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Inhibition of Egress of Adenosine 3′, 5′-Monophosphate from Pigeon Erythrocytes

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SUMMARY

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The formation of adenosine 3′,5′-monophosphate (cyclic AMP) is a response of nucleated pigeon erythrocytes that appears to be specific to catecholamines and is associated with the rapid appearance of the nucleotide in the incubation medium. Norepinephrine, 10 μM, produced a maximal response in 30–60 min of about 1 nmole of cyclic AMP accumulating both inside and outside cells when 25 μl of packed erythrocytes were incubated in 2 ml of buffered 0.154 M NaCl at 37°. The extrusion of cyclic AMP was primarily unidirectional and was inhibited by iodoacetate, prolonged incubation, and a high concentration of cyclic AMP in the medium. Extrusion was also inhibited by certain drugs. Notably potent was vinblastine (0.3 μM), which did not affect norepinephrine-induced intracellular accumulation of cyclic AMP. Colchicine (300 μM) and papaverine (100 μM) inhibited extrusion. These drugs also caused augmentation of intracellular cyclic AMP, probably as a result of inhibition of red cell cyclic nucleotide phosphodiesterase. Griseofulvin (300 μM) inhibited extrusion while partly reducing the norepinephrine-induced accumulation of intracellular cyclic AMP. Other agents which inhibited extrusion were quinine, quinidine, phenol red, chloroform, and toluene. Since most of the agents tested have been shown to bind microtubule protein or inhibit mitosis at metaphase, it is suggested that cyclic AMP extrusion from nucleated avian erythrocytes depends upon the integrity of microtubules or of analogous components in the plasma membrane. However, observations such as inhibition of extrusion by vinblastine without enhancement of intracellular accumulation of cyclic AMP indicate that drugs which affect extrusion may influence other related processes—cyclic AMP synthesis or degradation—and that alteration of one of these processes in turn influences the others.

INTRODUCTION

Davoren and Sutherland (1) showed that pigeon erythrocytes possess a receptor which is analogous to a *beta* andrenergic receptor: after stimulation by catecholamines the cells synthesized cyclic 3′,5′-AMP, and this response could be blocked by *beta* adrenergic antagonists. The same authors also showed that the pigeon erythrocyte extruded cyclic AMP into the incubation medium after stimulation with a catecholamine. This extrusion process occurred against an apparent concentration gradient

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and was inhibited by probenecid; these findings have been taken to indicate that the extrusion process may be an active one (1, 2).

Microtubules have been implicated in several mechanisms whereby substances are transported across cell membranes (3, 4). Because avian erythrocytes possess a marginal band of microtubules which lies just beneath the cell membrane (5), we investigated the effects of several agents which interact with microtubular protein upon the extrusion of cyclic AMP from pigeon erythrocytes. These agents were found to inhibit extrusion. Papaverine, a potent inhibitor of cyclic nucleotide phosphodiesterase (6), was found to have a similar effect.

MATERIALS AND METHODS

Adult pigeons were bled by cardiac puncture. The heparinized blood was centrifuged, and the plasma and buffy coat were removed. The red cells were washed twice in incubation medium (0.145 M NaCl–10 mM Tris-HCl, pH 7.4); the packed cells were then suspended in 3 volumes of chilled medium. Smears stained with Wright's stain confirmed that only erythrocytes were present. The experiment was begun by pipetting 25 μl of packed cells (diluted to 100 μl) into tubes containing 1.9 ml of incubation medium and the drug(s) to be studied at 0° and then transferring the tubes to a 37° water bath for periods of 0.5–120 min. The incubation was terminated (1) by chilling the tubes in ice. After centrifugation for 3 min at 0°, the sedimented cells were hemolyzed in 2.0 ml of distilled water at room temperature and then immediately chilled in ice. Both the hemolyzed cells and the supernatant fluid from the incubations were then heated for 5 min at 100° to destroy phosphodiesterase activity. The hemolyzed cells were centrifuged, and the sediment was discarded. The hemolysates and media were analyzed for cyclic AMP without further dilution or purification. The protein kinase activation method of Wastila *et al.* (7) was employed. The specificity and accuracy of this method for measuring avian erythrocyte cyclic AMP were tested as described for other tissues (7). The recovery of cyclic AMP (0.3–30 μM) added to suspensions of intact cells, hemolysates, or media was 90 ± 1.3% (SE). The recoveries were not altered if a supramaximal concentration of the phosphodiesterase inhibitor papaverine (1 mM) was present.

ATP was measured in trichloracetic acid extracts of cell lysates and media by the enzymatic fluorometric method described by Lowry and Passonneau (8). Each sample was assayed in triplicate.

For studies of red cell cyclic AMP degradative activity, 1 volume of washed, packed erythrocytes was homogenized in 1 volume of chilled 50 mM Tris-HCl, pH 8.0, in a Duall glass homogenizer (Kontes Glass Company) with 20 strokes of the pestle. The reaction was begun by adding 101 μl of homogenate to 200 μl of a 50 mM Tris-HCl–5 mM magnesium acetate buffer, pH 8.0, containing cyclic AMP in a range of concentrations from 0.3 μM to 3 mM and 200,000 cpm of cyclic [^{3}H]AMP. Drugs were dissolved in 50% (v/v) ethanol. The concentration of ethanol in the incubation, 1% or less, did not interfere with the reaction. The incubations were carried out at 37°, and the reaction was terminated by heating at 100° for 5 min. A homogenate heated prior to incubation served as a zero-time sample. After heating, the tubes were centrifuged and the supernatant fractions were incubated for 10 min with 0.5 mg/ml of *Crotalus atrox* venom. The total [^{3}H]nucleoside products were separated from cyclic [^{3}H]AMP on Dowex 1-2X (chloride, 100–200 mesh) as described by Rutten *et al.* (9) and measured by liquid scintillation spectrometry in a medium consisting of 5 ml of sample and 15 ml of solvent [2 parts toluene, 1 part Triton X-100 (Rohm & Haas), and 6 g/liter of Omnifluor (New England Nuclear)]. Phosphodiesterase activities were calculated from count rates that increased linearly with time of incubation (up to 30 min, depending on the cyclic AMP concentration). Count rates were corrected for the quenching that occurred (25–30%) in the medium relative to an unquenched ^{3}H-labeled standard and recovery of [^{3}H]adenosine (90%).

Tests for changes in red cell fragility induced by drugs were done according to Freeman and Spirtes (10). Statistical analysis of most of the data was performed with

Student's *t*-test for unpaired data. Enzyme kinetic data were subjected to a program for the Wang 462 calculator that provided analysis of linearity, least-squares fit to a straight line, and analysis of variance of x and y axis intercepts (Wang Laboratories, Tewkesbury, Mass.).

The following drugs were donated by the manufacturers: *l*-norepinephrine bitartrate, *l*-epinephrine bitartrate, and *dl*-isoproterenol HCl, by Sterling-Winthrop Research Institute; glucagon, papaverine HCl, vincristine, and vinblastine, by Lilly Research Laboratories; Ro 20-1724 and *dl*-4-(3-butoxy-4-methoxy-benzyl)-2-imidazolidone, by Hoffmann–La Roche; prostaglandins E_1 and $F_{1\alpha}$, by the Upjohn Company; chlorpromazine HCl and *d*-amphetamine sulfate, by Smith Kline & French Laboratories; and griseofulvin, by Ayerst Laboratories.

Purified cholera toxin, dried, lot 1071, was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, University of Texas Southwestern Medical School, Dallas.

Cocaine HCl was purchased from Mallinckrodt Chemical Works, and angiotensin II and colchicine, from Calbiochem. Sigma Chemical Company was the source of cyclic AMP, dopamine HCl, quinacrine HCl, quinine HCl, quinidine HCl, phenol red, and *C. atrox* venom.

Cyclic [^{3}H]AMP was purchased from Schwarz BioResearch or Amersham/Searle. It was purified by anion-exchange chromatography so that there was less than 1% contamination with [^{3}H]adenosine.

RESULTS

Properties of cyclic AMP accumulation process. In the absence of a catecholamine, little or no measurable cyclic AMP was found in either hemolysates[2] or medium. Of the drugs tested, only *l*-norepinephrine, *l*-epinephrine, and *dl*-isoproterenol caused formation of cyclic AMP. Cholera toxin (cells exposed for 1–2 hr), prostaglandins E_1 and $F_{1\alpha}$ amphetamine, glucagon, cocaine, angiotensin, and dopamine were ineffective. Saturating (11 μM) concentrations of isoproterenol, epinephrine, and norepinephrine caused equivalent responses. Norepinephrine was used throughout these experiments, always at a concentration of 10 μM. In its presence intracellular cyclic AMP rose linearly with respect to time for 15 min; it thereafter increased more slowly and reached a plateau after 40–50 min (Fig. 1). By contrast, an elevation of cyclic AMP in the medium could be detected as early as 3 min after the start of the reaction, and progressed linearly with time for 50–60 min (Fig. 2). These responses to norepinephrine are similar to those reported by Davoren and Sutherland (1). For studies of cyclic AMP accumulation in the cells and in the medium, an incubation time of 30 min was used unless otherwise noted.

The total amount of cyclic AMP produced in these experiments by 25 μl of cells was calculated to consume 1.5 nmoles of ATP in 1 hr, assuming that no cyclic AMP degradation took place with papaverine in the medium (Fig. 2). The amount of ATP in the cells decreased about 12% in 1 hr, from 76 nmoles/25 μl of cells prior to incubation (Fig. 1, inset). Therefore it is unlikely that in these experiments ATP became a factor limiting the synthesis of cyclic AMP, in spite of the absence of glucose in the incubation medium.

It was also determined that there was no measurable extracellular degradation of cyclic AMP. Cyclic [^{3}H]AMP (21 μM, 5×10^4 cpm/tube) was incubated with pigeon erythrocytes for 0, 60, or 120 min. Aliquots of the cell supernatant fractions were then subjected to ion-exchange chromatography

[2] All calculations of intracellular cyclic AMP are made on the basis of 25 μl of packed cells, with no corrections for trapped extracellular medium or inhomogeneity of the intracellular space. The distribution of cyclic AMP within the erythrocytes is not known. The cyclic AMP present in the incubation medium is referred to as "extracellular," while that in hemolysates prepared from washed cells is designated "intracellular." On rare occasions more than 0.05 nmoles of cyclic AMP accumulated in 25 μl of packed cells in the absence of norepinephrine. Such results were associated with loss of about 50% of the red cell ATP in 30 min of incubation and appearance of some of the ATP in the medium. Since such conditions were the exception and not the rule, these data have been omitted.

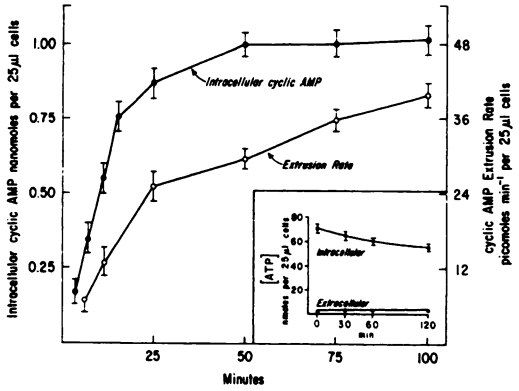

FIG. 1. *Comparison between intracellular content of cyclic AMP and ATP and rate of extrusion of cyclic AMP from pigeon erythrocytes*

The cells were incubated with 10 µM norepinephrine as described in MATERIALS AND METHODS. At 6, 12, 24, 49, 74, and 99 min of incubation the cells were rapidly chilled and centrifuged: the supernatant solutions were discarded, and the cells were resuspended in 0.25 ml of incubation medium containing 10 µM norepinephrine. They were incubated for 2 min at 37°; the reaction was then stopped, as described in MATERIALS AND METHODS, and the cell lysates and incubation media were analyzed for cyclic AMP. The extrusion rates shown thus represent the rates for minutes 6–8, 12–14, etc. The points represent the means ± 1 SE of three experiments. The inset shows the concentration of ATP in the red cells and medium before and during incubation with norepinephrine. The results represent the means of three experiments.

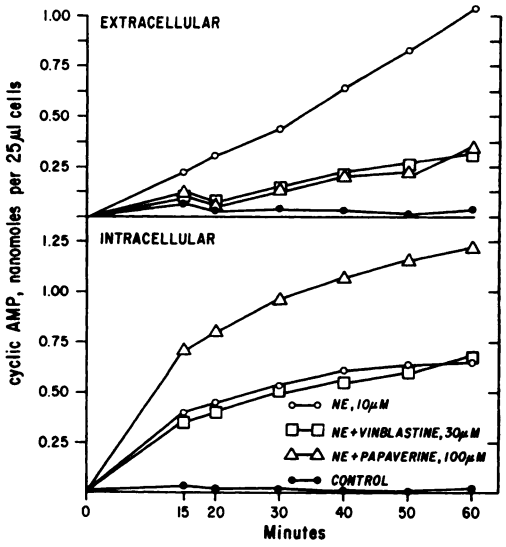

FIG. 2. *Time course of appearance of intracellular and extracellular cyclic AMP after norepinephrine, norepinephrine + vinblastine, and norepinephrine + papaverine*

The cells were incubated as described in MATERIALS AND METHODS. The drug concentrations used were: norepinephrine, 10 µM; vinblastine, 30 µM; papaverine, 100 µM. Control incubations: no drug added. Each point represents the mean of two determinations.

on anion (Dowex 1-Cl, 200–400 mesh) and cation (Dowex 50-H^+, 200–400 mesh) exchange columns with or without prior incubation with snake venom. More than 99% of the ^3H could be accounted for as cyclic [^3H]AMP; i.e., after up to 2 hr incubation of cyclic AMP with red cells no extracellular conversion of this nucleotide to 5′-AMP or nucleosides was demonstrable.

Davoren and Sutherland observed that when equal volumes of packed cells and medium were incubated with *l*-epinephrine the concentration of cyclic AMP in the medium exceeded that in the packed cells (1). Since the intracellular distribution of cyclic AMP could not be estimated, the results only suggested the possibility that cyclic AMP was extruded against a concentration gradient. In most of the experiments reported in the present paper pigeon erythrocytes were incubated with 80 volumes of medium, so that the concentration of cyclic AMP was always much lower in the medium.

Experiments were performed to determine the effect of cyclic AMP added to the medium on movement of the nucleotide into and out of the cells (Table 1). Incubation of erythrocytes with 16 µM cyclic [^3H]AMP for 30 and 60 min led to the apparent association of 3% of the added counts with the cell lysates. Owing to the 10-fold volume difference between medium and cells, the concentration of the counts would then be about 30% of the extracellular cyclic AMP concentration. However, no cyclic AMP was detected in the cells by the protein kinase assay. This assay was sensitive to 0.1 µM cyclic AMP in the lysates, or 0.6% of the concentration in the medium. The discrepancy between the tritium and direct assays may have been due to substantial influx of cyclic AMP followed by metabolic conver-

sion or concentration by the cells of a contaminant or an extracellular metabolite of the cyclic [^{3}H]AMP. This discrepancy has not been resolved, but the data in Table 1 do indicate that over a 60-min incubation period at 37° pigeon erythrocytes did not accumulate appreciable amounts of extracellular cyclic [^{3}H]AMP. No significant counts were associated with the ghosts (data not shown).

The cells in these experiments synthesized cyclic AMP in response to norepinephrine, and most of this was extruded. However, the concentration in the medium did not rise above that in the cells (Table 1). When both norepinephrine and cyclic AMP were added to the medium the intracellular concentration of the nucleotide was greater than when only norepinephrine was added. Since this was not associated with increased ^{3}H in the cell lysates, extracellular cyclic AMP presumably either inhibited extrusion of that cyclic AMP produced intracellularly or stimulated cyclic AMP production by the red cells. The results favor the hypothesis that the extrusion process was inhibited

TABLE 1

Effects of norepinephrine and cyclic AMP added to medium on intra- and extracellular cyclic AMP

In these experiments 25 µl of packed cells were suspended with 25 µl of 0.154 M NaCl and 0.01 M Tris-HCl, pH 7.4, and incubated for 0, 30, or 60 min in a total volume of this medium of 248 µl at 37° containing 50,000 cpm of cyclic [^{3}H]AMP and the additions noted below. The suspensions were then cooled rapidly to 0° and centrifuged. The cells were resuspended in 500 µl of medium and immediately centrifuged again. This washing procedure was repeated once more. The cells were then lysed in 250 µl of water. The lysate and cell supernatant fractions were assayed for cyclic AMP and ^{3}H. The cell ghosts were dissolved in 100 µl of 79% formic acid at 100° and then assayed for ^{3}H. This was neglible, and the results are not shown. The data in the table represent one of three experiments in which the absolute responses to norepinephrine differed but the relative changes in cyclic AMP were similar.

Time of incubation	Additions[a]		Lysate			Medium		
	NE, 10 µM	cAMP, 18 µM						
min			*cpm/25 µl*	*nmoles/25 µl*	*µM*	*cpm/250 µl*	*nmoles/250 µl*	*µM*
0	−	−	111	<0.00	<0.1	51,300	0.01	0.05
0	+	−	246	<0.00	<0.1	48,100	0.15	0.62
0	−	+	428	<0.01	<0.1	52,400	3.65	14.6
0	+	+	246	0.01	0.3	51,600	4.01	16.0
Mean ± SE			257 ± 65			50,800 ± 946		
30	−	−	1,265	<0.00	<0.1	53,600	0.00	0.00
30	+	−	740	0.70[b]	28.0[b]	50,420	1.23	4.92
30	−	+	2,218	0.00	0.0	46,600	3.75	15.0
30	+	+	1,117	1.42[b]	57.0[b]	50,000	4.10	16.4
Mean ± SE			1,335 ± 312[c]			50,200 ± 1,431		
60	−	−	675	0.00	0.0	49,600	0.01	0.03
60	+	−	2,103	0.64[b]	25.8[b]	44,400	1.97	7.90
60	−	+	1,313	0.00	0.0	50,500	3.95	15.8
60	+	+	2,169	1.70[b]	68.0[b]	43,700	4.25	17.0
Mean ± SE			1,565 ± 782[c]			47,000 ± 1,748		

[a] The abbreviations used in the tables and figures are: NE, norepinephrine; cAMP, adenosine 3′,5′-monophosphate.

[b] When the results for the three replicate experiments were normalized, the quantity and concentrations of cyclic AMP in the lysates of cells incubated with both norepinephrine and cyclic AMP were higher than those incubated with norepinephrine only ($p < 0.02$ by analysis of variance).

[c] The mean lysate counts at 30 and 60 min each differed from 0 min, with $p < 0.02$.

when 18 μM cyclic AMP was added to the medium. However, more extensive work is required to confirm this hypothesis.

The extracellular cyclic AMP accumulation depended upon the metabolic integrity of the red cells. Iodoacetate (10 mM) abolished extrusion while reducing the norepinephrine-induced intracellular accumulation of cyclic AMP by only 30% (Table 2). Furthermore, when the cells were first incubated at 37° for 100 min and norepinephrine was then added followed by incubation for 30 min more, the extracellular accumulation of cyclic AMP almost totally deteriorated. The accumulation of intracellular cyclic AMP in response to norepinephrine, however, was unimpaired. The extrusion process was not dependent upon Na^+ (extrusion of cyclic AMP was as effective in buffered isotonic KCl as in buffered NaCl) or upon Ca^{++} [extrusion was unhampered in an incubation medium containing 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid]. Nor was the process modified by ouabain (100 μM).

The cyclic AMP extrusion rate was measured by removing cells from a norepinephrine-containing medium after various times of incubation; the cells were then resuspended for 2 min in fresh medium containing 10 μM norepinephrine (Fig. 1). No maximal rate of extrusion was found; indeed, the rate of extrusion appeared to increase with time. These results are inconsistent with the observed linear accumulation of extracellular cyclic AMP (Fig. 2). The rate of extrusion as measured in the experiment of Fig. 1 evidently did not reflect the kinetics of extracellular cyclic AMP accumulation when cells were not washed and exposed for a brief period to fresh medium and norepinephrine.

TABLE 2

Effects of iodoacetate and prolonged incubation on cyclic AMP in erythrocytes and medium

Cells were incubated as described in MATERIALS AND METHODS; data are means ± 1 SE with $n = 3$.

Conditions	Intracellular cAMP	Extracellular cAMP
	nmoles/25 μl cells	
Control, 30 min	0 ± 0.01	0 ± 0.01
NE, 10 μM, 30 min	0.62 ± 0.08	0.54 ± 0.08
NE + iodoacetate, 10 mM, 30 min	0.42 ± 0.04	0 ± 0.00
Control incubation, 100 min; then NE, 10 μM, 30 min	0.79 ± 0.06	0.04 ± 0.03

Inhibition of cyclic AMP egress. The effects of vinblastine upon norepinephrine-stimulated accumulation of cyclic AMP in cells and medium are shown in Fig. 3. At a concentration of 100 μM vinblastine reduced intracellular cyclic AMP; this effect was no longer apparent at lower concentrations of the alkaloid, but at 30 and 100 μM concentrations vinblastine almost completely inhibited the extrusion of cyclic AMP into the incubation medium. Still lower concentrations inhibited the extrusion process to lesser degrees, in a concentration-dependent fashion. Extrusion was minimally but significantly inhibited by 0.3 μM vinblastine. Vincristine produced similar effects (data not shown).

Colchicine also inhibited extrusion; at the same time it potentiated the norepinephrine-induced elevation of intracellular cyclic AMP (Table 3). At a high concentration of colchicine (1 mM) intracellular cyclic AMP was elevated 37% over the amount seen with norepinephrine alone, but colchicine also sharply reduced (78%) the egress of cyclic AMP from the erythrocytes. The inhibition of extrusion was progressively less pronounced at lower concentrations of the drug.

Griseofulvin (1 mM) reduced the norepinephrine-induced intracellular accumulation of cyclic AMP, and even more markedly diminished the extrusion of cyclic AMP into the incubation medium (Table 3). Extrusion was also inhibited relatively more than cyclic AMP accumulation in the presence of 0.3 mM griseofulvin.

Papaverine (100 μM) markedly augmented the norepinephrine-induced increase of intracellular cyclic AMP; almost none of this large accumulation of cyclic AMP, however, was extruded into the medium (Table 3). Lower concentrations of papaverine also potentiated the effect of norepinephrine on intracellular cyclic AMP and, at 30 μM, extrusion continued to be moderately in-

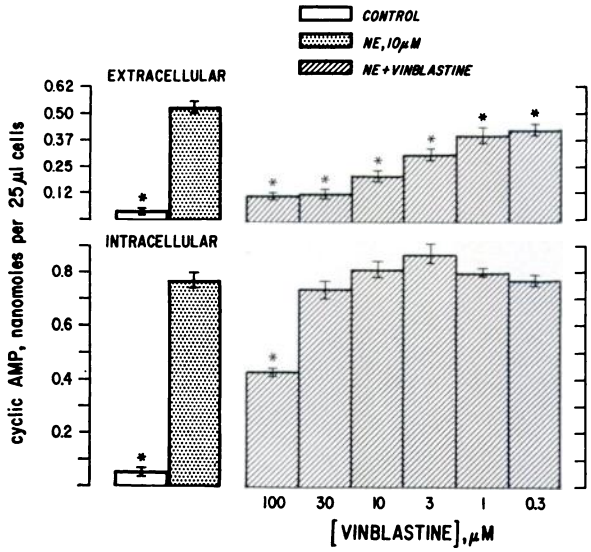

FIG. 3. *Effects of vinblastine upon norepinephrine-induced changes of intracellular and extracellular cyclic AMP*

The pigeon erythrocytes were incubated for 30 min as described in MATERIALS AND METHODS. Control incubations contained neither norepinephrine nor vinblastine. The concentration of cyclic AMP in cells exposed to 10 μM norepinephrine is calculated as 31 nmoles/ml of packed cells, and the concentration in the medium, as 2.2 nmoles/ml. The concentrations of vinblastine added to the medium are indicated below the appropriate bars in the figure. The bars represent means ± standard errors: control, $n = 17$; norepinephrine, NE $n = 25$; norepinephrine + vinblastine, $n = 6$. Asterisks indicate values which are significantly different ($p < 0.05$) from those obtained with norepinephrine alone.

hibited. At 10 μM, however, the amount of cyclic AMP extruded was back to that found with norepinephrine alone (Table 3). Papaverine alone (i.e., without norepinephrine) did not cause any detectable change of red cell cyclic AMP content. This was also found with the other extrusion inhibitors examined in this report.

The effects of vinblastine and papaverine were studied in cells incubated for periods other than 30 min (Fig. 2). The concentration of vinblastine chosen (30 μM) did not change the norepinephrine-induced buildup of intracellular cyclic AMP as compared with norepinephrine alone, while papaverine (100 μM) augmented the norepinephrine-induced increase of intracellular cyclic AMP content over cells treated with norepinephrine alone. Both drugs markedly inhibited the extrusion of cyclic AMP into the medium at all time intervals studied.

Incubation of the erythrocytes for 10–20 min with the drugs mentioned, prior to exposure to norepinephrine, did not alter the qualitative effects of the drugs. It did, however, expose certain complexities of drug action not seen otherwise. The intracellular accumulation of cyclic AMP in cells first incubated with vinblastine (100 μM), washed, and then exposed to norepinephrine was greater than the accumulation in cells exposed to norepinephrine alone; in sham-washed cells after incubation with vinblastine the intracellular accumulation of cyclic AMP remained depressed. Inhibition of cyclic AMP extrusion into the medium from cells first incubated with vinblastine occurred with both washed and sham-washed cells (Table 4). The inhibition by vinblastine of cyclic AMP extrusion persisted even after two or three washes. This was also true with vincristine (100 μM). In cells initially incubated with papaverine (100 μM) and then with norepinephrine, the intracellular accumulation of cyclic AMP was greater than in cells exposed to norepinephrine alone; this effect was present in sham-washed cells and was even more pro-

TABLE 3

Effects of colchicine, griseofulvin, or papaverine upon norepinephrine-induced changes of intracellular and extracellular cyclic AMP

The pigeon erythrocytes were incubated for 30 min as described in MATERIALS AND METHODS. The norepinephrine concentration was 10 μM. In control incubations no drug was present. The values are means ± standard errors (n = 6).

Conditions	Intracellular cAMP	Extracellular cAMP
	nmoles/25 μl cells	
Control	0.04 ± 0.01[a]	0.025 ± 0.01[a]
NE	0.82 ± 0.06	0.80 ± 0.05
NE + colchicine, 1 mM	1.12 ± 0.08[a]	0.18 ± 0.01[a]
NE + colchicine, 0.3 mM	0.98 ± 0.03[a]	0.62 ± 0.02[a]
NE + colchicine, 0.1 mM	0.81 ± 0.02	0.69 ± 0.04
Control	0.03 ± 0.01[a]	0.02 ± 0.01[a]
NE	0.55 ± 0.06	0.38 ± 0.04
NE + griseofulvin, 1 mM	0.32 ± 0.01[a]	0.07 ± 0.01[a]
NE + griseofulvin, 0.3 mM	0.42 ± 0.02[a]	0.12 ± 0.01[a]
NE + griseofulvin, 0.1 mM	0.54 ± 0.02	0.38 ± 0.02[a]
Control	0.02 ± 0.00[a]	0.01 ± 0.00[a]
NE	0.55 ± 0.03	0.64 ± 0.02
NE + papaverine, 100 μM	2.41 ± 0.13[a]	0.05 ± 0.01[a]
NE + papaverine, 30 μM	1.82 ± 0.05[a]	0.46 ± 0.04[a]
NE + papaverine, 10 μM	1.55 ± 0.06[a]	0.66 ± 0.02

[a] $p < 0.05$ compared with incubations carried out with norepinephrine alone.

TABLE 4

Effects of incubation with vinblastine or papaverine and subsequent washing upon response of pigeon erythrocytes to norepinephrine

Cells were first incubated for 5 min with vinblastine or papaverine (100 μM), centrifuged, and then resuspended either in fresh medium ("wash") or in the original incubation medium ("sham wash"). Norepinephrine (10 μM) was then added, and the cells were incubated for 30 min. n = 6 in each group.

Conditions	Intracellular cAMP	Extracellular cAMP
	nmoles/25 μl cells	
NE	0.70 ± 0.06	0.60 ± 0.10
NE + vinblastine, sham wash	0.48 ± 0.04[a]	0.05 ± 0.00[a]
NE + vinblastine, wash	1.11 ± 0.08[a]	0.10 ± 0.01[a]
NE	0.76 ± 0.07	0.75 ± 0.11
NE + papaverine, sham wash	1.60 ± 0.09[a]	0.00 ± 0.01[a]
NE + papaverine, wash	2.41 ± 0.10[a,b]	0.72 ± 0.05

[a] $p < 0.05$ compared with incubations carried out with norepinephrine alone.

[b] $p < 0.05$ compared with incubations including norepinephrine and papaverine, followed by sham washing.

nounced in washed cells. Cyclic AMP extrusion was completely blocked in the sham-washed cells, but this effect was eliminated by one washing of the cells (Table 4). The effects of colchicine and griseofulvin (both 1 mM) upon extrusion were also eliminated by a single washing of the cells. Thus the extrusion blockade induced by the *Vinca* alkaloids was not readily reversed, while the extrusion blockade induced by the other drugs was readily reversible.

Inhibition of cyclic AMP degradation. The finding that colchicine and papaverine potentiated the elevation of intracellular cyclic AMP caused by norepinephrine suggested that both agents inhibited the red cell system whereby cyclic AMP is degraded. Furthermore, the finding that washing the cells after incubation with vinblastine and papaverine enhanced the subsequent norepinephrine-induced rise of intracellular cyclic AMP suggested that sufficient drug remained within the cells after washing to inhibit cyclic AMP degradation. Therefore the extrusion inhibitors were tested in red cell homogenates for possible inhibitory effects on cyclic AMP phosphodiesterase.

Homogenates of pigeon erythrocytes, like many other tissues, contain two components of cyclic AMP phosphodiesterase activity as a function of cyclic AMP concentration (Fig. 4). At low substrate concentrations a K_m of 12 μM was estimated. Activity continued to increase at high cyclic AMP con-

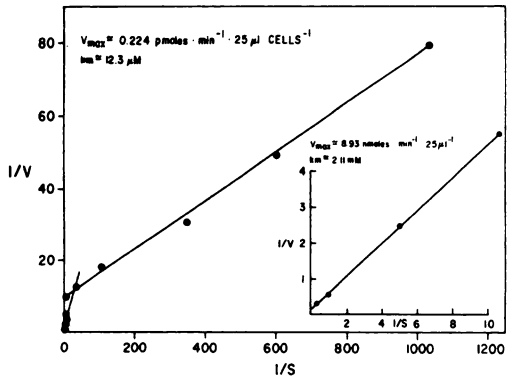

FIG. 4. *Lineweaver-Burk plots of phosphodiesterase activity of pigeon red cell homogenates*

The inset displays the activity partly plotted on the extreme left side of the larger graph. Cell contents were diluted 6-fold in the assay. The unit of velocity is picomoles per minute per 25 μl of cells. Substrate concentrations ranged from 0.29 μM to 3.0 mM. V_{max} and K_m were calculated from a linear regression analysis program for the Wang 462 calculator. Incubation conditions are described further in MATERIALS AND METHODS.

centrations to an estimated V_{max} 40 times greater, with a K_m of 2 mM. Drugs were tested with a substrate concentration (30 μM) similar to the cyclic AMP concentration in the cells stimulated by norepinephrine for 30 min (Table 5). Papaverine was found to be the most potent phosphodiesterase inhibitor, with an estimated IC_{50} of 11 μM, followed by the imidazolidone Ro 20-1724. This substance appeared to be less effective as an inhibitor of pigeon erythrocyte phosphodiesterase than has been reported for the rat erythrocyte enzyme (11). Theophylline was a very weak inhibitor. Both vinblastine and colchicine were weak inhibitors. The nature of inhibition by vinblastine was examined in more detail. This agent had some of the characteristics of a noncompetitive inhibitor at concentrations of 100 μM or less, but at a higher concentration the data were not interpretable on a Dixon plot (data not shown).

These results with papaverine are consistent with its capacity to raise intracellular cyclic AMP in norepinephrine-stimulated cells (Fig. 2; Tables 3 and 4). The inhibitory action of colchicine on phosphodiesterase at high concentrations is associated with a

TABLE 5

Inhibition of pigeon red cell phosphodiesterase

Activity was measured at 37° on homogenates of cells in the presence of 30 μM cyclic AMP. Values are the means of determinations at four time intervals during incubation up to 30 min. Product formation was always linear with time. See MATERIALS AND METHODS for further description of the assay conditions.

Inhibitor	Concentration	Velocity	Inhibition	Estimated IC_{50}
	μM	*pmoles min⁻¹ 25 μl cells⁻¹*	%	μM
None		92.5±4.7[a]	0	
Papaverine	10	61.0	44	
	100	24.0	74	11
	1000	16.6	82	
Ro 20-1724	10	70.3	24	
	100	27.0	69	33
	1000	7.5	92	
Theophylline	10	98.1	0	
	100	78.3	15	>1000
	1000	63.5	31	
Vinblastine	10	79.6	14	
	100	61.0	34	350
	1000	34.5	63	
Colchicine	10	72.2	22	
	100	53.8	42	270
	1000	36.4	61	

[a] Six replicate experiments ± standard error.

similar effect on intact cells (Table 3). However, in norepinephrine-stimulated cells vinblastine retarded the buildup of intracellular cyclic AMP at the highest concentration used, 100 μM (Fig. 3), which is about one-fourth its estimated IC_{50} for phosphodiesterase (Table 5). It should be noted that the inhibitory effects of both vinblastine and colchicine on phosphodiesterase were demonstrable at 37°, the temperature at which all intact cells experiments were performed, but not at 30° (data not shown).

Effects of other drugs on cyclic AMP extrusion and red cell fragility. The drugs mentioned above have all been reported to be

capable of producing metaphase arrest (12) and, except for papaverine, can either bind to microtubules or precipitate microtubular protein (13–15). Although probenecid has not been reported to have such effects, at 100 μM it inhibited the egress of cyclic AMP from pigeon red cells, as reported by Davoren and Sutherland (1) and confirmed by us. Other agents were tested for possible ability to inhibit cyclic AMP extrusion from pigeon erythrocytes. Some (quinacrine, quinine, quinidine, chloroform, and toluene) are known to produce metaphase arrest (11); others, like probenecid and phenol red, are actively secreted by the renal tubules (16). Three of these agents—quinine, quinidine, and phenol red (all at 100 μM)—greatly inhibited cyclic AMP extrusion from the pigeon red cell in the presence of norepinephrine (71%, 71%, and 60%, respectively). Phenol red simultaneously doubled the norepinephrine-induced buildup of intracellular cyclic AMP. The latter was unchanged after quinine and quinidine. Two others—chloroform and toluene (both at 1 mM)—also inhibited extrusion, but in addition inhibited (to a lesser degree) the intracellular accumulation of cyclic AMP. These agents did not inhibit the degradation of cyclic AMP by red cell homogenates.

In tests of red cell fragility it was found that papaverine, even in low concentrations, stabilized the red cells to hypo-osmotic hemolysis; at a high concentration papaverine induced hemolysis (Fig. 5). Chlorpromazine also had this effect. Furthermore, 1 mM colchicine also produced a degree of stabilization of the red cell. It is improbable, however, that papaverine and colchicine reduce cyclic AMP extrusion by "nonspecific" membrane stabilization. Were such a mechanism operative, chlorpromazine should also inhibit the extrusion process; it did not, at concentrations of 10 and 100 μM. Furthermore, no protection against hypo-osmotic hemolysis was conferred upon the cells by vinblastine (Fig. 5) or by norepinephrine.

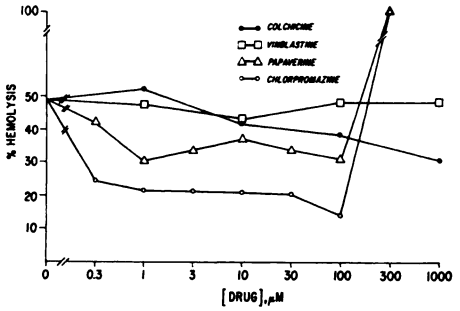

FIG. 5. *Effects upon red cell fragility of colchicine, vinblastine, papaverine, and chlorpromazine*

The incubation medium consisted of 0.40% NaCl in 1 mM Tris-HCl, pH 7.4. Washed erythrocytes (100 μl) were added to 1.9 ml of medium; the resulting suspension was mixed gently and then incubated at room temperature for 30 min. The samples were then centrifuged, and the optical densities of the supernatant solutions were measured at 540 nm. Control hemolysis was 49 ± 2% (mean of six determinations ± standard error); at other data points $n = 2$. For 100% hemolysis, cells were incubated in distilled water.

DISCUSSION

The extrusion of cyclic AMP from pigeon erythrocytes should be examined in the context of the over-all turnover of the nucleotide in these cells. There are certain limitations in determining cyclic AMP turnover from the data obtained here. One of these is that we made no direct measurements of cyclic AMP turnover based on labeling of the intracellular ATP. In fact, we have not excluded the possibility that adenylate cyclase, as a membrane enzyme, can form cyclic AMP on both sides of the membrane. Drugs may selectively inhibit extracellular cyclic AMP generation. This would be incorrectly interpreted as inhibition of cyclic AMP extrusion. We do not have the evidence to support such speculations.

Another limitation is that we do not know the volume of distribution of intracellular cyclic AMP. This limits the reliability of determining synthesis, degradation, and extrusion rates. The apparent velocities of these components of cyclic AMP turnover (Table 6) must be considered with these limitations in mind. The capacity to convert cyclic AMP to 5′-AMP appears to be adequate for all the cyclic AMP formed in response to norepinephrine. Extrusion nevertheless occurs at approximately half the rate of synthesis, and more cyclic AMP

TABLE 6

Processes involved in cyclic AMP turnover by pigeon erythrocytes

Process	Rate
	pmoles min^{-1} *25 μ cells*$^{-1}$ *at 37°*
1. Formation with NE	
Intracellular accumulation (Fig. 1)	33[a]
Adenylate cyclase [Øye and Sutherland (17)]	25
2. Degradation	
In intact cells	—[b]
Phosphodiesterase	92
3. Extrusion	
Extracellular accumulation (Fig. 2)	15[a]
Rate measurements (Fig. 1)	25[a]

[a] Based on changes measured during the first 30 min of incubation.

[b] No data.

appears in the medium than remains in the cells within 0.5 hr after the addition of norepinephrine. The rate of extrusion is difficult to estimate because of the discrepancy between the results obtained when cells were exposed continuously to norepinephrine and when extrusion was measured after exposure to fresh medium containing the catecholamine (Fig. 1). No attempt was made to explore further the reasons for this inconsistency as it might be related to inactivation of norepinephrine or formation of a diffusible inhibitor of cyclic AMP extrusion during incubation.

The extrusion mechanism is a labile one, but direct evidence that it is an active process, against a concentration gradient, was not obtained. Extrusion of cyclic AMP could be inhibited either by iodoacetate or by prolonged incubation. These effects are probably not the result of ATP depletion, because extrusion occurred in the absence of glycolysis and ATP was well maintained during incubation. Extrusion was also inhibited when the extracellular concentration of cyclic AMP was raised to 18 μM (Table 1), suggesting that efflux rates are controlled by the concentration gradient across the cell membrane. Although our data add to the previous evidence concerning the nature of cyclic AMP extrusion, the thesis that it is an active process still awaits secure proof. Further work, therefore, must be done to establish the point.

How do the extrusion inhibitors produce their effect? There are several possibilities. One possibility—nonspecific membrane stabilization—seems ruled out. Another possibility is binding of the drugs to the erythrocyte microtubules. Further support for the microtubule hypothesis is circumstantial. The *Vinca* alkaloids and colchicine have been shown to bind to microtubule subunits, and griseofulvin may also bind to tubulin subunits (18) and can disrupt the mitotic spindles of dividing cells (19). Finally, certain other extrusion inhibitors—papaverine, quinine, quinidine, chloroform, and toluene—can arrest mitosis at metaphase, a cellular mechanism mediated by microtubules. It seems possible, then, that the extrusion of cyclic AMP from the pigeon erythrocyte depends upon the integrity of the microtubules. If so, the finding that several of the extrusion inhibitors are also phosphodiesterase inhibitors may be more than coincidental. There are data which suggest that the polymerization of microtubules *in vivo* can be promoted by cyclic AMP (20–24). If so, a close association between microtubules and enzymatic activity which degrades cyclic AMP might be anticipated, especially since microtubules are evanescent organelles, quickly formed from pre-existing subunits and quickly dissipated. Thus drugs which bind to microtubules may well be expected to affect the activity of an enzyme, such as phosphodiesterase, which might serve to regulate the moment-to-moment status of microtubule structure. This entire argument is placed in jeopardy by the finding that microtubules can be assembled *in vitro* without any apparent need for cyclic AMP (25–27). The conditions of these experiments *in vitro*, however, can scarcely be conceived to exist in living cells. It remains to be demonstrated that the regulation of microtubule assembly and disassembly *in vivo* requires adenylate cyclase, cyclic AMP, and phosphodiesterase.

We do not wish to overstate the micro-

tubule hypothesis, however. The submembrane layer of microtubules of pigeon erythrocytes does not encompass the entire cell: it forms a ring, a marginal bundle, at the cell's point of widest diameter (5). Elsewhere the cell membrane is not sublayered with microtubules. Several recent communications have emphasized that functions modified by *Vinca* alkaloids and colchicine cannot automatically be explained as functions mediated by microtubules.

Another possibility is that the extrusion inhibitors act by binding to non-tubulin proteins within the erythrocyte membrane itself. Colchicine-binding protein is present in the membranes of many cells (28, 29). Furthermore, as Wunderlich *et al.* (30) have shown, the binding of colchicine to cell membranes renders the membrane rigid, impairing the movement of membrane components; the same authors suggested that this result may be due to the binding of colchicine to membrane protein(s) with colchicine-binding properties similar to the subunits of microtubules. Such protein(s) could also, then, bind *Vinca* alkaloids and other agents which bind to microtubule subunits. This kind of interaction between ligand and membrane could well be the basis of the effects we have observed. The likelihood of this is enhanced by the observations of Mizel and Wilson (31) that colchicine can inhibit nucleoside uptake into mammalian cells.

The drug effects we have observed may be too complex to be attributed to a single site of action. The level of complexity of the different drug actions themselves appears to vary. Papaverine and vinblastine offer clear examples of this. Both drugs inhibited cyclic AMP extrusion and inhibited phosphodiesterase. In addition, vinblastine, in high concentration, also inhibited the norepinephrine-induced buildup of intracellular cyclic AMP; this effect was reversed by one washing of the cells. The same washing did not, however, reverse the extrusion blockade by vinblastine; indeed, it unmasked the evidence that in the intact cell vinblastine either inhibited phosphodiesterase or augmented the response of adenylate cyclase. However, the finding that cyclic AMP extrusion was inhibited by vinblastine, without a cellular increase in cyclic AMP over control values (Fig. 2) [in fact there was a decrease, (sham wash, Table 4)], indicates inhibition of cyclic AMP formation by vinblastine. Thus the drugs may be operative at multiple sites, both within the cell and probably at the level of the cell membrane; these multiple sites of operation may in turn influence one another.

Not all the extrusion inhibitors attenuated the breakdown of cyclic AMP. One, phenol red, has not been reported to bind to microtubular protein or to inhibit mitosis.

Regardless of mode of action, our data clearly indicate that elevated tissue cyclic AMP concentrations in response to treatment with a drug such as papaverine may be due not only to inhibition of phosphodiesterase (6) but also to inhibition of the egress of cyclic AMP from the cell. A similar dual effect may be predicted for colchicine. Many types of cells can form cyclic AMP and then release the nucleotide into the incubation medium (2). It will be of interest to see whether the drugs described in this report also affect the release of cyclic AMP from cells other than the pigeon erythrocyte.

The function of the very active pigeon red cell adenylate cyclase system remains unclear. It may be related to Na^+ transport (32). The function of the extrusion process is also unclear. Is the extrusion simply a means whereby the cell rids itself of excess cyclic AMP? Or might the extruded cyclic AMP carry a message from the red cell to some other cell type? If so, to what cell, and what is the message?

It may well be that cyclic AMP extrusion can be inhibited in several ways by drugs; one of these ways may involve actions within the cell membrane and/or interactions with microtubules, and may thus be sensitive to such agents as the *Vinca* alkaloids and colchicine. Another mechanism may also exist whereby drugs can inhibit extrusion, and this process might be the one affected by probenecid and phenol red.

REFERENCES

1. Davoren, P. R. & Sutherland, E. W. (1963) *J. Biol. Chem.*, **238**, 3009–3015.
2. Broadus, A. E., Hardman, J. G., Kaminsky,

N. I., Ball, J. H., Sutherland, E. W. & Liddle, G. W. (1971) *Ann. N. Y. Acad. Sci.*, **185**, 50–66.
3. Rasmussen, H. (1970) *Science*, **170**, 404–412.
4. Poisner, A. M. & Bernstein, J. (1971) *J. Pharmacol. Exp. Ther.*, **177**, 102–108.
5. Behnke, O. (1970) *J. Ultrastruct. Res.*, **31**, 61–75.
6. Triner, L., Vulliemoz, Y., Schwartz, I. & Nahas, G. G. (1970) *Biochem. Biophys. Res. Commun.*, **40**, 64–69.
7. Wastila, W. B., Stull, J. T., Mayer, S. E. & Walsh, D. A. (1971) *J. Biol. Chem.*, **246**, 1996–2003.
8. Lowry, O. H. & Passonneau, J. V. (1972) *A Flexible System of Enzymatic Analysis*, p. 153, Academic Press, New York.
9. Rutten, W. J., Schoot, B. M. & DePont, J. J. H. H. M. (1973) *Biochim. Biophys. Acta*, **315**, 378–383.
10. Freeman, A. R. & Spirtes, M. A. (1963) *Biochem. Pharmacol.*, **12**, 47–53.
11. Sheppard, H. & Wiggan, G. (1971) *Mol. Pharmacol.*, **7**, 111–115.
12. Biesele, J. J. (1958) *Mitotic Poisons and the Cancer Problem*, Elsevier, Houston.
13. Borisy, G. G. & Taylor, E. W. (1967) *J. Cell Biol.*, **34**, 525–533.
14. Malawista, S. E. Sato, H. & Bensch, K. G. (1968) *Science*, **160**, 770–772.
15. Bensch, K. G., Marantz, R., Wisniewski, H. & Shelanski, M. (1969) *Science*, **165**, 495–496.
16. Weiner, I. M. & Mudge, G. H. (1964) *Am. J. Med.*, **36**, 743–762.
17. Øye, I. & Sutherland, E. W. (1966) *Biochim. Biophys. Acta*, **127**, 347–354.
18. Creasey, W. A., Bensch, K. G. & Malawista, S. E. (1971) *Biochem. Pharmacol.*, **20**, 1579–1588.
19. Malawista, S. E., Sato, H. & Bensch, K. G. (1968) *Science*, **160**, 770–772.
20. Goodman, D. B. P., Rasmussen, H., DiBella, F. & Guthrow, C. E., Jr. (1970) *Proc. Natl. Acad. Sci. U. S. A.*, **67**, 652–659.
21. Roisen, F. J., Murphy, R. A. & Braden, W. G. (1972) *Science*, **177**, 809–811.
22. Kram, R. & Tomkins, G. M. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 1659–1663.
23. Murray, A. W. & Froscio, M. (1971) *Biochem. Biophys. Res. Commun.*, **44**, 1089–1095.
24. Kirkland, W. L. & Burton, P. R. (1972) *Nature New Biol.*, **240**, 205–207.
25. Weisenberg, R. C. (1972) *Science*, **177**, 1104–1105.
26. Borisy, G. G., Olmsted, J. B. & Klugman, R. A. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 2890–2894.
27. Wiche, G., Zomzely-Neurath, C. & Blume, A. J. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 1446–1450.
28. Feit, H. & Barondes, S. H. (1970) *J. Neurochem.*, **17**, 1355–1364.
29. Stadler, J. & Frank, W. W. (1972) *Nature New Biol.*, **237**, 237–238.
30. Wunderlich, F., Müler, R. & Speth, V. (1973) *Science*, **182**, 1136–1138.
31. Mizel, S. B. & Wilson, L. (1972) *Biochemistry*, **11**, 2573–2578.
32. Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P. & Aurbach, G. D. (1973) *J. Biol. Chem.*, **248**, 5590–5597.