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The Adenylate Kinase of Human Plasma, Erythrocytes and Platelets in Relation to the Degradation of Adenosine Diphosphate in Plasma

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1. Adenylate kinase (EC 2.7.4.3) has been shown to be present in human plasma obtained by conventional means and the adenylate-kinase activities of plasma and of lysed and intact human platelets and erythrocytes have been measured at 37° by sensitive spectrophotometric methods. 2. The activities found in plasma ranged from 2·7 to 22·9 μmoles of ADP formed/min./l. and in lysed platelets and lysed erythrocytes mean values of 0·79 and 12·0 μmoles of ADP formed/min./10^9 cells respectively were found. Intact platelets and erythrocytes showed little or no activity. 3. The apparent K_m of plasma adenylate kinase for ADP was found to be 1·4–1·6 mM. 4. The adenylate-kinase activity of plasma was correlated with the free haemoglobin present and the larger part of the activity could be accounted for by haemolysis occurring either during the withdrawal of the blood or *in vivo*. 5. Aggregation of platelets by ADP, collagen fibres or thrombin released up to 16% of the platelet adenylate kinase into the suspending medium. 6. Measurement of the rate of breakdown of 1·6 μM-ADP in plasma gave values of about 0·1 mμ-mole/min./ml. This was not increased by addition of sufficient erythrocyte lysate to increase the activity of plasma adenylate kinase five to ten times. 7. It was concluded that the activity of adenylate kinase found in plasma, even after aggregation of the platelets, is insufficient to account for the rate of breakdown of low concentrations of ADP usually observed, and that another enzyme is responsible for this process.

The aggregation of blood platelets by ADP is thought to play a part in both haemostasis and thrombosis (for reviews see Mustard, Rowsell & Murphy, 1964; Marcus & Zucker, 1965). In platelet-rich plasma this aggregation is spontaneously reversible (Born, 1962). The reversal process can be greatly accelerated by addition of enzymes which remove ADP, such as pyruvate kinase (in the presence of phosphoenolpyruvate), potato apyrase, snake-venom adenosine diphosphatase and adenylate kinase (Haslam, 1966). From these observations and the fact that rapid breakdown of ADP occurs in plasma, whether or not platelets are present (Ireland & Mills, 1964; 1966), it is inferred that degradation of ADP is responsible for the spontaneous reversal of aggregation. Adenylate kinase (EC 2.7.4.3), which catalyses reaction (1):

$$2ADP^{3-} \rightleftharpoons AMP^{2-} + ATP^{4-} \qquad (1)$$

has been observed in erythrocytes, leucocytes, platelets and plasma (Tatibana, Nakao & Yoshikawa, 1958; Cerletti & Bucci, 1960; Gross, 1961; Holmsen & Stormorken, 1965; Todd, Bell & Baron, 1964). In conjunction with enzymes which degrade AMP and ATP, adenylate kinase could in theory play a part in the irreversible breakdown of any ADP accessible to it, and a number of recent reports have suggested that adenylate kinase in platelets and plasma is in fact involved in the metabolism of ADP added to platelet-rich plasma (Kerby & Taylor, 1964; Holmsen & Stormorken, 1965; Turtle & Firkin, 1965). The results reported here indicate that adenylate kinase is always present in small amounts in platelet-free plasma, although largely originating from damaged erythrocytes. The adenylate-kinase activities of plasma and of lysed and intact platelets and erythrocytes have been measured by a sensitive assay procedure and evidence is presented that adenylate

kinase is not the principal enzyme involved in the breakdown of ADP at the low concentrations ($< 5\,\mu$M) which produce readily reversible platelet aggregation.

MATERIALS AND METHODS

Special chemicals. ATP, ADP, AMP, reduced glutathione and collagen were obtained from Sigma (London) Chemical Co. Ltd., London, S.W. 6. NADH, $NADP^+$, sodium phosphoenolpyruvate and all enzyme reagents were supplied by Boehringer Corp. (London) Ltd., Ealing, Middx. [8-^{14}C]ADP (specific activity 34 mc/m-mole) was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Benzidine hydrochloride ('for blood testing'; E. Merck A.-G., Darmstadt, Germany) was supplied by May and Baker Ltd., Dagenham, Essex, heparin (Pularin) by Evans Medical Ltd., Speke, Lancashire, bovine thrombin by Parke, Davis and Co., Detroit, Mich., U.S.A., and saponin (white) by British Drug Houses Ltd., Poole, Dorset. Triton X-100 was a gift from Lennig Chemicals Ltd., London, W.C. 1.

Silicone M441 (Imperial Chemical Industries Ltd., Stevenston, Ayrshire) was used for preparing silicone-treated glassware.

Venepunctures. Blood samples were obtained from healthy adult volunteers of both sexes by two procedures.

(*a*) Syringe bleeding. Blood was withdrawn into a dry 20 ml. plastic disposable syringe through a 19 gauge needle from a superficial vein in an arm in which the venous return was occluded by a sphygmomanometer cuff. After disconnection of the needle from the syringe the blood was ejected into a silicone-treated glass centrifuge tube containing 0·01 vol. of 1000 units of heparin/ml. of 0·154 M-NaCl.

(*b*) Free-flow venepuncture. Silicone-treated 17 gauge needles obtained from Fenwal Laboratories (Morton Grove, Ill., U.S.A.) were attached to short lengths of silicone rubber tubing [Esco (Rubber) Ltd., Seal Street, London, E. 8]. Each was inserted into a superficial arm vein after an intradermal injection of local anaesthetic. After the first 5 ml. of blood had flowed to waste the sphygmomanometer cuff was deflated and the blood was allowed to flow slowly into heparin solution in a silicone-treated glass centrifuge tube as described.

Platelet-rich and platelet-free plasma. The blood was immediately centrifuged at room temperature for 10 min. at 230*g* and the supernatant platelet-rich plasma was then carefully removed with a silicone-treated Pasteur pipette. Platelets were removed from platelet-rich plasma by centrifuging the plasma twice in an angle head at 10000*g* for 15 min. Both platelet-rich and platelet-free plasma were used within 4 hr. and were kept at room temperature to avoid the aggregation of platelets which occurs when heparinized platelet-rich plasma is cooled below 15–20°.

Lysates of platelets and erythrocytes. Platelet adenylate-kinase activity was usually measured as the difference between the adenylate-kinase activities of platelet-rich plasma treated with either 0·2% (w/v) saponin or 0·33% (v/v) Triton X-100 (final concentrations) and platelet-free plasma from the same donor. Neither saponin nor Triton X-100 at these concentrations affected the adenylate-kinase activity of platelet-free plasma. Alternatively platelets were isolated from blood containing EDTA as anticoagulant and were washed twice at 5° with buffered saline containing EDTA as described by Haslam (1964). These platelets were finally resuspended in a medium containing 135 mM-NaCl, 3·85 mM-KCl, 11·5 mM-tris–HCl buffer, pH 7·4, and 5·0 mM-glucose, and were lysed with 0·33% (v/v) Triton X-100. Platelets were counted by microscopy in a haemocytometer with 1 in 20 dilutions of platelet-rich plasma or of suspensions of washed platelets in 3·8% (w/v) sodium citrate.

Erythrocytes were washed three times at 10–15° by centrifugation at 500*g* and resuspension in 10 vol. of 0·154 M-NaCl, the buffy coat being rejected each time, and the packed cells were finally resuspended again in 10 vol. of 0·154 M-NaCl. Adenylate kinase was determined in lysates prepared by diluting this suspension 1 in 100 with water and the erythrocytes were counted in 1 in 100 dilutions in saline. Erythrocyte ghosts were prepared by addition of 9 vol. of 8·6 mM-EDTA to the suspension and were isolated by centrifugation at 20000*g* for 40 min. (C. Long, personal communication). The ghosts were washed four times by resuspension in 0·154 M-NaCl, dilution with 5 vol. of water and centrifugation, and were finally resuspended in 0·154 M-NaCl containing 0·5% (v/v) Triton X-100.

Measurement of adenylate kinase released from platelets during their aggregation. Samples of heparinized platelet-rich plasma or of suspensions of washed platelets (1·9–2·0 ml.) were incubated at 37° with stirring in the apparatus used for turbidimetric recordings of platelet aggregation (Haslam, 1964). After 5 min. temperature equilibration the aggregating agent under study was added to give a final volume of 2·0 ml. and the incubation was continued for a further 10 min. Samples were then centrifuged twice at 1750*g* for 10 min. at room temperature. Adenylate kinase was assayed in the supernatants from test samples and from controls treated in the same manner apart from the omission of the aggregating agent. The difference in adenylate-kinase activity between test and control samples was expressed as a percentage of the adenylate-kinase activity released by 0·33% (v/v) Triton X-100.

Assay of haemoglobin. Haemoglobin was estimated by the benzidine method (Hanks, Cassell, Ray & Chaplin, 1960) against standards of lysed erythrocytes from the same individual and was expressed as the equivalent number of erythrocytes. The method was modified slightly in that the maximum extinction at 550 mμ rather than the value after a standard time was used, since the colour developed more rapidly in plasma samples than in the standards.

Assay of adenylate kinase. Adenylate kinase was estimated in the forward direction by a method developed from the enzymic assay for ATP (Lamprecht & Trautschold, 1963), with conditions adjusted so that adenylate kinase was rate-limiting.

$$2\,ADP^{3-} \xrightarrow{\text{adenylate kinase}} AMP^{2-} + ATP^{4-}$$

$$ATP^{4-} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate}^{2-} + ADP^{3-} + H^+$$

$$\text{Glucose 6-phosphate}^{2-} + NADP^+ \xrightarrow{\text{glucose 6-phosphate dehydrogenase}} \text{glucono-}\delta\text{-lactone 6-phosphate}^{2-} + NADPH + H^+$$

The assay mixture (2·5ml.) contained 4·0mM-ADP, 10mM-glucose, 10mM-$MgCl_2$, 0·2mM-$NADP^+$, 100mM-triethanolamine–HCl buffer, pH7·5, 100μg. (14e.u.) of hexokinase, 20μg. (2·8e.u.) of glucose 6-phosphate dehydrogenase and up to 0·4ml. of sample for assay. The reduction of $NADP^+$ was followed at 340mμ at 37° in 1·0cm. cells in a Hilger–Gilford recording spectrophotometer. The ADP used in the assay was largely freed from ATP and AMP, which inhibit rabbit-muscle adenylate kinase (Callaghan & Weber, 1959), by chromatography on a DEAE-cellulose column (Staehelin, 1961). Addition to the reaction mixture of AMP equivalent to 10% of the ADP used caused a 21% inhibition of plasma adenylate-kinase activity, which is close to the value expected (18·5%) from the kinetic data of Callaghan & Weber (1959). The amounts of AMP produced during the course of the assay were negligible. After addition of the enzyme reagents to the assay mixture there was a rapid rise in extinction caused by residual traces of ATP in the ADP, followed by a very slow increase, which was probably due to impurities in the enzymes. The latter was allowed for by subtracting a reagent blank. Another blank in which the sample was added to reaction mixture from which ADP was omitted was sometimes included, but showed no increased rate of reduction of $NADP^+$. One unit of adenylate-kinase activity in the forward direction (F unit) was defined as 1μmole of ADP removed/min. at 37° under the conditions of assay described above. This assay procedure was shown to be linear with respect to adenylate-kinase concentration in the range of activities studied, with commercially purified rabbit-muscle adenylate kinase (Fig. 1).

The estimation of adenylate kinase in the backward direction was developed similarly from the enzymic assay for ADP (Adam, 1963).

$$AMP^{2-} + ATP^{4-} \xrightarrow{\text{adenylate kinase}} 2\,ADP^{3-}$$

$$ADP^{3-} + \text{phosphoenolpyruvate}^{3-} + H^+ \xrightarrow{\text{pyruvate kinase}} \text{pyruvate}^- + ATP^{4-}$$

$$\text{Pyruvate}^- + NADH + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate}^- + NAD^+$$

The assay mixture (2·5ml.) contained 2·0mM-AMP, 2·0mM-ATP, 1·0mM-phosphoenolpyruvate, 25mM-$MgSO_4$, 80mM-KCl, 0·1mM-NADH, 20mM-triethanolamine–HCl buffer, pH7·5, 100μg. (12·5e.u.) of pyruvate kinase, 50μg. (18e.u.) of lactate dehydrogenase and the sample under assay. In both the forward and backward assays the buffer concentrations were chosen so as to make the reaction mixture approximately iso-osmotic with plasma, making possible the measurement of the available adenylate-kinase activity of suspensions of intact cells. The extinction was followed at 340mμ as in the forward assay. After stabilization of the extinction after removal of ADP contaminating the ATP, samples of the material under assay were added to the test cell and to a blank from which AMP was omitted. The latter permitted correction for adenosine triphosphatase and other possible enzymes leading to NADH oxidation. One unit of adenylate-kinase activity in the backward direction (B unit) was defined as 1μmole of ADP formed/min. at 37°. This assay also was linear over the range used (Fig. 2). Dilute solutions of purified adenylate kinase showed a progressive loss of activity. When this was prevented by the addition of 0·2–0·5mg. of reduced glutathione/ml. of enzyme solution successive determinations of the forward and backward activities gave a forward to backward (F/B) ratio 1·5:1.

RESULTS

Metabolism of high concentrations of ADP and ATP in plasma. When heparinized plasma was incubated with 0·3–1·0mM-ADP at 37° the P_i released was equivalent to only about one-half of the ADP removed. The missing phosphate was found to be present in ATP. The results of such an experiment are given in Table 1 (incubation 1). Most of the ADP which disappeared was accounted for by the formation of AMP and ATP, and the difference probably represents the further breakdown of AMP which occurs in plasma (Jørgensen,

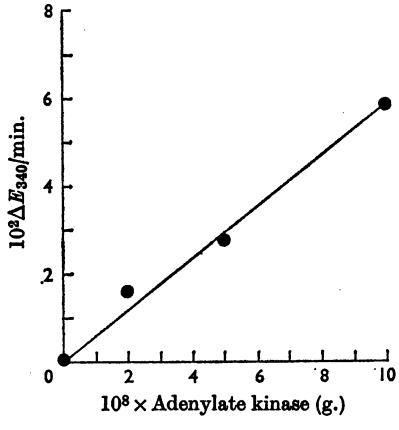

Fig. 1. Linearity of assay of purified rabbit-muscle adenylate kinase in the forward direction.

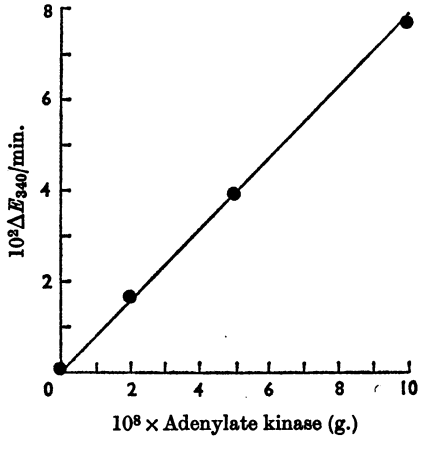

Fig. 2. Linearity of assay of purified rabbit-muscle adenylate kinase in the backward direction.

Table 1. *Breakdown of high concentrations of ADP and ATP in fresh and dialysed heparinized plasma*

Some plasma was used on the day of preparation (incubations 1 and 2) and the rest the following day (incubations 3 and 4) after dialysis overnight at 4° against Krebs–Henseleit Ringer bicarbonate lacking phosphate and including 5 units of heparin/ml. Portions (3·6ml.) of plasma were incubated at 37° for 2hr. with 0·4ml. of 0·154M-NaCl containing ADP (final concn. 0·80mM; incubations 1 and 3) or ATP (final concn. 0·46mM; incubations 2 and 4). The reactions were terminated by addition of 1·0ml. of ice-cold 2N-$HClO_4$. After centrifugation P_i was determined in the supernatant by the method of Allen (1940) and adenine nucleotides by enzymic methods (Adam, 1963). The results are expressed in mμmoles/ml. of incubation mixture.

	Undialysed		Dialysed	
Incubation	1	2	3	4
Substrate	ADP	ATP	ADP	ATP
Initial concn. ...	800	460	800	460
Nucleotide changes				
ATP	+137	−175	+182	−112
ADP	−445	+ 63	−442	+ 75
AMP	+272	+ 30	+254	+ 25
Nucleotide not accounted for	36	82	6	12
Calculated phosphate changes				
ATP	+411	−525	+546	−336
ADP	−890	+126	−884	+150
AMP	+272	+ 30	+254	+ 25
Phosphate not accounted for as nucleotides	207	369	84	161
Observed P_i release	222	245	160	185

1956; Ireland & Mills, 1964, 1966). The release of P_i expected from the nucleotide changes (Table 1) corresponds closely with that found. The simultaneous formation of ATP and AMP from ADP suggested that adenylate kinase was present in the plasma. More AMP than ATP accumulated during the incubation, but this can be attributed to the breakdown of ATP in plasma, which was measured in a parallel incubation (Table 1, incubation 2). In this case about half of the ATP removed appeared as ADP and AMP, and half was apparently broken down further. The expected release of P_i was considerably greater than that observed, confirming an earlier observation of Jørgensen (1956), who suggested that the phosphorylation of an unknown acceptor occurred. Dialysis of the plasma overnight to remove possible phosphate acceptors did not affect the rate of disappearance of added ADP, but ATP breakdown was decreased by 36% (Table 1, incubations 3 and 4). However, subsequent work has shown that plasma contains a pyrophosphate-forming adenosine triphosphatase (Mills, 1966), which can account for the discrepancy in phosphate release in incubation 2. This enzyme is apparently inactivated by dialysis, which increased the ratio of ATP to AMP formed from ADP from 0·50 to 0·72. The latter value is closer to the ratio (1·0) expected from the action of adenylate kinase. The presence of adenylate kinase in plasma was confirmed by the specific assay procedures.

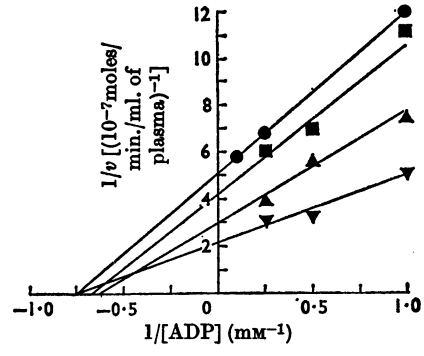

Fig. 3. Lineweaver–Burk plots of the plasma adenylate-kinase activities of four normal males assayed in the forward direction.

Assay of adenylate kinase in plasma, platelets and erythrocytes. In the first instance adenylate-kinase activity was measured by both the forward and backward assays in 0·2ml. samples of plasma from blood taken by syringe from eight normal males. The mean values found were respectively 22·3 F units/l. (range 12·3–48·5) and 11·4 B units/l. (range 5·7–22·9) and the correlation coefficient between the two assays was 0·96. The ratio of the mean forward and backward activities was 1·96, which is somewhat higher than the value obtained with purified muscle adenylate kinase. The

forward assay was used with ADP concentrations from 1 to 10mM to determine the apparent K_m for ADP of the adenylate kinase in the plasma of four individuals (Fig. 3). The values found (1·4–1·6mM) are close to the value (1·58mM) obtained for muscle adenylate kinase by Callaghan & Weber (1959).

Platelet adenylate kinase was assayed with 0·05–0·1ml. samples of lysates of platelet-rich plasma. A mean value $0·79 \pm 0·052$ (S.E.M.) B unit/10^9 platelets was found in a group of donors comprising nine males and three females. No difference in platelet adenylate-kinase activities was detected between the sexes. Measurement of adenylate kinase in the backward direction in samples of unlysed platelet-rich plasma gave the same results as with the corresponding platelet-free plasma, indicating that platelet adenylate kinase is not normally available to extracellular AMP and ATP (Table 2). The average activity of lysed washed platelets from four donors was 1·03 (range 0·74–1·25) B units/10^9 platelets, which is slightly higher than found in platelets in platelet-rich plasma, and in one experiment (Table 3) the activity of adenylate kinase in washed platelets was actually shown to be higher than that in the corresponding platelets in their original plasma. This may represent selective recovery of the larger platelets during the washing procedure. Assay of adenylate kinase in suspensions of intact washed platelets revealed the presence of 0–6% of the activity found after lysis. Even after incubation of washed platelets with stirring for 15min. at 37° in the presence of Ca^{2+} ions only about 10% of the total platelet adenylate-kinase activity could be assayed in unlysed suspensions. Most of this activity had leaked into the suspending medium and did not represent available activity within the platelets (see, e.g., Table 3).

Assay of adenylate kinase in 0·1ml. samples of water lysates of washed erythrocytes from a group of 12 normal males gave a mean value of $12·0 \pm 0·64$ (S.E.M.) B units/10^9 cells (see Table 4). Addition of 0·1ml. samples of saline suspensions containing approx. 10^8 intact erythrocytes to both the backward and forward assay systems indicated that the externally available adenylate-kinase activity, if any, was very small compared with that of the same cells when lysed (<0·8%). Larger numbers of erythrocytes could not be added because of their effect on the extinction of the assay mixture. Preparations of erythrocyte ghosts contained variable amounts of haemoglobin but the

Table 2. *Adenylate-kinase activities of corresponding platelet-free and lysed and unlysed platelet-rich plasma*

Subject	Adenylate-kinase activity of platelet-free plasma (B units/l.)	Adenylate-kinase activity of unlysed platelet-rich plasma (B units/l.)	10^{-6} × Platelet count (platelets/ml.)	Lysing agent	Adenylate-kinase activity of lysed platelet-rich plasma (B units/l.)	Platelet adenylate-kinase activity (B unit/10^9 platelets)
1	10·8	8·0	249	Triton X-100	236	0·90
2	10·9	11·8	161	Triton X-100	173	1·00
3	7·4	7·4	250	Triton X-100	197	0·76
4	4·8	3·7	373	Saponin	314	0·83
5	4·2	<2	364	Saponin	320	0·87

Table 3. *Activities of adenylate kinase in washed and unwashed platelets from the same donor*

Twice-washed platelets were prepared from platelet-rich plasma containing EDTA as described in the Materials and Methods section. The suspension was incubated with a final concentration of 1·1mM-$CaCl_2$ for 15min. at 37°, and was stirred for the last 12½min. Adenylate kinase was assayed in the incubated suspension and in supernatant prepared from it by centrifuging twice at 1750*g* for 10min. at room temperature. Total platelet adenylate-kinase activity was determined after lysis of both platelet-rich plasma and washed suspension with 0·33% Triton X-100. The platelet-rich plasma and suspension of washed platelets contained 322 and 202 × 10^6 platelets/ml. respectively.

	Activity of platelet suspension (B units/l.)	Activity of suspending medium (B units/l.)	Activity of lysed suspension (B units/l.)	Activity of platelets (B units/10^9 platelets)
Washed platelet suspension	25·3	18·7	232	1·06
Platelet-rich plasma	—	8·2	278	0·84

Table 4. *Activities of adenylate kinase in erythrocytes and in plasma and amounts of haemoglobin in plasma from 12 normal males bled by free-flow venepuncture*

The activity of adenylate kinase equivalent to the plasma haemoglobin of each subject was calculated as the product of his own erythrocyte activity and plasma haemoglobin, and the activity of adenylate kinase in plasma which cannot be accounted for by complete lysis of erythrocytes was calculated as the difference between the observed plasma activity and that calculated from the plasma haemoglobin.

Subject	Erythrocyte adenylate-kinase activity (B units/10^9 erythrocytes)	Observed plasma adenylate-kinase activity (B units/l.)	Plasma haemoglobin (erythrocytes equiv. $\times 10^{-9}$/l.)	Adenylate-kinase activity equivalent to plasma haemoglobin (B units/l.)	Adenylate-kinase activity in plasma not accounted for by complete lysis of erythrocytes (B units/l.)
1	12·6	5·2	0·37	4·7	0·5
2	16·1	5·3	0·27	4·3	1·0
3	8·5	3·9	0·38	3·2	0·7
4	14·1	10·6	0·50	7·1	3·5
5	8·8	9·1	0·53	4·7	4·4
6	13·6	6·6	0·47	6·4	0·2
7	13·2	5·4	0·42	5·5	−0·1
8	10·3	2·7	0·13	1·3	1·4
9	10·5	3·8	0·20	2·1	1·7
10	13·0	5·2	0·17	2·2	3·0
11	11·4	8·0	0·44	5·0	3·0
12	12·3	4·7	0·20	2·5	2·2
Mean	12·0	5·9	0·34	4·1	1·8
S.E.M.	0·64	0·70	0·041	0·54	0·42

ratio of adenylate-kinase activity to haemoglobin content was always less than or equal to that of the erythrocytes from which they were derived. For example, one preparation was found to contain 6·9% of the original cellular protein (estimated by the method of Lowry, Rosebrough, Farr & Randall, 1951), 5·0% of the haemoglobin and 2·2% of the adenylate-kinase activity of the original lysate. These findings suggest that adenylate kinase is not specifically associated with the erythrocyte membrane.

Source of plasma adenylate kinase. The high adenylate-kinase activities in platelets and particularly in erythrocytes suggested that part at least of the adenylate kinase in plasma might be derived from them, as for example 0·01% haemolysis could account for the average activity found. This was confirmed by the finding that the activities in different plasma samples from the same individual varied widely and correlated with the plasma haemoglobin concentrations. For example, one donor bled by syringe had plasma adenylate kinase and haemoglobin values of 15·8 B units/l. and $1·22 \times 10^9$ erythrocytes equivalent/l. respectively, but when the same donor was bled by the free-flow venepuncture technique to reduce haemolysis to a minimum the corresponding figures were 5·8 B units/l. and $0·58 \times 10^9$ erythrocytes equivalent/l. Even when every possible precaution was taken to prevent haemolysis there was still a correlation between plasma adenylate kinase and haemoglobin. This is indicated in Fig. 4, in which are plotted the plasma adenylate-kinase and haemoglobin values from the 12 blood samples used for assay of erythrocyte adenylate kinase, which were taken by free-flow venepuncture (see also Table 4). The correlation coefficient between plasma adenylate

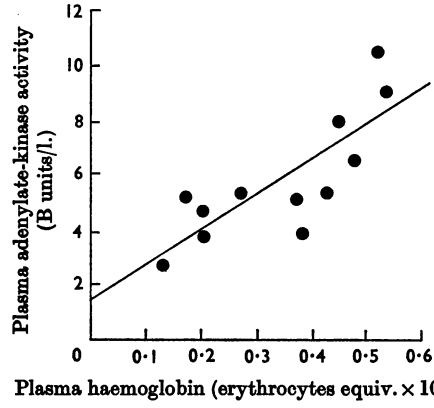

Fig. 4. Correlation between the adenylate-kinase activities and free haemoglobin in heparinized platelet-free plasma from twelve normal males bled by free-flow venepuncture (see also Table 4). The regression of plasma adenylate kinase against plasma haemoglobin is plotted.

kinase and plasma haemoglobin is 0·80, which is significant at the 0·1% level. However, the regression of adenylate kinase against haemoglobin intersects with the adenylate-kinase axis at 1·4 B units/l. (Fig. 4), suggesting that on average this amount of the enzyme in plasma was not associated with free haemoglobin and was probably derived from a source other than the erythrocytes. The value for the activity of adenylate kinase equivalent to the amount of free haemoglobin in the plasma, calculated from the erythrocyte activity of the same individual, was smaller than that actually found in 11 of the 12 donors (Table 4). The mean value for this excess of adenylate-kinase activity was $1·8 \pm 0·42$ (S.E.M.) B units/l., which could include not only enzyme derived from sources other than the erythrocytes but also contributions from a preferential release of adenylate kinase from erythrocytes or a preferential removal of haemoglobin from the circulation. However, the slope of the regression of plasma adenylate kinase against plasma haemoglobin (Fig. 4) suggests that these last two factors are relatively unimportant. In conclusion the results suggest that on average some 70% of the low adenylate-kinase activity found in plasma from blood taken by free-flow venepuncture is liberated from erythrocytes, either *in vivo* or during the preparation of the plasma samples. The results do not indicate whether or not the remainder is derived from the platelets.

Release of adenylate kinase from platelets under physiological conditions. Platelets are known to release many of their constituents into the suspending medium during their aggregation by thrombin or collagen fibres, but it has been generally supposed that ADP alone does not induce such a release (see e.g. Marcus & Zucker, 1965). These three aggregating agents were therefore examined for activity in releasing from platelets adenylate kinase which might contribute to the activity of the enzyme in plasma either *in vivo* or *in vitro*.

Variable results were obtained on addition of ADP to heparinized platelet-rich plasma (Fig. 5). Material from each donor studied showed some release of adenylate kinase in the presence of a sufficiently high concentration of ADP, but the concentration required to initiate release of the enzyme ranged from 0·5 μM to more than 10 μM, whereas 2 μM-ADP was always sufficient to induce the maximum rate and extent of platelet aggregation. The percentage of adenylate kinase released even with the highest concentration of ADP studied (25 μM) never exceeded 16%. The liberation of the enzyme appeared to be only loosely correlated with the extent of platelet aggregation as measured by the maximum decrease in extinction of the suspension but it was inversely related to

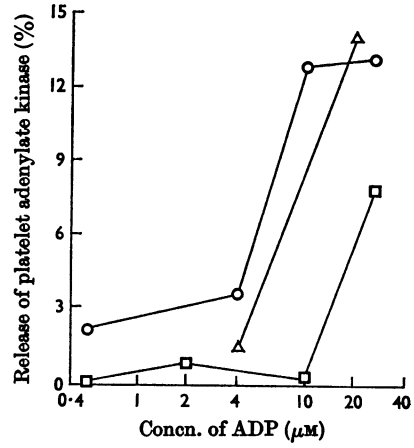

Fig. 5. Release of adenylate kinase from platelets into heparinized plasma during their aggregation by different concentrations of ADP. After 5 min. preincubation of 1·95 ml. samples of platelet-rich plasma at 37°, 0·05 ml. of 0·154 M-NaCl containing various concentrations of ADP was added. ○, □ and △, Experiments with three different platelet-rich plasma samples containing respectively 381, 280 and 670×10^6 platelets/ml. Other details are described in the Materials and Methods section.

the extent to which the platelets disaggregated spontaneously. This suggests that platelets which had lost adenylate kinase were not easily separated after the breakdown of the ADP in the plasma, perhaps as a reflection of their loss of structural integrity. The reason for the variable release of the enzyme is not clear. In another experiment not shown in Fig. 5 addition of 0·2 mM-2-chloroadenosine 5 min. before 4 μM-ADP almost abolished the aggregation of the platelets and reduced the liberation of adenylate kinase from 12·0% to zero. Similar amounts of adenylate kinase were released on addition of ADP to citrated platelet-rich plasma prepared from blood containing 1 vol. of 3·8% (w/v) trisodium citrate/10 vol. These results show that ADP itself can induce a limited release of adenylate kinase from platelets. Whether there is a compartment of the enzyme in each platelet which is readily liberated or whether a fraction of the platelets are particularly susceptible to complete lysis by ADP remains uncertain.

The addition to heparinized platelet-rich plasma from two donors of sufficient of a fine suspension of collagen fibres in 0·154 M-sodium chloride to cause complete platelet aggregation within 3 min. also induced the release of 10·6% and 15·9% of platelet adenylate kinase. Similarly when bovine thrombin was added to two suspensions of washed platelets containing 1·1 mM-calcium chloride to give a final concentration 0·1 N.I.H. unit/ml.,

which is sufficient to cause rapid and complete aggregation of the platelets, 6·6% and 13·6% of the platelet adenylate kinase was liberated. It thus appears that the maximum percentage of the platelet adenylate kinase which is available for release is the same with these two aggregating agents as with ADP.

In other experiments the amount of enzyme released into serum was measured after clotting of whole blood collected without anticoagulant and also after clotting of recalcified citrated platelet-rich plasma (Table 5). The amount released was variable but was never greater in serum from whole blood than in serum from the same donor's platelet-rich plasma (allowing for dilution on recalcification). This suggests that most of the adenylate kinase released into serum formed from whole blood is derived from the platelets rather than from the erythrocytes. In some experiments (e.g. Table 5, Expt. 2) appreciably more than 16% of the platelet adenylate kinase was released during clotting of platelet-rich plasma. It may be that fibrin formation in the plasma causes a greater disruption of the platelet structure with liberation of adenylate kinase than aggregation of platelets by ADP, thrombin or collagen fibres.

Effect of haemolysis on the breakdown of micromolar concentrations of ADP in plasma. To determine whether the release of adenylate kinase from erythrocytes (or platelets) could affect the rate at which low concentrations of ADP are degraded in plasma, the breakdown of 1·6 μM-[8-^{14}C]ADP was studied in plasma supplemented with either a small quantity of water or of erythrocyte lysate from the same donor. In each of three experiments (Table 6) there was no change in the amount (approx. 1 mμmole) of ADP removed in 10 min. on increasing the activity of adenylate kinase in plasma five to ten times. Both AMP and adenosine were formed from the [8-^{14}C]ADP during the incubations but, in marked contrast with the results obtained with high ADP concentrations (see Table 1), no ATP accumulated. The [8-^{14}C]ADP used in these experiments was contaminated with 15–25% of AMP but, in view of the high K_i for inhibition of

Table 6. *Effect of addition of erythrocyte lysate on the breakdown of* 1·6 μM-[8-^{14}C]*ADP in heparinized plasma*

Nine volumes of platelet-free plasma were mixed with 1 vol. of either (a) water or (b) erythrocyte lysate. Samples (0·45 ml. or 0·9 ml.) of each of these mixtures were incubated with 0·05 ml. or 0·1 ml. of 16 μM-[8-^{14}C]ADP at 37° for 10 min. and the reactions were then stopped by addition of 5·0 ml. of ice-cold 10% (w/v) trichloroacetic acid containing carrier nucleotides. Blanks in which acid was added immediately after the ADP were included. The radioactive compounds were extracted by means of charcoal, separated by paper electrophoresis and assayed for radioactivity in a liquid-scintillation counter. The amount of ADP remaining after each incubation was calculated from the amount added and the proportion of the total count recovered as ADP, after application of factors to correct for variations in the recovery of individual compounds from the charcoal. The methods used have been described in detail by Ireland & Mills (1966).

Expt. no.	Adenylate-kinase activity in incubation mixture (B units/l.)	ADP removed (mμmoles/ml.)
1(a)	2·1	0.63
(b)	18·4	0·64
2(a)	4·3	1·08
(b)	18·9	1·03
3(a)	5·0	1·02
(b)	39·7	1·01

Table 5. *Release of adenylate kinase during clotting of whole blood and platelet-rich plasma*

A mixture of 9 ml. of whole blood obtained by free-flow technique and 1 ml. of 0·154 M-NaCl was allowed to clot in a glass tube incubated at 37° for 2 hr. and the serum above the retracted clot was then assayed for adenylate kinase. Blood from the same donor (9 vol.) was also mixed with 1 vol. of 3·8% (w/v) trisodium citrate. Platelet-rich and platelet-free plasma samples were prepared from this material as described in the Materials and Methods section. The activities of adenylate kinase in the platelet-free plasma and in platelet-rich plasma lysed with Triton X-100 were both assayed. Platelet-rich plasma, mixed with 0·3 vol. of 0·11 M-$CaCl_2$, was incubated in a glass tube at 37° for 2 hr. and the adenylate kinase in the supernatant serum was then assayed. In calculating the percentage of the enzyme released during clotting of platelet-rich plasma corrections were applied both for the activity in platelet-free plasma and for the dilution on recalcification.

Expt. no.	Activity of serum from blood (B units/l.)	Activity of platelet-free plasma (B units/l.)	Activity of lysed platelet-rich plasma (B units/l.)	Activity of serum from platelet-rich plasma (B units/l.)	Platelet adenylate kinase released into serum from platelet-rich plasma (%)
1	18·4	6·5	270	16·5	5·5
2	50·2	6·7	432	83·7	23·6

adenylate kinase by AMP (0·5mM; Callaghan & Weber, 1959), no significant inhibition would be expected. The results therefore indicate that adenylate kinase does not play a significant role in the breakdown of ADP which occurs under the conditions of these experiments, and they provide evidence for the existence of another enzyme in plasma capable of converting ADP into AMP. It also follows that the amounts of adenylate kinase released from erythrocytes and platelets under physiological conditions are unlikely to accelerate appreciably the breakdown of the ADP involved in platelet aggregation.

DISCUSSION

Assay of adenylate kinase by three-stage enzymic methods. Assays for adenylate kinase based on the coupled enzyme reactions which we have used have also been described by others (Kornberg & Pricer, 1951; Oliver, 1955; Todd *et al.* 1964) but theoretical objections have been raised by Bergmeyer (1963), who maintains that, to obtain accurate results, each enzyme in the reaction chain must have more than 100 times the activity of the preceding enzyme. This view appears to be based on the assumption that the reaction catalysed by the enzyme under assay is first-order and that a significant change in substrate concentration occurs. In fact, in most coupled enzyme assays, including the present ones, the first reaction is effectively zero-order and, provided that the K_m values of the auxiliary and indicator enzymes for the intermediate substrates are low compared with the concentration of the initial substrate, a moderate excess of their activities over the enzyme under assay is sufficient to prevent a significant accumulation of intermediates before a steady state is reached. After addition of adenylate kinase to either the forward or backward assay there was a short lag period representing the time required for the intermediate substrates to reach steady-state concentrations; then the change in extinction with time was linear. The difference between the projection of the linear part back to zero time and the true initial extinction gave a measure of the accumulated intermediates in terms of the equivalent amount of $NADP^+$ or NADH. This was always less than 1% of the initial substrate concentration, even with the highest adenylate-kinase activities measured. Neither a 20-fold increase in glucose 6-phosphate dehydrogenase in the forward assay nor a fivefold increase in lactate dehydrogenase in the backward assay increased the measured adenylate-kinase activity.

Activities of adenylate kinase in constituents of the blood. The adenylate-kinase activity of lysed erythrocytes has been assayed by a number of workers using different methods. The values obtained (see Table 7) are appreciably lower than those reported here although exact comparison is not possible because of differences in the experimental conditions, including the temperature. Kashket & Denstedt (1958) claimed that adenylate kinase is present in the erythrocyte membrane and available to external substrates, whereas Askari & Fratantoni (1964) found adenylate kinase in erythrocyte ghosts which was activated by Na^+ or K^+ ions. However, others (Cerletti & De Ritis, 1962; Sen & Post, 1964) have prepared erythrocyte ghosts free of adenylate-kinase activity. The present work shows that if there is erythrocyte-adenylate-kinase activity accessible to extracellular substrates it is very small compared with the total activity in the cells. No selective binding of the enzyme to erythrocyte ghosts was found, but Green *et al.* (1965) have shown that, at least with glycolytic enzymes, the degree of binding to the stromal fraction varies widely with the method of preparation.

Previous measurements of the adenylate-kinase activity in platelets (Table 7) have given values close to that reported here, allowing for differences in the temperature of assay. Turtle & Firkin (1965) recently described the conversion of high concentrations of ADP (2–4mM) into AMP and ATP and vice versa in platelet-rich plasma and in suspensions of washed platelets and attributed this effect to the action of adenylate kinase situated in or on the platelets. This conclusion is not supported by our results, which indicate that platelet adenylate kinase is not normally available to external AMP and ATP and that the activity in platelet-rich plasma or suspensions of washed platelets is extracellular. A small proportion (up to 16%) of the platelet adenylate-kinase activity was released by agents that caused platelet aggregation, such as ADP, collagen fibres and thrombin, and variable amounts were released during clotting of platelet-rich plasma or blood. The release of the enzyme by relatively low concentrations of ADP was unexpected and indicates that ADP alone may induce more gross changes in the platelet than has been previously supposed, although evidence has already been presented that micromolar concentrations of ADP can release platelet nucleotides (Haslam, 1966).

While this work was in progress Todd & Baron (1965) reported values for normal plasma adenylate kinase, assayed in the backward direction at 25°, in the range 1–35 units/l. (mean 13 units/l.). This range of activities embraces those found with both our techniques of taking blood and the higher values are larger than any found in this study. In contrast with our results, Todd & Baron (1965) found no correlation between plasma adenylate

Table 7. *Available information on the adenylate-kinase activity of human blood constituents*

F (forward direction) and B (backward direction) are discussed in the Materials and Methods section.

	Method of assay			Erythrocyte adenylate-kinase activity		Platelet adenylate-kinase activity		Plasma adenylate-kinase activity	
Reference	Principle	Conditions	F/B	Units of original paper	μmoles/min./10^9 cells	Units of original paper	μmoles/min/10^9 cells	Units of original paper	μmoles/min./l.
This paper	Direct enzymic coupling to NADH oxidation	ATP 2mM, AMP 2mM, pH7·5, 37°	B	—	12·0	—	0·79	—	2·7–22·9
Overgaard-Hansen (1958)	Assay of residual ADP after incubations; adenylate-deaminase method	ADP 0·62mM, pH7·4, 37°	F	3000μmoles/min./l. of erythrocytes	0·3	—	—	—	—
Tatibana *et al.* (1958)	Incorporation of ^{32}P from [^{32}P]ADP into glucose 6-phosphate in the presence of hexokinase	ADP 1·0mM, pH7·0–9·0, 30°	F	0·097μmole/min./mg. of protein	3·9	—	—	—	—
Cerletti & Bucci (1960)	Estimation of incubation products after chromatographic separation	38°, pH7·4 ADP 2mM	F	0·35μmole/8min./0·5mg. of haemoglobin	2·8	0	0	0*	0
		38°, pH7·4 ATP 1mM AMP 1mM	B	0·40μmole/8min./0·5mg. of haemoglobin	3·2	—	—	—	—
Gross (1961)	Direct enzymic coupling to NADH oxidation	Optimum substrate concn.; pH7·5, 25°	B	500μmoles/hr./g.	0·8	600μmoles/hr./g.	0·2	—	—
Holmsen & Stormorken (1965)	Assay of residual ADP after incubations; coupled enzymic method	ADP 0·72mM, pH8·75, 37°	F	—	—	—	—	2·8%/min.†	20
Todd & Baron (1965)	Direct enzymic coupling to NADH oxidation	ATP 1mM, AMP 1mM, pH7·5, 25°	B	20μmoles/min./g.	2·0	14μmoles/min./g.	0·28	13μmoles/min./l.	13

* Value found in serum.

† Value given in corrigendum (personal communication).

kinase and haemoglobin in the range 3–18 units/l., and they believe that plasma adenylate kinase arises by variable damage to the platelets during preparation of heparinized platelet-free plasma. The evidence presented in this paper indicates that the low activity found in heparinized plasma prepared from freely flowing blood as described above is derived mainly from erythrocytes, although there is a proportion (on average 30%) of a separate origin which may include some released from platelets. This discrepancy probably arises from differences in the methods of preparation of heparinized platelet-free plasma. On the other hand, our results support the view of Todd & Baron (1965) that adenylate kinase in serum is largely derived from platelets. Holmsen & Stormorken (1965) have also reported the presence of adenylate kinase in plasma, although the value they give (11·1% of ADP removed/min. at 37° and pH 8·75 in the first-order range of ADP concentration) is very high as a result of lysis of residual platelets on freezing and thawing of the plasma (personal communication). Their corrected value for platelet-free plasma (2·8% of ADP removed/min.) is of the same order as that reported here. The rate of breakdown of a high concentration of ADP found in the experiment described in Table 1 is much lower than that previously reported by Ireland & Mills (1966) in experiments in which syringe bleeding almost certainly resulted in a greater degree of haemolysis. Kerby & Taylor (1964) have found evidence that adenylate kinase is concerned in the metabolism of high concentrations of ADP in sonically treated platelet-rich plasma, but this is unlikely to be relevant to conditions *in vivo* (see below).

The question arises whether the adenylate kinase in plasma which is associated with free haemoglobin is released by haemolysis *in vivo* or during preparation of the plasma. The amount of plasma haemoglobin can be no higher than the lowest value found with the best technique. Hanks *et al.* (1960) found normal plasma haemoglobin values in the range 0·16–0·58 mg./100 ml., which corresponds to $0{\cdot}05–0{\cdot}19 \times 10^9$ erythrocytes equivalent/l., i.e. somewhat lower than the range we found ($0{\cdot}13–0{\cdot}53 \times 10^9$ erythrocytes equivalent/l.). Calculating from the regression of plasma adenylate kinase against plasma haemoglobin (Fig. 4) and the mean value for plasma haemoglobin found by Hanks *et al.* (1960) an average value of 3·0 B units/l. for the activity of adenylate kinase in plasma *in vivo* is obtained. As this is likely to be an overestimate it can be concluded that the activity found in plasma prepared in the usual way is largely an artifact.

Relevance of adenylate kinase to the enzymic breakdown of ADP in plasma. If the rate of removal of ADP from plasma by adenylate kinase declines linearly from the apparent K_m of the enzyme (1·4–1·6 mM) to micromolar concentrations, the activities of the enzyme found can account for the observed rate of disappearance from plasma of high concentrations (0·1–1·0 mM) of ADP but not for the removal of the lowest concentration used (1·6 μM). This conclusion is confirmed by our failure to stimulate the breakdown of 1·6 μM-ADP by the addition of small amounts of erythrocyte lysate to plasma. The results show that μM-ADP is broken down in plasma at a rate of about 0·1 mμmole/min./ml. This would require an adenylate-kinase activity, as assayed under our conditions, of about 250 F units/l. or 128 B units/l., which is much higher than found in any plasma samples, even after aggregation of platelets by ADP or collagen fibres. The results indicate therefore that it is very unlikely that adenylate kinase plays a part in the breakdown of the low concentrations of ADP that are probably involved in aggregation of platelets *in vivo*. Indeed, investigation of the degradation of ADP at very low concentrations has revealed the existence of another enzyme in plasma which may play an important role in the control of platelet adhesiveness (Mills, 1966).

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REFERENCES

Adam, H. (1963). In *Methods of Enzymatic Analysis*, pp. 539, 573. Ed. by Bergmeyer, H. U. London: Academic Press (Inc.) Ltd.

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.

Askari, A. & Fratantoni, J. C. (1964). *Proc. Soc. exp. Biol., N.Y.*, **116**, 751.

Bergmeyer, H. U. (1963). In *Methods of Enzymatic Analysis*, p. 10. Ed. by Bergmeyer, H. U. London: Academic Press (Inc.) Ltd.

Born, G. V. R. (1962). *Nature, Lond.*, **194**, 927.

Callaghan, O. H. & Weber, G. (1959). *Biochem. J.* **73**, 473.

Cerletti, P. & Bucci, E. (1960). *Biochim. biophys. Acta*, **38**, 45.

Cerletti, P. & De Ritis, G. (1962). *Clin. chim. Acta*, **7**, 402.

Green, D. E., Murer, E., Hultin, H. O., Richardson, S. H., Salmon, B., Brierley, G. P. & Baum, H. (1965). *Arch. Biochem. Biophys.*, **112**, 635.

Gross, R. (1961). In *Blood Platelets*, p. 410. Ed. by Johnson, S. A., Monto, R. W., Rebuck, J. W. & Horn, R. C. London: J. and A. Churchill Ltd.

Hanks, G. E., Cassell, M., Ray, R. N. & Chaplin, H., jun. (1960). *J. Lab. clin. Med.* **56**, 486.

Haslam, R. J. (1964). *Nature, Lond.*, **202**, 765.

Haslam, R. J. (1966). *American Lecture Series: Symp. Physiology of Haemostasis & Thrombosis, Detroit.* Ed. by Johnson, S. A. & Seegers, W. Springfield, Ill.: Charles C. Thomas (in the Press).

Holmsen, H. & Stormorken, H. (1965). *Scand. J. clin. Lab. Invest.*, **17**, suppl. 84, 138.

Ireland, D. M. & Mills, D. C. B. (1964). *Biochem. J.* **92**, 30P.

Ireland, D. M. & Mills, D. C. B. (1966). *Biochem. J.* **99**, 283.

Jørgensen, S. (1956). *Acta pharm. tox., Kbh.*, **12**, 294.

Kashket, S. & Denstedt, O. F. (1958). *Canad. J. Biochem. Physiol.* **36**, 1057.

Kerby, G. P. & Taylor, S. M. (1964). *Thromb. Diath. Haemorr.* **12**, 510.

Kornberg, A. & Pricer, W. E., jun. (1951). *J. biol. Chem.* **193**, 481.

Lamprecht, W. & Trautschold, I. (1963). In *Methods of Enzymatic Analysis*, p. 543. Ed. by Bergmeyer, H. U. London: Academic Press (Inc.) Ltd.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Marcus, A. J. & Zucker, M. B. (1965). *The Physiology of Blood Platelets*, pp. 65–72. New York: Grune and Stratton Inc.

Mills, D..C. B. (1966). *Biochem. J.* **98**, 32P.

Mustard, J. F., Rowsell, H. C. & Murphy, E. A. (1964). *Amer. J. med. Sci.* **469**, 127.

Oliver, I. T. (1955). *Biochem. J.* **61**, 116.

Overgaard-Hansen, K. (1958). *Acta pharm. tox., Kbh.*, **14**, 67.

Sen, A. K. & Post, R. L. (1964). *J. biol. Chem.* **239**, 345.

Staehelin, M. (1961). *Biochim. biophys. Acta*, **49**, 11.

Tatibana, M., Nakao, M. & Yoshikawa, H. (1958). *J. Biochem.* **45**, 137.

Todd, J. K., Bell, J. L. & Baron, D. N. (1964). *Biochem. J.* **90**, 7P.

Todd, J. K. & Baron, D. N. (1965). *Research in Muscular Dystrophy*, p. 212. London: Pitman Medical Publishing Co.

Turtle, J. R. & Firkin, B. G. (1965). *Australas. Ann. Med.* **14**, 282.