Proteins of the Turkey Erythrocyte Membrane†

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ABSTRACT: A new and simplified method is described for preparation of turkey erythrocyte membranes which are essentially devoid of supernatant or nuclear contamination, but retain catecholamine-sensitive adenylate cyclase activity. These membranes have been solubilized in sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis and the major protein components identified. The turkey erythrocyte membranes exhibit a protein profile very similar to that of the human erythrocyte membrane, but contain a protein component of apparent molecular weight of 50 000 which is not present in the human membranes. Three surface glycoprotein components of the turkey erythrocyte membranes (apparent molecular weights of 90 000, 41 000, and 26 000) have been identified by periodic acid–Schiff staining of polyacrylamide gels and by cell surface ^{125}I labeling using lactoperoxidase followed by polyacrylamide gel electrophoresis. After deoxycholate solubilization of membranes prepared from iodinated cells, glycoproteins with molecular weights of 90 000 and 41 000 bind to an affinity column of concanavalin A–Sepharose 4B and elute upon application of methyl α-D-mannopyrannoside. The lowest molecular weight glycoprotein component, however, does not bind to the insolubilized concanavalin A.

Although the avian erythrocyte has been known for many years to possess a catecholamine-sensitive adenylate cyclase (Davoren and Sutherland, 1963; Oye and Sutherland, 1966), which is not found in human erythrocyte, investigators have only recently begun to utilize this convenient system. Studies have largely concentrated on two related areas: the isolation and characterization of the membrane bound β-adrenoreceptor protein (Schramm et al., 1972; Bilezikian and Aurbach, 1973a,b; Cuatrecasas et al., 1974; Levitski et al., 1974; Atlas et al., 1974; Aurbach et al., 1974); and the regulation of the β-adrenoreceptor coupled adenylate cyclase (Bilezikian and Aurbach, 1974; Field, 1974; Puchwein et al., 1974; Steer and Levitski 1975a,b; Pfeuffer and Helmreich, 1975). In addition, a number of studies have explored the characteristics of sodium and potassium transport, a process in part regulated by cAMP[1] (Gardner et al., 1973, 1974a, 1974b, 1975).

Despite the number of studies utilizing the turkey erythrocyte, published procedures for isolating pure avian erythrocyte membranes are tedious and time consuming (Zentgraf et al., 1971; Bilezikian and Aurbach, 1973a; Puchwein et al., 1974) and in some studies sufficient data have not been presented to verify the purity of the final membrane product. Furthermore, detailed electrophoretic analysis of the proteins from purified avian erythrocyte membranes has not been attempted. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Blanchet (1974) has compared the profiles of solubilized nuclear and plasma membranes from adult and embryonic chicken erythrocytes. Jackson (1975) has labeled the exterior surface of chicken erythrocytes with the cationic reagent *p*-nitrophenyl *N,N,N*-trimethyl[^{125}I]iodotyrosinate and noted two surface glycoproteins on sodium dodecyl sulfate–polyacrylamide gels.

Identification of membrane protein and glycoprotein components is potentially important in further elucidating membrane events associated with catecholamine binding and regulation of adenylate cyclase activity. Well established techniques exist for the solubilization and separation of membrane proteins on polyacrylamide gels containing sodium dodecyl sulfate (Fairbanks et al., 1971). A number of workers have used lactoperoxidase-catalyzed iodination to label surface proteins containing exposed tyrosine residues in a variety of cell types (Marchalonis et al., 1971; Kennel and Lerner, 1973; Czech and Lynn, 1973; Trosper and Levy, 1974; Nachman et al., 1973; Hubbard and Cohn, 1972, 1975; Tsai et al., 1973; Philips and Morrison, 1971; Reichstein and Blostein, 1973), followed by separation on polyacrylamide gel electrophoresis. Surface glycoproteins have also been identified by affinity chromatography on the appropriate insolubilized lectin (Allan et al., 1972; Hunt and Marchalonis, 1975; Findlay, 1974), usually concanavalin A, which binds to terminal α-D-mannose or α-D-glucose residues of surface glycoproteins.

This communication reports a simple and rapid procedure for isolation of purified turkey erythrocyte membranes. These membranes have been analyzed by the techniques discussed above and the major protein and glycoprotein components identified.

Experimental Section

Preparation of Purified Turkey Erythrocyte Membranes. Five to twenty milliliters of blood was drawn from the wing vein of either adult male or non-egg-laying female domestic turkeys into a heparinized syringe, chilled immediately, and centrifuged for 5 min (600*g*, 4 °C). After removing the plasma and buffy coat, the cells were resuspended in ice-cold 150 mM NaCl buffered with 10 mM Tris-HCl to pH 7.4. The cells were centrifuged again for 5 min (600*g*, 4 °C), and the supernatant and top 1-mm layer of cells removed. After two more similar washes, the erythrocytes were hemolyzed in 10 mM potassium phosphate buffer, pH 7.4, containing 4 mM $MgSO_4$, using a ratio of 40 ml of hemolyzing buffer to 1 ml of packed cells. In all low ionic strength buffers, Mg^{2+} was necessary to preserve the integrity of the nuclei. After centrifugation of the hemolysate (25 000*g*, 4 °C, 10 min), a two layered pellet was visible:

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[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; PAS, periodic acid–Schiff; Con A, concanavalin A.

the upper pink pellet contained nucleated erythrocyte ghosts, and the lower small, white pellet contained cells rich in granular material as viewed under phase contrast microscopy. This lower pellet probably represents protease-rich leukocytes which are not removed even by extensive initial washings, as noted by other workers (Fairbanks et al., 1971). Care must be exercised to prevent contamination of the erythrocyte ghost fraction with these leukocytes since this results in proteolytic degradation of the final membrane product. The supernatant was aspirated and the erythrocyte ghosts were gently resuspended by swirling in the above hemolyzing buffer (20 ml/ml initial packed cells) to which 0.3 M sucrose had been added. The nucleated erythrocyte ghosts were decanted, leaving behind the firmly adherent leukocyte pellet and a proportion of the erythrocyte ghosts. After standing on ice for 15 min, the resuspended nucleated ghosts were centrifuged (25 000*g*, 4 °C, 10 min). The resultant homogeneous pellet of creamy to pink nucleated ghosts was resuspended in 50 mM NaCl, 10 mM potassium phosphate, pH 7.4 (5 ml/ml initial packed cells), and homogenized with a Polytron PT-10 (Kinematica) at a setting of 10 for 10 s. Mg^{2+} was not included in the buffer used in this step because it promotes vesicle formation and unsealed membranes with both sides accessible to substrate were desired. NaCl (50 mM) was found to be the lowest molarity salt that protected the nuclear membrane from significant disruption during homogenization in the absence of Mg^{2+}. The homogenized ghosts were centrifuged (50 000*g*, 15 min, 4 °C) and the supernatant was removed; the adherent, viscous bottom layer contained nuclei. The upper two layers containing the membranes and the unbroken ghosts were easily resuspended in 1–4 ml of 5 mM potassium phosphate buffer, pH 7.4, containing 2 mM $MgSO_4$, and recentrifuged in 1-ml glass centrifuge tubes (Pyrex) for 10 min (12 500*g*, 4 °C). The final pellet was clearly separated into two thick layers: a creamy pellet of nucleated ghosts covered by a thick band of white translucent membranes. After aspiration of the supernatant, the membrane layer was readily resuspended by Pasteur pipet in 5 mM potassium phosphate buffer, pH 7.4, 2 mM $MgSO_4$, to a final concentration of approximately 1 mg of membrane protein/ml. In those experiments where adenylate cyclase activity was measured, the same procedure was followed except that 1 mM dithiothreitol was included in the homogenization buffer (50 mM NaCl, 10 mM potassium phosphate, pH 7.4) and buffer for subsequent resuspensions was changed to 25 mM Tris-HCl, pH 7.4, 5 mM $MgSO_4$, 1 mM dithiothreitol.

Preparation of Human Erythrocyte Ghosts. This was performed as described by Dodge et al. (1963).

Lactoperoxidase-Catalyzed Iodination. This was performed as described by Marchalonis et al. (1971), using 5×10^7 washed erythrocytes in a total volume of 250 μl. For those experiments where $^{125}I^-$ was incorporated into purified membranes, the same procedure was followed using a total volume of 50 μl, containing approximately 25 μg of membrane protein.

Enzyme and Chemical Assays. Acetylcholinesterase was assayed as described by Steck and Kant (1974). 5′-Nucleotidase was assayed as described by Avruch and Wallach (1971). Adenylate cyclase was assayed as described by Salomon et al. (1974). The 100-μl incubation mixture contained 25 mM Tris, pH 7.4, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM cAMP, 1 mM α-[^{32}P]ATP (approximately 10^6 cpm/tube), 4.2 mM phosphoenolpyruvate, 2.95 units of pyruvate kinase, and other agents as indicated in the figure legends. Cytochrome oxidase was assayed as described by Schnaitman and Greenawalt (1968). Lactate dehydrogenase was assayed as described by Nielands (1955). Hemoglobin was determined spectrophotometrically using Soret band absorption at 410 nm as follows: suitable aliquots of various fractions containing 0.1% sodium dodecyl sulfate and 30–60 μg of DNase I/ml were incubated at room temperature for an hour. Samples were read at 410 nm and the amount of hemoglobin present was calculated using the known absorption 0.863 at 540 nm for a 1 mg/ml solution of hemoglobin (Williams and Tsay, 1973). Neither sodium dodecyl sulfate nor DNase affected the absorption properties of the hemoglobin. DNA was assayed as described by Kissane and Robins (1958) as modified by Waldman and Alm (1970) for determination in the presence of sucrose. Using bovine serum albumin as a standard, protein was assayed as described by Lowry et al. (1951) and, in cases where dithiothreitol was present, as modified by Geiger and Bessman (1972).

Affinity Chromatography on Concanavalin A–Sepharose 4B. Using the method of Allan et al. (1972), purified membranes from ^{125}I iodinated cells or membranes ^{125}I iodinated after preparation were solubilized by resuspension in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, containing 1% deoxycholate at a ratio of approximately 1 mg of membrane protein/ml of 1% deoxycholate solution. After 30 min at 4 °C with frequent vortexing, the mixture was centrifuged for either 30 min (100 000*g*, 4 °C) or 15 min (12 500*g*, 4 °C). The two centrifugation procedures were found to give identical results with respect to percent solubilization of either radioactivity or protein. The supernatants containing the solubilized and ^{125}I-iodinated membrane proteins were applied to 1-ml (packed volume) columns of concanavalin A–Sepharose 4B which had previously been washed with 10 ml of 1% deoxycholate in Tris–saline buffer, pH 7.4, 10 ml of 5% methyl α-mannopyrannoside in the 1% deoxycholate, Tris–saline buffer, and then finally 20 ml of 1% deoxycholate in Tris–saline. Following application of the sample, columns were washed with 1% deoxycholate in Tris–saline until radioactivity in the 0.25-ml effluent fractions had returned to background. Bound glycoproteins were then eluted with 5% methyl α-mannopyranoside in 1% deoxycholate, Tris–saline. Fractions containing radioactive peaks of both unbound and bound material were pooled and aliquots subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. This was performed as described by Fairbanks et al. (1971). Membrane aliquots were solubilized in a buffer containing 1% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10% sucrose and heated at 100 °C for 3 min. Bromphenol blue was added and the soluble membrane proteins (20–50 μg) were electrophoresed on 5.6% gels (6 × 110 mm) containing 1% sodium dodecyl sulfate. Gels were fixed and stained with either Coomassie blue or periodic acid–Schiff reagent as previously described (Fairbanks et al., 1971). Stained gels were scanned in a Canalco gel scanner utilizing the appropriate filter for either Coomassie blue or PAS stain. Gels containing ^{125}I-iodinated proteins were sliced into 1.25-mm slices using a razor blade stack and counted in a Packard Auto-gamma spectrometer. Molecular weight markers used were: phosphorylase A, 94 000; lactoperoxidase, 77 500; bovine serum albumin, 68 000; catalase, 60 000; glutamic dehydrogenase, 53 000; ovalbumin 43 000; aldolase, 40 000; light chain, 23 000; and trypsin inhibitor, 14 000.

Materials. Unless otherwise stated, all substrates, coenzymes, nucleotides, enzymes, and other agents were obtained from Sigma, Calbiochem, or Boehringer. Phosphoenolpyruvate was obtained from Fine Chemicals of Australia; propranolol

TABLE I: Recovery of Markers in Fractionation of Turkey Erythrocytes.[a]

	^{125}I Radioactivity		Lactate Dehydrogenase Act.		Hemoglobin		DNA	
	% Recovery[b] (100)[g]	Spec Radioact.[c] (61)	% Recovery (100)	Spec.[d] Act. (19.0)	% Recovery (100)	Spec[e] Quantity (0.60)	% Recovery (100)	Spec[f] Quantity (5.05)
Hypotonic hemolysis (25 000g, 10 min)								
Supernatant	20	16	101	18.6	83	0.68	0	0
Nucleated erythrocyte ghosts	60	419	2.2	4.0	6.2	0.28	66	36.5
Leukocytes	18	269	0.5	4.7	1.1	0.24	21	46.8
Resuspension and wash (25 000g, 10 min)								
Supernatant	2	54	1.4	14.0	2.1	0.63	0	0
Nucleated erythrocyte ghosts	50	592	0.1	0.5	0.4	0.04	58	56
Polytron homogenization (50 000g, 15 min)								
Supernatant	3.5	582	0.4	16.8	0.2	0.21	0	12.0
Nucleated erythrocyte ghosts and membranes	16	1237	<0.1	0.9	<0.1	0.03	3.5	15.8
Nuclei	ND	ND	ND	ND	ND	ND	ND	ND
Resuspension (12 500g, 10 min)								
Supernatant	<0.1	100	<0.1	1.9	0	0	0.2	5.5
Nucleated erythrocyte ghosts	3.8	830	0	0	0	0	1.5	112
Membranes	8.5	1322	0	0	0	0	0.1	5.5

[a] Values of zero indicate that no activity or material was detected under the assay conditions used; ND means not determined. [b] Values represent the mean of four experiments. [c] Expressed as cpm $\times 10^{-3}$ mg^{-1} protein; values represent the mean of two experiments. [d] Specific activity expressed as nmol of NADH produced min^{-1} mg^{-1} protein; values represent the mean of three experiments. [e] Specific quantity expressed as mg of hemoglobin/mg of protein; values represent the mean of two experiments. [f] Specific quantity expressed as μg of DNA/mg of protein; values represent the mean of two experiments. [g] Totals are numbers in parentheses.

was from ICI; lactoperoxidase was a gift from Dr. J. J. Marchalonis; concanavalin A–Sepharose 4B was obtained from Pharmacia; deoxycholate was from Merck or Sigma; sodium dodecyl sulfate was from either BDH (specially pure grade) or Pierce (99% grade). All radioisotopes were obtained from the Radiochemical Centre, Amersham. All chemicals were of the highest grade available.

Results

Recovery of Turkey Erythrocyte Membranes. Using ^{125}I incorporated into accessible cell surface tyrosine residues as a plasma membrane marker, recovery of turkey erythrocyte plasma membranes was approximately 10% (Table I). Specific radioactivity increased 21 times in association with membrane purification.

The high percentage of total radioactivity (20%) present in the first supernatant probably represents ^{125}I incorporation into serum proteins adherent to the fresh, well-washed erythrocytes, a phenomenon documented for immunoglobulin (Nossal et al., 1972) and albumin (Demus, 1973). Electron microscopic autoradiography (not shown) suggests that only surface proteins are iodinated by the conditions used in this communication. Significant losses of membrane material occur in the initial stages of purification in association with the discarded leukocyte pellet and at the later step of Polytron homogenization. Losses of membrane material and associated radioactivity which occur at each step of the preparation are probably due to nonspecific absorption to the plastic centrifuge tubes and glass pipets. The most critical variable governing percent cell disruption and membrane yield, however, is the efficiency of Polytron homogenization, a factor found to be dependent on the individual machine and wear of the rotor shaft.

No membrane enzymes were found which gave realistic estimates of membrane yield. Acetylcholinesterase, an external membrane enzyme in human erythrocytes, possessed such low activity in both turkey erythrocytes and purified membranes that it could not be used as a plasma membrane marker. In three experiments human erythrocyte membranes contained acetylcholinesterase activity of 2.23 μmol min^{-1} mg^{-1} protein whereas purified turkey erythrocyte membranes contained activity of only 0.0266 μmol min^{-1} mg^{-1} protein, which was not increased by incubation in 0.1% Triton X-100.

No activity of the plasma membrane enzyme, 5′-nucleotidase, was detected in either the initial hemolysate of turkey erythrocytes or in the final membrane product. Furthermore, it was not feasible to use Lubrol-WX (0.5%) solubilized, fluoride-stimulated adenylate cyclase activity as a membrane marker because significant inhibition of activity occurred in those fractions containing an appreciable amount of cytoplasm.

Purity and Functional Integrity of Turkey Erythrocyte Membranes. Table I demonstrates the absence of significant contamination of the purified membranes by either nuclear or cytoplasmic components. Neither hemoglobin nor activity

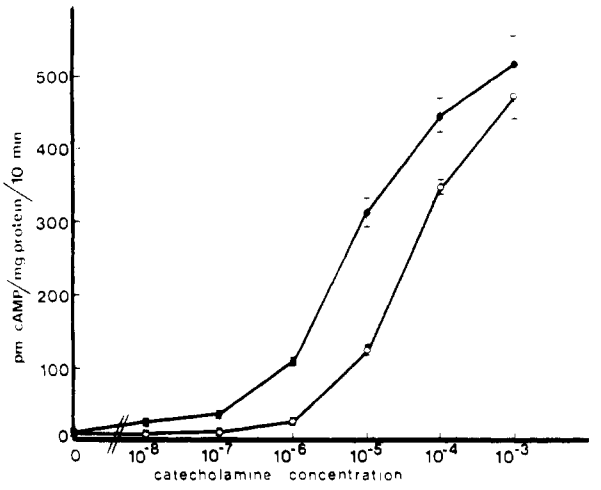

FIGURE 1: Adenylate cyclase activity of turkey erythrocyte membranes as a function of l-isoproterenol (●) or l-norepinephrine (○) molar concentration. Duplicate determinations were performed as described in the Experimental Section. Values represent the mean ± standard deviation for three separate experiments.

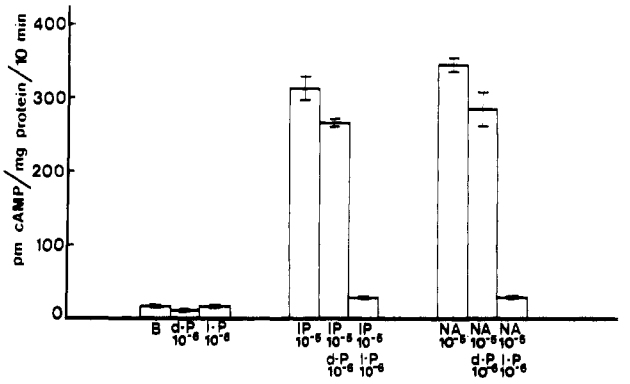

FIGURE 2: Adenylate cyclase activity of turkey erythrocyte membranes in presence of catecholamines and propranolol. Duplicate determinations were performed as described in the Experimental Section in presence of the following agents at the indicated molar concentrations: d-P, *d*-propranolol; l-P, *l*-propranolol; IP, *l*-isoproterenol; NA, *l*-norepinephrine. Basal activity is indicated by B. Values represent the mean ± standard deviation for three separate experiments.

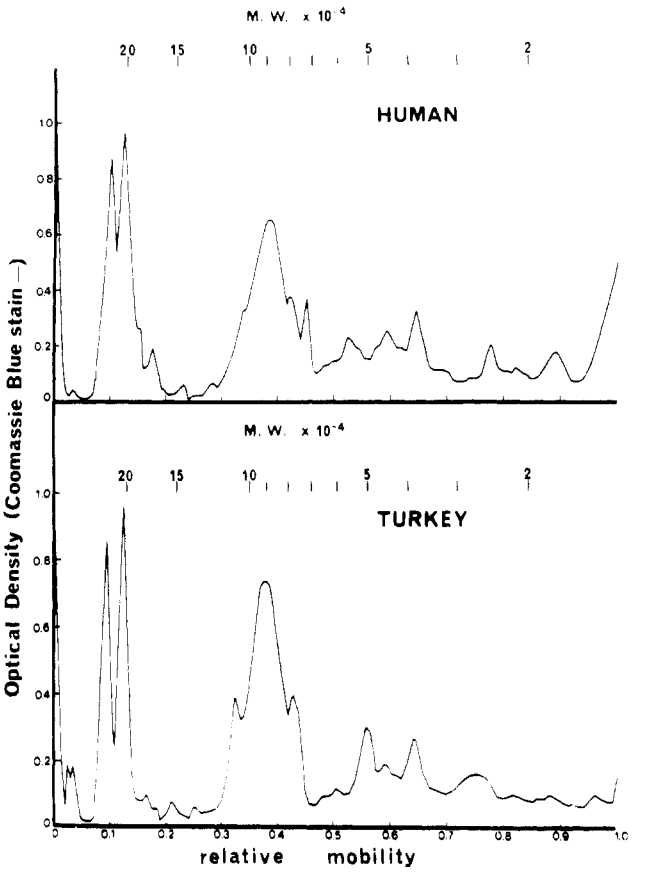

FIGURE 3: Densitometric scans of polyacrylamide gels of human and turkey erythrocyte membranes. Sodium dodecyl sulfate solubilized membranes prepared from human and turkey erythrocyte membranes were electrophoresed in parallel with each other and the molecular weight markers were as described in the Experimental Section. Optical density is an arbitrary scale.

of the cytoplasmic enzyme lactate dehydrogenase was detected in the final membrane product. Minimal contamination with DNA (0.1% of total amount of DNA present initially) was observed, although electron microscopy of the Polytron homogenate (not shown) revealed slight disruption of the nuclear envelope. The final membrane product appeared in the form of large fragments in electron micrographs (not shown) and there was no evidence of contaminating nuclei or nucleated ghosts. Cytochrome oxidase was not detected in Lubrol solubilized ghosts obtained after the first or second centrifugation, suggesting that, if mitochondria are present as Harris and Brown (1971) propose, they function poorly, if at all.

Functional integrity of the membrane product was assessed by adenylate cyclase activity as a function of catecholamine concentration (Figure 1). The curves are similar to those reported by others with half-maximal activity occurring at 6×10^{-6} M isoproterenol and at higher concentration, 5×10^{-5} M, for norepinephrine. Figure 2 confirms the β-adreno nature of the catecholamine stimulated adenylate cyclase activity. The *l* isomer of propranolol effectively abolished isoproterenol stimulated activity whereas the *d* isomer was essentially inactive. Neither *d*- nor l-propranolol exhibited significant cyclase stimulatory properties on their own.

Protein Composition of Turkey Erythrocyte Membranes. In Figure 3, typical densitometric patterns obtained from Coomassie blue stained gels of human and turkey erythrocyte membranes show marked similarities. Both possess a high molecular weight doublet of proteins which comprises a significant proportion of membrane proteins. In addition, the other major membrane component appears to have a similar apparent molecular weight of 88 000–90 000 in the two preparations. Turkey erythrocyte membranes possess a unique component with molecular weight of approximately 105 000, and a major unique component of 50 000–52 000. The human erythrocyte membranes contain at least one unique protein moiety of molecular weight 72 000–74 000 and several minor unique components with molecular weights of 55 000–60 000.

When nucleated ghosts are subjected to electrophoresis (not shown), seven distinct low molecular weight nuclear proteins are added to the profile of turkey membrane proteins, obscuring the electrophoretic detail of membrane components with molecular weights up to 65 000. When turkey erythrocyte membranes are intentionally contaminated with proteases, the electrophoretic patterns (not shown) exhibit two new bands with a relative mobility of 0.20. Neither distinct nuclear proteins nor changes associated with membrane proteolysis are found in the electrophoretic profile of purified membranes.

Glycoproteins and Iodinated Surface Components of Turkey Erythrocyte Membranes. Although turkey erythrocyte

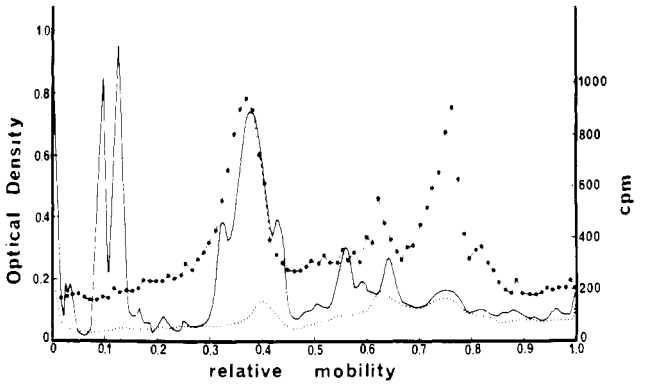

FIGURE 4: Distribution of ^{125}I in turkey erythrocyte membranes prepared from surface ^{125}I-iodinated cells. Cells were iodinated and membranes prepared, solubilized, and electrophoresed as described in the Experimental Section. Duplicate gels were stained either for protein with Coomassie blue (—) or for glycoprotein with PAS (. . .). Following scanning, gels were sliced and radioactivity was determined. Both Coomassie blue and PAS stained gels gave identical results in terms of radioactive profile so only one pattern of ^{125}I counts (● - - - ●) is presented. Optical density is an arbitrary scale.

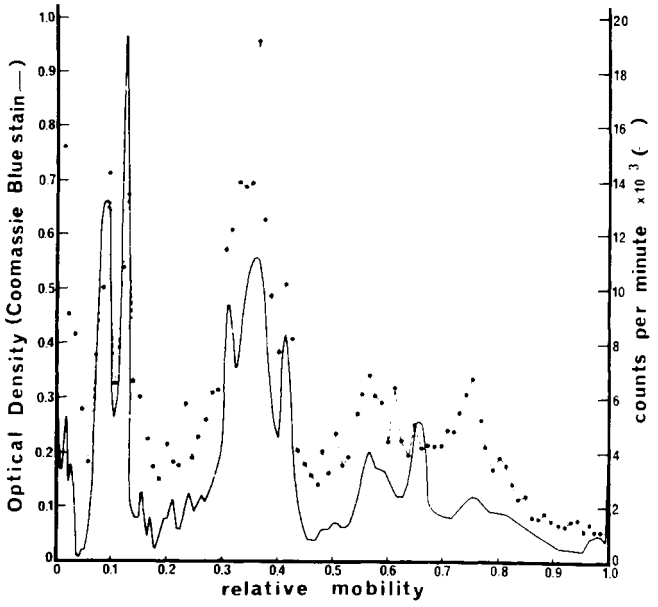

FIGURE 5: Distribution of ^{125}I in ^{125}I-iodinated turkey erythrocyte membranes. Membranes were first prepared and then iodinated as described in the Experimental Section. Solubilization, electrophoresis, and staining were as described in the Experimental Section. Optical density is an arbitrary scale.

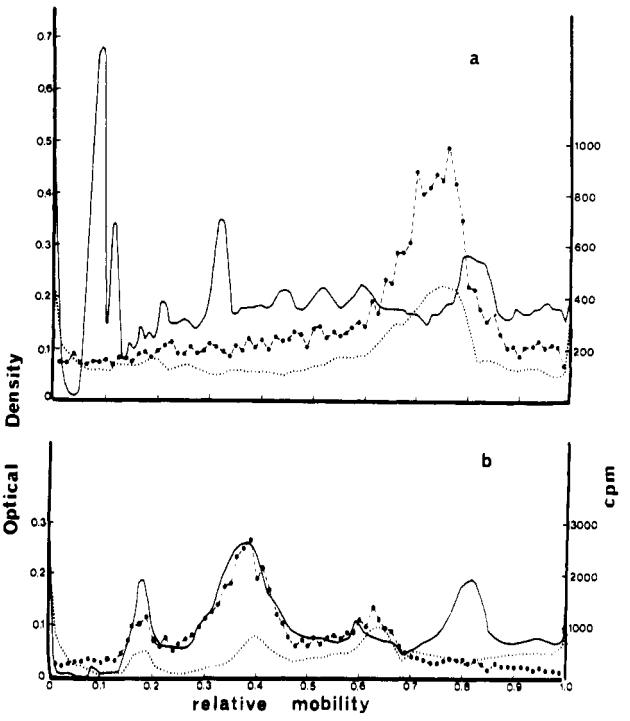

FIGURE 6: Densitometric scans and distribution of ^{125}I in polyacrylamide gels of aliquots obtained from (a) the pooled unbound fractions and (b) the pooled bound fractions obtained from concanavalin A-Sepharose 4B affinity chromatography of membranes prepared from surface ^{125}I-iodinated turkey erythrocytes. Duplicate aliquots from each pool were electrophoresed in parallel and then stained either for protein with Coomassie blue (—) or for glycoprotein with PAS (. . .) as described in the Experimental Section. Following scanning, gels were sliced and radioactivity was determined. Both Coomassie blue and PAS stained gels gave identical results in terms of radioactive profile so only one pattern of ^{125}I counts (● - - - ●) is presented for each set of gels. Optical density is an arbitrary scale.

membranes stain poorly with the carbohydrate stain, PAS, in comparison with human erythrocyte membranes (human pattern not shown, but essentially the same as reported by Steck (1974)), three glycoprotein components with molecular weights of approximately 90 000, 41 000, and 26 000 are readily detectable (Figure 4), two of which (mol wt 90 000 and 26 000) co-electrophorese with Coomassie blue stained components. Lactoperoxidase-catalyzed iodination of intact turkey erythrocytes resulted in incorporation of label into surface proteins with essentially the same molecular weights as the PAS-stained glycoproteins (Figure 4). The amount of radioactivity incorporated into each peak varied from experiment to experiment but, in general, the protein with molecular weight 26 000 contained the majority of total radioactivity, a fact somewhat surprising if its poor staining with PAS and Coomassie blue accurately represents its proportional contribution to total membrane protein. It is most improbable that this peak represents anything but protein-bound ^{125}I, as both free iodide and glycolipid run well ahead of the dye marker in the electrophoretic system used. The erythrocyte membrane proteins unique to the turkey were not iodinated.

Iodination of Isolated Membranes. When purified membranes rather than whole cells were iodinated, significantly more radioactivity was incorporated into membrane proteins. Furthermore, all proteins and glycoprotein components appear to be labeled (Figure 5), and the amount of ^{125}I contained in each protein in general corresponds to the stain density of that protein. Some poorly staining minor components, however, appear as distinct proteins only on the basis of their ^{125}I content. The most striking difference in ^{125}I incorporation between whole cells and isolated membranes is in the labeling of the high molecular weight doublet. The amount of $^{125}I^-$ incorporated into the 26 000 glycoprotein is a significantly smaller proportion of the total incorporated radioactivity in the membrane iodination than in the cell iodination.

Solubilization of Membrane Proteins Affinity Chromatography on Concanavalin A-Sepharose 4B. Polyacrylamide Gel Analysis of Unbound and Specifically Bound Proteins. Membranes prepared from iodinated cells which were to be subjected to affinity chromatography on concanavalin A-Sepharose 4B were first solubilized in 1% deoxycholate as described under the Experimental Section. Eighty-two percent of membrane-associated radioactivity was solubilized by treatment with deoxycholate, whereas only 65% of protein as determined by the method of Lowry was solubilized. Electro-

phoretic analysis of deoxycholate solubilized membranes (not shown) produced very similar patterns to those in Figure 6 with the following exceptions: the 210 000 molecular weight component solubilized much less readily than the 240 000 molecular weight component as is shown in the electrophoretic pattern of the unbound effluent from the concanavalin A column (Figure 6a); relatively poor solubilization of the major 89 000 molecular weight component was obtained, although sufficient material was still present to permit further analysis; the 26 000 molecular weight glycoprotein solubilized most readily, resulting in a relative enrichment in this component. All other solubilized protein and glycoprotein components were present in approximately the same proportions to each other as in the standard membrane electrophoretic pattern (Figures 4 and 5).

Approximately 88% (average of five experiments) of counts contained in solubilized membranes prepared from ^{125}I-iodinated cells does not bind to a concanavalin A–Sepharose 4B affinity column. Eight percent of total counts applied was bound and eluted specifically with α-methyl mannopyrannoside. Figures 6a and 6b represent the gel electrophoretic patterns obtained from aliquots of pooled unbound and bound fractions collected during the affinity chromatography on insolubilized Con A. In both Figures 6a and 6b, the large peak of Coomassie blue stained protein migrating with a relative mobility of 0.80–0.85 represents free concanavalin A which eluted from all batches of commercially prepared Con A–Sepharose used, regardless of the extent of prior washing. As shown in Figure 6a, the majority of Coomassie blue stained membrane proteins do not bind to the concanavalin A affinity column. In addition, the glycoprotein with molecular weight of approximately 26 000 did not bind as indicated by the presence of a PAS positive peak containing ^{125}I. The only membrane components which were bound to the Con A affinity column were the two remaining PAS positive glycoproteins (mol wt 90 000 and 41 000). In addition to these glycoproteins, two Coomassie blue stained components were present with relative mobilities slightly different from the PAS stained glycoproteins. It is therefore possible that each of the two ^{125}I peaks is a composite of several glycoproteins with different staining properties. The peak of ^{125}I which stains with both PAS and Coomassie blue and migrates with a relative mobility of 0.15–0.20 is probably a dimer of the 89 000 molecule weight material, a documented occurrence with human erythrocyte membranes (Steck, 1972). Identical results (not shown) were obtained when this series of experiments was performed using turkey erythrocyte membranes with ^{125}I incorporated into all membrane components. Similar experiments were performed with surface iodinated, solubilized human erythrocyte membranes and the findings of Findlay (1974) confirmed, namely, that the only glycoprotein which binds to concanavalin A is the minor glycoprotein (Fairbanks et al., 1971; component 3).

Discussion

A simplified method for preparing purified turkey erythrocyte membranes which does not involve density gradient centrifugation is reported in this communication. Recovery of membranes utilizing this method (about 10%) compares favorably with yields of purified membranes obtained from other cell types (De Pierre and Karnovsky, 1973; Crumpton and Snary, 1974) and adequate levels of purification are readily attained. Because membranes retain catecholamine-responsive adenylate cyclase activity, it is probable that the preparative method outlined in this paper does not entail functionally serious disruption of membrane architecture. Hormonally responsive adenylate cyclase is labile enzyme complex composed of at least three separate components (Rodbell et al., 1975) and is highly sensitive to perturbations in membrane structure (Perkins, 1973).

Both removal of contaminating leukocytes and conditions chosen for solubilization and electrophoresis (Fairbanks et al., 1971) in this study minimize protolytic degradation of membranes. Electrophoretic patterns reported for chicken erythrocyte membranes by Blanchet (1974) exhibited two prominent bands with approximate relative mobilities of 0.20 which have been found by both Jackson (1975) and myself to represent products of membrane proteolysis. Jackson was unable to prevent these changes unless he included a potent inhibitor of proteolytic enzymes in his ghost preparation procedure. It is probable that the proteolysis problems experienced by both Blanchet and Jackson in chicken erythrocyte membranes arose from contaminating leukocytes which are not always removed even by the most careful initial washing of the erythrocytes. It is also possible that the chicken erythrocyte membrane harbors more active intrinsic proteases than the turkey erythrocyte membrane.

Although proteolytic changes were contained in the report for chicken erythrocyte membranes by Blanchet and nuclear proteins obscured the membrane electrophoretic patterns reported by Jackson, in general, the patterns obtained by these workers were found to be similar to both human erythrocyte membrane patterns and to patterns reported in this paper for turkey erythrocyte membranes. These marked general similarities in erythrocyte protein components suggest that the fundamental structure of the erythrocyte membrane was determined relatively early in vertebrate evolution and remained highly conserved over the 200 million years since the two reptilian lines giving rise to birds and mammals diverged (Romer, 1954).

The most striking difference between the human and turkey erythrocyte membranes is a protein which comprises a significant proportion of total turkey erythrocyte membrane protein, as judged by intensity of Coomassie blue staining, with an apparent molecular weight of 50 000. There is no corresponding component in the human pattern and it is tempting to speculate on the possible relationship of this protein to the adenylate cyclase enzyme complex present in the turkey erythrocyte but not in the human. Pfeuffer and Helmreich (1975) recently reported isolation from pigeon erythrocyte membranes of a protein which binds GTP and various GTP analogues not susceptible to phosphate group hydrolysis and presumed to represent the regulatory unit of adenylate cyclase enzyme complex, known to be exquisitely sensitive to nonhydrolyzable derivatives of GTP (Londos et al., 1974; Lefkowitz, 1974; Pfeuffer and Helmreich, 1975). This protein was found to have intact molecular weight of approximately 230 000 and to be composed of subunits not greater than 60 000. It is possible that the 50 000 molecular weight protein unique to the turkey erythrocyte membrane represents these subunits of the GTP binding and regulatory portion of the adenylate cyclase complex. Another possibility is that tubulin, with molecular weight in the range of 50 000–60 000, or a tubulin-like protein, is an integral membrane component and is responsible for the binding of GTP which is critical in the regulation of hormonally responsive adenylate cyclase activity.

The three major glycoprotein components of the turkey erythrocyte are exposed surface proteins susceptible to iodination with the nonpenetrating reagent, lactoperoxidase. Under conditions used in this study, only surface proteins were labeled as evaluated by autoradiography. Furthermore, the high mo-

lecular weight doublet, known to reside on the inner surface of the human erythrocyte (Steck, 1974), was not labeled during the cell surface iodination procedure. All major protein components, however, contained accessible tyrosines which were iodinated when purified membranes, rather than whole cells, were incubated with lactoperoxidase. These findings suggest that surface exposure of proteins is of major importance in determining protein labeling during cell iodination procedures. In contrast to the turkey erythrocyte, Jackson (1975) found six chicken erythrocyte glycoprotein components which ranged from 36 000 to 190 000 in apparent molecular weight. Only two of these components (molecular weights 96 000 and 55 000) were labeled when washed cells were incubated with the cationic membrane reagent *p*-nitrophenyl *N,N,N*-trimethyl[^{125}I]iodotyrosinate.

Further analysis of turkey erythrocyte surface glycoproteins using affinity chromatography on concanavalin A–Sepharose 4B distinguished the three glycoprotein components. Two surface components with molecular weights of 90 000 and 41 000 contained sufficient quantity of terminal α-D-glucose or α-D-mannose residues to bind to the insolubilized concanavalin A, and to be specifically eluted with methyl α-D-mannopyrannoside. Because each of these components stained with both Coomassie blue and PAS and exhibited slightly different mobilities with each stain, it is possible that each of these two components represents a complex of proteins of very similar molecular weights but containing differing proportions of carbohydrate. There is some precedence for this in the human erythrocyte where the major sialoglycoprotein which stains well with PAS and contains 64% carbohydrate has an apparent molecular weight of approximately 86 000. The minor glycoprotein of the human erythrocyte membrane on the other hand stains well with Coomassie blue, contains less than 10% carbohydrate, and has apparent molecular weight of 88 000 (Fairbanks et al., 1971). Both proteins are capable of being iodinated (Reichstein and Blostein, 1973; Morrison et al., 1974) but only the minor glycoprotein binds to concanavalin A (Findlay, 1974). In contrast to the human erythrocyte membranes, all but one turkey erythrocyte membrane glycoprotein component was bound to insolubilized Con A and this fact, combined with the relatively poor staining of the glycoproteins by PAS, suggests that the surface components of the turkey erythrocyte membrane may be less heavily sialated than the glycoproteins of the human erythrocyte membrane. The glycoprotein component of the turkey erythrocyte membrane which did not bind to the insolubilized Con A contained the majority of ^{125}I incorporated into the cell surface, although this component stained poorly with both Coomassie blue and PAS. Similar low molecular weight glycoproteins which label well and stain poorly have been found in other cell types (Czech and Lynn, 1963; Trosper and Levy, 1974; Warr and Marchalonis 1976), but the significance of these glycoproteins is unknown.

Based on the findings reported in this paper, studies are continuing on the membrane events associated with catecholamine action, particularly in relation to the identification and control of the adenylate cyclase enzyme regulatory unit.

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