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ADENOSINE ANALOGS AND HUMAN PLATELETS—II.

INHIBITION OF ADP-INDUCED AGGREGATION BY CARBOCYCLIC ADENOSINE AND IMIDAZOLE-RING MODIFIED ANALOGS. SIGNIFICANCE OF ALTERATIONS IN THE NUCLEOTIDE POOLS*

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Abstract—A number of adenosine analogs modified in the imidazole portion of the purine ring or in the carbohydrate moiety have been examined for their ability to inhibit the aggregation of human blood platelets induced by 10 μM ADP. Also, the effects of incorporation of adenosine analogs into the nucleotide pools, or of alteration of the natural adenine nucleotide levels, were studied. In accord with earlier findings, alterations in the ribose moiety of adenosine markedly diminished effectiveness in blocking ADP-induced aggregation. The C-nucleoside, formycin and its 1-methyl and 2-methyl derivatives, displayed little inhibitory activity and α-D-ribofuranosyl adenine and α-L-lyxofuranosyl adenine were without activity. Replacement of the 5′-hydroxyl group of the ribose by carboxyl, amino or *S*-methyl groups decreased capacity to inhibit aggregation. In contrast, carbocyclic adenosine (wherein an oxygen atom of the ribofuranosyl ring is replaced by a methylene group) retained full ability to inhibit ADP-induced aggregation. The effects of modifications in the imidazole portion of the purine ring of adenosine are complex. Tubercidin (7-deazaadenosine), 4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside (4-APP-ribonucleoside) and 8-azaadenosine displayed negligible or weak inhibitory activity. However, analogs related to tubercidin, i.e. 6-aminotoyocamycin and sangivamycin (5-carboxamide tubercidin), as well as the 3-carboxamide derivative of 4-APP ribonucleoside, displayed inhibitory activity approaching that of adenosine. No relation was established between the incorporation of adenosine analogs into platelet nucleotide pools and their ability to inhibit ADP-induced aggregation. Analogs such as formycin, which in the presence of the adenosine deaminase inhibitor, deoxycoformycin, readily enter the platelet nucleotide metabolic pool with replacement of a substantial portion of the natural adenine nucleotides, cause only weak inhibitory effects, whereas analogs such as 6-aminotoyocamycin, and sangivamycin, which did not form analog nucleotides or formed only small quantities of the 5′-monphosphate nucleotides, display inhibitory activity comparable to that of adenosine. When platelets were incubated with 2-fluoroadenosine, a potent inhibitor of aggregation, large amounts of 2-fluoroadenosine, 5′-mono, di- and triphosphate nucleotides were formed with a coincident marked decrease in the ATP concentrations. When these platelets were washed free of extracellular 2-fluoroadenosine and resuspended in platelet-free plasma, normal aggregation was produced by ADP. However, incubation of this suspension for 10 min with 2-fluoroadenosine resulted in an almost complete inhibition of ADP-induced aggregation.

The recent availability of a wide variety of adenosine analogs with modifications in the purine ring structure and exocyclic substituents, as well as in the sugar moiety (Fig. 1), has made structure–activity studies on the inhibition of ADP-induced platelet aggregation possible. These investigations have been facilitated greatly by the development of potent inhibitors of key enzymes of adenosine metabolism, e.g. 2′-deoxycoformycin (a tight-binding inhibitor of adenosine deaminase) and N^6-phenyladenosine (N^6-ϕ-Ado, a potent inhibitor of adenosine kinase). Another significant development is the application of high pressure liquid chromatography (h.p.l.c.) to the detailed analysis of intracellular nucleotide pools. Earlier investigations [1] demonstrated that

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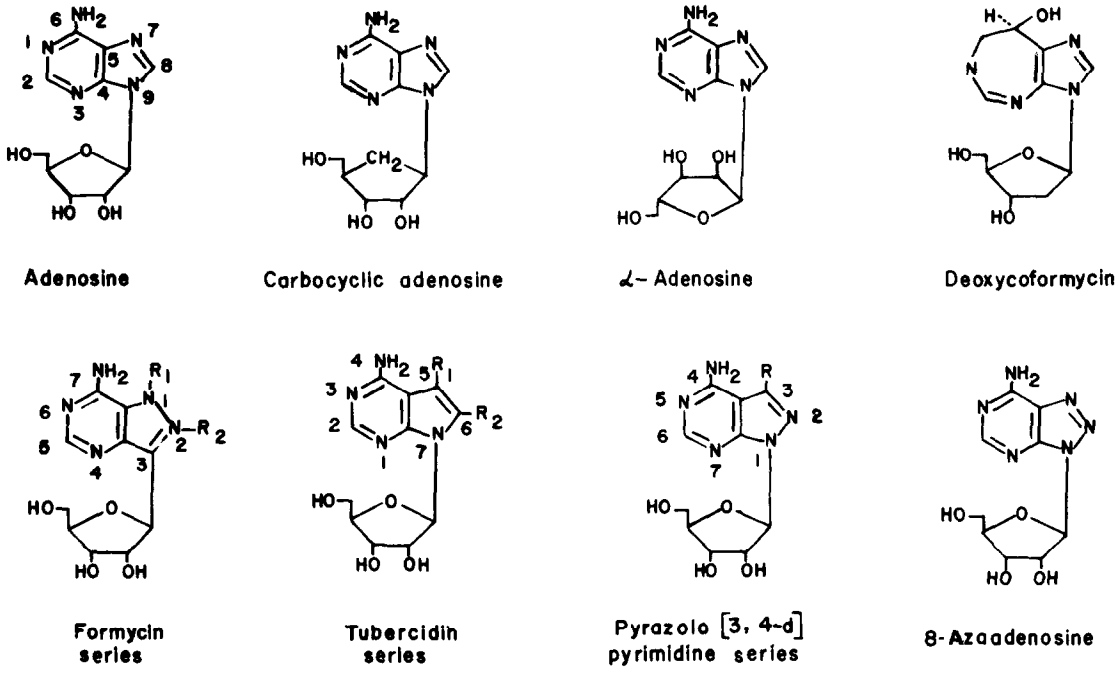

Fig. 1. Structural formulae of adenosine and analogs.

various adenosine analogs, e.g. F-Ado* and N^6-ϕ-Ado, are potent inhibitors of the aggregation phenomenon. Also, in the presence of an ADA inhibitor, the activity of adenosine, itself, is prolonged markedly. Especially significant was the apparently crucial role of the ribose moiety, since compounds such as arabinosyladenine, 2′-deoxyadenosine and 3′-deoxyadenosine (cordycepin) displayed only weak ability to inhibit ADP-induced aggregation. Furthermore, formycin, a C-nucleoside analog of adenosine in which the ribose moiety is sterically altered in comparison with adenosine [2–5], was essentially devoid of inhibitory activity. Of particular interest were the investigations that employed h.p.l.c. and demonstrated extensive incorporation of analogs such as F-Ado into the metabolic pools of platelets, largely replacing the natural nucleotide, ATP. When such F-ATP-loaded platelets were treated with thrombin, a normal discharge of granular contents and aggregation occurred. However, the nucleotides released consisted predominately of natural purine nucleotides while the newly incorporated F-adenine nucleotides remained within the platelets.

A number of important questions were left unanswered by the earlier investigations. For example, it was not established whether analogs such as F-Ado blocked ADP-induced aggregation through interaction with an ADP-responsive receptor or by incorporation into the nucleotide pools with alteration of the normal ATP:ADP ratio. Although these prior studies provided important new information about the structure–activity relations of the purine ring and its substituents, this analysis was far from complete. The present investigations represent an attempt to further clarify these and other questions regarding the phenomenon of ADP-induced platelet aggregation. Preliminary reports of these studies have been presented [6, 7].

MATERIALS AND METHODS

Adenosine 5′-diphosphate (ADP) and 2-chloroadenosine (Cl-Ado) were purchased from Sigma Chemical Co., St. Louis, MO. The carbocyclic analog of adenosine (C-Ado), α-adenosine and lyxofuranosyl adenine were obtained from Dr. L. Lee Bennett, Jr. of Kettering Meyer Laboratory, Southern Research Institute, Birmingham, AL. C-Ado was synthesized by the method of Shealy and Clayton [8]. 5′-Deoxy-5′-substituted adenosine analogs were provided by Dr. S. H. Chu of this laboratory. F-Ado was synthesized by Dr. John Montgomery and K. Hewson [9] and obtained through the courtesy of Dr. Harry B. Wood, Jr. from the Drug Development Branch, Division of Cancer Treatment of

* Abbreviations in the text are as follows: 2-fluoroadenosine (F-Ado); carbocyclic adenosine (C-Ado), 9-[β-DL-2α, 3α-dihydroxy-4-β-(hydroxymethyl)-cyclopentyl]adenine; formycin, 7-amino-3-(β-D-ribofuranosyl)pyrazolo [4,3-*d*]pyrimidine; tubercidin, 4-amino-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; sangivamycin, 4-amino-5-carboxamido-7-(β-d-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; toyocamycin, 4-amino-5-cyano-7-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; 2′-deoxycoformycin, (R)-3-(2-deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol (covidarabine); α-adenosine (α-Ado), 9-(α-D-ribofuranosyl)adenine; arabinosyladenine, 9-(β-D-arabinofuranosyl) adenine; 4-APP riboside, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*]pyrimidine; 4-APPR-3-carboxamide, 4-amino-1-(β-D-ribofuranosyl)pyrazolo[3,4-*d*] pyrimidine-3-carboxamide; 5′-deoxy, 5′-substituted adenosines, 5′-CH_2-COOH-adenosine, 5′-CH_2-NH_2-adenosine and 5′-CH_2-S-CH_3-adenosine; and ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4).

the National Cancer Institute. Formycin was kindly supplied by Dr. Hamao Umezawa of the Institute of Microbial Chemistry, Tokyo [10]. The adenosine deaminase (ADA) inhibitor, 2′-deoxycoformycin, was a gift from Dr. H. W. Dion of Parke, Davis & Co., Detroit, MI. Analogs of formycin, tubercidin and the pyrazolo-[3,4-*d*] pyrimidine nucleosides (Fig. 1) were synthesized by published procedures [11–17].

Isolation and washing of platelets. Human blood was collected in 0.1 vol. of 3.8% sodium citrate. Platelet-rich plasma (PRP) was separated by centrifugation of the whole blood at 377 *g* for 10 min at room temperature. This PRP was employed for platelet aggregation studies. To obtain the platelet pellet, 0.15 vol. acid–citrate–dextrose (ACD, U.S.P. formula A) was added to PRP, which then was centrifuged at 1350 *g* for 20 min. The pellet was washed once by resuspension in a buffer solution containing sodium phosphate buffer, pH 7.5, 20 mM; NaCl, 128 mM; $MgCl_2$, 2 mM; and glucose, 10 mM. After centrifugation, the platelet pellets were resuspended in the same buffer to achieve a platelet count of about 2 to 3 × 10^9/ml.

Incorporation of analogs into nucleotide pools of platelets. Adenosine analogs were dissolved in 0.85% NaCl, and then added in appropriate concentrations to the washed platelet suspension. The incubation was carried out in a shaking water bath at 37°. After the appropriate period of incubation, acid-soluble extracts were prepared by adding an equal volume of ice-cold perchloric acid (PCA, 6%); each extract was then subjected to several mixings on a Vortex mixer and allowed to stand at 0–4° for 15 min. The sediments were removed by centrifugation. An aliquot of the supernatant fluid was neutralized with KOH, and pre-

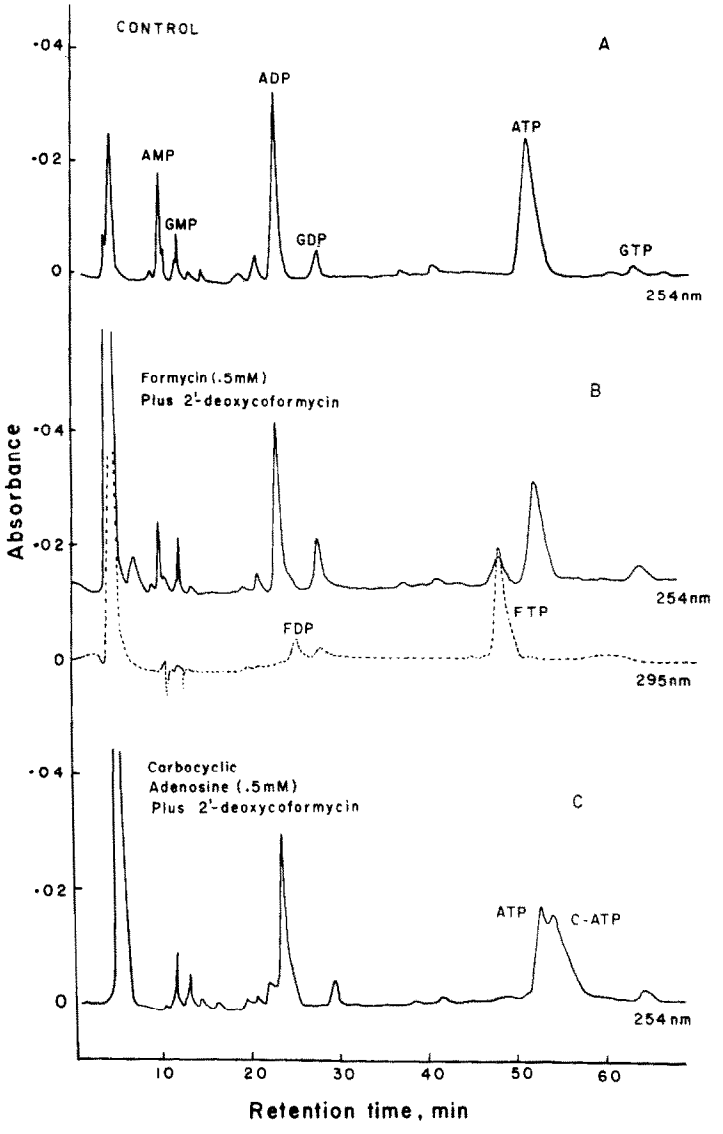

Fig. 2. Nucleotide profiles of human platelets incubated with or without adenosine analogs. Washed platelet suspension was incubated at 37° with (A, control) saline replacing the analog solution; (B) formycin, 0.5 mM plus 2′-deoxycoformycin, 1 μg/ml; and (C) carbocyclic adenosine, 0.5 mM plus 2′-deoxycoformycin, 1 μg/ml. After 1 hr of incubation, acid-soluble extracts were prepared as described in Materials and Methods. Aliquots (50 μl) were subjected to high pressure liquid chromatography.

Table 1. Incorporation of adenosine analogs into nucleotide pools and effects on ATP:ADP ratio and ADP-induced platelet aggregation*

Analogs	Analog nucleotides			ATP:ADP ratio	% Inhibition†
	Mono-	Di-	Tri		
None				1.7:1	
Adenosine	+	+	+	2.3:1	85
2-Fluoroadenosine (F-Ado)	+	+	+	0.9:1	95
2-Chloroadenosine (Cl-Ado)	−	−	−	1.5:1	95
Carbocyclic adenosine (C-Ado)	+	+	+	1.1:1	78
Formycin	+	+	+	1.3:1	10
6-NH_2-toyocamycin	±	−	−	1.7:1	78
Sangivamycin	±	−	−	1.7:1	67
4-APPR-3-carboxamide	±	−	−	1.6:1	65

* Washed platelet suspension (about 2×10^9 platelets/ml) was incubated with the analog (0.5 mM) in a shaking water-bath at 37°. After 1 hr of incubation, acid-soluble extract was prepared and an aliquot of 50 µl was analyzed on h.p.l.c. for the nucleotides.

† Platelet-rich plasma was incubated with the analog (100 µM) for 15 min and then the aggregation was induced by ADP (10 µM). Platelet aggregation was measured as described in Materials and Methods. In cases of adenosine, formycin and C-Ado, the platelet suspension and PRP were preincubated with 2′-deoxycoformycin (1.4 µg/ml) for 5–10 min before the addition of the analog.

cipitated $KClO_4$ was removed by centrifugation. The neutralized supernatant solutions were stored at −20° until analyzed by h.p.l.c.

Nucleotide analysis. High pressure liquid chromatography was performed on a Varian LCS 1000 (Varian Aerograph, Palo Alto, CA.) equipped with a Reeve–Angel Partisil-10 SAX (25 cm × 4.6 mm) column (Whatman, Inc., Clifton, NJ) [18]. The low and high concentrate eluents were 0.002 and 0.5 M KH_2PO_4 (pH 4.5). Flow rates were 50 ml/hr for both column and gradient pumps. Aliquots (20–50 µl) of the neutralized acid-soluble extracts were subjected to h.p.l.c. analyses. Nucleotide concentrations were estimated by comparing the h.p.l.c. chromatogram peak areas with those of known nucleotide standards. It should be noted that under these conditions, the F-adenine nucleotides eluted just before the adenine nucleotides, whereas in the earlier studies [1, 19] which employed a different column and flow rate [20], the F-adenine nucleotides had a longer retention time and appeared just after the guanine nucleotides. Measurements of absorbancy other than at 254 nm (e.g. formycin at 295 nm) were made by passing the effluent of the column through a model SF 770 Spectroflow Monitor (Schoeffel Instrument Co., Westwood, NJ) set at the appropriate wavelength.

Aggregation studies. Platelet aggregation was carried out at 37° in PRP by the turbidometric method of Born and Cross [21] with the use of a Payton aggregometer (Payton Associates, Inc., Buffalo, NY). The extent of aggregation was estimated by the per cent increase in light transmission in 4 min after the addition of ADP to PRP. Cell-free plasma was employed as the blank to represent 100 per cent light transmission.

RESULTS

Incorporation of adenosine analogs into platelet nucleotide pools. Figure 2 presents several examples of h.p.l.c. nucleotide profiles that illustrate the incorporation of adenosine analogs into the nucleotide pools of human platelets. Adenosine analogs that have absorbancy maxima at a characteristic wavelength, e.g. 295 nm for formycin, make possible the specific identification of analog nucleotide formation. High pressure liquid chromatography chromatograms of acid-soluble extracts of untreated platelets do not display absorbancy peaks at 295 nm, whereas incubation with formycin (0.5 mM) and 2′-deoxycoformycin (1.0 µg/ml) for 1 hr yielded distinct peaks at 295 nm consistent with the formation of the di- and triphosphate derivatives of formycin. In agreement with earlier findings with F-Ado [1], the ratio of tri- to diphosphate nucleotides of formycin is approximately 7:1 whereas the ATP:ADP ratio in the control extract is approximately 1.7:1. This is strong evidence that, during this incubation period, formycin entered principally the metabolic nucleotide pool of the platelet. Also, as in earlier studies with F-Ado [1], the synthesis of formycin nucleotides caused a decrease in the platelet ATP concentration, with little change in the ADP level, and an ATP:ADP ratio of about 1.3:1. Many of the adenosine analogs examined in the present study either have absorbancy maxima significantly different from that of adenosine or form nucleotides that have distinctive retention times on h.p.l.c., which permitted us to separate them cleanly from the natural adenine nucleotides. However, some compounds are encountered, such as C-Ado (Fig. 2C), where the absorbancy maxima are identical to that of adenosine and the analog nucleotides elute from h.p.l.c. with retention times similar to those of adenine nucleotides. In this case, precise analyses are very difficult by the techniques employed here. However, even with C-Ado, it was possible to detect a notched peak in the region of ATP and a decrease in the area of the ATP. This is strong evidence for the formation of analog nucleotides of carbocyclic adenosine with partial replacement of the ATP of the platelet metabolic nucleotide pool. However, in order to evaluate more precisely the quantity of C-Ado nucleotide formed, other techniques may be required such as the use of radioisotope-labeled analogs.

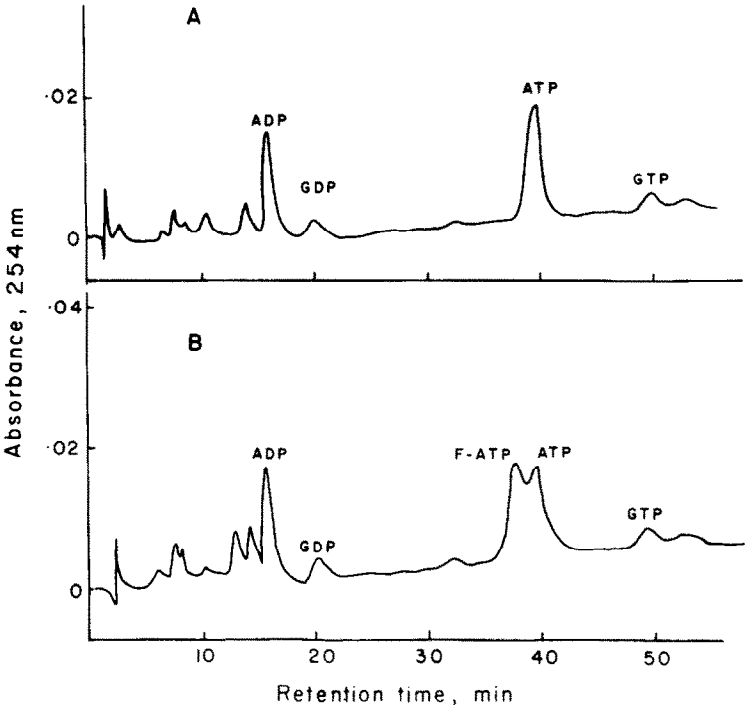

Fig. 3. Nucleotide profiles of human washed platelets after incubation with or without F-Ado. Fresh platelet-rich plasma (PRP) was incubated at 37° with (A) saline replacing the analog solution; (B) F-Ado, 0.5 mM. After 30 min of incubation, 0.15 vol. ACD (acid–citrate–dextrose) was added to the PRP and centrifuged. The platelet-free plasma was discarded and the platelets were washed by resuspension in phosphate-buffered saline medium and by centrifugation. Acid-soluble extracts were prepared as described in Materials and Methods, and aliquots (100 μl) were subjected to high pressure liquid chromatography.

Table 2. Inhibition of ADP-induced platelet aggregation by various adenosine analogs*

Adenosine analogs	Substitutions	Aggregation inhibition (%)
Formycin series	(Imidazole modified)	
Formycin	$R_1 = H$; $R_2 = H$	10
1-Methylformycin	$R_1 = CH_3$; $R_2 = H$	0
2-Methylformycin	$R_1 = H$; $R_2 = CH_3$	13
Tubercidin series		
Tubercidin	$R_1 = H$; $R_2 = H$	0
5-Br-tubercidin	$R_1 = Br$; $R_2 = H$	28
5-I-tubercidin	$R_1 = I$; $R_2 = H$	20
Toyocamycin	$R_1 = CN$; $R_2 = H$	25
6-NH_2-toyocamycin	$R_1 = CN$; $R_2 = NH_2$	78
Sangivamycin	$R_1 = CONH_2$; $R_2 = H$	67
6-NH_2-sangivamycin	$R_1 = CONH_2$; $R_2 = NH_2$	32
6-Br-sangivamycin	$R_1 = CONH_2$; $R_2 = Br$	36
Pyrazolo[3,4-*d*]pyrimidine series		
4-APP riboside	$R = H$	24
4-APP riboside-3-carboxamide	$R = CONH_2$	65
8-Azaadenosine		20
	(Sugar modified)	
α-D-Ribofuranosyl adenine (α-Ado)		0
α-L-Lyxofuranosyl adenine		0
Carbocyclic adenosine (C-Ado)		78
5′-CH_2COOH-adenosine		6
5′-CH_2-NH_2-adenosine		14
5′-CH_2-S-CH_3-adenosine		8

* Platelet-rich plasma was incubated with the analog (100 μM) for 15 min and then the aggregation was induced by ADP (10 μM). Platelet aggregation was measured as described in Materials and Methods. In cases of formycin, C-Ado, 8-azaadenosine and control samples, PRP was preincubated with 2′-deoxycoformycin (1.4 μg/ml) for 5–10 min before the addition of the analog.

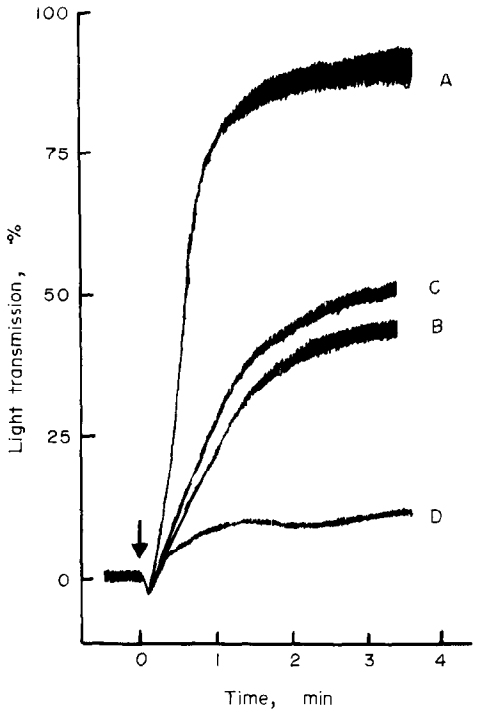

Fig. 4. ADP-induced platelet aggregation of washed platelet suspension prepared after incubation with or without F-Ado. Details of the experiment are described in the legend of Fig. 3. After incubation and washing, the washed platelets were resuspended in cell-free plasma to obtain platelet-rich plasma (about 0.5×10^9 platelets/ml). Aggregation was initiated by the addition of ADP (10 μM) to (A) the original PRP; (B) PRP prepared after incubation (without addition of F-Ado), washing and resuspension; (C) PRP prepared after incubation with F-Ado (0.5 mM, 30 min), washing and resuspension; and (D) PRP (employed in C) after incubation with F-Ado (10 μM) for 10 min.

A number of other adenosine analogs were examined in addition to those presented in Fig. 2. With many of these compounds, the formation of analog nucleotides was readily accomplished and the effects of the analog incorporation on the natural nucleotide pools were observed. Table 1 presents some selected examples of this study. Unexpectedly, Cl-Ado, a compound previously established as a potent ADP-induced aggregation blocker [22, 23], does not enter the nucleotide pools in quantities sufficient for detection by this technique nor does it cause a measurable alteration in the ATP:ADP ratio. By contrast, as reported previously [1], F-Ado readily enters the nucleotide pools in substantial quantities with a marked decrease in the ATP:ADP ratio. On the other hand, analogs such as 6-amino-toyocamycin (5-CN,6-amino-tubercidin) and 4-APPR-3-carboxamide (both relatively potent inhibitors of platelet aggregation) formed only measurable amounts of analog 5'-monophosphate nucleotides with no detectable synthesis of the analog di- and triphosphate nucleotides and also with no effect on the ATP:ADP ratio. These results and others have raised questions about the role of the formation of analog nucleotides and of the alteration of the normal ATP:ADP ratio in the blockade of ADP-induced platelet aggregation.

ATP:ADP ratio, intracellular analog nucleotide formation, and platelet aggregation. In an earlier report from this laboratory [1], it was shown that F-Ado is capable of forming large quantities of analog nucleotides in human platelets with the replacement of a substantial portion of the natural adenine nucleotides in the metabolic pool. Furthermore, platelets that contain large quantities of F-Ado nucleotides responded normally to thrombin, with aggregation and discharge of the contents of the storage granules into the medium. Here, principally natural nucleotides were released and the F-Ado nucleotides were retained in the aggregated platelets. The ratio of F-ATP : F-ADP, however, decreased from about 7:1 to about 3:1 after thrombin treatment. This suggested that the F-Ado nucleotides are capable of replacing the natural adenine nucleotides in the metabolic events that accompany the thrombin-induced aggregation phenomenon. These earlier studies, however, left unanswered the question of whether inhibition of ADP-induced aggregation by F-Ado was the result of analog nucleotide formation. Therefore, experiments were designed to examine this question.

Figures 3 and 4 present the results of an experiment in which human platelet-rich plasma was incubated with 0.5 mM F-Ado for 30 min, whereupon aliquots were centrifuged at 1570 *g* for 15 min, the supernatant fluid was discarded and the pellet resuspended in about 5 vol. of phosphate-buffered saline. Aliquots were again centrifuged and the pellets resuspended in either 1 vol. of platelet-free plasma or phosphate-buffered saline. A similarly treated control sample and an aliquot of platelets resuspended in phosphate-buffered saline were extracted with PCA as described above. Figure 3 presents the h.p.l.c profiles of these acid-soluble extracts measured at 254 nm. As seen in Fig. 3A, the experimental manipulations did not cause significant alterations in the nucleotide profile of the control sample. Figure 3B shows that substantial amounts of F-Ado nucleotides were formed, in accord with earlier findings [1] with a significant depletion in the concentration of natural ATP. Figure 4 presents tracings from aggregation studies performed with the platelet suspension in response to the addition of ADP (10 μM). Tracing A shows the normal aggregation obtained prior to incubation with F-Ado. Tracings B and C show the aggregation that occurred in the control and the F-Ado-incubated platelets, respectively, after washing and resuspension in platelet-free plasma. In this case, there was essentially identical ADP-induced aggregation in the control and the F-Ado nucleotide-containing platelets. However, when these F-Ado nucleotide-containing washed platelets were incubated with F-Ado (10 μM) for 10 min, ADP-induced aggregation was markedly inhibited as seen in tracing D. These results are consistent with the concept that F-Ado inhibits ADP-induced aggregation through interaction with an ADP-responsive receptor rather than through alterations in the platelet metabolic nucleotide pool.

Effect of adenosine analogs modified in the imidazole ring on ADP-induced aggregation. We have had the opportunity to examine, as inhibitors of ADP-induced aggregation, a variety of adenosine analogs (100 μM) modified in the imidazole ring, i.e. formycins, tubercidins, pyrazolo[3,4-*d*]pyrimidines and 8-azaadenosines (see Fig. 1). As reported earlier, only slight inhibition was observed with the C-nucleoside formycin. The methylated formycins (1-methylformy-

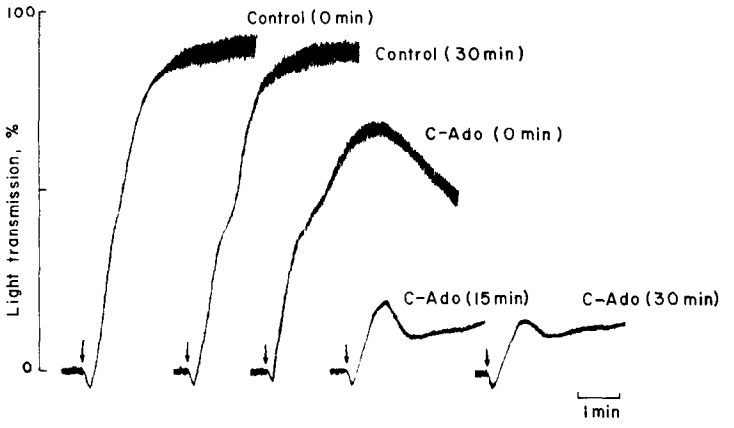

Fig. 5. Inhibition of ADP-induced platelet aggregation by carbocyclic adenosine (C-Ado). Controls (0 and 30 min): ADP (10 μM) was added to PRP before and after 30 min of incubation of PRP at 37°; C-Ado (0 min): ADP (10 μM) and C-Ado (100 μM) were added simultaneously to PRP; and C-Ado (15 and 30 min): PRP was incubated with C-Ado (100 μM) for 15 or 30 min and ADP (10 μM) was added to aggregate platelets.

cin and 2-methylformycin, i.e. comparable to substitutions on the 7 and 8 positions of the purine ring), did not modify the inability of the formycin-type structure to inhibit aggregation. In contrast, strikingly different results were observed in the tubercidin series (7-deazaadenosines). No inhibition of aggregation was observed with the parent compound, tubercidin (Fig. 1). On the other hand (see Table 2), substitutions on positions 5 and 6 (comparable to purine positions 7 and 8) provided analogs with varying capacities to inhibit ADP-induced aggregation. The most potent inhibition was seen with sangivamycin and 6-aminotoyocamycin (67 and 78 per cent inhibition respectively). Only two compounds of the pyrazolo[3,4-*d*]pyrimidine series were available for study. The parent compound (4-APPR) gave moderate inhibition (24 per cent), whereas the 3-carboxamide of this series (a structure that resembles sangivamycin) inhibited ADP-induced aggregation about 65 per cent, i.e. comparable to sangivamycin. 8-Azaadenosine was examined for inhibition of ADP-induced aggregation in the presence of the ADA inhibitor, 2′-deoxycoformycin (1.4 μg/ml), and displayed only moderate inhibition (about 20 per cent). As expected [1], various sugar-modified derivatives (α-D-arabinosyl; β-D-xylosyl) of 8-azaadenosine at 100 μM concentrations had no effect on ADP-induced aggregation. Also, only weak inhibition (6–14 per cent) was observed with several 5′-substituted adenosine analogs (Table 2).

Additional studies on sugar-modified adenosine analogs including carbocyclic adenosine. Earlier studies demonstrated the crucial importance of the stereochemistry of the ribose moiety in the inhibition of ADP-induced aggregation by adenosine and its analogs [1, 24, 25]. Both the 2′ and 3′ carbons of the ribose moiety play key roles since 2′-deoxyadenosine, β-D-arabinosyladenine and 3′-deoxyadenosine (cordycepin) displayed only weak inhibitory activity in comparison with compounds such as adenosine or F-Ado. Also, the C-nucleoside, formycin, which has a normal ribose moiety altered in its stereochemical relationship to the adenine-like moiety (in comparison with adenosine), had negligible inhibitory activity, as did several methylated formycins shown in Table 2. In other studies, we have examined certain additional adenosine analogs (see Table 2) modified in the sugar moiety. Here α-D-ribofuranosyladenine (α-Ado) and α-L-lyxofuranosyladenine did not block ADP-induced aggregation, further emphasizing the importance of the correct stereochemistry of the ribose moiety for the binding of adenosine and its analogs to the ADP-reactive aggregation receptor. However, of the compounds examined, the carbocyclic analog of adenosine (C-Ado) (see Fig. 1), in which the oxygen bridge of the ribofuranosyl ring is replaced by a methylene group, displayed potent inhibition of ADP-induced aggregation (Fig. 5). As noted above (Fig. 2), C-Ado is also incorporated into the platelet nucleotide pools, indicating that it is a substrate for the enzymes necessary to convert adenosine to ATP.

DISCUSSION

These investigations extend our earlier observations [1] of the structure–activity relationships of adenosine, its analogs, and the nucleotide pools to the phenomenon of ADP-induced platelet aggregation. The findings indicate that most, if not all, of the adenosine analogs examined which inhibit platelet aggregation do *not* function through the formation of analog polyphosphate nucleotides or by alteration of the natural adenine nucleotide pools. For example, platelets in which 2-fluoroadenine-containing nucleotides replaced significant amounts of the natural adenine nucleotides in the metabolic pool aggregated normally in response to ADP. On the other hand, the C-nucleoside formycin, which in the presence of 2′-deoxycoformycin (a tight-binding ADA inhibitor), enters the metabolic nucleotide pools in large quantity with marked replacement of the natural adenine nucleotides, did not inhibit aggregation. Furthermore, several adenosine analogs that do not form intracellular nucleotides, or form only small quantities of analog monophosphate nucleotides, are potent aggregation inhibitors, e.g. N^6-ϕ-Ado [1], sangivamycin and 6-aminotoyocamycin (see Table 2). Therefore, findings to date are consistent with the hy-

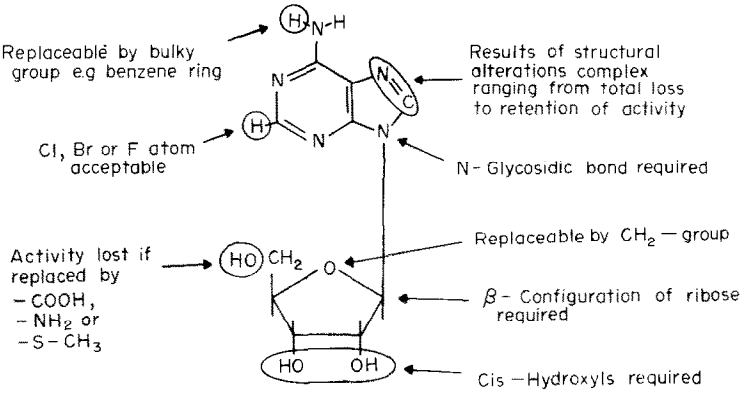

Fig. 6. Some structure–activity relationships of inhibition of ADP-induced platelet aggregation by adenosine analogs.

pothesis that adenosine and its analogs do not inhibit platelet aggregation primarily through effects on polyphosphate nucleotide metabolism. Of course, one cannot rule out the possibility that adenosine analogs will As expected [1], various sugar-modified derivatives (α-D arabinosyl; β-D-xylosyl) of 8-azaadenosine at 100 μM concentrations had no effect on ADP-induced aggregation. Also, only weak inhibition (5–15 per cent) was observed with several 5′-substituted adenosine analogs (Table 2).
be identified that affect platelet functions through the formation of analog polyphosphate nucleotides.

Present evidence suggests that the possible actions on platelets of adenosine and its analogs will prove to be highly complex, with effects occurring at various levels, e.g. on the interaction between ADP and a membrane-bound receptor, on adenylate cyclase and other factors that affect cyclic nucleotide function and metabolism; and on phenomena that result from the formation of "fraudulent" adenine nucleotides.

In earlier studies, the importance of the stereochemistry and conformation of the carbohydrate moiety of adenosine on inhibition of ADP-induced aggregation was emphasized. Further, because compounds such as N^6-φ Ado and 2-Cl-Ado are potent aggregation inhibitors (but do not form analog nucleotides), it appears that substitution of a bulky moiety, e.g. a chlorine atom on C-2, or a large aromatic ring on the 6-NH_2 group, enhances the inhibitory ability [26]. The present study has revealed several interesting structure–activity relationships that involve the imidazole portion of the purine ring. For example, tubercidin (7-deazaadenosine) is devoid of inhibitory activity, suggesting that a nitrogen atom in the 7-position of the purine ring is essential. However, if the 7-nitrogen of adenosine is replaced by a substituted carbon atom, as in sangivamycin or 6-aminotoyocamycin (see Table 2), potent inhibitory activity is observed. Furthermore, in the pyrazolo[3,4-*d*]pyrimidine series, the parent adenosine analog (N-7 replaced by C, C-8 replaced by N) displayed only moderate (about 20 per cent) inhibitory activity, whereas the carboxamide derivative (N-7 replaced by $C{-}\overset{\overset{O}{\|}}{C}{-}NH_2$) displays potent inhibitory activity. It is also of interest that 8-azaadenosine (C-8 replaced by N) is a much weaker inhibitor than adenosine. Some of the more significant structure–activity relationships are illustrated in Fig. 6.

Because of their chemotherapeutic potential, adenosine analogs, especially when used in combination with apotent ADA inhibitor, have been receiving increasing attention. As evidence accumulates on these compounds, it is becoming obvious that they display a wide spectrum of biological activities. Many of these adenosine analogs have marked toxicity. One may not assume, however, that all adenosine analogs are toxic. It is entirely likely that it will be possible to identify or design one or more adenosine analogs or related structures with potent inhibitory activity against ADP-induced platelet aggregation, but with low cytotoxicity. Such compounds could prove useful in the treatment of thromboembolic disease or various disorders of platelet metabolism. It is possible that with increasing knowledge, rational combinations of analogs could be developed. For example, in order to exert cytotoxicity, many adenosine analogs must first be converted to the nucleotide level by the enzyme, adenosine kinase. Thus, the use of an adenosine kinase inhibitor, e.g. N^6-φ-Ado [27] or 5-iodotubercidin [28], might prevent the cytotoxic actions of an adenosine analog, while not impeding its platelet aggregation-inhibitory activity. It is hoped that structure–activity studies such as those described in this and our preceding paper [1] will aid in the identification of useful therapeutic agents.

An important consideration discussed in earlier publications [24, 29–31] is whether adenosine and ADP interact with, and perhaps compete for, the same receptor on the platelet membrane. Evidence in the literature is too limited to permit evaluation of the structure–activity relationships of ADP and it analogs. Competitive inhibition has been described at low concentrations of ADP and adenosine [32]. Also it has been reported [33–35] that 2-Cl-ADP and 2-methylthio-ADP have equal or greater potency than ADP in the induction of aggregation, whereas, 2′-deoxyADP, 3′-deoxyADP, 1-*N*-oxideADP, and α-β-methyleneADP are 100- to 1000-fold less active than ADP. These data, although limited, are in accord with the findings of this paper and are consistent with the hypothesis that adenosine and ADP can interact with the same receptor. Therefore, when additional analog ADP's, e.g. 2-fluoroADP, arabinosyladenine diphosphate, carbocyclic-ADP, and formycin diphosphate, become availa-

ble, it will be of interest to compare the structure–activity relationships of ADP in the induction of aggregation with those of adenosine for blocking this phenomenon. This could yield important further insights into the interactions of these metabolites. Recently, this laboratory has developed methods for the biosynthesis (by washed human erythrocytes), isolation and purification of a variety of analog polyphosphate nucleotides, including some of those cited above *. Thus, it should be possible in the near future for this laboratory to undertake such investigations.

A reasonable alternative or complementary hypothesis is that adenosine inhibits ADP-induced aggregation by stimulating adenylate cyclase, thus increasing the cyclic-AMP levels of the cell [36]. Of considerable interest in this regard are the observations that various adenosine analogs, e.g. F-Ado, formycin and C-Ado, can enter the nucleotide pools in large quantities with replacement of substantial amounts of the metabolic ATP without interfering with ADP-induced aggregation. One wonders whether adenosine cyclic AMP analogs can be formed in platelets, as has been found in mitogen-stimulated lymphocytes [37]. Recently, a report has appeared [38] in which a variety of adenosine analogs (including several of those examined in the present study) were tested for their effects on adenylate cyclase from several tissues, including human blood platelet membranes. Certain of these compounds, including adenosine itself, were stimulatory to platelet adenylate cyclase at low concentrations but inhibitory at higher concentrations. In general, the compounds that stimulated adenylate cyclase are related to those that we and others have found to inhibit ADP-induced aggregation. On the other hand, a number of analogs, including 2′- and 3′-deoxyadenosines, and arabinosyladenine, i.e. analogs of adenosine with modified ribose moieties, inhibited platelet adenylate cyclase, whereas in our studies these compounds did not block ADP-induced aggregation. In the present experiments, however, 10 μM ADP was employed throughout, a concentration selected to induce optimal aggregation. Therefore, it is of considerable interest to determine whether adenosine analogs that do not block ADP-induced platelet aggregation, but which are inhibitors of platelet adenylate cyclase, are capable of potentiating the aggregative activity of ADP through decreases in the steady state concentrations of cyclic AMP.

A recent finding† which supports the concept that adenosine-type compounds may act at sites other than a membrane-bound ADP-receptor, is that arachidonic acid-induced aggregation of platelets is inhibited by F-Ado. In a number of studies [39–43] it has been reported that the addition of agents such as ADP, arachidonic acid, collagen or serotonin to platelets causes a progressive elevation in the level of cyclic GMP with no significant change in the concentration of cyclic AMP. Furthermore, it has been reported that cyclic AMP, or agents that stimulate its synthesis, inhibit aggrega tion [44]. Since these observations are in accord with the hypothesis that, in many biological systems, cyclic AMP and cyclic GMP promote opposing cellular events [45], an important subject for future study is the effect of adenosine analogs, such as those discussed above, on the steady state levels of cyclic AMP and cyclic GMP in human blood platelets as well as examination of possible modifications in the responses of the cyclic nucleotide levels to various substances that trigger platelet aggregation.

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* G. W. Crabtree, C. A. Bell and R. E. Parks, Jr., unpublished results.

† K. C. Agarwal and R. E. Parks, Jr., unpublished results.

REFERENCES

1. K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **24,** 2239 (1975).
2. D. C. Ward and E. Reich, *Proc. natn. Acad. Sci. U.S.A.* **61,** 1494 (1968).
3. D. C. Ward, A. Cerami, E. Reich, G. Acs and L. Altwerger, *J. biol. Chem.* **244,** 3243 (1969).
4. D. C. Ward, W. Fuller and E. Reich, *Proc. natn. Acad. Sci. U.S.A.* **62,** 581 (1969).
5. D. C. Ward, E. Reich and L. Stryer, *J. biol. Chem.* **244,** 1228 (1969).
6. R. P. Agarwal, G. W. Crabtree, K. C. Agarwal, R. E. Parks, Jr. and L. B. Townsend, *Proc. Cancer Res.* **17,** 214 (1976).
7. K. C. Agarwal, R. E. Parks, Jr. and L. B. Townsend. *Fedn Proc.* **61,** 379 (1977).
8. Y. F. Shealy and J. D. Clayton, *J. Am. chem. Soc.* **88,** 3885 (1966).
9. J. A. Montgomery and K. Hewson, *J. Am. chem. Soc.* **82,** 463 (1960).
10. M. Ishizuka, T. Sawa, T. Kayama, T. Takeuchi and H. Umezawa, *J. Antibiot., Tokyo* (Ser. A) **21,** 1 (1968).
11. J. A. Montgomery, S. J. Clayton and W. E. Fitzgibbon, Jr. *J. heterocyclic Chem.* **1,** 215 (1964).
12. R. L. Tolman, R. K. Robins and L. B. Townsend, *J. heterocyclic Chem.* **6,** 215 (1969).
13. G. R. Revankar and L. B. Townsend, *J. chem. Soc.* (C), 2440 (1971).
14. R. L. Tolman, R. K. Robins and L. B. Townsend, *J. heterocyclic Chem.* **8,** 703 (1971).
15. R. A. Earl and L. B. Townsend, *J. heterocyclic Chem.* **11,** 1033 (1974).
16. L.B. Townsend, R. A. Long, J. P. McGraw, D. W. Miles, R. K. Robins and H. Eyring, *J. org. Chem.* **39,** 2023 (1974).
17. K. H. Schram and L. B. Townsend, *J. chem. Soc.*, Perkin I, 1253 (1975).
18. P. R. Brown, *J. Chromat.* **52,** 257 (1970).
19. R. E. Parks, Jr., G. W. Crabtree, C. M. Kong, R. P. Agarwal, K. C. Agarwal and E. M. Scholar, *Ann. N.Y. Acad. Sci.* **255,** 412 (1975).
20. R. E. Parks, Jr. and P. R. Brown, *Biochemistry* **12,** 3294 (1973).
21. G. V. R. Born and M. J. Cross, *J. Physiol., Lond.* **168,** 178 (1963).
22. G. V. R. Born, *Nature, Lond.* **202,** 95 (1964).
23. G. V. R. Born, R. J. Haslam, M. Goldman and R. D. Lowe, *Nature, Lond.* **205,** 678 (1965).
24. S. Clayton, G. V. R. Born and M. J. Cross, *Nature, Lond.* **200,** 138 (1963).
25. G. V. R. Born, *XII Cong. Interl. Soc. Hemat. N.Y., U.S.A.* (plenary session papers), 95 (1968).
26. K. Kikugawa, H. Suehiro and M. Ichino, *J. med. Chem.* **16,** 1381 (1973).
27. A. Y. Divekar and M. T. Hakala, *Molec. Pharmac.* **7,** 663 (1971).

28. J. F. Henderson, A. R. P. Paterson, I. C. Caldwell, B. Paul, M. F. Chan and K. F. Lau, *Cancer Chemother. Rep.* **3** (Part 2), 71 (1972).
29. G. V. R. Born and M. J. Cross, *J. Physiol., Lond.* **166,** 29 (1962).
30. E. W. Salzman, T. P. Ashford, D. A. Chambers, L. L. Neri and A. P. Dempster, *Am. J. Physiol.* **217,** 1330 (1969).
31. D. C. B. Mills and D. E. Macfarlane, in *Platelets in Biology and Pathology* (Ed. J. I. Gordon), p. 159. North-Holland, Amsterdam (1976).
32. G. V. R. Born, *J. Physiol., Lond.* **209,** 487 (1970).
33. A. Gaarder and S. Laland, *Nature, Lond.* **202,** 909 (1964).
34. G. Gough, M. H. Maguire and F. Penglis, *Molec. Pharmac.* **8,** 170 (1972).
35. H. Horak and P. G. Barton, *Biochim. biophys. Acta* **373,** 471 (1974).
36. R. J. Haslam and G. M. Rosson, *Molec. Pharmac.* **11,** 528 (1975).
37. T. P. Zimmerman, J. L. Rideout, G. Wolberg, G. S. Duncan and G. B. Elion, *J. biol. Chem.* **251,** 6757 (1976).
38. C. Londos and J. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **74,** 5482 (1977).
39. R. J. Haslam, in *Platelet Function, in Biochemistry and Pharmacology of Platelets* (Ed. J. Knight), p. 121. Elsevier, Amsterdam (1975).
40. T. Davies, M. M. L. Davidson, M. D. McClenagham, A. Say and R. J. Haslam, *J. Thromb. Res.* **9,** 387 (1976).
41. N. D. Goldberg and M. K. Haddox, *A. Rev. Biochem.* **46,** 823 (1977).
42. D. B. Glass, J. M. Gerrard, D. Townsend, D. W. Carr, J. G. White and N. D. Goldberg, *J. Cyclic Nucleo. Res.* **3,** 37 (1977).
43. K. C. Agarwal and M. Steiner, *Biochem. biophys. Res. Commun.* **69,** 962 (1976).
44. E. W. Salzman and L. Levine, *J. clin. Invest.* **50,** 131 (1971).
45. N. D. Goldberg, R. F. O'Dea and M. K. Haddox, in *Advances in Cyclic Nucleotide Research* (Eds. P. Greengard and G. A. Robinson), Vol. 3, p. 155. Raven Press, New York (1973).