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Analogues of Adenosine 5′-Diphosphate—New Platelet Aggregators

Influence of Purine Ring and Phosphate Chain Substitutions on the Platelet-Aggregating Potency of Adenosine 5′-Diphosphate

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SUMMARY

A number of previously unreported analogues of adenosine 5′-diphosphate have been synthesized in order to evaluate their effects as aggregators of mammalian blood platelets. These compounds include 2-methoxyadenosine 5′-diphosphate, 2-methylthioadenosine 5′-diphosphate, and 2-ethylaminoadenosine 5′-diphosphate, which are shown to be more powerful aggregating agents than ADP, 2-chloro-N^6-methyladenosine 5′-diphosphate (which is less potent than the unsubstituted nucleotide), and 2-chloroadenosine 5′-methylenediphosphonate (which is inactive). The 2-methylthio analogue proved to be exceptionally active and aggregated sheep platelets at concentrations 30 times less than equieffective concentrations of ADP. Evidence is presented that all these substances act at the same receptor site. On the basis of calculated analogue-receptor affinity constants and the observed lack of activity of the two analogues of ADP in which the anhydride oxygen is replaced by a methylene group, namely, adenosine 5′-methylenediphosphonate and 2-chloroadenosine 5′-methylenediphosphonate, some speculations concerning the requirements of the platelet ADP receptor have been made.

INTRODUCTION

Adenosine 5′-diphosphate is known to cause aggregation of mammalian blood platelets both *in vitro* (1) and *in vivo* (2), and it has been suggested that the nucleotide plays a key role in the process of platelet clumping, which invariably constitutes the first observable phase of thrombus formation in response to mural damage of blood vessels (2–4). Certain analogues of ADP, such as 3′-deoxyadenosine 5′-diphosphate and adenosine 1-*N*-oxide 5′-diphosphate, have been shown to mimic the effects of the parent compound, but only at higher dose levels (5), and the only nucleotide so far described as being more active than ADP has been 2-chloroadenosine diphosphate (6).

A number of new analogues incorporating modifications of both the adenine moiety and the diphosphate side chain have now been synthesized, and their properties are reported in this communication. Although the precise mechanism whereby ADP and its congeners promote the platelet "stickiness" which is antecedent to aggregation remains unknown, these compounds afford some insight into the requirements of the hypothetical platelet nucleotide receptor.

MATERIALS AND METHODS

Paper chromatography was carried out in the following solvent systems: 2-propanol–

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0.25 M aqueous ammonium bicarbonate (2:1 by volume) (solvent I) and isobutyric acid–1 M ammonia (5:3 by volume) (solvent II). Adenosine 5′-methylenediphosphonate was purchased from Miles Laboratories, Inc.

Synthetic Procedures

The starting materials used were the appropriate adenosine 5′-monophosphate analogues in the form of the free acids. The preparation and characterization of 2-chloro- and 2-methylthio-AMP[3] have been reported (7, 8), and the syntheses of 2-methoxy-, 2-ethylamino-, and 2-chloro-N^6-methyl-AMP will be reported in detail elsewhere.[4] 2-Methoxy-, 2-methylthio-, and 2-ethylamino-AMP were allowed to react with morpholine in the presence of dicyclohexylcarbodiimide to form the corresponding 5′-phosphoromorpholidates, in accordance with the general procedure of Moffatt and Khorana (9). Each phosphoromorpholidate was obtained in excellent yield as its 4-morpholine- *N,N′*-dicyclohexylcarboxamidinium salt.

2 - Methoxyadenosine 5′ - phosphoromorpholidate. R_F in solvent I, 0.49.

$C_{32}H_{54}N_9O_9P \cdot 4H_2O$

Calculated: C 47.34, H 7.69, N 15.53, P 3.82
Found: C 46.96, H 7.33, N 16.31, P 4.10

2 - Methylthioadenosine 5′ - phosphoromorpholidate. R_F in solvent I, 0.54.

$C_{32}H_{54}N_9O_8PS \cdot 2H_2O$

Calculated: C 48.53, H 7.38, N 15.92, P 3.91
Found: C 48.09, H 7.43, N 16.50, P 4.05

2 - Ethylaminoadenosine 5′ - phosphoromorpholidate. R_F in solvent I, 0.62. This material was not analyzed.

[3] The abbreviations used are: 2-chloro-AMP, 2-methylthio-AMP, 2-methoxy-AMP, 2-ethylamino-AMP, and 2-chloro-N^6-methyl-AMP, the 5′-monophosphates of 2-chloroadenosine, 2-methylthioadenosine, 2-methoxyadenosine, 2-ethylaminoadenosine, and 2-chloro-N^6-methyladenosine; 2-methoxy-ADP, 2-methylthio-ADP, 2-ethylamino-ADP, 2-chloro-ADP, and 2-chloro-N^6-methyl-ADP, the 5′-diphosphates of 2-methoxyadenosine, 2-methylthioadenosine, 2-ethylaminoadenosine, 2-chloroadenosine, and 2-chloro-N^6-methyladenosine.

[4] G. Gough, M. H. Maguire and F. Penglis, manuscript in preparation.

Nucleoside 5′ - phosphoromorpholidate (0.15 mmole) was dried by three successive additions and evaporations of 10 ml of anhydrous pyridine. Mono(tri-*n*-butylammonium)orthophosphate (0.45 mmole) was dried in the same way to give an oily residue, which was dissolved in anhydrous pyridine (5 ml) and added to the phosphoromorpholidate. Two further evaporations were carried out to free the reaction mixture from traces of water. The residue was dissolved in pyridine (1.5 ml), and the solution was allowed to stand at room temperature for 3 days with exclusion of moisture. Pyridine was then removed under vacuum, and water (10 ml) was added. The cloudy solution thus obtained was applied to a column of DEAE-cellulose (2.5 × 20 cm, bicarbonate form) and eluted with a linear gradient of ammonium bicarbonate (0–0.4 M in 3 liters); 15-ml fractions were collected. In each case the diphosphate emerged as a homogeneous peak in tubes 80–100. The pooled fractions were concentrated to dryness, and ammonium bicarbonate was removed by several additional evaporations of water. The residue was subjected to further purification by preparative chromatography on sheets of Whatman No. 3 paper in solvent II. At the conclusion of each chromatographic run, strips containing the diphosphates were cut out and washed with 2-propanol to remove ammonium isobutyrate; the nucleotide material was then eluted with water. Lyophilization of the aqueous eluates gave the following products.

2-Methoxyadenosine 5′-diphosphate, diammonium salt. Yield, 70%.

$C_{11}H_{17}N_5O_{11}P_2 \cdot (NH_3)_2 \cdot 5H_2O$

Calculated: P 10.66, ratio of P to 2-methoxy-AMP, 2.00
Found: P 10.62, ratio of P to 2-methoxy-AMP, 2.04

2 - Methylthioadenosine 5′ - diphosphate, diammonium salt. Yield, 76%

$C_{11}H_{17}N_5O_{10}P_2S \cdot (NH_3)_2 \cdot 4H_2O$

Calculated: P 10.68, ratio of P to 2-methylthio-AMP, 2.00
Found: P 10.65, ratio of P to 2-methylthio-AMP, 2.14

TABLE 1

Paper chromatographic and spectral properties of ADP analogues

Compound	R_F in solvent II[a]	λ_{max} in 0.1 N HCl	ϵ
		nm	$\times 10^{-3}$
ADP	0.43	257	14.9
2-Chloro-ADP	0.43	266	14.0
2-Methoxy-ADP	0.47	247, 274	7.6, 11.0
2-Methylthio-ADP	0.50	268	15.5
2-Ethylamino-ADP	0.68	254.5, 298	12.4, 8.0
2-Chloro-N^6-methyl-ADP	0.54	272.5	14.9
2-Chloroadenosine 5′-methylenediphosphonate	0.51	264	12.5

[a] Solvent II is isobutyric acid–1 M NH_4OH(5:3 by volume).

2 - Ethylaminoadenosine 5′ - diphosphate. Yield, 85%; crystallizable from aqueous acetone.

$C_{12}H_{20}N_6O_{10}P_2 \cdot (NH_3) \cdot 2 \cdot 5H_2O$

Calculated: C 27.07, H 5.30, N 18.42, P 11.64

Found: C 27.09, H 5.15, N 19.11, P 11.17

Chromatographic and spectral data for these compounds are shown in Table 1.

Preparation of phosphoromorpholidates from 2-chloro analogues of AMP was complicated by the formation in significant quantity of by-products which exhibited blue fluorescence under ultraviolet light (7). These have been identified as 2-morpholino derivatives formed by nucleophilic substitution of the chlorine atom at position 2. An alternative to the phosphoromorpholidate procedure was therefore chosen for synthesis of diphosphates in the case of 2-chloro analogues. This utilized the formation of phosphorimidazolidates as reactive amidate intermediates for pyrophosphate bond synthesis (10). 2-Chloro-N^6-methyladenosine 5′-monophosphoric acid (0.15 mmole) was dissolved in anhydrous pyridine. After the addition of tri-*n*-butylamine (36 μl, 0.15 mmole), the mixture was concentrated to dryness and freed from traces of moisture by coevaporation with pyridine. Residual pyridine was removed by evaporation with benzene. The resulting oil was taken up in dry *N,N*-dimethylformamide (1 ml) and treated with 1,1′-carbonyldiimidazole (120 mg, 0.75 mmole). The mixture was allowed to stand overnight at room temperature in a stoppered flask.

Chromatography in solvent I showed that quantitative transformation into the phosphorimidazolidate (R_F 0.77) had taken place. Methanol (50 μl) was added, and after 30 min the solution was treated with mono(tri-*n* - butylammonium)orthophosphate (0.75 mmole, rendered anhydrous as above) in dry *N,N*-dimethylformamide (5 ml). After 24 hr at room temperature with exclusion of moisture, the mixture was filtered. The filtrate was evaporated to dryness, and the residue was chromatographed on DEAE-cellulose as described above. 2-Chloro-N^6-methyladenosine 5′-diphosphate emerged in fractions 78–96 and was finally isolated as a white, lyophilized solid (65% yield) following preparative paper chromatography.

$C_{11}H_{16}ClN_5O_{10}P \cdot (NH_3)_2 \cdot 4H_2O$

Calculated: P 10.64, ratio of P to 2-chloro-N^6-methyl-AMP, 2.04

Found: P 10.48, ratio of P to 2-chloro-N^6-methyl-AMP, 2.02

A similar procedure resulted in 67% yield of 2-chloroadenosine 5′-diphosphate identical with an authentic sample prepared as reported previously (7).

2 - Chloroadenosine 5′ - methylenediphosphonate. This compound was synthesized by treating 2 - chloro - 2′,3′ - *O* - isopropylidene adenosine (7) with methylenediphosphonic acid in the presence of dicyclohexylcarbodiimide, a method analogous to that of Myers *et al.* (11). After removal of the isopropylidene blocking group and purification by DEAE and preparative chromatography as outlined above for the diphosphates, the product was isolated as the lyophilized diammonium salt in 70% yield.

$C_{11}H_{16}ClN_5O_9P_2 \cdot (NH_3)_2 \cdot 2 \cdot 5H_2O$

Calculated: C 24.52, H 5.06, N 18.20, P 11.49

Found: C 25.00, H 4.81, N 18.80, P 10.97

Platelet Aggregation

The aggregation of sheep platelets in citrated platelet-rich plasma was studied using a technique based on the turbidimetric method of Born (12). The modified procedure and the preparation of platelet-rich plasma have been described in detail elsewhere (6, 13). Changes in the optical density of platelet-rich plasma induced by the various aggregators were measured using a potentiometric recorder. In order to quantitate platelet clumping, the initial rate of aggregation (slope of the absorbance curve during the first 30 sec) at 37° was used to plot log dose-response curves for each aggregator. Comparative experiments to determine the potency of each analogue relative to ADP were always carried out on the same batch of plasma.

RESULTS

All the analogues of ADP possessing an unmodified diphosphate side chain caused aggregation of sheep blood platelets. The clumping induced by low doses of each of the analogues was reversible, and doses which elicited a maximal response caused irreversible aggregation. The time course of the response to 2-methylthio-ADP was somewhat more prolonged than that of ADP, as a comparison of aggregation curves typical of the two nucleotides indicates (Fig. 1). 2-Methoxy-, 2-ethylamino-, and 2-chloro-N^6-methyl-ADP also displayed somewhat extended aggregation time courses, which were intermediate between those of ADP and 2-methylthio-ADP. In contrast, platelet aggregation mediated by 2-chloro-ADP had a time course similar to that of ADP (6). The potency of ADP itself was arbitrarily assigned a value of unity, and the potencies of the analogues relative to the parent nucleotide are shown in Table 2 together with results obtained with the methylenediphosphonate analogues of ADP and 2-chloro-ADP. These two substances did not initiate aggregation, nor did they potentiate or inhibit the effect of ADP or 2-chloro-ADP in concentrations up to 200 μM (14).

Data obtained from aggregation studies on the various substituted nucleotides are expressed in the form of log dose-response

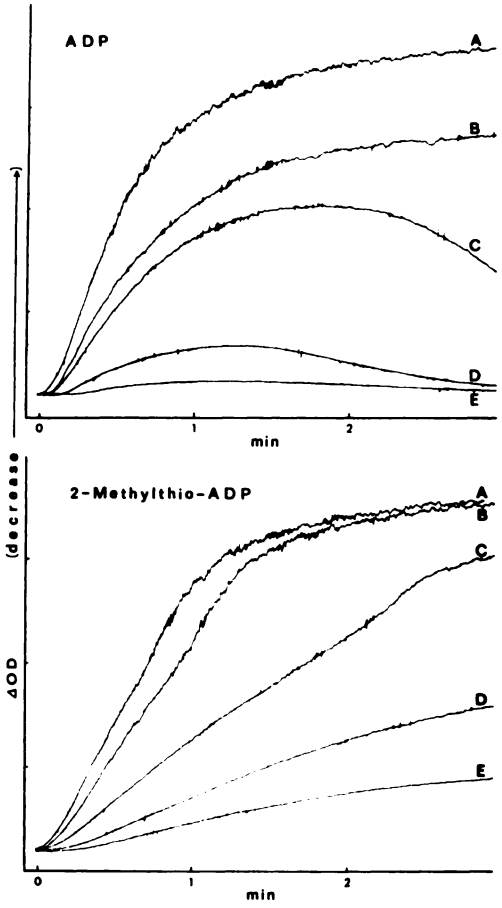

FIG. 1. *Aggregation of sheep blood platelets induced by ADP and 2-methylthio-ADP*

Aggregation was followed by recording the change in optical density which occurred after the addition of ADP or 2-methylthio-ADP to sheep platelet-rich plasma, as described previously (6, 13). Aggregators were added at zero time to aliquots of the same batch of platelet-rich plasma. The upper tracings show the responses to the following concentrations of ADP: *A*, 2.64 μM; *B*, 1.32 μM; *C*, 0.66 μM; *D*, 0.33 μM; *E*, 0.16 μM. The lower tracings depict the corresponding responses to 2-methylthio-ADP: *A*, 46 nM; *B*, 23 nM; *C*, 11.4 nM; *D*, 5.7 nM; *E*, 2.9 nM.

curves in Fig. 2. Affinity constants (K_a) of the analogues for the platelet ADP receptor were also calculated from these data as described by Maguire and Michal (6), and are included in Table 2. The absolute values of K_a given here for ADP and 2-chloro-ADP are somewhat lower than those previously

TABLE 2

Potency of ADP analogues as aggregators of sheep blood platelets

The molar potency of each ADP analogue with respect to ADP was calculated from the log dose-response curves for each batch of plasma, as described in the text. The affinity constants, K_a, of ADP and the ADP analogues for the platelet ADP receptor were obtained similarly; K_a equals the reciprocal of the molar concentration of the compounds which elicited 50% of the maximum aggregation response (5).

Compound	Molar potency[a]	K_a[a]
	$\pm SE$	$\times 10^{-6}$ ($\pm SE$)
ADP	1	1.14 ± 0.04
2-Chloro-N^6-methyl-ADP	0.62 ± 0.05	0.78 ± 0.08
2-Ethylamino-ADP	2.97 ± 0.19	3.07 ± 0.05
2-Methoxy-ADP	5.10 ± 0.20	5.44 ± 0.42
2-Chloro-ADP	7.62 ± 0.08	9.49 ± 0.41
2-Methylthio-ADP	30.71 ± 1.80	33.15 ± 2.80
Adenosine 5′-methylenediphosphonate[b]	0	
2-Chloroadenosine 5′-methylenediphosphonate[b]	0	

[a] These values are means of no fewer than three determinations.

[b] Results of Michal (14).

reported (6). However, the potency ratio and relative affinity of 2-chloro-ADP with respect to ADP remain very similar to values obtained from the earlier experiments, and the variation probably reflects a change of unknown nature in the sensitivity of the platelets themselves.

DISCUSSION

A number of hypotheses have been advanced in attempts to delineate the mechanism by which ADP in the presence of calcium ions causes the aggregation of mammalian blood platelets, These have been reviewed by Mustard and Packham (15), and range from the early concept of platelet-platelet bridges via ADP, calcium, and a protein cofactor (5) to hypotheses involving ADP in energy-producing reactions at the platelet membrane. The possible presence of a platelet enzyme which hydrolyzes ADP to AMP to produce the energy to clump platelets was suggested by Spaet and Lejnieks (16). Salzman *et al.* (17) found that platelet ecto-ATPase was inhibited by ADP, and hypothesized that the action of this ATPase maintained the platelet in a nonsticky state. Recent observations, particularly those elucidating the ultrastructural properties of platelets (18, 19), have resulted in a more detailed understanding of the phenomenon of platelet aggregation, and in a refinement of the earlier hypotheses of the mechanism of ADP-induced platelet aggregation. The aggregation of blood platelets is now known to take place in three stages. The first is a shape change in which the discoid platelet extends pseudopodia (19, 20). It is triggered by ADP, has no calcium requirement (21), and is followed rapidly by the second stage, that of reversible aggregation, which requires calcium (22), and which merges into the third stage, the release reaction. In this stage a contractile wave within the platelet results in the extrusion of adenine nucleotides and amines and in irreversible aggregation of the platelets (23–25).

The initiating stimulus in the aggregation process is the interaction of ADP with the platelet. Born (21) has suggested that this interaction occurs via specific ADP receptor sites on the platelet membrane, and results in energy-requiring changes in the platelet structures which are responsible for maintaining the disc shape of normal platelets. The parallel nature of the log dose-response curves for ADP and the aggregating analogues of ADP described in this communication indicates that these analogues also act at the ADP receptor site, triggering shape changes and subsequent aggregation. The great differences observed in the potencies of the compounds can be ascribed to differences in their affinities for the ADP receptor site and not to differences in efficacy, as all analogues, like ADP, elicited maximal platelet aggregation. In our studies on sheep platelet clumping, it has not been possible to isolate the shape change from the other steps which lead finally to irreversible aggregation, but work in progress[5] shows that the relative potencies of these analogues in

[5] F. Penglis, unpublished observations.

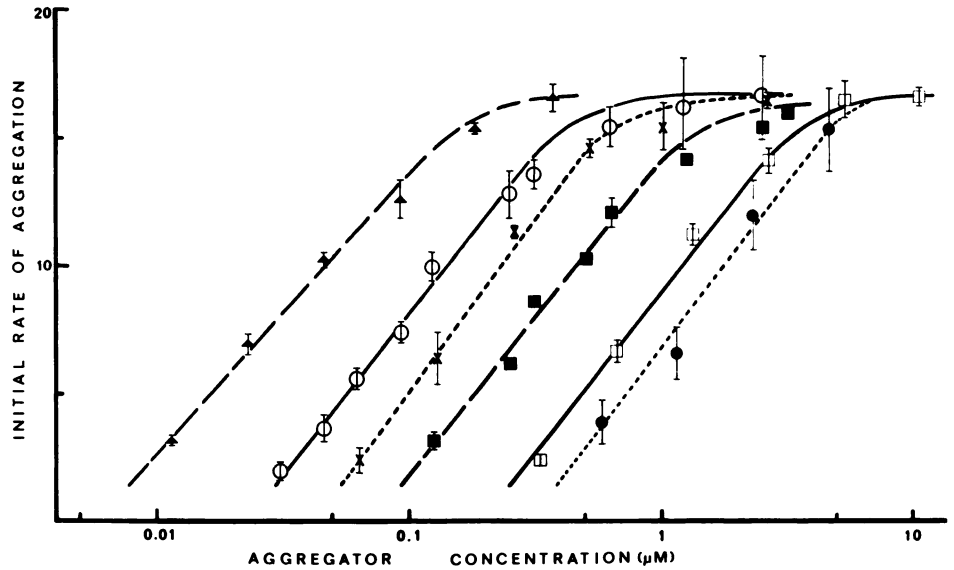

FIG. 2. *Log dose-response curves of ADP and ADP analogues as platelet aggregators*

The log dose-response curves for the aggregation of sheep platelet-rich plasma by ADP (□), 2-methylthio-ADP (▲), 2-chloro-ADP (○), 2-methoxy-ADP (×), 2-ethylamino-ADP (■), and 2-chloro-N^6-methyl-ADP (●) were obtained by the turbidimetric technique described in the text. Initial rates of aggregation were obtained from the slopes using the first 30 sec of the aggregation curves. The points on each curve represent means of no fewer than three experiments. The standard errors of the means are indicated by the vertical lines, but where these were too small to be shown graphically they have been omitted.

eliciting shape changes in rabbit platelets are similar to those reported here for sheep platelet aggregation.

All the ADP analogues with substituents only at position 2 of the adenine moiety had greater aggregating properties than ADP itself. However, N^6-methylation of 2-chloro-ADP caused a 12-fold reduction in the potency of this aggregator, suggesting that the functional integrity of the 6-amino group is a prerequisite for strong activity. The enhancing effect of the 2-substituent on the aggregating potency of ADP is, in rank order, methylthio > chloro > methoxy > ethylamino, and may be considered in terms of the space-filling properties of the substituents and their effects on the electronic structure of the purine ring. In spite of the space-filling and conformational differences among the groups, a general trend in decreasing size of the substituent is ethylamino > methylthio > methoxy > chloro; this trend does not correlate with the rank order of potency of the analogues.

The inductive effect of the substituents on the adenine moiety of ADP will be similar to the effect that the groups exert on adenine itself when substituted on position 2. The pK_{a1} of adenine is 4.12 (26), and 2-methylthioadenine and 2-chloroadenine have pK_{a1} values of 3.26 and less than 2, respectively (27). 2-Aminoadenine has a pK_{a2} of 5.05 (27), and the 2-ethylamino group will be even more base-strengthening. The pK_{a1} of 2-methoxy-N^6-methyladenine is 3.70, and that of 2-methylthio-N^6-methyladenine is 3.54,[6] indicating that in ADP itself the 2-methoxy substituent will be less base-weakening than the 2-methylthio substituent. The inductive influence of the four substituent groups on the electronic structure of the purine ring of ADP will increase in the order ethylamino < methoxy < methylthio < chloro. The increasing potency of only three of the ADP analogues, 2-ethylamino-, 2-methoxy-, and 2-chloro-ADP, can thus be

[6] M. H. Maguire and M. K. Sim, unpublished results.

correlated with the increasing inductive character, and the decreasing size, of the 2-substituent. It would appear that the topography of the ADP receptor in the platelet membrane is such that ADP analogues with certain substituents on position 2 of the purine ring actually meet the binding requirements of the receptor better than ADP itself.

The extreme affinity of 2-methylthio-ADP for the ADP receptor appears to be anomalous, as it cannot be included in the trend established for the other three agonists. However, the potency of 2-methylthio-ADP may be accounted for by the high polarizability of the sulfur atom, and by free rotation of the methylthio group about the sulfur-purine bond, which would allow adoption of a conformation in which steric interference by the methyl group would be minimal.

ADP-mediated platelet aggregation is inhibited by AMP (8, 12), and the greater aggregating potency conferred on 2-methylthio-ADP by the methylthio group can be compared with the influence of this substituent on the inhibitory potency of 2-methylthio-AMP, which has 20 times the potency of AMP as an inhibitor of ADP-mediated sheep platelet aggregation, and which appears also to act at the ADP receptor (8).

The sheep platelet ADP receptor appears to be extremely sensitive to alterations in the pyrophosphate moiety of ADP. Thus the 5′ - methylenediphosphonate analogues of ADP and 2-chloro-ADP were devoid of aggregating activity. As the 5′-methylenediphosphonates cannot be hydrolyzed to AMP or 2-chloro-AMP because of the great stability of the carbon-phosphorus bond (28, 29), these findings may appear to support the proposal of Spaet and Lejnieks (16) that the breakdown of ADP to AMP and inorganic phosphate is a key energy-providing reaction in platelet aggregation, even though the hydrolysis of ADP is not normally an energy source in biological systems. However, if resistance to hydrolytic cleavage were the only factor involved in the lack of aggregating activity of the 5′-methylenediphosphonates, they would be expected to act as competitive inhibitors of ADP. The observation that they do not indicates that these compounds have been rendered unacceptable to the receptor.

The aliphatic phosphonic acids are usually weaker than their phosphate isosteres (28). In keeping with this general rule, Myers *et al.* (11) found that the pK_a of the secondary phosphonyl hydroxyl group of adenosine 5′-methylenediphosphonate was 8.0, while that of the analogous group of ADP was 7.0. Thus, at the pH of the citrated platelet-rich plasma used in these studies, 7.4–7.6, 75% of ADP was fully ionized, but only 25% of the methylenediphosphonates was present in the form of the trianion. Born (21) showed that the rate and maximum of the ADP-induced platelet shape change were unaltered in the pH range 5.8–9.2, indicating that both the di-and trianionic forms of ADP are equally acceptable to the ADP receptor. Therefore it is unlikely that the weaker acidity of the methylenediphosphonates is responsible for their lack of agonist or antagonist activity.

Calcium ions are essential for platelet aggregation, and calcium-bound ADP has been postulated to be an active entity in the aggregation process (30). The inactivity of the methylenediphosphonates may possibly be due to a decrease in their binding capacity for calcium compared to that of ADP. However, such an explanation is unlikely, for the following reasons. Although a stability constant for calcium adenosine 5′-methylenediphosphonate has not been reported, comparison of the apparent binding constants of ATP and its β, γ-methylene isostere for calcium at pH 7.4, 1800 M^{-1} and 4800 M^{-1} respectively (31), indicates that the methylenediphosphonate analogues will actually bind calcium somewhat better than ADP itself. Moreover, calcium is not a requirement for the initial shape change induced by ADP, and recent evidence (32) suggests that ADP need not be complexed with calcium for participation in platelet aggregation.

The most likely reason for the inactivity of the methylenediphosphonates is that these compounds do not bind to the ADP receptor, either because the methylene group imposes

some steric distortion on the phosphate chain, as has been demonstrated for the β, γ-methylene isostere of ATP (33), or because the chain possesses a hydrophobic group in place of the oxygen of the normal agonist.

The results of the investigations of the platelet-aggregating properties of the seven ADP analogues reported here lend support to Born's hypothesis of specific ADP receptors on the platelet membrane (21), and have allowed some advance in the definition of the binding requirements of the sheep platelet ADP receptor site. Those analogues which are potent aggregators should prove valuable tools in platelet studies, and they point the way to the exciting possibility of affinity labeling (34) of the ADP receptor. Thus substitution of alkylating groups, such as iodoacetamide, at position 2 of ADP should give compounds which will still bind to the receptor, but which may also result in irreversible alkylation of the receptor (35).

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