarded and the bottom layer taken to dryness in a rotary vacuum evaporator at a maximum temperature of 60° C. This extract was stored *in vacuo* at −30° C., usually for not more than 1 or 2 days.

*Chromatography.*—A modification of the method of Lea, Rhodes, and Stoll[1] was employed. The chloroform used (Fisher, A.C.S.) was washed with water and filtered, and 2 per cent methanol (v/v) was added as a preservative. The silicic

acid (Mallinckrodt, A.R., 100 mesh) was activated by heating at 110°–120° for 18 hours and was added to the column as a slurry of 5 ml. of chloroform per gram of silicic acid. The serum extract was added in 2.5 ml. of chloroform per gram of silicic acid. Elution was then accomplished with the following amounts of solvent mixtures per gram of silicic acid: 7.5 ml. of chloroform; 15 ml. of 20 per cent methanol in chloroform (v/v); 60ml. of 20 per cent methanol in chloroform (v/v) containing 1.25 per cent water; and 12.5–15 ml. of methanol. Air pressure of 14 cm. mercury was applied to the column during elution. Fractions ranging from 3 to 5 ml. per gram of silicic acid were collected with an automatic fraction collector. Silicic acid–impregnated filter paper was prepared as described by Lea, Rhodes, and Stoll,[1] and the lipid extract was chromatographed in the descending manner with 20 per cent methanol in chloroform (v/v).

The column fractions were analyzed for phosphorus by the method of Fiske and Subbarow;[2] for total nitrogen by digestion with a mixture of sulfuric acid, copper sulfate, and potassium sulfate, followed by direct Nesslerization; for amino nitrogen by the ninhydrin method of Lea and Rhodes;[3] and for ester bonds by the method of Rapport and Alonzo.[4] The paper chromatograms were developed by (1) spraying with ninhydrin in butanol, followed by heating at about 110° for 5 minutes; (2) the phosphomolybdic acid stain of Chargaff, Levine, and Green;[5] and (3) the Schiff stain as described by Hack.[6]

## RESULTS

All the serum specimens examined by the above chromatographic procedure showed four peaks of phosphorus concentration (Table 1). Chemical analyses

TABLE 1

CHROMATOGRAPHY OF SERUM EXTRACTS ON SILICIC ACID
(Per Cent Total Phosphorus)

| Sample | Peak I | Peak II | Peak III | Peak IV |
|---|---|---|---|---|
| 1* | 4.7 | 73.7 | 15.7 | 5.9 |
| 2† | 6.2 | 69.0 | 17.9 | 6.9 |
| 3† | 5.4 | 64.1 | 20.9 | 9.7 |
| 4‡ | 4.5 | 71.0 | 16.7 | 7.9 |

* Pooled fresh normal sera (3 young male donors). Serum lipid P was 9.8 mg/100 ml.
† Pooled patients' sera.
‡ Column run at 0°–1° C. Fresh normal serum (1 young male donor). Serum lipid P was 7.4 mg/100 ml.

of the first three peaks and comparison with synthetic materials[7] revealed that the first peak (Peak I), eluted with 20 per cent methanol in chloroform, was predominantly phosphatidyl ethanolamine, and that the second (Peak II) and third (Peak III), eluted with 20 per cent methanol in chloroform containing 1.25 per cent water, were largely lecithin and sphingomyelin, respectively.[8] The fourth peak (Peak IV), which was eluted by methanol, was rechromatographed on the column with the same system and also on silicic acid–impregnated paper and found by both procedures to consist of three main components, two smaller ones with the mobilities of lecithin (Peak II-A) and sphingomyelin (Peak III-A) and a larger one (Peak IV-A) (Table 2). Chromatography of the Peak IV-A material on silicic acid–impregnated paper resulted in a single phosphomolybdic acid–staining spot corresonding to the third and slowest phosphomolybdic acid–staining spot

seen on chromatographing serum extracts (see below). The cause for the carryover of some lecithin and sphingomyelin into Peak IV is not known, and attempts to eliminate this phenomenon by variations in the technique were unsuccessful.

On chromatographing the extract of normal pooled sera, 5.9 per cent of the total lipid phosphorus appeared in Peak IV, and, on rechromatographing Peak IV, 80 per cent of it, or 4.7 per cent of the total lipid phosphorus, seemed to be a single component (Tables 1 and 2). In both of these chromatographic runs, the recovery

TABLE 2

RECHROMATOGRAPHY OF PEAK IV ON SILICIC ACID
(Per Cent Total Phosphorus)

| Sample* | Peak I-A | Peak II-A | Peak III-A | Peak IV-A | (Per Cent) Peak IV-A/Total P |
|---|---|---|---|---|---|
| 1 | 1.7 | 11.4 | 7.3 | 79.8 | 4.7 |
| 2 | 2.0 | 11.9 | 5.9 | 80.4 | 5.5 |
| 3 | 1.1 | 6.5 | 17.1 | 75.4 | 7.3 |
| 4 | 1.2 | 14.6 | 12.0 | 72.2 | 5.7 |

* See notes to Table 1.

of the phosphorus added to the column was 96 per cent. Performing the chromatographic separation at 0°–1° C. on an extract of normal serum and using chloroform which was washed, dried, and distilled did not appear to affect significantly the isolation of this component (Tables 1 and 2). The Peak IV-A material was also obtained with methanol elution, following elution of the second and third peaks of a serum extract with 35 per cent methanol in chloroform (v/v), i.e., without adding water to the eluants.

The Peak IV-A material was subjected to various tests. On silicic acid–impregnated paper, it was stained with the phosphomolybdic acid method but not with the Schiff stain, whereas lecithin and sphingomyelin were stained with the former and lecithin (weakly) and "cephalin" (strongly) with the latter. The reaction with the Schiff stain was probably due to the presence of plasmalogens. On hydrolysis with 6 *N* HCl at 110° C. for 18 hours, followed by chromatography on filter paper (Whatman No. 1) with butanol–acetic acid–water elution (4:1:4, using butanol layer), it consistently showed one spot, with the same mobility and staining reaction with the phosphomolybdic acid method as choline. Chemical analysis showed a molar nitrogen-to-phosphorus ratio and ester-bond-to-phosphorus ratio of approximately 1 (Table 3) and a small amount of ninhydrin-reacting material.

TABLE 3

CHEMICAL ANALYSIS OF PEAK IV-A

| Sample | N/P* | Ester Bond/P* |
|---|---|---|
| 1† | 1.10 | 1.05 |
| 3‡ | 1.05 | 1.02 |

* Molar ratio.
† Average of 2 determinations.
‡ Average of 3 determinations.

The Peak IV and Peak IV-A materials were found to be hemolytic on incubation for 2 hours at 37° with 2 ml. of a 1 per cent suspension of washed sheep red blood cells suspended in isotonic saline. Hemolysis could be seen with as little as 0.01 micromole of two different samples of Peak IV-A material. None of the other peak materials produced any hemolysis.

The above tests indicated that the material in Peak IV-A was lysolecithin. To show that this substance did not arise from lecithin during the isolation procedure, the lecithin peak obtained on one chromatographic run was rechromatographed. Only 1.5 per cent of the total phosphorus was eluted with the methanol, and no lysolecithin could be detected. Similarly, by chromatographing synthetic L-$\alpha$-dimyristoyl lecithin, 2 per cent was eluted with the methanol, but no lysolecithin was found. In addition, Peaks I, II, and III from one chromatographic run were recombined, put through the extraction procedure, and rechromatographed, but still no lysolecithin could be recovered. Likewise, when material from Peak I, Peak II, or Peak III, or synthetic phosphatidyl ethanolamine, phosphatidyl serine, or lecithin, was chromatographed on silicic acid–impregnated paper, each produced a single spot, but none with the mobility of the Peak IV-A material.

When material from Peak II (lecithin) emulsified in phosphate buffer was treated with cobra venom and the product chromatographed on the silicic acid column, most of the phosphorus-containing material was eluted with the methanol and was hemolytic.

The results obtained by chromatography on silicic acid columns were corroborated by chromatography on silicic acid–impregnated filter paper. Chromatography of the serum extract on paper produced four spots, one staining with the ninhydrin method and three with the phosphomolybdic acid method. The Rf of each substance varied with the batch of paper used and to some extent with the amount of the substance applied to the paper; the absolute Rf values, therefore, had little reproducibility. The ninhydrin-staining spot had a mobility corresponding to synthetic phosphatidyl ethanolamine, synthetic phosphatidyl serine, and the Peak I material from the column. The fastest phosphomolybdic acid–staining spot was by far the largest in amount and had a mobility corresponding to synthetic lecithin and to the Peak II material from the column. The second phosphomolybdic acid–staining spot was next in size and had a mobility corresponding to the Peak III material from the column. The third phosphomolybdic acid–staining spot had a mobility corresponding to the material from Peak IV-A and the bulk of the material from Peak IV.

The same spot sequence as above was observed on chromatography of the unprocessed filtrate from (1) a mixture of 1 volume of serum and 15 volumes of 1:1 (v/v) methanol-chloroform and (2) a mixture of 1 volume of serum and 20 volumes of 3:1 (v/v) alcohol-ether, thus making it unlikely that any of the spots found arose from the heating or water washing of the extraction procedure or were limited to the chloroform-methanol method of extraction.

## DISCUSSION

The analysis of human serum by the chromatographic methods described permitted the isolation and quantitation of the main phospholipid components. In addition to the known components, another phospholipid was consistently found, which, by staining properties, chromatographic mobility, chemical analysis, and hemolytic activity, appeared to be lysolecithin. Evidence for the presence of lysolecithin in animal tissues has recently been reported. Lea, Rhodes, and Stoll[1] isolated lysolecithin from egg yolk by chromatography on silicic acid. Marinetti, Witter, and Stotz,[9] using chromatography on silicic acid–impregnated filter

paper, reported evidence for the presence of lysolecithin in rat tissues. Moreover, Titus, Weiss, and Hajdu,[10] employing chromatography on Florisil, were able to isolate palmitoyl lysolecithin from beef adrenal glands.

Further studies indicated that the lysolecithin probably was not formed as a result of the isolation procedure. On chromatographing the isolated serum lecithin or synthetic L-$\alpha$-dimyristoyl lecithin, either on the silicic acid column or on silicic acid–impregnated paper, no lysolecithin could be found. When the serum "cephalin," lecithin, and sphingomyelin fractions obtained by the column method were recombined, put through the serum-extraction procedure, and rechromatographed, lysolecithin again was not detected. Although it thus appears unlikely that the lysolecithin arose from the breakdown of lecithin, it is possible, nevertheless, that a certain fraction of the serum lecithin was more labile and split off a fatty acid moiety during the isolation procedure.

Another possible source of lysolecithin was choline-containing plasmalogen. The concentration of lysolecithin in serum found in the present investigation, however, is more than twice the reported total serum plasmalogen concentration.[11] A considerable proportion of the serum plasmalogen, in addition, is probably of the "cephalin" type.[8]

Although the chemical state and physiological role of lysolecithin in the plasma is conjectural, Titus, Weiss, and Hajdu[10] have demonstrated that the lysolecithin isolated from beef adrenal gland had a digitalis-like effect on the frog heart.

## SUMMARY

The analysis of a lipid extract of human serum by chromatography on silicic acid columns or on silicic acid–impregnated filter paper revealed, in addition to the known phospholipids, the presence of a component which was characterized as lysolecithin. The phosphorus of this component was found to comprise 4.7 per cent of the total lipid phosphorus of a sample of pooled sera from normal young males.

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[7] The author is grateful to Dr. E. Baer, University of Toronto, Toronto, Canada, for generously supplying samples of synthetic L-$\alpha$-dimyristoyl lecithin, L-$\alpha$-dimyristoyl phosphatidyl ethanolamine, and L-$\alpha$-distearoyl phosphatidyl serine.

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