Isolation of a ^{32}P-Labeled Polypeptide of Low Molecular Weight from Phosphorylated Human Erythrocyte Membranes†

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ABSTRACT: The incorporation of ^{32}P into well washed human erythrocyte membranes was studied in a medium containing [γ-^{32}P]ATP, Mg^{2+}, and EGTA. Following phosphorylation, the membranes were completely solubilized in 1% sodium dodecyl sulfate and subjected to gel electrophoresis in dodecyl sulfate polyacrylamide. A large incorporation of radioactivity was observed in a band which migrated faster than component 7 (nomenclature of T. L. Steck, (1972), *J. Mol. Biol. 66,* 295) but slower than the bromophenol blue tracking dye, and did not stain with Coomassie Blue. Isolation of this band by preparative gel electrophoresis revealed that 41% of the radioactivity was associated with a ^{32}P-labeled polypeptide. This polypeptide was further purified by gel chromatography on Sephadex LH-20 in chloroform–methanol–HCl, and Bio-Gel A 1.5m in dodecyl sulfate. Its amino acid composition is characterized by a high content of acidic residues. The calculated minimal molecular weight is 15084. Based upon the recovery of amino acids, the polypeptide fraction comprises at least 1.8% by weight of the total erythrocyte membrane proteins. An apparent molecular weight of 15000 was estimated by gel chromatography in dodecyl sulfate, while a range of 14000–16000 was estimated by electrophoresis in dodecyl sulfate polyacrylamide. The state of phosphorylation of this peptide may reflect a physiological function in the intact red cell.

Results from several laboratories have amply demonstrated that well washed human erythrocyte membranes will catalyze the transfer of ^{32}P from ATP to several polypeptide and phospholipid membrane components (Avruch and Fairbanks, 1972, 1974a,b; Guthrow et al., 1972; Rubin et al., 1972; Rubin and Rosen, 1973; Roses and Appel, 1973). Both the phosphate receptors and the kinase enzymes catalyzing the reactions appear to be endogenous to the membranes. Unlike the well-defined role of phosphorylation reactions in the activation of phosphorylase (Krebs and Fischer, 1956; Rall et al., 1956), however, the physiological function of phosphorylation reactions in cell membranes is still unclear. In a recent report in this direction, Rudolph and Greengard (1974) using intact turkey erythrocytes showed that β-adrenergic agents stimulated the incorporation of ^{32}P into a single membrane component with the same time course as the increase in membrane sodium permeability.

Guthrow et al. (1972) studied the phosphorylation of human erythrocyte membranes in the presence and absence of cAMP,[1] using gel electrophoresis in dodecyl sulfate polyacrylamide to resolve the labeled membrane components. They identified several radioactive bands, including a fast moving band migrating in the lipid region of the gel. This band did not stain with Coomassie Blue and was characterized by them as consisting of ^{32}P labeled di- and triphosphoinositides. Avruch and Fairbanks (1974,b), in a particularly detailed study of the effect of various ions on the pattern of the phosphorylation reaction, also observed a fast moving band of radioactivity which they identified as ^{32}P-labeled phospholipids.

In our studies on the phosphorylation of human erythrocyte membranes a similarly fast moving radioactive band was also observed. This band migrated behind the bromophenol blue tracking dye and did not stain with Coomassie Blue. Isolation by preparative gel electrophoresis on dodecyl sulfate polyacrylamide has revealed that about 41% of the radioactivity consists of a ^{32}P-labeled polypeptide with an estimated molecular weight of 15000. It is the aim of this report to describe the isolation and partial characterization of this polypeptide, a previously unreported constituent of the human erythrocyte membrane.

Materials and Methods

Source of Materials. [γ-^{32}P]ATP with a specific activity of 5–10 μCi/nmol was purchased from New England Nuclear Corp.; Sephadex G-100 and Sephadex LH-20 were from Pharmacia Fine Chemicals Inc.; Bio-Gel A 1.5m, 100–200 mesh, was from Bio-Rad Laboratories. Polypeptide markers were from Sigma Chemical Co. All other chemicals were of reagent grade and were utilized without prior purification.

Preparation of Erythrocyte Membranes. The procedure of Dodge et al. (1963) was used. Fresh blood was diluted with 2 volumes of cold isotonic saline containing 0.15 *M* NaCl and 20 mosm sodium phosphate (pH 7.4) and centrifuged at 2000*g* for 10 min. The supernatant was removed by aspiration and the surface of the packed red cells was thoroughly aspirated to remove the buffy coat. The washing, centrifugation, and aspiration procedure was repeated

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[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; cAMP, cyclic adenosine 3',5'-monophosphate.

three times. The packed red cells were hemolyzed by rapid dilution with 30 volumes of cold 20 mosm sodium phosphate (pH 7.4) hypotonic buffer. The dark red suspension was centrifuged at 50000*g* for 10 min, and the supernatant decanted by aspiration. The pellet was washed with 40 volumes of cold hypotonic buffer and recentrifuged, and the supernatant was decanted by aspiration, taking care to remove the hard packed cream colored "button" under the loose layer of erythrocyte membranes, as described by Fairbanks et al. (1971). The washing, centrifugation, and decantation procedure was repeated assiduously seven times in order to remove last traces of hemoglobin. The packed erythrocyte membranes were finally washed with nine volumes of hypotonic buffer containing 11 m*M* $MgCl_2$ and 0.33 m*M* EGTA, and set aside on ice for phosphorylation experiments.

Phosphorylation of Erythrocyte Membranes. Freshly prepared erythrocyte membranes were suspended at a concentration of 1 mg of membrane protein per ml in a reaction medium containing 20 mosM sodium phosphate (pH 7.4), 10 m*M* $MgCl_2$, and 0.3 m*M* EGTA, at 30°. Phosphorylation was initiated by the addition of 50 nmol (in 50 μl) of [γ-^{32}P]ATP (0.1–0.2 μCi/nmol) per mg of membrane protein, and stopped after 5 min by the rapid addition of 5% Cl_3CCOOH. The reaction mixture was then centrifuged at 50000*g* for 5 min, and the supernatant discarded. The tightly packed pellet was broken up with a stirring rod, suspended in 40 volumes of cold water, and centrifuged. The washing procedure was repeated a second time, and the pellet of phosphorylated membrane was solubilized in 1% dodecyl sulfate containing 10 m*M* sodium phosphate (pH 7) and 50 m*M* dithiothreitol. The solution was quickly brought to 80° for 2 min and stirred for a few hours at room temperature to bring about complete solubilization, at a final protein concentration of 4–5 mg/ml. The solution was frozen and saved for analytical and preparative gel electrophoresis.

Analytical Gel Electrophoresis. Gel electrophoresis was performed according to the procedure of Weber and Osborn (1969) in 10% polyacrylamide gels containing 0.1 *M* sodium phosphate (pH 7), 0.1% dodecyl sulfate, and an acrylamide to methylenebisacrylamide weight ratio of 37:1. Gels were cast in 5 × 125 mm dimensions. Samples subjected to electrophoresis contained 50–150 μg of membrane protein dissolved in a maximum volume of 100 μl containing 10 m*M* sodium phosphate (pH 7), 1% dodecyl sulfate, 50 m*M* dithiothreitol, and 10% sucrose. Electrophoresis was carried out for 17 hr at room temperature at a current of 2.5 mA/gel tube. Some of the gels were stained with Coomassie Blue, while others were sliced directly and the slices counted for radioactivity. For staining, the gels were transferred to a solution containing 25% v/v 2-propanol, 10% v/v acetic acid, 0.25% Coomassie Blue, and allowed to stain for 6 hr at room temperature. They were destained by diffusion in a solution containing 40% v/v methanol and 10% v/v acetic acid. For counting, the gels were cut into 5-mm slices, and the slices transferred into 15 × 50 mm polyethylene counting vials filled with water and counted by Cerenkov radiation. The counting efficiency under these conditions was about 35%.

Preparative Gel Electrophoresis. Gel electrophoresis on a preparative scale was carried out in a Shandon Model MK II apparatus using a 50-ml gel bed of 10% polyacrylamide in 0.1 *M* sodium phosphate (pH 7) and 0.1% dodecyl sulfate and polymerized according to the formulation of Weber and Osborn (1969). Samples for electrophoresis contained 10–20 mg of membrane protein at a concentration of 5 mg/ml in 10 m*M* sodium phosphate (pH 7), 1% dodecyl sulfate, 50 m*M* dithiothreitol and 10% sucrose. The upper and lower buffer reservoir were filled with 0.1 *M* sodium phosphate (pH 7) and 0.1% dodecyl sulfate. Following a preelectrophoresis run for 4 hr at 50 mA, the sample was slowly layered over the gel surface using a peristaltic pump. Electrophoresis was started at 50 mA and carried out for 40 hr. The elution chamber at the bottom of the gel column was swept continuously with 0.1 *M* sodium phosphate (pH 7) and 0.1% dodecyl sulfate at a flow rate of 20 ml/hr using a peristaltic pump; fractions of 10 ml were collected. Due to large shifts in pH in the upper buffer reservoir, buffer was circulated between the upper and lower reservoir with a peristaltic pump. The collected fractions were monitored for radioactivity by Cerenkov counting and the appropriate fractions under the radioactive peaks were pooled. Aliquots were subjected to gel chromatography on Sephadex G-100 and the remaining solution was dialyzed exhaustively against cold water, lyophilized to dryness, and saved for further characterization.

Determination of ^{32}P-Labeled Serine Phosphate. ^{32}P-labeled fractions were analyzed for ^{32}P-labeled serine phosphate by partial hydrolysis in 6 *N* HCl, followed by cochromatography with carrier serine phsophate on Dowex 1-X8 according to the procedure reported by Barela and Kizer (1974). Samples to be analyzed were hydrolyzed in 3 ml of 6 *N* HCl at 108° for 5 hr in sealed, evacuated tubes. The hydrolysates were rotary evaporated to dryness, dissolved in 2 ml of 0.1 *M* formic acid containing 4 μmol of carrier serine phosphate, and applied to a 1.5 × 7 cm column of Dowex 1-X8 equilibrated with 0.1 *M* formic acid. The column was first eluted with 0.1 *M* formic acid and 15 fractions of 3 ml were collected at a flow rate of 1 ml/min. Elution was then continued with a solution containing 0.25 *M* formic acid and 0.25 *M* pyridine, and a total of 40 fractions was collected. Aliquots of 1 ml from each fraction were subjected to ninhydrin assay according to the procedure of Stein and Moore (1948). The remaining 2 ml was counted for radioactivity in 10 ml of scintillation fluid prepared by dissolving 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, and 0.375 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of dioxane.

Gel Chromatography in Chloroform–Methanol–HCl. Sephadex LH-20 was equilibrated with methanol and packed in a 2.5 × 90 cm column equipped with solvent resistant Teflon fittings (Glenco Scientific, Inc.). The column was then equilibrated with a solvent mixture consisting of 2 volumes of chloroform and 1 volume of methanol, containing 10 m*M* HCl, and eluted with the same solvent. The collected fractions were monitored for radioactivity by Cerenkov counting, rotary evaporated to dryness, and saved for further characterization.

The column was calibrated with a solution containing a mixture of cytochrome *c*, [γ-^{32}P]ATP, and $^{32}P_i$. This solution was prepared by dissolving 5 mg of cytochrome *c* (Sigma, C-2506) in 10 ml of chloroform–methanol–HCl, followed by the addition of 25 nmol of [γ-^{32}P]ATP and 25 nmol of $^{32}P_i$ in 1 μl of water. It was clarified at 20000*g* for 5 min, and applied to the column. The elution of cytochrome *c* was monitored by optical density measurement at 400 nm, while the elution of [γ-^{32}p]ATP and $^{32}P_i$ was monitored by Cerenkov counting. Moreover, to obtain information on the elution of phospholipids from the column, a so-

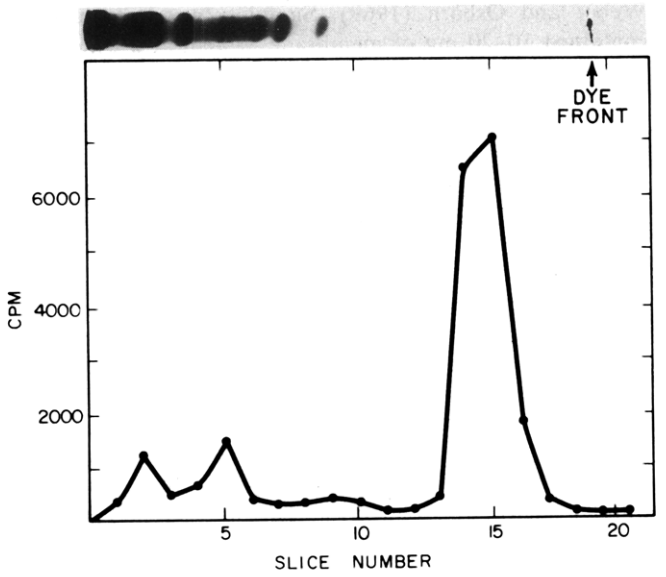

FIGURE 1: Radioactivity profile of phosphorylated erythrocyte membranes after disc gel electrophoresis on 10% polyacrylamide containing 0.1% dodecyl sulfate. Aliquots containing 135 μg of membrane protein were applied to each gel. After electrophoresis, four parallel gels were sliced into 5-mm segments and counted for radioactivity as described under Materials and Methods. A parallel gel was stained with Coomassie Blue and the staining pattern is shown at the top of the figure.

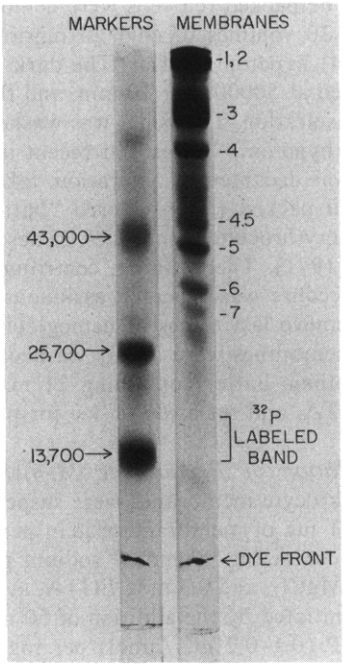

FIGURE 2: Coomassie Blue staining pattern of phosphorylated erythrocyte membranes after disc gel electrophoresis in 10% polyacrylamide containing 0.1% dodecyl sulfate. An aliquot containing 135 μg of membrane protein was subjected to electrophoresis (right-hand gel). The bracket shows the position of the fast moving band of radioactivity (slices 14 and 15 in Figure 1) in relation to the other polypeptide components of the erythrocyte membrane. The major bands are labeled according to the numerical system of Steck (1972). The left-hand gel shows the staining pattern of three polypeptide markers of known molecular weight; ovalbumin, 43000; chymotrypsinogen, 25700; ribonuclease a, 13700. The position of the bromophenol blue dye front was marked by piercing the gel with a needle dipped in india ink.

lution of phosphatidylcholine was chromatographed in a separate run. Its elution from the column was monitored by evaporating the collected fractions to dryness and examining the residues for phosphatidylcholine by thin-layer chromatography. By this procedure, phosphatidylcholine was found to elute from the LH-20 column in a volume coinciding with that of ATP.

Gel Chromatography in 0.1% Dodecyl Sulfate. Columns of 2.5 × 90 cm packed with Sephadex G-100, or Bio-Gel A 1.5m, were equilibrated and eluted with 0.1 *M* sodium phosphate (pH 7) and 0.1% dodecyl sulfate. The effluent line was attached to a peristaltic pump and the column eluted at a flow rate of 20 ml/hr in the case of Sephadex G-100, and 40 ml/hr in the case of Bio-Gel A 1.5m. Fractions of 10 ml were collected and each fraction was monitored for radioactivity by Cerenkov counting. The appropriate fractions were pooled, dialyzed exhaustively against cold water, lyophilized to dryness, and saved for further characterization.

Amino Acid Analysis. Analyses were performed on a Spinco Model 120C automatic amino acid analyzer. Samples for analysis were hydrolyzed in 3 ml of 6 *N* HCl for 24 hr at 108° in sealed, evacuated tubes. The hydrolysates were rotary evaporated to dryness under vacuum, the residue was dissolved in 0.2 *N* citrate buffer (pH 2.2), and aliquots were subjected to amino acid analysis in duplicate.

Other Methods. Protein concentration was determined with the biuret reagent of Gornall et al. (1949) by optical density measurement at 320 nm, using bovine serum albumin as a standard. Membrane protein was solubilized with dodecyl sulfate prior to assay. In a typical assay, 100 μg of protein in 100 μl when reacted with 400 μl of biuret reagent in a 1-ml capacity cuvette gives an optical density reading of 0.240.

All radioactivity measurements were performed in a Nuclear Chicago Unilux II scintillation counter.

Results and Discussion

Phosphorylation of Erythrocyte Membranes. The radioactivity profile of phosphorylated erythrocyte membranes after disc gel electrophoresis on 10% polyacrylamide is shown in Figure 1. A major incorporation of ^{32}P can be seen in a broad, fast moving band (slices 14 and 15), and a lesser incorporation in slower moving bands corresponding to components 3 and 4.5 of the erythrocyte membrane. When 5% Cl_3CCOOH was first added to the reaction mixture followed by the addition of [γ-^{32}P]ATP, no ^{32}P was incorporated in the membrane, thereby demonstrating the ability of Cl_3CCOOH to stop the reaction. Moreover, when [γ-^{32}P]ATP and $^{32}P_i$ were added to nonlabeled membranes dissolved in 1% dodecyl sulfate and the solution was subjected to electrophoresis, all the radioactivity migrated well ahead of the bromophenol blue tracking dye, a control experiment which demonstrates the absence of anomalous binding of radioactivity during electrophoresis. Figure 2 is a Coomassie Blue staining profile of phosphorylated erythrocyte membranes after gel electrophoresis in 10% polyacrylamide, and shows the pattern of migration of the ^{32}P-labeled fast moving band in relation to the known polypeptide components of the erythrocyte membrane. As can be seen in the figure, this band migrates faster than component 7 but slower than the bromophenol blue tracking dye, with an apparent molecular weight larger than that of ribonuclease a (13700). It does not stain with Coomassie Blue but appears as a white, opalescent, band characteristic for phospholipids (Lopez and Siekevitz, 1973). Although the broadness of the

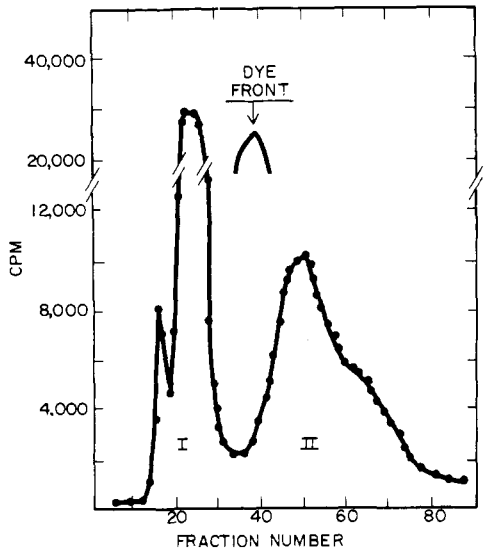

FIGURE 3: Preparative gel electrophorese of phosphorylated erythrocyte membranes on 10% polyacrylamide containing 0.1% dodecyl sulfate. An aliquot containing 11.5 mg of membrane protein solubilized in 1% dodecyl sulfate was subjected to electrophoresis as described under Materials and Methods. Fractions 18-28 (peak I) and fractions 40-70 (peak II) were pooled.

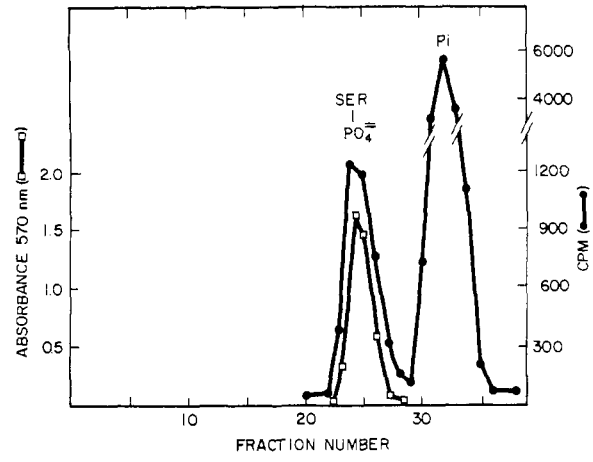

FIGURE 4: Determination of ^{32}P-labeled serine phosphate in a partial hydrolysate of peak II from Figure 3. An aliquot containing 0.5 nmol of ^{32}P was hydrolyzed in 6 *N* HCl for 5 hr at 108° and cochromatographed with 4 μmol of carrier serine phosphate on a 1.5 × 7 cm column of Dowex 1-X8. For more details see Material and Methods. (●) ^{32}P counts; (□) absorbance at 570 nm after ninhydrin assay.

band introduces uncertainty in the location of the polypeptide component in the gel, and precludes accurate estimation of its molecular weight, a range of 14000–16000 is indicated.

Addition of 2 μ*M* cAMP to the reaction mixture did not enhance its phosphorylation, in accord with the observations of Guthrow et al. (1972), and Avruch and Fairbanks (1974a,b).

Based upon the time dependence of ^{32}P incorporation, the reaction appeared to reach a plateau after 20 min. In view of the high ATP to membrane ratio in the reaction mixture, ATP depletion is unlikely (Rubin and Rosen, 1973).

Preparative Gel Electrophoresis of Phosphorylated Erythrocyte Membranes. The ^{32}P elution profile of 11.5 mg of phosphorylated erythrocyte membranes subjected to preparative gel electrophoresis on 10% polyacrylamide containing 0.1% dodecyl sulfate is shown in Figure 3. Two main peaks of radioactivity can be seen: peak I, a sharp peak migrating ahead of the bromophenol blue tracking dye in the gel, and peak II, a broad peak migrating behind the tracking dye. Small aliquots from peaks I and II were subjected to gel chromatography on Sephadex G-100 in 0.1 *M* sodium phosphate and 0.1% dodecyl sulfate. Peak I emerged in the salt volume of the column and was presumed to contain mainly unreacted [γ-^{32}P]ATP and $^{32}P_i$, compounds known from control experiments to migrate ahead of the tracking dye during electrophoresis; no further examination of peak I was undertaken. Peak II emerged from the column in a volume well separated from the salt volume, suggesting that it is macromolecular in nature. Rechromatography of peak II on the same column in the presence of 1% dodecyl sulfate did not split the peak or change its position of elution. The total radioactivity under peak II corresponded to 3.55 nmol of ^{32}P. Fractions 40–70 were pooled and dialyzed exhaustively against cold water. The dialyzed solution was divided into aliquots and lyophilized to dryness. Analytical gel electrophoresis on an aliquot showed the presence of a broad band similar to the band in Figure 1 (slices 14 and 15), and the total absence of Coomassie Blue staining material.

Amino acid analysis revealed the presence of all the common amino acids, while partial acid hydrolysis in 6 *N* HCl at 108° for 5 hr revealed the presence of ^{32}P-labeled serine phosphate. The results of the serine phosphate determination are shown in Figure 4. Two radioactive peaks may be seen: the first peak eluting between fractions 23 and 27 with a profile identical with 570 nm ninhydrin absorbance profile of added serine phosphate carrier, and a second peak eluting between fractions 30 and 35 and corresponding to the elution position of inorganic phosphate. Of the radioactivity added to the column, 90% was recovered. The yield of ^{32}P-labeled serine phosphate after correction for 65% loss during hydrolysis amounted to 41% of the radioactivity pooled under peak II before dialysis and lyophilization.

The remaining radioactivity in peak II consists most likely of ^{32}P-labeled phosphoinositides. This conclusion is based upon the findings of several investigators. Guthrow et al. (1972) studied the phosphorylation of human erythrocyte membranes in a medium containing 3–4 nmol of [γ-^{32}P]ATP/mg of membrane protein, using a reaction time of 5 min at 30°. They observed a fast moving band of radioactivity which they characterized as ^{32}P-labeled di- and triphosphoinositides by thin-layer chromatography. Avruch and Fairbanks (1972), using 4 nmol of [γ-^{32}P]ATP/mg of membrane protein, and a reaction time of 5 sec, observed a similar band of radioactivity which they ascribed to ^{32}P-labeled phospholipids, but raised the possibility that it might also contain ^{32}P-labeled polypeptide of low molecular weight. Finally, several studies (Palmer and Verpoorte, 1971; Schneider and Kirschner, 1970; Hokin and Hokin, 1964), dealing with the phosphorylation of erythrocyte membranes more from a standpoint of the metabolism of phospholipids, have also demonstrated the rapid formation of ^{32}P-labeled di- and triphosphoinositides.

Sephadex LH-20 Chromatography of Peak II (Figure 3) in Chloroform–Methanol–HCl. The ^{32}P radioactivity in an aliquot of dialyzed and lyophilized peak II was readily extractable in 5–10 ml of chloroform–methanol–HCl by stirring for 30 min at room temperature, or in the cold. The solution was clarified by centrifugation at 20000 *g* for 15 min and the clear chloroform–methanol solution was chromatographed on a column of Sephadex LH-20 as shown in

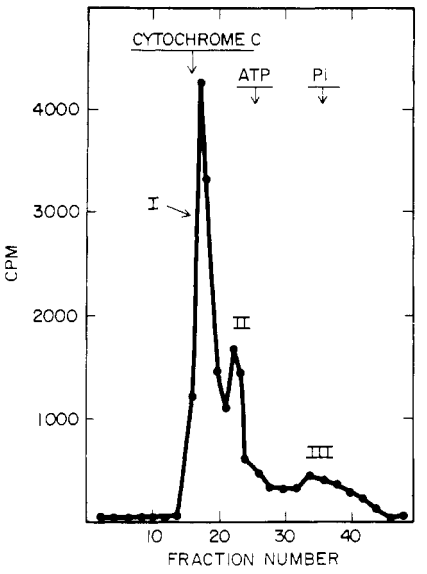

FIGURE 5: Gel chromatography on Sephadex LH-20 in chloroform–methanol–HCl of an aliquot of peak II obtained by preparative gel electrophoresis. The 2.5 × 90 cm column was eluted with chloroform–methanol, 2:1, containing 10 m*M* HCl and fractions of 10 ml were collected at a flow rate of 1 ml/min. Fractions were monitored for ^{32}P by Cerenkov counting, and fractions 17, 18, and 19 were pooled. The elution positions of cytochrome *c*, [γ-^{32}P]ATP, and $^{32}P_i$ are shown by the arrows at the top of the figure, and were determined in a separate calibration run. All the ^{32}P counts added to the column were recovered. See Materials and Methods for more details.

Table I: Amino Acid Composition of Peak I from the Bio-Gel A 1.5m Column.[a]

Amino Acid	nmol of Amino Acid in 26.2 μg of Polypeptide[b]	mol of Amino Acid in 15000 g of Polypeptide[c]
Aspartic acid	24.6	14
Threonine[d]	10.5	6
Serine[d]	20.5	12
Glutamic acid	30.2	17
Proline	9.0	5
Glycine	35.0	20
Alanine	23.8	14
Valine	13.5	8
Methionine	2.8	2
Isoleucine	14.5	8
Leucine	22.7	13
Tyrosine	7.2	4
Phenylalanine	10.6	6
Histidine	1.7	1
Lysine	9.3	5
Arginine	12.5	7

[a] Based on 24-hr hydrolysis in 6 *N* HCl at 108°. [b] Original amino acid composition data showing the recovery of polypeptide. [c] Amino acid composition calculated for estimated molecular weight of 15000 and rounded to the nearest integer. [d] Not corrected for destruction. Cystine and tryptophan were not determined.

Figure 5. Of the total radioactivity added to the column, 50% emerged as a sharp peak (peak I) in the void volume of the column, while the remaining 50% of the radioactivity emerged as lower molecular weight material in peaks II and III. We examined peak I for its serine phosphate content and found that now 80% of the radioactivity in that peak consisted of ^{32}P-labeled serine phosphate, in contrast with 41% before chromatography. These results suggest that peak II from Figure 3 contains ^{32}P-labeled phospholipids in tight association with polypeptide, and that this phospholipid can be separated by gel chromatography in chloroform–methanol–HCl. The ability of the LH-20 column to remove contaminating phospholipids is based on the observation that phosphatidylcholine coelutes from the column with ATP, in a volume well separated from that of the main peak (Figure 5). The possibility of contamination by more complex lipid material of higher molecular weight, however, cannot be ruled out. Gel chromatography in chloroform–methanol mixtures has been particularly utilized for the "delipidation" of protein–lipid complexes from brain tissues (Mokrasch, 1972).

Peak I fractions from several chromatographic runs were pooled and rotary evaporated to dryness under water suction, and the residue was dried under high vacuum in order to remove last traces of HCl. The radioactivity in the residue was insoluble in aqueous buffer solutions, even in the presence of 8 *M* urea, but dissolved readily upon addition of dodecyl sulfate.

Chromatography of Peak I (Figure 5) on Bio-Gel A 1.5m in 0.1% Dodecyl Sulfate. An aliquot from peak I was chromatographed on a 2.5 × 90 cm column Bio-Gel A 1.5m equilibrated with 0.1 *M* sodium phosphate (pH 7) and 0.1% dodecyl sulfate. Two peaks were obtained: peak I, a sharp, symmetrical peak, comprising 80% of the radioactivity applied to the column, and peak II, a smaller peak comprising 20% of the radioactivity and emerging in the salt volume of the column. It seems likely that the radioactivity seen in this peak originated either from the breakdown of peptide bound, acid labile, ^{32}P in the chloroform–methanol–HCl environment, or from some lower molecular weight contaminant not removed by gel chromatography on LH-20.

The molecular weight of the main peak was estimated by calibrating the column of Bio-Gel with polypeptides of known molecular weights. Its elution volume was very close to that of hemoglobin subunits, and an apparent molecular weight of 15000 was estimated. This value is in basic agreement with the molecular weight range of 14000–16000 estimated by disc gel electrophoresis on 10% polyacrylamide.

Amino Acid Composition of Peak I from the Bio-Gel Column. An aliquot containing 1.6 nmol of ^{32}P was thoroughly dialyzed against cold distilled water, lyophilized, and subjected to amino acid analysis. The results are shown in Table I and demonstrate the polypeptide nature of peak I. The left-hand column shows the original amino acid analysis data, from which was calculated a polypeptide recovery of 26.2 μg. Since the hydrolyzed sample originated from an aliquot equivalent to 1.5 mg of erythrocyte membrane, peak I polypeptides comprise at least 1.8% by weight of the total protein content of the membranes. The right-hand column shows the amino acid composition data for a calculated minimal molecular weight of 15084 based upon the histidine content. Also readily calculable from the radioactivity content of the analyzed sample is a ^{32}P incorporation stoichiometry of 0.11 mol per 15000 g of peak I polypeptide. The low stoichiometry of ^{32}P suggests that the polypeptide may be already partially phosphorylated in the intact red cell, or that it is contaminated with nonlabeled polypeptides. We also cannot discount the possibility that it is being dephosphorylated by an endogenous, membrane bound, protein phosphatase (Delorenzo and Greengard, 1973).

We find that when normal erythrocyte membranes are completely solubilized in 1% dodecyl sulfate and subjected to electrophoresis in dodecyl sulfate polyacrylamide, the

membrane phospholipids migrate behind the tracking dye as a broad band 0.5–1.0 cm wide that does not stain with Coomassie Blue, but appears as a white opalescent band upon destaining in 40% v/v methanol containing 10% v/v acetic acid. After phosphorylation of the membranes with [γ-^{32}P]ATP, this band is seen as a broad zone of radioactivity (Figure 1, slices 14 and 15) comprising of a mixture of ^{32}P-labeled polypeptides and phospholipids. It is suggested that its failure to stain with Coomassie Blue probably stems from two possible reasons: (a) the small quantity of ^{32}P-labeled polypeptide in the gel (1.8 μg per 100 μg of membrane protein, and (b) the acidic nature of the polypeptide, polypeptides rich in acidic amino acids being known to stain poorly with Coomassie blue (Fairbanks et al., 1971). The possibility that the polypeptide is a proteolytic degradation product of a larger protein cannot be ruled out. To minimize this, hemolysis and washing of the membranes were carried out at ice cold temperature, and the membranes were dissolved in 1% dodecyl sulfate by heating at 80° for 2 min.

In preliminary experiments, whole blood has been phosphorylated by incubation with $^{32}P_i$ as described by Palmer and Verpoorte (1971). The isolated membranes revealed a ^{32}P labeling pattern similar to the one in Figure 1, an indication that the same polypeptide may be labeled in the intact red cell. Whether its state of phosphorylation reflects a physiological function in the cell would be of great interest for future investigation.

Acknowledgment

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