Judah, J. D., Ahmed, K., and McLean, A. E. M. (1962), *Biochim. Biophys. Acta 65,* 472.
Kant, J. A., and Steck, T. L. (1972), *Nature (London), New Biol. 240,* 26.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Marchesi, V. T., and Palade, G. E. (1967), *J. Cell Biol. 35,* 385.
Marchesi, V. T., and Steers, E., Jr. (1968), *Science 159,* 203.
Miyamoto, E., Ko, J. F., and Greengard, P. (1969), *J. Biol. Chem. 244,* 6395.
Nicolson, G. H., Marchesi, V. T., and Singer, S. J. (1971), *J. Cell Biol. 51,* 265.
Palmer, F. B. St. C., and Verpoorte, J. A. (1971), *Can. J. Biochem. 49,* 337.
Penniston, J. T. (1972), *Arch. Biochem. Biophys. 153,* 410.
Penniston, J. T., and Green, D. E. (1968), *Arch. Biochem. Biophys. 128,* 339.
Resch, K., Imm, W., Ferber, E., Wallach, D. F. H., and Fischer, H. (1971), *Naturwissenschaften 58,* 220.
Rodnight, R., and Lavin, B. E. (1964), *Biochem. J. 93,* 84.
Rosenthal, A. S., Kregenow, F. M., and Moses, H. L. (1970), *Biochim. Biophys. Acta 196,* 254.
Roses, A. D., and Appel, S. H. (1973), *J. Biol. Chem. 248,* 1408.
Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972), *J. Biol. Chem. 247,* 6135.
Rubin, C. S., and Rosen, O. M. (1973), *Biochem. Biophys. Res. Commun. 50,* 421.
Skou, J. C. (1965), *Physiol. Rev. 45,* 596.
Steck, T. L. (1972), *J. Mol. Biol. 66,* 295.
Steck, T. L., Weinstein, R. S., Strauss, J. H., and Wallach, D. F. H. (1970), *Science 168,* 255.
Suelter, C. H. (1970), *Science 168,* 789.
Weed, R. I., LaCelle, P. L., and Merrill, E. W. (1969), *J. Clin. Invest. 48,* 795.
Wolf, H. U. (1970), *Biochim. Biophys. Acta 219,* 521.

Phosphorylation of Endogenous Substrates by Erythrocyte Membrane Protein Kinases. II. Cyclic Adenosine Monophosphate-Stimulated Reactions†

Grant Fairbanks* and Joseph Avruch

ABSTRACT: Cyclic AMP stimulated protein kinase activity in the human erythrocyte membrane is associated with the phosphorylation of endogenous polypeptide substrates. Several features of this reaction distinguish it from the salt-stimulated phosphorylation of the major polypeptide 2 (mol wt 215,000) of "spectrin." The preferred substrates in the cyclic AMP stimulated reaction are minor components, among which polypeptides 2.1 (mol wt 200,000) and 4.5 (mol wt 52,000) are most prominent. These phosphopeptides are tightly held in the membrane and are not eluted at low ionic strength with "spectrin." The response to cyclic AMP is strongly inhibited by monovalent ions at physiologic concentrations and by 1 mM Ca^{2+}. The phosphorylation of polypeptide 3 (mol wt 105,000) is similarly regulated by monovalent ions and Ca^{2+}, and may represent a basal function of the cyclic AMP stimulated enzyme. Relative to polypeptide 2 phosphorylation, these reactions are less strongly stimulated by an increase in the Mg^{2+} concentration from 1 to 20 mM. A portion of the cyclic AMP stimulated activity is eluted with "spectrin." The phosphorylation of endogenous substrates by the free enzyme in such eluates is indiscriminate, but the labeling pattern is normalized when the eluate is admixed with vesicles under the conditions of the reaction.

Protein kinase activity associated with the human erythrocyte membrane (Rubin *et al.,* 1972) has been implicated in the selective phosphorylation of endogenous polypeptide substrates observed both in isolated membranes (Guthrow *et al.,* 1972; Roses and Appel, 1973; Rubin and Rosen, 1973) and intact cells (Palmer and Verpoorte, 1971). In a preceding publication (Avruch and Fairbanks, 1974), we described the reaction in which a single high molecular weight polypeptide, the 215,000-dalton component of "spectrin" (Marchesi and Steers, 1968), is phosphorylated. We drew attention to its high substrate specificity, its stimulation by monovalent cations and Ca^{2+}, and its relative unresponsiveness to cyclic AMP. In this paper we present the contrasting results obtained in the parallel analysis of another set of endogenous phosphorylations—reactions highly stimulated by cyclic AMP, inhibited by monovalent cations and Ca^{2+}, and utilizing multiple polypeptide substrates. These and other features of the reactions suggest that a separate enzyme, or enzyme complex, may be involved.

Methods

The methods of erythrocyte membrane preparation, incubation with [γ-^{32}P]ATP, and electrophoretic analysis of la-

† From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545 (G.F.), and the Diabetes Unit, Massachusetts General Hospital, Boston, Massachusetts 02114 (J.A.). *Received July 18, 1974.* Supported by U. S. Public Health Service Grant No. 12708 to the Worcester Foundation and a grant from the John A. Hartford Foundation, Inc., to the Diabetes Unit, Massachusetts General Hospital. G.F. was aided by a Cancer Research Scholar Award from the American Cancer Society, Massachusetts Division, Inc. J. A. was a recipient of a Research and Development Award from the American Diabetes Association, and is presently an investigator of the Howard Hughes Medical Institute.

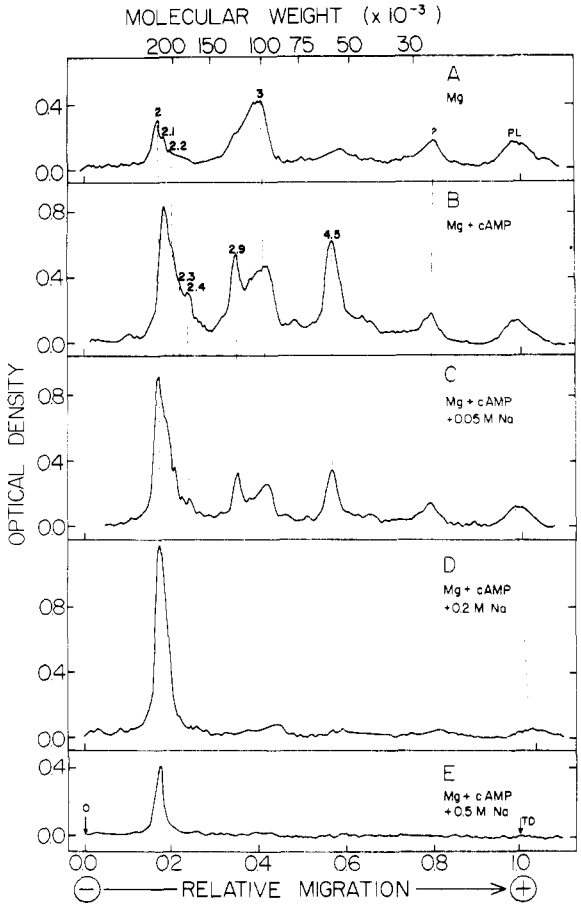

FIGURE 1: Differential effects of cyclic AMP and monovalent ions on the phosphorylation of endogenous substrates. Membranes were phosphorylated in 10-sec incubations with 2 μM [γ-^{32}P]ATP in 20 mM imidazole chloride (pH 7.4)–1 mM $MgCl_2$. In B–E, cyclic AMP was present at 20 μM; in C–E, NaCl was present at 0.05, 0.2, or 0.5 M, respectively. The membranes were precipitated with perchloric acid, washed, and fractionated electrophoretically as described (Avruch and Fairbanks, 1974). The distributions of radioactivity in fixed, stained gels were determined by scanning radioautographs of dried longitudinal slices (Fairbanks *et al.*, 1965).

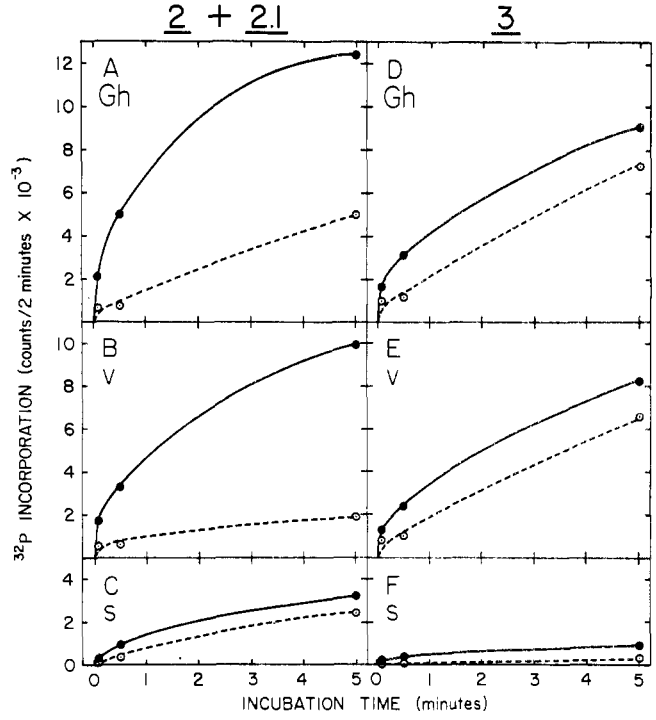

FIGURE 2: Cyclic AMP effects on kinetics of incorporation into elutable and nonelutable phosphopeptides. Phosphorylation reactions were terminated after the indicated times by the addition of 30 vol of cold 5 mM sodium phosphate, 1 mM EDTA, and 1 mM ATP (pH 8). The membranes were sedimented in 15 min at 27,000g_{max} and washed twice in the same buffer. Each pellet was resuspended in 19 vol of warm (37°) 0.2 mM EDTA–0.25 mM dithioerythritol (pH 8), allowed to stand for 15 min at 37°, and homogenized by two passages through a 26 gauge hypodermic needle. A portion of the suspension was removed; this contained the complete complement of "ghost" polypeptides (Gh). Vesicles (V) were separated from the "spectrin" eluate (S) by centrifugation for 45 min at 81,500g_{max}. The membrane fractions were subjected to electrophoresis in one of the discontinuous sodium dodecyl sulfate–Tris-borate systems of Neville (1971), with the lower gel buffered at pH 9.18 (nominal running pH 9.50) and containing 8% acrylamide monomer. The 200-μl samples applied were equivalent to about 25 μg of total ghost protein. Radioactivity was determined by counting transverse slices from gels frozen without fixation and staining. In this experiment, the [^{32}P]ATP specific activity was 17.3 cpm/fmol: (A–C) incorporation into phosphopeptides associated with unfractionated membranes (Gh), vesicles (V), or "spectrin" eluate (S) and migrating in the zone including bands 2 and 2.1; (B–D) incorporation into phosphopeptides associated with unfractionated membranes (Gh), vesicles (V), and "spectrin" eluate (S) and migrating with component 3.

beled phosphopeptides were described previously (Avruch and Fairbanks, 1972, 1974). Minor variations are given in legends to tables and figures. A Joyce-Loebl microdensitometer was used in scanning radioautographs of dried longitudinal gel slices. In one experiment (Figure 2 and Table III), a discontinuous sodium dodecyl sulfate–Tris-borate electrophoresis system described by Neville (1971) was used. The correspondence between major bands in these gels with those produced by electrophoresis at pH 2.4 (Avruch and Fairbanks, 1972) has been verified through comparisons of electrophorograms of membrane subfractions prepared by elution at low and high ionic strength.

Crystalline cyclic AMP was obtained from Sigma (A-9501) and Schwarz/Mann (907405).

Results

Substrate Specificity of Cyclic AMP Stimulated Reactions. The use of polyacrylamide gel electrophoresis in analyzing the substrate specificities of the endogenous phosphorylation reactions was discussed in the preceding paper (Avruch and Fairbanks, 1974). We noted therein that the high-resolution radioautographic technique is helpful in revealing the complex response to cyclic AMP. This is illustrated in Figure 1, which shows that, when cyclic AMP is added to the standard incubation medium, consisting of washed membranes in 1 mM Mg^{2+}, 20 mM imidazole (pH 7.4), and 2 μM [γ-^{32}P]ATP, phosphopeptide labeling in 10-sec incubations is increased overall and the relatively simple pattern of "basal" phosphorylations is altered. The most striking feature of the cyclic AMP response is the preferential utilization of two substrates, 2.1 (mol wt 200,000) and 4.5 (mol wt 52,000), that are relatively minor components in the Coomassie Blue staining profile (Avruch and Fairbanks, 1974; Fairbanks *et al.*, 1971). Significant increases in the incorporation into four other minor components—2.2, 2.3, 2.4, and 2.9—are also observed (Figure 1B). In most experiments, cyclic AMP slightly stimulates the labeling of polypeptide 3 and causes traces of ^{32}P to appear in bands 1, 4, and 5. No labeling of component 6 is detected under these conditions.

Because the separation between bands 2 and 2.1 in electrophorograms is small, these components are incompletely

TABLE I: Divalent Cation Dependence of Cyclic AMP Stimulated Phosphorylations.[a]

Additions	Membrane Component: 2.1 (+2) Net Incorp. (Counts/4 min)	2.1 (+2) Increment with cAMP (%)	3 Net Incorp. (Counts/4 min)	3 Increment with cAMP (%)	4.5 Net Incorp. (Counts/4 min)	4.5 Increment with cAMP (%)
Mg^{2+}	1,639		2,381		803	
Mg^{2+} + cyclic AMP	6,955	324	5,047	112	3,326	314
Ca^{2+}	626		2,025		586	
Ca^{2+} + cyclic AMP	733	17	2,070	2	555	−5
Co^{2+}	2,786		3,631		1,124	
Co^{2+} + cyclic AMP	13,112	371	5,872	62	3,794	238
Mn^{2+}	2,061		4,296		936	
Mn^{2+} + cyclic AMP	6,985	239	5,228	22	1,550	66
Zn^{2+}	646		1,659		463	
Zn^{2+} + cyclic AMP	538	−17	1,597	−4	414	−11
Fe^{2+}						
Fe^{2+} + cyclic AMP	*b*	*b*	*b*	*b*	*b*	*b*

[a] Membranes were phosphorylated in 10-sec incubations with [γ-^{32}P]ATP in 20 mM imidazole chloride (pH 7.4). Divalent cation chlorides were present at 1 mM. The phosphorylated membranes were precipitated with perchloric acid, washed, and fractionated electrophoretically as described (Avruch and Fairbanks, 1974). Incorporations into individual membrane polypeptides were determined by liquid scintillation counting of transverse slices from gels frozen without fixation and staining. [b] In the presence of Fe^{2+}, perchloric acid washing did not remove free [^{32}P]ATP, which obscured the peptide-bound radioactivity. After staining and fixation, negligible radioactivity was detected in these components.

resolved in densitometer tracings of radioautographs. Thus, under conditions in which labeling of polypeptide 2 predominates, the radioactivity in 2.1 appears as a shoulder on the leading edge of 2 (Figures 1A and 1C). On the other hand, when incorporation into 2.1 is selectively stimulated, phosphopeptide 2 is submerged and is detectable only as a shoulder on the trailing edge of 2.1 (Figure 1B). These adjacent bands are clearly distinguishable in photographs of radioautographs (Figures 1 and 5 of Avruch and Fairbanks, 1974; Figure 3 of this paper) and their identification in Figure 1 was unambiguous when radioautographs were superimposed on the corresponding dried longitudinal slices.

In several experiments (Figure 2, Table III and IV, below), polypeptides 1, 2, and 5 were eluted from phosphorylated membranes so that separate quantitative analyses of the incorporations into 2 and 2.1 could be performed using the electrophoretic techniques. In some cases, the results indicate that the labeling of 2 can be stimulated to a degree by cyclic AMP. However, the response of 2.1 phosphorylation under the same conditions is invariably much higher, and it will be seen that, for the cyclic AMP stimulated enzyme, a consistent pattern of preferential utilization of 2.1 and other tightly bound polypeptides has been demonstrated.

Divalent Cation Requirements and Effects of Cyclic GMP, Colchicine, and Cytochalasin. The divalent cation requirements of the cyclic AMP stimulated phosphorylations were examined, with the results presented in Table I. At 1 mM, both Co^{2+} and Mn^{2+} appear to be effective substitutes for Mg^{2+} in supporting the basal phosphorylations and the stimulation by cyclic AMP. Cyclic AMP stimulation in the presence of Ca^{2+} or Zn^{2+}, alone, is negligible, and the basal levels of incorporation are depressed.

The pattern of endogenous phosphorylation elicited by cyclic GMP is qualitatively indistinguishable from that shown by cyclic AMP. However, the magnitude of the stimulation by cyclic GMP is fourfold lower when both cyclic nucleotides are applied at 20 μM (Table II). In the case of cyclic AMP, this concentration (which was standard in all experiments described here) is probably saturating (Guthrow *et al.*, 1972).

The effects of colchicine and cytochalasin B were also explored, using conditions described previously (Avruch and Fairbanks, 1974). Neither agent significantly altered the cyclic AMP stimulated phosphorylation reactions (data not shown).

Inhibition by Monovalent Ions. Figure 1 depicts the inhibition of cyclic AMP stimulated phosphorylations by monovalent ions. The chlorides of Na^+, K^+, Li^+, and NH_4^+ were tested at concentrations of 0.001, 0.01, 0.05, 0.2, and 0.5 M. When present at concentrations of 0.05 M and higher, each inhibits cyclic AMP activation and depresses the phosphorylation of polypeptide 3 and lipid (Figure 1C–E). This inhibition by salts is one of several characteristics that serve to distinguish these reactions from the phosphorylation of polypeptide 2, which proceeds at its maximal rate in the presence of monovalent ions at 0.2 M (Avruch and Fairbanks, 1974). At this salt concentration, activation by cyclic AMP is negligible (Figure 1D), and the labeling pattern produced with cyclic AMP added is virtually indistinguishable from that seen in its absence.

Differential Incorporation into Elutable and Nonelutable Proteins. In investigating the ionic regulation of the major phosphorylation reactions and the physical relationship between protein kinases and their endogenous substrates, we have made use of selective elution at low ionic strength. This procedure effects a separation of polypeptides 1, 2, and 5 from vesicles bearing membrane lipids and the remaining proteins including 2.1 and 3 (Fairbanks *et al.*, 1971). Figure 2 presents kinetic data on the labeling of

TABLE II: Cyclic GMP Stimulation of Endogenous Phosphorylation Reactions.[a]

	Membrane Component					
	2.1 (+2)		3		4.5	
	Net Incorp. (Counts/10 min)	Increment (%)	Net Incorp. (Counts/10 min)	Increment (%)	Net Incorp. (Counts/10 min)	Increment (%)
Mg^{2+}	1,101		1,292		453	
Mg^{2+} + cyclic AMP	4,405	300	2,393	85	1,205	166
Mg^{2+} + cyclic GMP	1,917	74	1,552	20	636	40

[a] Membrane phosphorylation and electrophoretic analysis of incorporations into individual polypeptides were carried out as described in Table I. Mg^{2+} was present at 1 mM and cyclic nucleotides at 20 μM.

TABLE III: Differential Effects of Magnesium Ions and Cyclic AMP.[a]

			Components 1 + 2 + 2.1			Component 3			Component 4.5	
Incubation Time (sec)	Condition	Fraction	Incorp. (Counts/2 min)	Increment (%)	Distrib. of Increment[b] (%)	Incorp. (Counts/2 min)	Increment (%)	Distrib. of Increment[b] (%)	Incorp. (Counts/2 min)	Increment (%)
10	Basal	Gh	669			979			(84)	
	(1 mM Mg^{+})	V	530			796			(130)	
		S	(222)			(107)			(50)	
	+Cyclic AMP	Gh	2,124	217		1,565	60		445	(430)
		V	1,678		79	1,233		75	213	
		S	(276)		(4)	(234)		(22)	(108)	
	+20 mM Mg^{2+}	Gh	5,383	705		1,232	26		(119)	(42)
		V	1,876		29	977		72	(89)	
		S	3,531		70	(382)		(109)	(42)	
	+20 mM Mg^{2+}	Gh	8,214	53[c]		1,890	53[c]		332	(179)[c]
	+Cyclic AMP	V	3,067		42[c]	1,471		75[c]	(151)	
		S	4,233		25[c]	539		24[c]	(103)	
30	Basal	Gh	734			1,140			(100)	
	(1 mM Mg^{2+})	V	578			981			(77)	
		S	372			(91)			(33)	
	+Cyclic AMP	Gh	4,992	580		3,106	172		1,054	(954)
		V	3,265		63	2,346		69	302	
		S	962		14	410		16	(196)	
	+20 mM Mg^{2+}	Gh	14,652	1896		3,094	53		(203)	(103)
		V	4,139		26	2,558		81	(185)	
		S	9,234		64	852		39	(127)	

[a] Membranes were phosphorylated and "ghost" (Gh), vesicle (V), and low ionic strength "spectrin" eluate (S) fractions were prepared and analyzed electrophoretically as described under Figure 2. The [^{32}P]ATP specific activity was 17.3 cpm/fmol. Responses are expressed as per cent increments in incorporations over those measured after incubation with 1 mM Mg^{2+}, except as indicated (footnote *c*). Figures in parentheses are based on levels of gross counts below two times background. [b] (Radioactivity increment observed in vesicle or eluate fraction)/(total increment in corresponding polypeptides in unfractionated membranes). [c] Increments relative to incorporations in 20 mM Mg^{2+}.

certain polypeptides in the two fractions. It can be seen that incorporation continues for at least 5 min and that the initial reaction rate is stimulated by cyclic AMP.[1] When phosphorylated ghosts are subfractionated into vesicles (V) and "spectrin" eluate (S) by low ionic strength extraction, the radioactivity is apportioned unequally. Polypeptide 3 is tightly bound (Fairbanks *et al.*, 1971) and, after incubation either in the presence or absence of cyclic AMP, most of the radioactivity transferred to it is recovered with the vesicles.[2]

[1] As pointed out previously, the progressive reduction in labeling rate appears to follow hydrolysis of ATP by the membrane ATPases and does not represent saturation of the endogenous polypeptide substrates (Avruch and Fairbanks, 1974). Incorporation in 10-sec incubations is taken as a measure of the initial rate.

[2] Note that, in this experiment, electrophoresis and sample preparation were performed under conditions that hydrolyze the acyl phosphate bonds in the labile intermediate of the Na^{+},K^{+} transport ATPase reaction (Avruch and Fairbanks, 1972). This accounts for the differences in the kinetics of polypeptide 3 labeling between that shown here and that previously shown in Figure 2 of Avruch and Fairbanks (1974).

TABLE IV: Differential Effects of Calcium Ions and Cyclic AMP.[a]

Conditions	Fraction	Components 2 + 2.1			Component 3			Component 4.5		
		Incorp. (Counts/ 4 min)	Increment (%)	Index of Retention[b]	Incorp. (Counts/ 4 min)	Increment (%)	Index of Retention[b]	Incorp. (Counts/ 4 min)	Increment (%)	Index of Retention[b]
Basal	Gh	841			2,069			334		
(1 mM Mg^{2+})	V	794		0.77	3,054		0.96	330		0.89
	S	232			(122)			(40)		
+Cyclic AMP	Gh	3,489	+315		3,122	+51		847	+154	
	V	3,524	+344	0.88	3,703	+21	0.97	734	+122	0.89
	S	477	+106		(119)	(−2)		(95)	(+138)	
+Ca^{2+}	Gh	1,497	+78		1,286	−38		262	−22	
	V	617	−22	0.44	1,315	−57	0.91	(168)	(−49)	(0.57)
	S	776	+234		(126)	(−3)		(129)	(+222)	
+Cyclic AMP +Ca^{2+}	Gh	2,208	−37[c] +47[d]		1,780	−43[c] +38[d]		411	−51[c] +57[d]	
	V	1,795	−49[c] +82[d]	0.67	1,930	−48[c] +47[d]	0.95	400	−46[c] +138[d]	0.72
	S	868	+191[c] +12[d]		(102)	(−14)[c] (−19)[d]		(153)	(+61)[c] (+19)[d]	

[a] Membranes were phosphorylated and "ghost" (Gh), vesicle (V), and low ionic strength "spectrin" eluate (S) fractions were prepared and fractionated electrophoretically as described under Figure 3. Incorporations into individual zones were determined by liquid scintillation counting of segments from fixed, stained gels. Responses are expressed as per cent increments above basal incorporations in 1 mM Mg^{2+}, except as indicated (footnotes *c* and *d*). Figures in parentheses are based on levels of gross counts below two times background. [b] For each component, or group of components: index of retention = (counts retained in vesicles)/(counts retained plus counts eluted). [c] Response to Ca^{2+} with cyclic AMP present; increment relative to incorporation with Mg^{2+} plus cyclic AMP. [d] Response to cyclic AMP with Ca^{2+} present; increment relative to incorporation with Mg^{2+} plus Ca^{2+}.

Although the phosphorylated polypeptides, 2 and 2.1, cannot be resolved readily by transverse slicing, their separation by elution of "spectrin" prior to electrophoresis reveals that a large proportion of the basal activity is directed against 2, while the increment due to cyclic AMP is largely accounted for by a selective increase in the labeling of 2.1.

Differential Effects of Magnesium Ions and Cyclic AMP. The same approach was used to demonstrate differential effects of Mg^{2+} and cyclic AMP on these reactions. Thus, in Table III it can be seen that raising the Mg^{2+} concentration from 1 to 20 mM stimulates several-fold the total incorporation into the zone comprising polypeptides 2 and 2.1. In this experiment, 64–70% of the total radioactivity increment in this zone was eluted with "spectrin," indicating that increasing Mg^{2+} at this level preferentially stimulates polypeptide 2 phosphorylation. As shown above, cyclic AMP has the opposite effect; in this case, 63–79% of the increment was retained in the vesicles, which is consistent with preferential utilization of polypeptide 2.1 as substrate. It is of interest that the per cent stimulation by cyclic AMP is relatively low at 20 mM Mg^{2+}. The absolute increment is increased slightly over that at 1 mM Mg^{2+}, but, at the same time, the level of basal phosphorylation of nonelutable proteins is increased at least three- to fourfold (Table III).

The phosphorylation of polypeptide 4.5 is stimulated more than twofold by 20 μM cyclic AMP (Tables III and IV). This may substantially underestimate the degree of stimulation, because the background seen in this region after phosphorylation in the absence of cyclic AMP does not correspond precisely to the phosphorylated component 4.5. We have noted some variation both in the magnitude of this response to cyclic AMP and in the sharpness of band 4.5 in radioautographs (*cf.* Figures 1 and 5 of Avruch and Fairbanks (1974) and Figures 1 and 4 of this paper). However, selective stimulation of this reaction by cyclic AMP is invariably seen, though the rate of incorporation into 4.5 is usually significantly lower than that into 2.1.

Calcium Ion Antagonism of Cyclic AMP Effects. Differential effects of Ca^{2+} on these reactions are shown in Figure 3 and Table IV. In the absence of cyclic AMP, 1 mM Ca^{2+} increases the net incorporation into high molecular weight polypeptides. However, from the separate analyses of the "spectrin" eluate and vesicle fractions, it can be seen that the Ca^{2+} stimulation is selective for polypeptide 2 and that the basal incorporation into 2.1 is simultaneously depressed. As is consistent with the data presented above, the addition of cyclic AMP to this preparation activated preferential utilization of polypeptide 2.1 as a substrate and resulted in a larger increment in the vesicle fraction than in the "spectrin" eluate. When cyclic AMP is present, the addition of Ca^{2+} inhibits net incorporation into the nonelutable high molecular weight polypeptides. A similar antagonistic effect of Ca^{2+} is seen in the cyclic AMP stimulated phosphorylation of component 4.5 (Table IV) although in this case the net incorporations are much lower. Inhibition of lipid phosphorylation by Ca^{2+} is also observed (Figure 3).

Table IV introduces an "index of retention" as an alternative mode of presenting data on the substrate specificities of these reactions. This number is the proportion of the total counts in a particular polypeptide size class that is recovered in the vesicle fraction after extraction at low ionic strength. The "integral" polypeptide 3 (Fairbanks *et al.*, 1971; Singer and Nicolson, 1972; Bretscher, 1971; Steck and Yu, 1973) would be expected to exhibit a high index of retention; in this experiment, the index was greater than 0.9

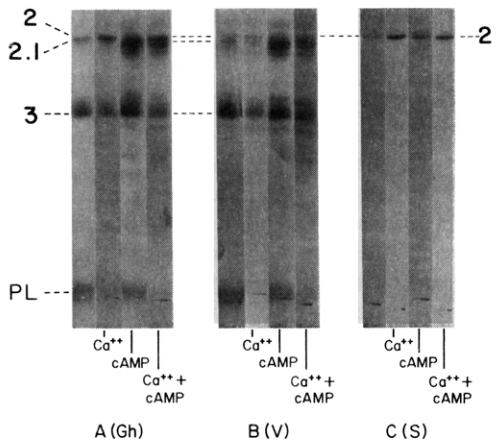

FIGURE 3: Effects of Ca^{2+} and cyclic AMP on endogenous phosphorylation reactions. Membranes were phosphorylated in 10-sec incubations, with 1 mM $CaCl_2$ and/or 20 μM cyclic AMP present as indicated. The reactions were terminated by the sudden addition of 20 vol of cold 5 mM sodium phosphate–1 mM EDTA (pH 8), and the membranes washed twice in the same buffer. Low ionic strength extraction, electrophoretic fractionation at pH 2.4, and radioautography of dried longitudinal slices were performed as described in the preceding paper (Avruch and Fairbanks, 1974). The gels were loaded in proportion to the recovery of protein in the corresponding membrane subfraction: (A) patterns of ^{32}P incorporation into components of total "ghost" membranes (Gh), about 40 μg of protein applied; (B) distribution of ^{32}P in membrane components recovered in vesicles (V) after extraction, 30 μg of protein; (C) distribution of ^{32}P in membrane polypeptides eluted with "spectrin" (S), 10 μg of protein.

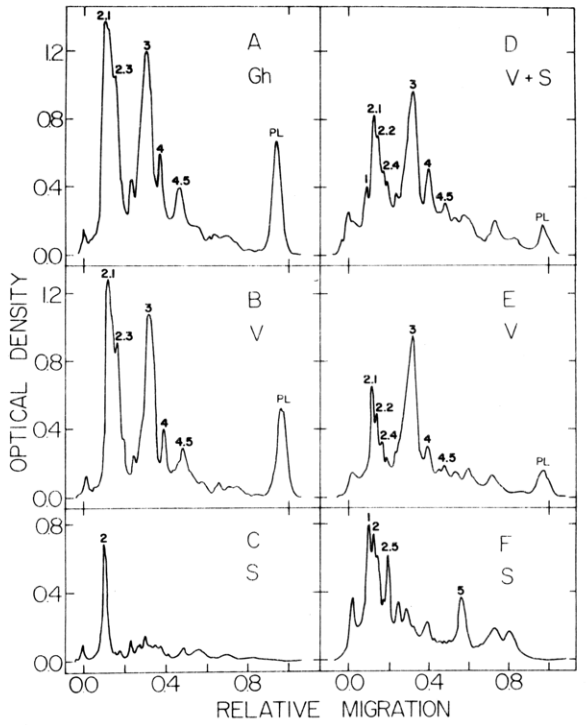

FIGURE 4: Cyclic AMP dependent phosphorylation in the low ionic strength eluate. (A–C) Phosphorylation *before* elution. Membranes were phosphorylated in the presence of 20 μM cyclic AMP and were washed to remove excess [γ-^{32}P]ATP. A portion of the preparation was extracted at low ionic strength. Unfractionated "ghosts" (Gh) and the vesicle (V) and "spectrin" eluate (S) fractions were subjected to electrophoresis at pH 2.4 in sodium dodecyl sulfate-containing polyacrylamide gels. The sample loading was adjusted so that the gels of the eluted and retained proteins contained these components at the same levels as the gel of the unfractionated membranes. Radioautographs of dried longitudinal slices were scanned using a microdensitometer. Methods are detailed in the preceding paper (Avruch and Fairbanks, 1974). (D–F) Phosphorylation *after* elution. Membranes were extracted at low ionic strength as described by Avruch and Fairbanks (1974). The "spectrin" eluate (S) was concentrated by ultrafiltration to the same volume as the resuspended vesicles (V). A 1:1 admixture (S + V) of these fractions contained the endogenous substrates in the same proportions as unfractionated membranes. Phosphorylation reactions were carried out in the presence of 20 μM cyclic AMP and were terminated with perchloric acid. Unlabeled vesicles were then added to eluate fractions to aid in the precipitation of the phosphorylated proteins. Electrophoresis and densitometric analysis of radioautographs were carried out as described above.

for ^{32}P-labeled material migrating in this zone, regardless of the conditions of labeling (Table IV). By contrast, the differential effects of Ca^{2+} and cyclic AMP on the labeling of elutable and nonelutable polypeptides result in large changes in the index of retention for ^{32}P in the zone comprising components 2 and 2.1. In this case, added Ca^{2+} lowers the index substantially, while cyclic AMP increases it. By the same criterion, component 4.5 appears to be relatively tightly bound to the membrane and thus resembles 3 and 2.1 (Table IV).

Partial Elution of Cyclic AMP Stimulated Protein Kinase Activity. As described in the preceding paper, we found that the salt-stimulated protein kinase was not eluted from the membrane with its specific substrate, polypeptide 2 of "spectrin." Figure 4 (D–F) presents evidence that the same conditions do release protein kinase activity that is expressed in the presence of cyclic AMP. In these experiments, we have carried out the elution first, and have then analyzed the endogenous phosphorylation reactions separately in the isolated "spectrin" (S) and vesicle (V) fractions, as well as in the mixture (V + S) reconstituting the membrane proteins in their original proportions. Figures 4E and 4F are densitometer scans of radioautographs showing the patterns of phosphorylation produced in the vesicle and eluate fractions, respectively, upon incubation with [γ-^{32}P]ATP in the presence of cyclic AMP. The profile in Figure 4F reflects a tenfold stimulation by cyclic AMP of total self-phosphorylation in the eluate and thus clearly demonstrates the presence of a cyclic AMP dependent enzyme. A rigorous estimate of the amount of eluted enzyme cannot be derived from these data because of the multiple ambiguities arising from differences in the substrates and in physical constraints on the enzyme in the "spectrin" eluate and vesicle fractions.

The pattern of incorporation in the vesicle fraction (Figure 4E) resembles that in intact ghosts (Figure 4A,B), though the proportion of total radioactivity in components 2.1 and 4.5 relative to 3 is somewhat lower. It is interesting to note, however, that phosphorylation by the eluted enzyme in the presence of cyclic AMP is virtually indiscriminate (*cf.* Figure 4C,F). Under normal circumstances, components 1, 2.5, and 5 are not major substrates and the stimulation of polypeptide 2 phosphorylation by cyclic AMP is relatively weak. The occurrence of these reactions in the eluate suggests that the process of extraction releases the enzyme from major constraints on its access to potential substrates. Our data indicate that this process may be reversible, in that admixture of the two fractions and incubation in the presence of buffer plus Mg^{2+} appear to normalize the pattern of incorporation (Figure 4D) by depressing those phosphorylation reactions that can be recognized as aberrant in the eluate incubated alone.

Discussion

In the experiments described here and in the preceding paper (Avruch and Fairbanks, 1974), we stressed analysis

of the substrate specificities of the endogenous phosphorylation reactions and explored the physical relationships between the protein kinase activities and their principal substrates in the erythrocyte membrane. By means of high-resolution electrophoretic analysis of the labeled phosphopeptides, we have shown that the effect of cyclic AMP in this system is more complex than was hitherto recognized. In particular, a high molecular weight substrate in the cyclic AMP dependent reaction is clearly distinguished from polypeptide 2, which is, in contrast, the principal substrate in the presence of monovalent ions and in the absence of cyclic AMP. We have also shown selective effects of monovalent ions and calcium, in that the cyclic AMP dependent phosphorylations are inhibited by these ions at concentrations that stimulate the phosphorylation of polypeptide 2. Finally, we have observed partial elution of the cyclic AMP dependent activity under conditions that do not result in release from the membrane of the salt-stimulated polypeptide 2 kinase activity.

The pattern of phosphorylation we describe is similar overall to that described by others, but our results depart in some significant details from the earlier findings. Thus, in contrast to our results, Guthrow *et al.* (1972) and Roses and Appel (1973) reported only inhibitory effects of Ca^{2+} on the endogenous reactions. In addition, Roses and Appel (1973) detected significant incorporation into the zone of polypeptide 1 and did not find a response to monovalent ions. In the report by Rubin and Rosen (1973), cyclic AMP stimulation of the rate of incorporation into component 4.5 was shown, but there appeared to be no increase in the reaction rate for substrates in the zone of polypeptides 1, 2, and 2.1. Finally, the extent of cyclic AMP stimulated incorporation into components other that 4.5 appears smaller in the experiments of Guthrow *et al.* (1972) and Rubin and Rosen (1973).

Our experience suggests that these variations in the published findings will be found to reflect differences in the conditions under which the phosphorylation reactions have been carried out. The chemical character of the buffer, its pH and ionic strength, the Mg^{2+} concentration, and the time and temperature of the incubation must all be considered. We have already shown here that some of these factors have significant roles in regulating the endogenous reactions.

On the basis of the observation that ^{32}P incorporated into 4.5 exhibits the same mobility in sodium dodecyl sulfate gels as a covalent affinity label for cyclic AMP binding sites, it has been suggested that component 4.5 is the regulatory subunit of the cyclic AMP dependent protein kinase (Guthrow *et al.*, 1973). If this proves to be the case, the properties of the other preferred substrates, particularly 2.1, will be of considerable interest, because these components may be elements in pathways for functional expression of the cyclic AMP stimulated protein kinase other than phosphorylation of its own subunit. Little is known about the localization or function of these membrane polypeptides. As Guthrow *et al.* (1972) suggested, it is conceivable that incorporation into the high molecular weight region might arise through aggregation of the phosphorylated component 4.5. In this connection, however, it is important to note that our observations are not tied to the use of the pH 2.4 sodium dodecyl sulfate-polyacrylamide gel system alone. Incorporation into high molecular weight substrates that are tightly bound to the membrane and not elutable with "spectrin" has been detected in experiments using several electrophoretic techniques (Figure 2, Table III, and data not shown). Inasmuch as sample preparation in these cases did not involve perchloric acid precipitation and did permit thorough reduction with dithioerythritol in the presence of EDTA, it appears unlikely at present that these components are artifacts generated by acid treatment or through the formation of disulfide or cation bridges.

Our observations draw attention to the possibility that two or more independent enzymes are involved in erythrocyte membrane protein phosphorylation. The elution results are particularly suggestive of this. However, because only partial resolution of the two activities has been achieved at this stage, and because the endogenous phosphorylation reactions in membrane subfractions are not susceptible to unambiguous quantification, the results remain open to several interpretations. For example, the data can be rationalized in part on the basis of the model proposed by Brostrom *et al.* (1971). In this vein, we would postulate that (1) a single membrane-associated protein kinase is involved; (2) the association of its regulatory and catalytic subunits is controlled by the ionic strength, as well as by cyclic AMP binding; and (3) the free and complexed forms of the catalytic subunit are both active but exhibit radically different substrate specificities. Corbin *et al.* (1973) have described salt effects on the association of regulatory and catalytic subunits of the adipose tissue enzyme. Similar ideas might be invoked in interpreting Jergil and Dixon's (1970) finding that salt nonspecifically stimulated phosvitin phosphorylation by a partially purified trout testis enzyme while at the same time depressing the activity against histones.

This unitary model would explain satisfactorily the observations that cyclic AMP independent activity is extracted from erythrocyte membranes with high salt (Rubin *et al.*, 1972; G. Fairbanks and J. Avruch, unpublished observations) and that, after such extraction, the cyclic AMP binding activity is recovered with the membranes (Rubin *et al.*, 1972). However, the model requires an additional contrivance to make it consonant with our data. It must be supposed that elution at low ionic strength selectively destroys the capacity of the eluted enzyme to phosphorylate polypeptide 2 in the presence of salt, while the same enzyme bound to the membrane retains that activity.

Although we have divided the major phosphorylation reactions into two classes on the basis of differential responses to several conditions, the assignment of polypeptide 3 phosphorylation in this scheme is not straightforward. This reaction is depressed by salts and stimulated by cyclic AMP; in these respects it resembles the reactions utilizing components 2.1 and 4.5. However, because the basal incorporation rate is high and the response to cyclic AMP relatively weak, it does not fit precisely into this class and might represent a separate process altogether. On a tentative basis, we have taken the response to monovalent ions as the defining factor and regard the phosphorylation of polypeptide 3 as a basal function of the cyclic AMP stimulated enzyme.

As other investigators have pointed out (Guthrow *et al.*, 1972; Roses and Appel, 1973), the existence of a cyclic AMP dependent protein kinase in human erythrocyte membranes seems anomalous in the light of evidence that adenylate cyclase is not present in these membranes (Sheppard and Burghardt, 1969; Wolfe and Shulman, 1969). The activity might represent a vestige from a presursor in erythropoeisis or the presence of a contaminant in the cell preparations. Rubin *et al.* (1972) noted, however, that reticulocyto-

sis in the blood donor had no effect on the levels of protein kinase activity in isolated membranes and we observed no changes in the pattern or level of cyclic AMP dependent membrane phosphorylation in intact erythrocytes prepared without removal of the "buffy coat" (G. Fairbanks and J. Avruch, manuscript in preparation). The possibility that the erythrocyte *in vivo* is responsive to circulating levels of cyclic AMP lacks appeal because of the low plasma levels reported (Broadus *et al.*, 1970) and the recent demonstration that the cyclic AMP binding protein is located on the inner surface of the membrane (Kant and Steck, 1973). It is conceivable that minute amounts of cyclic AMP, either produced by hormonal activation or encountered in the plasma, are sequestered locally near the membrane and thereby brought to bear on the protein kinases at an effective concentration. But it is clear that none of these explanations is compelling and that an understanding of the complex phenomenology may require the elucidation of a mode of regulation as yet unknown.

When eluted at low ionic strength with "spectrin," the cyclic AMP stimulated enzyme phosphorylates major polypeptides indiscriminately. This is a profound change from the specificity for minor substrates it exhibits in its original state of integration in the membrane. Such a breach in specificity might result from partial denaturation of the enzyme or its potential substrates during the elution process. Alternatively, release from the membrane may allow the enzyme to gain access to substrates from which it is normally shielded due to its localization or orientation *in situ*. In either case, our results seem to bear out the suggestion of Rubin *et al.* (1972) that the membrane-associated protein kinases should be useful as "endogenous probes" of membrane structure.

The cyclic AMP stimulated endogenous phosphorylation reactions occur at maximal rates under ionic conditions that deviate markedly from those pertaining *in vivo*. Intact human erythrocytes carry Mg^{2+} at about 2.5 mmol/l. of cell water and monovalent cations at 155 mequiv/l. (Gary-Bobo and Solomon, 1968). Our results suggest that, in this ionic milieu, the cyclic AMP stimulated activity will be depressed in favor of the phosphorylation of polypeptide 2. Ca^{2+} is present at only 4.1×10^{-2} mM (Schatzmann and Vincenzi, 1969), but most is bound to the membrane (Harrison and Long, 1968), where it might exert a similar differential effect on the membrane-associated protein kinases. Preliminary studies of membrane phosphorylation in erythrocytes subjected to long incubations with ^{32}P-labeled inorganic phosphate indicate that, in the *steady state*, labeling of polypeptide 2 predominates (Palmer and Verpoorte, 1971; G. Fairbanks and J. Avruch, manuscript in preparation). Further work with intact cells and reconstituted ghosts is needed to test the proposition that the *rate* of membrane protein phosphorylation *in vivo* is responsive to cyclic AMP, Ca^{2+}, and monovalent ions in the manner described for the endogenous reactions in the isolated membrane.

Acknowledgments

We thank Ms. Caroline I. Brown, Mr. Edward Phillips, and Ms. Karen I. Talbert for expert technical assistance and Dr. J. King for the use of his microdensitometer.

References

Avruch, J., and Fairbanks, G. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1216.

Avruch, J., and Fairbanks, G. (1974), *Biochemistry 13,* 5507.

Bretscher, M. S. (1971), *J. Mol. Biol. 59,* 351.

Broadus, A. E., Kaminsky, N. I., Hardman, J. G., Sutherland, E. W., and Liddle, G. W. (1970), *J. Clin. Invest. 49,* 2222.

Brostrom, C. O., Corbin, J. D., King, C. A., and Krebs, E. G. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2444.

Corbin, J. D., Soderling, T. R., and Park, C. R. (1973), *J. Biol. Chem. 248,* 1813.

Fairbanks, G., Jr., Levinthal, C., and Reeder, R. H. (1965), *Biochem. Biophys. Res. Commun. 20,* 393.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Gary-Bobo, C. M., and Solomon, A. K. (1968), *J. Gen. Physiol. 52,* 825.

Guthrow, C. E., Jr., Allen, J. E., and Rasmussen, H. (1972), *J. Biol. Chem. 247,* 8145.

Guthrow, C. E., Rasmussen H., Brunswick, D. J., and Cooperman, B. S. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3344.

Harrison, D. G., and Long, C. (1968), *J. Physiol. 199,* 367.

Jergil, B., and Dixon, G. H. (1970), *J. Biol. Chem. 245,* 425.

Kant, J. A., and Steck, T. L. (1973), *Biochem. Biophys. Res. Commun. 54,* 116.

Marchesi, V. T., and Steers, E., Jr. (1968), *Science 159,* 203.

Neville, D. M., Jr. (1971), *J. Biol. Chem. 246,* 6328.

Palmer, F. B. St. C., and Verpoorte, J. A. (1971), *Can. J. Biochem. 49,* 337.

Roses, A. D., and Appel, S. H. (1973), *J. Biol. Chem. 248,* 1408.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972), *J. Biol. Chem. 247,* 6135.

Rubin, C. S., and Rosen, O. M. (1973), *Biochem. Biophys. Res. Commun. 50,* 421.

Schatzmann, H. J., and Vincenzi, F. F. (1969), *J. Physiol. 201,* 369.

Sheppard, H., and Burghardt, C. R. (1969), *Biochem. Pharmacol. 18,* 2576.

Singer, S. J., and Nicolson, G. L. (1972), *Science 175,* 720.

Steck, T. L., and Yu, J. (1973), *J. Supramol. Struct. 1,* 220.

Wolfe, S. M., and Shulman, N. R. (1969), *Biochem. Biophys. Res. Commun. 35,* 265.