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PHOSPHORYLATION AND DEPHOSPHORYLATION OF MEMBRANE PROTEINS AS A POSSIBLE MECHANISM FOR STRUCTURAL REARRANGEMENT OF MEMBRANE COMPONENTS*

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SUMMARY

A correlation was found between dephosphorylation of chicken erythrocyte membrane proteins, aggregation of intramembrane particles, increase in the lipid bilayer phase of the membrane and exposure of membrane phospholipids toward phospholipase A and trinitrobenzene sulfonic acid. Most of the covalently bound phosphate of the membrane proteins turns over and is associated with 5 major bands. It is suggested that phosphorylation and dephosphorylation of these proteins causes changes in their charge and conformation. Such changes might affect the interaction of these proteins with the neighbouring lipids or lipoprotein complexes and results in the aggregation of intramembrane particles and relative increase in the exposed free lipid bilayer phase of the membrane.

INTRODUCTION

In a previous work it has been demonstrated that in several species a fraction of intact erythrocyte membrane phospholipids is not available to phospholipase C (*Bacillus cereus*) activity. The degree of protection found in different species appears to be a function of the relative content of sphingomyelin and the presence of a certain type of membrane organization, which was found to be correlated with the ATP content of the cells [1, 2]. Examination of freeze fractured membranes of rat erythrocytes has shown that upon ATP-depletion, intramembrane particles become aggregated with concomitant increase in the relative extent of free lipid bilayer area and susceptibility toward phospholipase C [2]. Based on these findings it was suggested that the association between lipids and proteins within the membrane of intact cells, prevents the hydrolysis of part of the lipids by phospholipase C (*B. cereus*, 2). Deple-

Abbreviation: TNBS, trinitrobenzene sulfonic acid.

* Preliminary results of this work were presented at the annual meeting of the Israeli Biochemical Society (January 1975) [30].

tion of ATP might induce changes in the phosphorylation of membrane protein, thereby affecting this interaction.

The interpretation of the data obtained with phospholipase C encounters some difficulties, since upon its action on the membrane phospholipids, the cells lyse and the enzyme might penetrate and attack lipids also present on the inner side of the membrane. In order to avoid this complication in the present work, the effect of ATP-depletion on exposure of phospholipids was studied, using phospholipase A_2 and trinitrobenzene sulfonic acid. As opposed to phospholipase C, the activity of these reagents does not induce singificant lysis of intact cells [3–6]. The results support the concept that a phosphorylation-dependent protein-lipid interaction might play a role in the exposure or masking of membrane phospholipids towards these agents.

MATERIALS AND METHODS

The medium for cell handling and enzymatic treatment (Solnk) contained 130 mM KCl/5.4 mM NaCl/0.8 mM $MgCl_2$ in 30 mM Tris · HCl buffer (pH 7.4).

Erythrocytes were collected from the neck of decapitated chickens into an Erlenmeyer containing heparin (100 U/ml blood). The cells were washed (3 times) in Solnk and the buffy coat was carefully removed.

Enzymatic treatment. Phospholipase A_2 obtained from Sigma (bee venom) was added (final concentration of 4 IU/ml) to a suspension of cells in Solnk (10 % v/v), containing 1 mM $CaCl_2$. Incubation was carried out at 37 °C with gentle shaking. The reaction was stopped by dilution (3 times) with cold Solnk, followed by centrifugation at $10\,000 \times g \times 10$ min at 4 °C. The supernatant was used for measuring the degree of hemolysis [7], and the pellet was resuspended in 3–6 vol. of Solnk. The phospholipids were then extracted according to Burger et al. [8]. The extracted phospholipids were finally dissolved in 1 ml chloroform/methanol (2 : 1 v/v) and the phospholipid phosphorous was determined according to Bartlett [9]. Treatment with sphingomyelinase (Sphase, *Staphylococcus aureus* 0.1 IU/ml of 10 % v/v cells) was carried out as described [1]. Phospholipid separation and analysis by two-dimensional thin-layer chromatography was carried out as reported previously [1].

Treatment with trinitrobenzene sulfonic acid. Trinitrobenzene sulfonic acid (TNBS, Pierce Chemicals, U.S.A.) was added to reaction mixtures containing 5 ml of cells (5 % v/v) suspended in Solnk in which the Tris buffer was replaced by 20 mM phosphate buffer (pH 8.0). Incubation was carried out at 20 °C for periods of time as indicated. The reaction was stopped by dilution (5 times) and the cells were freed from unbound TNBS by washing 3 times with cold Solnk. The phospholipids were then extracted and analyzed as above. The phosphatidylethanolamine derivate of TNBS was clearly separated from unreacted phosphatidylethanolamine. Under the experimental conditions used, phosphatidylserine did not react with TNBS at pH 8.0, as demonstrated by use of lipid micelles containing lecithin and phosphatidylserine [5].

The reaction of phosphatidylethanolamine was determined from the proportion of reacted to unreacted phosphatidylethanolamine, as compared to the total amount of phosphatidylethanolamine in a non-treated system. The data from different experiments were compared, using the membrane lecithin as an internal standard.

Labeling of erythrocytes with $^{32}P_i$. For labeling of erythrocyte membranes with $^{32}P_i$ and determination of the radioactivity content of various membrane proteins,

120 ml of fresh chicken erythrocytes (5 % v/v) were washed and suspended in Solnk containing 5 mM glucose. The cells were incubated at 37 °C with gentle shaking. At zero-time, $^{32}P_i$ was added ($2.4 \cdot 10^4$ cpm/pmol) to give a final concentration of 0.01 mM potassium phosphate buffer. Aliquots of 0.5 ml were taken at intervals of 30 min. After 240 min incubation, KCN (1 mM) and NaF (10 mM) were added (depletion period). Aliquots were taken again after 2 and 4 h from the beginning of the ATP-depletion period.

The aliquots were washed with phosphate buffer (5 times) in the cold, to remove excess radioactivity and 0.2 ml of the washed cells were taken for ATP determination as described [7]. The remaining 0.3 ml were hemolyzed in a buffer containing 10 mM Tris · HCl (pH 7.4) and 5 mM $MgCl_2$ (hemolysis buffer). After hemolysis the cells were washed in hemolysis buffer (3 times) and freed of hemoglobin. The final pellet was suspended in 2 ml of 10 % cold trichloroacetic acid and incubated at 0 °C for 5 min. Thereafter the suspension was pelleted and the supernatant was discarded. The pellet was dissolved in 0.1 ml of 0.5 M NaOH and the solubilized material precipitated with 3 ml of 10 % cold trichloroacetic acid. The procedure was repeated 3 times. The final trichloroacetic acid precipitate was digested with 1 ml mixture of soluen (Packard) and isopropanol (v/v) at 50 °C for 2 h. The solution was transferred to scintillation vials and 0.1 ml of H_2O_2 (Merck) was added to bleach hemoglobin. The vials were counted after addition of Insta Gel (Packard) scintillation fluid and radioactivity of different samples was calculated using internal standards for quenching. The radioactivity of these aliquots was considered as ghost radioactivity.

Cell fractionation. Cell fractionation was carried out using 20 ml of the cell suspension taken from the incubation mixture after different periods of time from the onset of the experiment. A modification of the method described by Blanchet [10] was used. The samples were washed as above, concentrated to 30 % cells (v/v) and homogenized in a French press in the cold under nitrogen pressure of 500 lb/inch^2. The homogenate was immediately loaded on a discontinuous sucrose gradient of equal volumes (8 ml) of 0.5, 1 and 2 M sucrose in 10 mM Tris · HCl (pH 7.4) and centrifuged, using a Spinco SW 25 rotor at 25 000 rev./min for 30 min. The plasma membrane fraction was collected (about 50 % yield) from the interphase between 1 and 2 M sucrose. The pellet contained the nuclear fraction. The two fractions were washed (2 times) in hemolysis buffer and finally pelleted. Each fraction was dissolved in 4 % sodium dodecyl sulfate (Matheson Collman and Bell, U.S.A.) and the protein content was determined in the presence of 0.01 % of sodium dodecyl sulfate according to Lowry et al. [11].

Acrylamide gel electrophoresis. Acrylamide gels were prepared, run and stained with Coomassie brilliant blue R-250 (Serva) according to Fairbanks [12]. About 200 μg of protein was applied to each gel.

Electron microscopy. The plasma membrane and nuclear fraction were fixed and embedded for thin sectioning as previously described [13]. Preparation of erythrocytes for freeze etching was done as reported before [2]. Electron micrographs were taken with a Philips E.M. 300 electron microscope.

RESULTS

1. Effect of phospholipase A_2 on fresh and ATP-depleted chicken erythrocytes

Phospholipase A_2 has no effect on fresh cells, whereas in the case of ATP-

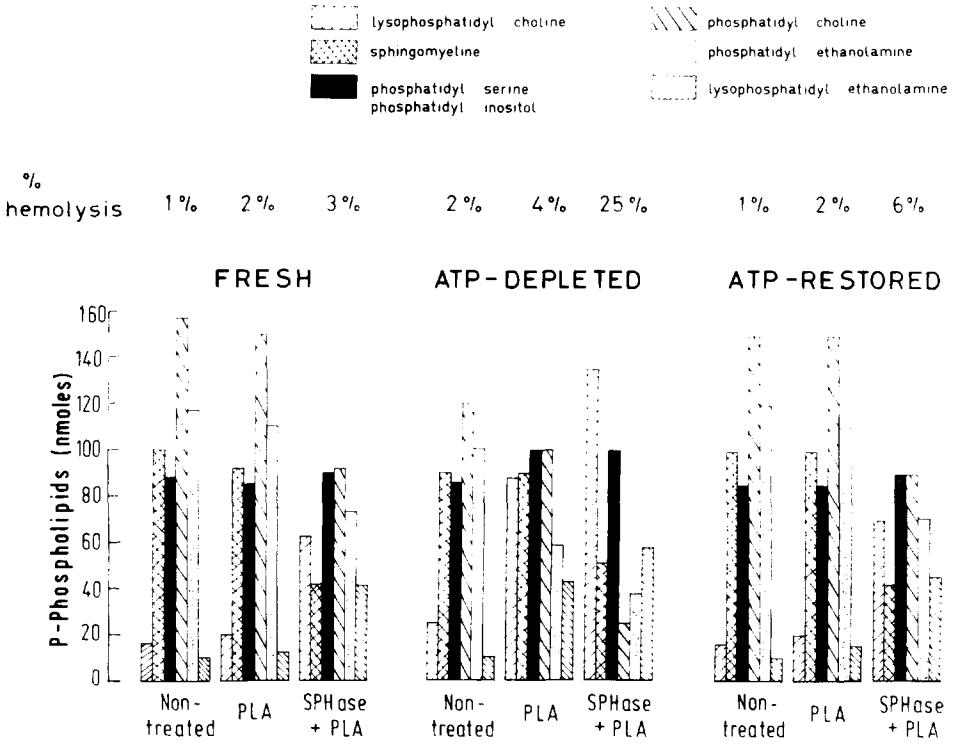

Fig. 1. Susceptibility of chicken erythrocytes toward phospholipase A_2 (bee venom) and sphingomyelinase, following ATP depletion and restoration. The enzymes were incubated with the different types of cells for 1 h as described in Methods. The phospholipids were extracted and dissolved in 1.0 ml chloroform/methanol (2:1 v/v) and 0.1 ml aliquots were applied on thin-layer chromatography plates. Phospholipid separation and phosphorous determination were carried out as described in Methods.

depleted cells incubation with this enzyme results in the hydrolysis of about 33–40 % of the total phosphatidylcholine and ethanolamine of the cell, without lysis. The hydrolysis of ester-bonds caused by phospholipase A_2 is accompanied by the appearance of lysocompounds within the cell membrane. Upon restoration of the ATP level of the cells, the effect of phospholipase A_2 and sphingomyelinase was reduced to that found in fresh cells (Fig. 1). Pretreatment of fresh cells with sphingomyelinase, which hydrolyzes about 50 % of the cell sphingomyelin enables phospholipase A_2 to hydrolyze about 40 % of the phosphatidylcholine and 20 % of the phosphatidylethanolamine of the membrane phospholipids, without causing lysis. Treatment of ATP-depleted cells with both enzymes, causes 25 % lysis and the degree of hydrolysis is increased to about 85 % for phosphatidylcholine and about 65 % for phosphatidylethanolamine. This effect is reversible upon restoration of ATP (Fig. 1).

2. Effect of ATP-depletion on exposure of membrane phosphatidylethanolamine to TNBS

As expected under the experimental conditions used, TNBS reacts with some of the membrane phospholipids, mainly with phosphatidylethanolamine [4–6]. The

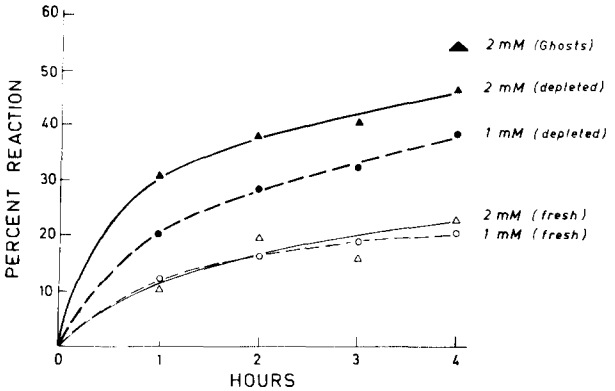

Fig. 2. Binding of TNBS to fresh and ATP-depleted chicken erythrocytes. The phospholipids were extracted and dissolved in 0.5 ml of chloroform/methanol (2:1 v/v); equal aliquots of 0.1 ml were applied on thin-layer chromatography plates. For further details see Methods.

reaction with fresh cells is completed within 2 h when using a concentration of 1 mM or 2 mM of TNBS. In this case about 10–15 % of the phosphatidylethanolamine have reacted without causing any lysis (Fig. 2). The reaction of TNBS with ATP-depleted cells shows basically similar kinetics, however, after 1 h of incubation with 2 mM of the reagent, which does not induce lysis, 20 % and 32 %, respectively, of the phosphatidylethanolamine have reacted. When 1 mM TNBS is used, the reaction with ATP-depleted cells continues at a lower rate after the first hour and after 4 h 35 % of the phosphatidylethanolamine has reacted without lysis (Fig. 2). After 4 h of incubation of ATP-depleted cells with 2 mM TNBS, less than 5 % lysis was detected.

3. Aggregation of intra-membrane particles upon ATP-depletion

Replicas of freeze-fractured plasma membranes of intact chicken erythrocytes show the usual two types of faces, an outer face consisting essentially of a flat surface studded with randomly dispersed particles and a fracture face heavily covered with numerous particles, leaving very small stretches of flat smooth areas between them. The outer surface of both fresh and ATP-depleted cells (Fig. 3) appears to be identical. The number of particles per μm^2 is 245 ± 30 and 300 ± 32, respectively. The smooth surface of the membrane is gently ridged with faint protrusions which appear to be slightly more pronounced in the ATP-depleted cells (Fig. 3b). The fracture face of both types of cells contains equal amounts of particles, their number being 3340 ± 270 and 3390 ± 255 per μm^2 membrane, respectively, in fresh and ATP-depleted cells. However, a clear difference is observed between the fresh and ATP-depleted cells with regard to the distribution pattern of these particles. In membranes of fresh cells the particles are loosely packed, leaving small, smooth membrane areas between them (Fig. 4a), whereas in ATP-depleted cells, the degree of aggregation and packing of intra-membrane particles is enhanced. The clusters are more tightly packed and accordingly the area of smooth membrane between them is increased (Fig. 4b). The change in the degree of packing and aggregation is clearly shown when the densitometer tracings of the two types of membranes are compared (Fig. 4). When the number of absorbance peaks representing single isolated particles on these densitograms are

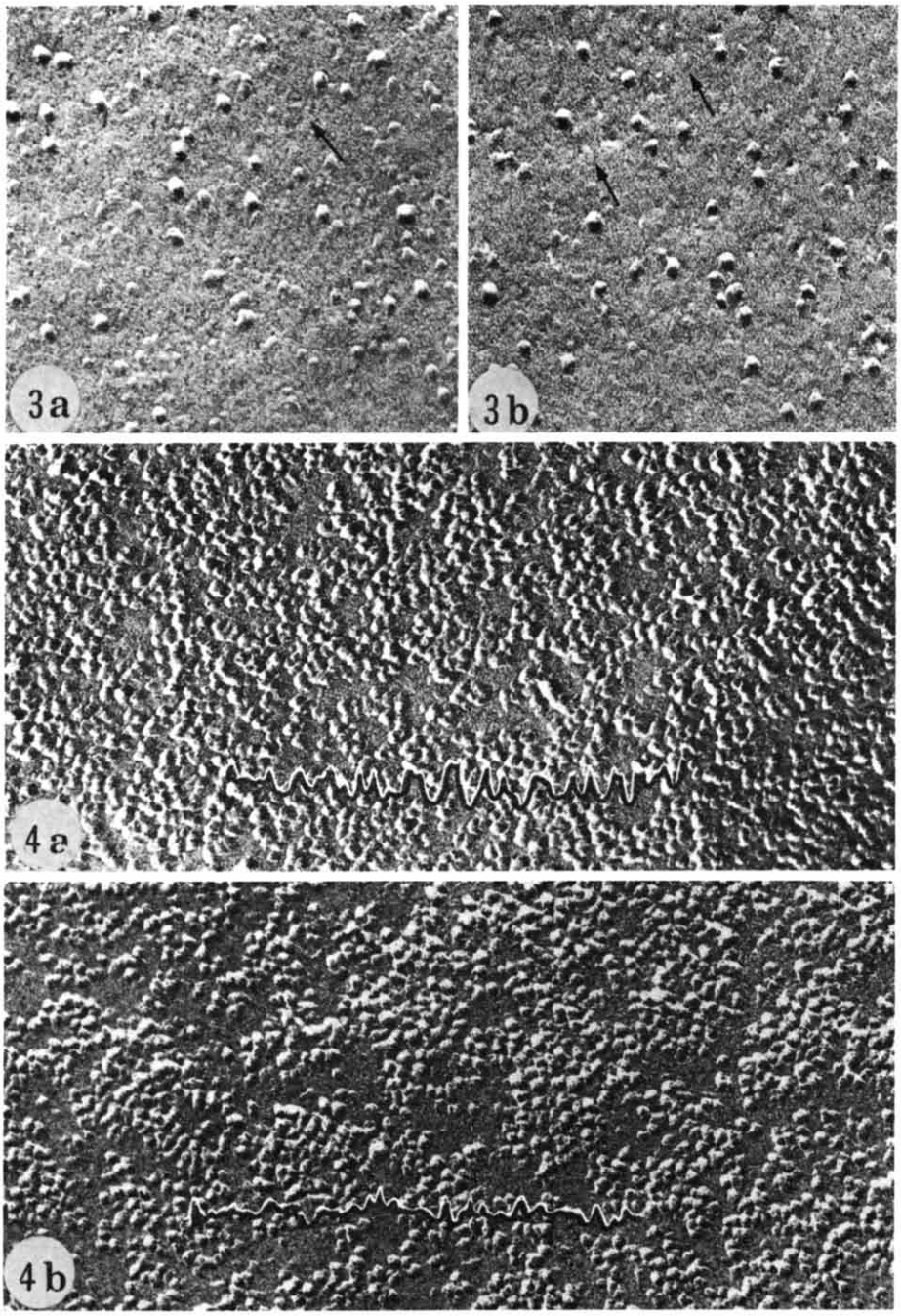

Fig. 3. Replicas of the membrane outer face of freeze-fractured chicken erythrocytes. Notice the random distribution of particles which are mostly free in both fresh (Fig. 3a) and ATP-depleted cells (Fig. 3b) and the slight protrusions of the flat, smooth surface between the particles which are better seen in the membranes of ATP-depleted cells (arrows, Fig. 3b). The depletion of ATP

TABLE I

NUMBER AND DISTRIBUTION OF MEMBRANE PARTICLES SEEN IN FREEZE-FRACTURED MEMBRANES OF FRESH AND ATP-DEPLETED CHICKEN ERYTHROCYTES

The numbers represent averages of measurements made on micrographs using at least 8 randomly-selected areas of 6 × 6 cm for the outer face and 16 areas of 1.5 × 1.5 cm for the fracture face. In all cases micrographs of a final magnification of × 150 000 were used. Densitometer tracings of negatives magnified × 75 000 of replica from fractured face, as shown in Fig. 4 were used for measurements of the distribution of single particles and average lengths of smooth areas.

	Particles/μm^2		Single particles/μm	Average length
	Outer face	Fracture face		of smooth area (Å)
Fresh cells	300 ± 32	3340 ± 270	33 ± 2	110 ± 15
ATP-depleted cells	245 ± 30	3390 ± 255	20 ± 2	240 ± 35

counted, one finds about 33 ± 2 single particles/1 μm actual length of membrane in fresh cells as compared with only 20 ± 2 single particles/1 μm actual length of membrane in ATP-depleted cells. The average length of the smooth areas between particles calculated from the same type of densitograms is 110 ± 15 Å for the fresh cells, and 240 ± 35 Å in ATP-depleted cells. The numerical values of the above-measured parameters are summarized in Table I.

4. Phosphorylation and dephosphorylation of erythrocyte membranes

When chicken erythrocytes were incubated with $^{32}P_i$ in the presence of glucose, a rapid increase in the specific radioactivity of the internal ATP was observed, which reached a plateau within 2.5 h of incubation (Fig. 5). Upon the addition of NaF and KCN, the ATP level of the cells was reduced to 2 % of the initial content after 4 h of incubation. At the same time the specific radioactivity of the residual ATP increased dramatically (Fig. 5).

To be able to find a correlation between the ATP level of the cell and phosphorylation of membrane components, it was necessary to obtain pure plasma membranes. Following the procedure described in Methods, it was possible to obtain a fraction consisting of plasma membrane vesicles, which are shown in Fig. 6. The membrane preparation (Fig. 6a) is free of whole nuclei or debris. The nuclear fraction (Fig. 6b) shows the presence of intact nuclei, some still contained within a closed membrane, residual from the original plasma membrane. Thus, it is possible to conclude that in this fractionating procedure, large parts of the plasma membrane were shed giving rise to pure plasma membrane vesicles.

was carried out for 8 h. For details see Methods. × 150 000.

Fig. 4. Replicas of membrane fracture face of chicken erythrocytes. Notice the loose packing and the presence of small areas of smooth membrane between the particles found in fresh cells (Fig. 4a), as compared with the tight packing and relative increase in smooth membrane area of ATP-depleted cells (Fig. 4b). The differences between fresh and ATP-depleted cells is also clearly visible in the densitometer tracings (inserts) which were obtained by scanning of 0.33 μm of actual length of membrane on negative micrographs (× 75 000). The sharp peaks on the densitometer tracing represent single particles or aggregates, while the flat part of the line represents the smooth area between particles [2].

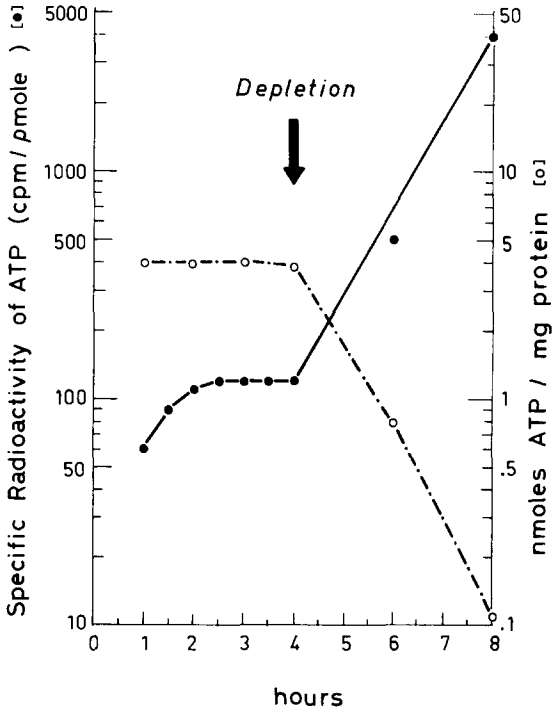

Fig. 5. Labeling of internal ATP pool of chicken erythrocytes with $^{32}P_i$ and the effect of ATP depletion. Fresh cells were incubated as described in Methods. Aliquots were taken from the incubation mixture for ATP determination. Total ATP was determined by the luciferin/luciferase method; radioactive ATP was separated from the mixture of nucleotides by the charcoal method or by paper chromatography (Whatman paper No. 3, developed in 7:3 v/v ethanol 95%/ammonium acetate 1 M pH 7.5, containing 1 mM EDTA) which gave identical results. The specific radioactivity of ATP was calculated from the ratio of the counts of labeled ATP to total ATP in the same volume of the cell hemolysate.

Gel electrophoresis of the fractions obtained show indeed a clear difference between the protein pattern of the plasma membrane fraction, as compared with that of the nuclear fraction (Fig. 7). Essentially comparable results were obtained by Blanchet [10] and Shelton [14] using a similar technique. Based on this procedure, the plasma membrane fraction and nuclear fraction were isolated from the cells at different time points of their labeling with $^{32}P_i$ during incubation with glucose and during the ATP-depletion period. The results shown in Fig. 8 demonstrate that the proteins of the plasma membrane fraction become much more labeled with $^{32}P_i$ than the nuclear fraction. Loss of $^{32}P_i$ from the fractions occurs during ATP depletion. Analysis of $^{32}P_i$ distribution in the various plasma membrane peptides by use of electrophoresis (Fig. 9), shows that most of the radioactivity is located in distinct peptide bands of a molecular weight from 300 000 to 60 000. During ATP depletion the radioactivity of these membranes was almost completely lost (Fig. 8).

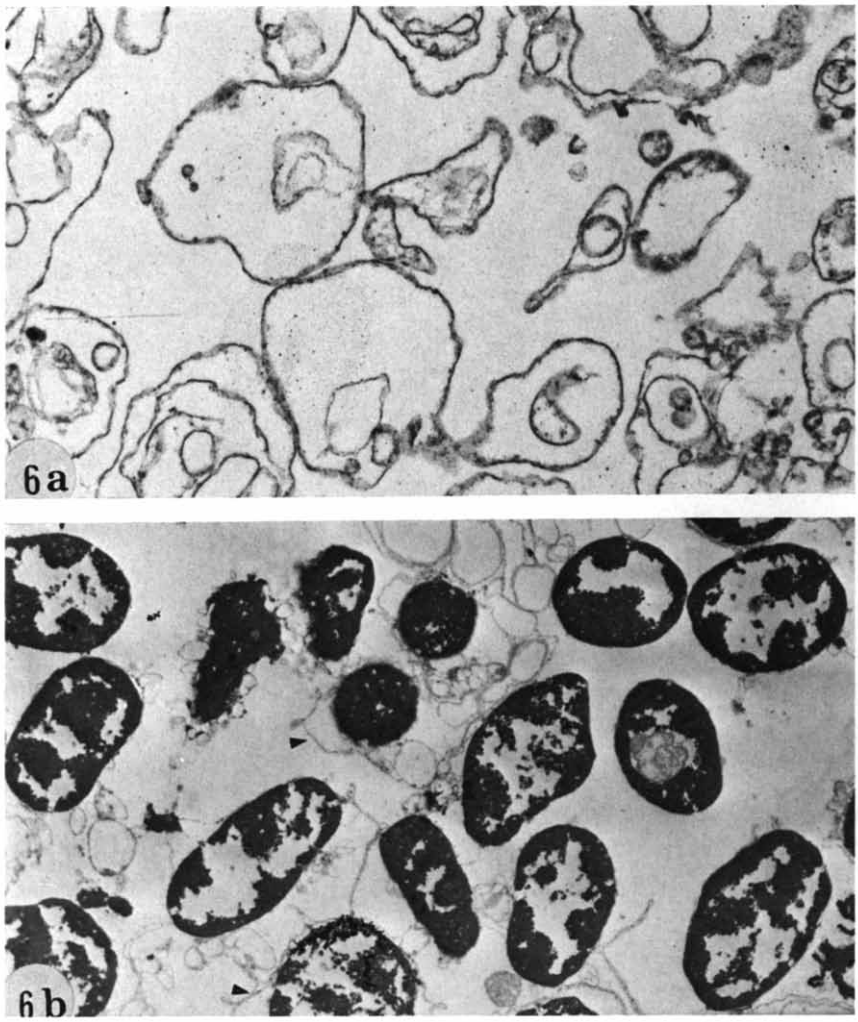

Fig. 6. Electron micrographs of the plasma membrane and nuclear fractions of chicken erythrocytes. (a) Section of purified plasma membrane fraction, magnified × 18 000. (b) Section of the nuclear fraction, magnified × 6 800. Notice the presence of intact nuclei, many being still contained within the vesicles formed by residual plasma membrane (arrow).

DISCUSSION

It has been reported that ATP depletion of erythrocytes in a variety of species renders them susceptible to phospholipase C attack acting either directly or following pretreatment with sphingomyelinase [1]. We have recently shown that the phospholipids of rat erythrocytes are exposed quite extensively to phospholipase C in fresh cells in the absence of cell lysis. However, following ATP depletion, an additional fraction of the membrane phospholipids becomes exposed to hydrolysis, resulting in cell lysis [2, 15]. A quantitative analysis of the distribution of intramembrane particles visualized by the use of freeze fracture technique, in fresh and ATP-depleted rat cells,

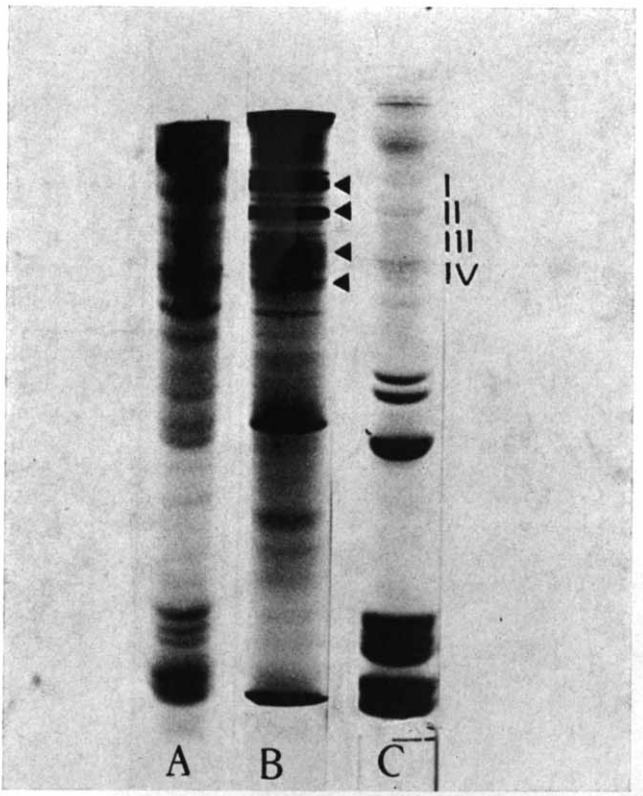

Fig. 7. Electrophoretic pattern on sodium dodecyl sulfate acrylamide gels, of total ghosts (A), purified plasma membrane (B), and nuclear fraction (C). About 200 μg of protein was loaded on each gel. The acrylamide concentration was 10%. For other experimental details, see Methods. Arrows indicate the region of bands I–IV corresponding to 250, 180, 100 and 85 kdaltons, respectively.

has led us to postulate that the degree of intramembrane particle aggregation is altered by ATP-depletion. This results in an increase of the free lipid bilayer phase susceptible to phospholipases.

It was also shown that the phospholipids of fresh chicken erythrocytes are refractile to phospholipase C even after hydrolysis of sphingomyelin, but a latent sensitivity toward the enzyme is developed in ATP-depleted cells, which can be revealed by prior treatment of the cells with sphingomyelinase [1]. As a possible mechanism for the ATP-dependent exposure of phospholipids and intramembrane particles aggregation, one might consider phosphorylation and dephosphorylation of membrane protein. Thus, upon incubation of the cells with $^{32}P_i$, the ATP pool of the cells and specific membrane-peptides become labeled, whereas upon ATP depletion the radioactivity of the membranes is lost. This would indicate a rapid turnover of the phosphate covalently bound to membrane peptides. Similar results have recently been reported by Cobbs and Shelton [16]. The fact that the specific radioactivity of the ATP pool of the cells is significantly lower than that of $^{32}P_i$ added to the incubation medium, might be due to lack of equilibration between the phosphate

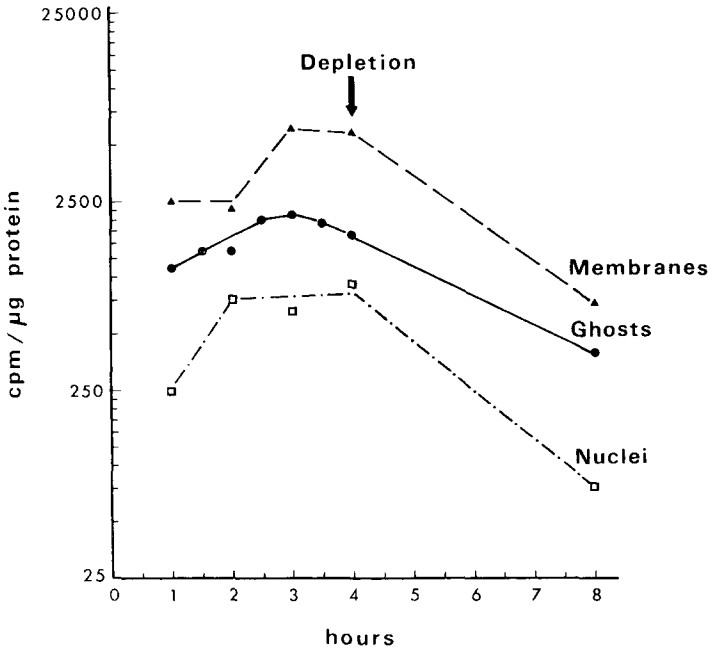

Fig. 8. Changes in the $^{32}P_i$ content of cell ghosts, isolated nuclei and plasma membrane fraction, upon incubation of erythrocytes followed by ATP depletion.

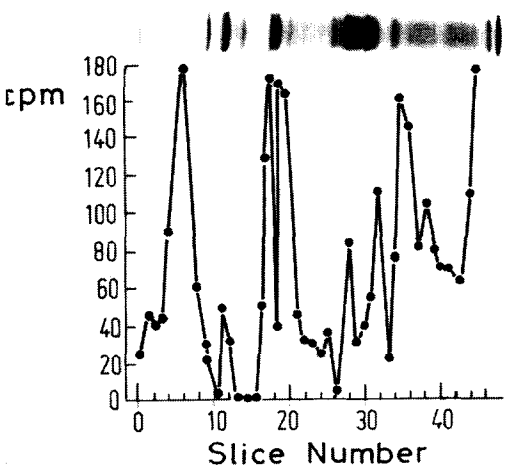

Fig. 9. Distribution of radioactive phosphate in various peptide bands of plasma membrane. The curve represents the amount of $^{32}P_i$ incorporated into the plasma membrane fraction during 3 h incubation of fresh cells with glucose and $^{32}P_i$. In this experiment, 7.5 % gels were used. The gels were stained and then sliced from top to bottom of the gel in 2 mm slices. The slices were placed in minivials and digested by a mixture of 0.1 ml of 1 % sodium dodecyl sulfate and 0.5 ml of H_2O_2 for 10 h at 60 °C and counted using 3 ml of scintillation fluid (33 % Triton X-100, 0.5 % PPO and 0.05 % POPOP in toluene). Plasma membrane fraction was prepared as described in Methods.

present in the medium at the very low concentration used (0.01 mM) and the internal phosphate of the cells which, if similar to that of human cells, might be a hundred-times higher [17]. At such concentrations, a phosphate efflux might be obtained and the rate of phosphate uptake might be extremely low [17]. The rise in the specific radioactivity of the ATP during the depletion period, can be explained if one considers the possibility that exchange might occur between phosphorylated membrane proteins and residual small amounts of ATP. This will be the case especially if the specific radioactivity of the membrane-bound phosphate is higher than that of the ATP pool. Such a possibility should be considered if one assumes that a direct exchange might occur also between membrane polypeptide phosphorous and $^{32}P_i$ of the medium whose specific radioactivity is about 200-times higher than that of the internal ATP (Milner, Y., unpublished data).

The $^{32}P_i$ covalently bound to the membrane peptides is rapidly lost during the ATP-depletion period. Most of the membrane $^{32}P_i$ was found by gel electrophoresis to be located in five distinct bands. The phosphorylated bands include peptides which can be identified tentatively as spectrin, band III (100 kdaltons) and band IV (85 kdaltons) of human erythrocytes [12]. In addition, a peptide of about 180 kdaltons, which is not present in human erythrocyte membranes was found. A similar peptide is present in the electrophoretic pattern of chicken erythrocytes in the data published by Blanchet [10]. Radioactivity was also found in a very faintly stained peptide of about 300 kdaltons. At the present time, it is not known whether it is a membrane protein or a contamination.

Spectrin has been reported to play a major role in the stabilization of the membranes [18–20]. One of the proteins belonging to the band III is known to span the membrane and hydrophobically interact with the membrane lipids [21]. A recent report by Jenkins and Tanner [22] indicates that this molecule might have an S shape and thus traverse the membrane twice. It is possible that one or several of these phosphorylated bands might control the masking and unmasking process of the membrane phospholipids. At the present time, it is not possible to decide which of these proteins is responsible for the changes observed in the lipid-protein interaction. However, it is tempting to speculate that the chicken-specific 180 kdaltons protein might be involved in the above-mentioned phenomenon.

Protein-lipid interactions acting between membrane proteins and at least one layer of the surrounding lipid, has been reported [23]. Thus, it seems reasonable to assume that alteration of the charge and conformation induced by dephosphorylation of the proteins, might affect their interaction with the lipid annulus, which is adjacent to the intramembrane particles. The lipid annulus surrounding membrane proteins, such as the sarcoplasmic reticulum ATPase was calculated to be of about 30 molecules [24]. Considering that the number of intramembrane particles per 1 μm^2 of membrane is about 3500, and assuming that each particle interacts with 30–40 phospholipid molecules, the number of lipids bound to these particles will be approx. 10^5 molecules per 1 μm^2. If the average area occupied by a lipid molecule in a mixed bilayer is 70–80 $Å^2$ [25], one might speculate that about 10 % of the lipid bilayer is found in the annulus directly interacting with the particles, and another 15–20 % could be calculated for a second lipid layer located around the first. Thus, a large fraction of the total membrane lipid could be considered to be immediately affected by the ATP depletion of the cells. The effect of protein dephosphorylation might be expressed in

terms of changes in the compressibility of the lipid bilayer which was shown by Demel et al. [26] to influence the exposure of phospholipids towards phospholipases. One should mention that about 5 % of the total $^{32}P_i$ found in the membrane is extractable by chloroform/methanol and thus the possibility that the effect of ATP depletion is also due to the dephosphorylation of lipids such as phosphatidylinositol which amount to about 3 % of the total lipids, should not be excluded.

In the present work we have used phospholipase A_2 to detect lipid exposure following ATP depletion. Since its action on depleted cells does not induce lysis, even when extreme hydrolysis occurs, one can conclude that, indeed, a large amount of the membrane lipids are directly exposed to the enzyme acting on the outer membrane surface. This interpretation of the data is supported also by the results obtained with TNBS which show that more NH_2 groups of phosphatidylethanolamine, which is known to be located on the inner face of the membrane [3–6, 27], become available to the reagent in ATP-depleted cells. Similar results have been reported for human erythrocytes by Deuticke [6]. About the same amount of phosphatidylethanolamine is either hydrolyzed by phospholipase A_2 or reacted with TNBS (35 % of the total phosphatidylethanolamine) in ATP-depleted cells.

The aggregation of the intramembrane particles and the relative increase in the smooth free lipid phase, as demonstrated by the freeze-fracture technique, are in agreement with previous data obtained with ATP-depleted rat cells [2].

The data presented in this work establish a correlation between ATP depletion, dephosphorylation of membrane proteins, aggregation of intramembrane particles, relative increase in the free lipid bilayer phase and exposure of phospholipids toward phospholipases and TNBS. Certainly one can consider that this correlation is accidental. However, a myosin-actin type of interaction between spectrin and an actin-like membrane protein [20] and a Ca^{2+}-dependent ATPase-like activity of spectrin-actin complex [19] have been described. These activities have been considered to play a role in the regulation of translational movements of membrane integral proteins [18]. Combined with the data demonstrating strong interactions between membrane intrinsic proteins and lipids [23, 24], these observations support the concept that the above-described correlations are related by cause-effect relationships. The ATP-dependent alteration of membrane structure in intact cells appears to be of a more general nature and is not restricted to erythrocytes. Increased agglutination of ATP-depleted transformed fibroblasts has been reported [28], which apparently is also correlated with an increased susceptibility to phospholipase C-induced lysis [29] and aggregation of intramembrane particles (unpublished results).

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