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An Analysis of the Autophosphorylation of Rabbit and Human Erythrocyte Membranes†

M. Marlene Hosey and Mariano Tao*

ABSTRACT: The autophosphorylation of rabbit and human erythrocyte membranes has been studied under various experimental conditions. The phosphopeptides of the erythocyte membranes were identified using sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis followed by radioautography. The pattern of phosphorylation of membrane components differs with respect to the phosphoryl donor used (ATP or GTP) and to the pH at which the reaction is carried out. Both species appear to contain at least two distinct membrane-bound protein kinases. The human erythrocyte membrane contains a cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinase and several substrates for this kinase. Only ATP can be used as a phosphoryl donor for this kinase. In contrast, the rabbit erythrocyte membrane does not contain a cyclic AMP dependent protein kinase but does contain a kinase which utilizes only ATP as the phosphoryl donor and is specific for certain endogenous substrates at low pH. Both the human and rabbit erythrocyte membranes contain a kinase which utilizes GTP, perhaps also ATP, as the phosphoryl donor. The substrates of these kinases are similar in both species.

Considerable interest has been focused on the problem of whether the enzymic phosphorylation and/or dephosphorylation of membrane proteins can result in altered functional properties of those proteins. Recent observations have implicated membrane phosphorylation in such diverse cellular processes as neuronal transmission (Greengard, 1975), ATPase activation (Knauf et al., 1974; Katz and Blostein, 1975), and insulin-stimulated glucose transport (Chang et al., 1974). In addition, it has been reported that certain muscular dystrophies may be associated with altered erythrocyte membrane phosphorylation (Roses and Appel, 1975; Roses et al., 1975).

In an attempt to further elucidate the factors controlling the processes of membrane protein phosphorylation, we have initiated studies dealing with rabbit erythrocyte membrane preparations. In contrast to results obtained with human erythrocyte membranes (Guthrow et al., 1972; Fairbanks and Avruch, 1974), we have not been able to detect any cyclic adenosine 3′,5′-monophosphate (cyclic AMP)[1]

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[1] The abbreviations used are cAMP, cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; NaDodSO$_4$, sodium dodecyl sulfate; mol wt, molecular weight.

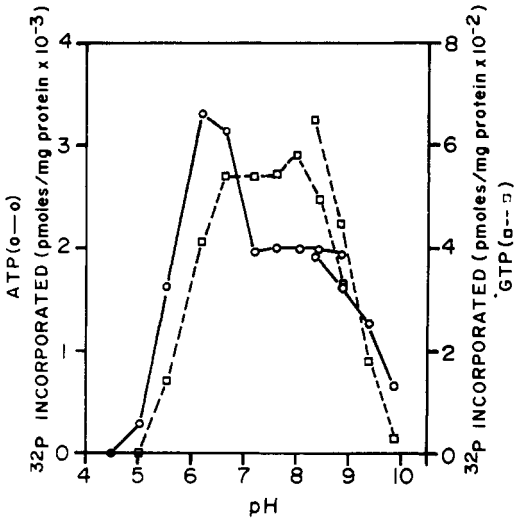

FIGURE 1: Effect of pH on the autophosphorylation of rabbit erythrocyte membrane proteins. Membranes were phosphorylated as described for 5 min at 37 °C with either [γ-^{32}P]ATP (25 cpm/pmol) or [γ-^{32}P]GTP (33 cpm/pmol). The buffers (0.1 M) used were sodium acetate, pH 4.5, 5.0, and 5.5; Tris-acetate, pH 6.0 and 6.5; Tris-Cl, 7.0, 7.5, 8.0, 8.5, and 9.0; and glycine-NaOH, pH 8.5, 9.0, 9.5, and 10.0.

dependent protein kinase activity in rabbit erythrocyte membrane preparations. However, two distinct patterns of autophosphorylation of membrane proteins are obtained depending on the phosphoryl donor used, ATP or GTP. This communication reports on the properties of the rabbit erythrocyte membrane phosphorylating system. In addition, we have compared this system with that of the human erythrocyte membrane.

Experimental Procedures

Preparation of Cell Membranes. Hemoglobin-free ghosts of rabbit or human erythrocytes were prepared from freshly drawn blood as previously described (Hosey and Tao, 1975) according to the method of Dodge et al. (1963).

Protein Phosphorylation. The incorporation of ^{32}P into proteins from either [γ-^{32}P]ATP or [γ-^{32}P]GTP was determined by a modification of the procedure described by Tao and Hackett (1973). In addition to membranes, the incubation mixture contained the following in a final volume of 0.1 ml: 0.1 M Tris-acetate, pH 6.0; 10 mM $MgCl_2$; 0.2 mM [γ-^{32}P]ATP; ±2 μM cyclic AMP or guanosine cyclic 3′,5′-monophosphate; and ± exogenous protein substrates. When [γ-^{32}P]GTP (0.2 mM) was used as the phosphoryl donor, the buffer was replaced by 0.1 M glycine-NaOH, pH 8.5.

The study of autophosphorylation was conducted at substrate amounts (100–200 μg) of membrane proteins. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. When incorporation of ^{32}P into exogenous substrates was to be determined, the incubation mixture contained 1.8 mg/ml of added substrate and catalytic amounts (approximately 20 μg) of membrane proteins.

The reaction was initiated by the addition of membranes and the incubation was carried out at 37 °C. To terminate the reaction, 2 ml of 10% trichloroacetic acid and 30 μl of 12 mg/ml of bovine serum albumin were added in rapid succession to each tube. The samples were then processed as previously described (Tao and Hackett, 1973).

Electrophoretic Analysis. Analysis of phosphorylated membrane components was carried out by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) followed by radioautography. Electrophoresis was performed according to the method of Fairbanks et al. (1971) at pH 7.4 on 5 or 5.6% acrylamide gel slabs containing 5% sucrose and either 0.2 or 1% $NaDodSO_4$. Each of the gel slabs (10 × 14 × 0.15 cm; Hoeffer Scientific Instruments) contained ten wells for sample application. The membranes were phosphorylated as described above except that the reaction volume was reduced by half. In these experiments, the amounts of membrane proteins applied ranged from 20 to 60 μg and the specific activities of the nucleotides used were 150–600 cpm/pmol. The reaction was terminated by adding 25 μl of a solution containing 15% sucrose; 6% $NaDodSO_4$; 30 mM Tris-HCl, pH 8.0; 3 mM EDTA; and 12 mg/ml dithiothreitol. The samples were immediately placed in a boiling water bath for 2 min and 5 μl of tracking dye (50 mg % bromophenol blue) was added to each sample. The entire mixture was applied to the gel and electrophoresed at 45–60 mA/gel slab until the dye front approached the bottom of the gel (4–6 h). During the run, the electrophoresis unit was cooled with circulating tap water.

The gels were stained for proteins with Coomassie blue and for glycoproteins with periodic acid–Schiff reagent according to the procedures of Fairbanks et al. (1971) as modified by Steck and Yu (1973). The destained gels were dried under vacuum and placed on Kodak No-Screen Medical x-ray film (No. NS-2T) for 2–8 days, depending on the specific activity of the radioisotope used. Densitometric scanning of both stained gels and radioautograms were performed using a Zeineh Soft-Laser densitometer.

Molecular weight determinations were made according to the method of Weber and Osborn (1969) using β-galactosidase (130 000), phosphorylase *b* (93 000), urease (83 000), bovine serum albumin (68 000), hexokinase (51 000), pepsin (35 000), hemoglobin (16 000), and cytochrome *c* (13 000) as standards.

Materials. All reagents for the polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. Radioisotopes were purchased from New England Nuclear. ATP and GTP were obtained from P-L Biochemicals. Cyclic AMP, cyclic GMP, hemoglobin, bovine serum albumin, urease, phosphorylase *b*, pepsin, β-galactosidase, cytochrome *c*, calf thymus histones (type II-A), protamine, and phosvitin were supplied by Sigma Chemical Co. Hexokinase was obtained from Boehringer Mannheim and casein from Schwarz/Mann.

Results

General Properties of Rabbit Erythrocyte Membrane Autophosphorylation. The rabbit erythrocyte membranes contained both protein kinase and phosphoryl acceptor activities. Incubation of erythrocyte membranes with either [γ-^{32}P]ATP or [γ-^{32}P]GTP resulted in the incorporation of radioactivity into trichloroacetic acid insoluble materials. The autophosphorylation of rabbit erythrocyte membranes was dependent on Mg^{2+}. The optimal concentration of Mg^{2+} using ATP as the phosphoryl donor was 10–20 mM, whereas using GTP, Mg^{2+} was optimal at 4–10 mM. For purposes of comparison, all further assays of membrane-bound kinase activity contained 10 mM Mg^{2+}.

Figure 1 shows the effect of pH on the autophosphorylation of rabbit erythrocyte membrane proteins. Broad but distinct pH–activity profiles were obtained for ATP and GTP. The rate of phosphorylation in the presence of ATP

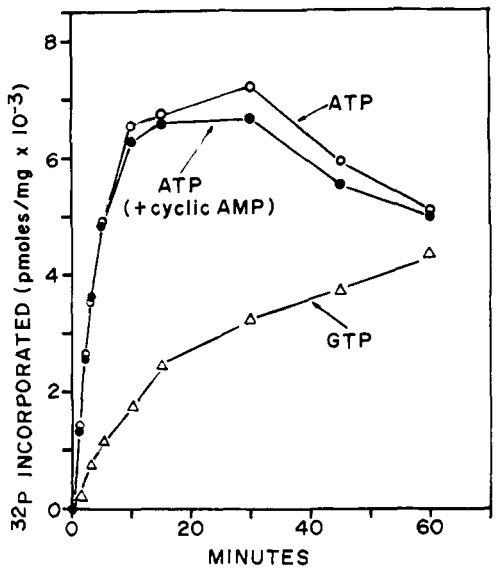

FIGURE 2: Time course of autophosphorylation of rabbit erythrocyte membrane proteins. The phosphorylation of rabbit erythrocyte membranes using either [γ-^{32}P]ATP (20 cpm/pmol) or [γ-^{32}P]GTP (35 cpm/pmol) was carried out in the presence of either 152 or 133 μg of membrane proteins, respectively. Other experimental details are described under Experimental Procedures.

appeared to exhibit a pH optimum between 6.0 and 6.5. This was different from that observed in the presence of GTP where a slight peak of actitivity was obtained at about pH 8.5. The addition of cyclic AMP or cyclic GMP did not alter the pH–activity profiles nor did it increase the amount of phosphate incorporated into the membranes (data not shown). The effect of pH on the autophosphorylation of rabbit erythrocyte membranes in the presence of ATP somewhat resembled that reported for human erythrocyte membranes where a similar activity peak at about pH 6.5 was observed in the presence of cyclic AMP (Guthrow et al., 1972). Since the two phosphoryl donors exhibited different pH–activity profiles, the subsequent experiments (except as noted) were conducted in either Tris-acetate, pH 6.0 (for ATP), or glycine-NaOH, pH 8.5 (for GTP), depending on the substrate used.

The time course of membrane autophosphorylation is illustrated in Figure 2. The incorporation of ^{32}P from [γ-^{32}P]ATP was quite rapid and linear for about 10 min. A decline in ^{32}P incorporated from ATP was observed after 30 min of incubation which might result from a depletion of the substrate due to hydrolysis and from the action of phosphatase on phosphoproteins. Cyclic AMP had no significant effect on the rate of phosphorylation of erythrocyte membranes. The incorporation of ^{32}P from [γ-^{32}P]GTP into membrane proteins was less rapid but remained approximately linear for 15 min.

Effect of NaF on Membrane Phosphorylation and Nucleotide Hydrolysis. The effects of varying concentrations of NaF on ATP:protein phosphotransferase and GTP:protein phosphotransferase activities were determined. Low concentrations of NaF (5–10 mM) appeared to enhance phosphorylation approximately twofold with ATP as the phosphoryl donor. A similar enhancement of phosphorylation was not observed when the phosphoryl group was provided by GTP. In both instances, membrane phosphorylation was inhibited by concentrations of NaF greater than 25 mM (data not shown).

Table I: Phosphorylation of Exogenous Substrates by Rabbit and Human Erythrocyte Membranes in the Presence of ATP.[a]

	Rabbit[b]		Human[b]	
Addition	Control	+ Cyclic AMP	Control	+ Cyclic AMP
None	85	85	30	30
Histone	106	108	44	132
Casein	89	93	53	51
Protamine	55	45	17	25
Phosvitin	107	99	62	57
Bovine serum albumin	113	114	30	35

[a] Phosphorylation was carried out in the presence of 20 μg of membrane proteins at 37 °C for 5 min as described in Experimental Procedures. The specific activity of [γ-^{32}P]ATP was 28 cpm/pmol. [b] In picomoles of ^{32}P incorporated/5 min.

Since NaF is a well-known inhibitor of phosphohydrolyase activity, the effect of NaF on the hydrolysis of ATP and GTP by the rabbit erythrocyte membrane was determined. Membranes were incubated with either [γ-^{32}P]ATP or [γ-^{32}P]GTP in the presence or absence of 5 mM NaF under the conditions described for phosphorylation. The reaction mixture (0.1 ml) was incubated at 37 °C for 5 min, after which 50 μl of 1 N HCl was added to terminate the reaction. Carrier ATP or GTP was then added to each sample. The samples were chromatographed on PEI-cellulose (Brinkman MN polygram cel 300 PEI) using 0.52 M potassium phosphate, pH 7.8, as the solvent (Anthony and Spector, 1972). The radioactivity remaining in the spot corresponding to ATP or GTP was determined and expressed as a percentage of the total radioactivity recovered. Approximately 60% of ATP was hydrolyzed after 5 min of incubation. The presence of 5 mM NaF completely inhibited the breakdown of ATP. Thus the enhancement of the phosphorylation of membrane proteins in the presence of ATP and NaF may be explained, in part, by the preservation of the phosphoryl donor. In contrast, GTP was not appreciably hydrolyzed by the membrane preparations either in the absence or presence of NaF. This was consistent with the observation that NaF had no stimulatory effect on membrane phosphorylation using GTP as the phosphoryl donor.

Phosphorylation of Exogenous Protein Substrates by Membrane-Bound Kinase(s). The possibility that the membrane-bound kinase(s) might also catalyze the phosphorylation of exogenous protein substrates was examined. These studies were carried out using catalytic amounts of membrane proteins. In each of the experiments shown in Table I, correction was made for any nonspecific retention of radioactivity on the filter due to added membrane and exogenous proteins in the absence of a reaction. The results presented in Table I suggest that histones, phosvitin, and bovine serum albumin may be phosphorylated by rabbit erythrocyte membranes in the presence of ATP as the phosphoryl donor. When a similar experiment employing GTP as the phosphorylating agent was performed, only casein was phosphorylated to some degree (data not shown). In no instance did cyclic AMP increase the phosphorylation of the exogenous substrates using either ATP or GTP as the phosphoryl donor.

These results differ from those obtained with human erythrocyte membranes (Table I). Using [γ-^{32}P]ATP as the phosphoryl donor, the phosphorylation of histones by

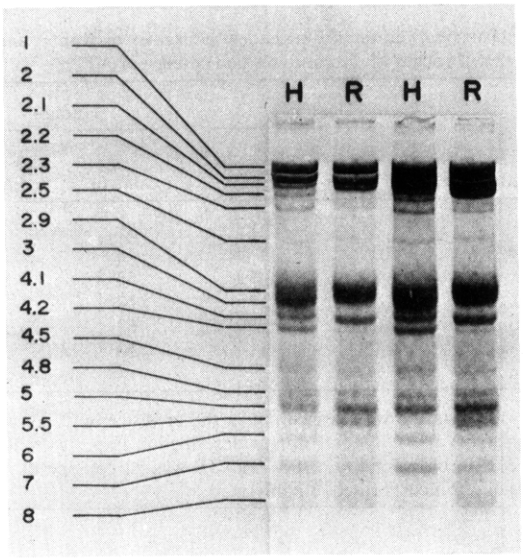

FIGURE 3: Electrophoretogram of the polypeptide components of human (H) and rabbit (R) erythrocyte membranes. Each of the two samples on the left received 11 μg of membrane proteins while those on the right received 22 μg. The electrophoresis was carried out in 5% acrylamide and 0.2% $NaDodSO_4$.

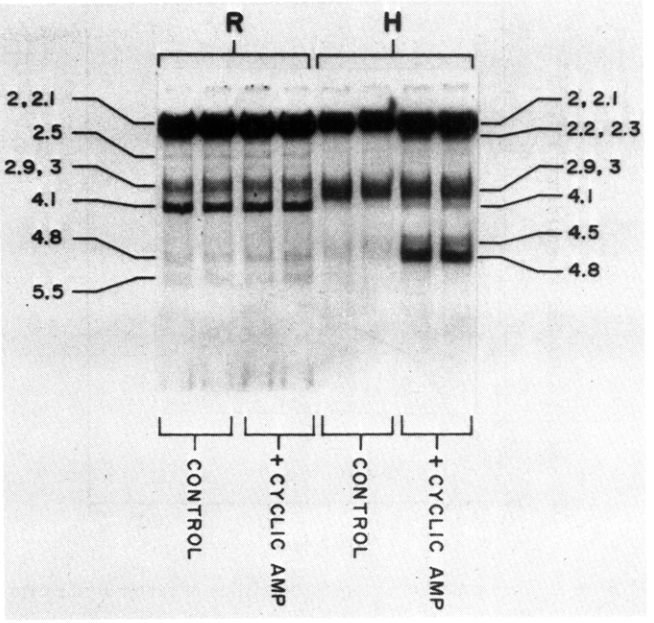

FIGURE 4: Comparison of the radioautographic profiles of rabbit and human erythrocyte membrane components phosphorylated in the presence of [γ-^{32}P]ATP. About 40 μg of either rabbit (R) or human (H) erythrocyte membranes was phosphorylated for 5 min at pH 6.0 in the presence and absence of cyclic AMP. The specific activity of [γ-^{32}P]ATP was 270 cpm/pmol. $NaDodSO_4$–polyacrylamide gel electrophoresis and radioautography were performed as described under Experimental Procedures. The radioautogram was exposed for 3 days.

human erythrocyte membrane-bound kinase was stimulated by cyclic AMP. Although casein and phosvitin could also serve as substrates for the human erythrocyte enzyme, the phosphorylation of these substrates was not affected by cyclic AMP. Whether or not a different kinase is responsible for the phosphorylation of casein and phosvitin or whether these substrates may cause the activation of cyclic AMP-dependent protein kinase by a phenomenon of protein–protein interaction as described previously (Miyamoto et al., 1971; Tao, 1972) remains unknown. In the presence of GTP as the phosphoryl donor, the phosphorylation of exogenous proteins by the human erythrocyte membranes was below measurable levels. Although the results of Table I suggest that certain exogenous proteins may be phosphorylated by the membranes, it must be emphasized that we have not determined whether the addition of exogenous proteins results in an alteration of the phosphorylation of the membrane components.

Identification of Rabbit Erythrocyte Membrane Components by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Although the protein and glycoprotein components of human erythrocyte membranes have been fractionated on $NaDodSO_4$–polyacrylamide gels and extensively studied (for review, see Steck, 1974), little information is available regarding the membrane components of rabbit erythrocytes. Figure 3 shows a $NaDodSO_4$–polyacrylamide slab gel electrophoretogram of human and rabbit erythrocyte membranes performed at two different amounts of protein. In order to facilitate comparison between human (H) and rabbit (R) erythrocyte membrane components, the wells in each pair contained equal amounts of protein. The major polypeptides were numbered according to the system suggested by Steck (1972) based on mobility and molecular weight. Except for a few differences, the electrophoretic pattern of the Coomassie blue stained polypeptides of rabbit erythrocyte membranes had the general appearance of that of the human. The major difference between the Coomassie blue stained polypeptides of the rabbit and human erythrocyte membranes appeared in the area of bands 4.1 and 4.2. While two bands of similar staining intensity were found in this region of the human erythrocyte membrane in 0.2% $NaDodSO_4$, one major band appeared in the rabbit erythrocyte membrane (in both 0.2 and 1.0% $NaDodSO_4$). Minor differences between the membranes of the two species were found in bands 2.2, 5.5, 6, 7, and 8. Bands 6 and 7 of human erythrocytes appeared to be more prominent while bands 5.5 and 8 less prominent than those of rabbit erythrocytes. Band 2.2, which was found in human erythrocyte membranes, appeared to be absent in rabbit erythrocyte membranes. However, several other minor bands appeared in the region between 2.1 and 2.3 of the rabbit erythrocyte membranes. The mobility of band 3 in the human erythrocyte membranes was slightly greater than that of the corresponding polypeptide in the rabbit erythrocyte membranes.

The electrophoretic pattern of periodic acid–Schiff (PAS) stained components of the rabbit erythrocyte ghosts differed markedly from that of the human erythrocyte ghosts (data not shown). The PAS profile of the human membranes showed four easily discernible bands as previously reported (Steck, 1974). However, only two faintly PAS-stained bands were present in an equal amount of rabbit erythrocyte membranes. Our results thus confirm those of Hamaguchi and Cleve (1972) who could not detect a major glycoprotein in rabbit erythrocyte membranes.

Comparison of Endogenous Substrates of Human and Rabbit Erythrocyte Membrane Kinase. Membranes from human and rabbit erythrocytes were prepared and phosphorylated under identical conditions. A radioautogram comparing the electrophoretic patterns of ^{32}P-labeled components of human and rabbit erythrocyte membranes is shown in Figure 4. Each of the phosphorylation reactions was carried out in duplicate at pH 6.0 using [γ-^{32}P]ATP as the phosphoryl donor and in the presence or absence of cyclic

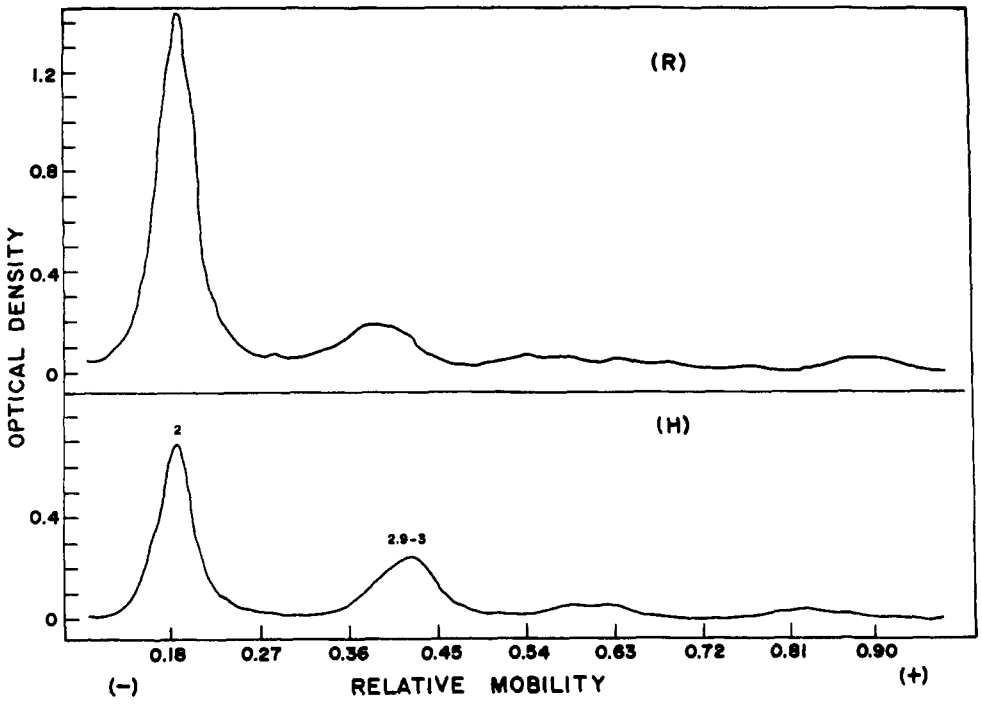

FIGURE 5: Comparison of the labeling patterns of rabbit and human erythrocyte membrane components phosphorylated in the presence of [γ-^{32}P]GTP. The reaction was carried out at 37 °C for 5 min in the presence of either rabbit (R) or human (H) erythrocyte membranes and other components as described under Experimental Procedures. The specific activity of [γ-^{32}P]GTP was 333 cpm/pmol. The radioautogram was exposed for 5 days and scanned using a Zeineh Soft-Laser densitometer.

AMP. The major phosphopeptides of rabbit erythrocyte membranes were in area 2 and bands 2.9, 3, and 4.1. Bands 2.5, 4.8, and 5.5 were also phosphorylated but to a lesser degree. In addition, a minor radioactive band (designated as 2.7), which appeared between bands 2.5 and 2.9 and could not be identified with any of the Coomassie blue stained protein components of Figure 3, was also observed. Figure 4 does not clearly show which of the bands migrating in the area of bands 1, 2, and 2.1 are phosphorylated. However, in separate experiments where the radioautograms are exposed for shorter periods of time, we have been able to discern the phosphorylation pattern and determine that only bands 2 and 2.1 are labeled.

In comparing the phosphorylation patterns of rabbit and human erythrocytes in the absence of cyclic AMP, marked differences occurred in the area designated as band 4.1. While band 4.1 was a major phosphopeptide in the rabbit erythrocyte membranes, it was only slightly phosphorylated in the human erythrocyte membranes. Furthermore, there was comparatively less labeling of bands 2.5, 2.7, and 5.5 in the human than in the rabbit erythrocyte membranes. In both rabbit and human erythrocytes, the area designated 2–2.1 appears to be the major substrate.

The phosphorylation of several membrane components of the human erythrocytes was stimulated by cyclic AMP while the cyclic nucleotide had no visible effect on the phosphorylation of any component of the rabbit erythrocyte membranes. The phosphorylation of bands 2.1, 2.3, 4.1, 4.5, and 4.8 of human erythrocyte membranes was significantly enhanced in the presence of cyclic AMP.

In contrast, the phosphorylation of human and rabbit erythrocyte membrane components in the presence of [γ-^{32}P]GTP at pH 8.5 appeared to be very similar. Figure 5 shows no significant difference in the radioactivity profiles of human and rabbit erythrocyte membranes. The major products of the phosphorylation reaction using GTP as the phosphoryl donor appeared in the areas of band 2 and 3. Diffuse minor radioactive components (not numbered in the figure) were also detected in the area between 4.1 and 5. Both cyclic AMP and cyclic GMP did not alter the phosphorylation pattern (data not shown). Band 2 of the rabbit erythrocyte membranes appeared to contain slightly more radioactivity than that of the human erythrocyte membranes.

The amount of phosphate incorporated into the protein bands has been roughly estimated by analyzing the radioactivity present in 1-mm gel slices (data not shown). The gels were derived from membrane proteins phosphorylated for 30 min and electrophoresed in cylindrical gels using the same system as described for slab gels. In the absence of cyclic AMP, the amount of phosphate incorporated ranged from less than 0.1 mol/mol of protein for bands 2.9–3 and 4.8 to about 0.6 mol/mol of protein for bands 2–2.1. However, the amount of phosphate incorporated into several of the cyclic AMP dependent protein kinase substrates was increased by about fourfold in the presence of the cyclic nucleotide. Because of the limitations of the densitometric procedure in the determination of the amount of protein in each stained band, these values must be considered approximate. The low level of phosphate incorporated into certain protein components suggests that these proteins may be already partially phosphorylated. On the other hand, it is also possible that the phosphorylation of these proteins results from a small denatured fraction. As shown by Bylund and Krebs (1975), certain proteins may become substrates for protein kinase following denaturation.

Effect of pH on the Autophosphorylation of Rabbit and Human Erythrocyte Membrane Components. The results in Figure 1 indicate that changes in pH can differentially affect the amount of ^{32}P incorporated from either [γ-^{32}P]ATP or [γ-^{32}P]GTP into rabbit erythrocyte membranes. In order to determine whether the phosphorylation

of specific proteins varied with pH, rabbit erythrocyte membranes were phosphorylated at several pH values and analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis followed by radioautography. The buffers chosen for this experiment were Tris-acetate, pH 6.0, Tris-HCl, pH 6.5, 7.5, and 8.5, and glycine–NaOH, pH 8.5. The results of these experiments using [γ-^{32}P]ATP are shown in Figure 6. This figure represents a densitometric tracing of a radioautogram of the phosphopeptides 2.5 through 5.5 of rabbit erythrocyte membranes. The slower migrating components were not included in the scan since they exhibited no visible variation in phosphorylation with pH. Several points should be noted. The phosphorylation of band 4.1 and band 5.5 was greater at pH 6.0–6.5 than at pH 7.5–8.5. In the area of band 3, two components were phosphorylated. The phosphorylation of the faster migrating species (band 3) was greatly enhanced at pH 8.5, whereas the phosphorylation of the slower migrating species (band 2.9) appeared to be less dependent on pH. Interestingly at pH 8.5, there was a significantly greater degree of labeling of band 3 in glycine-NaOH buffer than in Tris-HCl buffer. In addition, minor changes also occurred, such as the disappearance of the phosphopeptide designated 2.7 (relative mobility at about 0.25) at pH 8.5.

The results of a similar experiment using ATP and the human erythrocyte membranes are illustrated in Figure 7. The buffer systems used for this experiment were Tris-acetate, pH 6.0, Tris-HCl, pH 7.5 and 8.5, and glycine-NaOH, pH 8.5. Each pair represents samples incubated in the presence (left) and absence (right) of cyclic AMP. At all pH values studied, the phosphorylation of components 2.1, 2.3, 4.1, 4.5, and 4.8 was stimulated by cyclic AMP. However, the phosphorylation of the latter three components was also significantly enhanced at pH 8.5 in the absence of cyclic AMP. Although the pattern of phosphorylation in the area of 2.9 and 3 differed from that of rabbit erythrocyte membranes, there was an overall increase in the labeling of this area at pH 8.5. However, no significant difference in the labeling of human erythrocyte membrane components was found between reactions carried out in Tris-HCl and in glycine-NaOH. It was curious that the phosphorylation of most components of both the human and rabbit erythrocyte membranes using ATP as the phosphoryl donor were lowest at pH 7.5.

The phosphorylation of the rabbit erythrocyte membranes at various pH values using GTP as the phosphoryl donor was also determined (data not shown). The results were confirmatory of Figure 1 in that GTP was not used efficiently at low pH. The phosphorylation of the major substrate, bands 2–2.1, increased slightly with increasing pH. More noticeably, however, was the phosphorylation of component 2.9 and, to a lesser extent, component 3 which were significantly greater at pH 8.5. The composition of the buffer also had a slight effect on the phosphorylation of these two components, such that the labeling of 2.9 and 3 at pH 8.5 appeared to be somewhat better in Tris-HCl than in glycine-NaOH. The results were in contrast to those obtained using ATP as the phosphoryl donor where band 3 was a better substrate than band 2.9 at pH 8.5 and phosphorylated to a greater extent in glycine-NaOH than in Tris-HCl buffer.

Discussion

The results of experiments presented in this communication strongly suggest that the rabbit erythrocyte membrane contains at least two protein kinases, both of which appear to be insensitive to cyclic nucleotides. One kinase preferentially uses ATP as the phosphoryl donor, exhibits a pH optimum between 6.0 and 6.5, and can be distinguished on the basis of specificity for certain membrane polypeptides, especially bands 4.1, 4.8, and 5.5. A second kinase which uses GTP, perhaps also ATP, as the phosphoryl donor, appears slightly more active toward endogenous substrates at pH 8.5. However, the rate of ^{32}P incorporated from [γ-^{32}P]ATP into membrane components is about two to five times greater than from [γ-^{32}P]GTP, each measured at its pH optimum. This may, in part, reflect the fact that rabbit erythrocyte membranes contain a greater number of phosphoryl acceptors for the enzyme utilizing ATP (Figure 4) than for the enzyme utilizing GTP (Figure 5). Under our experimental conditions, we are presumably dealing mainly with the phosphorylation of seryl and threonyl residues of proteins. This has been adequately demonstrated by other laboratories (see Roses and Appel, 1973; Avruch and Fairbanks, 1974). Whether or not there are other kinases in rabbit erythrocyte membranes which may catalyze the formation of unstable phosphate remains to be determined.

The phosphorylation of several rabbit erythrocyte membrane polypeptides appears to be markedly affected by pH (Figures 6 and 7). The effect of pH on phosphorylation may be amenable to several interpretations. For example, a change in the structure of the protein substrate with pH may result in an alteration in its acceptor capacity. Alternatively, there may be several kinases in the rabbit erythrocyte membrane preparations. A change in the degree of labeling of certain substrate(s) with pH may simply reflect the activity of one or more of these kinases. In human erythrocytes, there is evidence to indicate that several protein kinases may be involved in the autophosphorylation of membrane components (Avruch and Fairbanks, 1974; Fairbanks and Avruch, 1974). The study of the solubilized enzymes and their substrates from red cell membranes should permit us to further evaluate these observations and interpretations. The physiological significance of the effect of pH on membrane phosphorylation remains unknown. However, it would be of interest to determine whether different phosphoproteins were formed under abnormal conditions such as acidosis or alkalosis.

Several laboratories (Rubin et al., 1972; Guthrow et al., 1972; Fairbanks and Avruch, 1974) including our own (this communication) have reported the presence of a cyclic AMP dependent protein kinase activity in human erythrocyte membranes. However, a similar enzymic activity appears to be absent in rabbit erythrocyte membranes. In no instance did the autophosphorylation of rabbit erythrocyte membrane components exhibit a dependency on cyclic AMP or cyclic GMP. Furthermore, we have also failed to detect any stimulation of the membrane-catalyzed phosphorylation of exogenous substrates, such as histone, by the cyclic nucleotides. The phosphorylation of histone by the soluble cyclic AMP-dependent protein kinases of rabbit erythrocytes has previously been shown to be stimulated by cyclic AMP (Tao and Hackett, 1973). Despite our failure to demonstrate a cyclic AMP-dependent protein kinase in rabbit erythrocyte membrane, it is still conceivable that such an enzyme may be present in the membrane preparation. It is possible that an appropriate substrate for the membrane-bound, cyclic AMP dependent protein kinase has not been identified. Recently, we have obtained evidence to indicate that rabbit erythrocyte membranes contain substrates for

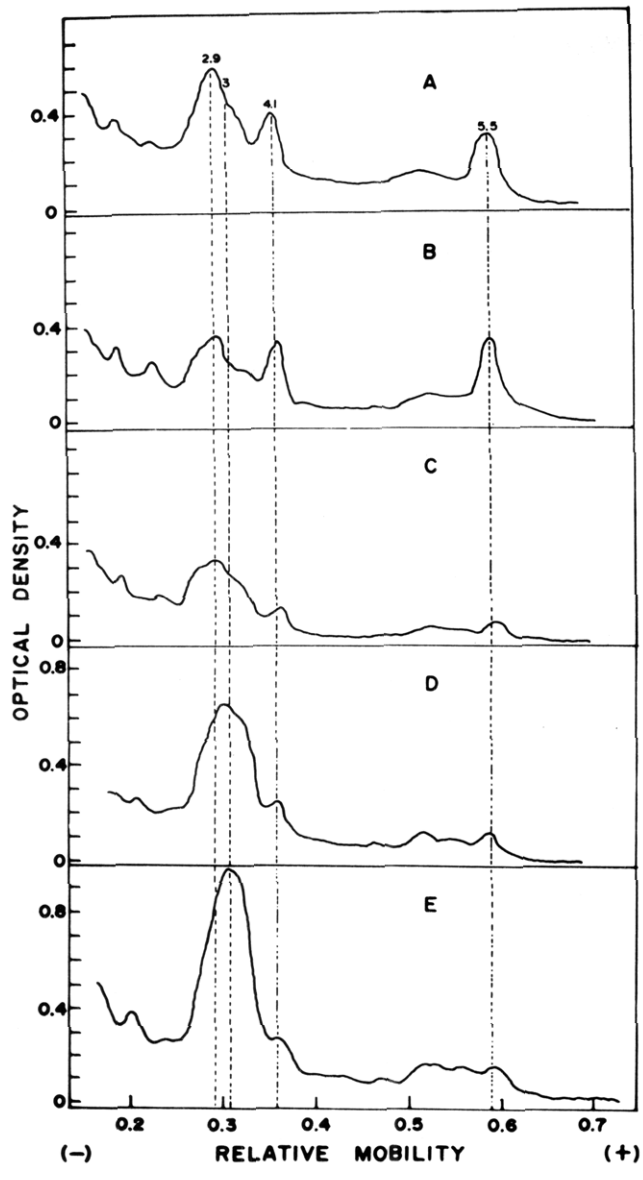

FIGURE 6: Effect of pH on the phosphorylation of rabbit erythrocyte membrane components in the presence of [γ-^{32}P]ATP. Rabbit erythrocyte membranes (23 μg) were phosphorylated at 37 °C for 5 min in the presence of [γ-^{32}P]ATP (330 cpm/pmol). Electrophoresis and radioautography (exposure time, 4 days) were performed as described under Experimental Procedures. Only the portion of the radioautogram showing a marked variation in the phosphorylation patterns with pH was scanned and presented in the figure. (A) Tris-acetate, pH 6.0; (B) Tris-HCl, pH 6.5; (C) Tris-HCl, pH 7.5; (D) Tris-HCl, pH 8.5; (E) glycine-NaOH, pH 8.5.

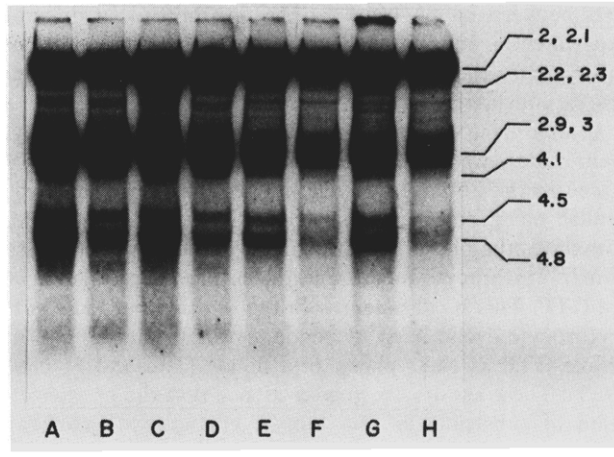

FIGURE 7: Radioautogram demonstrating the effect of pH on the autophosphorylation of human erythrocyte membranes using ATP as the phosphoryl donor. Membranes (22 μg) were phosphorylated using [γ-^{32}P]ATP (280 cpm/pmol). (A) Glycine-NaOH, pH 8.5 with cyclic AMP; (B) same as A without cyclic AMP; (C) Tris-HCl, pH 8.5 with cyclic AMP; (D) same as C without cyclic AMP; (E) Tris-HCl, pH 7.5 with cyclic AMP; (F) same as E without cyclic AMP; (G) Tris-acetate, pH 6.0 with cyclic AMP; (H) same as G without cyclic AMP.

the soluble cyclic AMP dependent protein kinases (Hosey and Tao, manuscript in preparation). However, the substrate specificity of the membrane-bound enzyme may be different from that of the soluble enzymes. Another possibility which must be considered is that only the catalytic moiety of the cyclic AMP dependent protein kinase is associated with the membrane. In the absence of the regulatory subunit, the enzyme is insensitive to regulation by cyclic AMP (Gill and Garren, 1970; Tao et al., 1970; Walsh and Krebs, 1973). Unfortunately, experiments utilizing isolated regulatory subunits from soluble cyclic AMP dependent protein kinase I of rabbit erythrocytes (Tao and Hackett, 1973) or the heat-stable protein inhibitor from rabbit muscle (Walsh et al., 1971) to inhibit the membrane-bound kinase activity failed to yield conclusive results due to impurities in these preparations (Hosey and Tao, unpublished observations). That the regulatory subunit of a cyclic AMP dependent protein kinase may be preferentially removed during the preparation of the membranes seems unlikely in view of the findings of Rubin et al. (1972) that the catalytic moiety of human erythrocyte membrane of cyclic AMP dependent protein kinase appears to be more loosely associated with the membranes than the regulatory subunit. Furthermore, repeated washing of the rabbit erythrocyte membrane with 0.15 M KCl did not alter the pattern of membrane phosphorylation. This suggests that the kinase activity associated with the cell membranes did not arise from the artefactual translocation of the catalytic subunits of soluble cyclic AMP dependent protein kinases during cell fractionation (Keely et al., 1975).

The pattern of phosphorylation which we obtained with cyclic AMP in the human erythrocyte membrane differs slightly from previous studies (Rubin and Rosen, 1972; Guthrow et al., 1972; Fairbanks and Avruch, 1974) in the area between bands 4 and 5. As illustrated in Figure 4, the phosphorylation of two bands, designated 4.5 (apparent mol wt 58 000) and 4.8 (apparent mol wt 50 000), is stimulated by cyclic AMP. The principal substrate of the cyclic AMP dependent protein kinase in the human erythrocyte membrane migrates closer to band 5 and, thus, has been designated 4.8 in these studies. Earlier studies determining cyclic AMP binding to human erythrocyte membranes have suggested that the binding subunit of the cyclic AMP dependent kinase is located between bands 4 and 5 and comigrates with a cyclic AMP stimulated phosphopeptide (Guthrow et al., 1973; Rubin, 1975). Recently, Haley (1975), using photoaffinity labeling techniques, has reported the presence of two cyclic AMP binding proteins in the human erythrocyte membrane. These proteins have apparent molecular weights of 55 000 and 49 000, and most likely correspond to the phosphopeptides we have designated 4.5 and 4.8. Both Haley and ourselves have utilized longer gels (20 and 10 cm, respectively) for separation. It may be that improved resolution has led to the demonstration of two cyclic AMP stimulated phosphopeptides and two cyclic AMP binding

sites between bands 4 and 5. The possibility that different experimental conditions have given rise to an additional substrate for the cyclic AMP dependent protein kinase must also be considered.

In analyzing the phosphorylation of human erythrocyte membranes, we found the pattern of phosphorylation obtained in the absence of cyclic nucleotides to be somewhat similar using either ATP or GTP. However, cyclic AMP stimulates the phosphorylation of several components of the human erythrocyte membrane in the presence of ATP but not GTP. This is consistent with the finding that the soluble erythrocyte cyclic AMP dependent protein kinases can not utilize GTP as their phosphoryl donor (Tao and Hackett, 1973). These results are good evidence that the phosphorylation of substrates of the human erythrocyte membrane which can use either ATP or GTP as phosphoryl donors does not reflect the basal, unstimulated state of the cyclic AMP dependent enzyme.

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