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Kinetics of ADP-induced human platelet shape change: apparent positive cooperativity¹

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The kinetics of ADP-induced human platelet shape change have been examined. Initial velocities of platelet shape change were estimated by two methods: (1) the slope of the initial decrease in light transmission through stirred, citrated platelet-rich plasma, and (2) direct examination of platelet morphologies by phase-contrast microscopy. In both cases, a value of the Hill coefficient, N_H , significantly greater than 1 is obtained (2.0 ± 0.2 and 1.8 ± 0.2 , respectively). The observed elevated value of N_H is not due to a substantial fraction of the ADP being platelet bound, the presence of factors in the plasma, platelet heterogeneity, or the influence of the rate of platelet shape change reversion. Our observations suggest that ADP-induced platelet shape change may be a positively cooperative or "threshold" type response.

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On étudie la cinétique du changement de forme des plaquettes humaines induit par de l'ADP. Les vitesses initiales du changement de forme des plaquettes sont estimées selon deux méthodes: (1) la pente de la diminution initiale de la transmission de la lumière à travers du plasma agité, citraté et riche en plaquettes, et (2) l'examen direct de la morphologie des plaquettes par microscopie à contraste de phase. On obtient dans les deux cas une valeur du coefficient de Hill, N_H , qui est plus grande que celle normalement obtenue (2.0 ± 0.2 et 1.8 ± 0.2 , respectivement). La valeur élevée de N_H qui est observé n'est pas due à une fraction substantielle d'ADP qui serait liée aux plaquettes, pas plus qu'à la présence de facteurs dans le plasma, à l'hétérogénéité des plaquettes ou à l'influence du taux de réversion du changement de forme des plaquettes. Nos observations suggèrent que le changement de forme des plaquettes induit par de l'ADP pourrait constituer une réponse positivement coopérative ou de type "à seuil."

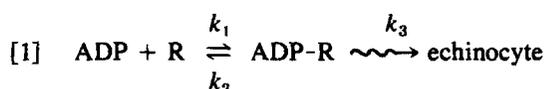
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Introduction

Platelet shape change from smooth, discoid-shaped platelets (discocytes) to more spherical forms with pseudopodal projections (echinocytes) induced by adenosine diphosphate (ADP) is generally regarded as an example of a receptor-mediated process (Born 1965, 1970; Born and Feinberg 1975; Mills and Macfarlane 1975; Nachman and Ferris 1974). Recently, Bennett et al. (1978) have demonstrated that the ADP receptor for shape change may be a membrane polypeptide of 100 000 molecular weight. The nature of the mechanism by which the binding of ADP molecules to membrane-bound receptors is translated into the morphological event of shape change is not precisely known.

The basis for present day discussions of the action of molecules at the receptor level is formed by models first introduced by Clark (1926) which closely resemble those used to describe the interaction of an enzyme with

its substrate. In view of these considerations, the kinetics of ADP-induced platelet shape change can be described in terms of a "Michaelis-Menton-like" model:



where k_1 , k_2 , and k_3 are rate constants, R is a receptor for platelet shape change located on a discocyte, ADP-R is the ADP-receptor complex, and the symbol " $\xrightarrow{k_3}$ " indicates that the conversion of discocyte to echinocyte may involve additional steps subsequent to the binding of ADP to its receptor, to yield

$$[2] \quad V = \frac{V_{\max} [\text{ADP}]_f}{K' + [\text{ADP}]_f}$$

where V and V_{\max} are, respectively, the initial and maximal initial velocities for shape change, $[\text{ADP}]_f$ is the concentration of free ADP, i.e., that which is not receptor bound, and K' is the apparent Michaelis-Menton constant obtained from the x -intercept of a plot of V^{-1} versus $[\text{ADP}]_f^{-1}$.

Previous studies with rabbit (Born 1970) and human (Favis and Colman 1978) platelets have suggested that

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the kinetics of ADP-induced platelet shape change are consistent with that predicted by Eq. 2. However, the lowest value of V considered in these studies corresponded to only approximately 30% of V_{\max} . Departures from the description offered by Eq. 2 are anticipated to be most significant at low [ADP], i.e., low V . Moreover, the changes in light transmission utilized by these authors to estimate V may not accurately reflect the rate of platelet shape change, as, for example, changes in platelet refractive index will also affect light transmission (Frojmovic 1978). It is clearly necessary to try to extend the kinetic measurements of shape change to lower values of V with a more direct measure of shape change before any conclusions concerning the nature of the process can be safely drawn.

Here we directly measure V by examining changes in platelet morphology with phase-contrast microscopy. The results are compared with those when V is measured from changes in light transmission. In both cases, it is shown that over a range of [ADP] which extends the measurement of V from V_{\max} to approximately 10% of V_{\max} , Eq. 2 does not provide an adequate description of the kinetics of ADP-induced platelet shape change. A Hill plot, i.e. $\log(V/(V_{\max}-V))$ versus $\log([ADP])$, is performed. Whereas Eq. 2 predicts that the Hill coefficient, N_H , equals 1, our data yield $N_H > 1$. The significance of this observation is discussed.

Methods

Preparation of platelet-rich plasma (PRP), platelet-free plasma (PFP), and washed platelets

Normal donors were chosen from healthy men and women between the ages of 20 and 30 years. Blood was drawn by venipuncture into 3.8% citrate (one volume to nine volumes blood) at 37°C and PRP prepared as described previously (Tang and Frojmovic 1977). PFP was prepared by first centrifuging PRP for 15 min at $1900 \times g$, 25°C, and then centrifuging the supernatant at $30\,000 \times g$, 4°C, for 15 min. PFP was allowed to incubate at 37°C for an additional 30 min before use to remove residual ADP. Washed platelets were prepared by the method of Mustard et al. (1972) and were generously supplied by Dr. H. L. Goldsmith, McGill University.

Kinetic studies

ADP (Sigma Chemical Co.) was dissolved in Ca^{+2} - Mg^{+2} -free Tyrodes buffer. One to $10 \mu\text{L}$ ADP was quickly injected using a Hamilton syringe into 0.4 mL platelet suspension which was contained in a siliconized cuvette (6.9 mm \times 45 mm) with stir bars (6 mm \times 1 mm) spun at 1000 rpm, 37°C. Injection of the dye, azure A, indicated that under these conditions mixing was complete in less than 1 s. The initial velocity of shape changes, V , was estimated by the following two methods.

(a) Changes in light transmission

Changes in light transmission were monitored using the aggregometer apparatus described previously (Frojmovic 1973), using a rapid chart speed (10 inches/min; 1 inch = 25.4 mm). A rapid decrease in light transmission is observed following the addition of ADP to stirred PRP. The slope of the initial

decrease in light transmission was utilized as a measure of V . Several other authors (Born 1970; Favis and Colman 1978; Bennett et al. 1978) have utilized this measurement to estimate V .

(b) Phase-contrast microscopy

At 5 s following the addition of ADP to stirred PRP, 0.05-mL samples were withdrawn and fixed with four volumes of 1.3% glutaraldehyde at 37°C as described previously (Frojmovic and Panjwani 1976; Milton and Frojmovic 1979). This sampling time was chosen as it was found that for all [ADP]'s used in this study, the increase in the fraction of shape-changed platelets, f_E , was linear for at least the first 6 s following ADP addition (Fig. 1). The value of f_E measured at 5 s following ADP addition was taken as a measure of V . Shape-changed platelets were identified with phase-contrast microscopy (Zeiss universal microscope; magnification $\times 800$) as those which possessed at least one pseudopod and (or) whose axial ratio, i.e., the ratio of thickness to diameter, exceeded 0.5 (Milton and Frojmovic 1979). A third criterion which had to be used to identify platelets in the early stages of shape change was the appearance of a rougher or "fuzzy" cell outline under phase-contrast microscopy. This contrasted with the very smooth outline of unactivated platelets. Dark-field microscopy observation indicated that the earlier stages of platelet shape change were associated with the development of short pseudopods and membrane undulations. Control experiments demonstrated that f_E was not altered by the above fixation procedure. In determining f_E , at least 400 platelets were randomly selected.

Platelet shape change reversion

The rate of platelet shape change reversion was estimated as follows. Following the addition of ADP to stirred PRP, the stirring was stopped after complete mixing had occurred to prevent aggregation, 0.025-mL samples were removed as a function of time and fixed with glutaraldehyde, and f_E was determined as described above.

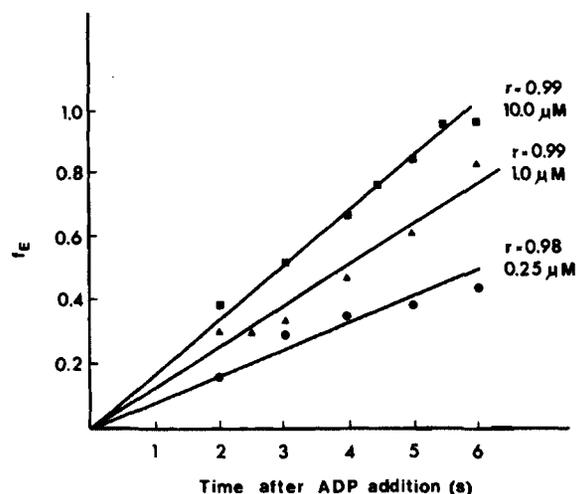


FIG. 1. Fraction of shape-changed platelets (f_E) during the first 6 s after ADP addition demonstrating the linearity with time over the ADP concentration range used in this study. Correlation coefficients (r) of the three corresponding linear regression lines are shown. ■, 10.0 μM ADP; ▲, 1.0 μM ADP, ●, 0.25 μM ADP.

Results

The results of a typical experiment showing the effect of total ADP concentration, $[ADP]$, on V as measured by direct examination of platelet morphologies under phase-contrast microscopy are given in Fig. 2A. Although over the concentration range of 0.25 – $10 \mu M$ ADP the plot of V^{-1} versus $[ADP]^{-1}$ is approximately linear (see dotted line in Fig. 2B), a linear relationship does not hold over the whole concentration range investigated. In particular, there is a pronounced upward curvature as $[ADP]$ becomes smaller. Qualitatively similar trends are observed when V is measured as the initial slope of the decrease in light transmission (see Fig. 2C and D). This demonstrates that the results in Fig. 2A and B are not dependent on the method utilized to measure V . The observation that a plot of V^{-1} versus $[ADP]^{-1}$ is not linear demonstrates that the kinetic de-

scription implied by Eq. 2 does not provide a valid description for the kinetics of ADP-induced platelet shape change.

Closer examination of the data presented in Fig. 2A and C suggests that the curves are sigmoid. Hill plots for these data are shown in Fig. 3. In each case, N_H is significantly greater than 1 ($p < 0.05$). It is important to note that the linear range of the Hill plots includes V 's measured in the more linear range of the plots in Fig. 2B and D. N_H and the concentration of ADP required for $\frac{1}{2} V_{max}$, $[ADP]_{1/2}$, for a number of different donors are summarized in Table 1.

A value of $N_H > 1$ is suggestive of some kind of cooperative process or "threshold-type" response for ADP-induced platelet shape change. However, there are other possibilities which may result in a spurious departure from Eq. 2.

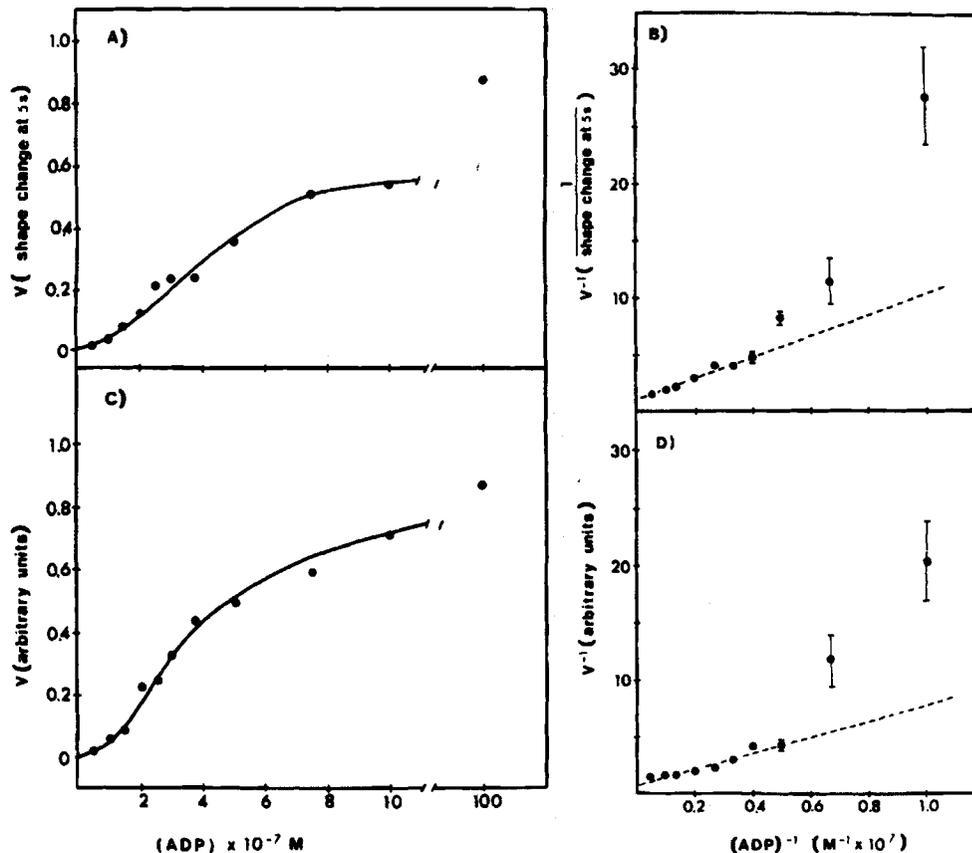


FIG. 2. Effect of ADP concentration on the velocity (V) of the change in shape of human platelets observed in a typical experiment. Donor (donor 1) was female, age 22 years, platelet count $330\,000 \mu L^{-1}$. V has been measured by the light microscope (A) and by the light transmission (C) methods which are described in Methods. Double-reciprocal plots of these results are given in (B) and (D), respectively. Error bars in (B) and (D) give the highest and lowest values measured, the point corresponding to the mean of measurements made (usually 2 or 3). For the remaining points in (B) and (D), the deviations in the measurements were less than the width of the point used in preparing the figure. K' determined by extrapolating the dotted line to $V^{-1} = 0$ was $1.0 \times 10^{-6} M$ (B) and $8.6 \times 10^{-7} M$ (D). K' determined for a number of donors in this way was summarized in the text of Discussion.

TABLE 1. Comparison of $[ADP]_{1/2}$ and N_H for washed platelets and platelets in PRP when V is measured by the light microscope and light transmission methods

	Method used to measure V			
	Light microscope		Light transmission	
	$[ADP]_{1/2}$ $\times 10^7 M$	N_H	$[ADP]_{1/2}$ $\times 10^7 M$	N_H
PRP				
Donor 1	6.4(8) ^e	1.6(0.99) ^{b,c,d}	4.7(8) ^e	1.8(0.97) ^{b,c,d}
	—	—	3.4(6)	2.0(0.90)
	—	—	5.1(6)	1.7(0.98)
Donor 2	4.4(5)	2.0(0.99)	—	—
Donor 3	2.9(7)	1.9(0.97) ^d	4.7(6)	2.1(1.00) ^d
Donor 4	4.1(3)	1.7(0.99)	—	—
Donor 5	—	—	3.3(10)	2.1(0.99)
Donor 6	4.6(7)	2.0(0.99)	—	—
Donor 7	—	—	4.6(10)	2.0(1.00)
Donor 8	—	—	3.1(4)	2.3(1.00)
Mean values	4.5 ± 1.3 ^c	1.8 ± 0.2 ^f	4.1 ± 0.8 ^c	2.0 ± 0.2 ^f
Washed platelets				
Donor 7	—	—	1.8(9)	1.9(0.97)
Donor 8	—	—	1.6(6)	2.5(0.94)
Donor 9	1.2(6)	1.7(0.97) ^e	1.6(7)	2.2(0.96) ^e
Mean values	—	—	1.7 ± 0.1	2.2 ± 0.3

^aThe number of data points falling in the linear range of the Hill plot is given in parentheses. In general only a single measurement was made at each ADP concentration.

^bThe correlation coefficient for the points falling in the linear range of the Hill plot is given in parentheses.

^cA *t*-test of the form $t_{N-3} = N_H - 1.0/S_0$, where S_0 is the standard error of the regression coefficient and *t* has $N-2$ degrees of freedom, was used to test if $N_H > 1$ (Sokal and Rohlf 1969). In all cases, N_H was significantly greater than 1 ($p < 0.05$).

^dAn analysis of covariance (Sokal and Rohlf 1969) was utilized to establish that the values of N_H determined by the two methods were not significantly different ($0.25 < p < 0.50$).

^eA *t*-test was used to show that the mean $[ADP]_{1/2}$ values obtained by the two methods are not significantly different ($0.5 < p < 0.9$).

^fA *t*-test was used to show that the mean N_H values obtained by the two methods are not significantly different ($0.1 < p < 0.2$).

^gThe values of N_H determined by the two methods were not significantly different. ($0.1 < p < 0.25$). Analysis same as for footnote *d*.

(a) Fraction of ADP bound

In testing Eq. 2 by plotting V^{-1} versus $[ADP]^{-1}$, it is implicitly assumed that the total ADP concentration, $[ADP]$, is approximately equal to $[ADP]_f$, i.e., $[ADP] \approx [ADP]_f$. An upward curvature for a plot of V^{-1} versus $[ADP]^{-1}$ can occur when a large fraction (>25%) of the ADP is platelet bound (Rodbard et al. 1974). A Hill plot at a platelet count of $700\,000\ \mu\text{L}^{-1}$ is compared with that obtained at a count of $40\,000\ \mu\text{L}^{-1}$ in Fig. 4. The fact that N_H is not significantly different at the two platelet counts ($0.1 < p < 0.25$) strongly suggests that only a negligible amount of the ADP is platelet bound under the conditions of our experiment.

(b) Plasma factors

N_H and $[ADP]_{1/2}$ for washed platelets and PRP are compared in Table 1. As can be seen, N_H remains elevated for washed platelets, but $[ADP]_{1/2}$ is smaller. Al-

though these results indicate that the increased sensitivity of washed platelets to ADP may be related to the absence of factors normally present in plasma, it is clear that the elevated value of N_H cannot be explained in these terms.

(c) Platelet shape change reversion

Under appropriate conditions following ADP-induced platelet shape change, human echinocytes revert spontaneously back to discocytes (Ehrman et al. 1978). The upward curvature shown in Fig. 2 B and D could be explained by assuming that an increased contribution from platelet shape change reversion lowers V at low $[ADP]$. A plot of f_E versus time following ADP addition when $[ADP]$ is 0.5 and 0.1 μM is shown in Fig. 5. The former $[ADP]$ corresponds to approximately K' and the latter to a concentration which yields V on the upswing portion of a plot of V^{-1} versus $[ADP]^{-1}$ (see Fig. 2B).

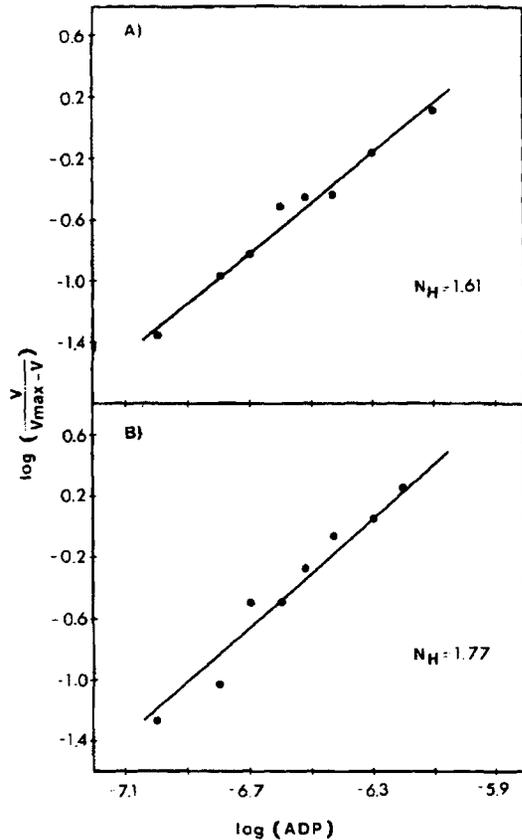


FIG. 3. Comparison of Hill plots for ADP-induced platelet shape change when V is measured by the light microscope method (A) and by the light transmission method (B). Data are taken from Fig. 1. In each case only the linear portion of the Hill plot has been shown. An analysis of covariance as described in Sokal and Rohlf (1969) was used to show that the slopes of these two Hill plots are not significantly different ($0.5 > p > 0.25$).

If at a low $[ADP]$ there is an increased contribution of rate of platelet shape change reversion, then we would expect to see that the time which corresponds to maximum f_R is shorter at the lower $[ADP]$. This expectation is clearly not consistent with the data presented in Fig. 5. Therefore, the upward curvature of a plot of V^{-1} versus $[ADP]^{-1}$ does not arise because of contributions due to platelet shape change reversion. A more detailed analysis of the kinetics of platelet shape change reversion will be presented in a separate communication (Milton, J. G., Glushak, C., and Frojmovic, M. M.: in preparation).

Discussion

Our results demonstrate that the kinetics of ADP-induced platelet shape change cannot be adequately described by Eq. 2. In particular, our kinetic data yield a value of $N_H > 1$ whereas Eq. 2 predicts $N_H = 1$. Fur-

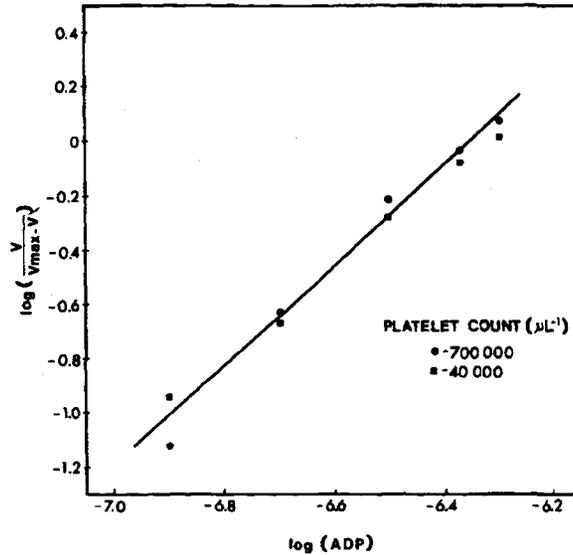


FIG. 4. Comparison of Hill plots for ADP-induced platelet shape change obtained at two different platelet concentrations (donor 2, female). N_H for a platelet count of $700\,000\ \mu\text{L}^{-1}$ was 1.98 and that for a platelet count of $40\,000\ \mu\text{L}^{-1}$ was 1.68. An analysis of covariance as described in Sokal and Rohlf (1969) was used to show that the slopes of these two Hill plots are not significantly different ($0.25 > p > 0.10$). Solid line is that obtained when the data are pooled; $N_H = 1.83$.

thermore, we have shown that the property $N_H > 1$ is not due to the influence of plasma-borne factors, nor to a large fraction of ADP being platelet bound, nor to an increased rate of platelet shape change reversion at low $[ADP]$. Karpatkin (1969a, 1969b) has demonstrated the existence of subpopulations of platelets which differ with respect to density, size, release reaction, and aggregation. However, binding sites of differing and independent affinities can only lead to $N_H < 1$.⁵ These observations strongly indicate that the kinetics we are observing reflect processes at the level of the platelet ADP interaction leading to shape change. However, our results do not specify the exact nature of the phenomena at the level of the platelet ADP receptor which result in an elevated N_H .

At face value, $N_H > 1$ suggests that ADP-induced platelet shape change is a positively cooperative process. This can imply that the binding of an ADP mole-

⁵ Indeed when

$$[3] \quad V = \sum_i \frac{f_i V_{\max_i} [ADP]}{[ADP] + K'_i}$$

where f_i is the fraction of receptors (platelets) with distinct V_{\max_i} , K'_i , then it can be shown that

$$d^2 \left(\frac{1}{V} \right) / d \left(\frac{1}{[ADP]} \right)^2 < 0 \text{ for all positive } f_i, K'_i, V_{\max_i}.$$

Hence a plot of V^{-1} versus $[ADP]^{-1}$ will curve downwards.

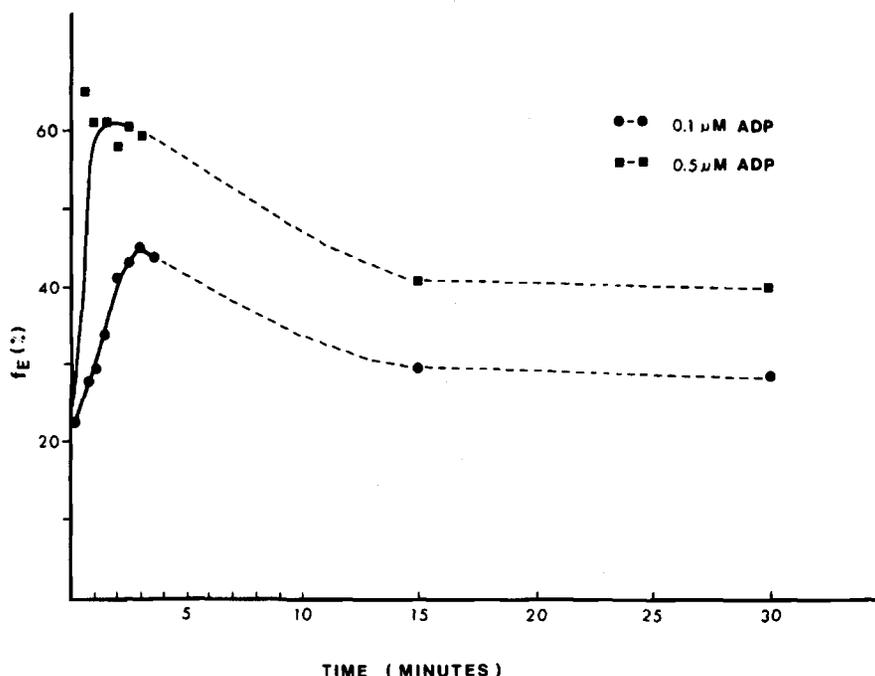


FIG. 5. Fraction of shape-changed platelets (f_s) expressed as a percent, as a function of time following addition of ADP.

cule at one receptor site somehow increases the likelihood of binding of ADP molecules at other receptor sites or that there is a cooperative enzymatic process leading to shape change subsequent to the binding of ADP to the receptor site. Positive cooperativity in membrane receptor mediated processes has been suggested previously (Podleski 1973; Rodbard et al. 1974; Jard et al. 1975).

An elevated value of N_H does not necessarily indicate a positively cooperative process. A value of $N_H > 1$ can arise when there is a threshold or "all-or-none" type response either at the level of binding to the receptor (Dixon and Webb 1964) which can arise when the binding of at least two molecules of ADP per receptor site is required to initiate shape change or at the level of the sequence of reactions subsequent to binding which leads to shape change (Rodbard 1973; Goldstein et al. 1974).

Binding studies of ADP to isolated membranes (Nachman and Ferris 1974) and intact platelets (Born and Feinberg 1975) suggest that there is no positive cooperativity at the level of the ADP-receptor binding. This suggests that the apparent positive cooperativity observed in our kinetic measurements arises at a step subsequent to the binding of ADP to the receptor. Previous authors (Podleski 1973; Rodbard et al. 1974) who have observed positive cooperativity in membrane receptor mediated processes have also tended to conclude that the cooperativity is due to processes subsequent to

the binding at the receptor. However, Bennett et al. (1978) have demonstrated that the number of "true" ADP receptors, ~ 2500 per platelet, is much smaller than the number of receptors determined in the earlier studies, e.g., 80 000 - 90 000 per platelet (Born and Feinberg 1975) and 100 000 - 200 000 per platelet (Nachman and Ferris 1974). It is possible that the presence of these additional receptors in the above binding studies masked the cooperativity of the binding of ADP to its "true" receptor. More careful binding studies will be required before the question of the cooperativity of the ADP-receptor binding process, or lack of, can be answered.

Moyle et al. (1976) have pointed out that an elevated N_H may also arise in a situation where there are "spare" receptors, i.e., when maximal response can be elicited when only a small fraction (e.g., 1-2%) of the available receptor sites are occupied. It is unlikely that the value of $N_H > 1$ observed here for ADP-induced platelet shape change can be explained in these terms. Bennett et al. (1978) observed that the maximal velocity of ADP-induced platelet shape change was decreased by the irreversible binding of the inhibitor, 5'-*p*-fluorosulfonyl benzoyl adenosine, to the ADP receptor sites only after at least 200 such binding sites were occupied by the inhibitor. As these authors estimated that there are ~ 2500 "true" ADP receptor sites per intact platelet, this suggests that V_{max} corresponds to greater than 90% occupancy of the receptor sites.

The kinetics of ADP-induced human and rabbit platelet shape change have been examined previously by light transmission methods (Born 1970; Favis and Colman 1978). Although Favis and Colman (1978) do not report a value of K' for ADP-induced human platelet shape change, the value of K' which can be obtained from the data presented in their communication, $4 \times 10^{-7} M$, is in excellent agreement with that obtained in our studies from the linear regions of the plots of V^{-1} versus $[ADP]^{-1}$ (corresponding to $[ADP] \geq 4 \times 10^{-7} M$), namely, $5.2 \times 10^{-7} \pm 2.6 \times 10^{-7}$ (microscope, eight donors) and $4.2 \times 10^{-7} \pm 1.1 \times 10^{-7} M$ (light transmission, six donors). It is of interest to note that closer examination of the data presented by these authors with emphasis placed on the values of V measured at low $[ADP]$ suggested an upward curving V^{-1} versus $[ADP]^{-1}$ plot similar to that reported in this communication.

Although the two methods we have used to measure V are in good agreement (compare, for example, $[ADP]_{1/2}$ in Table 1), it cannot be said that they agree exactly. In particular, N_H determined from light transmission data is typically slightly greater than that determined from data based on platelet morphologies (see Table 1). These differences may in part be due to the relative sensitivity of the two techniques for detecting platelet shape change. With the apparatus at our disposal it was technically more difficult to measure the initial slope of a small decrease in light transmission as opposed to determining directly whether a fraction of platelets have undergone shape change. A limitation of light transmission measurements is that the presence of pseudopods leads to a theoretically predicted increase in light transmission, opposite from the decrease expected for shape change, which is normally associated with pseudopod formation at the earliest stages of activation (Frojmovic 1978). On the other hand, the major limitation of measuring V by direct microscopic observation of platelet morphologies is that it ultimately rests on a subjective decision made on the part of the investigator. Our results do suggest that the differences in kinetics observed between the two methods are small enough that they can be used interchangeably for most practical purposes, as, for example, determining $[ADP]_{1/2}$.

A number of phenomena have been observed and (or) suggested to occur in conjunction with ADP-induced platelet shape change including the dissolution of the circumferential band of microtubules (White 1968), mobilization of intracellular Ca^{2+} (Grette 1962; Kinlough-Rathbone et al. 1975), and phosphorylation of ADP to ATP (Guccione et al. 1971; Mustard et al. 1975). Whichever of these processes is important for platelet shape change, it is clear that any proposed mech-

anism for shape change should also provide an explanation for the observed kinetics of the process. It is anticipated that careful studies with the actual ADP receptor molecule, possibly the 100 000 molecular weight membrane polypeptide isolated by Bennett et al. (1978), in light of the results reported here will greatly facilitate the search of the mechanism for platelet shape change.

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