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USE OF IATROSCAN FOR THE ANALYSIS OF RED CELL MEMBRANE LIPIDS

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Abstract

Hemolysate was prepared by adding H_2O (1.0 ml) to washed packed red cell (0.9 ml). Aliquot of 20 μl of the hemolysate was taken to assay the concentration of hemoglobin. The rest of the hemolysate was extracted by Rose's method (isopropanol 11.0 ml + Chloroform 7.0 ml). The extract are divided into two parts, A and B. Both A (0.4 ml) and B (7.0 ml) were evaporated. The residue of A was dissolved in n-propanol (50 μl) and the solution thus produced was measured for its free cholesterol concentration enzymatically. The residue B was dissolved in 0.4 ml of Folch'schloroform : methanol (2:1, v/v), and a portion of the solution (2 μl) was applied on thin layered silica gel sintered on a slender cylindrical quartz rod (chromarod : 0.9 mm in diameter, 150 mm in length, silica gel grain of 5 μm diameter). The chromarods were placed into a glass tank containing a solvent mixture of chloroform, methanol, and H_2O for development. The erythrocyte membrane lipids will be separated into five components, that is free cholesterol (FC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC) and sphingomyelin (SM). After drying the chromarods, detection of lipid components was performed by passing them through the flame ionization detector (FID) of the Iatroscan. The electric current produced bythe ionized carbon atoms in the flame was amplified electrically to be recorded as peaks appearing on a graphic paper in an autorecorder. Integrals of the peaks relevant to the individual components were calculated by a computer system to obtain the ratio of the amounts of their ionized carbon atoms, namely FC : PE : PS : PC : SM.

If the values of the peak areas referring to the quantity of ionized carbon atoms produced per unit weight of purified lipid have been checked with individual standard lipid specimens preliminarily, it is able to convert the aforementioned peak area ratio into the ratio of lipid weight ratio FC : PE : PS : PC : SM.

The lipid weight per 1 g of hemoglobin is also calculable, by colla-

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ting the free cholesterol concentration (mg/dl) of hemolysate to the hemoglobin concentration (g/dl) of the hemolysate.

Analytical result of normal red cell membrane lipid components (mg/g Hb) obtained in this way was as follows : FC : 4.69±0.57, PE : 2.94 ± 0.4, PS : 1.80 ± 0.34, PC : 2.85 ± 0.39, and SM : 2.52 ± 0.34. These estimations agreed well with those values got by the conventional chemical analysis of the red cell membrane lipid. Coefficients of variation of the analytical values of membrane lipids i. e. FC, PE, PS, PC and SM by this method were 1.5−2.9, 4.5−8.7, 5.6−12.6, 2.3−6.7, 3.0−8.7(%), respectively.

INTRODUCTION

Chemical analysis of normal lipid composition of erythrocyte membrane has been studied enthusiastically by Cooper and other investigators,[1)-4)] and its overall aspects have become elucidated to a considerable extent. Namely, the major lipid components of normal human erythrocyte membrane are free cholesterol (FC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC), and sphingomyelin (SM), while lysolecithin, globosides, phosphatidyl inositol and so forth form the minor or trace components.

However, the lipid analysis is not so simple in procedure as is thought beside the table. It requires silica gel column chromatography or silica gel thin layer chromatography. These methods demand meticulous attention to fairly complicated operations. The reproducibility is occasionally unsatisfactory.

Recently, a new technique has become available for lipid analysis. This is the Iatroscan technique.[5)-10)] Lipid extract is applied to a chromarod which is a silica gel thin layer sintered onto a quartz rod, followed by chromatography with developer solution put in an air-tightly fixed glass container. The chromarod is passed through a FID of the Iatroscan to make them produce ionized carbon atoms by combustion in a hydrogen burner of this equipment from the lipid components which had been developed on it. The ionized carbon atoms generate electric current in FID. This is amplified and sent to a graphic system to be recorded on a graph paper. The Iatroscan is therefore a combination of thin layer chromatography and gas chromatography.

We intended to use the Iatroscan for the analysis of red cell membrane lipids. After trials and errors with this equipment we have recognized that the Iatroscan techniques have many advantages over the traditional procedures, particularly in the simplicity of operation, capability of repetition of analysis with the same sample of lipid extract which is minute in amount, and good reproducibility of estimation values. However, it is true that it does not get rid of the demerits inherent in the thin layer chromatography and the gas chrom-

atography. In our laboratory when these shortage were successfully controlled, the analytical values of normal human red cell membrane lipids were in good agreement with those obtained by the conventional techniques. The purpose of this paper is to introduce our experience with the Iatroscan particularly with reference to its pertinent manipulation.

METHODS

1. Extraction of red cell membrane lipids

a) Washing of the red cells

Three ml of whole blood which is prevented from coagulation by addition of a droplet of anti-clot ET is centrifuged (3,000 rpm, 10minutes) to separate the erythrocyte sediment. Plasma component and buffy coat are removed from the sediment. The sedimented red cell layer is washed three times with isotonic saline solution and centrifuged to obtain packed red cells.

When anticoagulated blood is not available, blood clots can be used as samples. In this case, blood clots are crushed with glass rod over the funnel which is covered with a sheet of gauze, and streams of red cells flowing down from the funnel are collected in a centrifuge tube. They are washed and centrifuged in the same way as described above to obtain packed cells.

b) Preparation of hemolysate

Take the centrifuge tube of 50 ml. Setting a rubber cap to the head of the volumetric pipette of 1.0 ml which is graduated to 0.1 ml moisten its inner lumen with isotonic saline solution preliminarily by aspiration. Then, aspirate 0.9 ml of the washed packed cell (prepared above) into the volumetric pipette, hold it straight and insert it in the centrifuge tube. Immediately, take the rubber cap off from the volumetric pipette so that the washed packed cells may flow into the centrifuge tube freely. After the red cells having been introduced into the centrifuge tube, take 1.0 ml of H_2O precisely with the same volumetric pipette by resetting the rubber cap on it. Thereafter, leaving to stand the volumetric pipette in the same position in the centrifuge tube, aspirate and disgorge the content of the tube repeatedly into the pipette to hemolyze the red cells with H_2O and to rinse the inner lumen of the pipette with hemolyzed solution. In this way, all the red cells pipetted are transferred in the centrifuge tube and subjected to complete hemolysis by stirring the centrifuge tube on a Vortex mixer.

c) Measurement of hemoglobin concentration of hemolysate[11]

Measure the hemoglobin concentration of the hemolysate before

extraction procedure. Take 20 μl of hemolysate to determine its hemoglobin concentration [Hb (g/dl)] by cyanmethemoglobin method and calculate the following equation

$$[\text{Hb(g/dl)} \times \frac{1.9}{0.9} = \text{X (g/dl)}\ ]$$

to get the hemoglobin concentration of the packed red cells.

d) Extraction procedure[12)]

Add 11 ml of isopropanol (special grade [$(CH_3)_2$ CHOH]) to the container (centrifuge tube) of the hemolysate by shaking it vigorously, and mix completely on a Vortex mixer. It is desirable that fine movable floating coagulants are seen in the centrifuge tube. After allowing it to stand for an hour, add 7.0 ml of chloroform (for chromatography) and stir up sufficiently on a Vortex mixer. An hour later, add 4.0 ml of isotonic saline solution to the centrifuge tube, shake and keep in a refrigerator, at 4°C overnight. On the next day, the content of the centrifuge tube will separate into three layers, that is, 1) water, 2) red cell ghost, and 3) mixture of isopropanol and chloroform (membrane lipid is extracted in it) from the top to the bottom. Then centrifuge it (3,000 rpm, 20 min) and remove the top layer of water by aspiration. The mixture of isopropanol and chloroform are filtered through the funnel set with a Toyo filter paper No. 7 on it and make it flow into a centrifuge tube of 12 ml. This filtered solution is called the extract.

e) Evaporation of the extract

After keeping the extract for two hours in a refrigerator at -20°C, take it out from the refrigerator and allow it to stand at the room temperature. Then, remove the top layer of water by aspiration. Next, take 0.4 ml of the extract for free cholesterol assay and also 7.0 ml of it for lipid analysis. Both are evaporated (40°C in a warm bath). The residue for free cholesterol assay is dissolved in 50 μl of n-propanol and measured enzymatically.

2. Separation and development of the lipid extract.

a) Activation of the chromarods

The chromarods (thin layered silica gel sintered on a quartz rod which is 0.9 mm in diameter, 150 mm in length, silica gel grain of 5 μm diameter) are set on a scanning holder and they are passed through the hydrogen burner of 720°C. This operation makes the silica gel of the chromarods activated.

b) Application of samples to chromarods

Set the activated chromarods in the holder. The residue of the

lipid extract is dissolved in Folch's solution (chloroform : methanol = 2 : 1 mixture ; 0.4 ml). Take 2 μl of this solution by microdispenser and apply it to a chromarod just above 3 cm from its end (in duplication or triplication).

c) Development

Place the chromarods which are set in the holder in a developer tank (glass tank of $11.5 \times 3.0 \times 18.5$ cm in size, erect a rolled thick filter paper inside of the tank so as to fence the chromarod holder). Introduce 70 ml of mixture of chloroform : methanol : H_2O (70 : 35 : 3.5 in volume) into the glass tank and cover the tank on its top with a glass plate tightly to separate the lipid components by the gradual ascension of developer on the chromarods. (at 18°C for 20 minutes)

3. Analytical operation by use of Iatroscan

(1) Apparatus and its principle

Iatroscan[5-10] is an apparatus equipped with a thin layer chromatography (TH-10, Iatron Lab) assembly and a pen recorder (Hitachi 055). After separation and development of lipid extracts on chromarods, they were dried at 115°C for five minutes, and then passed slowly through a hydrogen burner with a constant speed to make them produce ionized carbon atoms from the lipid components by combustion. The ionized carbon atoms of each lipid component generate electric current in FID. This is recorded as a peak area individually on a graph paper (Fig. 1). Each peak area drawn on the graph paper corresponds to each of the relevant lipid components separated on the

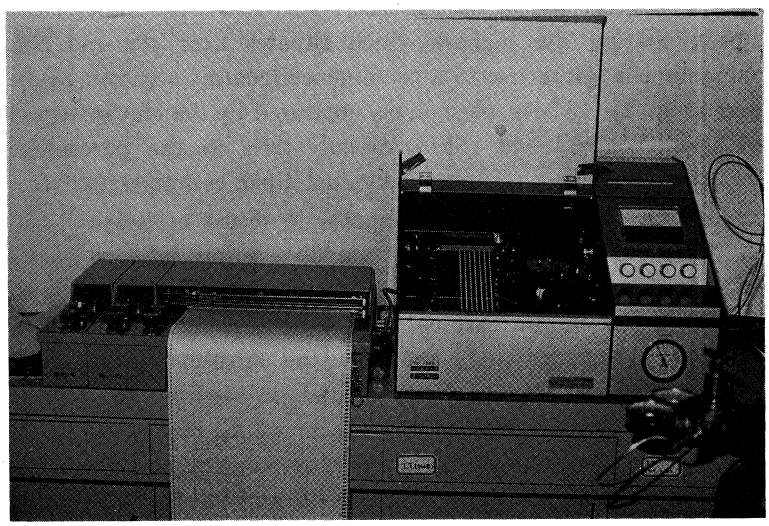

Fig. 1. Analytical assembly of Iatroscan TH-10.

chromarod.

Areas circumscribed between these peaks and the base line are proportionally large to the number of ionized carbon atoms emitted from each lipid component by combustion.

The sizes of these areas can be calculated by chromatopack E IA computer (Shimazu). Lipid composition of the extract (expressed in terms of the ionized carbon atoms) is obtainable by calculating the ratios (in percentage) of the areas of individual peaks to the sum of the peak areas which arise on the graph paper.

(2) Operation

The chromarods on which lipid components have been separated by development in the tank are transferred into an oven. They are placed in a steel frame work (a holder) in parallel and dried at 115°C for five minutes. Then, the holder with its chromarods is applied to the Iatroscan (TH-10, Iatron Lab) for the purpose of combustion in the flame of its hydrogen burner. The condition of combustion and recording the electric current generated by the ionized carbon atoms are as follows :

hydrogen control : 160 u/min, air control : 2.0 l/min, scanning speed : 32 sec/scan, and recording chart speed : 120 mm/min.

4. Calculation of the lipid component

a) Express the ratio of lipid components in terms of the number of ionized carbon atoms

Usually there appear five peaks (FC, PE, PS, PC, SM) on the graph paper of the recorder of the Iatroscan (Fig. 2). Free cholesterol produces a peak soaring the highest which situates near the end point, while sphingomyelin gives rise to a prominence locating adjacent to the starting point (Fig. 2). These peak areas measured by the chromatopack ([FC], [PE], [PS], [PC] and [SM]) directly refer to the percentage of the ionized carbon atoms of the individual lipid components.

b) Express the ratio of lipid components in terms of weight

Take 100 μl aliquots of 10 mg/dl solutions of each pure specimen of lipid component (FC, PE, PS, PC, SM) and mix them. Two μl of the mixture is put on the chromarods. After their development in the tank as described above, apply them to the Iatroscan in order to get the chromatopack readings a, b, c, d and e of lipid component peaks. These readings are proportional to the amounts of ionized carbon atoms produced by the individual lipid components. Therefore, we can calculate the ratio expressed in terms of weight of the lipid components separated on chromarods by employing the reciprocals of these readings

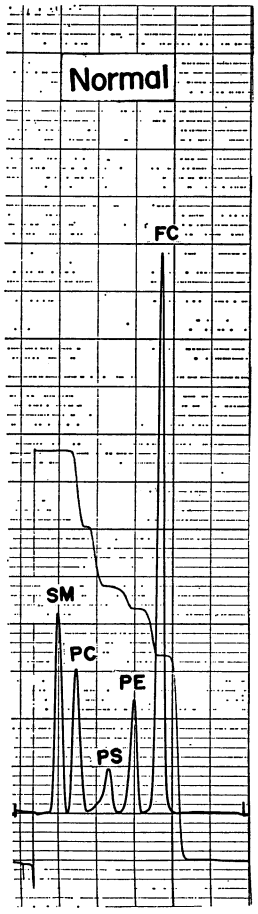

Fig. 2. Normal Iatroscan graphic pattern of the analysis of red cell membrane lipids. Note five peaks (FC, PE, PS, PC and SM) in the curve.

($\frac{1}{a}$, $\frac{1}{b}$, $\frac{1}{c}$, $\frac{1}{d}$ and $\frac{1}{e}$).

For example, the percentage of free cholesterol (FC%) is obtained by the following equation.

$$FC\% = \frac{\frac{1}{a}[FC]}{\frac{1}{a}[FC] + \frac{1}{b}[PE] + \frac{1}{c}[PS] + \frac{1}{d}[PC] + \frac{1}{e}[SM]} \times 100^*$$

The percentages of other lipid components are calculated similarly.

5. Determination of red cell membrane free cholesterol content by enzymatic

method.[13,14]

Add 1.0 ml of H_2O to 0.9 ml of packed cells to extract lipids from them with 18 ml of the lipid solvent (isopropanol : chloroform = 11 : 7). Then, an aliquot of 0.4 ml of the extract is evaporated to get its residue (Follow the procedure 1 [a - e] until this stage) and dissolve it in 50 μl of n-propanol (sample). Concurrently, prepare free cholesterol standard solution (100 mg/dl, dissolved in n-propanol). Add cholesterol oxidase reagent solution (Iatron) to both of the sample (50 μl) and standard solution (50 μl) to produce red quinone substance by the chemical reaction between H_2O_2 generated from cholesterol enzymatically and 4-amino-antipyrine. The optical density of the colored solution is measured at 505 nm in a spectrophotometer. (As to the procedure the instruction sheet of the Iatron kit of cholesterol determination is followed). In this procedure, $\frac{0.4}{18}$ of 0.9 ml or 0.02 ml (20 μl) of packed cells was employed as a sample for lipid extraction, and the color produced by 20 μl of lipid extract is compared with that generated by 50 μl of free cholesterol standard solution (100 mg/dl). Therefore, for the purpose of getting the free cholesterol content it is necessary to multiply the cholesterol values obtained in this method by 2.5 ($= \frac{50}{20}$) to get the free cholesterol content (mg) of 100 ml of packed cells.

6. Determination of the content of each lipid in terms of weight

If the free cholesterol content (mg) per 100 ml (dl) of packed cells has been determined as described above, the free cholesterol content (mg) per 1 g of hemoglobin will be calculated by dividing free cholesterol content (mg/dl) with X g/dl of hemoglobin concentration of the packed cells. Once the free cholesterol content (mg) per 1 g of hemoglobin concentration has been obtained, the contents of other lipid components per 1 g of hemoglobin concentration are calculable by the following equations.

$$PE = \frac{1}{b} \times [PE] \times FC \text{ mg/gHb}$$

$$PS = \frac{1}{c} \times [PS] \times FC \text{ mg/gHb}$$

$$PC = \frac{1}{d} \times [PC] \times FC \text{ mg/gHb}$$

$$SM = \frac{1}{e} \times [SM] \times FC \text{ mg/gHb}$$

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*Indices employed for calculating the ratio of individual lipid components in terms of weight are as follows : $\frac{1}{a}=1, \frac{1}{b}=2.2, \frac{1}{c}=2.8, \frac{1}{d}=2.18$ and $\frac{1}{e}=1.73$.

and, as a corollary, the total lipid content (TL) of packed cells are given by
$TL = FC + PE + PS + PC + SM$ (mg/gHb).

TABLE 1 Analytical values of lipids of the normal red cell membrane (Mean±SD).

Lipids / analytical values	FC	PE	PS	PC	SM
Peak areas (%)	49.8 ±0.6	14.1 ±1.6	6.8 ±1.3	13.9 ±1.8	15.4 ±2.0
mg/g Hb	4.69 ±0.57	2.94 ±0.40	1.80 ±0.34	2.85 ±0.39	2.52 ±0.34
mg/10^{10} RBC	1.41 ±0.17	0.88 ±0.12	0.54 ±0.10	0.85 ±0.12	0.76 ±0.10
Weights (%)	31.7 ±3.8	19.9 ±2.7	12.1 ±2.2	19.3 ±2.6	17.0 ±2.2

RESULTS

In normal adult subjects (23 males and 5 females) red cell membrane lipids are separated into five peaks (FC, PE, PS, PC, and SM) from the top to the base of the chromarod as shown in Fig. 2. Analytical values of normal human red cell membrane lipids by the Iatroscan method are listed in Table 1.

DISCUSSION

Analytical quantitation of lipids is not so simple.[15] Its process consists of ① extraction with organic solvent of all the lipids from the samples, ② extracted lipid components are separated individually by adequate methods, and ③ appropriate chemical and enzymatic reaction is applied to each separated lipid components to get colorized solution for spectrophotometry. The color (optical density) thus obtained is compared with that of standard lipid component treated with the same reagent in entirely the same way to obtain the concentration of relevant lipid component. Accordingly, three steps comprising ① extraction, ② separation and ③ quantitation are required for the lipid analysis. Conventionally, Folch's solution (chloroform : methanol = 2 : 1)[16] is widely used for the extraction of red cell membrane lipids. Free cholesterol, which is detected by specific reaction, for example by the red-brown coloration with Zak's[17] reagent (ferric chloride solution in concentrated sulfuric acid) can be assayed spectrophotometrically without preliminary separation by adding the

reagent to the extract directly. Sphingomyelin can be determined in the similar principle (for instance, by the Anthron sulfuric acid[18] or by the color reaction of choline[19] contained in SM). In contrast the group of phospholipids containing phosphoric acid as one of their ingredients can not be discriminated individually by their own specific color reactions. Therefore, it is necessary for them to be separated into PE, PS and PC by silica gel or alumina column chromatography before they are determined. Recently, thin layer chromatography[20] is preferred for the separation of phospholipids. The batches of phospholipid components separated by either column or thin layer chromatography are digested by oxidation with sulfuric acid and H_2O_2. Then, the phosphoric acid remaining after digestion in the residue (in H_2SO_4) is colored with ammonium molybdate (Fiske-SubbaRow method)[23] to determine the phosphate content of the individual phospholipid components of the extract. Since the percentage of the phosphate content of each phospholipid component is already known, the concentration of each components (PE, PS and PC) is calculable by dividing the relevant phosphate concentration by its own phosphate percentage.

However, if silica gel thin layer chromatography is used for analysis, meticulous attention should be paid to each step of procedures. In the first place the quality of silica gel is the most important point, and the thickness of the silica layer, heating of the silica plate and its storage should not be neglected. The angle by which the silica plate is erected in the developer tank, the temperature and the vapor in the developer tank and so forth should carefully be controlled. Various conditions exerts influence on the results of the lipid component separation. After development, however, lipid components are individually colored by spray with their own color reagent (ferric chloride–H_2SO_4,[16] molybdate solution,[23] Anthron's solution,[18] etc.), to produce their colored spots which are measured by spectrophotometry. However, the accuracy of quantitation of these components in this way is frequently unsatisfactory. Therefore silica gel column chromatography[21,22] is preferred to thin layer silica gel chromatography[20] so far as the precision or accuracy is concerned, although it is cumbersome in procedure.

We tried, for the first time, to employ the Iatroscan as a tool for the erythrocyte membrane lipid analysis in November, 1975, and nowadays, we attained to the level of technical skill with which we recognize both the merit and the demerit of this instrument and are able to handle it freely, after experiencing various miscarrages. The limitation of usefulness of the Iatroscan has become also well known to us.

1. Properties of chromarods to which meticulous attention is to be paid.

Shortly after we were skilled in discrete separation and graphic demonstration of five lipid component peaks (FC, PE, PS, PC and SM) on chromarod by Iatroscan and evaluated the coefficients of variation of analytical data we became aware of important factors which affect the result of assay. For instance, ten chromarods were set in the holder A and another batch of ten chromarods which belonged to the same lot product in the holder B for Iatroscanning. Theoretically, if the same sample is analyzed with the chromarods in the holder A and those in the holder B separately, the same analytical values should be obtained. Surprisingly, in reality there was a considerable difference in averaged analytical values between the holder A and the holder B notwithstanding that the same samples were applied to the two sets of chromarods in A and B in the same manner. During the time of keeping chromarods in the holders, the properties of chromarods in the holder A might have been altered differently from those in the holder B due to delicately different ambient conditions between the A and the B. Accordingly, it was decided to make it routine preparatory work to check chromarods before it was employed for actual analysis by observing whether or not they gave normal separation of lipid components with pertinent analytical results when an extract of normal adult red cell membrane lipids was applied to them, developed in the developer tank and subjected to the burner of the Iatroscan. Unless the results are normal in pattern and estimation, the relevant chromarods are not used. They are tamed by heating in the hydrogen burner of the Iatroscan repeatedly until they become satisfying ones.

The lipid analytical values are affected by room temperature, atmospheric moisture and other miscellaneous factors which are not exactly known to us. However, usual alterations of the properties of chromarods during storage are able to be controlled adequately and to be made useful for analysis by regulating the composition of the developer solution as well as the time and temperature of development. Once we have succeeded in taming the chromarods to the right condition, the chromarods thus tamed can be used successively seven or eight times for analysis. (To each of the holders A and B are set 10 chromarods. If these two holders are used interchangeably at a time, and five chromarods are employed for pentaplicate application of the same single specimen of lipid extract, four specimens are able to be dealt with in pentaplication within an hour.)

The chromarods should be put in the holders, and the holders are kept in a desiccator (about 18 cm in diameter) which installs 100 ml beakers containing water inside (install one beaker when the air is moist ; two beakers when the air is dry). The silica gel sintered to the chromarods is moistened

with water vapor adequately to maintain its activity by this treatment.

2. Correlation between the lipid components separated on the chromarods and the electric current produced by each of the lipid components.

Prepare the chloroform solutions of various concentration of PC and FC (0.5, 1.0, 2.0, and 3.0 mg/dl) with their pure specimens. Take 1 μl aliquots of these solutions and apply them to chromarods individually, and develop them on the chromarods in the way described in the section of method. Then, pass them through the hydrogen burner of the Iatroscan to record the peak areas of PC or FC on a graphic recorder paper by ionized carbon electric currents which represent the amounts of PC or FC contained in the solutions. Take the peak areas on the ordinates and the lipid (PC or FC) content of the solutions on the abscissa and collate them on a graphic paper with respect to lipid solutions of various concentration. Fig. 3 shows the result of such trials. A straight line passing the origin of graphic axes is seen. This is interpreted as an evidence for the presence of a rectilinear relationship between the areas of peaks and the concentrations of lipids (PC or FC). Therefore, it is possible to draw rectilinear calibration curves in order to read the concentrations of lipid from the peak areas obtained on the graphic paper of the Iatroscan.

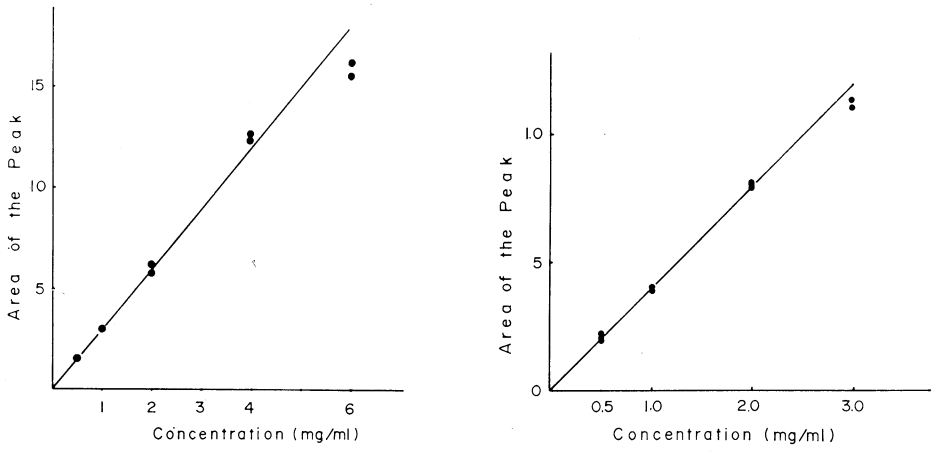

Fig 3. Calibration curve of free cholesterol determination (right)
Calibration curve of phosphatidyl choline determination (left).

Prepare 5 different sorts of 10 mg/dl lipid solutions by dissolving the standard specimens of FC, PE, PS, PC and SM in chloroform individually. Mix 5 ml aliquots of these 5 kinds of lipid solutions to get a mixture in which each lipid component is 2 mg/dl in concentration. Apply 2 μl volume of this mixture to the chromarods and subject to Iatroscan analysis so that the

peak areas of ionized carbon electric current produced by 5 kinds of lipids of the same concentration may be compared with each other. On a rectiangular graph, taking the peak area of FC on the abscissa and other lipid components on the ordinate plot the peak areas pertaining to PE, PC, PS and SM. In this way, it is possible to collate the ionized carbon electric current produced by FC with those of other lipids (PE, PC, PS and SM). Similar studies were carried out with various concentrations (such as 0.5, 1.0, 2.0 and 3.0 mg/dl) of the mixtures of chloroform solutions of 5 kinds of lipids. Fig. 4 is the

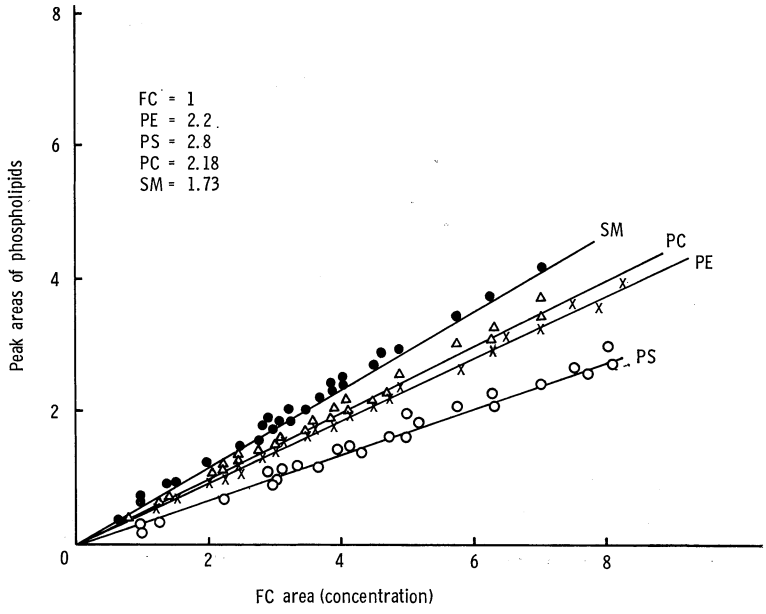

Fig 4. Correlation between areas of the peaks of individual phospholipids (SM, PC, PE and PS) and concentration of FC. Indices employed for calculating the ratio of individual lipid components in terms of weight are shown in the left upper corner of the figure.

summary of those studies. From the scrutiny of this figure it will be seen that if the ionized carbon electric current of FC is assumed to be 1, those produced by other lipid components will be read as the following indices :

$$PE=\frac{1}{2.2,}\quad PS=\frac{1}{2.8,}\quad PC=\frac{1}{2.18}\quad \text{and}\quad SM=\frac{1}{1.73.}$$

Therefore, if the concentration of FC of red cell membrane has been known to us by enzymatical analysis, it is possible to calculate the concentrations of the red cell membrane lipids (mg/dl) by multiplying the ionized carbon electric current of relevant lipid by multiplying the ionized carbon electric current of

relevant lipid (PE, PS, PC and SM) by the reciprocals of indices (2.2, 2.8, 2.18 and 1.73, respectively). One of the important things to be taken into consideration is that the infallibility of those indices is dependent upon the grade of purity of the standard lipids purchased from the suppliers of pure chemicals. In this respect the indices obtained by us are thought to be exact to a satisfactory extent.

As has been described above, the properties of chromarods change delicately by the storage condition. For this reason, in every analysis it is recommended to use a batch of ten well-checked chromarods at a time for the purpose of accuracy and precision. To one of the ten chromarods apply 2 μl* of the solution of pure standard lipids.

The rest nine chromarods are used for analyzing the lipid extract of samples. From the analytical values obtained by the checked chromarod, calculate the indices every time and use them to get the analytical values of samples. However, this was not always feasible, because pure standard lipid specimens were at times unavailable. Therefore, we think it to be the second best way which is practical to check the properties of chromarods with an extracts of normal human red cell membrane lipids every time when analysis is done.

3. Cautions to be paid in the procedure on Iatroscanning

i) Graphical patterns of the peaks

One of the example of patterns of lipid component peaks which are seen on the graph paper are summarized in Figure 2. Special attenion should be paid to the free cholesterol peak soaring upward straight from the base line as illustrated in Figure 5. This is suggestive of concomitance of all the lipid components without satisfactory separation to the head (or top) of the developer solution risen on the chromarod. In this case around the top of developer ascension on the chromarod not only free cholesterol but also other lipid components may be present forming mixture. This pattern refers, therefore, to insufficient separation of lipids on the chromarod.

ii) Developer solutions

The conventional systems of chloroform ($CHCl_3$) : methanol (CH_3OH) : water (H_2O) system was employed by us for development of the components of lipid of the extract applied to the chromarod. The composition

*Five ml aliquots of the solutions of FC, PE, PS, PC and SM (each 10 mg/dl in concentration) are mixed, and the resultant mixture is divided into 100 μl portions in individual test tubes and stoppered tightly. They were kept in a refrigerator at −20°C. Everytime on use one of them is taken out of the refrigerator and brought to the room temperature. A 100 μl mixture in a test tube was useful for analysis for a week after it has been opened.

or mix ratio should be changed or adjusted adequately taking the varied atmospheric conditions of environment such as seasons into consideration. However, $CHCl_3$: CH_3OH : H_2O of 70 : 35 : 3.5 (volume ratio) should be regarded to be the standard ratio. It is necessary to modify the standard ratio appropriately depending on the difference in the lot numbers of the chromarods supplied by the producers as well as on the variation of the temperature and humidity of the ambience.

As a rule it is recommended to decrease chloroform composition of the developer soluton when it is dry in the ambient air (for instance, $CHCl_3$: CH_3OH : H_2O = 60 : 35 : 3.5) and increase chloroform when it is humid in the air (for example, $CHCl_3$: CH_3OH : H_2O = 80 : 35 : 3.5).

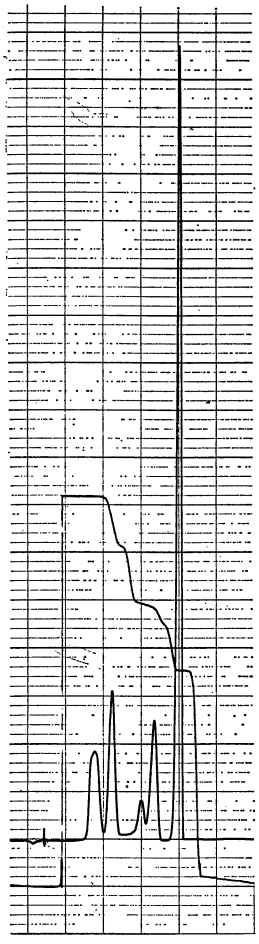

Fig. 5 One of the examples of "top pattern separation".

When it is noticed by graphic recording that the development is insufficient around the free cholesterol peak areas, it is necessary to increase the ratio of chloroform. If phospholipid peaks are not well separated because of unsatisfactory developing and, as a result, PC and SM occupy more than 20 percent of total lipids of normal extracts of red cell membrane, that is an event which suggests poor separation of phospholipids, is witnessed, it is indicated to increase methanol in the composition of developer solution.

About 65–70 ml of developer solution should be placed in the developer tank. Smaller volume of developer in the tank frequently results in appearance of the "top pattern separation" (Fig. 5).

iii) Developing time and the temperatue for developing

As short as twenty minutes are sufficient for complete developing and separation of lipids on the chromarods. This speedy developing is one of the merit of Iatroscanning. Checking the patterns of the peaks on the graph, it is possible to change the time for developing longer or shorter than 20 minutes by a minute or so for the purpose of better separation of the lipid peaks. The temperature of 18°C–20°C is suitable for developing. Usually the best result is obtained in Iatroscanning when 20 minutes are spent for developing so that the developer may ascend from scale 0 cm to scale 7.5–8.0 cm of the chromarod. Ideal separation of lipid peaks is obtained when the developing time is distinctly shorter or longer than this.

iv) Amount of extracts applied onto the chromarods

When the chromarods are new and well prepared for developing the lipid components the analytical reproducibility is not impaired by a certain degree of the variation of the amount of extracts applied to it. However, decreased older chromarqds of low adsorption activity results in poor precisions. It is desirable to apply exact amount of extract. Excess amount (volume) gives rise to small peak areas of FC (free cholesterol). Conversely, insufficient amounts make peak areas of FC unduely larger.

v) The maximum of repeated use of chromarods

The chromarods can be used for analysis seven or eight times successively in a day. However, it is recommended that further use is obviated, and the chromarods are put into a desiccator again in order to revive their absorption activity. The chromarods may be used over and over even one hundred and thirty times successively, but the coefficients of variation of analytical results will inevitably become gradually larger with increase in succession of use.

vi) Preservation of the chromarods

After Iatroscanning, it is necessary to put the chromarods in the desiccator at 110°C for five minutes to eliminate all the organic solvents which was infiltrated during the former use for development. On Iatroscanning, if other organic solvents are used in the same room, the chromarods adsorb them and, as a result, the base line of the graphic peaks of the lipid components rises up unduely from the true one. Therefore, separation patterns of lipid components become untruthful. To keep the atmosphere of the laboratory from contamination by the floating gas of organic solvent should not be forgotten in the performance of Iatroscanning.

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