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AUTOMATED COLUMN CHROMATOGRAPHIC ANALYSIS
OF DEACYLATED PHOSPHOLIPIDS

By

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U. S. ARMY AEROMEDICAL RESEARCH LABORATORY
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ABSTRACT

A procedure is fully described for isolation and deacylation of phospholipids from serum or tissue. Control experiments are described that ensure maximum yield with minimum degradation. A completely automated system is described for column chromatographic resolution and quantitative analysis of the fractions of a complex mixture of deacylated phospholipids. Control experiments are described that ensure maximum efficiency of chromatographic separation of and maximum sensitivity of analysis of the fractions. Elution profiles for human serum and red cells and for rat liver deacylated phospholipids are shown. All of the seven fractions are identified.

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Commanding

AUTOMATED COLUMN CHROMATOGRAPHIC ANALYSIS OF DEACYLATED PHOSPHOLIPIDS

INTRODUCTION

Combat operation of Army aircraft for extended periods may stress the aviator in terms of physical, fatigue, and psychic factors. The physiological stress may have short and long term effects on the aviator. The cumulative nature of this stress forms the basis for the restrictions on the number of flying hours for Army aviators. Restrictions based on statistics, however, must endanger certain susceptible individuals while severely restricting a greater number of aviators.

A biochemical measure of the cumulative effect of stress upon an individual would greatly aid the flight surgeon in his task of determining whether an aviator was fit to continue flying. Such a test, unlike performance tests, would be beyond the control of the aviator. Polis, et. al. (1968) report a factor which may be used as a biochemical measure of stress. They report interesting data concerning Navy pilots operating off of Vietnam. The data center around the phospholipids, phosphatidyl glycerol and phosphatidic acid, and the phospholipids, phosphatidyl ethanolamine and phosphatidyl serine. The work described in this and subsequent reports is an extension of their observation.

This report deals with the isolation of and analysis of deacylated phospholipids by column chromatography. The system is faster and more reliable than the paper chromatographic system used by Polis, et. al. (1968). The chromatography and analysis is fully automated, eliminating the possibility of bias by the observer.

ISOLATION AND DEACYLATION OF PHOSPHOLIPIDS

A modification of the procedure of Dawson (1960) was used. 10 ml of a mixture of 2 parts chloroform to 1 part methanol were used for each gram of tissue or ml of plasma. A maximum volume of 150 ml was permitted. Rat liver tissue was homogenized in a Waring blender for 10 minutes at low speed. Erythrocytes were blended with methanol before adding the chloroform and blending again.

The mixture was then refluxed in a Soxhlet extractor in a 75°C water bath for 30 minutes. The solvent was removed by aspiration. The phospholipids were redissolved in methanol in one-third the previous volume.

For deacylation, 30% (7.5 M.) sodium hydroxide was added to bring the final base concentration to 1.0 M. After 10 minutes at 22°C, 0.25 grams/ml of Rexyn 102 (H) (Fisher Scientific Company) were added to stop the deacylation. The pH was tested and additional Rexyn added if necessary to produce neutralization.

Debris plus Rexyn was removed by clinical centrifugation. The volume was reduced to less than 10 ml by aspiration. 10/7/13 water/isobutanol/chloroform were mixed and allowed to settle. 1 ml of the upper layer and 4 ml of the lower layer were added to each ml of sample. The mixture was shaken and centrifuged for 10-15 minutes at 4000 rpm. The upper (aqueous) layer was removed while avoiding the white interface material. The final volume of yellowish liquid was normally 10 to 15 ml.

Soxhlet extraction for less than 30 minutes led to a decrease in the final yield of phospholipid, whereas further extraction did not appreciably increase the yield.

Use of 1.0 M. sodium hydroxide for deacylation overwhelmed the buffer capacity of the sample and aided reproducibility. The amount of time and temperature of deacylation are critical. Decreasing the time or the temperature decreased the final yield. Increasing the time or temperature resulted in significant degradation. This point is illustrated in the section entitled "Identification of Fractions".

The samples were stored at 0°C for up to a week with no appreciable deterioration.

AUTOMATED COLUMN CHROMATOGRAPHY OF DEACYLATED PHOSPHOLIPIDS

A chromatographic system similar to that of Wells and Dittmer (1965) and an analysis system similar to that of Whitley and Alburn (1964) were interconnected to provide a fully automated apparatus for analysis of the components of a mixture of deacylated phospholipids. A Technicon Autoanalyzer flow diagram appears in Figure 1. The recorder speed was 0.3 inches per minute. The flow rate through the column was 0.6 ml per minute. The system requires a 95° heating bath, a colorimeter, a recorder, and two proportioning pumps from Technicon as well as a laboratory oven, a magnetic stirrer, and miscellaneous small parts. The glass wool

PHOSPHOLIPID CHROMATOGRAPHIC FLOW

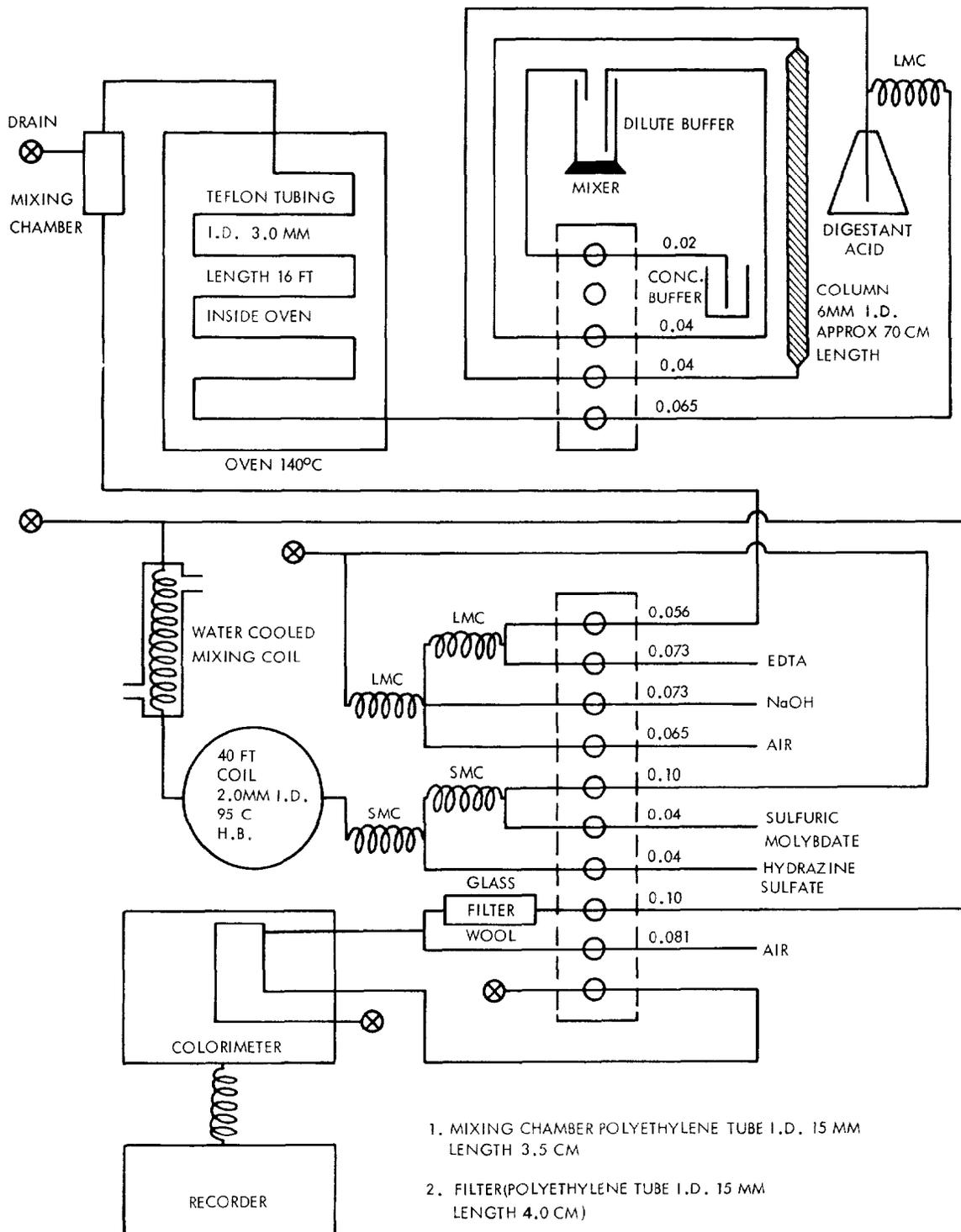


FIGURE 1

filter was used to trap particles produced during digestion. This point is discussed further in the section entitled "Identification of Fractions". Figure 2 shows the characteristic color reaction and the optical filters used. 830 m_μ filters have become available since beginning this work and would improve the sensitivity significantly. We observed that Beer's Law was followed up through an absorbance of 1.

A precipitate was formed when the digestant acid was mixed with the ammonium formate elution solution of Wells and Dittmer (1965). Ammonium formate was partially replaced by sodium formate in our solutions. The reagents used in this procedure are given in Table 1. EDTA was used to extend the pH range of the color reaction (Bartlett 1959).

The sample (5 - 10 ml) is introduced through the line leaving the mixer. The gradient elution is begun by placing both lines in the mixer immediately following the loading of the sample. The volume in the mixer at the start of the procedure is 240 ml. The gradient may be calculated using the following formula obtained using unsteady state material balance properties:

$$C = 3C_0 - 2C_0 \sqrt[3]{1 - \frac{3}{4} \frac{A}{V_0}}$$

- where
- C_0 is the concentration of the dilute buffer
 - V_0 is the initial volume of dilute buffer
 - A is the volume passed through the column
 - C is the concentration of buffer when A ml have passed through the column

A gradient is plotted in Figure 3.

The gradient needs to have the described shape and volume in order to obtain peak separation in our system. The pH of the buffer solution is important, as the order of elution depends on pH.

Control experiments show that an oven temperature of 140°C with the length of teflon tubing shown in Figure 1 was sufficient to ensure complete digestion. If the final solution (for color reaction) becomes too basic, a spurious

TABLE 1
SOLUTIONS USED FOR PHOSPHOLIPID ANALYSIS *

- | | |
|-----------------------|--|
| 1. Digestant Acid: | 800 ml 97% Sulfuric acid
80 ml water
10 ml of 0.4 g. vanadium pentoxide in 1.0 M.
sodium hydroxide
80 ml 70% perchloric acid |
| 2. Acid Molybdate: | 3% (w/v) ammonium molybdate tetrahydrate in
1.0 M. sulfuric acid |
| 3. Hydrazine Sulfate: | 0.2% (w/v) hydrazine sulfate |
| 4. Sodium Hydroxide: | 27.5% (w/v) (6.75 M.) sodium hydroxide |
| 5. EDTA: | ethylenediaminetetraacetate, disodium, 0.025 M. |
| 6. Stock Buffer: | |
| | A. 250 ml of ammonium formate solution made of 45 ml formic acid,
77 ml concentrated ammonium hydroxide, per liter, pH 8.9. |
| | B. 350 ml of sodium formate solution made of 60 ml 1.0 M. ammonium
borate, pH 9.4, 45 ml formic acid, titrated to pH 9.6 with sodium
hydroxide, per liter, pH 9.6. |
| | C. 400 ml water. The final pH of the Stock Buffer was 9.2. |
| 7. Conc. Buffer: | 3/4 stock buffer, 1/4 water |
| 8. Dil. Buffer: | 1/4 stock buffer, 3/4 water |
| 9. The Column: | Bio-Rad AG1 Anion Exchange Resin (Formate Form) |

* All reagents were analytical grade reagents. Distilled water was used throughout.

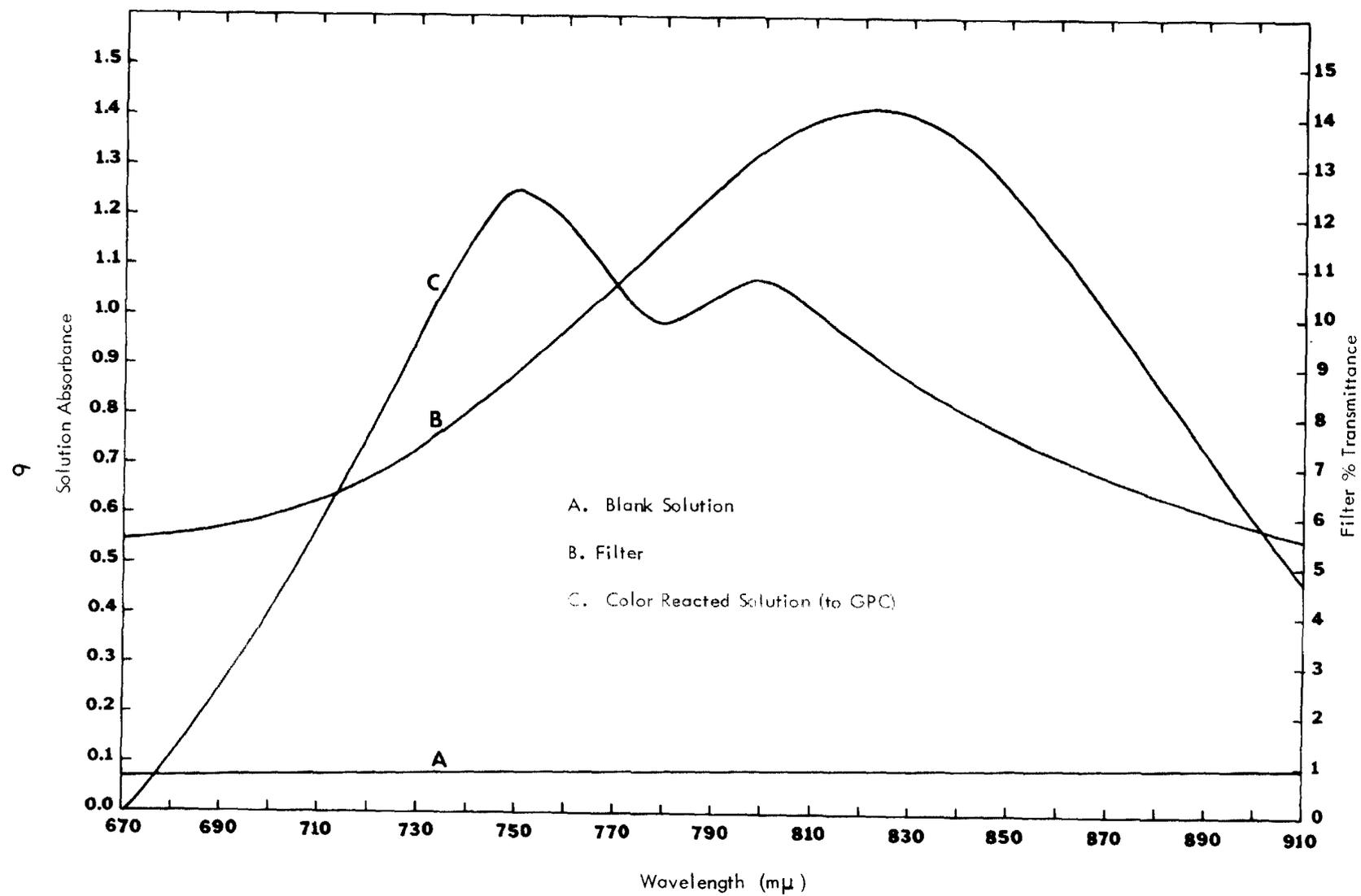


FIGURE 2

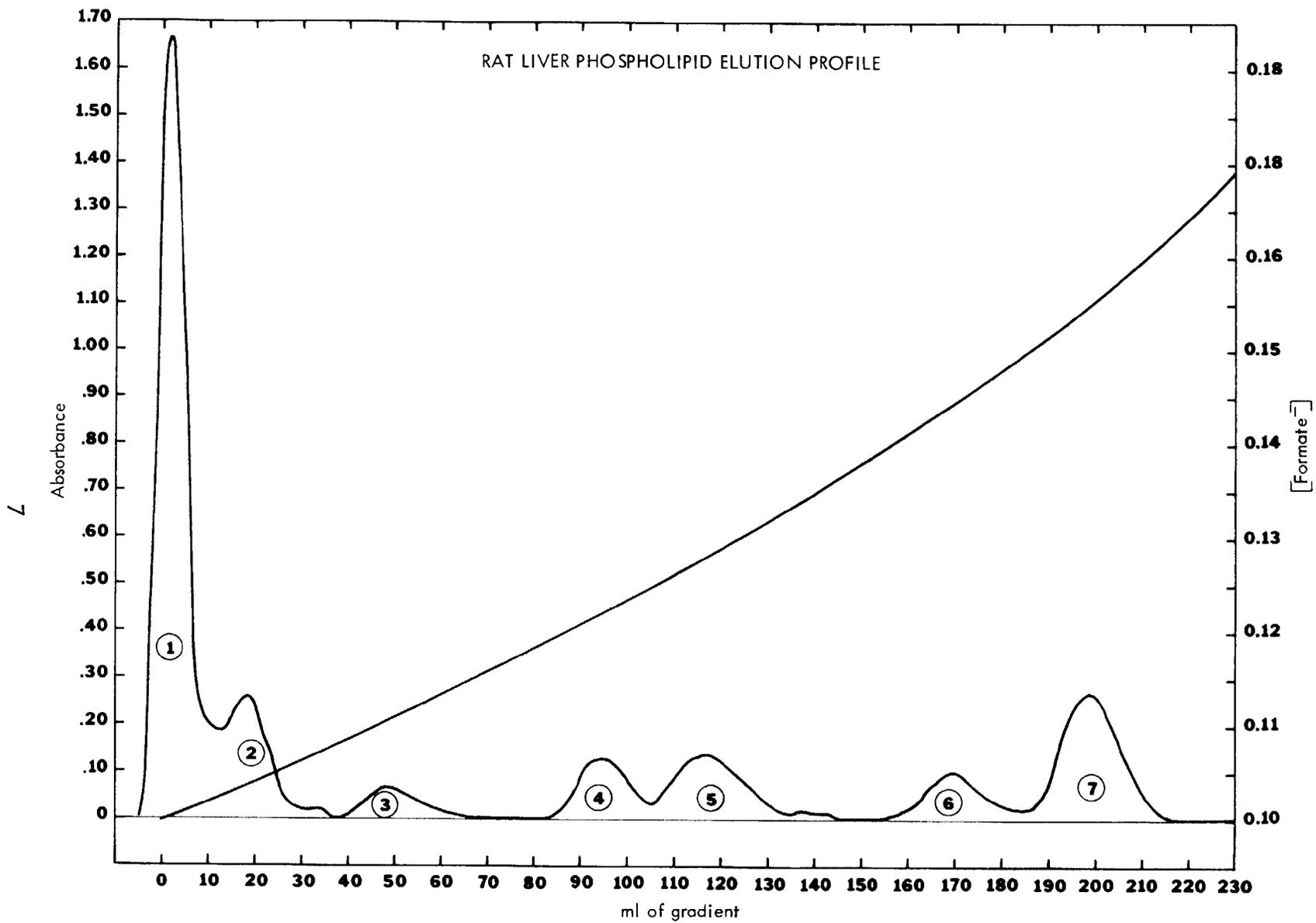


FIGURE 3

blue color is produced. The acid molybdate solution should thus be entered only after flow has otherwise become stabilized. When the lines handling acidic solutions become old, their flow rates may decrease. In this case, the baseline begins to rise, and it becomes necessary to replace the tubing. If the final solution becomes too acidic, no color is produced. This possibility is more remote, as normal drift of pH with time is toward increasing basicity. pH effects are discussed in detail by Chen, Toribara and Warner (1956) and Bartlett (1959).

A solution of 4.5 mg inorganic phosphorus (as phosphate) per ml produces an absorbance of 1.0 in our system. Our plasma and liver phospholipid results agree quantitatively with total values reported in the literature (Kraml, 1966).

The entire system was run repeatedly on the same sample. The relative and absolute quantities of each of the deacylated phospholipids did not vary significantly.

The glass wool filter should be cleaned and repacked between runs. The Bio-Rad AG-1 Anion Exchange Resin (Formate Form) column could be used repeatedly with no ill effects. The column is washed with more than 10 ml of conc. buffer and then with dilute buffer before reuse.

IDENTIFICATION OF FRACTIONS

Attempts to use two dimensional chromatography (Dawson, 1960) were only successful in the direction using the phenol solvent. As ionophoresis (Dawson, Hemington and Davenport, 1962) was not available, identifications were made by comparing elution profiles of deacylated phospholipids with data in the literature. Figures 3 and 4 show elution profile for deacylated phospholipids from the livers of two different rats. Similar profiles for human serum and human red cells are shown in Figures 5 and 6. Seven fractions are observed.

Wells and Dittmer (1965) and Lester (1963) give a description of this type of chromatographic system. Our identifications are compared with those of Lester (1963) in Table 2. We essentially agree with his results. The relative concentrations of all the fractions for rat liver and human serum are also consistent with the data of Polis (1968). The detailed identification of the fractions is given below.

TABLE 2
IDENTIFICATION OF FRACTIONS

Fraction Number	Identification of Lester (1963)	Our Identification
1	GPC	GPC
2	GPE	GPE
3	----	Cyclic Glycerophosphate
4	GPI	GPI
5A	GPG	GPG plus GP
5B	GP	
6	GPS	GPS
7A	GPGPG	GPGPG plus inorganic phosphate
7B	----	

where GP represents the deacylation product of phosphatidic acid and GPGPG represents the deacylation product of cardiolipin and where GP of GPX represents glycerylphosphoryl and

C: choline
E: ethanolamine
I: inositol
G: glycerol
S: serine

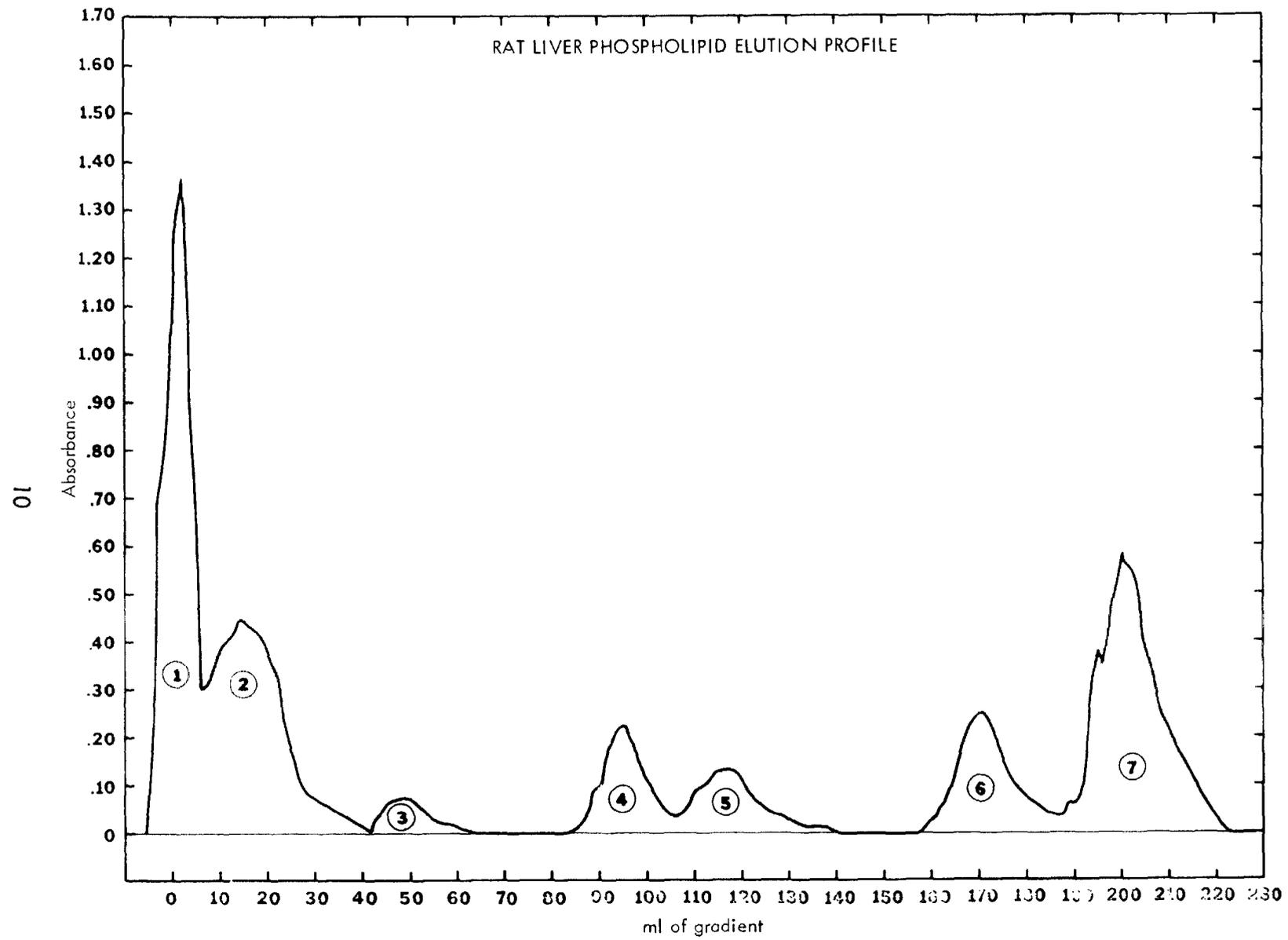


FIGURE 4

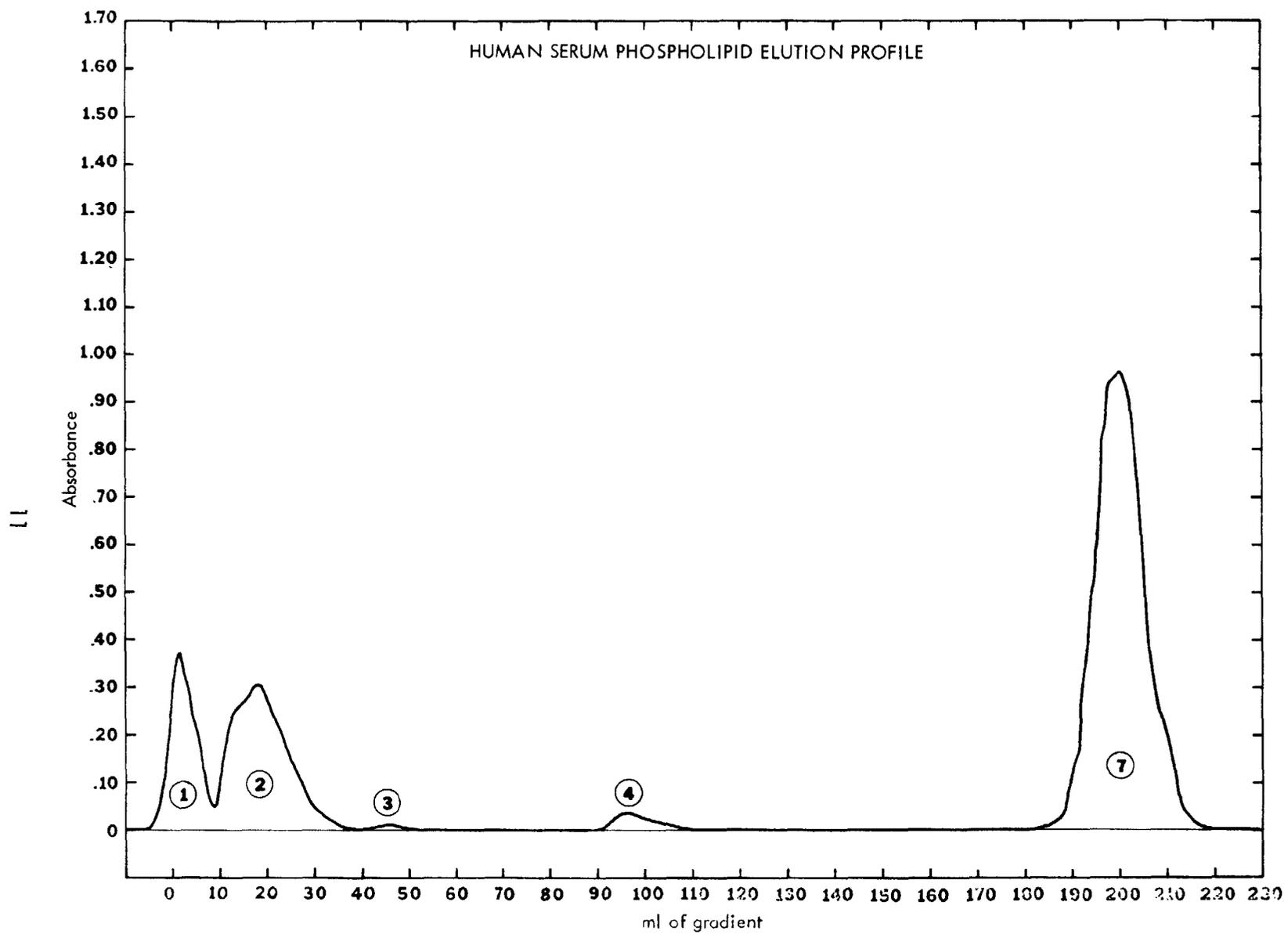


FIGURE 5

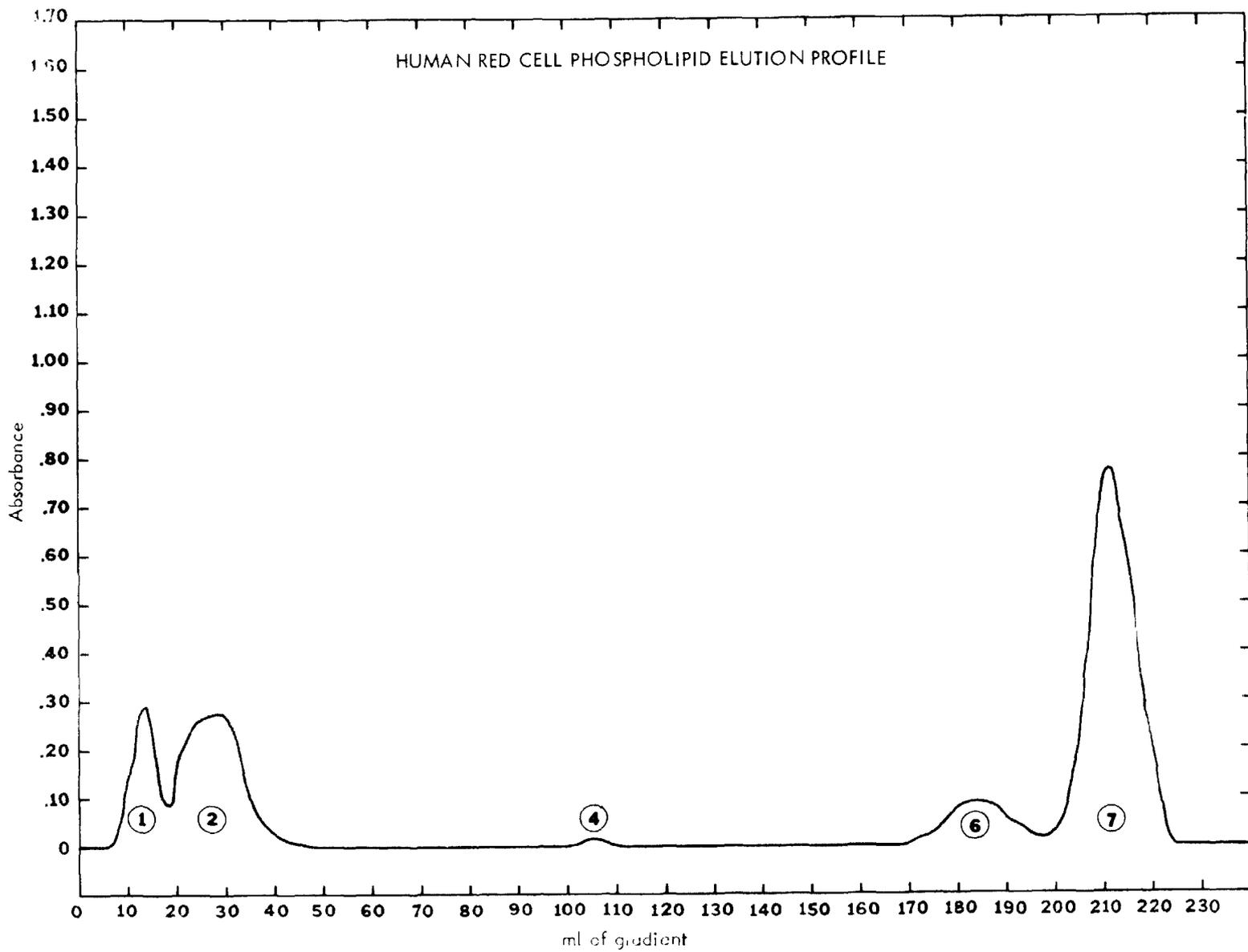


FIGURE 6

Fraction 1: GPC. The size of this fraction in liver, serum and cells leads to its only possible identification as deacylated lecithin (See also Dawson, Hemington and Lindsay, 1960).

Fraction 2: GPE. The size of this second largest fraction in all cases agrees with its identification as deacylated phosphatidyl ethanolamine. The absolute values obtained as fraction 2 are maximum values, as a large fraction of digested organic particulate material accompanies this fraction. Even the glass wool filter cannot trap all the particles accompanying this fraction. This fraction is the only one so obscured.

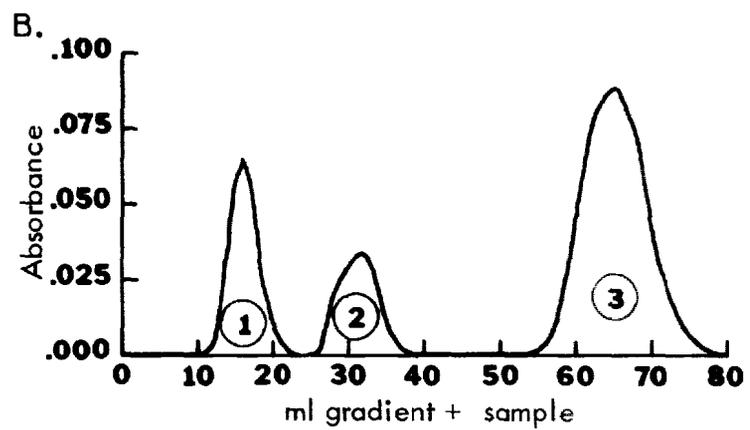
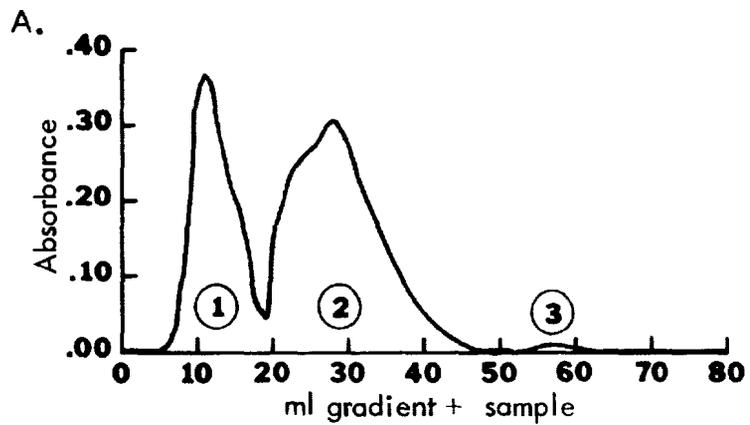
Fraction 3: Cyclic Glycerophosphate. Cyclic glycerophosphate is known to be formed from GPC and GPI following excessive treatment with alkali. Figure 7 demonstrates the results of such a treatment of human serum phospholipids and the resultant conversion of fraction 1 material into fraction 3 material. The low values normally obtained for cyclic glycerophosphate are consistent with sufficient but not excessive treatment with alkali. Dawson (1960) found 6% of GPC converted into cyclic glycerophosphate as well as sufficient degradation of GPI to give a 70% yield.

Fraction 4: GPI. The quantity is consistent with the identification. Fraction 4 disappears following excess alkali treatment. The elution order, except for the additional identification of the cyclic compound, is still the same as that of Lester (1963).

Fraction 5: GPG plus GP. Lester (1963) next observed GPG and then GP. By relative fraction size and after sufficient identification of the other fractions, we conclude that fraction 5 contains both GPG and GP. The skew nature of the peak is consistent with non-resolution of two components.

Fraction 6: GPS. The appearance of a large fraction 6 with red cells is in agreement with GPS as the only possible choice (Dawson, Hemington and Lindsay, 1960).

Fraction 7: GPGPG plus inorganic phosphate. Inorganic phosphate added to a sample appeared with fraction 7. Inorganic phosphate was carried through our isolation and deacylation procedure as seen with the serum and red cell values. GPGPG was expected to appear here (Lester, 1963). The skew nature of some elution profiles of fraction 7 is consistent with identification of two components.



A. Standard preparation deacylated 10 minutes at 22° C

B. Deacylated 60 minutes at 37° C

FIGURE 7

CONCLUSIONS

A reproducible fully automated system has been tested for chromatographic separation and analysis of deacylated phospholipids from serum or tissue.

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