Photoaffinity Labeling of Adenosine 3′,5′-Cyclic Monophosphate Binding Sites of Human Red Cell Membranes†

Boyd E. Haley*

ABSTRACT: To identify and investigate the cAMP binding sites of human red cell membranes a photoaffinity analog of cAMP, 8-azidoadenosine 3′,5′-cyclic monophosphate (8-N_3cAMP), has been synthesized. This analog activates cAMP-dependent protein kinase(s) in the red cell membrane. It exhibits tight, but reversible binding to the membranes which is competitive with cAMP. Photolysis of [^{32}P]-8-N_3cAMP with red cell membranes results in covalent incorporation of radioactive label onto two specific membrane proteins. This incorporation requires activating light and is reduced to background levels with addition of low levels of cAMP. Prephotolysis of 8-N_3cAMP completely abolished its ability to photolabel membrane proteins. Both the reversible and photocatalyzed binding of 8-N_3cAMP show saturation kinetics. The molecular weights of the two primarily labeled proteins are approximately 49,000 and 55,000. The differential effects of cAMP, ATP, and adenosine on the photocatalyzed incorporation of [^{32}P]-8-N_3cAMP onto these two proteins suggest that they have biochemically different properties. The potential usefulness of this compound for investigating various molecular aspects of cAMP action is discussed.

Since the discovery of cAMP as a second messenger (Sutherland and Rall, 1957, 1958), this unique compound has been implicated in the regulation of several membrane functions such as growth and contact inhibition in mammalian cells (Johnson et al., 1971; Hsie and Puck, 1971; Sheppard, 1971), cellular permeability (Orloff and Handler, 1967; Prince and Berridge, 1973; Klyce et al., 1973; DeLorenzo et al., 1973), synaptic transmission (Maeno et al., 1971; Weiss and Kidman, 1968), aggregation and differentiation in slime mold (Bonner et al., 1969), and modulation of inflammation and immunity (Bourne et al., 1974, and references therein). cAMP-dependent protein kinases have been found associated with the plasma membrane of various cellular systems (Johnson et al., 1972; Cawthon et al., 1974; Lemay et al., 1974; Ueda et al., 1973; Kish and Kleinsmith, 1974; Corbin and Krebs, 1969; Miyamoto et al., 1969). Considerable evidence supports the hypothesis that these protein kinases are molecular receptors of cAMP and the mediators of cellular responses to several hormones (Robison et al., 1968, 1971; Kuo and Greengard, 1969; Langan, 1969; Walsh et al., 1971; Gill and Garren, 1971). Several recent investigations have indicated that regulation of phosphorylation or dephosphorylation of endogenous substrates is probably involved in the mechanism of action of cAMP (Rudolph and Greengard, 1974; Johnson et al., 1972; Cawthon et al., 1974; Lemay et al., 1974; Ueda et al., 1973; Kish and Kleinsmith, 1974). It is apparent that any compound that would give added insight to the mode of action of cAMP with proteins, especially membrane-bound proteins, would be of great interest.

Several investigators have recently used photoaffinity reagents to investigate the binding of ligands to proteins (Fleet et al., 1969; Kiefer et al., 1970; Brunswick and Cooperman, 1971; Converse and Richards, 1969; Guthrow et al., 1973; Ruoho et al., 1973; Staros et al., 1974). A previous report has shown the utility of a photoaffinity analog of ATP for labeling ATP binding sites of human red cell membranes (Haley and Hoffman, 1974). Since human erythrocyte membranes have been shown to contain a cAMP-dependent protein kinase (Guthrow et al., 1972; Rubin et al., 1972; Roses and Appel, 1973), they were chosen as a model system for studying the use of 8-azidoadenosine 3′,5′-cyclic monophosphate (8-N_3cAMP) as a specific photoaffinity membrane probe for cAMP binding sites. The proposed structure of this compound is shown in Figure 1. The data presented herein indicate that this compound will prove very useful for labeling cAMP binding sites. A brief account of this work has been previously presented (Haley, 1975).

Materials and Methods

[^{32}P]-8-N_3cAMP was prepared from [^{32}P]-cAMP (ICN) as follows. [^{32}P]cAMP (3.48×10^{-8} mol, 14 Ci/mmol) was added to 8 ml of 0.25 *M* sodium acetate (pH 3.8) containing 5 μl of Br_2. The bromination reaction was kept at room temperature in a sealed 10-ml test tube (Teflon-sealed cap). This is a modification of the procedure of Mumeyama et al. (1971). After 10 hr the excess Br_2 was removed by flushing with an air stream. The reaction mixture was then dialyzed against 8 l. of distilled H_2O followed by 2 l. of 0.25 *M* triethylammonium bicarbonate buffer using a Dow 100-ml Beaker Osmolyzer with cellulose acetate-A fibers (Bio-Rad Labs b/HFO-1). The dialyzed solution was evaporated to dryness under reduced pressure (1 mm) at 30°, dissolved in 4 ml of distilled H_2O, and applied to a 2×25 cm column of DEAE-cellulose in the HCO_3^- form. The column was washed with 50 ml of H_2O followed by a linear gradient of 2 l. of 0–0.2 *M* triethylammonium bicarbonate (pH 7.5). Two peaks were resolved which contained 8 and 92% of the total radioactivity. The major peak migrated on two different thin-layer chromatography (TLC) systems (given below) exactly like 8-Br-cAMP and had a λ_{max} of 264 nm

† From the Department of Physiology, Yale University, New Haven, Connecticut 06510, and the Division of Biochemistry, University of Wyoming, Laramie, Wyoming 82071. *Received March 21, 1975.* This work was supported by National Institutes of Health Grants HL-09906, AM-05644, and GM-21998 and National Science Foundation Grant GB-18924

* Send correspondence to this author at the University of Wyoming.

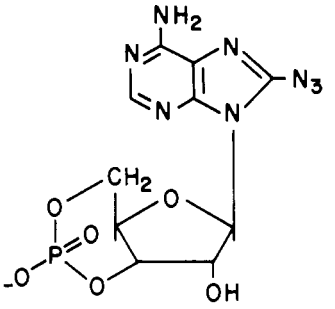

FIGURE 1: Proposed structure of 8-azidoadenosine 3′,5′-cyclic monophosphate (8-N_3cAMP).

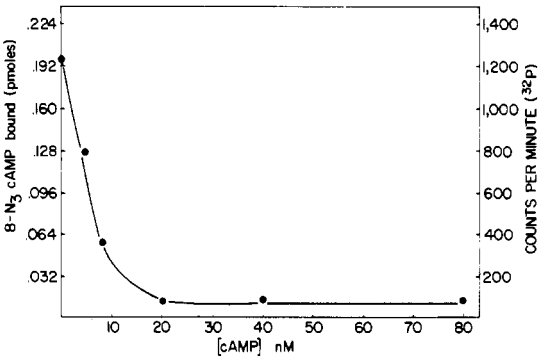

FIGURE 2: Effects of cAMP on reversible binding of 8-N_3cAMP to human red cell membranes. Red cell membranes (0.169 ± 0.012 mg of protein in 0.2 ml) were mixed with 0.025 μM [^{32}P]-8-N_3cAMP (specific activity = 6.2×10^9 cpm/μmol) and varying concentrations of cAMP. After incubation for 1 hr at 0°, the membranes (0.043 mg) were removed and washed three times with 10 ml of 10 mM TrisCl (pH 7.4), 40 mM NaCl, 20 mM KCl, and 2.5 mM $MgCl_2$. After washing the ghosts were dissolved in 0.25 ml of Nuclear Chicago solubilizer, placed in 10 ml of Fluor-toluene, and counted by liquid scintillation. The remaining membrane portion was used for other photolysis experiments.

which agrees with the λ_{max} expected for 8-Br-cAMP. The major peak, 8-Br-cAMP, was pooled and evaporated to dryness at 30°. It was then dissolved and coevaporated four times with 20-ml portions of absolute methanol to remove the excess triethylammonium bicarbonate. To the dried material was added 10 ml of dimethylformamide containing 1.0 mmol of triethylammonium azide (prepared by passing 50 mg of LiN_3 (1 mmol) dissolved in dimethylformamide through a 20-ml Dowex 50 column in the triethylammonium form also in dimethylformamide). The tube was tightly sealed and heated at 75° for 10 hr. The reaction mixture was evaporated to dryness (1-mm pressure, 18°), dissolved in 4 ml of distilled H_2O and chromatographed again as given above. One minor peak followed by a major peak (determined by the presence of radioactivity) were resolved. The major peak had an absorbance maximum of 281 nm which agreed with previous values given for 8-N_3cAMP (Mumeyama et al., 1971; Haley and Hoffman, 1974). This peak was pooled and evaporated to dryness at 18°. The excess triethylammonium bicarbonate was removed by four coevaporations with 20 ml of absolute methanol. The material isolated would bind irreversibly to cellulose when irradiated with ultraviolet light (254 nm). Thin-layer chromatography using Eastman TLC cellulose sheets 13254 was done in a solvent system of isobutyric acid–NH_4OH–H_2O (66:1:33 v/v). Most of the radioactivity (96%) in the major peak migrated with an R_f 0.78 (R_f's of cAMP, AMP, ADP, and ATP were 0.76, 0.70, 0.64, and 0.54, respectively). A minor portion, 4%, had an R_f 0.45.

A second solvent system of 1-butanol–acetic acid–H_2O (5:2:3 v/v) gave R_f values for 8-N_3cAMP, cAMP, AMP, ADP and ATP of 0.65, 0.62, 0.50, 0.35, and 0.18, respectively, with the 4% impurity having an R_f of 0.14. This impurity has a λ_{max} of 274 nm and does not display photoactivity. A product with similar physical characteristics may be produced from 8-N_3cAMP by photolysis, or thermal induced breakdown, in H_2O.

Hemoglobin-free red cell membranes were prepared from freshly drawn, heparinized, human blood and stored at −20° as described by Heinz and Hoffman (1965). On the day of use the ghosts were thawed and washed twice at 20° with 40 ml of a solution containing 40 mM NaCl, 20 mM KCl, 0.5 mM EGTA, 2.5 mM $MgCl_2$, and 20 mM Tris-Cl (pH 7.4). Photolysis of mixtures of 8-N_3cAMP and membrane were done for 60 min on 0.2-ml solutions containing approximately 0.16 mg of membrane protein, 10 mM Tris-Cl (pH 7.4), 40 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, and various concentrations of 8-N_3cAMP. The solutions being photolyzed were placed in glass serology plates which were kept cold by being partially immersed in an ice–water bath. The light source was a short wavelength (253.4 nm) UVS-11 mineralight (Ultra-violet Products, Inc.) located 9 cm from the solutions being photolyzed.

Autoradiography and gel electrophoresis were done as previously described (Haley and Hoffman, 1974) with the exception that slab gels were used instead of cylindrical gels. Densitometer tracings and peak integrations of gels stained for protein and their corresponding autoradiographs were done with an Ortec Model 4310 densitometer equipped with a digital integrator and printer. Gels were sliced and counted by liquid scintillation (Nuclear Chicago Mark I) as described by Knauf et al. (1974). Staining with Coomassie Blue and periodic acid-Schiffs reagent and notation of stained regions were done as given by Fairbanks et al. (1971).

Results

Figure 2 shows that the tight, but reversible, binding of [^{32}P]-8-N_3cAMP to red cell membranes is decreased by the presence of increasing cAMP concentrations. cAMP binds slightly tighter than 8-N_3cAMP with approximately 20 nM being required for prevention of binding due to 25 nM 8-N_3cAMP. Earlier work has shown that 8-N_3cAMP activates cAMP-dependent protein kinase of bovine brain (Mumeyama et al., 1971). With red cell membranes, using [^{32}P]-ATP, both cAMP and 8-N_3cAMP stimulate the phosphorylation of endogenous substrates. The increase of phosphorylation varies from 10 to 60% above control levels but neither cAMP nor 8-N_3cAMP gives consistent results from one membrane preparation to another (B. Haley, unpublished results).

Figue 3 shows that 8-N_3cAMP binds reversibly to red cell ghosts and saturates at approximately 75 nM concentration. This is 16.5 pmol/mg of membrane protein or about 3000 molecules/red cell membrane at pH 7.4. This corresponds closely to an earlier report by Rubin et al. (1972) that human red cell membranes have a maximum binding capacity of 12 pmol of cAMP/mg of membrane protein at pH 7.0.

The data in Figure 4A and B show that with uv photolysis [^{32}P]-8-N_3cAMP primarily labels a protein component located in band IVb. No noticeable labeling of membrane proteins occurs without photolysis or if the 8-N_3cAMP is

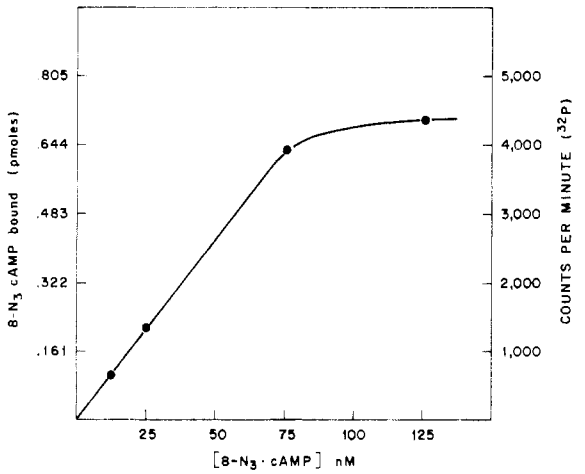

FIGURE 3: Reversible binding of 8-N_3cAMP to human red cell membranes. The procedure was the same as in Figure 2 except no cAMP was added and the concentration of 8-N_3cAMP was varied between 0.0125 and 0.125 μM. Samples counted contained 0.043 ± 0.003 mg of protein.

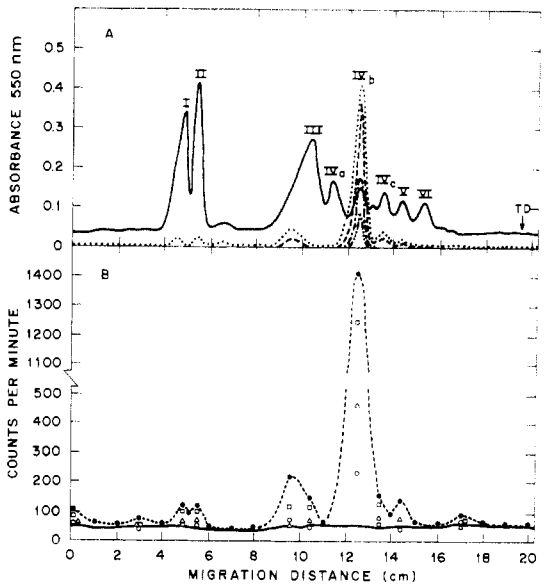

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membranes photolyzed in the presence of varying concentrations of [^{32}P]-8-N_3cAMP. Membranes (0.160 ± 0.012 mg of protein) were photolyzed as given in Materials and Methods except the concentration of [^{32}P]-8-N_3cAMP (6.2×10^9 cpm/μmol) was varied; 100 μg of membrane protein from each sample were electrophoresed, stained, dried, and autoradiographed, as well as sliced and counted by liquid scintillation. (A) The solid line is a densitometer tracing of the Coomassie Blue stain intensity of a representative gel on which membrane proteins have been electrophoresed. The bands are numbered as suggested by Fairbanks et al. (1971). The broken lines are densitometer tracings of autoradiographs of nearly identical gels from the same slab whose proteins have been photolabeled with [^{32}P]-8-N_3cAMP at concentrations of 0.0125 (·—···—·), 0.025 (·—·—·), 0.075 (— —), and 0.125 (···) μM, respectively. (B) The ordinate is the cpm ^{32}P present per gel segment as determined by liquid scintillation. The solid line represents within 10% the counts bound to: (1) control (no [^{32}P]-8-N_3cAMP added), (2) 0.125 μM [^{32}P]-8-N_3cAMP present without photolysis, and (3) 0.125 μM [^{32}P]-8-N_3cAMP present which was prephotolyzed 20 min before the membranes were added followed by a 60-min photolysis with membrane present. The markers ○, △, □, and ● represent the cpm values found on gels of membrane proteins photolyzed in the presence of 0.0125, 0.025, 0.075, and 0.125 μM [^{32}P]-8-N_3cAMP, respectively.

prephotolyzed before addition of membranes (see legend to Figure 4). Labeling of other membrane proteins, other than that in band IVb, is seen only after the 8-N_3cAMP concentration approaches 0.075 μM, a point at which the major labeled protein is almost saturated (Figure 4).

The data plotted in Figure 5 are taken from Figure 4B and show that the amount of [^{32}P]-8-N_3cAMP irreversibly bound to protein in band IVb by photolysis saturates at about 2.24 pmol/mg of membrane protein or approximately 410 molecules/red cell membrane. This is approximately 14% of the amount that was bound reversibly at saturation as shown in Figure 3.

The data in Figure 6 show that there are two proteins, A and B, which are primarily labeled by photolysis with higher concentrations of [^{32}P]-8-N_3cAMP (0.25 μM). Both proteins A and B were labeled in Figure 4 but protein A was labeled to a much greater extent at the lower 8-N_3cAMP concentrations. Additional experiments have shown that the quantity of label found in proteins A and B varies with different ghost preparations with the ratio of label incorporated varying from approximately 1 to 0.45 with protein A always being labeled to the greatest extent. Low concentrations of cAMP prevent [^{32}P]-8-N_3cAMP labeling of both proteins with protein B being protected most effectively by cAMP (see Figure 6B–D).

To investigate further the nature of the labeled proteins, membranes were photolyzed in the presence of [^{32}P]-8-N_3cAMP and other adenine containing compounds. The results in Table I show that the labeling of each protein is affected to a different degree by the addition of cAMP, adenosine, and ATP. Firstly, cAMP protects protein B against 8-N_3cAMP photolabeling better than it protects protein A. Thus, 0.087 μM cAMP decreased label incorporation in B by 92% but only by 52% in protein A. Secondly, 87 μM ATP protects protein A better than protein B with label incorporation decreased by 82% in protein A and 21% in protein B. Finally, adenosine, 87 μM, more than doubled the photolabeling of protein B (228% increase) whereas it increased the labeling in protein A by only 38%. All of the data in Table I were obtained using red cell membranes prepared from one donor, photolyzed at the same time, and electrophoresed on the same sodium dodecyl sulfate slab gel. This experiment has been repeated on three different membrane preparations with the same qualitative results.

The effect of photolyzing light on the incorporation of [^{32}P]-8-N_3cAMP is shown by considering reactions 2, 3, and 8 of Table I. Reaction 2 shows that no detectable irreversible incorporation of label occurs without activating light, whereas, with activating light considerable incorporation occurs (see reaction 3 and Figure 4B). In reaction 8, [^{32}P]-8-N_3cAMP was prephotolyzed before red cell membranes were added. Afterwards the sample was treated identically with reactions 1 through 7. This prephotolysis of 8-N_3cAMP abolished at least 99% of the [^{32}P]-8-N_3cAMP incorporation into proteins A and B. Other experiments have shown that the uv dependent incorporation of label is time dependent and maximum irreversible binding occurs within 12 min when the concentration of 8-N_3cAMP is below 2 μM and it is the only nucleotide present (B. Haley, unpublished results).

Discussion

The experiments reported in this paper provide evidence that 8-N_3cAMP will prove useful as a photoaffinity membrane probe for cAMP binding sites. To the extent that the binding is site specific, this compound should also prove

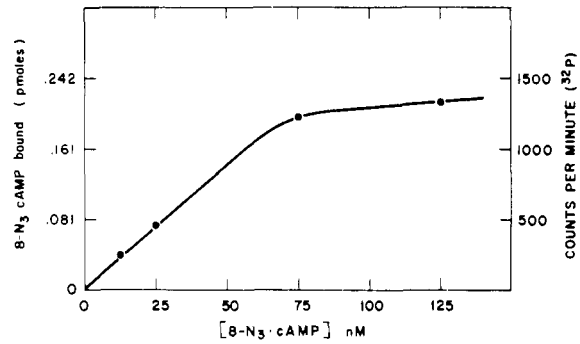

FIGURE 5: The effect of [^{32}P]-8-N_3cAMP concentration on photodependent covalent labeling of a protein component of human red cell membranes: This is a plot of the data obtained from Figure 4B with regard to the radioactivity incorporated into region which comigrates with staining band IVb.

Table I: Effect of cAMP, ATP, and Adenosine on the Photocatalyzed Covalent Binding of [^{32}P]-8-N_3cAMP to Human Red Cell Membrane Proteins.

Sample No.	Additions (μM) 8-N_3-cAMP	cAMP	ATP	Adenosine	$h\nu$	Relative Peak Areas of Densitometer Readings: Protein A	Protein B
1					+	0	0
2	0.25				−	1	0
3	0.25				+	100	100
4	0.25	0.087			+	48	8
5	0.25	0.87			+	0	0
6	0.25		87		+	18	79
7	0.25			87	+	138	228
8	0.25				+	0[a]	0.8[a]

[a] The [^{32}P]-8-N_3cAMP in sample 8 was prephotolyzed 20 min before being added to and photolyzed with the ghosts. Procedure: Membranes were photolyzed with [^{32}P]-8-N_3cAMP as given in Figures 3 and 5 and the ghost protein was separated by sodium dodecyl sulfate gel electrophoresis. The gels were stained with Coomassie Blue, dried on filter paper under vacuum, and autoradiographed. Densitometer analysis of radiation induced darkening of autoradiograph was done as given in the text.

useful for labeling and sequencing the polypeptide fragment of the cAMP binding site of purified protein kinases and other cAMP binding proteins. The results presented in Figure 4 are similar to that previously reported where the photoaffinity analog [^{3}H]-N^6-(ethyl-2-diazomalonyl)-cAMP was used (Guthrow et al., 1973). However, 8-N_3cAMP seems to have these advantages over N^6-(ethyl-2-diazomalonyl)-cAMP. (a) A larger percentage of the reversibly bound analog is covalently incorporated on photolysis (approximately 14%) and the label incorporation over background is much greater. (b) Photolysis may be done over the entire physiological pH range without noticeable formation of undesirable side products. (c) There does not appear to be any photolytic process occurring which promotes covalent binding of the analog to membrane proteins except conversion of the azide to a nitrene unless the system is irradiated to the degree where considerable protein cross-linking is also observed (B. Haley, unpublished results). This degree of photolysis is not necessary for photolabeling with 8-N_3cAMP. (d) Prephotolysis of 8-N_3cAMP abolishes the ability of the compound to label membranes with or without

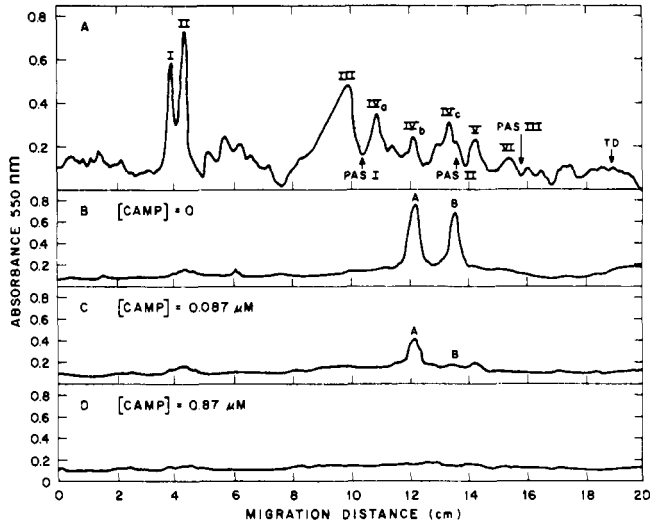

FIGURE 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of membranes photolyzed in the presence of [^{32}P]-8-N_3cAMP and varying concentrations of cAMP. Membranes (0.19 mg of protein) were photolyzed as given in Materials and Methods except 0.25 μM [^{32}P]-8-N_3cAMP was present along with varying concentrations of cAMP. (A) The densitometer tracing of the Coomassie Blue stain intensity of a representative gel on which membrane proteins have been electrophoresed. PAS I, II, and III marks the area where glycoproteins are located as determined by staining with periodic acid–Schiffs reagent (Fairbanks et al., 1971). The solid lines in B, C, and D are densitometer tracings of autoradiographs of nearly identical gels from the same slab whose proteins have been photolabeled with [^{32}P]-8-N_3cAMP as follows: (B) photolysis in the absence of cAMP; (C and D) photolysis with 0.087 and 0.87 μM amounts of cAMP present as indicated.

follow-up photolysis. Therefore, this analog is a true photoaffinity reagent and not a pseudophotoaffinity reagent.

The possibility that the label incorporation could be due to the 4% impurity may be eliminated by observing that photolysis is absolutely required for labeling and that the impurity shows no photoactivity. The reason for a relatively low level of label incorporation (14% of reversibly bound and approximately 2–4% of total 8-N_3cAMP available) may be due to several reasons such as: (a) preferential reaction of the nitrene with solvent within the binding site; (b) protective effects of photolysis products of 8-N_3cAMP which may also have an affinity for the binding site; (c) uv destruction of the binding site. A study of the effects of uv light on cAMP binding sites and protein kinase activity is currently under way.

The increased sensitivity obtained with 8-N_3cAMP has shown that there are two red cell membrane bound proteins which are primarily photolabeled with nM concentrations of 8-N_3cAMP. The first of these, protein A, coincides exactly with band IVb (Fairbanks et al., 1971) and has an approximate mol wt of 55,000. The other, protein B, has a molecular weight of approximately 49,000 and coincides with a region that stains positive with periodic acid–Schiffs reagent (PAS II, Fairbanks et al., 1971) and migrates slightly faster than band IVc. This may mean that 8-N_3cAMP (and cAMP) are binding to a glycoprotein. However, we have no further evidence at this time to support this possibility. Guthrow et al. (1972) and Rubin and Rosen (1973) have reported that cAMP stimulates phosphorylation of a protein in the region of band IVc, as well as other proteins. The observation that 8-N_3cAMP also labels in this region is consistent with the observations by others (Erlichman et al., 1974, Maeno et al., 1975) that the regulatory

subunit of cAMP-dependent protein kinase is an endogenous substrate of the catalytic subunit of this enzyme. In addition, 8-N_3cAMP labels a protein of 49,000 mol wt in several other membrane systems (Malkinson et al., 1975; Pomeranz et al., 1975). The level of labeling, especially of protein B, varies with each ghost preparation. The basis for this variation is not known at present but may be due to slight alteration in membrane preparation or to differences in each donor. That 8-N_3cAMP labels at least two bands may be expected for several reasons. First, other workers (Reiman et al., 1971) have presented evidence that there are two different cAMP regulatory or cAMP-binding subunits in purified protein kinase of rabbit skeletal muscle. Secondly, the thyroid contains at least three different cAMP stimulated protein kinases (Spaulding and Burrow, 1972). The red cell may be similar to either of these systems. It is also possible that 8-N_3cAMP may be labeling another nucleotide binding site, e.g., the ATP site on the catalytic subunit of a protein kinase or a cAMP regulatory site on another enzyme such as phosphofructokinase. The data in Table I show that the 8-N_3cAMP binding with these two proteins is differentially affected by the addition of cAMP, ATP, and adenosine. This supports the idea that these are two entirely different proteins with possibly different cellular functions.

In addition to proteins A and B it has been observed that 8-N_3cAMP labels four other proteins found in bands I, II, III, and V. However, the amount of label in these bands is always much less than that found in proteins A and B (see Figures 3 and 6) and may be due to some nonspecific labeling or other lower affinity cAMP binding sites. It should be noted that proteins in bands II, III, and V are also labeled using a photoaffinity analog of ATP (Haley and Hoffman, 1974).

The differential effect of the adenosine compounds on 8-N_3cAMP photolabeling of membrane components suggests that an examination using catecholamines, prostaglandins, and various drugs on the binding of this analog to other membranes should be of considerable interest. The effect of adenosine noted in Table I may be restricted to cAMP binding on red cell membranes. However, it may prove useful in solving various biological control problems involving adenosine and cAMP interactions. For example, it has been reported that both adenosine and cAMP prevent platelet aggregation (Packham et al., 1969; Marquis et al., 1969). Several hypotheses on the mechanism of this action of adenosine have been proposed (Born and Cross, 1963; Born, 1965; Salzman et al., 1966) and one of these is that adenosine may act by increasing adenylate cyclase activity (Mills and Smith, 1971). The human red cell does not contain adenylate cyclase (Sheppard and Burghardt, 1969) and since adenosine increases the 8-N_3cAMP binding in red cell membranes it is possible that an allosteric effector mechanism may be involved. This mechanism would require that adenosine increase the affinity of a specific protein kinase, or other protein, for cAMP (e.g., protein B) whereas it could have much less effect on other cAMP binding proteins (e.g., protein A) in the same membrane. With the use of this photoaffinity analog of cAMP it may be possible to measure the differential binding properties cAMP may have in a membrane system and the effects that other compounds, e.g., hormones, have on this binding.

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