STUDIES ON THE PHOSPHATIDE CONTENT OF HUMAN SERUM*

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Recent observations appear to indicate that there is an association between human atherosclerosis and gross changes in serum phosphatides (1, 2). Since this lipide fraction is heterogeneous, it would be desirable and of interest to distinguish any differential changes in the individual phosphatide components.

The examination of the literature dealing with the qualitative and quantitative aspects of phosphatide distribution in human blood plasma or serum reveals certain discrepancies. For example the work of Taurog et al. (3) indicates that practically all of the phosphatides of human plasma are of the choline-containing type. Hack (4) and Sinclair (5, 6) seem to have reached the same conclusions. Artom, on the other hand (7), although finding choline-containing phosphatides a major constituent of human plasma, also estimated that of the total phosphatide content about 21 and 7 per cent could be accounted for as ethanolamine- and serine-containing compounds respectively. The analytical data obtained by individual investigators can only be compared when viewed in terms of the fractionation or purification procedures employed. These procedures are largely dependent upon differential solubilities or adsorption prior to analysis for individual phosphatides. Accordingly a method is desirable by which the plasma phospholipide constituents in crude unfractionated lipid extracts can be separated and estimated. The methods developed by Levine and Chargaff (8-10) offer a basis for such a procedure. Their methods involve the use of unidimensional paper partition chromatography for the separation and ultimate estimation of the nitrogenous bases present in phosphatide hydrolysates. It is of interest to note that Bevan et al. (11) have reported the separation of crude mixtures of phosphatides into individual components by use of filter paper chromatography. Bevan's methods are valuable in that intact phosphatides can be chromatographically separated, but quantitative application would present difficulties.

In adapting the methods of Levine and Chargaff to the study of serum

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phosphatides, it seemed desirable to introduce certain modifications. This paper therefore is concerned with a description of the quantitative procedures for the estimation of several phosphatide components as developed in this laboratory and preliminary data on the application of these methods to the analysis of the phosphatide content of human serum.

The procedure involves (a) extraction of phosphatides from serum samples by use of organic solvents; (b) hydrolysis of the crude filtered extracts to liberate free serine, ethanolamine, and choline of phosphatide origin; (c) separation of the bases by unidimensional paper partition chromatography on filter paper strips; (d) quantitative estimation of serine and ethanolamine by development of adsorption zones by means of ninhydrin, and extraction of the resulting colored areas with aqueous pyridine, followed by quantitative colorimetry; (e) development and estimation of choline on separate chromatograms by the method of Levine and Chargaff (10).

This general procedure therefore offers a relatively simple means of estimating phosphatides in serum by a direct analysis of their corresponding bases. Minor phosphatide constituents can be determined without recourse to fractionation which might result in their being overlooked or completely lost. For example, after extraction of phosphatides and separation of choline- and non-choline-containing compounds by the method of Taurog et al. (12), the presence of minor components such as phosphatidylserine could readily be missed by estimating the cephalin fraction in terms of its phosphorus content only (13).

In human serum phosphatide extracts it has been possible by the procedure outlined above to detect small amounts of serine-containing phosphatides, thus confirming the original observations of Artom (7). Future publications from this laboratory will be concerned with the distribution of serum phosphatides in normal and pathological conditions.

**EXPERIMENTAL**

**Material**

DL-Serine (Merck), ethanolamine which had been purified by distillation in vacuo, and choline chloride, recrystallized from ethanol and dried, served for the preparation of standard solutions. All solvents were redistilled before use. The total nitrogen content of the phosphatide extracts was determined by a modification of the method of Sobel et al. (14) and phosphorus by the colorimetric method of Fiske and Subbarow (15). For quantitative planimetry of the choline spots, a polar planimeter (Keuffel and Esser Company, New York) was used.
Chromatographic Separation and Estimation of Serine, Ethanolamine, and Choline

Each chromatographic separation was carried out on an individual strip of Schleicher and Schuell filter paper No. 597 (3 cm. wide and 45 cm. long). Large numbers of strips could be machine-cut to the desired dimensions, and strips derived from the same sheet of paper were always kept together to be used in individual runs. In choline determinations in which spots representing 45 to 50 \( \mu \text{g} \) of material were expected, strips 7.5 cm. wide were used. Aside from greater ease in handling, the use of individual strips rather than multilaned sheets was found to be an advantage in that the danger of overlapping of adsorption zones was eliminated.

Solutions to be analyzed were dispensed at a starting point of 9 cm. from the end of a paper strip by means of a Gilmont ultramicro burette (Emil Greiner Company, New York) of 0.1 ml. total capacity. Aliquots as large as 150 \( \mu \text{l.} \) per strip could be employed if the material were dispensed in 10 \( \mu \text{l.} \) portions onto a single spot with intermittent drying. The material on the paper strips was then subjected to unidimensional descending chromatography in a "chromatocab" (Research Equipment Corporation, Oakland, California) for about 18 hours. The solvent system employed for the separation of serine, ethanolamine, and choline was a mixture of \( n \)-butanol, diethylene glycol, and water (4:1:1 by volume respectively) according to Levine and Chargaff (10).

Development and Estimation

Serine and Ethanolamine—For the development and estimation of serine and ethanolamine after chromatographic separation, the strips were first dried at room temperature and then passed slowly once through a mixture composed of equal volumes of 2 per cent ninhydrin solution (16), \( n \)-propanol, and water respectively. The strips thus treated were then subjected to unidimensional descending chromatography in a "chromatocab" (Research Equipment Corporation, Oakland, California) for about 18 hours. The solvent system employed for the separation of serine, ethanolamine, and choline was a mixture of \( n \)-butanol, diethylene glycol, and water (4:1:1 by volume respectively) according to Levine and Chargaff (10).

Development and Estimation

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paper residues, and the color intensities of the extracts, in terms of optical density, were determined by means of a Beckman model DU spectrophotometer. Readings were usually made within 1 hour after extraction, with the instrument set at 580 mμ. The extracts were read against blanks prepared by the extraction of paper segments equal in size which were cut from each strip at a point below the adsorption zone of ethanolamine. Blanks prepared in this manner gave very low readings and did not differ among themselves nor from blanks prepared by the extraction of segments identical in position to the respective adsorption zones but cut from complete blank strips subjected to the same chemical treatments.

The readings followed Beer's law when 2 to 15 γ of serine or ethanolamine standards were subjected to chromatography either individually or as mixtures containing choline in addition.

The color yields with ninhydrin per micromole of serine or ethanolamine, respectively, varied with the particular ninhydrin reagent used. In any one series of determinations, however, on a particular freshly prepared ninhydrin reagent, considerable uniformity was encountered. For example, in a series of ten experiments, as outlined above, the average color yields obtained in the range of 5 to 15 γ of pure serine or ethanolamine subjected to chromatography separately or as a mixture were as follows: serine 1917 ± 81, ethanolamine 983 ± 29.

Ammonia was found not to interfere with the estimation of serine and ethanolamine. Ammonium chloride solutions (ten to 60 γ samples) which were subjected to chromatography produced chromatograms devoid of colored adsorption zones after development under the conditions described above.

**Choline** Separate strips were used for the development and estimation of the choline adsorption zones after chromatographic separation. The paper strips destined for choline determination were first completely air-dried at room temperature, subjected to steam vapors, and then saturated with ammonia gas. Such pretreatment of the papers resulted in excellent choline spots when the adsorption zones in question were made visible by the development method of Levine and Chargaff (10) with phosphomolybdic acid and stannous chloride. The choline was estimated by planimetry.

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1 A complete survey of the spectral curve of the extracts indicated that a fairly sharp peak was present in the vicinity of 580 mμ.

2 Optical density (× 10³) at 580 mμ per μM of base. The presence of choline in mixtures subjected to chromatography did not affect the color yields of either serine or ethanolamine.

3 Ammonium chloride could, however, be demonstrated in chromatograms which were sprayed with 2 per cent ninhydrin solution and heated in an oven at 100° for a few minutes. It appeared as a compact purple area whose RF value approximated that of choline.
of the areas occupied by the individual spots as developed by Levine and Chargaff (10).

A linear relationship between the area and the logarithm of the choline concentration was found in the range of 30 to 50 \( \gamma \) of choline per spot. This relationship existed whether the choline standards were chromatographed individually or as mixtures containing ethanolamine and serine.

In confirmation of the findings of Levine and Chargaff (10), it was observed that the lines plotted from data obtained from individual chromatograms differed in slope when strips cut from different paper sheets were used. As a result, all analyses of serum phosphatide hydrolysates, including standards, were carried out with paper strips cut from the same sheet.

\( R_f \) Values—The following \( R_f \) values were recorded when a series of standard mixtures of serine, ethanolamine, and choline was subjected to paper chromatography with the n-butanol, diethylene glycol, water solvent mixture: serine 0.20, ethanolamine 0.37, choline 0.51. These substances displayed identical \( R_f \) values when they were also chromatographed individually.

**Extraction and Hydrolysis of Serum Phosphatides**

In order to demonstrate the applicability of the methods already described to the study of the phosphatides of human serum, the following procedures were employed.

*Extraction*—Usually 3 ml. of fresh unhemolyzed serum\(^4\) were treated for 1 hour, at 55–60\(^\circ\), with 10 volumes of a 3:1 alcohol-ether mixture. After cooling, the supernatant fluid containing the extracted phosphatides was separated by centrifugation and removed, and the residue was reextracted for 1 hour with 5 volumes of the same solvent. The second extract was separated in the same way, and the two extracts were combined and filtered. The filter was washed with about 5 ml. of hot solvent, and the clear wash fluid was added to the combined extracts. The extracts were then concentrated almost to dryness in an atmosphere of nitrogen, and the residual oily material was treated with three 10 ml. portions of hot petroleum ether.

After filtration as described previously, the combined petroleum ether extracts were concentrated to about 15 ml. and quantitatively transferred to a 25 ml. volumetric flask and diluted to mark with the same solvent. Aliquots of the petroleum ether extract were utilized to determine the total phosphorus content and total nitrogen content (usually 2 ml. for phosphorus, 5 ml. for nitrogen).

*Hydrolysis*—The remainder of the petroleum ether extract, after being

\(^4\) Obtained from adults after a 10 hour fast.
quantitatively transferred to a 50 ml. Erlenmeyer flask equipped with a standard taper ground glass joint, was evaporated to dryness in an atmosphere of nitrogen, and the resulting residue was taken up in 6 ml. of 6 N aqueous HCl. The mixture was heated for 48 hours at 100° under a reflux. The hydrolysate mixture was then evaporated to dryness in vacuo and the dried residue taken up in exactly 2 ml. of water with slight warming. The resulting acidic hydrolysate solution, after being filtered, was subjected to chromatographic analysis. Usually 80 to 100 µl. per strip served for the separation and estimation of serine and ethanolamine and 100 to 150 µl. for the separation and estimation of choline.

In each chromatographic series of serum phosphatide analyses, sets of standards were always included. Calculations were based upon the values obtained for these standards. As the serine and ethanolamine standard, a mixture containing both of these substances at a concentration of 0.1 per cent in 0.1 N HCl was used. In each analysis 5, 10, and 15 µl. aliquots of this mixture were dispensed onto individual paper strips and subjected to chromatographic separation. A solution of choline chloride in 0.1 N HCl served as the choline standard (equivalent to a 0.1 per cent solution in terms of choline) and was dispensed in aliquots of 30, 40, and 50 µl. Individual strips bearing no other substances were used for the choline standards.

It was of interest to determine the stability of the nitrogenous substances to be expected in serum phosphatide hydrolysates toward heating in the presence of hydrochloric acid. 0.1 per cent mixtures of serine, ethanolamine, and choline, respectively, in 6 N HCl were subjected to heating under reflux for 48 hours. After cooling, the solutions were evaporated to dryness in vacuo; the dried residues were dissolved in water and diluted to definite volumes. Aliquots of these acidic solutions were subjected to chromatographic separation, and the resulting estimations of serine, ethanolamine, and choline were compared with standard mixtures which were not subjected to hydrolytic treatment. As the result of a series of seventeen such experiments, each consisting of from four to seven chromatographic separations, the recoveries were as follows: serine, 97 ± 4 (standard deviation) per cent; ethanolamine, 94 ± 3 (s.d.) per cent; and choline, 100 ± 5 (s.d.) per cent.

Serine, ethanolamine, and choline were equally resistant to hydrolytic treatment when heated with 6 N HCl in the presence of serum phosphatides. In this experiment, known amounts of serine, ethanolamine, and

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5 When the same amount of choline contained in 50 µl. was dispensed in a total of 100 µl., no variations in spot area were observed.

6 The mixtures subjected to heating with 6 N HCl gave compact adsorption zones upon chromatographic separation, having the same \( R_f \) values as the standards.
choline were added to the residue resulting from the evaporation of a petroleum ether serum phosphatide extract prepared as described previously. After drying in vacuo, the mixture was hydrolyzed with 6 N HCl for 48

TABLE I

Acid Hydrolysis of Human Serum Phosphatides

<table>
<thead>
<tr>
<th>Duration of hydrolysis</th>
<th>Composition of hydrolysate fluid per 100 µl.</th>
<th>Inorganic P</th>
<th>Serine</th>
<th>Ethanolamine</th>
<th>Choline</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>0.12</td>
<td>0.03</td>
<td>0.05</td>
<td>0.33</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.21</td>
<td>0.04</td>
<td>0.05</td>
<td>0.39</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.25</td>
<td>0.05</td>
<td>0.07</td>
<td>0.46</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.36</td>
<td>0.05</td>
<td>0.07</td>
<td>0.47</td>
<td>0.59</td>
<td></td>
</tr>
</tbody>
</table>

* Total phosphorus, 0.55 µM per 100 µl. of hydrolysate fluid, equivalent to lipid phosphorus of 11.3 mg. per cent.

TABLE II

Distribution of Serine, Ethanolamine, and Choline in Hydrolysates of Human Serum Phosphatide Extracts

<table>
<thead>
<tr>
<th>Serum sample*</th>
<th>Composition of petroleum ether extract per 900 µl.†</th>
<th>Composition of hydrolysate fluid per 100 µl.</th>
<th>Serine</th>
<th>Ethanolamine</th>
<th>Choline</th>
<th>Total</th>
<th>Molar ratio, choline to total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>A1</td>
<td>0.60</td>
<td>0.37</td>
<td>0.07</td>
<td>0.06</td>
<td>0.29</td>
<td>0.42</td>
<td>0.69</td>
</tr>
<tr>
<td>A3</td>
<td>0.71</td>
<td>0.29</td>
<td>0.05</td>
<td>0.04</td>
<td>0.18</td>
<td>0.27</td>
<td>0.67</td>
</tr>
<tr>
<td>A4</td>
<td>0.69</td>
<td>0.31</td>
<td>0.08</td>
<td>0.06</td>
<td>0.23</td>
<td>0.37</td>
<td>0.62</td>
</tr>
<tr>
<td>B13</td>
<td>0.62</td>
<td>0.23</td>
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<td>0.00</td>
<td>0.24</td>
<td>0.24</td>
<td>1.00</td>
</tr>
<tr>
<td>C13</td>
<td>0.60</td>
<td>0.22</td>
<td>0.03</td>
<td>0.08</td>
<td>0.14</td>
<td>0.25</td>
<td>0.56</td>
</tr>
<tr>
<td>C18</td>
<td>1.19</td>
<td>0.22</td>
<td>0.02</td>
<td>0.08</td>
<td>0.16</td>
<td>0.26</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* Samples A1, A3, and A4, xanthomatosis; Sample B13, pregnant woman; Samples C13 and C18, coronary heart disease.
† Equivalent to 100 µl. of hydrolysate fluid.

hours, and the nitrogenous bases were separated and estimated. An identical sample of phosphatide extract containing no added bases served as the control. Recoveries of added bases were 93, 94, and 95 per cent for serine, ethanolamine, and choline respectively.
Experiments were also performed to ascertain the completeness of hydrolysis of serum phosphatide extracts with 6 N HCl. A typical experiment is summarized in Table I. Several portions of human serum were pooled and extracted with alcohol-ether and petroleum ether as described previously. Aliquots of the resulting petroleum ether extract were individually hydrolyzed for 4, 8, 24, and 48 hours respectively. A fifth aliquot served as a zero time control and also for the determination of the total phosphorus content. After preparation of each hydrolysate sample for chromatography as described previously, the serine, ethanolamine, and choline contents were determined as well as inorganic phosphorus. The zero time sample, prepared in an identical manner but not subjected to heating, was also analyzed for nitrogenous constituents and inorganic phosphorus. None of the above components could be detected in this sample. The appearance of serine, ethanolamine, and choline is maximal after 24 hours hydrolysis, and certainly after 48 hours, while the inorganic phosphorus after this time period is still only 66 per cent of the total phosphorus. This discrepancy between the inorganic phosphorus content and the total phosphorus present is not surprising in view of the well known resistance of hydrolytic fragments such as glycerophosphate to cleavage even in acid.

**Preliminary Analyses of Serum Phosphatides**—Finally, Table II summarizes typical analyses of human serum phosphatide hydrolysates as to their respective serine, ethanolamine, and choline contents.

**DISCUSSION**

Study of the non-choline phosphatides demonstrates the presence of at least two distinct types as evidenced by the occurrence of both serine and ethanolamine in hydrolysate solutions. The presence of serine-containing phosphatides in the cephalin fraction of human plasma phosphatides was demonstrated by Artom (7). Subsequent workers in the field have apparently not extended Artom's initial observations or have entirely overlooked the presence of phosphatidyl serine in blood plasma.

Since the unequivocal identification of the bases present in the phosphatide hydrolysates studied is in a sense preliminary, i.e. based upon $R_F$ values of unidimensional chromatograms, it must be assumed that the substances in question are identical with authentic serine, ethanolamine, and choline. However, the fact that only a few nitrogenous bases have been described which are genuine constituents of phosphatides makes the identification based upon migration values fairly accurate. Of the compounds studied by Levine and Chargaff in regard to their chromatographic behavior (17), only hydroxyproline and hydroxyglutamic acid have the same $R_F$ values as serine on chromatograms with n-butanol, diethylene...
glycol, and water as the solvent system. It should also be mentioned here that a very small ninhydrin-reacting spot has been observed at about $R_p$ 0.02 on chromatograms of 48 hour serum phosphatide hydrolysates. This adsorption zone, which is too small to estimate accurately, may be phosphoserine (17).

**SUMMARY**

Micromethods for the separation and estimation of serine, ethanolamine, and choline by means of filter paper chromatography as applied to the study of crude human serum phosphatide extracts are described. The 48 hour acid hydrolysis of crude extracts yields maximal amounts of choline, serine, and ethanolamine, whereas the liberation of inorganic phosphorus is incomplete. A few representative analyses of various serum phosphatide extracts demonstrate that the choline-containing phosphatides represent the major constituent.

**BIBLIOGRAPHY**

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