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ADENOSINE ANALOGS AND HUMAN PLATELETS

EFFECTS ON NUCLEOTIDE POOLS AND THE AGGREGATION PHENOMENON*

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Abstract—The interaction between human blood platelets and adenosine and various adenosine analogs was examined. Effects were found both on the pools of nucleotides, as examined by the technic of high pressure liquid chromatography, and on the phenomenon of ADP-induced platelet aggregation. Although the normal ratio of ATP and ADP in platelets is about 1·5:1, after incubation with adenosine-8-^{14}C, ATP and ADP were labeled in a ratio of about 7:1. This is consistent with a distribution of the nucleotides among storage and metabolic pools, with the adenosine-8-^{14}C entering principally the metabolic pool. After 2 hr of incubation with 0·5 mM 2-fluoroadenosine (F-Ado), the concentration of F-ATP was approximately 12 μmoles/10^{11} platelets. The ratio of F-ATP to F-ADP was approximately 7:1, indicating that it entered primarily the metabolic nucleotide pool. Also, during the first hr of incubation, as the F-ATP concentration increased, the ATP concentration fell. When F-ATP-containing platelets were treated with thrombin, an aggregator and storage granule releaser, the nucleotides released into the medium consisted principally of ATP and ADP in a ratio of about 0·8:1, with very little 2-fluoroadenine-containing nucleotides. After thrombin treatment, the washed platelet pellet contained most of the 2-fluoroadenine nucleotides, but with significant increases in the concentrations of F-AMP and F-ADP. This suggests that F-ATP can replace ATP as the energy donor for the aggregation and release phenomena.

As reported elsewhere, adenosine strongly inhibits platelet aggregation induced by ADP. However, this inhibitory effect disappears after preincubation for about 30 min. If the preincubation is carried out in the presence of coformycin, a tight-binding inhibitor of adenosine deaminase ($K_i \cong 1 \times 10^{-10}$ M), the inhibition of aggregation by adenosine is markedly prolonged, indicating that the loss of inhibition results from conversion of adenosine to inosine by adenosine deaminase. ADP-induced aggregation is powerfully inhibited by F-Ado, and the inhibition becomes more pronounced on prolonged incubation. This is consistent with the observation that F-Ado has very weak substrate activity with adenosine deaminase. The analog, N^6-phenyladenosine, an inhibitor of adenosine kinase that does not form analog nucleotides in platelets, inhibits aggregation strongly, and the inhibition is maintained during incubation of 1 hr. Several other adenosine analogs only weakly inhibit ADP-induced aggregation even in the presence of coformycin. These include 2′-deoxyadenosine, 3′-deoxyadenosine (cordycepin), arabinosyladenine, and formycin A, a C-nucleoside. However, significant quantities of nucleotides of formycin A are formed in platelets in the presence of coformycin.

In comparison with human erythrocytes and leukocytes, human blood platelets contain greater concentrations of adenine nucleotides [1,2] which are distributed between metabolic and storage pools [3–6]. Platelet aggregation can be initiated by low concentrations (1–10 μM) of adenosine diphosphate (ADP) as well as by a variety of other substances such as collagen, epinephrine or thrombin [7]. During the induction of aggregation by an agent such as thrombin, the nucleotide storage pools (principally ADP) as well as amines such as 5-hydroxytryptamine are released into the plasma [8,9]. Aggregation can be inhibited by AMP but more effectively by adenosine (Ado) [10,11]. Earlier reports indicated that 2-chloroadenosine also inhibits ADP-induced aggregation but that several other Ado analogs showed little activity [11].

This paper describes the effects on platelets of incubation with various adenosine analogs such as 2-fluoroadenosine (F-Ado), N^6-phenyladenosine (N^6-ϕ-Ado), formycin A and analogs that have variations in the sugar moiety, such as 2′-deoxyadenosine, 3′-deoxyadenosine (cordycepin) and arabinosyladenine. The responses to the adenosine-like compounds were examined in the presence and absence of coformycin, a tight-binding inhibitor of adenosine deaminase (ADAase) [12]. The adenosine analogs affected: (1) the composition of the platelet nucleotide pools, (2) ADP-induced platelet aggregation and (3) the release of nucleotides from the storage granules in response to thrombin treatment. These studies have been greatly facilitated by use of the technic of high pressure liquid chromatography [13]. An excellent review of the earlier work on platelet functions has been published [14]. Preliminary reports of portions of this work have been presented [15,16].

MATERIALS AND METHODS

The chemicals employed in this study were all of the highest grade of purity commercially available.

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Ado and ADP were purchased from P–L Biochemicals. Adenosine-8-^{14}C (50 mCi/m-mole) was obtained from Schwarz/Mann. Adenylate kinase, creatine kinase, phosphocreatine, 2′-deoxyadenosine and 3′-deoxyadenosine (cordycepin) were obtained from Sigma Chemical Co. F-Ado and 9-β-D-arabinofur-anosyladenine (arabinosyladenine) were obtained through the courtesy of Dr. Harry B. Wood, Jr., from the Drug Development Branch, Division of Cancer Treatment, of the National Cancer Institute. F-Ado was synthesized by Dr. John Montgomery [17]. Formycin A and coformycin were kindly supplied by Dr. Hamao Umezawa of the Institute of Microbial Chemistry, Tokyo [18,19] and N^6-ϕ-Ado by Dr. M. H. Fleysher of Roswell Park Memorial Institute, Buffalo, N.Y.

Isolation of platelets

Platelet-rich plasma (PRP) was obtained from fresh normal human blood (500 g) and collected in 75 ml acid-citrate-dextrose (ACD, U. S. P. formula A), by centrifugation at 4500 rev/min (5230 g) for 3·5 min. PRP was centrifuged again at 1500 rev/min (580 g) for 1·5 min to remove contaminating erythrocytes and leukocytes. To obtain the platelet pellet, an additional 0·15 vol. ACD was added to the PRP which then was centrifuged at 5000 rev/min (6460 g) for 10 min.

Aggregation studies

The aggregation studies were performed at 37° in citrated platelet-rich plasma. Platelet aggregation was measured by a technic based on the turbidometric method of Born [20] as described by Mustard and Glynn [21]. This method employs an aggregometer (Chrono-log, model 300) attached to a recorder. The extent of aggregation was estimated by the percentage of increase in light transmission (plasma freed of cells by centrifugation was used as the blank to represent 100 per cent light transmission) in 4 min after addition of the aggregating agent, ADP (20 μM).

Platelet incubation and extraction procedures

After centrifugation, as noted above, the platelet pellets were washed once with isotonic NaCl solution (154 mM) containing glucose (10 mM). The washed platelets were suspended in medium A, which consisted of phosphate buffer, 10 mM (pH 7·4); NaCl, 128 mM; $MgCl_2$, 1 mM; and glucose, 10 mM. Ado or the analogs was added in the appropriate concentrations, and the incubations were carried out in a Dubnoff shaking water bath at 37°. Acid-soluble extracts were prepared by dropwise addition of the platelet suspension into an equal volume of ice-cold perchloric acid (6%) stirred rapidly on a Vortex mixer. After centrifugation, aliquots of the supernatant fluid were neutralized with K_2CO_3 and precipitated $KClO_4$ was removed by centrifugation. The supernatant fluid was stored frozen until employed for the assay of nucleotides by high pressure liquid chromatography.

Release of nucleotides from the storage pool

Washed human platelets suspended in isotonic medium A were incubated with F-Ado (1·0 mM) at 37°. After incubation for 2 hr, thrombin (5 units/ml of suspension) was added, the mixture was incubated for an additional 5 min and then centrifuged at 3000 rev/min (2300 g) for 15 min at 4°. Nucleotides were extracted with perchloric acid (PCA) both from the supernatant fluid, which contains the released material (the storage pool), and from the pellet, which contains the metabolic pool of nucleotides. In the latter case, the pellet was first washed with isotonic medium A.

In the case of control platelet suspensions (not incubated with F-Ado), 1 ml of the washed platelet suspension was incubated with thrombin (5 units) for 5 min at 37°, followed by extraction of the supernatant fluid and pellet with perchloric acid as above.

Nucleotide analysis

Nucleotide profiles of the neutralized PCA extracts were determined by employing high pressure liquid chromatography (Varian LCS-1000) as described previously [13]. Nucleotide concentrations were estimated by comparing the peak areas with those of known nucleotide standards. Measurements of absorbancy at wavelengths in addition to 254 nm were made by passing the effluent of the Varian LCS-1000 column through a model SF 770 Spectroflow Monitor (Schoeffel Instrument Co.) set at the selected wavelength.

RESULTS

Studies of nucleotide pools

Incubation of platelets with adenosine. Figure 1a shows a typical high pressure liquid chromatogram of a PCA extract of human blood platelets. Several features distinguish the nucleotide profiles of the platelets from those of most other tissues. The total quantity of platelet adenine nucleotides is much greater than normally found in the other formed elements of human blood, i.e. erythrocytes or leukocytes. The most striking characteristic is the ratio of ATP to ADP, usually in the range of 5:1 to 10:1 in most normal tissues, which in platelets is of the order of 1·5:1. This is explained by the relatively large content of ADP in the storage granules of platelets. Also to be noted is the platelet ratio of GTP:GDP of approximately 1:1 rather than a value in the range of 5:1 to 10:1 usually found in most tissues. Another characteristic of nucleotide profiles of human platelets is the small peak of ultraviolet absorbing material (as yet unidentified) that emerges about 10 min after the GTP peak (Fig. 1). Attempts are currently being made to isolate sufficient quantities of this unidentified material for identification.

Figure 1b illustrates the effect of incubating human platelets for 1 hr with 0·5 mM Ado. No qualitative effects are seen in the nucleotide profile; however, the size of the ATP peak is significantly increased. This finding is in striking contrast to the effects of incubating normal human erythrocytes with Ado, where the adenine nucleotides are little affected. Rather a large new peak of inosinic acid (IMP) forms in the mononucleotide region. This emphasizes the role of ADAase in Ado metabolism in human erythrocytes [22]. Apparently, the enzymic mechanisms for rapidly converting Ado to IMP, so prominent in human erythrocytes, do not occur in human blood platelets.

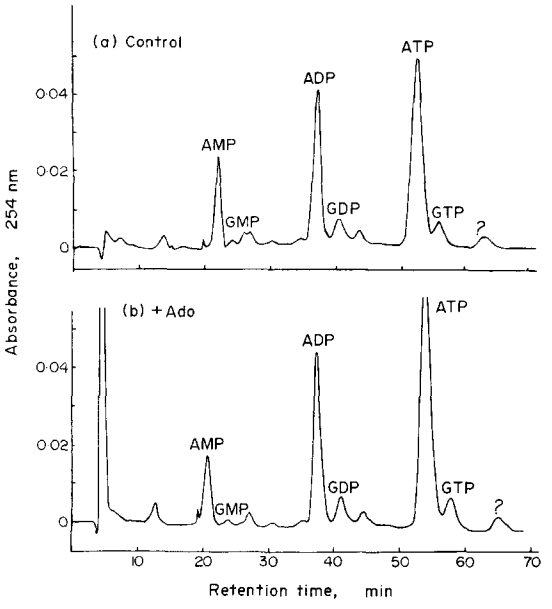

Fig. 1. High pressure liquid chromatographic nucleotide profiles of human platelets before and after incubation with adenosine. Washed human platelets were suspended in medium A (see Materials and Methods). Ado was added to a final concentration of 0·5 mM to the platelet suspension (2–3 × 10^9 platelets/ml), and the reaction mixture was incubated for 1 hr at 37°. After incubation, aliquots of the platelet suspension were added drop-wise to an equal volume of ice-cold 6% perchloric acid stirred rapidly on a Vortex mixer. After centrifugation, an aliquot of the supernatant fluid was neutralized by K_2CO_3 and 20-μl aliquots of the neutralized fluid were subjected to high pressure liquid chromatography. (a) Chromatogram before Ado addition (control); (b) chromatogram after 1 hr of incubation with Ado.

Figure 2 presents the results of incubating platelets with adenosine-8-^{14}C for 1 hr. After incubation, high pressure liquid chromatography was performed with concurrent collection of fractions for the measurement of radioactivity. It is seen that only four peaks of radioactivity emerged: that of unreacted adenosine-8-^{14}C, a small peak at about 20 min coincident with

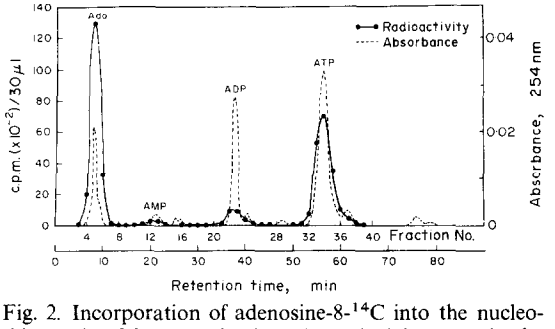

Fig. 2. Incorporation of adenosine-8-^{14}C into the nucleotide pools of human platelets. A washed human platelet suspension in medium A (0·8 ml) was incubated with 0·2 ml adenosine-8-^{14}C (2 μCi, sp. act., 50 mCi/m-mole) for 1 hr. After incubation, PCA extracts were prepared as in Fig. 1, and subjected to high pressure liquid chromatography; fractions were collected and radioactivity was measured by liquid scintillation counting. Both absorbancy and cpm are plotted vs retention time.

the AMP peak, a somewhat larger peak coincident with ADP, and a large peak of radioactivity in the position of ATP. Significantly, the unidentified peak that follows GTP (Fig. 1) contained no radioactivity. It is important to note that the ratio of labeled ATP to ADP is approximately 7:1 in contrast to the normal ATP:ADP ratio measured by absorbance of about 1·5:1. An explanation consistent with these findings is that the adenosine-8-^{14}C entered primarily the metabolic rather than the storage pool of nucleotides. These observations confirm those reported earlier by other laboratories [3–6]. It is possible that with prolonged incubation under similar conditions the ratio of ATP:ADP, as measured by radioactivity, would fall progressively, affording a method for measuring the transfer of adenine nucleotides from the metabolic pool into the storage pool. Also, as noted below, if such a nucleotide transfer does occur, one might expect to observe a progressive increase in the amount of labeled ADP expelled into the plasma by triggering granule release with an agent such as thrombin.

Formation of nucleotides of 2-fluoroadenosine. As noted in earlier reports from this laboratory, the Ado analog, F-Ado, is a valuable biochemical tool for the study of adenosine metabolism [22]. F-Ado has very weak, if any, substrate activity with adenosine deaminases (the V_{max} is approximately 0·01 per cent of that with Ado with human placental ADAase [23]), but reacts readily with various adenosine kinases [22,24,25]. Furthermore, the 2-fluoroadenine nucleotides formed are good substrates for several of the nucleotide kinases that normally react with adenine nucleotides [22]. Another great advantage is that 2-fluoroadenine nucleotides such as F-ATP separate cleanly from the normal adenine nucleotides in high pressure liquid chromatographs and are both readily recognizable and measurable [22].

Figure 3 presents the results of a time study of F-Ado incorporation into human platelets. The chromatographic elution system and the column employed in this experiment yielded nucleotide profiles that are somewhat different from those employed previously by this laboratory, e.g. ATP emerges at about 42 min (rather than at 55–60 min) and F-ATP emerges at about 47 min (rather than at 65–70 min). The F-ATP peak first appeared after 5 min of incubation and increased progressively until after 2 hr the F-ATP peak was several times greater than the normal ATP peak. Furthermore, the progressive formation of a peak of F-ADP occurred at an elution time of 28 min on the chromatograms. Significantly, the ratio of F-ATP:F-ADP is greater than 5:1 which indicates that the F-Ado entered primarily the metabolic nucleotide pool rather than the storage pool.

Figure 4 presents graphically the effects on the nucleotide concentrations of incubation of platelets with F-Ado. The nucleotide concentrations were estimated by comparing the areas under each peak with those of standard nucleotides. Since 1 × 10^{11} platelets are approximately equal to 1 ml or 1 g, the adenine nucleotide levels of platelets are 4- to 5-fold greater than those observed in normal human erythrocytes. The rate of F-ATP formation was approximately linear during the 2 hr of incubation with no evidence of a decrease in the synthetic rate at the termination

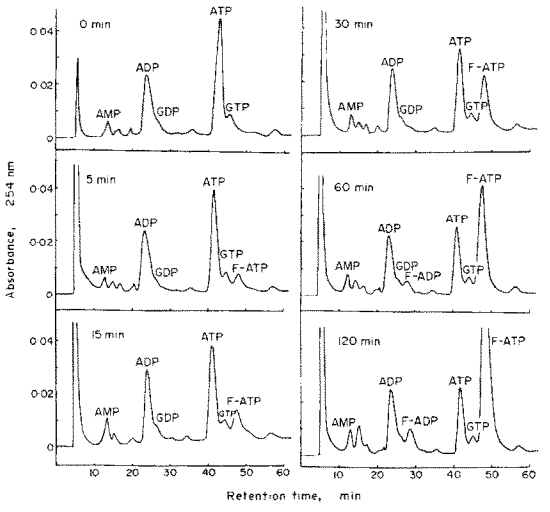

Fig. 3. Effect of 2-fluoroadenosine on the nucleotide pools of human platelets. Washed human platelets (2–3 × 10^9/ml) were suspended in medium A and incubated at 37° with 0·5 mM 2-fluoroadenosine. Aliquots were removed after incubation of 5, 15, 30, 60 and 120 min and added to equal volumes of ice-cold 6% per chloric acid as described in the legend of Fig. 1. The neutralized extracts were subjected to high pressure liquid chromatography.

of the experiment. However, during the first hr of incubation as the F-ATP peak increased in size, the ATP peak decreased, suggesting that, in the metabolic pool, ATP became replaced progressively by the analog, F-ATP. This is in striking contrast to findings with human erythrocytes, where the normal adenine nucleotide concentrations were essentially unaffected during the formation of very large quantities of F-ATP or of GTP [16,22]. It should be of interest to perform additional experiments with larger quantities of F-Ado incubated for longer periods of time in order to determine the full extent of F-ATP synthesis possible in this tissue. Note that the rate of F-ATP

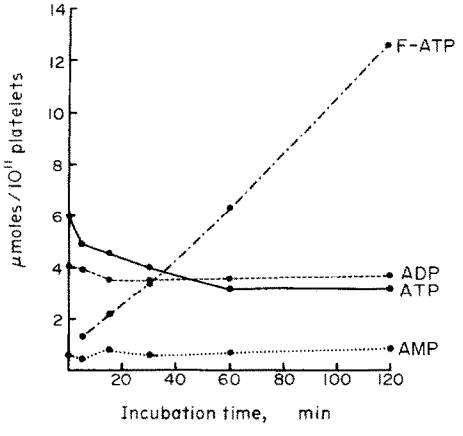

Fig. 4. Rate of formation of 2-fluoroadenosine-5′-triphosphate (F-ATP) and the effect of this synthesis on normal levels of nucleotides in human platelets. The nucleotide concentrations of Fig. 3 were estimated by comparing the peak areas with those of known nucleotide standards.

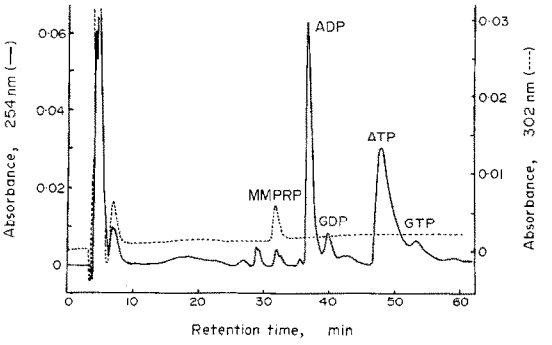

Fig. 5. Incorporation of 6-methylmercaptopurine ribonucleoside (MMPR) into the nucleotide pools of human platelets. Washed human platelets were suspended in medium A and incubated at 37° with 0·5 mM MMPR. After 1 hr of incubation, an acid-soluble extract was prepared as described in the legend of Fig. 1. Twenty μl of the neutralized extract was analyzed on high pressure liquid chromatography. The ratio between ATP and ADP peak areas is about 1·4:1. Column effluents were monitored at 254 and 302 nm. The latter wavelength was used to eliminate interference by natural nucleotides and to show specificity for MMPR nucleotides.

synthesis is about 6 μmoles/g of tissue/hr or about 0·1 μmol/min/g of platelets which is 3- to 4-fold greater than the rate of F-ATP synthesis observed in human erythrocytes [22].

Effect of various adenosine analogs on nucleotide pools. Many analogs of Ado have been synthesized and tested for antitumor, immunosuppressive, antiparasitic and antiviral activity. Preliminary experiments have been performed with a few of these Ado analogs with human blood platelets. Figure 5 shows the effect of the nucleotide profile of incubation platelets with 6-methylmercaptopurine ribonucleoside (MMPR) which in many tissues behaves as an Ado analog. In this experiment, the eluent in high pressure liquid chromatography was monitored at two wavelengths, i.e. 254 and 302 nm. Although MMPR and its nucleotides have substantial absorbancy at 302 nm, no peaks are observed at this wavelength in the nucleotide profiles of normal platelets. It may be seen that in platelets, as in erythrocytes [26,27], MMPR is incorporated readily into the nucleotide pools with accumulation of the 5′-monophosphate nucleotide, 6-methylmercaptopurine ribonucleoside-5′-phosphate (MMPRP). This finding indicates that the adenosine kinase of human platelets is capable of reacting with MMPR as a substrate. The accumulation of MMPR at the 5′-monophosphate nucleotide level indicates that MMPRP has very weak or no substrate activity with the adenylate kinase of platelets.

Recent studies in this laboratory have shown that the antibiotic, formycin A, which is a C-nucleoside analog of Ado, formed only trace amounts of nucleotides in human erythrocytes [28] despite the fact that, in a number of other tissues, formycin A nucleotides form readily and enter the nucleic acids [29–31]. Formycin A is subject to very rapid deamination by erythrocytic adenosine deaminase to form the inosine analog, formycin B [12] which is not phosphorylated by human erythrocytes. However, when formycin A was incubated with human erythrocytes in the presence of the tight-binding adenosine deaminase inhibitor,

coformycin, nucleotides of formycin A formed rapidly. These were readily detected by high pressure liquid chromatography with measurement at their specific absorbance maximum of 295 nm. In agreement with these earlier findings with erythrocytes, when formycin A was incubated with human platelets, only trace amounts of the nucleotides were detected. However, when similar incubations were performed in the presence of coformycin, large quantities of the mono-, di- and triphosphate nucleotides of formycin A were readily demonstrated. In agreement with the findings of Figs. 2 and 7a, the ratio of triphosphate to diphosphate nucleotides of formycin A was approximately 7 to 1, which indicates that these analog nucleotides entered the metabolic nucleotide pool. These observations suggest that the enzyme, ADAase, may play a role in the regulation of adenine nucleotide metabolism in human platelets.

Inhibition of F-ATP synthesis by N^6-phenyladenosine. The analog, N^6-ϕ-Ado, is a potent and relatively specific inhibitor of the enzyme, adenosine kinase [32], as shown in experiments with the isolated enzyme and with intact erythrocytes [22]. Upon incubation of human platelets with N^6-ϕ-Ado (0·5 mM) for 2 hr no new nucleotide formation was observed by high pressure liquid chromatography. Also in agreement with the behavior of human erythrocytes [22], when human platelets were incubated for varying time periods up to 2 hr with a mixture of 0·5 mM F-Ado and 0·5 mM N^6-ϕ-Ado, the rate of F-ATP accumulation in the platelets was inhibited approximately 65 per cent. This indicates that, as with the isolated enzyme and as in other tissues [32], N^6-ϕ-Ado is capable of inhibiting adenosine kinase in intact human platelets. Significantly, as discussed below, this adenosine analog is a strong inhibitor of the aggregation of human platelets induced by addition of ADP.

Effect of thrombin on the nucleotide pools of normal platelets. Figure 6 and Table 1 show the distribution of nucleotides released into the medium from the storage granules of normal platelets upon the addition of thrombin. Figure 6a presents a platelet nucleotide profile prior to thrombin addition which is typical of the nucleotide patterns normally seen with platelets. After the addition of thrombin, removal of the platelets by centrifugation and extraction of the supernatant fluid as described in Methods, the nucleotide profile of Fig. 6b was obtained. It is seen that a large quantity of ADP and a relatively smaller amount of ATP were released into the medium. Interestingly, the unidentified peak that appears about

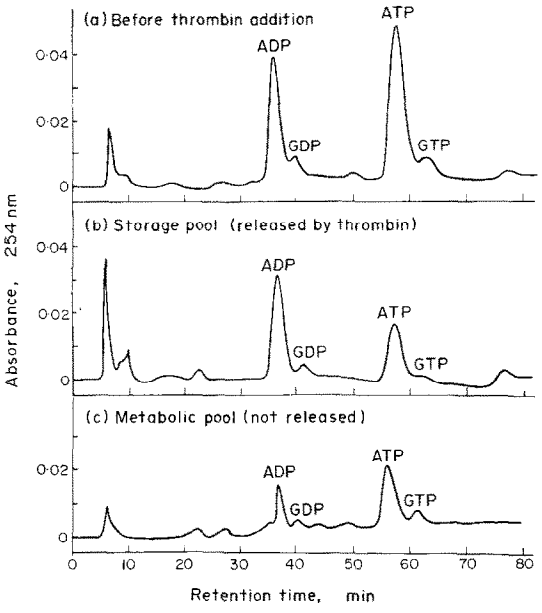

Fig. 6. Nucleotide profiles of normal human platelets before and after the addition of thrombin. Chromatograms of a PCA extract prepared from (a) the suspension of washed normal human platelets before addition of thrombin; (b) the supernatant fluid after addition of 5 units of thrombin and centrifugation; and (c) the platelet pellet after thrombin addition (5 units) and centrifugation. The platelet pellet was washed once by resuspension in medium A and centrifugation before preparation of the PCA extract.

10 min after GTP in the chromatograms (e.g. Fig. 1) is also released into the medium, suggesting that this unidentified substance is associated with the storage granules. Guanine nucleotides are also released into the medium. Figure 6c presents the nucleotide profile of the sedimented platelets after release of the contents of the storage granules and washing by centrifugation. The ratio of ATP:ADP in the platelet pellet (3·6:1) approaches but is lower than the ratios observed in most normal tissues. It should be appreciated that the apparently decreased (3·6:1) ratio of ATP:ADP (compared with the 7:1 ratio of ATP-^{14}C:ADP-^{14}C of Fig. 2) in the platelet pellet after thrombin treatment may reflect a decreased energy charge resulting from the consumption of high energy phosphate that occurs during the granules release and aggregation phenomena. This finding is consistent with results reported by other laboratories [3,6,8].

Table 1. Storage and metabolic pools of nucleotides in human platelets

	ADP	GDP	ATP	GTP	ATP/ADP	GTP/GDP
	(μmoles/10^{11} platelets)				(molar ratio)	
Before thrombin addition	3·5	0·9	5·1	1·0	1·5	1·1
Released by thrombin (storage pool)	3·0	0·5	1·9	0·3	0·6	0·6
Not released by thrombin (metabolic pool)	0·5	0·3	1·8	0·5	3·6	1·7

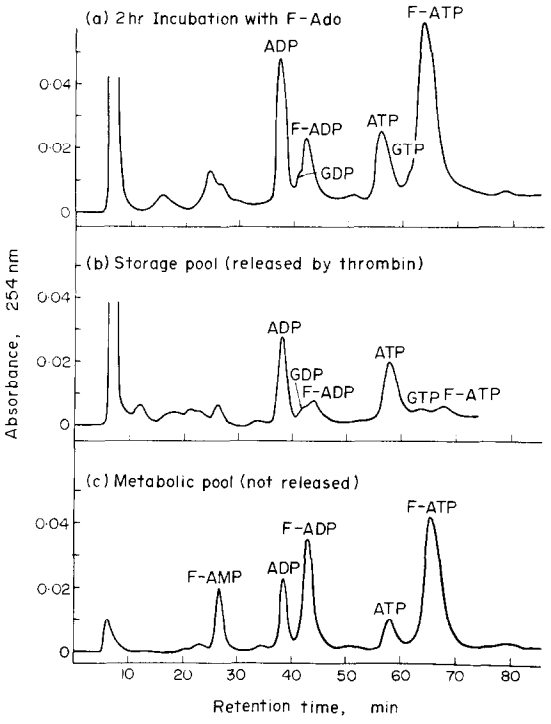

Fig. 7. Nucleotide profiles of 2-fluoroadenosine-treated human platelets before and after addition of thrombin. Chromatograms of a PCA extract prepared from (a) a suspension of washed human platelets after incubation with 1 mM F-Ado for 2 hr at 37°; (b) the supernatant fluid after the addition of 5 units of thrombin to the platelet suspension and centrifugation; and (c) the platelet pellet remaining after addition of thrombin and centrifugation. The platelet pellet was washed once with medium A before preparation of the PCA extract.

Figure 7 and Table 2 show the effects of thrombin treatment on platelets that have been preincubated with F-Ado for 2 hr. In Fig. 7a are seen the large peaks of F-ATP and F-ADP in a ratio of approximately 7:1 with a concomitant marked decrease in the level of ATP, resulting in an ATP:ADP ratio of about 0·8. Upon treatment with thrombin, the nucleotides released into the medium consist principally of ADP and ATP (Fig. 7b) with only very small quantities of 2-fluoroadenine nucleotides. On the other hand, high pressure liquid chromatography of the washed platelet pellet after treatment with thrombin reveals that the 2-fluoroadenine nucleotides have remained principally within the platelet. Furthermore, there is an apparent downward shift in energy charge so that the ratio of F-ATP:F-ADP is decreased from 7:1 to about 2·4:1. In addition, there is an increase in the peak of F-AMP. These observations indicate that the 2-fluoroadenine nucleotides formed during the 2 hr of incubation were incorporated principally into the metabolic nucleotide pool. Secondly, the downward shift in energy charge, as indicated by the increased concentrations of F-AMP and F-ADP, suggests that F-ATP is capable of efficiently replacing ATP in the energy-consuming reactions concerned with the aggregation and release phenomena after thrombin addition.

Figure 8 presents the result of a "peak-shift" experiment designed to characterize the nucleotides of Fig. 7c. An aliquot of the extract of Fig. 7c was incubated with crystalline rabbit muscle myokinase (adenylate kinase), crystalline rabbit muscle creatine kinase and an excess of creatine phosphate. Both of these enzymes are highly specific for adenine nucleotides or adenine-like nucleotides. After incubation, the peaks labeled F-AMP, ADP and F-ADP in Fig. 7c have disappeared and only ATP and F-ATP remain (Fig. 8), indicating that the mono- and diphosphate nucleotides of both adenine and 2-fluoroadenine were converted quantitatively to triphosphate nucleotides. The very great substrate specificity of the enzymes employed in this peak-shift clearly establishes the identity of the nucleotides in question. It should be

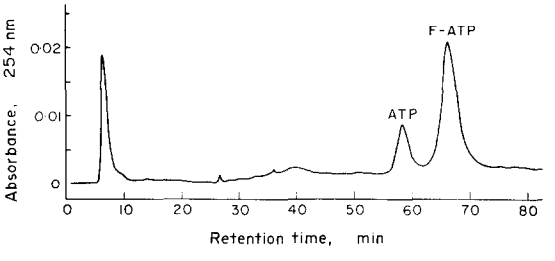

Fig. 8. Characterization of 2-fluoroadenine ribonucleotides (F-AMP, F-ADP and F-ATP) by an enzymic peak-shift technic. An aliquot (0·2 ml) of the neutralized PCA extract employed in Fig. 7c was incubated at room temperature for 10 min with 0·2 ml of 50 mM phosphate buffer (pH 7·5) containing crystalline adenylate kinase (5 units), creatine kinase (10 units), 20 mM creatine phosphate and 2 mM $MgCl_2$. The reaction was stopped by adding 0·2 ml PCA (6%) and the extract was neutralized by K_2CO_3. An aliquot was analyzed by high pressure liquid chromatography.

Table 2. Distribution of the analog nucleotides in storage and metabolic pools after incubation of human platelets with 2-fluoroadenosine for 2 hr

	ADP	F-ADP	ATP	F-ATP	ATP/ADP	F-ATP/F-ADP
		(μmoles/10^{11} platelets)			(molar ratio)	
Before thrombin addition	3·9	1·4	3·1	9·5	0·8	6·8
Released by thrombin (storage pool)	3·0	0·7	2·4	0·8	0·8	1·1
Not released by thrombin (metabolic pool)	1·5	2·3	1·0	5·6	0·7	2·4

noted that nucleosides and nucleotides of 2-fluoroadenine have little or no substrate activity with the deaminating enzymes, adenosine deaminase and 5′-AMP deaminase and, therefore, are incapable of degradation to inosine-type compounds as is the case with normal adenine nucleotides. This may explain the relatively large peak of F-AMP seen in Fig. 7c whereas no comparable peak of AMP occurs in the nucleotide profile of Fig. 6c.

Inhibition of platelet aggregation by adenosine and several adenosine analogs

Inhibition of ADP-induced platelet aggregation. Many substances, including such diverse agents as epinephrine, collagen, thrombin and adenosine diphosphate, can induce aggregation in human blood platelets, and this phenomenon is readily studied by measuring the increase in the light transmission of PRP at a visible wavelength such as 609 nm [21]. In most of the studies described below, platelet aggregation was induced by the addition of ADP at a final concentration of 20 μM to PRP. The results of typical experiments are shown in Fig. 9, which illustrates the rapid increase in light transmission that is initiated almost immediately after the addition of 20 μM ADP. If the PRP is preincubated with 10 μM Ado for 5 min prior to the addition of ADP, marked inhibition of aggregation occurs. However, if the preincubation with Ado is prolonged for 30 min, inhibition of aggregation by Ado is abolished. On the other hand, if the preincubation with Ado is carried out in the presence of the tight-binding ADAase inhibitor, coformycin [12], the inhibition by Ado is retained throughout the 30-min preincubation period. Also shown in Fig. 9 is the inhibition of aggregation induced by the Ado analog, F-Ado. As discussed above, F-Ado is not significantly deaminated by ADAase [12], but serves as a substrate for adenosine kinase and readily enters the nucleotide pools of human platelets (Fig. 3) as well as other cells [22,24]. Here it is seen that 10 μM

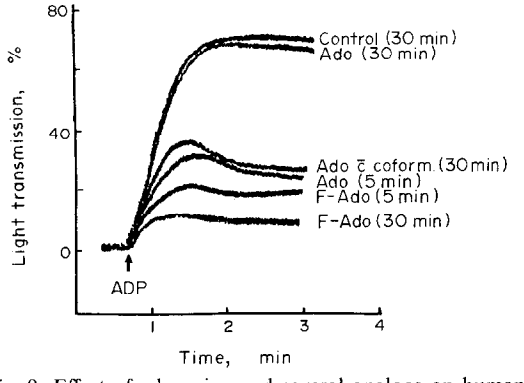

Fig. 9. Effect of adenosine and several analogs on human platelet aggregation induced by ADP. Fresh PRP was incubated at 37° for 5 or 30 min with 10 μM adenosine (Ado) or 10 μM adenosine plus 1·6 μg coformycin (Ado c̄ coform.) or 10 μM 2-fluoroadenosine (F-Ado). After the selected incubation period, 20 μM ADP was added to induce platelet aggregation and the increase in light transmission was recorded using an aggregometer (Chrono-log model 300) attached to a recorder. Zero per cent transmission is that of PRP and 100 per cent transmission is the light transmitted of 609 nm by the platelet-free plasma.

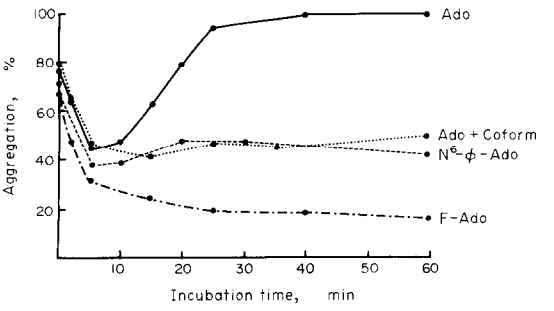

Fig. 10. Effect of adenosine and adenosine analogs on the aggregation of human platelets. Fresh PRP was incubated at 37° with 10 μM adenosine (Ado), 10 μM fluoroadenosine (F-Ado) or 20 μM N^6-phenyladenosine (N^6-ϕ-Ado). Aliquots were removed after various time intervals and subjected to aggregation studies immediately by addition of ADP (20 μM). In the zero time studies, Ado or the analog was added simultaneously with the ADP to PRP to induce aggregation. The results are expressed as the per cent of aggregation compared to that of control samples where the addition of the nucleoside solution to PRP was replaced by an equal volume of normal saline.

F-Ado, after 5 min of preincubation, causes greater inhibition than the equivalent concentration of Ado. Furthermore, when PRP is preincubated with 10 μM F-Ado for 30 min prior to addition of ADP, even greater inhibition of aggregation is observed, which is in striking contrast to the loss of inhibition seen with Ado. These observations emphasize further the importance of enzyme, ADAase, in terminating the inhibitory action of Ado.

The importance of ADAase is illustrated further in Fig. 10, where preincubation of PRP was performed for varying time periods up to 1 hr with 10 μM Ado in the presence and absence of coformycin, with 10 μM F-Ado and with 20 μM N^6-ϕ-Ado. Here it is seen that the inhibition by Ado, which is maximal after 5–10 min of preincubation, disappears completely after 30 min of preincubation. On the other hand, in the presence of coformycin, the inhibition by Ado remains essentially constant during 1 hr of preincubation. The inhibitory activity of N^6-ϕ-Ado is similar to that of Ado plus coformycin. It appears that the inhibition induced by F-Ado is greater than that caused by Ado (in the presence of the ADAase inhibitor, coformycin). This suggests that F-Ado binds more tightly to the aggregation receptor than does Ado or N^6-ϕ-Ado.

Dose–response relationship of inhibition of aggregation by adenosine, 2-fluoroadenosine and N^6-phenyladenosine. As discussed above, N^6-ϕ-Ado is capable of strongly inhibiting ADP-induced platelet aggregation. This compound is especially interesting because it does not enter the nucleotide pools of human erythrocytes [22] or of blood platelets as noted above. Figure 11 shows the inhibition of platelet aggregation caused by varying concentrations of Ado, F-Ado and N^6-ϕ-Ado. Since the preincubation period in this experiment was 30 min, the dose–response to Ado reflects the ADAase reaction and the quantities of Ado that have not yet been deaminated by the enzyme. Although both analogs inhibit the ADP-induced aggregation phenomenon, F-Ado appears more potent on a molar basis. It is noteworthy, however,

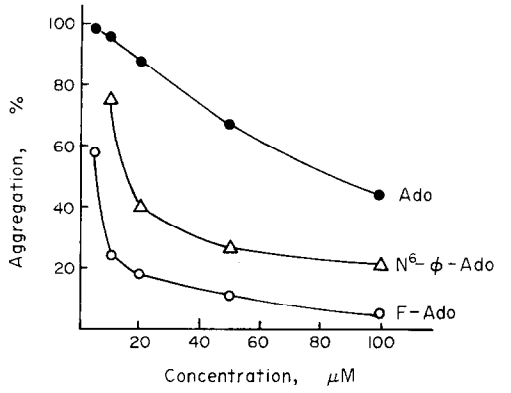

Fig. 11. Effect of adenosine, 2-fluoroadenosine and N^6-phenyladenosine on the aggregation of human platelets. Fresh PRP was incubated at 37° with various concentrations (5–100 μM) of adenosine (Ado), 2-fluoroadenosine (F-Ado) or N^6-phenyladenosine (N^6-ϕ-Ado). After 30 min of incubation, aggregation was induced by addition of ADP (20 μM). The results are expressed as the per cent of aggregation in comparison with that of the control sample as in Fig. 10.

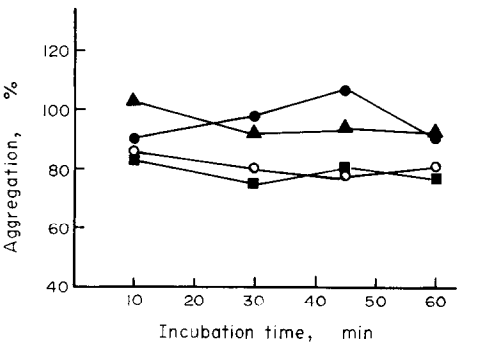

Fig. 13. Effect of various adenosine analogs on the aggregation of human platelets. Fresh PRP was incubated at 37° for 5 min with the adenosine deaminase inhibitor, coformycin (2 μg), followed by the addition of 100 μM of 2′-deoxyadenosine (▲), 3′-deoxyadenosine (■), arabinosyladenine (●) or formycin A (○). Aliquots were removed after various time intervals and subjected to aggregation studies immediately by addition of ADP (20 μM). The results are expressed as the per cent of aggregation compared to control samples as in Fig. 10.

that N^6-ϕ-Ado, which does not become incorporated into platelet nucleotides (see above), is a potent inhibitor of aggregation. This may be considered as support for the hypothesis that the blockade of ADP-induced aggregation by Ado analogs is caused by a reaction with a receptor (probably at or near the cell surface), rather than by interference with nucleotide metabolism through the formation of "fraudulent" nucleotides.

Figure 12 shows the aggregation-inhibitory actions of N^6-ϕ-Ado and F-Ado added either alone or together. No additive or synergistic effects were observed. Of interest is that N^6-ϕ-Ado, by inhibiting adenosine kinase, markedly decreases the rate at which F-Ado nucleotides are synthesized in the platelet as noted above. This is further evidence that the inhibition of aggregation caused by F-Ado is the result of binding to a specific receptor, rather than

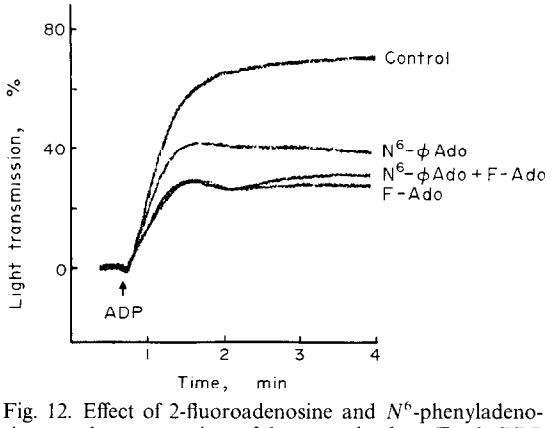

Fig. 12. Effect of 2-fluoroadenosine and N^6-phenyladenosine on the aggregation of human platelets. Fresh PRP was incubated at 37° with 5 μM 2-fluoroadenosine (F-Ado); 10 μM N^6-phenyladenosine (N^6-ϕ-Ado) or 5 μM F-Ado plus 10 μM N^6-ϕ-Ado. After 10 min of incubation, the aggregation was induced by ADP (20 μM), and the increase in light transmission was recorded as in Fig. 9.

through the formation of an analog nucleotide in the cell.

Significance of the ribose moiety of adenosine in the inhibition of ADP-induced platelet aggregation. In contrast to the inhibition of ADP-induced aggregation produced by Ado and several Ado analogs with purine ring substitutions, only weak inhibition of aggregation was caused by 2′-deoxyadenosine, 3′-deoxyadenosine or arabinosyladenine in agreement with results reported elsewhere [11]. Also formycin A caused only slight inhibition of aggregation. However, since these analogs are good substrates for ADAase from human erythrocytes [12], the possibility existed that rapid deamination, to form inactive inosine analogs, might have been responsible for the lack of inhibitory activity. This was shown not to be the case, because when PRP was preincubated with these analogs in the presence of the powerful ADAase inhibitor, coformycin, only weak inhibition of aggregation was observed, as seen in Fig. 13. These findings indicate that the nature of the carbohydrate moiety substituted on the N-9 of the purine ring plays a key role in the inhibition of ADP-induced aggregation and that, by the selection of appropriate Ado analogs (added in combination with an inhibitor of ADAase or adenosine kinase), it may be possible to characterize effectively some of the binding properties of the postulated aggregation receptor.

DISCUSSION

These investigations demonstrate the potential value of both the technic of high pressure liquid chromatography and the use of selected nucleoside analogs for the study of the physiology and biochemistry of human blood platelets. For example, the Ado analog, F-Ado, which has negligible substrate activity with adenosine deaminase but which is readily converted to intracellular nucleotides, has proved especially useful because its nucleotides are readily separable and recognizable by high pressure liquid chromatography [13]. Furthermore, analog nucleotides

such as F-ATP can accumulate to surprisingly high concentrations in the platelet metabolic pool, replacing the natural ATP. When the non-metabolic (storage) pool of nucleotides is released into the medium by addition of thrombin, principally natural nucleotides (mainly ADP) are extruded from the storage granules, whereas the newly formed 2-fluoroadenine nucleotides remain mostly within the platelet. Clearly these technics offer new opportunities for further examination of many facets of platelet nucleotide biochemistry.

In addition to events that occur at the nucleotide level, F-Ado as well as several other Ado analogs can block the aggregation phenomenon induced by the addition of small amounts of ADP. Inhibition of ADP-induced aggregation occurs with the analog, N^6-ϕ-Ado, which is an inhibitor of adenosine kinase and which does not form nucleotides within platelets. Furthermore, the addition of this adenosine kinase inhibitor (which also blocks 2-fluoroadenine nucleotide synthesis) does not interfere with the aggregation-inhibitory effect of F-Ado. Also, the inhibition of ADP-induced aggregation is first detectable within minutes after the addition of F-Ado, i.e. before significant quantities of the analog nucleotides have formed. All of these observations indicate that the inhibition of ADP-induced aggregation by Ado analogs does not result from the formation of analog nucleotides such as F-ATP. In fact, as seen in Fig. 7, platelets that contain large quantities of 2-fluoroadenine nucleotides are capable of an apparently normal response to thrombin addition, i.e. release of the nucleotides from the storage granules.

Particularly interesting are results with the adenosine analog, formycin A, which is a C-nucleoside [18]. Since this analog is a superior substrate for adenosine deaminase in tissues such as erythrocytes [12] and platelets, it may be converted rapidly to the inosine analog before nucleotide formation is possible. However, in the presence of the potent adenosine deaminase inhibitor, coformycin, formycin A readily forms mono-, di- and triphosphate nucleotides which indicates that it is a good substrate for platelet adenosine kinase, and that the nucleotides formed can react readily with platelet nucleotide kinases. On the other hand, formycin A, in relatively high concentrations (100 μM) and in the presence of coformycin, shows only weak inhibition of ADP-induced platelet aggregation. This suggests that the steric factors that distinguish Ado from formycin A [33–36] play a key role in the capacity of the nucleoside to bind with the platelet aggregation-receptor site.

As discussed elsewhere [33], the fact that formycins A and B (the adenosine and inosine analogs, respectively) have a C—C bond rather than the N—C glycosidic bond of nucleosides not only renders them insusceptible to cleavage by enzymes such as nucleoside phosphorylases [37], but also causes a significant difference in the steric relations between the purine-like ring and the ribose. For example, in the formycins, almost completely unhindered rotation of the sugar moiety about the C—C bond is possible, whereas with purine nucleosides the rotation about the nucleosidic linkage is markedly hindered, principally by interaction between C-2′ and its substituents on the ribose and C-8 and its proton on the purine ring. It appears that steric factors such as these may impede the binding of formycin A to the postulated receptor on the platelet.

The nature of the carbohydrate moiety linked to N-9 of the adenine ring appears to play a crucial role in the inhibition of ADP-induced aggregation. Since many adenosine analogs are substrates for adenosine deaminase [12], it is necessary to examine such analogs in the presence of a potent ADAase inhibitor such as coformycin. Interestingly, only weak inhibition was seen with 2′-deoxyadenosine, 3′-deoxyadenosine and arabinosyladenine, which indicates an important role (perhaps in inhibitor binding) for the hydroxyl groups in the normal *cis*-configuration on the 2′ and 3′ carbon atoms of the ribose.

Although the above studies of the interaction between human platelets and nucleoside analogs must still be considered in their initial stages, the results obtained to date indicate that this will be a fruitful area for future investigation. It seems likely that technics such as those described above may prove useful in the study of a number of diseases that involve blood platelets such as genetic conditions, e.g. the Wiscott–Aldrich Syndrome, platelet storage disorders and various thromboembolic diseases. Insights might be gained into the mechanisms by which agents such as aspirin, the prostaglandins, etc. affect platelet function. Since recent investigations have indicated an important role for the cyclic nucleotides, c-AMP and c-GMP, in the functioning of platelets [38–43], it will be of great interest to learn how the addition of various nucleoside analogs and the formation of analog nucleotides within platelets will affect the cyclic nucleotide response. Another area worthy of study is the interaction of platelets with guanine and hypoxanthine analogs such as 6-mercaptopurine and 6-thioguanine, since recent studies in this laboratory have shown that human erythrocytes are capable of incorporating surprisingly large amounts of the purine analogs into their nucleotide pools [16,44,45]. It is also possible that further studies such as these may lead to the development of new therapeutic agents of value in disorders that involve platelet disfunction.

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