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## Regulation of Membrane Flexibility in Human Erythrocytes<sup>†</sup>

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**ABSTRACT:** We have used spin-labels to detect prostaglandin E induced changes in erythrocyte membranes. The observed changes in spin-label resonance spectra can be mimicked in erythrocyte ghosts by loading them with cAMP or cGMP. These changes can also be observed by adding either of these cyclic nucleotides to intact cells. This entry of cyclic nucleotides into intact cells is blocked by an inhibitor

of the anion channel. We suggest that the observed changes in paramagnetic resonance spectra are due to changes in lipid "fluidity" that are brought about by changes in the biochemical state of membrane-associated proteins (such as spectrin) and in the direct or indirect biophysical interactions of these proteins with membrane lipids.

The morphology, chemical composition, and dynamical properties of the plasma membranes of most biological cells are doubtlessly subject to continuous regulation. The present paper is concerned with the membrane of the mature human erythrocyte. Recent studies by Allen and Rasmussen (1971) and by Kury et al. (1974) have shown that the rheological properties of erythrocytes at high hematocrit are affected by physiological concentrations of prostaglandins and epinephrine. These studies suggest but do not prove that the flexibility of an isolated erythrocyte can be controlled by these substances. Studies by Allen and Valeri (1974) indicate further that erythrocyte morphology is regulated by low concentrations of the prostaglandins PGE<sub>1</sub>,<sup>1</sup> or PGE<sub>2</sub>, which can decrease, or increase, respectively, the internal volume of the cell by a small amount (~3%). The paramagnetic resonance spectra of fatty acid spin-labels bound nonspecifically to these membranes show small but reproducible changes on the addition of low concentrations (10<sup>-10</sup>–10<sup>-12</sup> M; of the order of few molecules per cell) of the prostaglandins PGE<sub>1</sub> and PGE<sub>2</sub> (Kury et al., 1974), and also show reproducible changes on the addition of physiological concentrations of adrenaline and carbamyl choline (Huestis and McConnell, 1974). The spin-label concentration and resulting spectra suggest that the associated changes in membrane structure arise from small changes

throughout much of the membrane, rather than large changes localized to small regions of the membrane. The resonance spectra are a measure of the flexibility of the spin-label fatty acid chains in the bilayer region of the membrane. It is interesting that substances that increase the apparent single cell flexibility (e.g., PGE<sub>1</sub>) also increase this fatty acid chain flexibility, and vice versa (PGE<sub>2</sub> and epinephrine) (Kury et al., 1974). Recent studies of erythrocyte ghosts using circular dichroism also show changes in the presence of prostaglandins (Meyers and Swislocki, 1974).

The purpose of the present paper is to describe our paramagnetic resonance studies and to attempt to relate these biophysical changes to biochemical changes in the erythrocyte membrane that have been studied by other investigators.

### Materials and Methods

**Reagents.** The 10,3-fatty acid spin-label is the *N*-oxyl-4',4'-dimethyloxazoline derivative of 5-ketopalmitic acid.

**Ghost Preparation.** Human erythrocyte ghosts were prepared by a procedure adapted from Humphries and McConnell (1974). The cells were washed as described previously (Kury et al., 1974) and brought to 50% hematocrit in Allen's buffer (Allen and Rasmussen, 1971) (145 mM NaCl–5 mM KCl–1 mM MgSO<sub>4</sub>–3.5 mM Na<sub>2</sub>HPO<sub>4</sub>–1.5 mM NaH<sub>2</sub>PO<sub>4</sub>–10 mM glucose–1 mM CaCl<sub>2</sub>, final pH 7.0). A 1:4 dilution of Allen's buffer was prepared for the lysing solution. To 0.4 ml of 50% cells was added 2.0 ml of lysing solution. The cells were mixed and left on ice 10 min until translucent. They were centrifuged at g max 4300 for 10 min at 0°. Then 1.0 ml of the supernatant was carefully removed without disturbing the pellet. The membranes were resuspended in the remaining supernatant. Then 1.0 ml of resealing solution (252 mM KCl–10 mM NaCl–10 mM MgCl<sub>2</sub>–3.9 mM Na<sub>2</sub>HPO<sub>4</sub>–1 mM Na<sub>2</sub>ATP–2.4 mM

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<sup>1</sup> Abbreviations used are: cAMP, adenosine 3',5'-cyclic phosphate; cGMP, guanosine 3',5'-cyclic phosphate; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid.

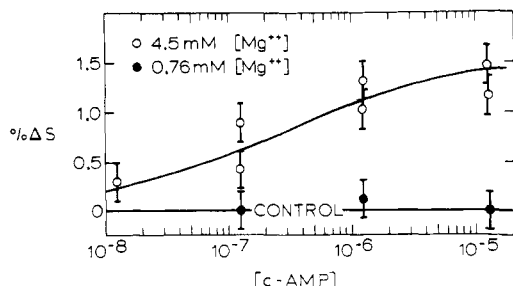


FIGURE 1: The effect of cAMP on %  $\Delta S$  on ghosts loaded with ATP. These experiments were performed on erythrocyte ghosts loaded with 0.4 mM ATP and varying concentrations of cAMP. (●) Internal  $Mg^{2+}$  concentration 0.76 mM; (○) internal  $Mg^{2+}$  concentration 4.5 mM. The control value of  $S$  is 0.67 at 37°.

theophylline, final osmolarity 586 mosm, pH 7.0) (we used 1 mM  $MgCl_2$  instead of 10 mM  $MgCl_2$  for the low  $Mg^{2+}$  concentration experiments) was added and the suspension was thoroughly mixed. The cells were left on ice 5 min and then incubated 1 hr at 37°. They were chilled on ice 5 min and then the ghosts were washed three times with Allen's buffer. This ghost preparation is only partially hemoglobin depleted because the lysis is performed under very mild conditions in order to minimize disruption of the membrane-associated proteins which solubilize at low ionic strength (proteins 1, 2, and 5) (Fairbanks et al., 1971). Greater than 95% of the membranes are resealed as determined by density gradient centrifugation (Bodemann and Passow, 1972). In the experiments with ghosts loaded with cyclic nucleotides, the appropriate amount of cyclic nucleotide is added just prior to addition of the resealing solution.

**Spin-Label Studies.** Washed erythrocytes were suspended in Allen's buffer at 70% hematocrit and incubated for 10 min at 37°. In experiments containing an inhibitor of the anion channel, 0.1 mM SITS was added first to the cells followed by the cyclic nucleotide at the appropriate concentration. The treated cells were incubated 10 min at 37° and then an aliquot was added to a test tube containing a film of the 10,3-fatty acid (final concentration of the spin-label is 0.1 mM) and incubated 10 min more at 37°. The film was obtained by blowing argon over the ethanolic solution of the spin-label. In experiments containing ghosts loaded with cyclic nucleotides, the loaded ghosts were incubated 10 min at 37° and then added to the spin-label as above. The labeled cells (or loaded ghosts) were kept at 12° after preparing the samples until the spin-label spectra could be determined. The spectra were determined at 37° on either a Varian E-4 or E-12 spectrometer. Because of the small changes in the electron spin resonance (ESR) spectra, the experiments were repeated several times.

In experiments where effectors were added to the extracellular solution, the concentration is expressed in terms of the entire volume even though 70% of the volume is cells.

The  $Mg^{2+}$  concentrations cited in the ghost experiments are calculated concentrations assuming (a) that the internal  $Mg^{2+}$  concentration in intact cells is 3.5 mM and (b) that the  $Mg^{2+}$  concentration fully equilibrates after addition of the lysing solution and the resealing solution.

Recently Bieri et al. (1974) have reported changes in erythrocyte morphology, including classic echinocyte morphology, due to the presence of various concentrations of the (12,3) fatty acid spin-label. Under our experimental conditions ( $10^7$  labels/cell, and the buffer described above) we have not been able to observe any significant change in

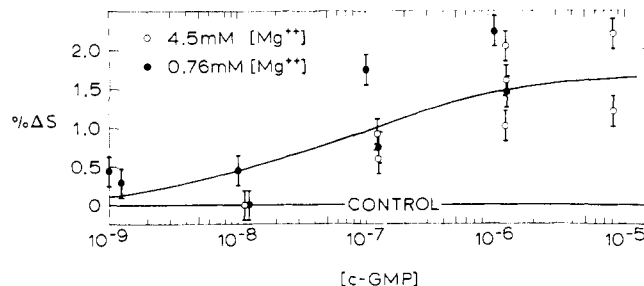


FIGURE 2: The effect of cGMP on %  $\Delta S$  on ghosts loaded with ATP. These experiments were performed on erythrocyte ghosts loaded with 0.4 mM ATP and varying concentrations of cGMP. (●) Internal  $Mg^{2+}$  concentration 0.76 mM; (○) internal  $Mg^{2+}$  concentration 4.5 mM. The control value of  $S$  is 0.67 at 37°.

erythrocyte morphology due to the (10,3) fatty acid label, using a light microscope.

## Results

**Order Parameter.** The present paper reports changes in order parameters of the fatty acid spin-label (10,3) in erythrocytes on addition of prostaglandins and cyclic nucleotides. The derivation of order parameters from spin-label resonance spectra has been discussed extensively in the literature (McConnell and McFarland, 1972). The order parameters are of course only measures of the hydrocarbon chain flexibility (lack of order) of the fatty acid spin-label itself. We shall later consider the possible relation between the spin-label order parameters and the "fluidity" of the erythrocytes membrane. Changes in order parameter are reported as %  $\Delta S$ , which is equal to  $100 \Delta S/S$  where  $\Delta S$  is the change in the order parameter,  $S$ , derived from changes in the paramagnetic resonance spectra. For a qualitative idea of what a certain %  $\Delta S$  represents, it may be noted that a phospholipid spin-label with the nitroxide on the fatty acid chain exhibits approximately a 20% change in  $\Delta S$  at the phase transition of dipalmitoyllecithin (Hubbell and McConnell, 1971). For much of the work described in the present paper, the order parameter is only a convenient spectral parameter, and a number of other spectral features could have been used equally well.

**Changes in Lipid Fluidity in Erythrocyte Ghosts Loaded with Cyclic Nucleotides.** In Figure 1, the percentage change in order parameter, %  $\Delta S$ , of the spin-label incorporated in the membrane of erythrocyte ghosts loaded with cyclic nucleotides is a function of the concentration of cAMP as long as the internal  $Mg^{2+}$  concentration is high (4.5 mM) but is independent of cAMP concentration at a lower internal  $Mg^{2+}$  concentration (0.76 mM). Therefore, at a high internal  $Mg^{2+}$  concentration cAMP increases the order parameter. The half-maximal change in %  $\Delta S$  occurs at  $2 \times 10^{-7}$  M cAMP.

In addition, cGMP causes a similar change in %  $\Delta S$ , but this effect is not dependent on the internal  $Mg^{2+}$  concentration over the same concentration range (Figure 2). The half-maximal change in %  $\Delta S$  occurs at  $6 \times 10^{-8}$  M cGMP.

The erythrocyte ghosts used for Figures 1 and 2 were prepared by loading the lysed cells with a final calculated concentration of ATP equal to 0.4 mM. If an equivalent concentration of GTP is added in place of the ATP in the resealing step (under the conditions of low internal  $Mg^{2+}$  concentration), then the cGMP effect remains essentially unchanged, but now cAMP decreases the order parameter

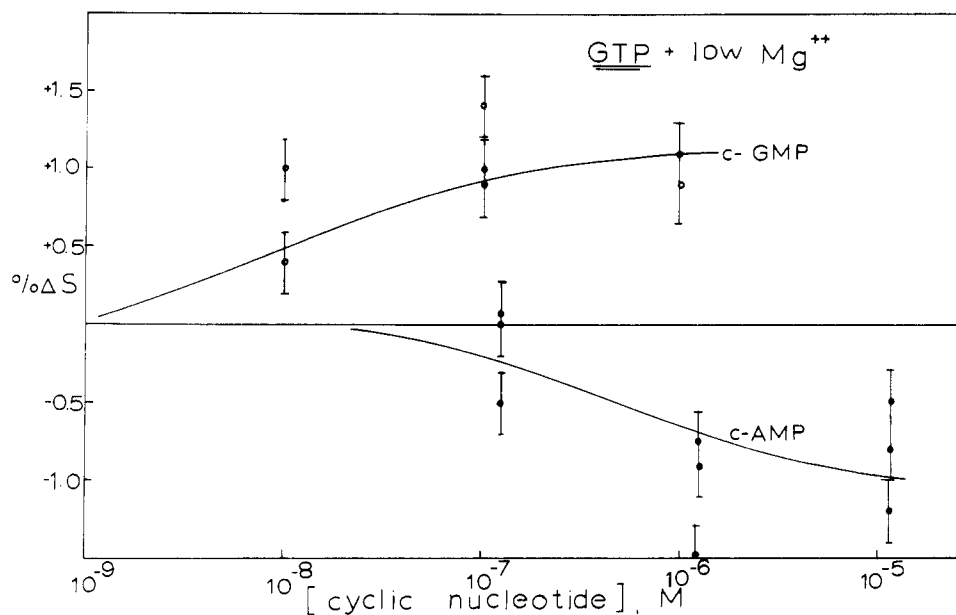


FIGURE 3: The effect of cyclic nucleotides on  $\% \Delta S$  on ghosts loaded with GTP. These experiments were performed on erythrocyte ghosts loaded with  $0.4 \text{ mM}$  GTP and varying concentrations of cyclic nucleotides. The internal  $\text{Mg}^{2+}$  concentration was  $0.76 \text{ mM}$ . The control value of  $S$  is  $0.68$  at  $37^\circ$ .

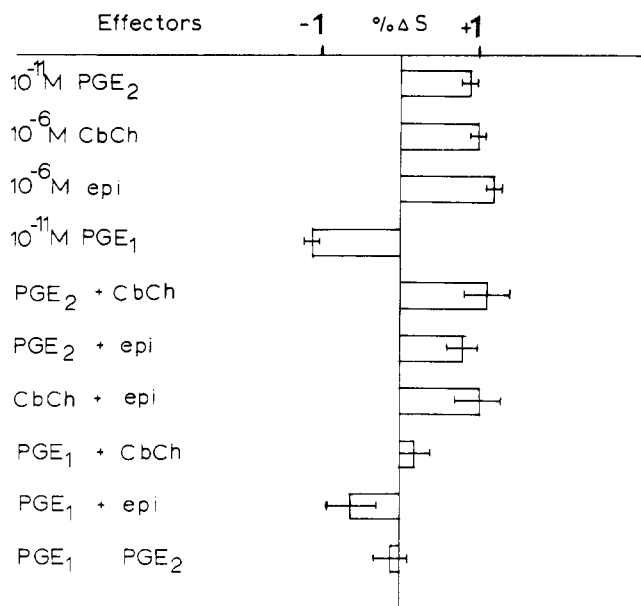


FIGURE 4: The response of two effectors on  $\% \Delta S$  with intact cells. The control value of  $S$  is  $0.68$  at  $37^\circ$ . Abbreviations: epi = L-epinephrine and CbCh = carbamyl choline.

with a maximal response in the  $\mu\text{M}$  concentration range (Figure 3).

Recently, we have found that the system is slightly more sensitive to cAMP when the concentration of ATP inside the ghosts is lowered from  $400$  to  $5 \mu\text{M}$ . We reduced the concentration of ATP because Guthrow et al. (1972) observed primarily cAMP independent phosphorylation at  $100 \mu\text{M}$  ATP and observed primarily cAMP dependent phosphorylation at  $5 \mu\text{M}$  ATP using human erythrocyte membranes. At  $5 \mu\text{M}$  ATP the half-maximal change  $\% \Delta S$  occurs at  $1 \times 10^{-8} \text{ M}$  cAMP, and the effect is maximal at  $1 \mu\text{M}$  cAMP (data not shown). The concentration of ATP itself does not seem to affect the order parameter.

**$\text{Mg}^{2+}$  Concentration Dependence of the PGE Effects.** The PGE effects on intact cells reported previously (Kury

Table I: Effect of PGEs on  $\% \Delta S$  at Various Internal  $\text{Mg}^{2+}$  Concentrations.<sup>a</sup>

Effector	$\% \Delta S$	
	$0.76 \text{ mM Mg}^{2+}$	$4.5 \text{ mM Mg}^{2+}$
$10^{-11} \text{ M PGE}_1$	-1.3	-1.0
	-0.9	-1.2
	-0.7	
$10^{-11} \text{ M PGE}_2$	+0.7	+0.6
	+1.0	+0.4
	+0.6	

<sup>a</sup> These experiments were performed on erythrocyte ghosts loaded with  $0.4 \text{ mM}$  ATP and varying concentrations of  $\text{Mg}^{2+}$ . The control value of  $S$  is  $0.68$  at  $37^\circ$ .

et al., 1974) can also be observed in resealed ghosts. Using ATP in the resealing solution, the PGE-induced change in membrane flexibility is independent of the internal  $\text{Mg}^{2+}$  concentration (Table I).

**Erythrocyte Volume.** We do not observe a change in order parameter when the osmolarity of the extracellular buffer is varied by as much as  $\pm 15\%$ . Also Rigand et al. (1974) have reported that the order parameter for the 12,3-fatty acid spin-label in erythrocyte is unaffected by changing the osmolarity from  $100$  to  $600 \text{ mosm}$ .

**Competition Experiments.** The concentration dependence of various effector compounds is biphasic (Allen and Rasmussen, 1971; Kury et al., 1974; Huestis and McConnell, 1974). We have added simultaneously two effector compounds, each at their most effective concentration, to determine if the combined effects would be additive (Figure 4). If two compounds individually increase the order parameter (for example  $\text{PGE}_2$  and epinephrine), then a combination of each at its most effective concentration does not produce an additive increase in order parameter. If the two compounds produce opposing effects (for example,  $\text{PGE}_1$  and  $\text{PGE}_2$ ), then the simultaneous addition of the two effectors, each at its most effective concentration, appears to have little effect on the order parameter.

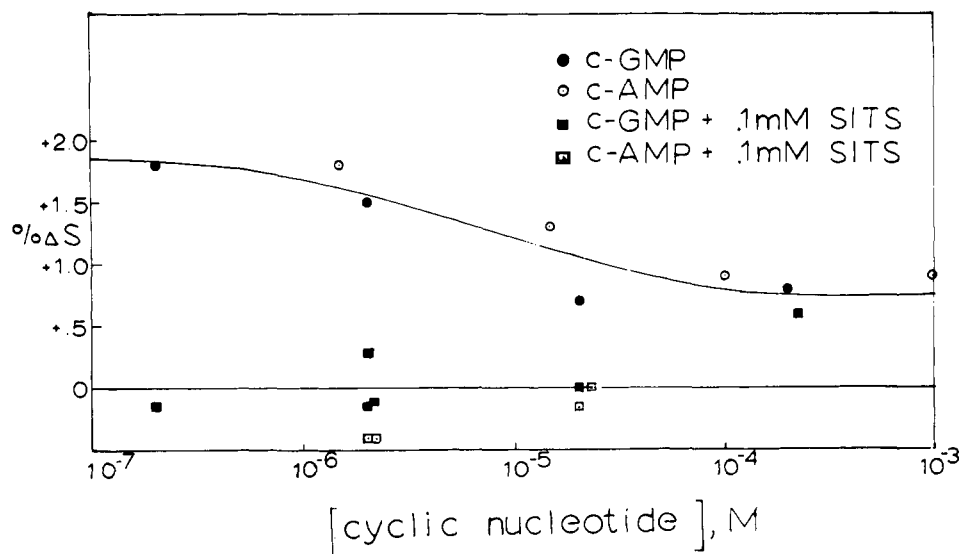
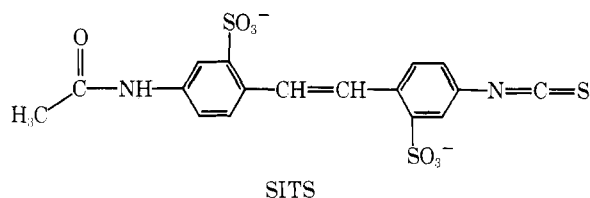


FIGURE 5: The effect of cyclic nucleotides on %  $\Delta S$  in intact erythrocytes. The control value of  $S$  is 0.68 at 37°.

*Cyclic Nucleotides Added Intra- and Extracellularly to Erythrocytes in the Absence or Presence of an Inhibitor of the Anion Channel.* We added cyclic nucleotides extracellularly to intact erythrocytes and observed reproducible increases in order parameter (Figure 5). There is a greater response at  $\mu M$  cyclic nucleotide concentrations than at  $mM$  concentrations. We find that it is possible to block this apparent entry of extracellular cyclic nucleotides by pretreating the cells with an inhibitor of the erythrocyte anion channel (Figure 5). The inhibitor is 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) (Cabantchik and



Rothstein, 1972, 1974). To ensure that a cyclic nucleotide-induced change in resonance spectra can still be observed when the anion channel is blocked, we loaded ghosts with a cyclic nucleotide, resealed, and then added or omitted SITS. We found that the changes in spin-label resonance spectra were not altered by the presence of SITS as long as the cyclic nucleotides were preloaded into the resealed ghosts.

#### Discussion

At an average concentration of a single prostaglandin molecule per intact erythrocyte, we have observed changes in the paramagnetic resonance spectrum of a membrane bound spin-labeled fatty acid (Kury et al., 1974). The fatty acid spin-labels bound to the membranes are present in rapid reversible equilibrium with labels present in solution. Labels are probably distributed more or less uniformly throughout the membrane; they are certainly not present in concentrated patches, otherwise they would show exchange broadened resonance spectra. We shall assume that the resonance spectra reflect the bulk flexibility of the membrane phospholipid fatty acid chains. We have, however, not ruled out the possibility that the change in the resonance spectra may arise from a change in the surface charge, pH, or ionic composition in the immediate region of the membrane sur-

face. It is likely that one or several prostaglandin effector molecules regulate the membrane flexibility of an entire erythrocyte. Thus, there must be an efficient amplification mechanism. It is likely that this mechanism involves cAMP and/or cGMP.

Although an erythrocyte guanyl cyclase has not yet been reported (Goldberg et al., 1973), there is increasing evidence that there is an adenyl cyclase in the mature human erythrocyte (Kaiser et al., 1974; Rubin and Rosen, 1973) although it has not yet been demonstrated that it is hormone sensitive. We observe changes in the spin-label spectrum when erythrocyte ghosts are loaded with cAMP or cGMP (Figures 1 and 2). The cAMP induced change depends critically on the magnesium ion concentration inside the ghost. The dependence on  $Mg^{2+}$  concentration could be due to a  $Mg^{2+}$ -sensitive protein kinase associated with the erythrocyte membrane which is activated by cAMP (Guthrow et al., 1972). This protein kinase is known to phosphorylate an erythrocyte protein called spectrin (Guthrow et al., 1972). We suggest that the changes in the paramagnetic resonance spectra arise from changes in the state of phosphorylation of membrane-associated proteins such as spectrin. There are at least two types of enzymes that can regulate the degree of phosphorylation of proteins. They are the ATP-requiring,  $Mg^{2+}$ -dependent protein kinases that phosphorylate protein substrates and the protein phosphatases that hydrolyze phosphoproteins (Taborsky, 1974). In avian erythrocytes the  $\beta$ -adrenergic agonist, *l*-isoproterenol, stimulates incorporation of  $^{32}P$  into a protein that is analogous to spectrin in human erythrocytes (Rudolph and Greengard, 1974). Extracellular cAMP (1  $mM$ ) mimics this effect. Huestis and McConnell (1974) find that the  $\beta$ -adrenergic agent, *L*-epinephrine, increases spin-label order parameters in human erythrocytes.

From the data of Guthrow et al. (1972) we calculate that under their experimental conditions (5  $\mu M$  ATP) only 2.5% of the spectrin is phosphorylated in the absence of cAMP and 3.6% phosphorylated in the presence of 1  $\mu M$  cAMP. At 5  $\mu M$  ATP we observe cAMP dependent changes in lipid order parameters. At higher concentrations of ATP (66–100  $\mu M$ ) an appreciable fraction of the spectrin molecules are phosphorylated (Guthrow et al., 1972; Rubin and Rosen, 1973), but the fraction of cAMP stimulation of  $^{32}P$  incorporation into protein decreases at higher ATP concen-

trations (Guthrow et al., 1973). The physiological concentration of ATP is approximately 0.7–1.0 mM (Bishop and Surgenor, 1964) so presumably in vivo most spectrin molecules are phosphorylated, and only a small percentage of those result from specific cAMP stimulation of phosphorylation. Fairbanks et al. (1971) have estimated from sodium dodecyl sulfate gels that there are approximately  $3.4 \times 10^5$  molecules of spectrin/cell. Kant and Steck (1973) estimate from binding studies that there are  $6 \times 10^3$  internal binding sites for cAMP per cell (see, however, Swillens et al., 1974). These cAMP molecules are thought to bind to the regulatory subunit of the protein kinase. Using these data we conclude that there are approximately 50 spectrin molecules/kinase regulatory subunit.

There are two lines of evidence that these changes in lipid order parameter are directly related to the phosphorylation of proteins. One is that we observe approximately the same concentration of cAMP that half-maximally increases the order parameter as Guthrow et al. (1972) observes for half-maximal stimulation of incorporation of phosphate into spectrin. The other is that the erythrocyte protein kinase (Guthrow et al. (1972) is sensitive to the same concentration of  $Mg^{2+}$  as the cAMP-induced change in order parameter (Figure 2). The change in the paramagnetic resonance spectrum induced in ghosts by cGMP is not sensitive either to the internal  $Mg^{2+}$  concentration or to whether ATP or GTP is used in the resealing solution (Figures 2 and 3). cGMP may regulate the activity of a protein phosphatase. The fact that prostaglandin-induced changes in erythrocyte ghost lipid fluidity are not sensitive to intracellular  $Mg^{2+}$  concentration (Table I) indicates that  $PGE_1$  and  $PGE_2$  may alter the guanyl cyclase activity rather than the adenyl cyclase activity.

Ryan and Hendrick (1974) and Guthrow et al. (1972) have reported a biphasic response for cAMP stimulation of  $^{32}P$ -incorporation into membrane proteins. The biphasic character of the phosphorylation might be due to dual effects of the two cyclic nucleotides, depending on their concentrations and on the intracellular ionic constituents.

Although it is commonly thought that cyclic nucleotides cannot enter intact cells, there are various systems reported in the literature where effects are observed upon addition of extracellular cyclic nucleotides (Rudolph and Greengard, 1974; Ryan and Hendrick, 1974; Ford and Omachi, 1972; Siggins et al., 1971; Bloom et al., 1975). We have presented evidence that  $\mu M$  concentrations of cyclic nucleotides readily enter the intact erythrocyte (Figure 5) at 37°. Our data are consistent with the possibility that cyclic nucleotides enter the cell via the anion channel since blockage of the channel prevents the response.

There are a number of protein-lipid interactions that can affect lipid fluidity. For example, Sefton and Gaffney (1974) have shown that the order parameter of fatty acid as well as phospholipid spin-labels bound to the membrane of Sindbis virus is decreased by 9% when the membrane proteins are removed. Also the insertion of intrinsic proteins into membranes (e.g., rhodopsin) increases the order parameters of phospholipid spin-labels (Hong and Hubbell, 1972). Thus one can imagine a variety of relatively simple, direct mechanisms whereby a change in the biochemical state of a membrane protein affects the label order parameter. For example, the degree of phosphorylation of spectrin could affect specific as well as nonspecific binding to the cytoplasmic surface of the erythrocyte. From the experiments of Sefton and Gaffney (1974), yielding a 9% reduction in *S*

on removing membrane proteins, we can readily see that even a relatively small change in the degree of spectrin binding (~10–20%) could produce a 1–2% change in *S*. Although this is only an order-of-magnitude argument, it does show clearly that a mere change in the degree of binding, or strength of binding, of spectrin to the cytoplasmic surface might well produce the observed changes in lipid flexibility. A second quite general and quite plausible mechanism is that the phosphorylation of cytoplasmic proteins results in a "muscle-like" change in the state of these proteins that alters the lateral compression or extension of the membrane lipids. Both effects may be simultaneously operative, so it may be difficult to design experiments that distinguish them.

#### Acknowledgments

The prostaglandins were a gift of Dr. Margaret Merritt of the Upjohn Company. The 10,3-fatty acid spin-label was a gift of Dr. Betty Jean Gaffney; we are particularly indebted to Dr. Gaffney for pointing out the possibility that fatty acid labels may exhibit spectral changes due to membrane surface charges. We thank Professor H. Rasmussen for suggesting the experiment of measuring %  $\Delta S$  on changing cell volume. We also thank the Hematology Department at Stanford University Medical School for supplying us with the human erythrocytes.

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## Aldosterone-Induced Membrane Phospholipid Fatty Acid Metabolism in the Toad Urinary Bladder<sup>†</sup>

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**ABSTRACT:** Aldosterone action in the isolated toad urinary bladder has been studied by incubation of the tissue with several specifically labeled lipogenic precursors. Within 30 min after hormone addition phospholipid synthesis is stimulated; the metabolism of oleic acid is particularly enhanced. Additionally, during this time interval a phospholipid deacylation-reacylation cycle is stimulated by aldosterone.

The addition of aldosterone to the isolated toad urinary bladder stimulates transcellular sodium transport (Sharp and Leaf, 1966; Edelman and Fimognari, 1968). Aldosterone treatment alters at least three other membrane-related processes. It increases (1) the permeability of the tissue to water and low-molecular solutes in the presence of antidiuretic hormone (Goodman et al., 1969; Handler et al., 1969); (2) the sensitivity of sodium transport to inhibition by the cardiac glycoside, ouabain, a known inhibitor of Na<sup>+</sup>-K<sup>+</sup>-activated ATPases (Goodman et al., 1969); and (3) the sensitivity of sodium transport to inhibition by increased partial pressures of oxygen (Allen et al., 1973). Because an alteration in membrane lipid composition could account for these diverse hormone-induced changes in tissue function, we initiated an investigation of the effects of aldosterone on membrane lipid metabolism. In our earlier studies we found that aldosterone treatment increased both lipid synthesis and the turnover of membrane phospholipid fatty acids within 30 min (Goodman et al., 1971). Additionally, pretreatment of the tissue with phospholipase A resulted in a significant reduction in the latent period between hormone addition and the inception of the physiological response, an increase in transcellular sodium transport. Aldosterone also induced an increase in the weight percentage of phospholipid long-chain polyunsaturated fatty acids. These studies thus provided the first biochemical evidence that a modifi-

After 4 hr aldosterone increases the oxidation of all fatty acids utilized, but enhances, specifically, the elongation and desaturation of oleic acid, as well as the recycling of [<sup>14</sup>C]acetyl-CoA derived from [1-<sup>14</sup>C]oleic acid into membrane phospholipid fatty acid. These data provide further evidence for a rapid and specific action of aldosterone on toad bladder membrane phospholipid fatty acid metabolism.

cation of lipid metabolism might be a significant effect of the action of aldosterone. The purpose of the present investigation was to characterize more fully and specifically the effects of aldosterone on lipid metabolism in the toad urinary bladder.

Utilizing specifically labeled [<sup>14</sup>C]- and [<sup>3</sup>H]fatty acids as precursors and analyzing tissue phospholipid fatty acids by radio-gas chromatography, we have shown that aldosterone stimulates specifically the elongation and desaturation of oleic acid (18:1ω9). By prelabeling tissue lipids with [<sup>14</sup>C]acetate, we have also demonstrated that aldosterone stimulates the release of <sup>14</sup>C from phospholipid into [<sup>14</sup>C]fatty acid.

### Materials and Methods

**Experimental Animals and Preincubation.** Urinary bladders were removed from female toads (*Bufo marinus*, National Reagents Co., Bridgeport, Conn.) treated as previously described (Goodman et al., 1971). Tissue was routinely preincubated overnight at room temperature in substrate-free aerated Ling-Ringer phosphate buffer (Ling, 1962), pH 7.4, containing 50 mg/l. of both penicillin G and streptomycin sulfate. In experiments where tissue lipids were prelabeled, 1 mM sodium acetate, containing 1.0 μCi/ml of sodium [1,2-<sup>14</sup>C]acetate, was included in the overnight incubation solution. The following morning experiments were performed.

**Tissue Incubations; [<sup>14</sup>C]Lipid Analysis.** Individual matched hemibladders from six toads were incubated for 1 hr in Ling-Ringer phosphate (pH 7.4) containing 4 mM glucose. The tissue was then transferred into a fresh Ling-Ringer glucose buffer containing either 0.1 mM sodium ac-

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