ACTA CHEMICA SCANDINAVICA 18 (1964) 2029–2043

Incorporation of Phosphorus into Human Erythrocyte Ghost Fractions

GUNNAR ÅGREN and GUNNAR RONQUIST

Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

The fraction of the red-cell membrane which is labelled by radioactive inorganic phosphate has been extracted and purified about 50 times. This fraction has the properties of a large lipoprotein complex. The labelling is a comparatively slow process, proceeding in several steps. The phospholipids of the complex seem to be not completely inert. The soluble fraction contains acetylcholinesterase activity, and is labelled by radioactive diisopropyl phosphofluoridate. Possible functions of the fraction are discussed.

It was demonstrated in a previous paper that about 30 labelled phosphopeptides and phosphorylserine could be isolated from red-cell ghosts which had been incubated with radioactive inorganic phosphate ($^{32}P^*$).[1] The rate of incorporation and the pH-dependence of the labelling were also studied. These processes have now been more extensively examined, and it has also been possible to solubilize the active fraction from the red-cell ghosts. Some of these results have been published in a preliminary communication.[2]

METHODS

Preparation of labelled red-cell ghosts. 2000–3000 ml samples of human blood were obtained twice a week from the University Hospital, Uppsala, where the blood had just been used in connexion with major operations. The cells were centrifuged and washed at least twice in a refrigerated Stock centrifuge, and all further procedures were carried out at 4°C. After each centrifugation, the leukocytes were removed by skimming and suction. Nine volumes of cold distilled water were added, and the pH adjusted to 6.0 by means of CO_2. The ghosts were washed first with 10^{-3} M Tris-acetate, pH 6.5, followed by washings with 2×10^{-2} M Tris-glycylglycine buffer, pH 8.2, as previously described.[1] The volume of the practically colorless ghosts, when prepared from about 1000 ml of red cells, usually amounted to around 300 ml, with a decrease in dry weight from about 6 to 2 g. Ghosts from 50 l of human red cells were prepared in this way, and used when the incorporation of ^{32}P into the membrane fraction was studied.

* ^{32}P = radioactive inorganic phosphate; Tris = tris(hydroxymethyl)aminomethane; TCA = trichloroacetic acid; cpm = counts per minute; UV = ultraviolet; mC = millicurie; SerP = phosphorylserine; ATP = adenosine triphosphate.

For labelling with ^{32}P, the ghosts (300 ml) were diluted to about 2 l with 2×10^{-4} M Tris-glycylglycine buffer at pH 7.4, and incubated with approximately 0.1 mC of ^{32}P per ml for different periods of time. The ghosts were then precipitated by addition of cold TCA to 10 % (w/v). The precipitate was centrifuged, and washed once or twice with 10 % TCA, containing about 5 mg of sodium mono-H-orthophosphate per 100 ml of TCA solution. The lipids were removed by extraction twice with ethanol-ether (3:1) at 45°C, followed by ether. The residue contained the Schneider rest protein and nucleic acid fractions.

Purification of the ^{32}P-incorporating fraction from red-cell ghosts. In the experiments described earlier,[1] it was observed that the clear centrifugates from washing of ghosts contained material which incorporated ^{32}P in about the same way as the ghosts. After some preliminary work, purification of the extract was achieved in the following way.

The ghosts from 1000 ml of packed red cells were prepared and washed with 10^{-3} M Tris-acetate buffer, as previously described, but never washed more than 5 times. To the ghosts were added 2 volumes of 2×10^{-2} M Tris-glycylglycine buffer, pH 8.2. After mixing, the suspension was centrifuged at 20 000 r.p.m. for 60 min in the Spinco ultracentrifuge (Model L, rotor No. 21). The precipitate was washed 5 times by resuspension in an equal volume of buffer and centrifugation. Tris-glycylglycine, 2×10^{-2} M, pH 8.2, was always used. This step usually took 2 days.

The combined clear buffer extracts were brought to pH 6.0 by careful addition of concentrated acetic acid. A heavy precipitate settled during storage overnight in the cold room. The clear supernatant was removed by centrifugation in the Stock centrifuge, and Tris-acetate buffer, pH 7.5, was added to a final concentration of 0.05 M.

The clear solution (3000—4000 ml) was concentrated by ultrafiltration [3] to less than 1/10 of the original volume. Ten aspirator bottles of 2 l volume, containing Visking dialysis tubing, size 8/32 in., generally of 2 m length, were used for each experiment. A negative pressure of about 620 mm Hg was applied to the aspirator bottles by a vacuum pump. The concentrated solution, together with rinsings from the dialysis tubings, was applied to Sephadex G-200 columns.

The protein concentration of all fractions was estimated by measuring the ultra-violet absorbancy/cm at 280 mμ in a Beckman model DU spectrophotometer. A solution which gave the absorbancy value 1.0 was regarded to contain 1 UV unit/ml.

Gel filtration was carried out mainly according to Flodin and Killander [4] on Sephadex G-200 (AB Pharmacia, Uppsala, Sweden). The dextran gel, with a particle size of 40—120 μ, was sieved to the 200—400 mesh fraction. Particles which did not settle from a suspension in 0.05 M Tris-acetate buffer (pH 7.5) within 1 h were removed by decantation. This procedure was repeated 6 times. The column dimensions were 6.5×52 cm. A 2 cm layer of glass beads and glass wool was placed above the column outlets. The columns had a total gel-bed volume of 1000 ml, and were run in a cold room at 4°C. The elution flow rate was 25—30 ml/h.

Application of the sample was made on the top of the gel bed, by layering between the bed surface and the buffer solution. The density of the sample was increased by addition of NaCl to a final concentration of 0.1 M. The sample was then applied by means of a syringe, to which 1 mm polyethylene tubing was connected. The volume of the applied sample never exceeded 1/10 of the total bed volume, and did not contain more than about 1500 UV units.

The eluate, usually from two columns (1800—2000 ml of red cells), was concentrated by ultrafiltration to 3—4 % of the original volume, and incubated with 0.1 mC/ml of ^{32}P in the presence of 1.0×10^{-4} M unlabelled phosphate. Labelled phosphate in excess was removed by gel filtration on a Sephadex G-25 (Medium) column. The column dimensions and total gel-bed volume were the same as described previously. The column was equilibrated with 0.05 M Tris-acetate buffer, pH 7.5. Purified material from about 2000 ml of red cells was applied to one column directly on the bed surface. The flow rate was 60—70 ml/h. The filtration of labelled phosphate could be followed directly on the column wall by means of a Panax GM Monitor, and the filtration of the protein fraction by UV analyses.

When the ^{32}P-incorporating pattern was analyzed, the protein fraction filtrated from the Sephadex G-25 column was precipitated by addition of cold TCA to a final concentration of 10 % (w/v). For incubation experiments with fractions from the red-cell content,

the protein fraction was concentrated by ultrafiltration to about 1/10 of the original volume, and then precipitated by addition of TCA.

The preparation of the TCA-soluble content of the erythrocytes will be described in a later paper.

Analytical ultracentrifugation was performed in a Spinco Model E ultracentrifuge.

Adenosine triphosphatase activity was determined according to Post *et al.*[5] Acid and alkaline phosphatase activity was estimated with the method of Engström.[6]

The procedures for isolation of SerP and phosphopeptides have been described in a previous paper.[1] Similar methods were used to analyze the material from the "phosphorylethanolamine" and "phosphorylcholine" peaks. Phosphorylethanolamine has previously been isolated in crystalline form at this laboratory,[7] and the rate of ^{32}P incorporation into this substance, as well as into phosphorylcholine, has been studied in the TCA precipitates from ^{32}P-labelled ascites tumour cells [8] and rat liver.[11]

Analytical methods. The purification of subfractions from the Dowex-1 formate columns, by high-voltage electrophoresis, as well as the scanning of the electrophorograms, have been described previously.[1] For final identification of phosphorylethanolamine and phosphorylcholine, one-dimensional paper chromatography was used. The solvent systems found to be most suitable were isobutyric acid-ammonia [9] and benzene-propionic acid.[10] When the specific activity of the labelled compounds was low, or had decreased whith time during the isolation procedures, the position of organic phosphate on the paper chromatogram could be detected with the ammonium molybdate-benzidine spot test, as modified by Ågren and Glomset.[10]

The qualitative amino-acid composition of purified phosphopeptides was determined by high-voltage electrophoresis or two-dimensional paper chromatography.[11]

Simultaneous automatic recording of radioactivity and UV-absorbancy was carried out by an apparatus built in this laboratory.[12] This device was used for following the labelling of nucleotides.

The radioactivity of the different fractions isolated by column chromatography of the hydrolyzed protein residues was measured in glass cups, using one commercial and one modified L.K.B. Robot Scaler. Determinations of specific activity were made on stainless steel planchettes with the Tracerlab Superscaler. At least 1000 counts were measured. The specific activity of the different phosphorylated compounds was calculated as cpm/μg of phosphorus. All activity values were recalculated to the time of incubation. Phosphorus was determined as previously described.[13]

For identification of different nucleotides, the ratios given in the Pabst Laboratories Circular OR-7 were used.[14] For identifying and controlling the purity of the UV peaks, a sample from each tube corresponding to a peak was analyzed by paper chromatography. The solvent system Nos. I and III in the Pabst Circular were used.

RESULTS

Rate of ^{32}P incorporation into red-cell ghosts. In a previous investigation,[1] the rate of the initial ^{32}P incorporation into red-cell ghosts was followed during the first 30 min of incubation. It was evident from the figures that the maximal incorporation might not have been obtained during that time. Several new experiments were therefore made with incubation times up to 16 h. In the previous experiments, the eluates of the partially hydrolyzed proteins of the red-cell ghosts, run on Dowex-50 columns, were collected until 5 column volumes of 0.01 N HCl had passed through. In the present experiments, elution was continued until 12 column volumes had passed through.

In this way, several new peaks were found, two in positions where in other connexions we had found phosphorylethanolamine [7] and phosphorylcholine [8,11] (*cf.* Fig. 4, peaks 5 and 8). It is evident from Table 1 that ^{32}P incorporation into the Schneider rest protein fractions of ghosts is a surprisingly slow reaction. Thus, in some cases, a maximum incorporation was not reached

Table 1. Red cell ghosts from 900 ml of red cells washed six times with CO_2-saturated water and six times with 0.02 M Tris-glycylglycine buffer, pH 8.2, in Spinco centrifuge. 3900 ml of extract concentrated by ultrafiltration to 300 ml. Ghost-residues suspended in the same volume of Trisbuffer. Both fractions divided into three samples of 100 ml. Incubation at pH 7.5 for different times with 10 mC ^{32}P added to each fraction. Activity as cpm per 100 mg of dry weight.

Phosphorylated fractions. Fraction No.	15 min		60 min		240 min	
	Ghost	Extract	Ghost	Extract	Ghost	Extract
1	220	2520	610	7 700	3030	33 000
2	470	2540	810	6 500	1660	9 850
3	320	1520	1690	9 200	2660	24 200
4	780	3060	2500	8 000	7650	34 600
5	57	7050	88	10 500	460	14 700
6	0	4820	110	10 500	200	14 100
7	0	0	0	540	0	4 520
8	0	6120	0	—	100	11 400
Dry weights (g)	0.65	1.90	0.64	1.60	0.77	1.90

even after incubation for 4 h. Of considerable interest is the labelling of the Tris-glycylglycine washings of the ghosts. Per 100 mg of dry weight, the labelling was higher in the extract than in the ghost-residue fraction, and it is obvious that the larger part of the ^{32}P-incorporating material can be solubilized.

It is also evident from Table 1 that the rate of ^{32}P-incorporation initially is a slower reaction for the labelled fractions which are eluted in a later part of the elution curve (peaks 5—8). A similar tendency is observed when the incubation time is increased.

From Table 2 it is clear that after 4 h incubation, a maximum of ^{32}P-incorporation is reached for the first four fractions containing phosphorylserine and phosphopeptides,[1] whereas a maximum may not have been attained even after 16 h for the fractions which are eluted later from the Dowex-50 column. These fractions appeared in the expected positions for phosphorylethanolamine (Fig. 4, peak 5) and phosphorylcholine (peak 8), respectively. Attempts were made to characterize these two fractions.

Incorporation of ^{32}P into the phospholipids of red-cell ghosts. The phospholipids present in the red-cell stroma have been studied by Prankerd *et al.*[15] who found about equal parts of cephalins, lecithins, and sphingomyelin. At about the same time, Dawson [16] published a new method for the identification of individual phospholipids in biological samples. We have used this method to study the occurrence of different phospholipids in red-cell ghosts. The results are given in Table 3. So far, we have been unable to find any traces of hydrolytic products of monophosphoinositide.

Table 2. Red cell ghosts from 1000 ml of red cells washed six times with CO_2-saturated water and six times with 0.02 M Tris-glycylglycine buffer, pH 8.2, in Spinco centrifuge. Ghost residues suspended in 180 ml of Trisbuffer, pH 7.5 and divided in three equal samples. Each fraction incubated with 15 mC ^{32}P for different times. Activity as cpm per 100 mg of dry weight.

Phosphorylated fractions. Fraction No.	Time of incubation, h		
	4	8	16
1	139 000	108 000	133 000
2	91 500	84 500	104 000
3	146 000	103 000	132 000
4	282 000	205 000	240 000
5	9 750	15 700	62 000
6	9 840	7 400	7 750
8	22 700	40 600	54 500

The material of peaks 5 and 8 (Fig. 4) from several preparations of red-cell ghosts were collected and run through small Dowex-1 columns with gradient elution 0 → 1 M formic acid. The results were as follows.

Fig. 1 shows the elution chromatogram of about 0.9 g of radioactive material eluted in a position corresponding to peak 5, Fig. 4. Separation was carried out on a 0.5 × 50 cm Dowex-1 formate column. A large number of peaks were obtained. Six of them were further analyzed by one-dimensional paper chromatography. Peaks 1 and 2 in Fig. 1 were given by ninhydrin-positive material. After hydrolysis of each fraction at 120°C for 20 h with 2 N HCl, the following amino acids were identified in both materials by two-dimensional paper chromatography: alanine, glycine, proline, serine, threonine, valine, probably hexosamine and basic amino acids. Glutamic and aspartic acid were not present.

Table 3. Individual phospholipids found by unidimensional chromatography of phospholipid hydrolysates from human red cell ghosts.

Hydrolysis product	Parent phospholipid	R_F	
		Presently found	Found by Dawson [16]
Glycerylphosphorylcholine	Lecithin	0.75	0.77
Glycerylphosphorylethanolamine	Phosphatidylethanolamine	0.58	0.63
Glycerylphosphorylserine	Phosphatidylserine	0.25	0.30
Glycerylphosphorylethanolamine	Ethanolamine plasmalogen	0.60	0.63
Glycerylphosphorylserine	Serine plasmalogen	0.29	0.30
Phosphorylcholine and sphingosylphosphorylcholine	Spingomyelin	0.74	0.79

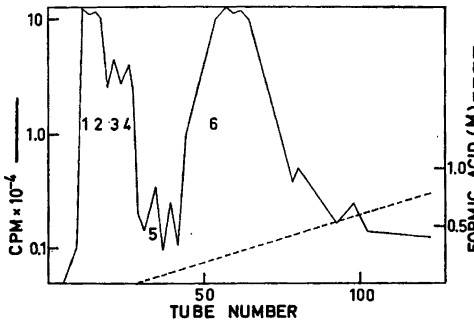

Fig. 1. Radioactivity curve of the material from peak No. 5, Fig. 4, separated on a Dowex 1 (2 % DVB) column. The radioactivity curve (the continuous line) was obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents formic acid concentration.

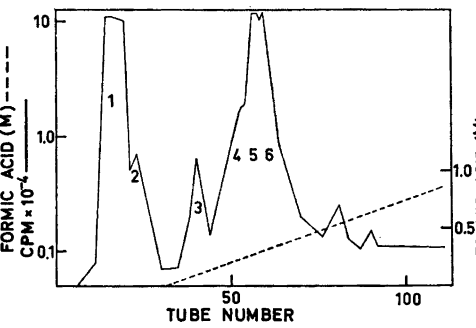

Fig. 2. Radioactivity curve of the material from peak No. 8, Fig. 4. See legend for Fig. 1.

Phosphorylethanolamine was the only substance, present in fractions 3 and 4 (Fig. 1) when they were analyzed by one-dimensional paper chromatography. We have previously found that both phosphorylethanolamine and phosphorylcholine can give double peaks on elution from Dowex-1 formate columns. A part of the amines will pass through with water, and the residue just in front of the formic acid gradient. About 9 mg of labelled phosphorylethanolamine were isolated. Fraction 5 was not further analyzed, and fraction 6 contained only UV-absorbing material.

50 mg of radioactive material corresponding to fraction 8 in Fig. 4 were run through a Dowex-1 formate column with gradient 0 → 1 M formic acid. The elution curve is given in Fig. 2. Subfraction 1 contained 25 mg of ninhydrin-positive material which was not further analyzed. Fraction 2 and 3, together 12 mg, consisted of phosphorylcholine. Fraction 3 appeared just in front of the formic acid gradient. Fractions 4 gave a positive ninhydrin reaction. After hydrolysis, two-dimensional paper chromatograms showed the presence of alanine, small amounts of aspartic acid, glycine, and serine, considerable amounts of basic amino acids and one unidentified spot.

Fractions 5 and 6 were completely separated. One-dimensional analyses of fraction 5 showed the presence of ninhydrin-positive material and one UV-absorbing fraction. After acid hydrolysis of fraction 5, paper chromatograms showed spots corresponding to the positions of alanine, glycine, basic amino acids, small amounts of aspartic acid, and an unidentified large, blue-coloured spot in a position with a low R_F value in pyridine/isoamyl alcohol. The UV-absorbing material in fractions 5 and 6 has not so far been further investigated.

Rate of ^{32}P incorporation into soluble fraction from red-cell ghosts. As previously mentioned, experiments had shown that the clear centrifugates from the washings of ghosts in the Spinco ultracentrifuge contained material which

Table 4. Red cell ghosts from 920 ml of red cells washed six times with CO_2-saturated water and six times with 0.02 M Tris-acetic acid buffer, pH 8.2, in Spinco centrifuge. Ultrafiltration of 2800 ml of extract to 200 ml. Dilution with 0.02 M Tris-acetic acid buffer, pH 7.5, to 650 ml. Ghost residues suspended in equal amount of buffer. Incubation for 8 h with 20 mC ^{32}P to each fraction. Activity as cpm per 100 mg of dry weight.

Phosphorylated fractions. Fraction No.	Ghost residues	Extract
1	48 000	103 000
2	23 400	26 000
3	20 000	55 000
4	91 000	98 000
5	10 000	20 000
6	5 500	26 000
7	580	6 400
8	30 000	72 500
Total activity for ghost and extract fractions per 100 mg dry weight	2.3×10^5	4.0×10^5
Dry weight (g)	3.8	5.1

Table 5. Red cell ghosts from 650 ml of red cells washed six times with CO_2-saturated water and six times with 0.02 M Tris-glycylglycine buffer, pH 8.2, in Spinco centrifuge. 1300 ml of extract was brought to pH 6.0 by addition of acetic acid. The washed precipitate was solved in 170 ml of Tris-acetic acid buffer, pH 7.5. The combined extracts were concentrated by ultrafiltration to the same volume. Incubation for 8 h with the addition of 8 mC ^{32}P to each fraction. Activity as cpm per 100 mg of dry weight.

Phosphorylated fractions. Fraction No.	Fraction precipitated at pH 6.0	Fraction soluble at pH 6.0
1	11 900	3 860 000
2	5 250	6 500 000
3	4 750	380 000
4	12 300	906 000
5	13 600	1 420 000
6	1 100	75 000
8	120 000	8 120 000
Total activities of precipitated and soluble fraction per 100 mg of dry weight	1.69×10^6	21.2×10^6
Dry weight (g)	1.07	0.92

incorporated ^{32}P in about the same way as the ghosts. Table 4 illustrates a typical elution pattern of the hydrolyzed protein residues of red-cell ghosts and concentrated Spinco washings, passing through Dowex-50 columns with 0.01 N HCl. It had been shown in separate experiments that the ghost extracts could be concentrated to very small volumes by ultrafiltration, without any loss of ^{32}P-incorporating activity.

It is evident from the figures in Table 4 and other experiments that the main part (from 70 to 90 %) of the ghost fraction which is labelled can be brought into solution, and that the split products after partial acid hydrolysis are qualitatively the same. Calculated per unit dry weight, the labelling is higher in the soluble fraction.

In the next step of purification, the soluble fraction was brought to pH 6.0 by addition of acetic acid during vigorous stirring. After 12 h at 4°C, the precipitate was centrifuged and washed once with a dilute solution of acetate/acetic acid buffer. The results of a typical experiment are given in Table 5. More than 90 % of the total ^{32}P-incorporating activity was recovered in the soluble phases from isoelectric precipitation. The split products obtained by partial acid hydrolysis and separation on Dowex-50 columns gave the same elution pattern as before. Per unit dry weight, the soluble fraction was about 15 times more active.

The ghost extract, purified by isoelectric precipitation at pH 6.0, was next run through a Sephadex G-200 column (Fig. 3). A ^{32}P-incorporating

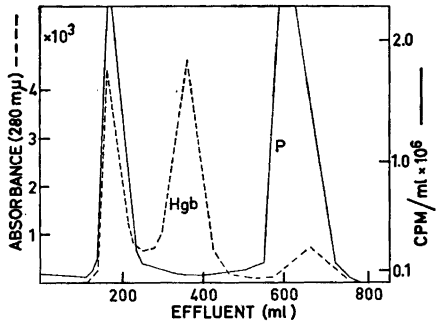

Fig. 3. 1000 ml of packed red cells. Extract isoelectrically precipitated. Centrifuged solution concentrated to 150 ml. 4 ml through a 100 ml bed volume of Sephadex G-200 with 0.05 M Tris-acetate buffer, pH 7.5. The radioactivity curve (continuous line) was obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The broken line represents absorbancy at 280 mμ.

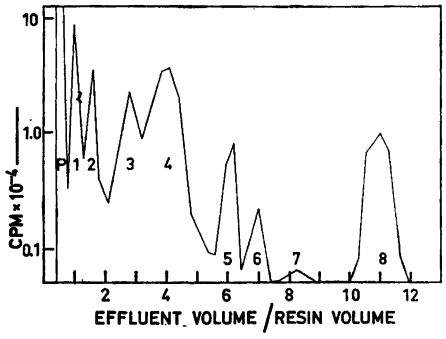

Fig. 4. Radioactivity curve from a partial hydrolysate of Sephadex filtered extract of red cell ghosts (Fraction No. 1, Fig. 3) separated on a Dowex 50 (8 % DVB) column. The curve was obtained by plotting the number of impulses per min (cpm) in 1 ml aliquots of each tube. The protein solution had been incubated with 0.1 mC of ^{32}P per ml for 16 h.

colorless protein peak (v_0 fraction) was the first to appear, followed by a haemoglobin peak and a third small protein peak, which also contained ^{32}P-incorporating activity.

The haemoglobin fraction could be completely separated from the ^{32}P-incorporating one by a second gel filtration on Sephadex G-200, when the activity came out in front of haemoglobin. The v_0 fraction still showed the typical ^{32}P-incorporating pattern, as demonstrated by Fig. 4. So far, the different fractions have not been submitted to any further fractionation.

Analytical ultracentrifugation showed that the v_0 fraction was far from homogeneous, with peaks containing components with sedimentation coefficients of about 19 S, 12 S, 9 S, and 7 S.

Since the ^{32}P-incorporating protein fraction could be a lipoprotein, experiments were carried out to concentrate it by preparative ultracentrifugation, as described by Wallenius *et al.*[17] The density of the ^{32}P-incorporating fraction from gel filtration, with density 1.003, was increased to 1.02 and 1.20 by the addition of NaBr. The Spinco preparative rotor No. 40 was used at 30 000 r.p.m. for 15 h. The top and bottom layer were removed, according to standard lipoprotein methods.[18] NaBr was removed by gel filtration on a Sephadex G-25 column from the fractions which had been separated at density 1.20. The ^{32}P-incorporation was inhibited at a NaBr molar concentration of 1.93 (density 1.20), but not at the molar concentration of 0.193 (density 1.02). The volume of the top layer was diluted to the same, as that of the bottom fraction, and incubated with ^{32}P. The results are given in Table 6, and demonstrate that the activity per unit weight is much higher in the two lipoprotein layers from both types of separation. Calculation based on the total activity of the starting material showed that about 50 % of this was accumulated in the top layer. The distribution of labelling in the hydrolyzed and separated split products of the hydrolyzed protein fractions differed somewhat in top and bottom layers, mainly with respect to fractions 5 and 8. As a regular step in purification, this procedure did not appear promising.

A better result was obtained by repetition of isoelectric precipitation after filtration on the Sephadex G-200 gel. The first two protein fractions (*cf.* Fig. 3) were brought to pH 5.2 by addition of acetic acid, and stored for 2 days at 4°C. The precipitates were washed once. They were easily brought

Table 6. Distribution of ^{32}P-incorporating activity in top and residual layers from preparative ultracentrifugation of soluble fraction from red cell ghosts purified by isoelectric precipitation and gelfiltration. Protein residues partially hydrolysed and hydrolysates separated on Dowex 50 columns. Activity as cpm per mg of dry weight. For each fraction activity in percentage of total activity is also calculated. The fractions are numbered as in Fig. 4.

Expt. No.	Fraction numbers 1	2	3	4	5	6	8
1. Density = 1.020							
Top-layer	3320 6 %	6100 10 %	4560 8 %	9300 16 %	33 983 58 %		1790 2 %
Residual layer	Fractions 1 to 4 = 640 40 %				50 3 %	140 9 %	760 48 %
2. Density = 1.20							
Top-layer		680 2 %	910 3 %	4770 17 %	2040 7 %		20 200 71 %
Residual layer		35 2 %	130 6 %	330 15 %	1160 52 %		560 25 %

Table 7. Extract from 800 ml packed red cells. The first two protein fractions (I and II) from Sephadex filtration brought to pH 5.2 by addition of acetic acid. The washed precipitates solved in Tris-acetic acid buffer, pH 7.5, to the same volume as the soluble fraction from precipitation. Incubation with 0.1 mC ^{32}P per ml for 16 h. Hydrolyzed protein split products (phosphopeptides) separated on Dowex 50 columns. Activity as cpm per mg dry weight. For each phosphorylated subgroup activity in per cent of total activity was also calculated. Subgroups numbered as in Fig. 4.

Hydrolyzed protein residues from	Isolated phosphorylated peptide fractions. Subgroup No.:					
	1	2	3	4	5	8
Protein fraction I, soluble fraction at pH 5.2. Dry weight 8 mg	24 380 8 %	18 300 6 %	16 890 6 %	44 250 14 %	32 880 11 %	106 900 55 %
Protein fraction I, pH 5.2-precipitate. Dry weight 96 mg.	1960 7 %	1434 6 %	2300 8 %	5230 17 %	1380 5 %	16 650 57 %
Protein fraction II, soluble fraction at pH 5.2. Dry weight 142 mg.	—	1240 8 %	1080 7 %	2180 15 %	660 5 %	9580 65 %
Protein fraction II, pH 5.2-precipitate. Dry weight 13 mg.	15 170 14 %	8080 7 %	9000 8 %	22 630 21 %	1180 3 %	50 690 47 %

into solution in 0.05 Tris-acetate buffer, pH 7.5. Incubation with ^{32}P was carried out for 16 h, with the same concentration of labelled phosphate in all solutions. The results are given in Table 7. Calculated per mg dry weight, the radioactivity of phosphopeptides from the soluble fraction on isoelectric precipitation and Sephadex filtration increased in average about 6 times. About 50 % of the total activity was still present in the different phosphorylated split products of the fraction insoluble at pH 5.2. The figures also demonstrate that a considerable part of ^{32}P-incorporating activity leaves the Sephadex column together with the haemoglobin peak when the material is separated on a Sephadex column.

Storing of the soluble fraction at low temperatures. When preparing larger amounts of material for further steps in purification, it was important to store the ^{32}P-incorporating lipo-protein fraction. For shorter times (up to one month), this could be attained by storage in a cold room at +4°C. For longer periods of time, other methods had to be tested. Storage at −16°C was not successful, as shown by Tables 8 and 9.

Different degrees of destruction of the ^{32}P incorporating activity were observed. The figures in Table 8 show a general decrease in activity both for red-cell ghosts and extracts of this material. Fraction 8 from the "phospho-

Table 8. Red cell ghosts from 2000 ml of red cells washed six times with CO_2-saturated water and six times with 0.02 M Tris-glycylglycine buffer, pH 8.2, in Spinco centrifuge. 4390 ml of extract concentrated by ultrafiltration to 480 ml. Ghost residues suspended in an equal amount of Tris-buffer, pH 7.5. Half portions of ghost suspension and extract at pH 7.5 kept frozen at −16°C for one month. Incubation for 8 h with the addition of 10 mC ^{32}P to each fraction (240 ml). Activity as cpm per 100 mg of dry weight.

Phosphorylated fractions. Fraction No.	Fresh material		Frozen material	
	Ghosts	Extract	Ghosts	Extract
1	27 600	32 600	6 900	10 000
2	4 600	6 940	6 320	7 050
3	24 800	25 800	11 800	5 950
4	36 000	14 400	11 500	9 700
5	15 900	31 800	10 400	5 950
6	2 100	2 980	560	810
8	63 600	31 100	0	0
Total activity for ghosts and extract fractions per 100 mg dry weight	1.48×10^5	1.41×10^5	0.72×10^5	0.44×10^5
Dry weight (g)	3.11	3.60	2.49	2.75

Table 9. Red cells ghosts from 1000 ml of packed red cells washed with CO_2-saturated water and extracted with 0.02 M Tris-glycylglycine buffer pH 8.2 in Spinco centrifuge. Extract concentrated by ultrafiltration to 200 ml. Ghost residues suspended in an equal amount of Tris-glycylglycine buffer. Half portions of ghost suspensions and extract at pH 7.5 kept frozen at −16°C for ten days. Incubation for 8 h with the addition of 10 mC ^{32}P to each fraction (100 ml). Activity as cpm per 100 mg of dry weight. Protein residues partially hydrolyzed and phoshorylated split products separated on Dowex 50 columns. Subgroups numbered as in Fig. 4.

Phosphorylated fractions	Fresh material		Frozen material	
	Ghosts	Extract	Ghosts	Extract
Fraction No. 1	85 000	1 930 000	0	0
Fraction No. 2	56 000	335 000	0	0
Fraction No. 3 to No. 8	144 000	5 090 000		
	285 000	7 355 000		
	4 % of total activity	96 % of total activity		

rylcholine" peak (Fig. 4) seemed to be the first to lose this activity completely. Sometimes the capacity to label peaks 5 and 8 was completely lost.

In attempts to preserve human red cells, rapid freezing down to −186°C in liquid nitrogen has met with some success. A similar technique was used in some experiments. The pH 8.2 extract of red-cell ghosts was purified by

isoelectric precipitation at pH 6.0. The soluble phase, adjusted to pH 7.5, was sprayed down into a small beaker with liquid nitrogen. The beaker was lowered into a jar with liquid nitrogen. The outlet of a large funnel ended in the beaker. The protein solution was sprayed down into the beaker. Small rose-coloured pearls with a diameter of about 1—2 mm were immediately formed. After 10 min, the pearls of frozen material were rapidly brought into solution by addition of a large volume of 0.01 M Trisacetate buffer at 40°C and pH 7.5. The results of an experiment are given in Table 10. When compared with the total activity of the control solution, 32 620 cpm/mg dry weight, both frozen fractions showed full activity, both when protected during freezing by a hexose mixture (total activity 42 780 cpm/mg dry weight) and without any protecting substances (total activity 42 680 cpm/mg).

Attempts were also made to deep-freeze on a larger scale, by rotating 500 ml flasks with 150 ml of purified extract from red-cell ghosts in liquid nitrogen. The frozen material was rapidly thawed, as previously described. The results are given in Table 11. The same phenomenon appeared here as when a similar extract was stored at −16°C (*cf.* Table 8). There was no labelling of peak 8 (Fig. 4). The total activity of the isolated subgroups from the material which had been stored for 15 h had decreased from 10 650 cpm/mg dry weight in the control to 8 790.

Enzyme activity. ATPase activity was determined in the ghost residue after extraction with 0.02 M Tris-glycylglycine buffer in the Spinco centrifuge, according to Post *et al.*[5] As already demonstrated by this group, the enzyme is located in the membrane, and our ghost-residue fraction from Spinco centrifugation contained activity, whereas it was not present in any of our soluble fractions.

Phosphatase activity was determined at pH 6.0. Most of the acid phosphatase activity was removed with the haemolysate. Most of the remaining part was

Table 10. Extract from 500 ml of packed red cells, isoelectrically precipitated at pH 6.0. The soluble fraction adjusted to pH 7.5 rapidly sprayfrozen at −186°C in the presence of glucose (final concentration 5 %) + lactose (final concentration 7.5 %) or without addition. After rapid thawing and concentration by ultrafiltration the protein solutions and control (90 ml volumes) were incubated with 0.1 mC ^{32}P per ml for 16 h. Activity as cpm per mg dry weight. Protein residues partially hydrolyzed and phosphorylated split products separated on Dowex 50 columns. For each phosphorylated subgroup from the Dowex column eluate activity in per cent of total activity was also calculated. The subgroups numbered as in Fig. 4.

Hydrolyzed protein residues from	Isolated phosphorylated peptide fractions. Subgroup No. 1	2	3	4	5	8
Fraction frozen with hexoses	2900 7 %	650 2 %	2920 7 %	5740 13 %	2800 5 %	28 480 66 %
Fraction frozen without hexoses	1 + 2 =	3500 8 %	3 + 4 =	11 420 27 %	8350 20 %	19 410 45 %
Control	1 + 2 =	4940 15 %	3220 10 %	7660 23 %	7080 22 %	9720 30 %

Table 11. Extract from 750 ml of packed red cells, isoelectrically precipitated at pH 6.0. The soluble fraction adjusted to pH 7.5. 150 ml portions rapidly frozen at −186°C in rotating 500 ml flasks. After rapid thawing and concentration by ultrafiltration the protein solution and control (118 ml fractions) were incubated with 0.1 mC ^{32}P per ml for 16 h. Activity in cpm per mg dry weight. Protein residues partially hydrolyzed and phosphorylated split products separated on Dowex 50 columns. For each phosphorylated subgroup from the Dowex column eluate activity in per cent of total activity was also calculated. The subgroups numbered as in Fig. 4.

Hydrolyzed protein residues from	Isolated phosphorylated peptide fractions. Subgroup No.					
	1	2	3	4	5	8
Fractions frozen for 20 min	1 + 2 =	2200 21 %	3 + 4 =	480 5 %	7960 74 %	0
Fraction frozen for 15 hours	1 + 2 =	164 2 %	1520 17 %	= 200 2 %	6910 79 %	0
Control	450 4 %	1140 11 %	620 6 %	2030 20 %	1120 11 %	5000 48 %

removed by the washings with CO_2-saturated water. Since the enzyme with this pH optimum is sensitive both to daylight and electric light,[19] it is not surprising that our purified fractions were practically devoid of phosphatase activity.

Acetylcholinesterase activity was determined as previously described.[20] This activity was difficult to separate from the ^{32}P-incorporating fraction, and cholinesterase activity was present even in the purified lipoprotein fraction from Sephadex filtration. It has previously been demonstrated that radioactive diisopropyl phosphofluoridate ($DF^{32}P$) reacts with the liver esterases and completely supresses their activity, by forming a covalent linkage to a serine residue in the active site of the enzymes.[21]

When a ^{32}P-incorporating extract of red-cell ghosts, purified by isoelectric precipitation, was incubated with 10^{-5}M $DF^{32}P$ for 30 min at 4°C and run through a Sephadex G-200 column, only the first protein peak was labelled (Fig. 3) and $Ser^{32}P$ could be isolated from the hydrolyzed protein.

DISCUSSION

The data obtained in the present investigation are consistent with the view that the ^{32}P-incorporating factor of the red-cell ghost might be a lipoprotein complex with a high molecular weight. When filtered on Sephadex G-200 columns, it appeared rather early. In preparative ultracentrifugation at density 1.02 and 1.20, the ^{32}P-incorporating activity was concentrated in the top layer like a lipoprotein, with an *s* rate of 7 Svedberg units. Analytical ultracentrifuge analysis demonstrated the presence of a 7 S fraction in the active protein peak from Sephadex filtration.

In this connexion, the destruction which occurs after storage at −16°C is of interest. The large ice crystals formed at this temperature might, by friction, influence the structure of a large lipoprotein molecule. At the temperature of liquid nitrogen, where the rapid lowering of water temperature prohibits any formation of ice crystals, the ^{32}P-incorporating activity seemed to remain intact.

The rate of this incorporation is a comparatively slow process, and of the same order as that previously found in several rat organs.[22] The maximum of ^{32}P incorporation is reached after 4 h incubation for only the first four phosphopeptide fractions eluted from the Dowex-50 columns. Later eluted protein split products containing phosphopeptides, nucleotides and acid split products from phospholipids, sometimes did not show maximum labelling even after 16 h incubation. The reason for this slow process is not known, although it has been suggested that phosphoprotein of this type may be involved in ion transport.

Some preliminary experiments have been carried out in which the lipoprotein fraction from Sepadex filtration, after incubation with ^{32}P for 8 h and removal of free ^{32}P by filtration on a Sephadex G-25 column, was incubated with TCA-soluble nucleotides of the red-cell haemolysate. A labelling of the ATP was observed after very short incubation times. For the time being, it is difficult to exclude the interesting possibility that the labelled ATP is formed by the lipoprotein complex, when it is dissociated from the ATPase activity left in the membrane residues.

Schauer and Hillman [23] recently obtained some evidence of the presence in the erythrocyte ghosts of an acid-labile, UV-absorbing substance, which they thought could play a role in the labelling of the membrane. In the present investigation, evidence has been found of the presence of acid-stable, UV-absorbing substances in the last two larger fractions (5 and 8) eluted from Dowex-50 columns. The chemical nature of these substances remains to be established.

These two fractions also contained phosphopeptides with some unusual combinations of amino acids. Glutamic acid and aspartic acid were always present in considerable amounts in the phosphopeptides isolated from the first part of the elution diagram of the hydrolyzed protein residues passing through a Dowex 50 column (peaks 1—4 in Fig. 4). By contrast, the phosphopeptides from peaks 5 and 8 contained considerable amounts of basic and neutral amino acids, including serine, and very small amounts of aspartic acid. Glutamic acid was not present.

The lipid fractions isolated from the washed red-cell ghosts contained considerable amounts of plasmalogens. This is in accordance with the recent results of Farquhar.[24] There has been some disagreement about the inertness of the red cell lipids. In more recent work, it is stated that any synthesis or exchange of lipid constituents in blood is a result of white-cell activity,[25] and that in mature cells the lipids are static. In the present work, due precautions were taken to remove the buffy layer of white cells after each centrifugation, first in removal of plasma and then in the washings. This was attained by skimming with spoon and cotton sticks. Care was taken to remove as small amounts as possible of the young red cells that are found in the centrifuged

top layer.[26] It was not possible to decrease the number of white cells any further by cotton-woll filtration.[27] After extraction of the ^{32}P-incorporating activity from red-cell ghosts and purification of the extract, labelled phosphorylethanolamine and phosphorylcholine could still be isolated from the lipoprotein fraction. It might well be that most of the phospholipids in the red-cell membranes are static. A certain extent of ^{32}P exchange with the surrounding medium seemed, however, to take place in the extracted and purified lipoprotein complex.

Acknowledgement. The authors are indebted to engineers S. Eklund and N. O. Hedlund, Miss M. Lagestrand and Mr. T. Fransson for skilful assistance. This investigation forms a part of a programme supported by grants from the *Swedish Medical Research Council.*

REFERENCES

1. Ågren, G., Hallberg, B. and Ronquist, G. *Acta Chem. Scand.* **16** (1962) 1770.
2. Ågren, G. *Acta Chem. Scand.* **17** (1963) 873.
3. Berggård, I. *Arkiv Kemi* **18** (1961) 291.
4. Flodin, P. and Killander, J. *Biochim. Biophys. Acta* **63** (1962) 403.
5. Post, R. L., Merrit, C. R., Kinsolving, C. R. and Albright, C. D. *J. Biol. Chem.* **235** (1960) 1796.
6. Engström, L. *Biochim. Biophys. Acta* **52** (1961) 36.
7. de Verdier, C.-H. and Ågren, G. *Acta Chem. Scand.* **12** (1958) 361.
8. Ågren, G. *Acta Chem. Scand.* **14** (1960) 2065.
9. Magasanik, B., Vischer, E., Doniger, R., Elson D. and Chargaff, E. *J. Biol. Chem.* **186** (1950) 37.
10. Ågren, G. and Glomset, J. *Acta Chem. Scand.* **7** (1953) 1071.
11. Ågren, G. *Acta Soc. Med. Upsalien.* **66** (1961) 297; **67** (1962) 55.
12. Sjöberg, C.-I. and Ågren, G. *Anal. Chem.* **36** (1964) 1017.
13. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **8** (1954) 1570.
14. Pabst Laboratories. Division of Pabst Brewing Company, 1037. W. Mc Kinsley Ave., Milwaukee S, Wisconsin. Circular OR-7, March 1955.
15. Prankerd, T. A. J. *The Red Cell.* Blackwell Sci. Publ., Oxford 1961.
16. Dawson, R. M. C. *Biochem. J.* **75** (1960) 45.
17. Wallenius, G., Trautman, R., Kunkel, H. G. and Franklin, E. C. *J. Biol. Chem.* **225** (1957) 253; *Chemical Analysis*, New York 1954, p. 141.
18. deLalla, O. F. and Gorfman, J. W. in Glick, D. *Methods of Biochemical Analysis*, New York **1** (1954) 459.
19. Ågren, G. and Eklund, S. *Unpublished results.*
20. Ramachandran, B. V. and Ågren, G. *Biochem. Pharmacol.* **12** (1963) 981.
21. Ramachandran, B. V., Engström, L. and Ågren, G. *Biochem. Pharmacol.* **12** (1963) 167.
22. Ågren, G., de Verdier, C.-H. and Glomset, J. *Acta Chem. Scand.* **7** (1954) 1971.
23. Schauer, R. and Hillman, C. *Z. physiol. Chem.* **225** (1962) 9.
24. Farquhar, J. W. *Biochim. Biophys. Acta.* **60** (1962) 80.
25. Buchanan, A. A. *Biochem. J.* **75** (1960) 315.
26. Marks, P. A., Johnsson, A. B., Hirschberg, E. and Banks, J. *Ann. N. Y. Acad. Sci.* **75** (1958) 95.
27. Fleming, A. Brit. *J. Exptl. Pathol.* **7** (1926) 281.

Received July 2, 1964.