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Cyclic 3′,5′-Adenosine Monophosphate in Human Blood Platelets

II. EFFECT OF N^6-2′-0-DIBUTYRYL CYCLIC 3′,5′-ADENOSINE MONOPHOSPHATE ON PLATELET FUNCTION

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ABSTRACT The relation of cyclic 3′,5′-adenosine monophosphate to platelet function has been studied by investigating the influence of this compound and of its N^6-2′-0-dibutyryl derivative on platelet aggregation and other aspects of platelet behavior after demonstration of adenyl cyclase activity in disrupted platelets.

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The action of dibutyryl cyclic AMP did not result from contamination with 5′-AMP, nor was it attributable to production of 5′-AMP by plasma enzymes. Dibutyryl cyclic AMP was degraded to 2′-0-monobutyryl cyclic AMP and to cyclic AMP in plasma, but plasma exhibited no cyclic nucleotide phosphodiesterase activity, and the production of 5′-AMP did not occur. The in vitro effects of dibutyryl cyclic AMP were associated with uptake of the compound by platelets.

Adenyl cyclase activity of platelet homogenates was demonstrated with production of 9.27×10^{-11} ($\pm 2.62 \times 10^{-11}$) mole cyclic AMP per min per 10^{10} platelets. The activity was increased by NaF and by prostaglandin PGE_1 and was decreased by epinephrine. The effect of epinephrine was blocked by phentolamine but not by propanolol. Adenyl cyclase activity was also inhibited by collagen, 5-hydroxytryptamine, and thrombin. ADP, dibutyryl cyclic AMP, and cyclic AMP did not alter adenyl cyclase activity.

These observations are consistent with the hypothesis that platelet aggregation is favored by a decrease in platelet cyclic AMP and inhibited by an increase in cyclic AMP.

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INTRODUCTION

Inhibition of platelet aggregation by 3′,5′-adenosine monophosphate (cyclic AMP, cAMP) was first reported by Marcus and Zucker in 1965 (1), but this observation attracted little attention for several years. Recently several laboratories have suggested a relation of cyclic AMP to platelet function and have studied the metabolism of this nucleotide in platelets. Adenyl cyclase activity in platelets was first described by Scott (2), who found the enzyme unresponsive to epinephrine. Abdullah (3) noted reversal of platelet aggregation by 10^{-4} M dibutyryl cyclic AMP but not by 10^{-3} M cyclic AMP, and he described stimulation of platelet cyclic nucleotide phosphodiesterase by imidazole and nicotinic acid. Butcher, Scott, and Sutherland (4) and Wolfe and Shulman (5) reported that adenyl cyclase activity in platelet homogenates was stimulated by prostaglandin PGE_1, and the latter authors also observed stimulation by NaF. These findings were confirmed by Zieve and Greenough (6), who also described stimulation of platelet adenyl cyclase by glucagon and inhibition by thrombin, norepinephrine, epinephrine, and serotonin. Marquis, Vigdahl, and Tavormina (7) reported confirmatory

results and observed inhibition of platelet phosphodiesterase by caffeine.

A reasonable hypothesis based on these observations (8) would be that agents that produce or augment platelet aggregation (e.g., epinephrine [9], nicotinic acid [3], and imidazole [3]) reduce the level of platelet cyclic AMP (by inhibition of adenyl cyclase or stimulation of phosphodiesterase), and that agents with the opposite effect on platelet clumping (e.g., caffeine [10], PGE_1 [11]) elevate platelet cyclic AMP. Direct measurement of the cyclic AMP content of platelets has supported this formulation; Salzman and Neri (12) found platelet cAMP increased by caffeine and reduced by epinephrine and by ADP. Robison, Arnold, and Hartmann (13) reported an increase in platelet cyclic AMP produced by PGE_1 and observed that the effect of epinephrine on the platelet content of cyclic AMP was inhibited by phentolamine, an α-adrenergic inhibitor, but not by the β-adrenergic inhibitor, propanolol. An analogous relation of α-adrenergic and β-adrenergic blocking agents has been described for platelet aggregation induced by epinephrine (9, 14).

In studies to be described, the relationship of cyclic AMP to platelet aggregation was further examined by use of the derivative compound, N^6-2′-0-dibutyryl cyclic 3′,5′-AMP (dibutyryl cyclic AMP, DB-cAMP) as employed by Posternak, Sutherland, Falbriard, and Henion (15, 16). In some circumstances acylated derivatives of cyclic AMP are known to have pharmacologic effects in vivo and in tissue slices greater than the parent compound (15, 17). The enhanced activity of dibutyryl cyclic AMP has been attributed to its resistance to cyclic nucleotide phosphodiesterase or, alternatively, by virtue of its nonpolarity, to a greater ability to penetrate lipophilic cell membranes (15, 17). Data on this latter point have not, to our knowledge, been reported.

The effect of agents influencing platelet aggregation was also explored by assessment of adenyl cyclase activity in platelet homogenates.

The data to be reported support the above mentioned hypothesis that platelet aggregation is accompanied by a fall in platelet cyclic AMP and inhibition of aggregation by the reverse.

METHODS

1. Blood was collected from human volunteers by venipuncture through siliconized needles (Siliclad, Clay-Adams, Inc., Parsippany, N. J.) and polyvinyl chloride tubing into polypropylene tubes containing 1/10 vol of 3.8 g/100 ml of trisodium citrate dihydrate or 1/6 vol of the acid citrate anticoagulant of Aster and Jandl (18) or sodium heparin (final concentration 5 U/ml). Platelet-rich plasma (PRP) was prepared by centrifugation at 150–200 *g* for 15–20 min at room temperature. Platelet-free plasma (PFP) was prepared from PRP by centrifugation at 31,000 *g* for 20 min at 4°C. Platelet counts were performed by the method of Brecher and Cronkite (19).

2. Platelet aggregation was studied by the method of Born (20). The optical density of PRP was measured at 600 mμ in a Coleman Jr. spectrophotometer (Coleman Instruments, Maywood, Ill.) modified to permit magnetic stirring, control of temperature at 37°C, and continuous recording.

3. Platelet factor 3 activity was measured by determination of the recalcified clotting time of PRP after activation with 10 mg/ml of kaolin according to the technique of Hardisty and Hutton (21).

4. Release of serotonin was studied by a modification of the method of Spaet and Zucker (22). To the citrate to be employed as anticoagulant (No. 1, above), was added 5-hydroxytryptamine-2-^{14}C (SA 5.2 μCi/mg; New England Nuclear Corp., Boston, Mass.) to a final concentration of 1.0 μCi/40 ml of blood, and after preparation of PRP the sample was separated into aliquots and incubated at 37°C. 30–60 min after collection of the blood, agents to be tested were added to aliquots of the PRP and further incubated at 37°C for 5 min. The PRP was then centrifuged at 20°C for 15 min at 2,000 *g*, and aliquots of the supernatant were added to scintillation fluid [1] and counted for radioactivity in a Nuclear-Chicago Mark I liquid scintillation counter (Nuclear-Chicago, Des Plaines, Ill.). Release of labeled serotonin by platelets was expressed as per cent of total uptake.

5. Platelet volume was measured at 37°C with the Coulter Counter Model B (Coulter Electronics Inc., Hialeah, Fla.) and histogram plotter according to our previously published technique (23).

6. The activated partial thromboplastin time (24) (Thrombofax; Ortho Pharmaceutical Corp., Raritan, N. J.), thrombin clotting time (25) (Thrombin Topical, Parke, Davis & Co., Detroit, Mich.), and one stage prothrombin time (25) (Thromboplastin; Dade Div., American Hospital Supply Corp., Miami, Fla.) were performed according to previously published techniques.

7. Platelet uptake of N^6-2′-0-dibutyryl cyclic 3′,5′-AMP-^{3}H (SA 2.8 Ci/mmoles; New England Nuclear) and of cyclic 3′,5′-AMP-^{3}H (SA 16.3 Ci/mmoles; Schwarz Bio Research Inc., Orangeburg, N. Y. was studied by a previously described technique (26). After incubation of 1.0 ml aliquots of PRP with the tracer and carrier nucleotide at specified concentrations, the samples were filtered through cellulose acetate Millipore filters (EAwp 02500, 1.2-μ pores) and the filters were immersed in scintillation fluid [1] and counted. Human albumin-^{131}I (Abbott Laboratories, N. Chicago, Ill.) (0.3 μCi added to each sample) was added to the tracer mixture as a plasma marker. After correction of the ^{3}H counts for overlap from the ^{131}I channel, the radioactivity retained by the Millipore filters following filtration of PRP was corrected for ^{3}H in entrapped plasma by reference to a plasma standard containing the ^{3}H cyclic compound together with albumin-^{131}I. The validity of the use of labeled albumin as a plasma marker, not significantly associated with platelets during short incubation periods, was previously established (26).[2]

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[1] Naphthalene, 375 g; 2,5-diphenyloxazole, 22.5 g; 1,4-bis-[2-(4-methyl-5-phenyloxazole)] benzene, 1.13 g; *p*-dioxane 3 liters.

[2] Chambers, D. A., and E. W. Salzman. 1970. Incorporation of fibrinogen-^{125}I by human blood platelets. Submitted for publication.

8. Adenyl cyclase activity was determined by a method derived from the techniques of Streeto and Reddy (27) and of Bar and Hechter (28). Platelets were separated by centrifugation at room temperature from 1.0 ml aliquots of PRP anticoagulated with acid citrate and after being washed twice with 0.145 M NaCl containing 1/10 vol of trisodium citrate, 3.8 g/100 ml, were centrifuged into a pellet. Each tube containing the platelets from 1.0 ml of PRP was quick frozen in acetone containing dry ice and was stored at −20°C. For the assay of adenyl cyclase activity, frozen platelet pellets were thawed at room temperature, were resuspended in 0.4 ml of cold 0.02 M glycylglycine buffer, pH 7.8, containing 10^{-3} M $MgSO_4$, were brought to 30°C by incubation in a water bath for a few moments, and then were added to 0.4 ml of 30°C Tris-HCl buffer (0.06 M), pH 7.8, containing 0.48×10^{-3} M 8-adenosine-5′-triphosphate-^{14}C (SA 44.4 mCi/mmoles; Schwarz Bio Research, Inc.), 6.7×10^{-2} M caffeine, 10^{-2} M $MgSO_4$, 5.7×10^{-3} M phosphoenolpyruvic acid (Calbiochem, Los Angeles, Calif.), and 0.016 mg of pyruvate kinase (Calbiochem). Blank tubes were prepared by boiling the platelet homogenates for 10 min before addition of the incubation mixture. After incubation of the tubes at 30°C for varying periods in a metabolic shaker, to each tube was added 0.2 μCi ^3H-labeled (SA 16.3 Ci/mmoles; Schwarz Bio Research) and 150 μg unlabeled adenosine-3′-5′-monophosphate and 150 μg adenosine-5′-monophosphate. The tubes were immediately placed in boiling water, boiled for 3 min, cooled, and centrifuged at approximately 1,500 *g* for 10 min at 4°C. $\frac{1}{10}$ ml of the supernatant was spotted on Whatman No. 40 filter paper and developed by descending chromatography in isopropanol: ammonia: H_2O (7:1:2 v/v) for 20 hr. Cyclic AMP spots were identified by ultraviolet absorption and reference to suitable standards and were eluted with water. The eluates were evaporated to dryness, redissolved in 0.2 ml H_2O, and once more spotted on Whatman No. 40 paper, and they were then subjected to descending chromatography in ethanol: 0.5 M ammonium acetate (5:2 v/v) for 16 hr. 2-cm segments were cut from the center of the cyclic AMP spots and counted in toluene (15 ml containing 3 mg of 2,5-diphenyloxazole and 0.6 mg of *p*-bis[2-(5-phenyloxazolyl)] benzene per ml) in a Nuclear Chicago Mark I liquid scintillation counter. Cyclic AMP-^{14}C production was corrected to 100% recovery by reference to the cyclic AMP-^3H internal standard, and after subtraction of the "blank" values obtained in the preboiled samples, the results were expressed in terms of per cent of substrate ATP-^{14}C converted to cyclic AMP. Blank values in the preboiled samples were never more than $\frac{1}{3}$ the values in the test samples after 2 min of incubation and did not increase with longer periods of incubation.

9. DB-cAMP-8-^{14}C (SA 16.8 mCi/mmole) and unlabeled DB-cAMP were purchased from Schwarz Bio Research. The collagen preparation employed was an acetic acid extract of human tendon obtained from Laboratoire Stago (Asnières-sur-Seine, France) and resuspended in Tris-HCl saline, pH 7.4. Other chemicals were reagent grade and were obtained from standard commercial sources.

RESULTS

Before considering the effects of DB-cAMP on platelet function, it was necessary to demonstrate that the commercially available compound was not contaminated with 5′-AMP or adenosine, which could account for many of the effects to be described. To examine this question, the commercial compound was purified by descending paper chromatography on Whatman 3MM paper in ethanol: 0.5 M ammonium acetate (5:2 v/v) (16) followed by elution in water, concentration by lyophilization, and redissolution in water. Concentration of the compound in solution was determined by absorption at 270 mμ (16). In studies of platelet aggregation, to be described below, the behavior of the chromatographed material was identical with that of the commercial preparation. In subsequent experiments, therefore, a single batch of the commercial preparation of DB-cAMP was used without further purification.

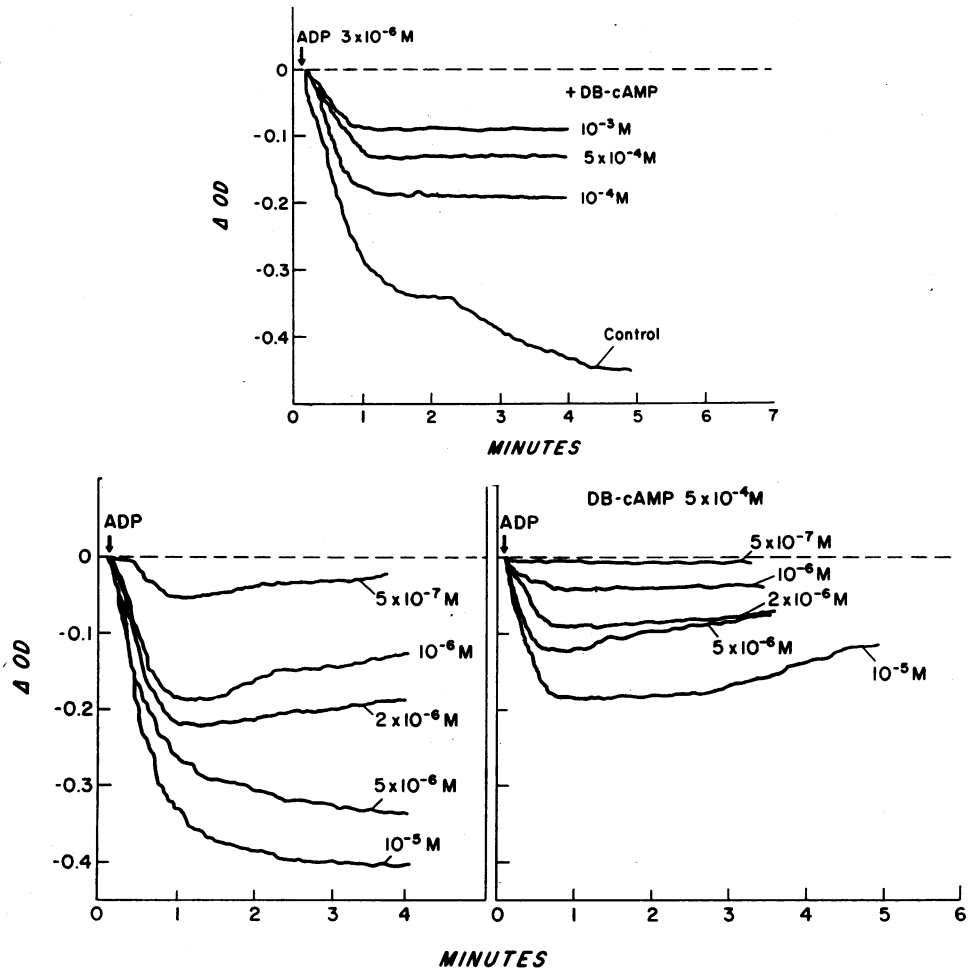
Platelet aggregation. ADP-induced platelet aggregation was impaired by dibutyryl cyclic AMP. The effect increased with the concentration of the inhibitor (Fig. 1). At low concentration of DB-cAMP, it was possible in some experiments to demonstrate selective inhibition of "secondary" aggregation, attributed to platelet "release" of ADP (29, 30); at higher concentrations "primary" aggregation was also affected. Inhibition of ADP-induced platelet aggregation by DB-cAMP varied with the concentration of ADP (Fig. 2).

The inhibitory effect increased with time of incubation of DB-cAMP in PRP before addition of ADP or the other aggregating agents which will be discussed. The effect of millimolar dibutyryl cyclic AMP reached a maximum after 30 min of incubation in PRP. At lower concentrations of inhibitor, the effect reached a peak after a shorter period and then declined with further incubation. Incubation of the PRP at 37° C for equivalent periods had only minor effects on the magnitude of platelet aggregation in the absence of DB-cAMP.

Inhibition of platelet aggregation by agents other than ADP was also demonstrated. The effect of DB-cAMP on epinephrine-induced platelet clumping is illustrated in Fig. 3. The inhibiting effect increased with the concentration of the inhibitor. Both primary and secondary aggregation were impaired. Platelet aggregation by epinephrine or ADP was also reduced by cyclic AMP, but the activity was much less than that of the dibutyryl derivative. In its requirement for incubation, cyclic AMP behaved like DG-cAMP in inhibition of platelet clumping.

Collagen-induced platelet clumping was also inhibited by dibutyryl cyclic AMP and to a lesser extent by cyclic AMP (Fig. 4). In contrast to the effect on ADP or epinephrine-induced platelet aggregation, the action of the inhibitors with collagen was first to delay aggregation; after longer periods of incubation with the cyclic nucleotides, the magnitude of aggregation was also inhibited.

Platelet aggregation by thrombin was also inhibited by cAMP and DB-cAMP (Fig. 5). The formation of fibrin was not delayed by these compounds.



FIGURES 1 and 2 Platelet aggregation by the turbidimetric technique of Born (20). 0.2 ml of ADP was added to 1.8 ml of citrated PRP continuously stirred in a cuvette at 37°C to a final concentration indicated, and the fall in optical density accompanying platelet aggregation was recorded. In some experiments, DB-cAMP was added 10 min before the ADP.

Other aspects of platelet function. Exposure of platelet-rich plasma to kaolin powder induces a clot-promoting activity known as platelet factor 3. The induction of platelet factor 3 activity was examined by the technique of Hardisty and Hutton, employing the kaolin-recalcification time of platelet-rich plasma (21). Incubation with kaolin suspension shortened the recalcification time of platelet-rich plasma. The effect varied with the platelet count (Fig. 6). Dibutyryl cyclic AMP added after the kaolin incubation had no effect on clotting time. If added to the platelet-rich plasma *before* kaolin, dibutyryl cyclic AMP led to a marked prolongation of the recalcification time. These data suggest a reduction in the availability of platelet factor 3 and indicate inhibition of the kaolin-induced platelet "release reaction" (31, 32) by DB-cAMP. DB-cAMP and cAMP (10^{-3} M) had no effect on the partial thromboplastin time, thrombin clotting time, or one-stage prothrombin time of platelet-poor plasma.

The platelet release reaction was studied more directly by labeling platelets with 5-hydroxytryptamine-2-^{14}C according to the technique of Spaet and Zucker (22). Incubation of the tracer in PRP led to platelet uptake of 60–85% of the label. Release of 25–45% of the isotope from platelets was induced by 10^{-6} M epinephrine or 10^{-6} M ADP. Uptake of serotonin was not inhibited, but release of the labeled compound was totally blocked by preliminary incubation of PRP with 10^{-3} M DB-cAMP.

Incubation of platelet-rich plasma with ADP leads to platelet swelling as well as to platelet aggregation (23, 33). These two responses of platelets to ADP are not inseparable, since the increase in platelet volume induced by ADP can be blocked without inhibition of

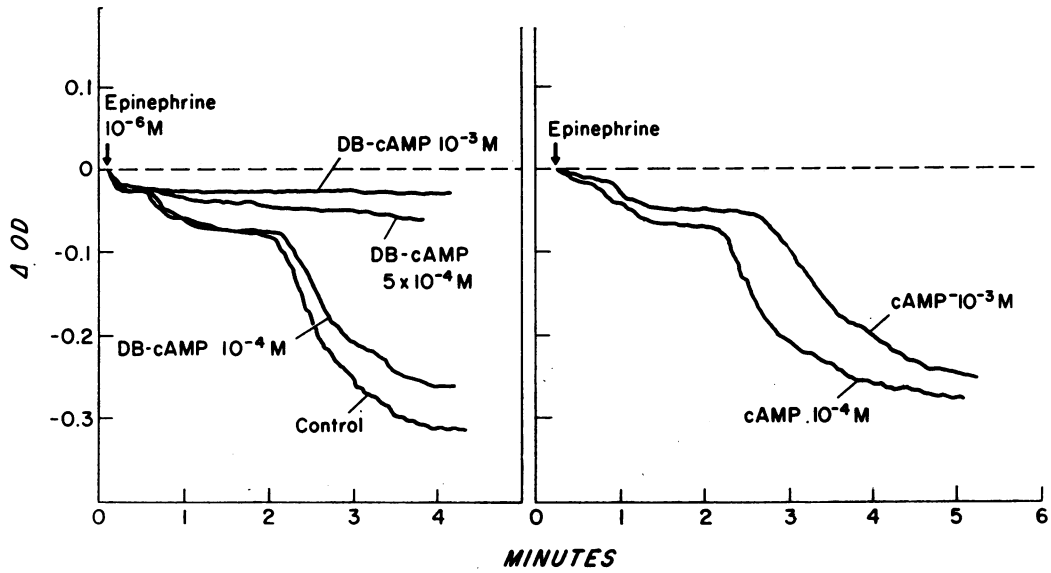


FIGURE 3 Platelet aggregation induced in PRP by epinephrine and its inhibition by DB-cAMP or cAMP incubated in PRP for 10 min before addition of the epinephrine.

platelet clumping (23), but under normal circumstances platelet swelling accompanies ADP-induced aggregation. Determination of platelet volume was carried out with the Coulter Counter (Table I). Dibutyryl cyclic AMP had no effect on platelet volume. In concentrations that totally blocked platelet clumping, the compound did not inhibit platelet swelling induced by ADP.

Mechanism of action of dibutyryl cyclic AMP. Except for the absence of an effect on ADP-induced platelet swelling, the actions of DB-cAMP described above are similar to those of 5′-AMP on platelets. It appeared possible that DB-cAMP might be converted to AMP by plasma enzymes during incubation in PRP and that AMP might account for some of the effects attributed to DB-cAMP. This possibility was examined by incubation of 10^{-3} M DB-cAMP in heparinized or citrated PRP for 30 min followed by precipitation with an equal volume of cold trichloracetic acid (10% w/v), centrifugation, and descending chromatography of the supernatant on Whatman No. 40 paper in ethanol: 0.5 M ammonium acetate (5:2 v/v). Products were located by ultraviolet absorption and identified by reference to suitable standards or in some experiments by radiochromatographic scanning after addition of DB-cAMP-^{14}C. Incubation of DB-cAMP in plasma led to the appearance of cAMP and of a product with the chromatographic mobility and ultraviolet absorption spectrum (maximum absorbance at 258 mμ) characteristic of 2′0-monobutyryl cAMP (16). No 5′-AMP was identified. In analogous experiments, incubation of 10^{-3} M cAMP in PRP or

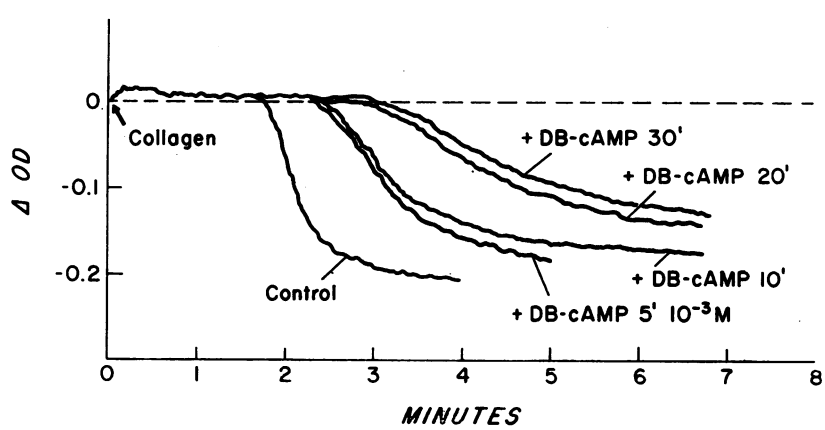


FIGURE 4 Platelet aggregation induced by 0.1 mg/ml collagen. Effect of DB-cAMP incubated for varying periods in PRP before the addition of collagen.

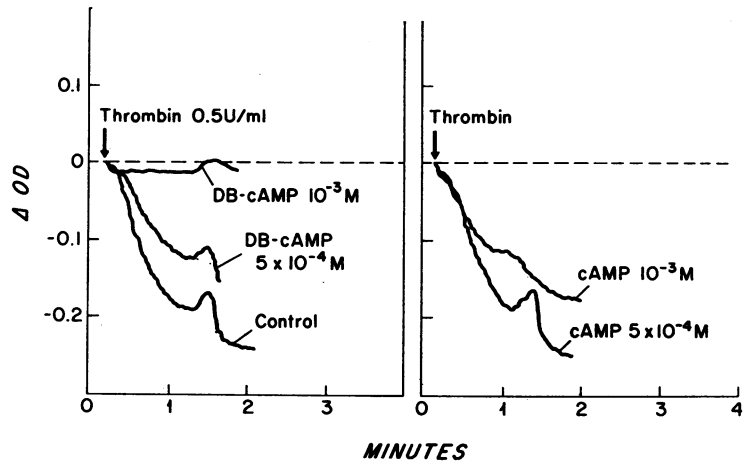


FIGURE 5 Platelet aggregation induced by 0.5 U/ml thrombin. The abrupt change in OD at about 1½ min indicates the appearance of fibrin.

PFP for 30 min failed to produce measurable 5′-AMP. Thus, it appears that plasma does not exhibit cyclic nucleotide phosphodiesterase activity and that platelet phosphodiesterase, whose activity is demonstrable in platelet homogenates (3, 7), is not available to plasma cyclic nucleotides in platelet-rich plasma.

The above studies of platelet aggregation indicate that when added to PRP dibutyryl cyclic AMP has a greater effect on platelet function than does cyclic AMP. Through the use of tritiated dibutyryl cyclic AMP of high specific activity, it was possible to demonstrate uptake of the label by platelets in PRP (Fig. 7). The platelet concentration of radioactivity increased with time of incubation. In similar experiments with tritium-labeled cyclic AMP, no platelet uptake could be shown, but the sensitivity of the method was not sufficient to exclude low levels of platelet uptake of the tracer.

Platelet adenyl cyclase activity. Demonstration of platelet uptake of dibutyryl cyclic AMP and inhibition of platelet clumping by this compound support the view that platelet aggregation is favored by agents that reduce platelet cyclic AMP and is inhibited by an increase in the cyclic nucleotide. This hypothesis was further examined by investigation of the action on platelet adenyl cyclase activity of agents known to influence platelet aggregation.

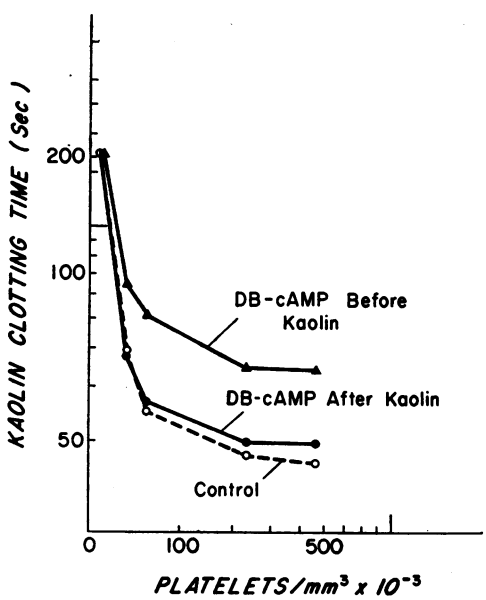


FIGURE 6 Effect on clotting time of recalcified platelet-rich plasma after incubation with kaolin (method of Hardisty and Hutton [21]) of variations in platelet concentration. The kaolin clotting time was prolonged by 10^{-3} M DB-cAMP added before incubation with kaolin but not if the DB-cAMP was added after the kaolin.

TABLE I
Platelet Volume, 37°C

	Modal volume	SD	Mean volume	SD
	μ^3		μ^3	
Control	4.94	0.83	9.11	1.31
10^{-6} M ADP	6.39	1.81	11.47	3.15
10^{-3} M, DB-cAMP, 10 min	4.82	0.51	7.79	0.73
DB-cAMP, then ADP	6.23	1.72	10.97	2.90

Platelet volume at 37°C determined with the Coulter Counter (23). To a 1:2,000 dilution of citrated PRP in Ringer's solution at 37°C was added ADP in the final concentration shown. Measurements of platelet volume were subjected to computer analysis by a published technique (23). In some experiments, 10^{-3} M DB-cAMP was incubated in the PRP 10 min at 37°C before dilution of the PRP.

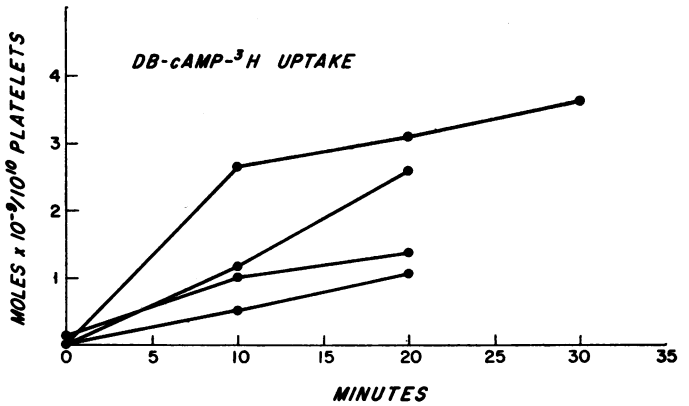


FIGURE 7 Uptake of radioactivity by platelets after incubation in citrated PRP of DB-cAMP-^{3}H and 10^{-3} M carrier DB-cAMP at 37°C for varying periods (four experiments). Results were corrected for trapped plasma by the use of human albumin-^{131}I as a plasma marker (see Methods) and are expressed as nanomoles uptake of DB-cAMP per 10^{10} platelets.

Washed human platelets were disrupted by freezing and thawing and were studied for adenyl cyclase activity as described under Methods. Since it was observed that disrupted platelet suspensions prepared from acid citrate-anticoagulated PRP after storage at 4°C for 24 hr or longer exhibited significantly less adenyl cyclase activity than when prepared from fresh PRP, all experiments to be reported were conducted with platelets frozen within 2 hr after collection of the blood sample.

Production of cAMP-^{14}C from ATP-^{14}C was proportional to platelet number and increased linearly with time up to 20 min. In 27 experiments, platelets disrupted by freezing and thawing produced 9.27×10^{-11} ($\pm 2.62 \times 10^{-11}$ SD) moles of cAMP per min per 10^{10} platelets. Adenyl cyclase activity was doubled by 10^{-2} M NaF (six experiments) and was increased 6–10 times by 10^{-6} M prostaglandin PGE_1 (10 experiments). A typical experiment is shown in Fig. 8. Epinephrine decreased adenyl cyclase activity 20–40% (Fig. 9) (eight experiments). The epinephrine effect was blocked by prior addition to the disrupted platelets of the α-adrenergic inhibitor, phentolamine, which itself stimulated activity above the basal level. Propanolol, a β-adrenergic inhibitor, produced an equivocal and statistically insignificant decrease in basal adenyl cyclase activity. It had no effect on the action of epinephrine.

Other agents that influence platelet aggregation were also studied. Suspensions of human collagen prepared by acid extraction (Laboratoire Stago) followed by suspension in Tris-saline, pH 7.4, (final concentration 0.1–1.0 mg/ml) had no adenyl cyclase activity but reduced the activity of disrupted platelets (Fig. 10) (eight experiments). 5×10^{-6} M 5-hydroxytryptamine inhibited adenyl cyclase activity 26–55% (four experiments). Bovine thrombin had no effect on adenyl cyclase activity at concentrations less than 10 U/ml, but at this concentration of thrombin the activity of disrupted platelets was inhibited 5–14% (two experiments), at 50 U/ml 27–39% (four experiments), and at 200 U/ml 39% (one experiment).

ADP, which causes platelet aggregation, has been shown to lower platelet-cAMP concentration (12). Its

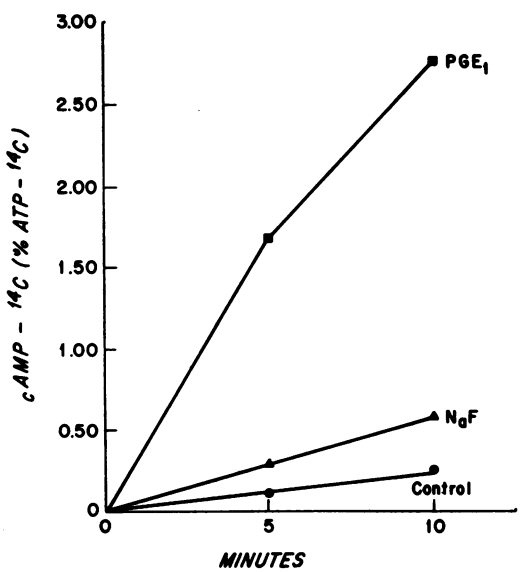


FIGURE 8 Conversion of 0.48×10^{-3} M ATP-^{14}C to cAMP-^{14}C by adenyl cyclase in disrupted platelets. Results are expressed as per cent conversion of substrate ATP to cAMP. Activity was stimulated by 10^{-2} M NaF or 10^{-6} M prostaglandin PGE_1 added to the platelet suspension immediately before the incubation mixture. Platelet count of original PRP 439,000/mm^3.

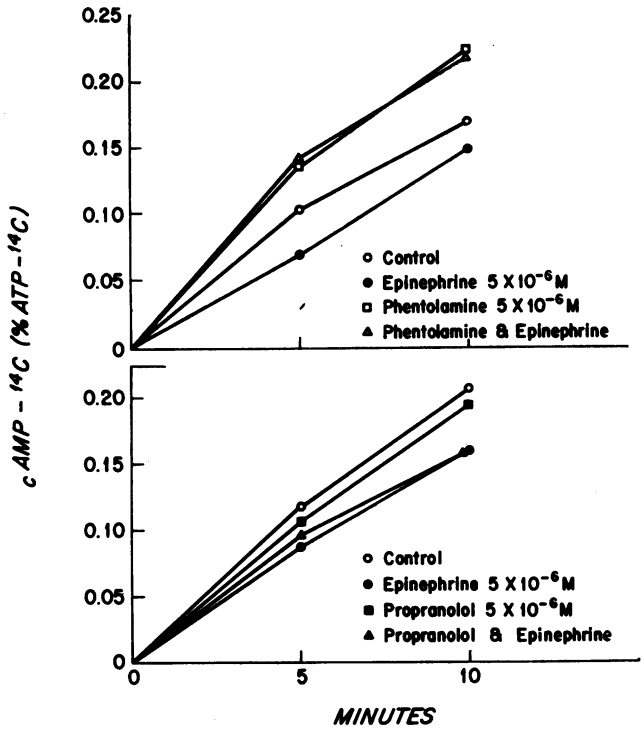


FIGURE 9 Effect of 5×10^{-6} M epinephrine on the adenyl cyclase activity of disrupted platelets. In the upper graph, the effect of 5×10^{-6} M phentolamine on adenyl cyclase activity and on the effect of epinephrine is examined. In the experiment shown in the lower graph, the effect of propanolol is shown. Epinephrine, phentolamine, and propanolol were added to the platelet suspension immediately before the incubation mixture. Platelet count of original PRP 463,000/mm^3 in the upper experiment and 547,000/mm^3 in the lower experiment.

effect on platelet adenyl cyclase activity could not be assessed in the standard incubation mixture because of pyruvate kinase and phospho-enol-pyruvic acid, added as an ATP-regenerating system to support the concentration of substrate ATP in the presence of competing ATPases. Since such a system could convert added ADP to ATP, the adenyl cyclase assay was also performed with an incubation medium from which pyruvate kinase and phospho-enol-pyruvate were omitted. Adenyl cyclase activity increased with time for at least 5 min in the absence of the ATP-regenerating system, and observations for incubation periods of this duration were possible. Addition of 10^{-6}–10^{-3} M ADP to a suspension of disrupted platelets in the absence of pyruvate kinase and phospho-enol-pyruvate failed to affect adenyl cyclase activity.

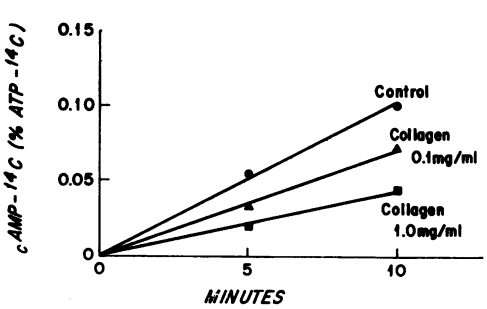


FIGURE 10 Effect of suspension of acid extractable human collagen on adenyl cyc'ase activity of disrupted platelets. No conversion of ATP-^{14}C to cAMP-^{14}C was produced by collagen alone (data not illustrated). Platelet count of original PRP 381,000/mm^3.

DB-cAMP and cAMP (10^{-5} M and 10^{-3} M) were studied in a system without phospho-enol-pyruvate and pyruvate kinase. They had no effect on platelet adenyl cyclase activity in a 2 min incubation but produced 15–30% inhibition at 5 min. If studied in an incubation mixture containing an ATP-regenerating system, no inhibition of adenyl cyclase activity was observed in incubation periods shorter than 10 min, but slight inhibition of activity was seen after this time. The significance of these observations is not clear, but since no inhibition of adenyl cyclase activity could be demonstrated during

TABLE II

Influence of Agents Afflicting Platelet Aggregation on Cyclic AMP and Its Enzymes

	Aggregation	Adenyl cyclase	Phospho-diesterase	cAMP
Epinephrine/norepinephrine	+(9)	↓(6, 50, *)		↓(12, 13)
α-blockade	↓(9)	↑(↓Epin.) (50, *)		↑(↓Epin.) (13)
β-blockade	↑(14)	±↓(0 Epin.) (50, *)		0(13)
5-HT	+(51)	↓(6*)		
Thrombin	+(52)	↓(6*)		
ADP	+(53)	0(*)		↓(12)
Collagen	+(54)	↓(*)		
Imidazole	↑(3, 55)		↑(3)	
Nicotinic acid	↑(3)		↑(3)	
PGE_1	↓(11)	↑(4, 5, 6*)	0(7)	↑(13)
Caffeine/theophylline	↓(10)		↓(7)	↑(12)
ATP	↓(56)	↑(57)		

In this table, production of an effect is indicated by +, augmentation of an effect by ↑, inhibition of an effect by ↓, and failure to influence an effect by 0. Data reported in this paper are cited by an asterisk. References are listed in parentheses.

Thus, e.g., α-adrenergic blockade increases adenyl cyclase activity but decreases the effect of epinephrine on adenyl cyclase.

short incubation periods, the results were not considered to show evidence of a direct effect on DB-cAMP or cAMP on the enzyme.

DISCUSSION

The identification by Sutherland and Rall (34) of 3′5′-adenosine monophosphate as an intermediate in the action of epinephrine on glycogenolysis in liver was the first of many reports that have appeared linking this compound to specific biological activities of different tissues. Several recent reviews have considered the role of cAMP in the regulation of cellular functions (2, 35–39).

That cyclic AMP might be involved in the aggregation of blood platelets was suggested to us (12) by the observation that many pharmacologic agents known to influence platelet clumping had been shown to owe their biological activity in other tissues to their effect on adenyl cyclase or cyclic nucleotide phosphodiesterase, the enzymes responsible for formation and destruction of cAMP. As studies of cyclic AMP and its regulating enzymes in blood platelets have begun to appear, a pattern has emerged whereby, at least insofar as data are at present available, agents that produce or augment platelet aggregation decrease the platelet content of cAMP, or, in instances in which such measurements have not yet been reported, reduce the activity of platelet adenyl cyclase or increase the activity of platelet phosphodiesterase. Conversely, inhibition of platelet clumping is accompanied by an increase in platelet cAMP and/or of adenyl cyclase activity or a decrease in activity of platelet phosphodiesterase. The evidence in support of this hypothesis is summarized in Table II.

Inhibition of platelet aggregation by dibutyryl cyclic AMP and to a lesser extent by cyclic AMP are also consistent with the above hypothesis. Platelet uptake of radioactivity after incubation with labeled DB-cAMP suggests that the compound owes its inhibitory activity to an increase in the platelet content of cAMP or of its acylated derivative. Impairment of platelet clumping by cAMP is probably the result of a similar mechanism; our inability to demonstrate platelet uptake of this compound is thought to have resulted from the insensitivity of methods available for recognition of the presumed low level of uptake of the isotope tracer. The greater biological activity of DB-cAMP than of cAMP has in other tissues been attributed either to the resistance of the acylated compound to destruction by phosphodiesterase or to its superior penetration of cell membranes, the result of its relative lipophilicity (15, 17). That the latter explanation is more likely correct is suggested by our demonstration of platelet radioactivity after incubation of DB-cAMP-^{3}H in PRP and by our failure to observe phosphodiesterase activity in plasma. The possibility of significant resistance of DB-cAMP to destruction by phosphodiesterase *within* platelets remains, but it would be a secondary effect requiring prior entrance of the compound into the platelet.

Inhibition of platelet clumping by DB-cAMP is accompanied by inhibition of the associated events, the release of 5-hydroxytryptamine (22) and the induction of activity of platelet factor 3 (21). Delayed or secondary platelet aggregation by ADP and epinephrine and platelet clumping induced by collagen and thrombin are

thought to be due to expulsion from platelets of a portion of their content of adenine nucleotides, especially ADP, another manifestation of the release reaction (32). These phenomena were reduced by DB-cAMP.

The initial or primary phase of platelet aggregation induced by ADP and epinephrine was also impaired by DB-cAMP, although this aspect of platelet clumping appears independent of the release reaction and is spared by agents that selectively inhibit secondary aggregation and release, such as aspirin (40, 41). Platelet swelling, on the other hand, which is a regular feature of platelet aggregation induced by ADP (23, 33) or by high concentrations of epinephrine (23) and is not affected by inhibition of release (23), was not affected by DB-cAMP in concentrations that totally blocked platelet aggregation.

Thus, the action of DB-cAMP and cAMP on platelets is complex and is not analogous to the effect of inhibitors of release, such as aspirin (40, 41) and phenylbutazone (42), or of EDTA, which totally blocks platelet response to aggregating agents and itself causes platelet swelling (34, 43) or of adenosine, which blocks all the known actions of ADP on platelets (44, 45). A natural analogy may occur in the platelets of patients with Glanzmann's thrombasthenia, in which there is total absence of platelet aggregation induced by epinephrine, ADP, collagen, and thrombin and of platelet factor 3 induction, but platelet swelling induced by ADP is unaffected (46–49). The metabolism of platelet cyclic AMP in patients with this disorder remains to be investigated.

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