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A NEW METHOD FOR THE *IN SITU* DETERMINATION OF PHOSPHOLIPIDS AFTER THIN-LAYER SEPARATION

THE PHOSPHOLIPID CONTENT OF Ca-ATPase AND Na,K-ATPase FROM HUMAN ERYTHROCYTE IN COMPARISON WITH THE PHOSPHOLIPID CONTENT OF HUMAN ERYTHROCYTES

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SUMMARY

A very sensitive method has been devised for the *in situ* determination of phospholipids after thin-layer chromatographic separation, which enabled us to investigate the phospholipid content of erythrocytes and ATPase preparations. The phospholipid compositions of the ATPase preparations and of the erythrocytes are different, and the relative phospholipid compositions of the Na,K-ATPase preparations are also different, which indicates that Na,K- and Ca-ATPase seem to be different with regard to their phospholipid composition.

An increase in temperature during the preparation procedure yields a Ca-ATPase preparation (II), which exhibits different kinetic properties and a different phospholipid composition from those of the normal Ca-ATPase preparation (I).

By changing the ionic strength and performing density gradient centrifugation, preparation (I) yields three fractions, one of which is composed of fibrils. The phospholipid composition of this fraction is approximately the same as that of preparation (II), and it contains ATPase activity and might possibly constitute one of the Ca-ATPase systems.

INTRODUCTION

In this paper, a method is described for determining minute amounts of phospholipids, and the practical application of this method to the determination of phospholipids of erythrocyte membranes in comparison with erythrocyte membrane preparations with high ATPase activity is discussed.

The sample required for determining the phospholipid content was obtained by extracting freeze-dried human erythrocytes, blood group O-Rh(+) with methanol–chloroform (2:1) (ref. 1). The solution was evaporated at 30° in a flash evaporator and the residue was extracted with a small volume of chloroform–methanol (2:1) and stored at −20°.

QUALITATIVE ANALYSIS OF PHOSPHOLIPIDS *

Qualitative analysis of phospholipids from erythrocytes was carried out by two-dimensional thin-layer chromatography (TLC) on pre-coated silica gel plates supplied by Merck (Fig. 1). The solvent used for the first dimension was chloroform–methanol–light petroleum (boiling range 50–75°)–acetone (8:8:6:1), while acetone–chloroform–methanol–acetic acid–water (8:6:2:2:1) was used for the second dimension. The separation was carried out in solvent-saturated chambers. The spots were detected by spraying with Phospray (Supelco).

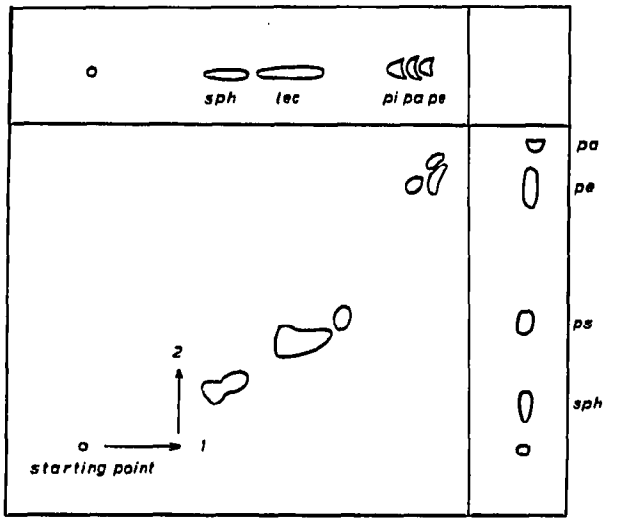


Fig. 1. Two-dimensional separation of a phospholipid extract from human erythrocytes. Solvents: chloroform–methanol–light petroleum (boiling range 50–75°)–water (8:8:6:1) in the first dimension and acetone–chloroform–methanol–acetic acid–water (8:6:2:2:1) in the second dimension.

In agreement with the results reported by other workers, we identified the following phospholipids: sphingomyelin, lecithin, phosphatidylserine, phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol.

For the direct quantitative determination of the phospholipids on the plate, sufficient separation in one row must first be achieved, and the fact that fluorimetric procedures are superior to absorptiometric procedures must be taken into account. It seemed therefore useful to develop a method that produces fluorescent phospholipid spots.

FLUORESCENCE METHOD

We have devised a simple yet very efficient procedure. After developing, the plate was air-dried, sprayed with concentrated sulphuric acid–ether (1:19) and heated for 10 min at 100° in a drying chamber. When exposed to ultraviolet light, the phospholipids treated in this way exhibit blue fluorescence.

One of the advantages of this method is that after spraying the plate with

* Abbreviations used: sph = sphingomyelin, ps = phosphatidylserine, pe = phospatidylethanolamine, lec = lecithin, pi = phosphatidylinositol, pa = phosphatidic acid.

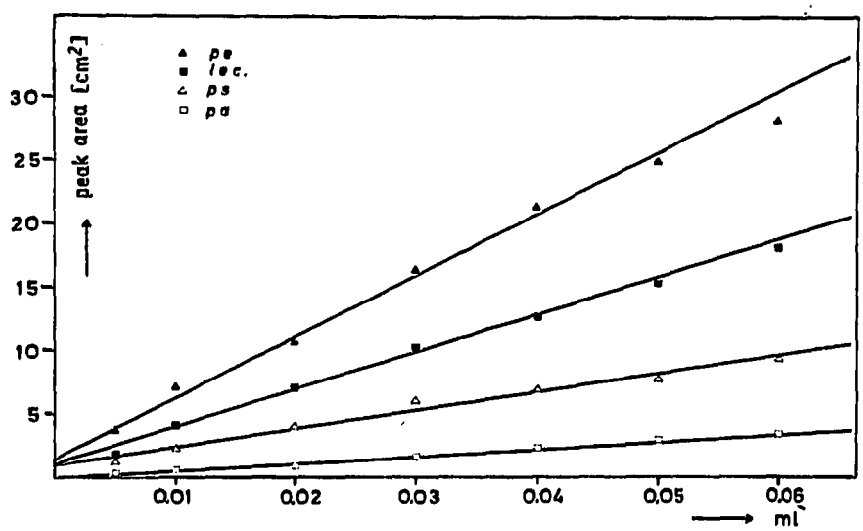


Fig. 2. Linear relationship between amount of phospholipid and peak area.

sulphuric acid–ether and heating, the phospholipid spots can still be quantitatively evaluated even after several months. Thereafter we checked whether or not the amount of lipid on the plate was proportional to the fluorescence produced. Fig. 2 shows that this indeed is the case. For these experiments, different amounts of the erythrocyte phospholipid extract were applied on to a Merck silica gel plate and were separated by using conditions that are specified later. In evaluating the fluorescence produced by the various spots, a Camag Z-scanner was used together with a Zeiss PMQ II spectrophotometer and a xenon high-pressure lamp. For activating, light was filtered through a 360 nm filter, while fluorescence was measured at 400 nm. The lipids were present in amounts ranging from 10^{-3} to 10^{-6} g.

Fig. 3 shows similar results for a mixture of pure lipids supplied by Supelco

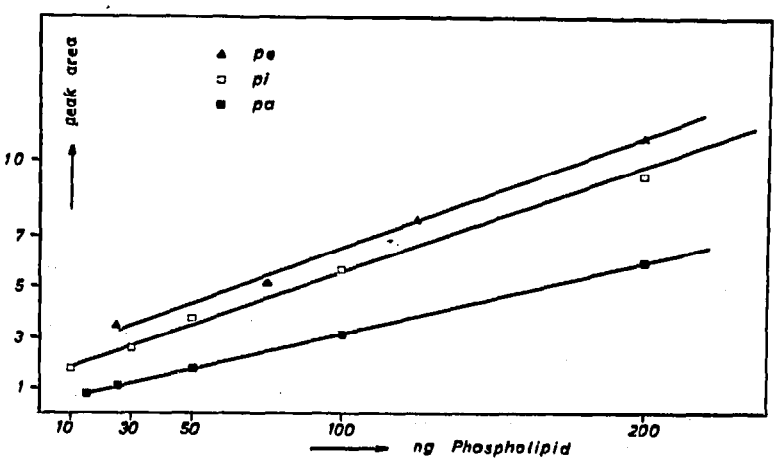


Fig. 3. Linear relationship between amount of phospholipid and peak area.

used in extremely small amounts. It can be seen that the lowest detection levels for the different phospholipids vary. For phosphatidylinositol, this level is 10 ng.

ONE-DIMENSIONAL SEPARATION OF PHOSPHOLIPIDS

When separating phospholipids by TLC, it is important to observe strictly defined conditions, the effect of separation depending to a great extent on the amount of moisture in the air. For this reason, we always used TLC plates that had been pre-treated with H_2SO_4–H_2O for 1 h, obtaining a special atmospheric humidity. The optimum atmospheric humidity was determined by varying the sulphuric acid concentration in a Vario KS chamber designed by Geiss and Schlitt. The effect of relative humidity on separations is shown in Fig. 4.

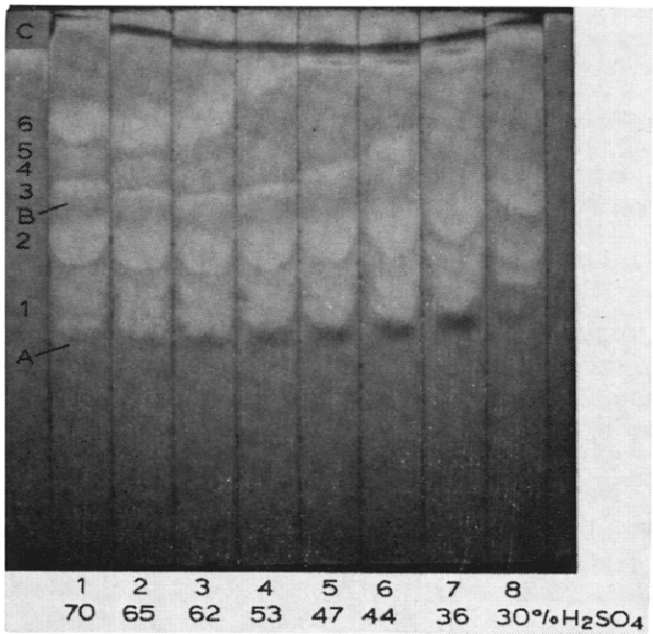


Fig. 4. Effect of varying relative humidity on separation. Solvent: chloroform–methanol–light petroleum (boiling range: 50–75°)–water (8:8:6:1).

Equal amounts of lipids extracted from erythrocytes were spotted on to a Merck silica gel plate. For all rows, the same solvent, light petroleum–chloroform–methanol–water (6:8:8:1), was used. Separations were also performed in a Vario KS chamber. The effect of relative humidity on the quality of separation is crucial. The first row was conditioned with 70% sulphuric acid, while 30% sulphuric acid was used for the eighth row, which did not yield a satisfactory separation. It can be seen that at high atmospheric humidity the separation of phospholipids is difficult to achieve without prior conditioning of the plate with sulphuric acid.

The upper non-fluorescent spot consists mainly of cholesterol (C). Of the phospholipids, phosphatidylethanolamine (6) moves the fastest. The two smaller spots

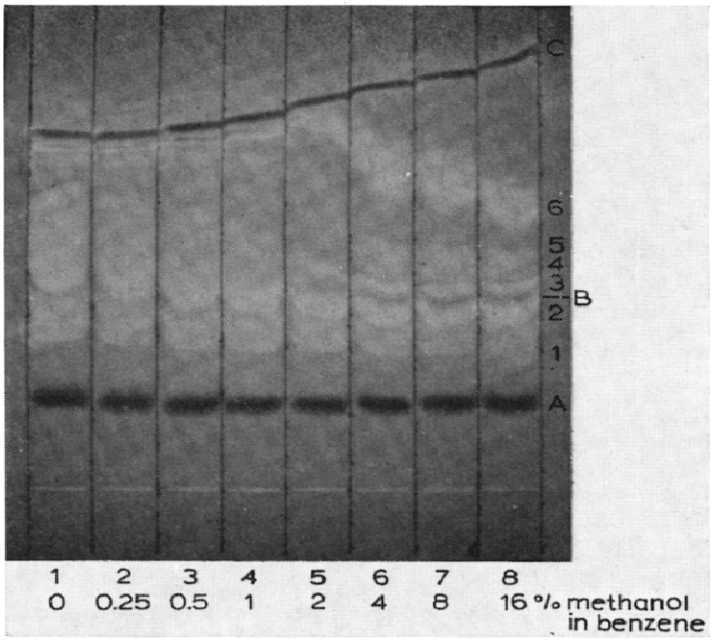


Fig. 5. Effect of pre-conditioning with methanol–benzene (plates previously pre-treated with 1:1 H_2SO_4–H_2O). Solvent: chloroform–methanol–light petroleum (boiling range 50–75°)–water (8:8:6:1).

are phosphatidylinositol (5) and phosphatidic acid (4). The separation of phosphatidylserine (3), lecithin (2) and sphingomyelin (1) is difficult to achieve. Two other non-fluorescent spots, A and B, occur, which are not caused by phospholipids and we therefore did not identify them.

When phospholipids received from the membrane preparations described later are applied to the plate, a thick spot appears at point A due to sucrose resulting from the solution in which the membranes had been suspended. For a direct quantitative evaluation, it is imperative to separate this spot from those of the phospholipids. For this purpose, the plate, pre-conditioned with concentrated H_2SO_4–water (1:1), was exposed to the vapour of different benzene–methanol mixtures for 5 min, and the results are shown in Fig. 5. In order to establish the optimum conditions, the methanol content of these mixtures was varied between 0% (row 1) and 16% (row 8). All of the rows show that the sucrose spot has definitely been separated, and the quality of the separation also depended on pre-conditioning with methanol–benzene.

Strikingly, sphingomyelin always occurs in two spots when the extract is applied in the form of bands. The sphingomyelin component, which moves more slowly, contains α-hydroxy fatty acids in its molecule, while the faster moving component contains saturated and monoenic fatty acids.

Figs. 6–8 show scanner recordings of the separations obtained with benzene–methanol mixtures of different ratios. The ratio of H_2SO_4–H_2O used for pre-conditioning was 1:1 in all instances.

Fig. 6 shows the results with benzene containing 2% of methanol. The first peak after the origin is spot A, which shortly after spraying with sulphuric acid–ether

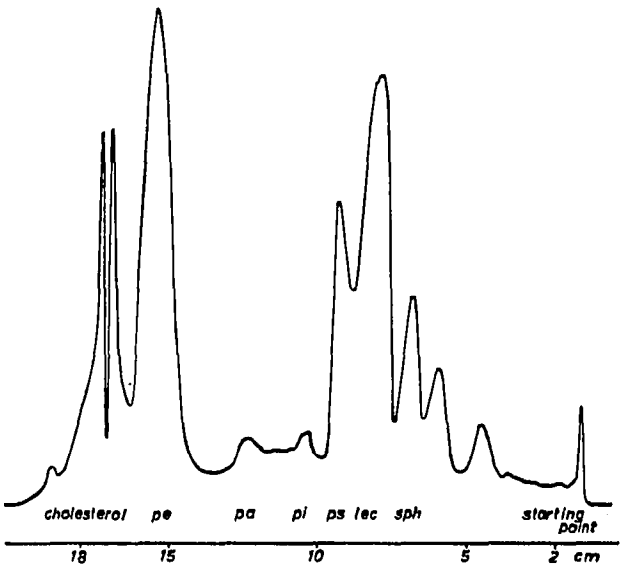


Fig. 6. Scanner recording of phospholipids separated on plates pre-treated with 98:2 benzene–methanol (plates previously pre-treated with 1:1 H_2SO_4–H_2O).

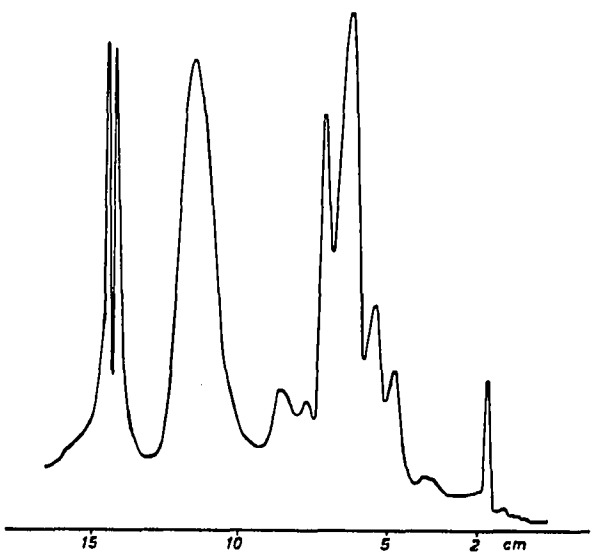


Fig. 7. Scanner recording of phospholipids separated on plates pre-treated with 92:8 benzene–methanol (plates previously pre-treated with 1:1 H_2SO_4–H_2O).

and heating always shows weak fluorescence. This peak is followed by the two bands of sphingomyelin and the lecithin peak. With this benzene–methanol ratio, phosphatidylserine and lecithin are poorly separated. The next peak is caused by phosphatidylinositol, which is followed by the phosphatidic acid and the phosphatidyl-

ethanolamine peaks. The fluorescence peak at the end, with an absorption peak embedded in it, is caused by cholesterol.

Fig. 7 shows that treatment with 8% of methanol in benzene causes the peaks to close up somewhat. Sphingomyelin is less well separated, while phosphatidylserine and phosphatidylethanolamine are better separated. For phosphatidylserine, the most favourable results were obtained by using 16% of methanol in benzene (Fig. 8).

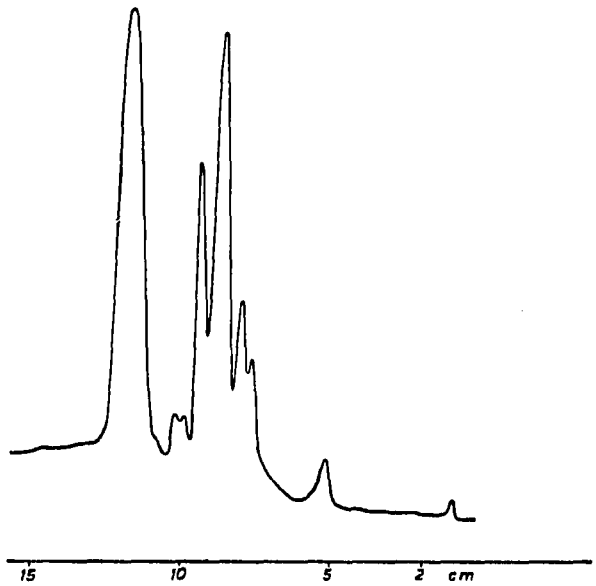


Fig. 8. Scanner recording of phospholipids separated on plates pre-treated with 84:16 benzene–methanol (plates previously pre-treated with 1:1 H_2SO_4–H_2O).

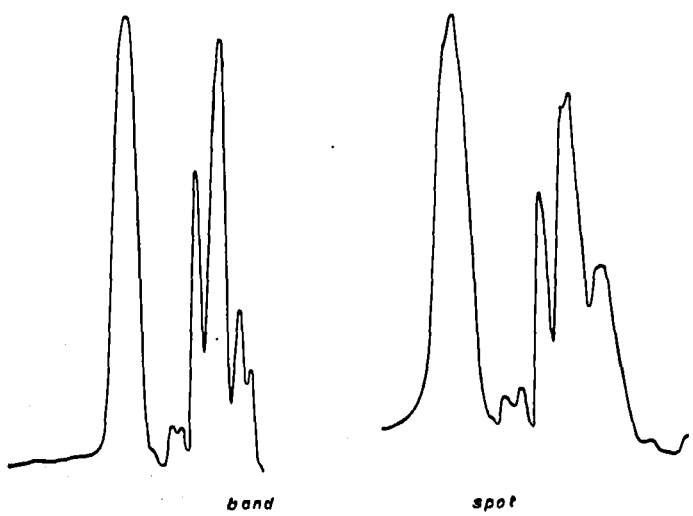


Fig. 9. Comparison between two different methods of application (band and spot). Separations were performed under identical conditions.

In all instances, the erythrocyte extract was applied in the form of bands by using the Camag Linomat. For quantitative determinations, it is generally preferable to use a mechanical device for application, as application by hand generally constitutes the major source of error.

Fig. 9 shows that the quality of separation is also better when the sample is applied in the form of bands by using the Linomat, because when the sample was applied in the form of spots, the two bands of sphingomyelin could never be observed under the experimental conditions used. In performing the quantitative determinations of the phospholipids, we therefore always applied the samples in the form of bands and exposed the plates to the vapour of a methanol–benzene mixture containing 2–16% of methanol. The methanol content depended on the phospholipids to be determined.

To summarize so far, the first improvement in the determination of phospholipids consists in using the fluorescent method. By using this method, we were able to extend the lowest detection level to 10^{-8} g. The second improvement consists in pre-conditioning the plates, which results in a one-dimensional separation of phospholipids, which is necessary for scanning the spots.

For quantitative determinations of phospholipids from human erythrocytes, we always carried out reference runs of phospholipids of known concentrations. After developing and treating the plates with sulphuric acid–ether, the spots were scanned and the phospholipids were then determined by relating the fluorescence produced by the different spots to the fluorescence produced by the reference spots.

We should emphasize that quantitative determinations can be achieved in any case by simply determining the peak area (height $\times$ width at half-height) even if single peaks are not completely separated.

PHOSPHOLIPID CONTENT OF HUMAN ERYTHROCYTES

Table I shows our results for lipid analysis compared with results reported by other workers[1–5]. It can be seen that there are some discrepancies that cannot easily be explained, but it seems likely that they are due to the different methods used for extracting and determining the lipids.

RELATIONSHIP BETWEEN PHOSPHOLIPIDS AND ATPases

In the Institut für Biochemie in Mainz we work on ATPases of human erythrocyte membranes. We used the present method for determining phospholipids in order to obtain new data on the phospholipid requirement of ATPases.

At least two ATPase systems exist: Na,K-ATPase and Ca-ATPase. Mg-ATP is the substrate of both enzyme systems and is split into Mg-ADP and P_i. The difference between both enzyme systems is mainly established by the varying extent of activation caused by monovalent and divalent cations and their response to ouabain and other cardiac glycosides, respectively. In particular, Na,K-ATPase is believed to participate in the active transport of Na and K across membranes[6,7], while the function of Ca-ATPase is at present much less understood. As stated by Schatzmann[8], however, it is possibly involved in the active transport of Ca. Up to the present, erythrocyte ATPases can only be isolated bound to membranes.

TABLE I

PHOSPHOLIPID CONTENTS (%) OF HUMAN ERYTHROCYTES

Phospholipid	*Reference*					
	1	*3*	*4*	*2*	*5*	*Our results*
Lcc	31.4	37.5	30.7	36.2	28.3	38.5
Pe	25.8	30.2	26.7	28.4	26.0	30.2
Sph	23.0	21.9	24.7	28.9	24.6	19.6
Ps	15.6	10.4	15.9	4.0	13.4	10.0
Pa	NR*	NR	NR	NR	2.1	0.8
Pi	4.2	NR	2.3	2.5	1.1	0.7

* NR = Not reported.

Several workers have reported that phospholipids are essential for Na,K-ATPase activity[9–14], while similar experiments with Ca-ATPase have not yet been performed. Upon complete removal of phospholipids from the membrane, irreversible inactivation of Na,K-ATPase is observed, while inactivation achieved by the partial removal of phospholipids can be reversed to a certain extent by the addition of phospholipids[14].

Similar results have been obtained by many other workers, *e.g.*, Taniguchi and Ilda[10]. They treated an Na,K-ATPase preparation with phospholipase A and, by adding phosphatidylserine and phosphatidylinositol, were able to restore the activity lost by the former treatment.

In investigating the effect of phospholipids on Na,K-ATPase and especially on Ca-ATPase, we chose an alternative approach. The choice of this method was based on the following considerations. The products formed by the action of phospholipase A still remain within the membrane and might possibly exert some effect there. Moreover, the organic solvents frequently used by other workers for extracting phospholipids always cause some denaturation of membrane proteins.

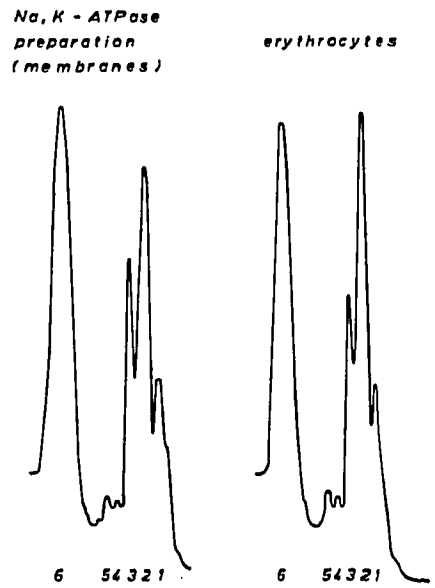
We therefore tried to obtain ATPase preparations with high activity by using as mild experimental conditions as possible. Provided that these preparations were sufficiently pure, their phospholipid composition would be expected to differ from that of the erythrocytes used, being characteristic for the ATPases.

Our method enabled phospholipids to be determined even from very small amounts of ATPase preparations compared with the erythrocyte extract in reference runs. The phospholipid extraction of ATPase preparations was carried out in the same way as described for the erythrocytes. The preparation of membranes with either high Na,K-ATPase or high Ca-ATPase activity was carried out by haemolysis of human erythrocytes and washing the ghosts under optimum conditions for Na,K-ATPase and Ca-ATPase, respectively. We have already described these procedures[15,16]. We particularly emphasize the use of deoxycholate (DOC) for Na,K-ATPase preparation. This compound removes phospholipids from the membranes so that Na,K-ATPase is inactivated[17]. The treating of the membrane with very low DOC concentrations yields a preparation with high Na,K-ATPase activity, while the Ca-ATPase activity, which is always associated with Na,K-ATPase activity, is reduced. It is possible that low DOC concentrations cause the different phospholipids to be removed to different extents, *i.e.*, that phospholipids essential for Na,K-ATPase

activity remain within the membrane, while inhibitory or inert phospholipids are removed.

PHOSPHOLIPID CONTENT OF A Na,K-ATPase PREPARATION

Fig. 10 shows that the the lipid compositions of the erythrocytes and the enzyme preparation are different. The lecithin and sphingomyelin contents have been decreased, while the phosphatidylserine and especially the phosphatidylethanolamine contents have increased.



	membranes	erythrocytes		membranes	erythrocytes
1 sph	12.4	19.6	4 pi	1.1	0.7
2 lec	22.9	38.5	5 pa	1.1	0.8
3 ps	13.7	10.0	6 pe	48.7	30.2

Fig. 10. Phospholipid content of erythrocytes in comparison to a membrane preparation rich in Na,K-ATPase activity.

PHOSPHOLIPID CONTENT OF Ca-ATPase PREPARATIONS

To obtain a Ca-ATPase preparation, we used Tween 20 instead of DOC and also used different ionic concentrations and a different pH from those used in the Na,K-ATPase preparation. When performing the standard procedure, the temperature was strictly controlled and maintained between 0 and 4°.

In order to check whether the temperature exerts some influence on the quality of the preparation, we made a Ca-ATPase preparation in such a way that during a centrifugation step the temperature increased to 18° for a period of 30 min (preparation II). Interestingly, fundamental differences with regard to the phospholipid composition can be observed with both preparations (Fig. 11), as was found by Arnold[22].

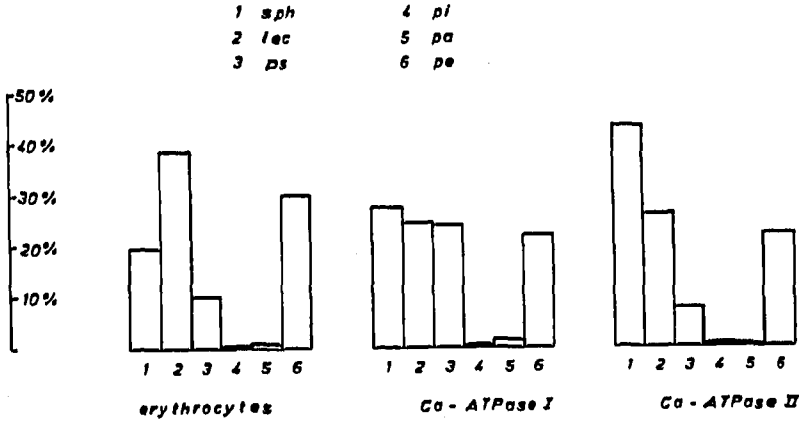


Fig. 11. Phospholipid content of erythrocytes and two different Ca-ATPase preparations.

Standard procedure I yields preparation I, which, with regard to the total phospholipid content, contains more sphingomyelin than the erythrocytes. In preparation II, the sphingomyelin content is even higher. On the other hand, the phosphatidylserine content of I is higher than that of II. We checked whether these significant differences in phospholipid composition also resulted in different kinetic behaviour, and it was found that the Lineweaver-Burk plots differ with both preparations (Fig. 12). Measurement was performed at 20°.

The conditions used in procedure I, with a temperature of 0°, are much milder,

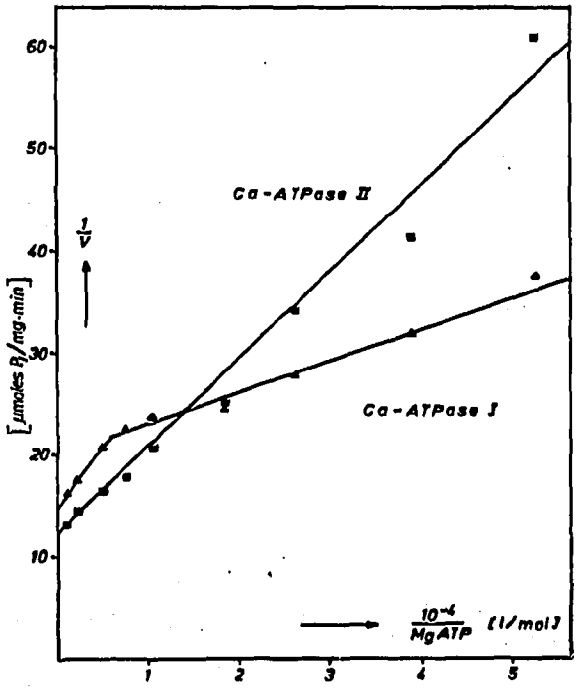


Fig. 12. Lineweaver-Burk plots of two different Ca-ATPase preparations.

of course, than those used in procedure II (18°). Therefore, the bend observed in the Lineweaver-Burk plot would be expected to characterize the normal behaviour of the enzyme, but the interpretation of this bend has to be considered. It might be that Ca-ATPase is composed of two different enzymes with extremely different affinities for ATP. An alternative explanation is offered by a substrate-induced conformational change in which the phospholipids are involved. The fact that this bend is missing for the Lineweaver-Burk plot of preparation II seems to support the assumption that either one of the two enzymes, with a different phospholipid composition, suspected to be present has been destroyed or removed, or because the absence of these phospholipids made a conformational change no longer possible.

A FIBRILLAR COMPONENT AS PART OF THE MEMBRANE

The above results caused us to try to isolate at least one of the two Ca-ATPases

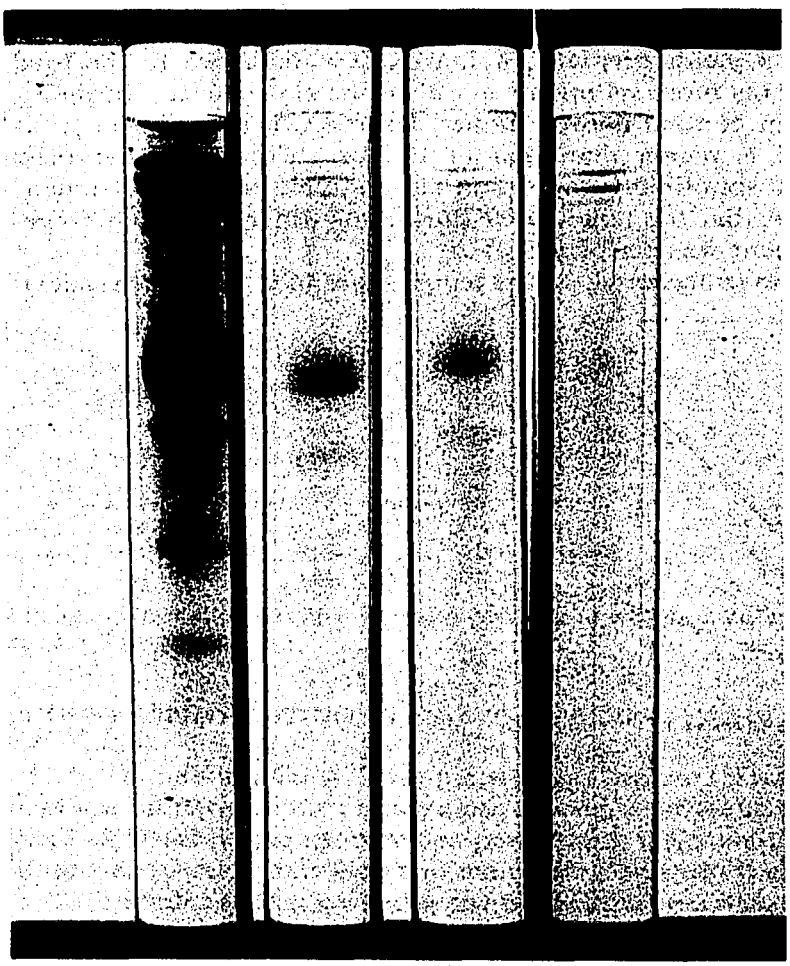


Fig. 13. Sodium dodecyl sulphate-gel electrophoresis according to the method of Weber and Osborn[21] of the three bands obtained by sucrose density gradient centrifugation of the membrane preparation. The first tube shows the patterns of the starting material.

possibly present by further degradation of the membranes. This treatment was carried out using as mild experimental conditions as possible by changing the ionic strength in the presence of all ions known to be effectors of Ca-ATPase. The enzyme preparation was treated at 20° with a solution containing 1 m*M* Mg^{2+}, 1 m*M* Mg-ATP^{2-}, 0.1 m*M* Ca^{2+} and 1 m*M* Tris (pH 8.1) and then centrifuged at 20,000 × *g*. This treatment was repeated with a solution of the same composition but containing in addition 800 m*M* Na^{+}. Analyses proved that phospholipids were split off during these treatments. The residue was subjected to sucrose gradient centrifugation with a gradient containing 150 m*M* Na^{+}, 2 m*M* Mg-ATP^{2-}, 2 m*M* Mg^{2+}, 0.1 m*M* Ca^{2+} and 10 m*M* Tris (pH 8.1). After centrifuging for 3 h at 126,000 × *g*, three bands appeared, the phospholipid and protein contents of which were investigated.

Protein determinations were performed by polyacrylamide gel electrophoresis and the results are shown in Fig. 13.

The characteristic double band associated with fraction No. 3 indicates that this fraction is probably composed of "spectrin", which has been described by Marchesi and co-workers[18,19]. Fig. 14 shows an electron micrograph of this fraction. It consists of fibrillar components with a length of about 1 μm and a diameter of 4–5 nm. So far, it has not been possible to isolate fibrils that exhibit these properties by this method. The two other fractions did not contain fibrils.

It now seems desirable to answer the question of whether the three fractions contain any Ca-ATPase activity and what their phospholipid composition is. Figs. 15–17 show the results of the phospholipid analyses of these three fractions. Interestingly, the fibrils fraction also contains phospholipids, which is in contrast to the

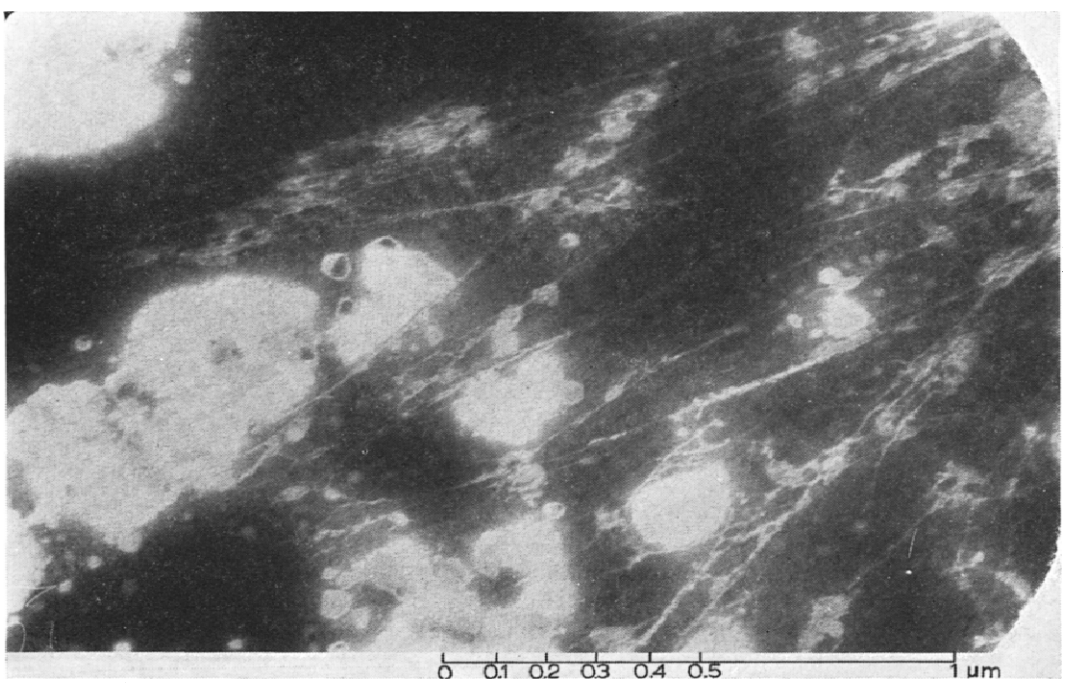
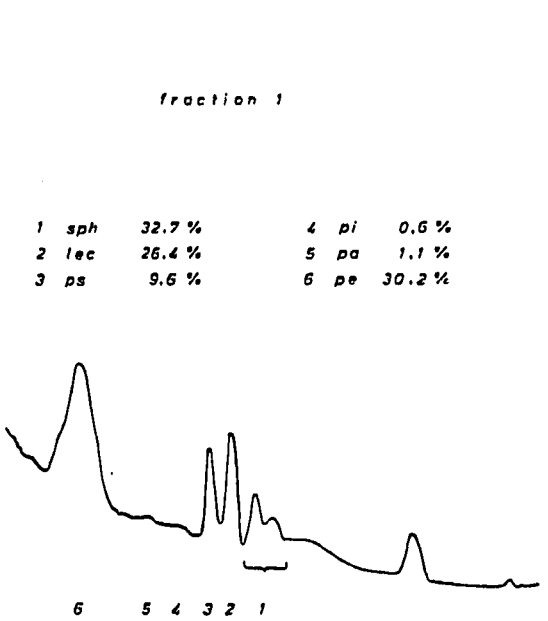


Fig. 14. Fibrillar proteins obtained from the third band of the sucrose density gradient centrifugation of the membrane preparation.



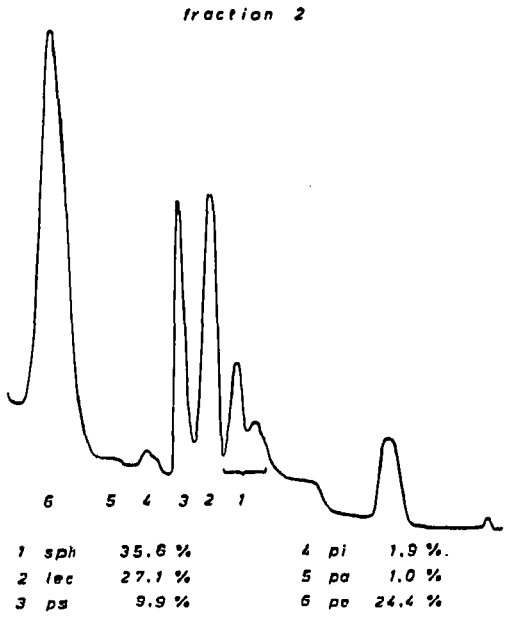


Fig. 15. Phospholipid analysis of the first of the three fractions obtained from the sucrose density gradient centrifugation of the membrane preparation.

Fig. 16. Phospholipid analysis of the second of the three fractions obtained from the sucrose density gradient centrifugation of the membrane preparation.

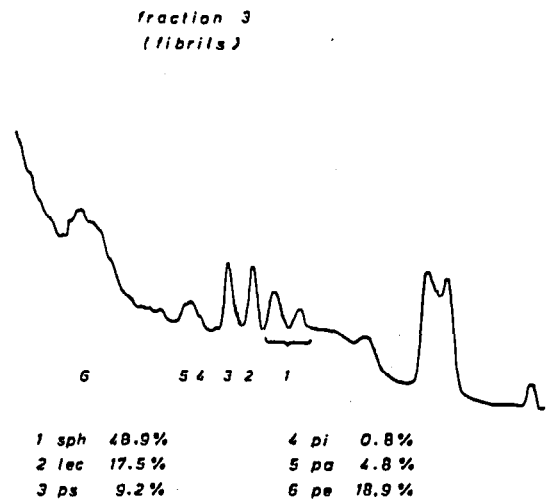


Fig. 17. Phospholipid analysis of the third of the three fractions obtained from the sucrose density gradient centrifugation of the membrane preparation.

results reported by other workers[19]. The phospholipid contents of the three fractions are shown in Fig. 18. Within the limits of experimental error, the phosphatidylserine content is constant in all three fractions. Compared with the total phospholipid content, the sphingomyelin content continuously increases from fraction 1 to 3, while the

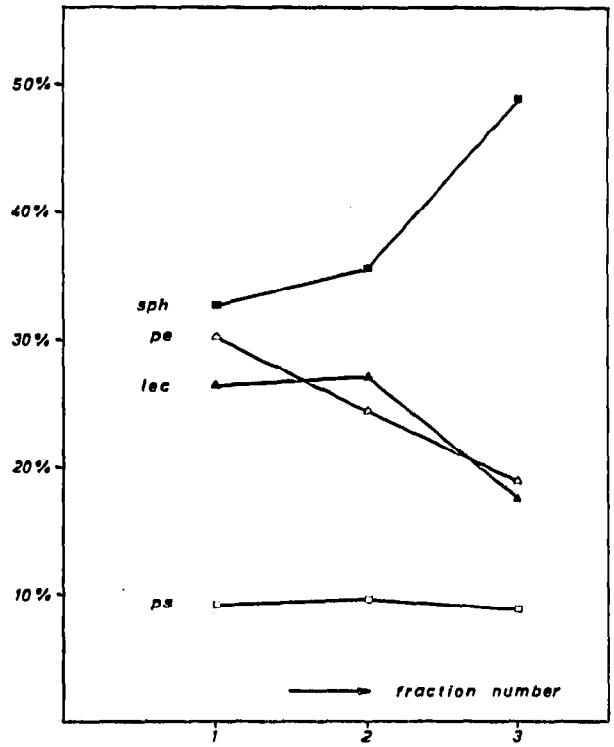


Fig. 18. Relative amounts of the four major phospholipids of the three fractions obtained from the sucrose density gradient centrifugation of the membrane preparation.

phosphatidylethanolamine content steadily decreases. The phospholipid composition of fraction 3, containing fibrils, corresponds approximately to the phospholipid composition of the Ca-ATPase ghost preparation (II), which did not show a bend in the Lineweaver-Burk plot.

Despite of the fact that the starting material for density gradient centrifugation exhibited very high Ca-ATPase activity, the first two fractions did not show any Ca-ATPase activity, while the third fraction, containing the fibrils, showed very low Ca-ATPase activity. This loss of activity is probably due to the removal of phospholipids from the membrane.

There are some clues for our assumption that the fibrils represent one of the Ca-ATPases, as follows.

(1) The phospholipid composition of the fibrils (the percentage of the individual phospholipids) agrees with that of one of our high-activity ghost preparations.

(2) For a long period of time, the fibrils are stable only in a medium that contains the same ions acting as Ca-ATPase effectors, which means that the specific binding sites of the fibrillar protein and of Ca-ATPase are probably identical.

(3) Williams has shown that after labelling bovine erythrocyte Ca-ATPase with [^{32}P]ATP, radioactivity was associated with one of the two spectrin peaks after polyacrylamide gel electrophoresis[20].

In our opinion, therefore, these fibrils, possibly containing Ca-ATPase

activity, might represent the contractile protein of erythrocytes, in a similar manner to the role of myosin ATPase in muscle.

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REFERENCES

1 C. F. Reed, S. N. Swisher, G. V. Marinetti and E. G. Eden, *J. Biol. Chem.*, 235 (1960) 1796.
2 H. J. Williams, M. Kuchmak and R. F. Witter, *Lipids*, 1 (1966) 391.
3 J. W. Farquhar, *Biochim. Biophys. Acta*, 60 (1962) 80.
4 P. Ways and D. J. Hanahan, *J. Lipid Res.*, 5 (1964) 318.
5 J. C. Turner and G. Rouser, *Anal. Biochem.*, 38 (1970) 423.
6 R. L. Post, C. R. Merritt, C. R. Kinsolving and C. D. Albright, *J. Biol. Chem.*, 235 (1960) 1796.
7 J. C. Skou, *Biochim. Biophys. Acta*, 23 (1957) 394.
8 H. J. Schatzmann, *Protides Biol. Fluids, Proc. 15th Colloq. Bruges*, 1966, 15 (1967) 251.
9 K. P. Wheeler and R. Whittam, *J. Physiol.*, 207 (1970) 303.
10 K. Taniguchi and S. Ilda, *Biochim. Biophys. Acta*, 274 (1972) 536.
11 A. Pitotti, A. R. Contessa, F. Dabbeni-Sala and A. Bruni, *Biochim. Biophys. Acta*, 274 (1972) 528.
12 T. Ohnishi and H. Kawamura, *J. Biochem.*, 56 (1964) 377.
13 R. Tanaka and L. G. Abood, *Arch. Biochem. Biophys.*, 108 (1964) 47.
14 J. C. Skou, *FEBS, 8th Meeting, Amsterdam*, 1972, Vol. 28, North-Holland, Amsterdam and Elsevier, New York, 1972, p. 339.
15 H. U. Wolf, *Biochim. Biophys. Acta*, 219 (1970) 521.
16 H. W. Peter and H. U. Wolf, *Biochim. Biophys. Acta*, 290 (1972) 300.
17 J. Philippot, *Helv. Physiol. Acta*, 429 (1967) CR429.
18 V. T. Marchesi and G. E. Palade, *Proc. Nat. Acad. Sci. U.S.*, 58 (1967) 991.
19 S. L. Marchesi, E. Steers, V. T. Marchesi and T. W. Tillack, *Biochemistry*, 9 (1970) 50.
20 R. O. Williams, *Biochem. Biophys. Res. Commun.*, 47 (1972) 671.
21 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
22 A. Arnold, private communication, 1972.