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PROTEIN PHOSPHORYLATION AND THE REGULATION OF CATION COTRANSPORT

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The turkey erythrocyte is a useful system in which to investigate the relationship between protein phosphorylation and membrane transport. This cell possesses a catecholamine- and cyclic AMP-stimulated Na^+–K^+ 'co-transport' system in its plasma membrane, and the physiological properties of this system have been partially elucidated. It is distinct from the Na^+–K^+ 'pump' and is insensitive to ouabain, but can be blocked by certain diuretic agents such as furosemide and bumetanide.

A plasma membrane protein of MW 230000, called goblin, has been identified as the major protein whose state of phosphorylation increases upon incubation of turkey erythrocytes with catecholamines or cyclic AMP. The kinetics of phosphorylation of goblin at its cyclic AMP-sensitive sites correlate well with the kinetics of Na^+–K^+ cotransport activation under various conditions. This protein may play a role in the regulation of the Na^+–K^+ cotransport system by catecholamines and cyclic AMP

At present it seems likely that some of the actions of a large number of regulatory agents and their associated second messengers are achieved through control of the state of phosphorylation of specific proteins present in various target tissues. This concept is illustrated schematically in Fig. 1, in which protein phosphorylation is envisaged as a final common pathway for the mediation of physiological responses to diverse cellular stimuli (for review see Greengard, 1978). One of the major tasks in this area of investigation is to determine which physiological effects of each regulatory agent are mediated through protein phosphorylation and which are not. In the case of those responses that do involve phosphorylation, the ultimate goal is identification of the relevant phosphoprotein substrate(s) and understanding of the molecular mechanism whereby substrate phosphorylation produces the particular physiological response characteristic of that regulatory agent.

With respect to certain enzyme systems of intermediary metabolism, such as those involved in the control of the glycogenolytic cascade by hormones, considerable progress has been made in the elucidation of the role of specific phosphorylation processes in the regulation of enzyme activity (for reviews see Nimmo & Cohen, 1977; Krebs & Beavo, 1979). In contrast, membrane-associated physiological processes that are under regulatory control, e.g. transport processes that are influenced by hormones and cyclic nucleotides, have received comparatively little attention and less is known of the particular phosphoproteins that may be involved and of the manner in which they may regulate the physiological response. In the case of the nervous system, where second messengers such as cyclic AMP and Ca^{2+} may regulate diverse

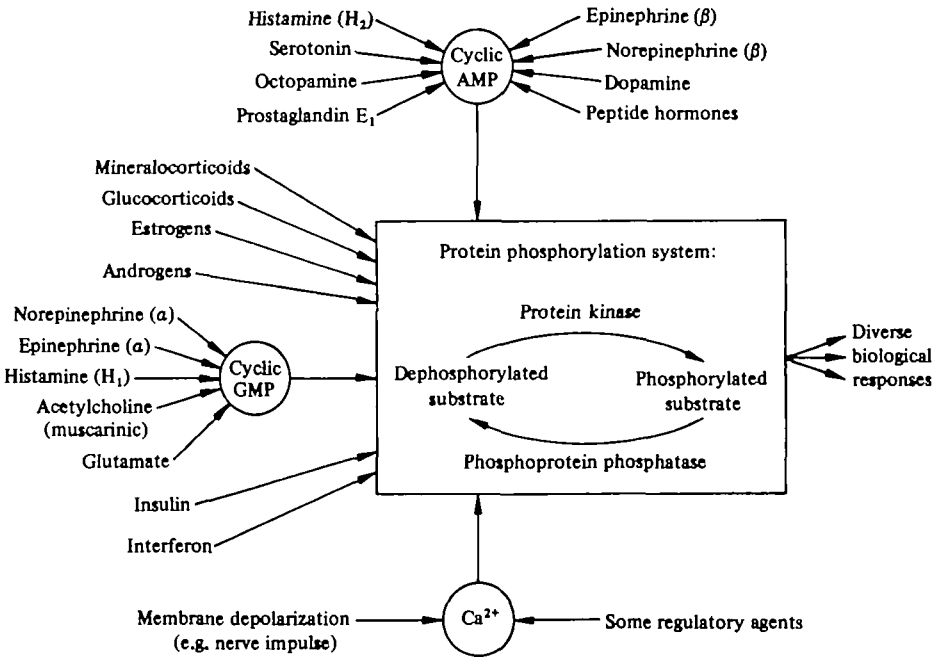


Fig. 1. Schematic diagram of postulated role played by protein phosphorylation in mediating some of the biological effects of a variety of regulatory agents. The diagram gives examples of regulatory agents, some of whose effects may be mediated through regulation of the phosphorylation of specific proteins, and is not intended to be complete. In addition to cAMP and a variety of neurotransmitters and hormones whose effects are mediated through cAMP, these regulatory agents include cGMP, a variety of neurotransmitters and hormones whose effects are mediated through cGMP, Ca^{2+}, and agents whose effects are mediated through Ca^{2+}, as well as several classes of steroid hormones, insulin, and interferon, For brevity, the numerous peptide hormones whose effects are known to be mediated through cAMP, and the various regulatory agents believed to act through translocation of Ca^{2+}, are not listed individually. As reviewed elsewhere, it seems likely that some, but not necessarily all, of the biological responses elicited by any given regulatory agent are mediated through the protein phosphorylation system; for simplicity, pathways from regulatory agent to biological response that do not involve protein phosphorylation are not shown. From Greengard (1978), with permission.

membrane events, this problem is particularly acute because of the enormous complexity of nervous tissue. Although a major interest of our research group during the past few years has been the role of cyclic nucleotides and protein phosphorylation in nerve cells, part of our effort has been directed towards a study of simpler preparations which might be more amenable to the investigation of the intermediary role of phosphoproteins in physiological processes.

One such preparation is the turkey erythrocyte, which has several advantages for this type of study. This cell possesses in its plasma membrane a monovalent cation cotransport system responsive to agents that elevate intracellular cyclic AMP, including β-adrenergic catecholamines. The kinetics of the response are sufficiently slow that a concurrent study of ion flux and protein phosphorylation can be made. Homogeneous preparations of turkey erythrocytes are easily obtained in large quantities suitable for biochemical and physiological analysis. Electron micrographs of intact cells (Fig. 2) reveal a rather simple intracellular structure with a prominen

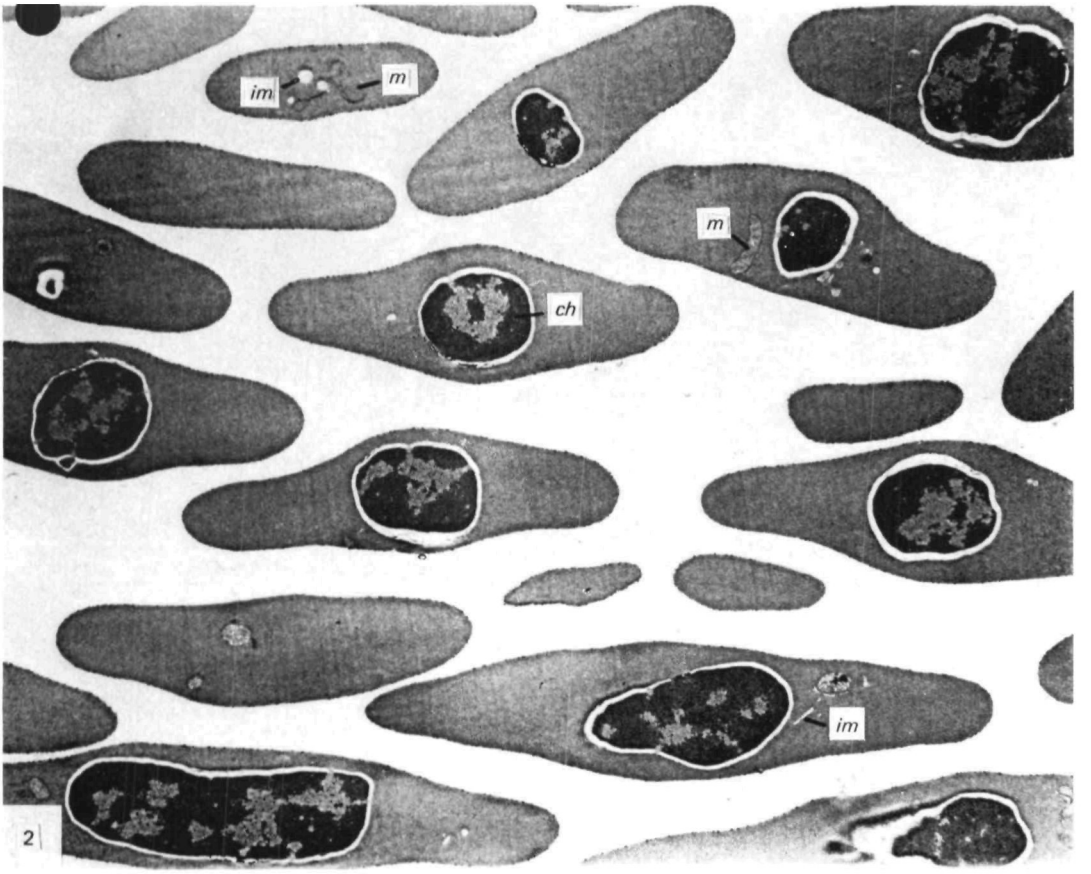


Fig. 2. Electron micrograph of turkey erythrocytes. The nucleus (*n*) contains compact chromatin masses (*ch*). A few mitochondrial profiles (*m*) and remnants of intracellular membranes (*im*) are seen in the cytoplasm. × 5300. From Beam *et al.* (1979), with permission.

Table 1. *Some properties of '$Na^+ + K^+$ cotransport' in turkey erythrocytes*

(1) Bidirectional transport of Na^+ and K^+ ions
(2) Influx saturable with respect to both cations in the bathing medium, with an almost absolute dependence of the influx of one cation on the presence of the second cation
(3) Insensitive to ouabain, but inhibited by certain 'loop' diuretics, e.g. furosemide and bumetanide
(4) Stimulated by catecholamines, cAMP, cAMP derivatives (e.g. 8-bromo-cAMP) and by certain other stimuli (e.g. hypertonic medium)
(5) Capable of leading to alterations in cell volume under appropriate ionic conditions

central nucleus and demonstrable but sparse internal membraneous structures such as mitochondria (Beam *et al.* 1979). This relatively simple organization makes subcellular fractionation and plasma membrane isolation straightforward. When these cells are suspended in a simple physiological salt solution and exposed to β-adrenergic catecholamines (e.g. isoproterenol), or to cyclic AMP itself, a specific '$Na^+ + K^+$ cotransport' system is activated. This article will review the physiological nature of this system briefly, and then consider the possible role of protein phosphorylation in its regulation.

Properties of '$Na^+ + K^+$ cotransport' in turkey erythrocytes

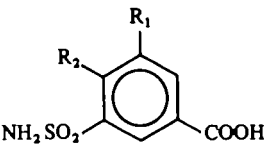
Table 1 lists some of the important physiological properties of this system in turkey erythrocytes (Gardner *et al.* 1976; Rudolph, Schafer & Greengard, 1977; Palfrey, Feit & Greengard, 1980; Palfrey & Greengard, 1980). Many of these properties also seem to be characteristic of a cotransport system described in duck erythrocytes by Kregenow (review, 1978) and McManus & Schmidt (review, 1978), and that we have also demonstrated in other avian erythrocytes such as goose and pigeon (Palfrey & Greengard, 1980).

Firstly, the system is bidirectional and can carry both Na^+ and K^+ into and out of the cell. Although the system is capable of performing net ion movements (see below), under the physiological conditions employed for most of the experiments described in this account it operates in an 'exchange diffusion' mode and no net gain or loss of ions by the cell occurs. Estimation of the state of activation of the system can be made using unidirectional ^{22}Na, ^{42}K or ^{86}Rb influx, the latter being a useful and convenient substitute for ^{42}K in this system.

Second, the influx of either Na^+ or K^+ is a saturable function of its own concentration in the bathing fluid, and of the concentration of the alternate ion in the medium. The saturation functions are hyperbolic and can be described by Michaelis–Menten kinetics. In such an analysis, the extracellular $[K^+]$ dependence of K^+ uptake yields a K_m value which is similar to that for the extracellular $[K^+]$ dependence of Na^+ uptake at constant external $[Na^+]$. This has led to an operational definition of the system as a 'Na^+ plus K^+ cotransport' system (McManus & Schmidt, 1978) in which the passage of one ion across the membrane is obligatorily dependent on the presence of the second ion on the same side of the membrane. For example, if Na^+ in the medium is replaced by the impermeant cation choline, little or no stimulation of K^+ influx by catecholamines or other stimuli can be detected, and analogously if K^+ is removed no Na^+ influx is observed.

Table 2. *Inhibitory effects of some 3-amino-5-sulfamoyl benzoic acid derivatives and furosemide on 8-bromo-cAMP-stimulated $Na^+ + K^+$ cotransport in turkey erythrocytes and a comparison with their diuretic activity in dogs* in vivo

(The half-maximal inhibitory effect of each substance on 8-bromo-cAMP (1 mM) stimulated ^{86}Rb uptake in turkey erythrocytes, and the diuretic activity, estimated as the mEquiv Na^+ excreted in the urine per 3 h following I.V. administration of 0·25 mg/kg to dogs, were determined as described by Palfrey *et al.* (1980). Note that minor structural modifications (compare Compound IV to Compound II) result in large parallel changes in both IC_{50} value and diuretic activity.)



	R_1	R_2	Turkey erythrocyte IC_{50} (M)	Diuretic activity in dogs
I.	$-NH(CH_2)_3CH_3$ (bumetanide)	—O—	$2{\cdot}5 \times 10^{-7}$	4·0
II.	$-NHCH_2$—	—S—	$4{\cdot}6 \times 10^{-8}$	3·6
III.	$-NH_2$	—O—	$> 10^{-3}$	< 0·1
IV.	$-NHCH_2$—OCH_3	—O—	3×10^{-4}	< 0·1
	Furosemide		$2{\cdot}5 \times 10^{-5}$	1·7

Thirdly, the system is insensitive to the well-known inhibitor ouabain, thus distinguishing it from the ubiquitous Na^+ pump that is also present in these cells. However, it can be inhibited by certain 'loop' diuretics such as furosemide and bumetanide. A detailed pharmacological analysis of a chemically related series of compounds of this class has been performed with the aim of finding agents with high inhibitory potency in the turkey erythrocyte (Palfrey *et al.* 1980). Some members o

his series are illustrated in Table 2, which shows that potent derivatives were indeed discovered, with IC_{50} values in the 30–300 nM range. These compounds were found to be relatively selective for the cation cotransport system at these low concentrations (no effects on other membrane-related phenomena, e.g. Na^+–K^+ pump activity, being found) and they may interact directly with the transport system itself. Radioactive derivatives of some of these agents are currently being synthesized in the hope that they may provide specific labels for the transport protein(s) involved. An interesting outcome of this investigation was the discovery that the efficacy of this group of compounds as inhibitors of the cation cotransport system in turkey erythrocytes correlated well with their activity as diuretics *in vivo* in dogs (Table 2, for a more detailed analysis see Palfrey *et al.* 1980) where their site of action is presumed to be the loop of Henle (Burg, 1976; Imai, 1977). This suggests that the transport mechanisms in the two tissues may be analogous in some respects. Furthermore, ion transport systems in other tissues which appear to involve coupled Na^+ plus Cl^- movements including various epithelia (Frizzell, Field & Schutz, 1979) and squid giant axon (Russell, 1979) are also sensitive to 'loop' diuretics, indicating that these may also be similar to the cotransport system in avian erythrocytes.

Fourthly, the system is stimulated by catecholamines and cyclic AMP, as well as by a number of stimuli that appear to act via a cyclic AMP-independent mechanism. An example of a cyclic AMP-independent class of stimulus is suspension of the cells in a hypertonic solution. This causes the cells to shrink osmotically, and switches on a cation cotransport system whose properties are indistinguishable from those of the system activated by cyclic AMP (Palfrey & Greengard, 1980; for review of this mechanism in duck red cells see Kregenow, 1978, and McManus & Schmidt, 1978). The mechanism of stimulation by hypertonic conditions is unknown, but no increase in intracellular cyclic AMP nor in protein phosphorylation could be demonstrated under these conditions (Palfrey & Greengard, 1980; see also Kregenow, Robbie & Orloff, 1976, who showed that hypertonic medium has no effect on cyclic AMP levels in duck red cells).

Finally, under certain conditions (e.g. elevated extracellular $[K^+]$), activation of cotransport leads to a change in cell volume following an induction of net cation flow across the membrane. Thus, when $[K^+] = 15$ mM in the medium, addition of isoproterenol leads to cell swelling until an upper stable volume is reached (Rudolph, Schafer & Greengard, 1977). The ability of these cells to regulate their volume using the cotransport system may point to its true physiological function *in vivo*, and has been examined in some detail in duck erythrocytes by Kregenow (1978).

This summary is far from exhaustive, and several aspects of the physiology of 'Na^+ plus K^+ cotransport' remain to be more clearly elucidated. For example, the essential role of chloride ions in the system, first described by McManus & Schmidt (1978) in duck cells and confirmed by us in turkey red cells (Palfrey & Greengard, 1980), must be investigated further, especially in the light of our finding that the pharmacology of the system resembles that of known Na^+ plus Cl^- cotransport systems. In addition, the stoichiometry of Na^+ transport with respect to K^+, and the energy source for transport need further study.

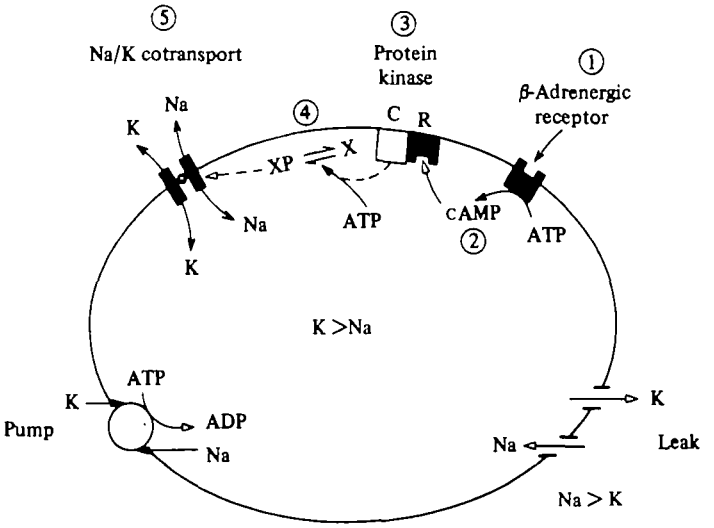


Fig. 3. A model of the turkey erythrocyte with particular reference to the monovalent cation transport systems present in the plasma membrane (leaks, Na^+ pump and $Na^+ + K^+$ cotransport) and a hypothesis on the mechanism of activation of the $Na^+ + K^+$ cotransport system by hormones. For details see text.

A model for activation of $Na^+ + K^+$ cotransport by hormones

With the knowledge that the Na^+ plus K^+ cotransport system is a well-defined physiological entity in the turkey erythrocyte membrane, it became possible to construct a working hypothesis of hormone activation of the system (Fig. 3). In turkey red cells, as in most other cells, the intracellular concentration of K^+ is higher, and that of Na^+ lower than in the extracellular fluid (Gardner *et al.* 1976). There are three major routes for monovalent cations to cross the plasma membrane of these cells: first, leak pathways which permit entry of Na^+ and exit of K^+ down their respective electrochemical gradients; second, a conventional ouabain-sensitive Na^+–K^+ pump counteracting the leak fluxes, and thirdly the Na^+ plus K^+ cotransport system whose properties were outlined above.

We hypothesize that the activation of cation cotransport in these cells proceeds as follows (numbers in brackets refer to Fig. 3): isoproterenol activates a β-adrenergic receptor coupling it to an adenylate cyclase present in the plasma membrane [1], which leads to an accumulation of cyclic AMP inside the cells [2]. This step can be by-passed by simple addition of cyclic AMP to the medium. In either case, the intracellular cyclic AMP then combines with the regulatory, i.e. inhibitory, subunit of a cyclic AMP-dependent protein kinase [3] freeing the catalytic subunit which is then able to phosphorylate various substrate proteins. We have used 'X' to designate a hypothetical membrane substrate protein [4] whose state of phosphorylation reversibly controls the activity of the Na^+ plus K^+ cotransport system [5]. This model thus predicts the existence of a hormone-sensitive phosphoprotein in the turkey erythrocyte membrane, which could be involved in cation cotransport. Previous work unequivocally established the presence of a β-adrenergic-sensitive adenylate cyclase in turkey red cells (e.g. Gardner *et al.* 1976). The existence of a type I cyclic AM[illegible]

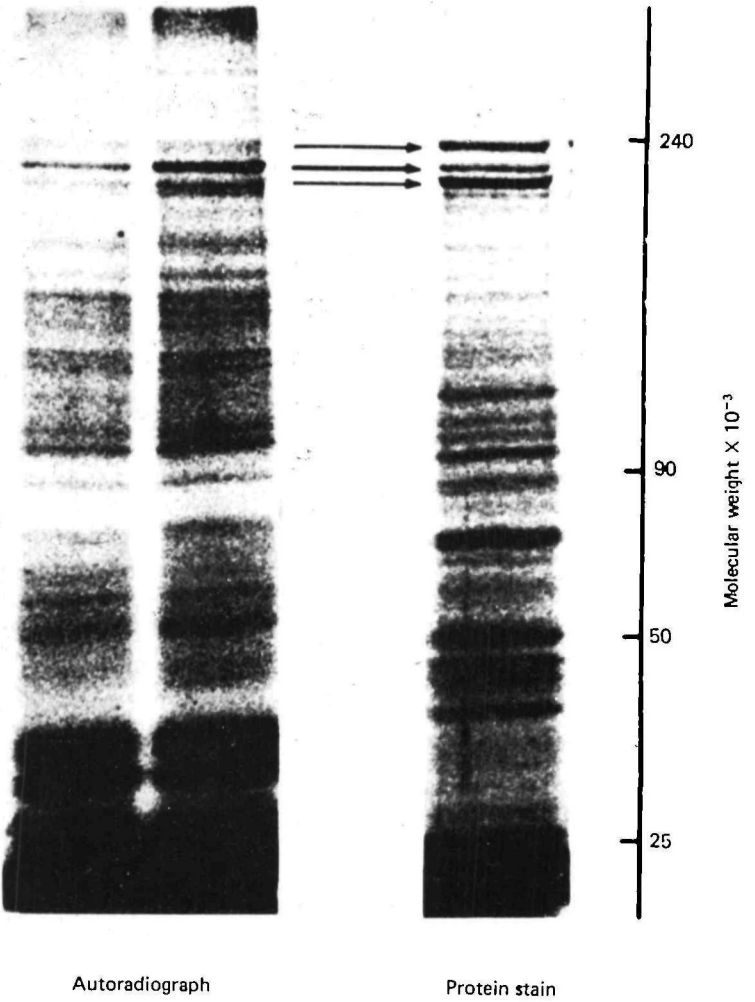


Fig. 4. Effect of DL-isoproterenol on the incorporation of ^{32}P into proteins of intact turkey erythrocytes as separated on SDS-polyacrylamide gels (6%, Laemmli system). Erythrocytes were prelabelled with $^{32}P_i$ for 3 h. Aliquots of cells were then incubated for an additional 20 min in the presence or absence of isoproterenol, and solubilized prior to electrophoresis. The outer two arrows indicate the spectrin doublet and the inner heavy arrow goblin. Lane 1, control; lane, 2, DL-isoproterenol (10^{-6} M). Modified from Beam *et al.* (1979).

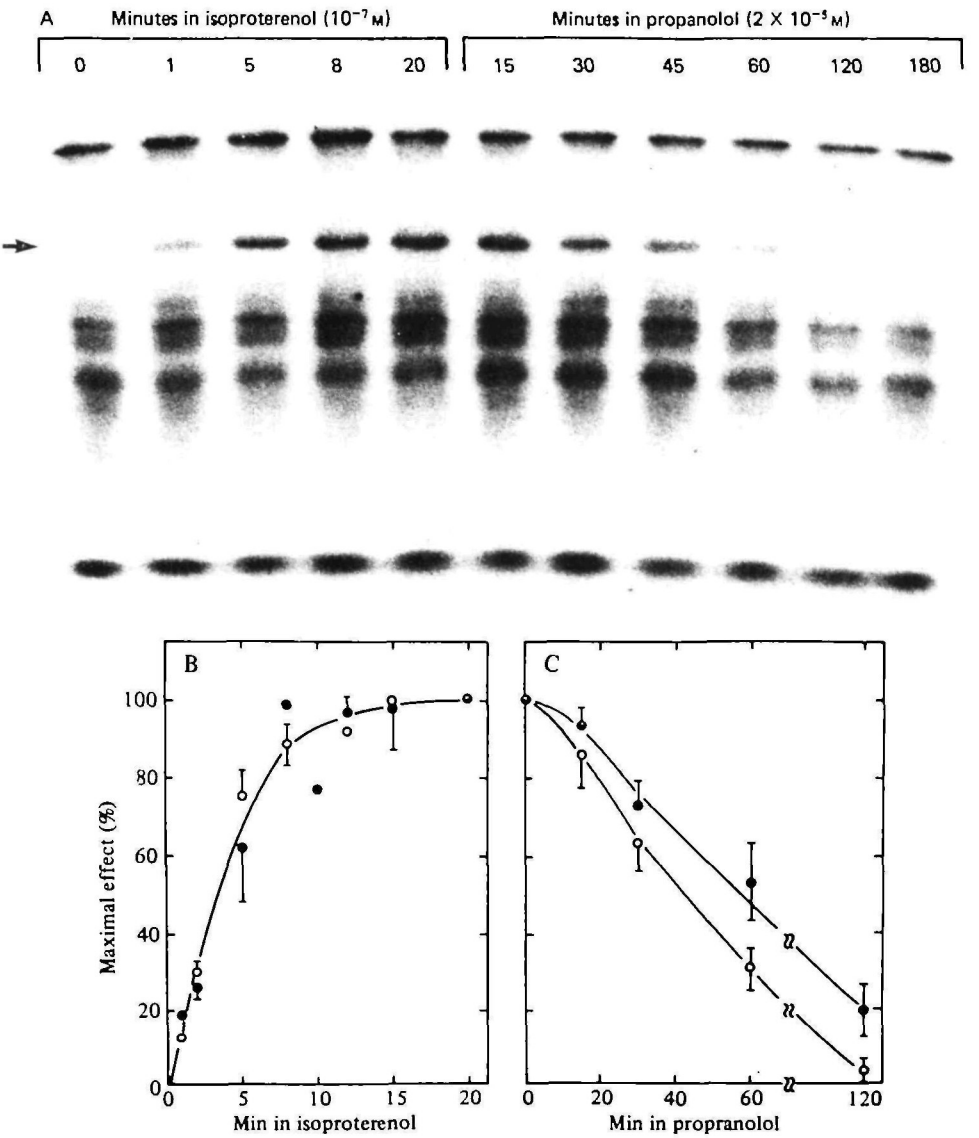


Fig. 5. (A) Autoradiograph of [^{32}P]peptides produced from goblin by limited proteolysis using *Staphylococcus aureus* V8 protease (1 μg/slot) using the procedure of Cleveland *et al.* (1977). Cells prelabelled with ^{32}P for 24 h were exposed to DL-isoproterenol (10^{-7} M), and then to a 200-fold excess of DL-propranolol. Samples were removed at the indicated times, solubilized, and proteins separated by SDS (15%)-polyacrylamide gel electrophoresis. The goblin band was localized by protein staining, excised from the gel, and proteolysed as described in the text. The major hormone-sensitive peptide of 37000 daltons is indicated by an arrow. (B, C) Comparison of the time-course of DL-isoproterenol (10^{-7} M)-induced onset and DL-propanolol (2×10^{-5} M)-induced reversal of $Na^+ + K^+$ cotransport as estimated by unidirectional ^{86}Rb influx (○) with that of ^{32}P content of the 37000 dalton fragment of goblin (●). Quantitation of the label in this peptide was achieved by densitometric scanning of autoradiographs such as that shown in (A). Data represent mean ± S.E. for four experiments. From Alper *et al.* (1980*a*), with permission.

dependent protein kinase, which appears to be the only intracellular receptor for cyclic AMP as determined by photoaffinity labelling using 8-azido-[^{32}P]cyclic AMP, has also been demonstrated in these cells (Alper *et al.* 1980*b*)]. The remainder of this article is devoted to a description of our efforts to determine the nature of Protein X and its possible relationship to the cation cotransport system.

Identification of a hormone-sensitive phosphoprotein in the turkey erythrocyte plasma membrane

The methodology employed in searching for hormone-stimulated phosphoproteins in the turkey erythrocyte was as follows: washed red cells were incubated for 3–24 h in a physiological medium containing $^{32}P_i$, leading to incorporation of label into the intracellular ATP pool. The cells were then divided into aliquots, and further incubated in the absence or presence of isoproterenol or other stimulatory agents for 10–20 min. Following this, cells were solubilized in a sodium dodecyl sulphate (SDS)-containing solution, boiled and subjected to SDS-polyacrylamide gel electrophoresis. The gels were then dried and subjected to autoradiography. Initial observations using the SDS-polyacrylamide gel system of Fairbanks, Steck & Wallach, (1971) indicated that a substantial increase in phosphorylation occurred in a protein of MW ~ 240000 (Rudolph & Greengard, 1974). Subsequent analysis (Beam *et al.* 1979) using the discontinuous buffer SDS-gel system of Laemmli (1970) has resolved this high MW region of the gel into three distinct protein staining bands; the phosphorylation of the middle band (M_r ~ 230000) appeared to be uniquely responsive to hormone addition. Such an experiment is shown in Fig. 4, where it can be seen that this protein is a major substrate for hormone-stimulated phosphorylation in these cells. This band was clearly separated from the outer two proteins of the triplet, believed to constitute the two subunits of spectrin, and was given an independent name, goblin. Further experiments using a rapid subcellular fractionation technique revealed that goblin, like spectrin, was a plasma membrane protein, but that, in contrast to spectrin, it failed to elute from isolated membrane preparations when these were exposed to either low or high ionic strength conditions. Thus, spectrin has the properties of a 'peripheral' membrane protein whereas goblin behaves more like an 'integral' membrane protein in some respects, e.g. it can be partially extracted from membranes using nonionic detergents (Beam *et al.* 1979).

Early experiments carried out by Rudolph & Greengard (1974) and by Rudolph *et al.* (1978) suggested that the increase in phosphorylation of the 240000 dalton protein now referred to as goblin correlated with the stimulation of cation influx under a number of conditions. For example, all agents that elicited an activation of cation cotransport also increased the phosphorylation of the 240000 dalton protein (Table 3). This raised the possibility that goblin might be the hypothetical protein X suggested in the working model (Fig. 3). However, the accuracy with which correlations between changes in phosphorylation and transport could be determined was limited by the fact that goblin was partially phosphorylated in control cells (Fig. 4, lane 1) so that the stimulation due to addition of hormone or cyclic AMP was maximally 2- to 3-fold (Fig. 4, lane 2; Table 2). Under some experimental regimes, e.g. when cells were preincubated with ^{32}P for 24 h in order to achieve isotopic equilibrium, the increased ^{32}P incorporation due to hormone addition appeared to be almost totally

Table 3. *Effect of various substances on net incorporation of ^{32}P into 240,000 molecular weight protein, on cAMP levels, and on sodium uptake in intact turkey erythrocytes*

(In each experiment, net ^{32}P incorporation into the 240000 molecular weight protein (separated on Fairbanks gels) during a 10 min incubation was calculated as percent of that found in erythrocytes incubated for 10 min in the absence of any addition; data represent the means ± S.E. for three or more experiments. Intracellular cAMP accumulated during a 10 min incubation at 37 °C was measured; data represent the means ± S.E. for four or more determinations. Sodium uptake was calculated from intracellular ^{22}Na accumulated during the interval between 8 and 12 min of incubation; data represent the means ± S.E. of triplicate determinations. In the absence of additions, sodium uptake was 2·84 ± 0·2 mmol/l of cells/h. From Rudolph and Greengard (1974), with permission.)

Additions	^{32}P Incorporation into 240,000 mol wt protein (% control)	Intracellular cAMP (μmol/l cells)	Sodium uptake (% control)
None	100	N.D.*	100
L-Isoproterenol, 10^{-6} M	236 ± 9	26·2 ± 1·9	311 ± 8
L-Epinephrine, 10^{-6} M	198 ± 20	19·5 ± 1·0	312 ± 7
L-Norepinephrine, 10^{-6} M	198 ± 14	22·3 ± 0·8	323 ± 6
Dopamine, 10^{-6} M	110 + 11	N.D.*	104 ± 1
L-Isoproterenol, 10^{-6} M, + DL-propranolol, 2×10^{-5} M	114 ± 6	N.D.*	106 ± 2
L-Isoproterenol, 10^{-6} M, + phentolamine, 10^{-4} M	211 ± 5	23·6 ± 0·8	296 ± 8
cAMP, 2×10^{-3} M	213 ± 8	—	302 ± 8
N^6-Monobutyryl cAMP, 2×10^{-3} M	238 ± 8	—	313 ± 14
N^6,$O^{2'}$-Dibutyryl cGMP, 2×10^{-3} M	105 ± 10	—	86 ± 3

* N.D., Not detectable (< 0·1 μmol/l of cells).

masked by the basal ^{32}P incorporation (Alper, Beam & Greengard 1980*a*). Such results suggested the existence of multiple phosphorylation sites on the goblin molecule, some of which are hormone- (and cyclic AMP-) independent and some of which are hormone- (and cyclic AMP-)dependent. In order to investigate this possibility further, proteolytic cleavage techniques were applied to goblin labelled *in vivo* with ^{32}P.

Multisite phosphorylation of goblin and correlations with cation cotransport

The limited proteolytic cleavage technique devised by Cleveland *et al.* (1977) was applied to goblin isolated by SDS-polyacrylamide gel electrophoresis (Alper *et al.* 1980*a*). This technique involves excising the goblin band from a gel such as that shown in Fig. 4, placing it in the well of a second Laemmli SDS-polyacrylamide gel and overlaying it with a solution of protease which remains active in SDS. During electrophoresis the protease cleaves goblin in a long stacking gel and the products are separated on a 15% resolving gel. An autoradiograph of such labeled cleavage peptides resulting from digestion using *Staphylococcus aureus* V8 protease is shown in Fig. 5. This represents an experiment in which prelabeled turkey erythrocytes were first exposed to isoproterenol for various periods of time and then to a 200-fold excess of the β-adrenergic antagonist propranolol to displace isoproterenol from its membrane receptors. Samples were taken at the indicated times, solubilized in SDS and subjected to conventional SDS-polyacrylamide gel electrophoresis, following which the goblin band was cut out from these gels and proteolysed. As can be seen in Fig. 5(*a*), th

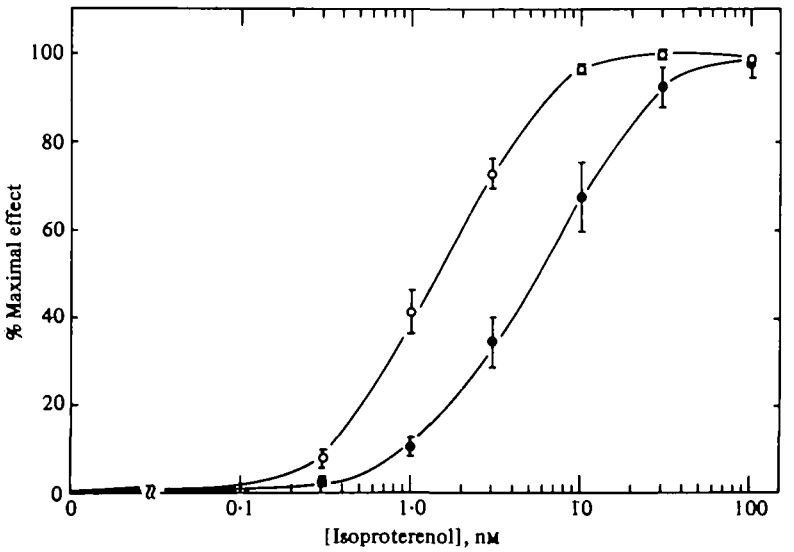


Fig. 6. Effect of various concentrations of DL-isoproterenol on $Na^+ + K^+$ cotransport and of ^{32}P incorporation into the 37000 dalton fragment of goblin. Analysis of these two parameters was similar to that described in the legend to Fig. 5. The ED_{50} value for phosphorylation was $5{\cdot}3 \times 10^{-9}$ M and for cotransport $1{\cdot}4 \times 10^{-9}$ M isoproterenol. Data represent mean ± S.E. for twelve experiments. From Alper *et al.* (1980*a*), with permission.

incorporation of ^{32}P into a peptide of ~ 37000 daltons was rapid on addition of isoproterenol, but was reversed relatively slowly on addition of propranolol. Addition of 8-bromo-cyclic AMP (1 mM) to the cells mimicked the effect of isoproterenol on the phosphorylation of this 37000 dalton fragment (Fig. 7*a*). The ^{32}P content of other fragments of lower molecular weight was not significantly affected by the hormone treatment and these probably contain the cyclic AMP-independent site(s) on this protein. A comparison of the degree of catecholamine-induced stimulation of phosphorylation obtained in the original goblin band (maximally 2- to 3-fold) with that in the 37000 dalton fragment (5- to 25-fold) indicates that the sensitivity with which changes in cyclic AMP-dependent phosphorylation can be detected has been substantially increased by application of this technique. The kinetic correlations between changes in goblin phosphorylation and stimulation of cation cotransport were thus assessed using this methodology.

The time course of phosphorylation and dephosphorylation of the 37000 dalton fragment, as shown in Fig. 5(*a*), was quantitated either by densitometric scanning of the autoradiograph or by excising the piece containing the peptide from the gel and subjecting it to liquid scintillation counting. These values were then compared with the onset and reversal of isoproterenol induced cation cotransport, whereupon the results shown in Fig. 5 were obtained. Both processes increased on addition of the hormone with $T_{\frac{1}{2}}$ ~ 3·5 min and were maximal at 10–15 min; following the addition of excess propranolol, phosphorylation and cation cotransport decreased roughly in parallel, with the former lagging somewhat behind the latter. Half-times of 57 min (phosphorylation) and 40 min (cotransport) were found for these reversal processes.

An analogous experiment illustrating the effect of various doses of isoproterenol on the two parameters is shown in Fig. 6. Stimulation of phosphorylation and transport occurred in the same range of isoproterenol concentrations with EC_{50} values of 5·3 and $1·4 \times 10^{-9}$ M, respectively. Cholera toxin, which causes an increased sensitivity of cation cotransport to isoproterenol (Rudolph *et al.* 1977) also led to a reduction in the concentration of hormone required to elicit a phosphorylation response. The shift in the half-maximal effective dose of isoproterenol required to activate cotransport was from 1·4 to 0·6 nM and the ED_{50} of isoproterenol for phosphorylation was reduced from 5·3 to 1·6 mM (Alper *et al.* 1980*a*). Taken together, these results indicate a close correlation between the state of phosphorylation of the cyclic AMP-dependent site(s) of goblin and the state of activation of cation cotransport.

It would be advantageous to be able to interfere directly with the *in vivo* process of phosphorylation itself so that a direct evaluation of the possible involvement of this reaction in the stimulation of transport by catecholamines or cyclic AMP could be made. Unfortunately, no agents which specifically inhibit the cyclic AMP-dependent protein kinase and can cross cell membranes are available. We thus searched for agents that might affect protein phosphorylation in a relatively non-specific manner in the hope that the possible correlation between phosphorylation and transport could be further evaluated. One reagent we have found useful in this regard is the thiol-oxidizing compound, diamide. This agent rapidly crosses cell membranes, and has been shown to oxidize intracellular glutathione (Kosower, Kosower & Wertheim, 1969) as well as protein sulphhydryl groups, sometimes leading to protein cross-linking (Hosey, Plut & Tao, 1977). It has been suggested that some of the biological effects of this reagent may be mediated by an inhibition of protein kinases (McClung & Miller, 1977). When turkey erythrocytes were prelabelled with ^{32}P, and then exposed to 8-bromo-cyclic AMP in the presence of increasing concentrations of diamide, a dose-dependent inhibition of cyclic AMP-stimulated phosphorylation of the 37000 dalton peptide of goblin was detected (Fig. 7*a*). Comparison of this inhibitory effect with the reduction by the compound of the stimulation of cation cotransport by 8-bromo-cyclic AMP revealed a very close correlation, with half-maximal effects occurring at about 0·3 mM diamide for both processes (Fig. 7*b*). However, as this thiol-oxidizing reagent is relatively non-specific it could have been inhibiting phosphorylation and transport by completely independent mechanisms. This is rendered unlikely by the observation that the severity of inhibition by diamide of cation cotransport stimulated by placing the cells in a hypertonic medium was considerably less than that obtained when stimulation was achieved using 8-bromo-cyclic AMP. As indicated above, the hypertonic effect almost certainly operates by a cyclic AMP-independent mechanism to activate transport; thus we conclude that diamide inhibits 8-bromo-cyclic AMP-stimulated transport by interfering with some aspect of the protein phosphorylation system. We are currently attempting to elucidate the precise mechanism whereby diamide achieves this effect.

Tryptic fingerprinting of goblin: further evidence for multisite phosphorylation

The one-dimensional peptide mapping procedure used for the experiments described above, in which the substrate was exposed to the protease for a short period of time, resulted in only a limited proteolytic cleavage of goblin to relatively large fragments

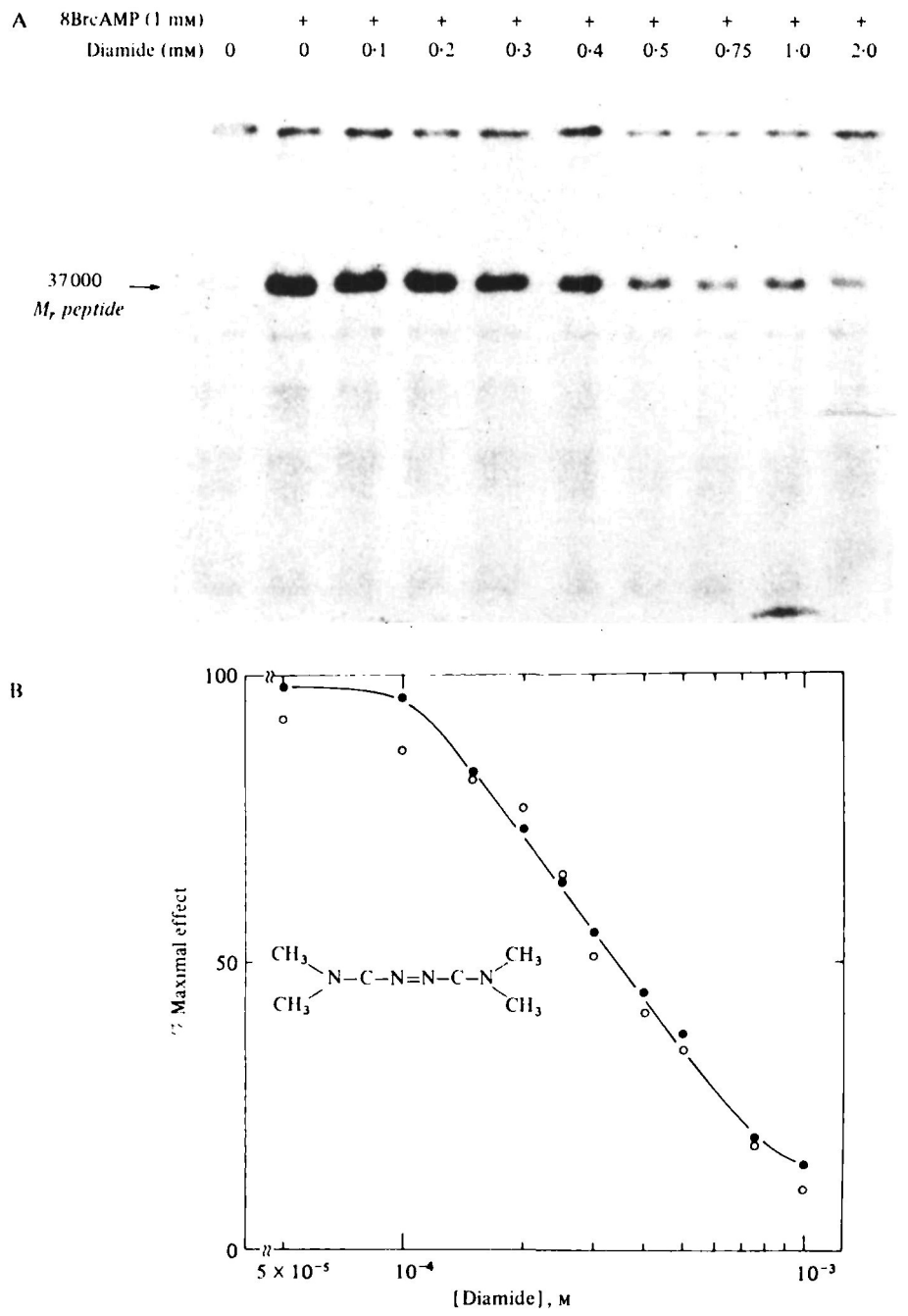


Fig. 7. (A) Inhibition by diamide of phosphorylation of the 37000 dalton peptide. The procedures used for incubation of cells and preparation of autoradiographs of peptide fragments produced by limited proteolysis of goblin were similar to those outline in the legend to Fig. 5. After preincubation in ^{32}P all cells were incubated for 15 min in the agents indicated. The left two lanes show that 8-bromo-cAMP (1 mM) stimulates phosphorylation of the 37000 dalton peptide in a manner analogous to that induced by isoproterenol (cf. Fig. 5*a*). Inclusion of increasing concentrations of diamide in the medium simultaneously with 8-bromo-cAMP progressively inhibited ^{32}P incorporation into this fragment. (B) Correlation between diamide inhibition of 8-bromo-cAMP-stimulated phosphorylation of goblin, as determined from ^{32}P content of the 37000 dalton fragment (●), and diamide blockade of 8-cAMP-activated cation co-transport, measured as unidirectional ^{86}Rb influx ( ). Cells were incubated for 15 min in the presence of both agents at the concentrations indicated.

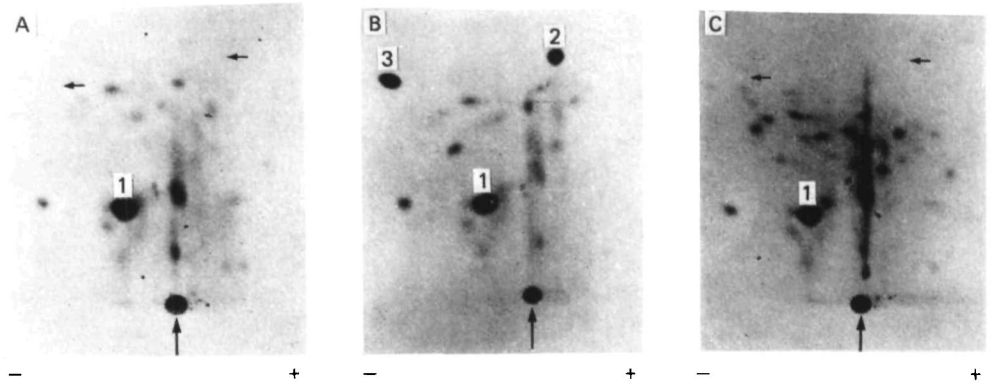


Fig. 8. Autoradiographs of two-dimensional tryptic fingerprints of ^{32}P-labelled goblin isolated by SDS-polyacrylamide gel electrophoresis. Prelabelled turkey erythrocytes were either not treated (A), exposed to DL-isoproterenol (10^{-7} M; 20 min) (B), or exposed to DL-isoproterenol (10^{-7} M; 20 min) followed by DL-propranolol (2×10^{-5} M; 90 min) (C). Goblin was excised from gels of all three samples and exhaustively digested with trypsin (50 μg.ml; 24 h). After centrifugation and lyophilization of the supernatant the sample was redissolved in electrophoresis buffer and a small volume was spotted at the origin (long arrow). The tryptic peptides were separated using electrophoresis (horizontal dimension) followed by ascending chromatography (vertical dimension) on cellulose TLC plates. These were dried and autoradiographed. For experimental details, see Alper *et al.* (1980*b*). Note that peptides 2 and 3 appeared when cells were incubated in isoproterenol (panel B) but were absent (short arrows) in control cells (panel A) or when cells were incubated in isoproterenol followed by propranolol (panel C). The state of phosphorylation of peptide 1 was not altered significantly by these manipulations.

The more rigorous tryptic fingerprinting technique which, when carried to completion, leads to digestion of the molecule to smaller peptides, was used in order to obtain further information on the number of phosphorylatable sites present. This method was adapted for use with acrylamide gel slices of goblin, obtained from experiments in which labeled whole cells were exposed to stimuli which can activate cotransport (Alper *et al.* 1980*b*). The results of a typical *in vivo* labelling experiment are shown in Fig. 8. Goblin from cells incubated in ^{32}P without added hormone yielded one major (Peptide 1) and several minor labeled peptides when the tryptic hydrolysate was subjected to two-dimensional peptide mapping (Fig. 8*a*). On exposure of the cells to isoproterenol for 20 min, phosphorylation of two other peptides was detected; these are labeled peptides 2 and 3 in Fig. 8*b*. Peptides of identical mobility were labeled when cells were exposed to cholera toxin, or to cyclic AMP or its analogues. Addition of a 200-fold excess of propranolol to hormone-treated cells resulted in dephosphorylation of peptides 2 and 3 (Fig. 8*c*). During this entire procedure, the ^{32}P content of peptide 1 did not change significantly; it can be concluded that this peptide does not contain a site capable of phosphorylation by a cyclic AMP-dependent protein kinase *in vivo*. Peptides 2 and 3 contain the cyclic AMP-dependent phosphorylation sites and can, in fact, be generated by tryptic proteolysis of the 37000 dalton peptide fragment produced by the one-dimensional peptide mapping discussed above (Fig. 5) (Alper *et al.* 1980*b*). We have also shown that these sites can be phosphorylated by [γ-^{32}P]ATP in isolated turkey erythrocyte membrane preparations by an endogenous cyclic AMP-dependent protein kinase (Alper *et al.* 1980*b*), thus lending support to the idea that goblin can be phosphorylated by a membrane-bound cyclic AMP-dependent protein kinase *in vivo*.

The discovery of three major phosphorylation sites in goblin is interesting in view of the role of multisite phosphorylation in the control of glycogen metabolism (Cohen, 1979). The kinetic relationship between the labeling of two cyclic AMP sites on goblin and the activation of cation cotransport is under investigation at present. Recent results (Alper *et al.* 1980*b*) further suggest that phosphorylation of peptides on the goblin molecule may be under the control of a Ca^{2+}-dependent protein kinase present in the turkey erythrocyte membrane. Dual phosphorylation at separate sites by Ca^{2+}- and cyclic AMP-dependent protein kinases has also been demonstrated recently in the case of the synaptic protein, Protein I (Hüttner & Greengard, 1979).

In summary, we have studied a hormone-sensitive transport process in turkey erythrocytes from both physiological and biochemical standpoints. Evidence has been presented supporting a close correlation between the phosphorylation of a plasma membrane protein termed goblin and activation of a specific membrane cation cotransport system. For this study to be carried out accurately, methods for studying individual phosphorylation sites on goblin were necessary. These methods may also prove useful in other contexts where evidence for the involvement of a particular phosphoprotein in a specific response is being sought. Our future plans include attempts to label the transport system itself using radioactive diuretics. This should enable us eventually to identify and purify the membrane components involved in ion transport. Ultimately, it is our aim to reconstitute the entire hormone-sensitive system in a form which would enable a more direct demonstration of the involvement of goblin and/or other phosphoproteins in the activation process.

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