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The influence of salicylate on platelets and whole blood adenine nucleotides

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1. The addition of sodium salicylate to freshly withdrawn human blood or native platelet-rich plasma significantly delays platelet aggregation *in vitro*.

2. The administration of acetylsalicylic acid to human subjects also significantly delays platelet aggregation in their whole blood and there is a short delay in the formation of fibrin but this is not statistically significant.

3. Salicylate, whether added to human blood *in vitro* as sodium salicylate or given by mouth as acetylsalicylic acid, significantly reduces the platelet clumping activity of adenosine diphosphate (ADP) added to whole blood *in vitro*.

4. The administration of aspirin in high doses for several days produces a marked increase in the total adenine nucleotide content of whole blood. The percentage of adenosine triphosphate (ATP) was increased, that of ADP decreased while there is an obvious increase in the ATP : ADP ratio.

5. There is little correlation between the plasma salicylate level and the delay aspirin produces in platelet aggregation *in vitro* or the changes that occur in the levels of ADP or ATP in whole blood during administration of aspirin.

6. Significant correlations do occur, however, between the delay in platelet aggregation *in vitro* and (*i*) the percentage increase in the ATP concentration, (*ii*) the percentage decrease in ADP concentration, (*iii*) the percentage change in the ATP : ADP ratio observed during aspirin administration.

Certain types of thrombotic disease usually presenting as pulmonary infarction but accompanied by focal myocardial necrosis are thought to result from intravascular platelet clumping and its sequelae (Hughes & Tonks, 1968a). Administration of aspirin in high dosage exerts remarkable benefit in the acute phase of this disease (Hughes & Tonks, 1968b) and will be further described (Hughes, 1969). In the light of these clinical observations it was decided to investigate the influence of salicylate on platelet aggregation both *in vitro* and during administration of high doses of aspirin to human subjects. Determinations of whole blood adenine nucleotide levels were performed *in vitro* together with the observations on platelet aggre-

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gation before and during aspirin administration. Preliminary accounts of this work have already appeared (Davies, Hughes & Tonks, 1968a, b).

Methods

Adenosine diphosphate (ADP). All solutions of the disodium salt of ADP (C. F. Boehringer and Soehne, Mannheim) were freshly prepared in ice cold 0·9% NaCl and stored at 2° C. Solutions more than 4 hr old were discarded.

Sodium salicylate B.P. (B.D.H.). Solutions for addition to whole blood *in vitro* were made isotonic with NaCl. Sodium salicylate solutions in concentrations above 3×10^{-1}M were hypertonic, in these cases sodium salicylate was dissolved in deionized water and used as such, no visible haemolysis was seen on addition to blood.

Acetylsalicylic acid. Enteric coated tablets (300 mg) were given.

Glassware and needles. All glassware was chemically cleaned by soaking overnight in chromic acid or in a 2% aqueous solution of RBS 125 (Medical Pharmaceutical Development Ltd.) followed by washing and rinsing in at least six changes of deionized glass distilled water before drying in a hot air oven.

Siliconed glassware and needles were prepared by immersion in a 2% solution of silicone fluid MS. 1107 (Hopkins and Williams) in ethyl methyl ketone. The glassware and needles were then air dried and cured in a hot air oven for 1 hr at 150° C.

Measurement of platelet aggregation in non-anticoagulated freshly withdrawn whole blood in vitro

Platelet aggregation was determined by a method previously described by Hughes & Tonks (1965, 1966). All experiments were carried out at room temperature (18°–23° C). Blood was withdrawn from the antecubital vein with a siliconed G-19 needle into a siliconed glass syringe. Immediately after withdrawal some of the blood was distributed into siliconed glass tubes containing either one-fiftieth of its volume of isotonic chloride which acted as a control or a similar volume of a solution of the drug under test. No anticoagulants were added. After centrifugation for 1 min at 450 *g* at room temperature the supernatant platelet-rich plasma was left in contact with the red cells and examined microscopically at 5 min intervals to determine the platelet aggregation time and the first appearance of fibrin. The remainder of the blood sample was used to harvest native platelet rich plasma by the method described below.

Measurement of platelet aggregation in native platelet-rich plasma

An aliquot of the blood was added to one-fiftieth of its volume of isotonic solutions of sodium chloride or sodium salicylate in plastic tubes and centrifuged for 10 min at 450 *g*. The supernatant platelet-rich plasma was then removed to siliconed glass tubes with a plastic syringe, avoiding contamination with red and white blood cells, and examined microscopically as above. No anticoagulants were used in this procedure.

Determination of adenine nucleotides

The adenine nucleotides adenosine monophosphate (AMP), ADP and adenosine triphosphate (ATP) were determined enzymatically by the methods of Adams (1965) utilizing the test combinations as supplied by C. F. Boehringer and Soehne, Mannheim, West Germany. The methods depend on the formation of nicotinamide adenine dinucleotide (NAD) in stoichiometric ratio to the amount of nucleotides present in the test solution which is measured spectrophotometrically as NADH oxidized at 340 mμ and 366 mμ. All measurements were made at 366 mμ on a Unicam SP.500 spectrophotometer to avoid interference caused by the absorption by salicylate at lower wavelengths (Gould, Huggins & Smith, 1963).

Salicylate has been shown to inhibit reversibly dehydrogenase enzymes (Bryant, Smith & Hines, 1963; Smith & Bryant, 1963; Smith, Bryant & Hines, 1964; Hines & Smith, 1964; Dawkins, Gould, Sturman & Smith, 1967; Davies, 1967); the inhibition involving competition with NADH, increasing concentrations of which reverse the inhibition. Since dehydrogenase enzymes are involved in the determination of the adenine nucleotides we felt that salicylate could interfere with the determinations by inhibiting these enzymes. However, when salicylate in concentrations in excess of those found in test solutions were added to solution of nucleotides they did not interfere with their determination (Davies, 1967).

Plasma salicylate

Plasma salicylate was determined by the method of Trinder (1954).

Results

Effect of sodium salicylate on platelet aggregation in freshly withdrawn human whole blood

As can be seen in Table 1, sodium salicylate caused a delay in platelet aggregation, which became significant at a final concentration of 2×10^{-3}M (28 mg/100 ml. whole blood) and above.

Figure 1 clearly demonstrates the linear relationship between salicylate concentration added to whole blood *in vitro* and the percentage delay in the time taken for 100 μ platelet clumps to appear.

Effect of sodium salicylate on platelet aggregation in freshly prepared non-anticoagulated platelet-rich plasma

Sodium salicylate added to whole blood in a final concentration of 3×10^{-3}M prolonged the platelet clumping time by 37·2% (see Fig. 1). Separation of platelet-

TABLE 1. *Effect of adding sodium salicylate to whole blood* in vitro *on the time taken for the appear ance of 100 μ platelet clumps*

No. of experiments	Whole blood salicylate concentration ($\times 10^{-3}$M)	Time (min. ± S.E.M.) for appearance of 100 μ platelet clumps in		Significance of difference *P*
		Blood and saline	Blood and salicylate	
10	1·00	27·5±5·1	31·0±4·9	N.S.
46	2·00	38·2±2·3	49·8±2·3	<0·005
9	3·00	46·5±5·4	64·8±6·8	<0·05
10	4·00	23·9±3·9	37·2±3·6	<0·05

rich plasma from the red and white blood cells in these whole blood samples resulted in a 33·6% prolongation of the clumping time with this concentration of sodium salicylate.

Removal of platelet rich plasma from red and white cells in itself delays the onset of platelet aggregation. Thus the first appearance of 100 μ platelet clumps in the control whole blood samples used here was in $46{\cdot}5 \pm 5{\cdot}4$ min (Table 1) and in the control platelet-rich plasmas separated from these blood samples, $59{\cdot}7 \pm 3{\cdot}29$ min. This prolongation is significant ($P<0.05$). Additionally, sodium salicylate, 3×10^{-3}M significantly ($P<0{\cdot}001$) prolonged the clumping time in these separated platelet-rich plasmas to $79{\cdot}8 \pm 3{\cdot}09$ min for the nine samples.

Effect of acetylsalicylic acid taken orally by men on platelet aggregation in whole blood in vitro

Venous blood was withdrawn from nineteen male subjects (34–58 yr) immediately before and 3–7 days after receiving 4 to 6 g acetylsalicylic acid by mouth daily when the mean plasma salicylate level was $24{\cdot}6 \pm 1{\cdot}3$ mg/100 ml.

At this plasma salicylate level there was a considerable delay in the time taken for platelets to form 100 μ clumps *in vitro* in whole blood: 54 ± 5 min compared with the pre-treatment clumping time of $28{\cdot}5 \pm 3{\cdot}5$ min ($P<0.001$).

The formation of fibrin was delayed from 43 ± 2 min to 47 ± 3 min after administration of aspirin but the difference is not statistically significant. This strongly suggests that in this experimental system salicylate does not inhibit platelet aggregation by preventing fibrin formation, since, if this were so, one would expect fibrin formation to be delayed by at least as much as platelet aggregation. That this delay in platelet aggregation was not a chance occurrence was shown by subjecting

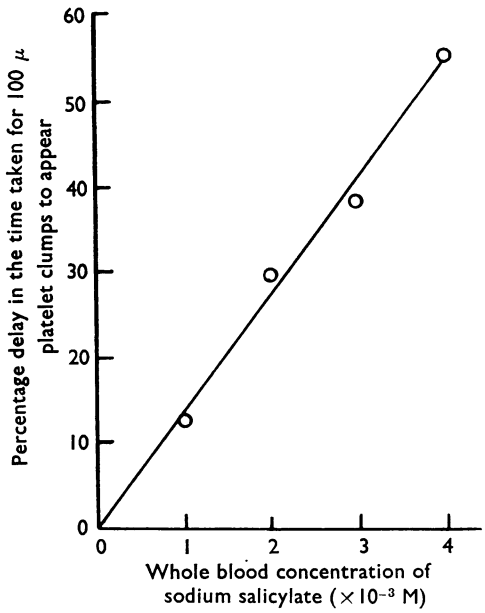

FIG. 1. Effect of sodium salicylate on the platelet clumping time of freshly withdrawn human venous blood. Each point represents the mean of nine or more separate experiments.

a control group of nineteen age-matched male subjects to two venipunctures at 3–7 day intervals and determining platelet aggregation time. A mean delay of 2·8% was seen on the occasion of the second puncture of the control group compared with a delay of 88% in the test group receiving aspirin. No significant changes occurred in the haematocrit readings of either group during the 3–7 day period.

Effect of sodium salicylate added to normal human blood on platelet aggregation induced by adenosine diphosphate in vitro

Hughes & Tonks (1966) showed that ADP (1×10^{-8}M) added to whole blood caused immediate platelet aggregation followed by a period of disaggregation when discrete platelets reappear and clumps disappear, a pattern which has been observed by Born (1962) and other workers using different test systems

Figure 2 shows the effect of sodium salicylate (3×10^{-3}M) on platelet aggregation induced by ADP (1×10^{-7}M).

In the whole bood sample added to saline alone the platelets remained discrete up to about 30 min ; aggregation then commenced, with large clumps being present by

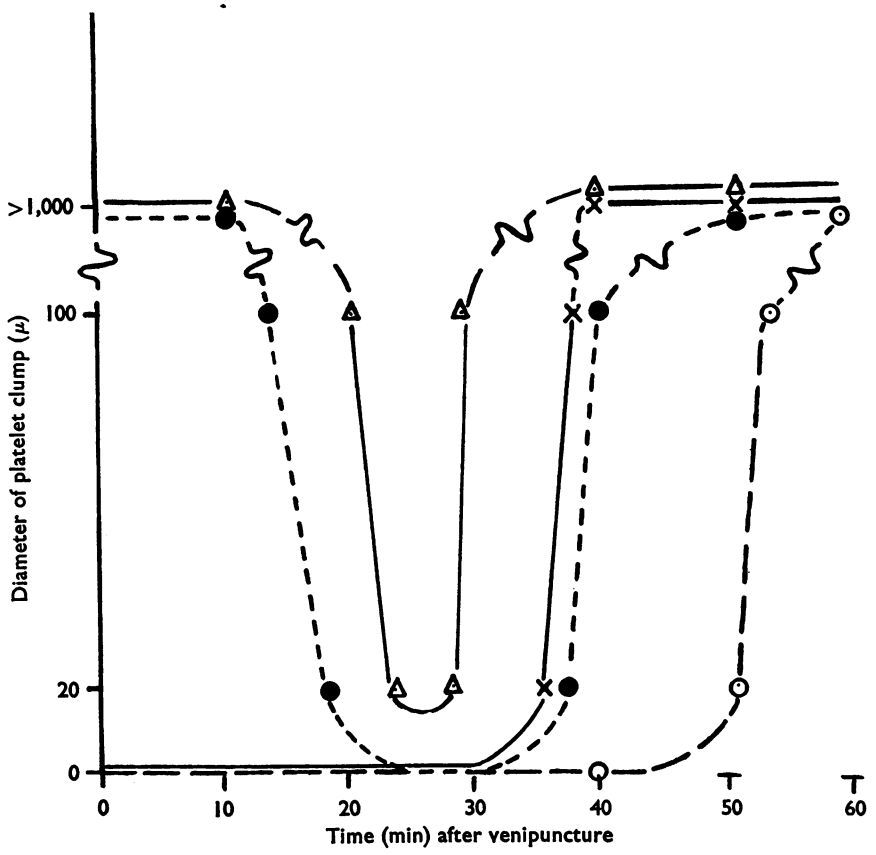

FIG. 2. Effect of sodium salicylate (in a final concentration of 3×10^{-3}M) on platelet clumping induced by adenosine diphosphate (final concentration 10^{-7}M). Each point is the mean of nine individual experiments. —×—, Blood and saline (control); —△—, blood and ADP; - - -○- - -, blood and sodium salicylate; - - -●- - -, blood and sodium salicylate + ADP.

about 40 min. In the case of sodium salicylate there was a delay in platelet clumping with no aggregation before 40 min; 55 min elapsed before the appearance of large clumps. ADP (1×10^{-7}M) caused immediate aggregation of platelets into large clumps followed by a period of disaggregation, then secondary irreversible aggregation ensued. When sodium salicylate was added together with ADP, it accelerated the disaggregation of platelets and also prolonged the period of disaggregation delaying the second phase of platelet aggregation beyond that seen in the saline control.

Effect of aspirin administration on platelet aggregation induced in vitro *by adenosine diphosphate and whole blood adenine nucleotide levels*

Platelet aggregation induced *in vitro* by adenosine diphosphate and the levels of blood adenine nucleotide were determined just before and during aspirin administration in eleven male patients (36 to 57 yrs) with thrombotic disease. The aspirin was administered in doses of 4–6 g daily for 3–7 days before performing the tests for the second time.

As shown in Fig. 2, ADP 1×10^{-7}M caused immediate platelet aggregation followed by disaggregation before secondary irreversible aggregation ensued. This also occurred with concentrations of $1 \times$ and 5×10^{-8}M ADP, but not with ADP in a concentration of 5×10^{-9}M. Therefore, in Fig. 3, which shows the effect of aspirin on ADP induced platelet aggregation in whole blood, the time taken for the first appearance of 100 μ platelet clumps is given for the saline controls and the

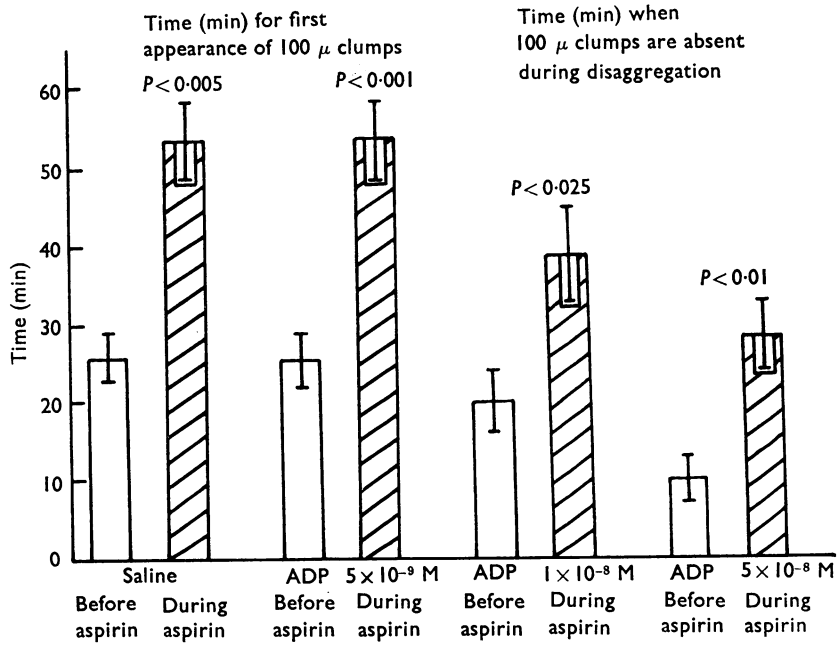

FIG. 3. Effect of aspirin administration on the response of platelets in whole blood to ADP *in vitro*. Aspirin was administered in form of 300 mg enteric coated tablets to eleven male patients (36–57 yr) in doses of 4–6 g daily. Platelet clumping was determined just before and after aspirin administration for 3–7 days at which time the patients had a mean plasma salicylate of 23·2 + 1·8 mg/100 ml. Vertical bars represent mean times ± S.E.M.

lowest concentration of ADP used. With the two higher concentrations of ADP the times during which 100 μ clumps were absent in the period of disaggregation are recorded. In all cases, there was a significant delay of platelet aggregation during aspirin administration.

Table 2 gives the values for the determinations of adenine nucleotide levels on aliquots of the same blood samples used in platelet aggregation experiments described above. The results are given in mg/100 ml. whole blood and also as the amount of each nucleotide expressed as a percentage of the total whole blood adenine nucleotides. During aspirin administration there was a significant increase

TABLE 2. *Effect of aspirin administration (4–6 g daily by mouth) on the whole blood adenine nucleotide levels of eleven male patients (36 to 57 yr)*

	Mean levels ± S.E.M.						
	ATP		ADP		AMP		Total adenine nucleotides
Time of estimation	mg/100 ml.	% of total	mg/100 ml.	% of total	mg/100 ml.	% of total	mg/100 ml.
Before aspirin administration	30·60±1·81	84·50±0·78	3·95±0·10	11·13±0·47	1·48±0 07	4·16±0·31	36·02±2·01
During aspirin administration	36·80±1·35	87·30±0·59	3·83±0·13	9·16±0·13	1·54±0 09	3·53±0·28	42·07±1·33
Significance of difference (*P*)	<0·02	<0·02	N.S.	<0·01	N.S.	N.S.	<0·05

Values are expressed as mg/100 ml. and as a percentage of total adenine nucleotide for the three nucleotides.

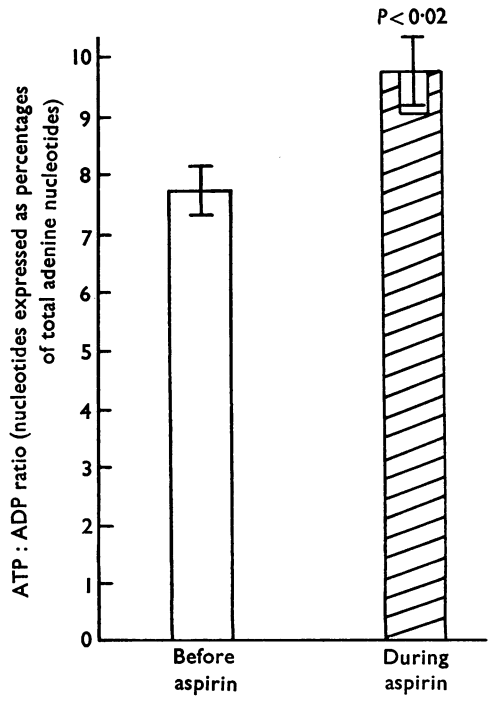

FIG. 4. Ratio of ATP to ADP in whole blood before and during aspirin administration to eleven male patients (36–57 yr). Adenine nucleotide levels were determined immediately before and 3–7 days after commencing aspirin therapy (4–6 g daily in the form of 300 mg enteric coated tablets). The eleven patients had a mean plasma salicylate of 23·2±1·8 mg/100 ml. when the adenine nucleotides were determined during aspirin administration. Vertical bars represent mean values ± S.E.M.

in ATP and total adenine nucleotide levels, while there was also a significant increase in the percentage ATP, a significant decrease in the percentage ADP and a significant increase in the ATP : ADP ratio from (7·76 to 9·75) during aspirin administration (Fig. 4).

No significant changes were observed in the haematocrit values of these patients during this period of aspirin administration.

Relation between plasma salicylate levels, adenine nucleotide levels and platelet aggregation in vitro

Table 3 shows the level of correlation between the delay in platelet clumping *in vitro*, the plasma salicylate level and the percentage changes in adenine nucleotide balance during aspirin administration.

The table demonstrates a significant positive correlation between the delay in platelet clumping and the percentage increase in ATP, the percentage decrease in ADP and the percentage change in ATP:ADP ratio. The correlations between these parameters and plasma salicylate levels were not significant.

Discussion

Various authors have described how platelets aggregate more readily in the presence of erythrocytes. When anticoagulants are absent, as in the methods described here, the influence of red cells is similar, that is, the platelet clumping time in supernatant platelet-rich plasma left in contact with red cells is considerably shorter than that of platelet-rich plasma previously separated from red cells. The addition of sodium salicylate *in vitro* to such samples produces in each a proportional prolongation of platelet clumping time. Thus it is unlikely that salicylate added *in vitro* to whole blood delays platelet clumping by its influence on red cell

TABLE 3. *Levels of correlation between the delay in platelet clumping* in vitro, *the plasma salicylate concentration and percentage changes in adenine nucleotide concentration during aspirin treatment* (4–6 *g daily by mouth*) *in eleven male patients* (36 *to* 57 *yr*)

Correlation between *X*	and *Y*	Correlation coefficient (*Y*)	Level of significance (*P*)
Delay in platelet clumping	Plasma salicylate level	+0·372	N.S.
Delay in platelet clumping	Percentage decrease in ADP (expressed as % of total adenine nucleotides)	+0·692	<0·01
Delay in platelet clumping	Percentage increase in ATP (expressed as % of total adenine nucleotides)	+0·665	<0·05
Delay in platelet clumping	Percentage change in ATP: ADP ratio	+0·676	<0·05
Plasma salicylate level	Percentage decrease in ADP (expressed as % of total adenine nucleotides)	+0·111	N.S.
Plasma salicylate level	Percentage increase in ATP (expressed as % of adenine nucleotides)	+0·307	N.S.
Plasma salicylate level	Percentage change in ATP: ADP ratio	+0·087	N.S.

metabolism. Morris (1967), Zucher & Peterson (1968) and O'Brien (1968b) have shown that salicylic and acetylsalicylic acids inhibit ADP induced platelet aggregation in citrated platelet-rich plasma in the absence of erythrocytes. Sodium salicylate and aspirin also reduce this activity of ADP in blood containing red cells but with no anti-coagulants as reported here.

Compared with *in vitro* results, salicylate given by mouth, as aspirin, has a greatly enhanced effect in delaying platelet clumping for a given concentration and a considerable influence upon red cell metabolism. Morris (1967) demonstrated that administration of acetylsalicylic acid reduces adhesiveness of platelets to glass in citrated platelet-rich plasma. Weiss & Aledort (1967) showed that aspirin administration reduced platelet aggregation by washed connective tissue fragments but not by ADP; this was confirmed by the observations of O'Brien (1968a). Further, O'Brien (1968b), Zucker & Peterson (1968) and Macmillan (1968) have shown that aspirin administration inhibits the second phase of platelet aggregation accompanied by release of platelet constituents induced by ADP in citrated platelet-rich plasma.

The effect of erythrocytes on platelet adhesiveness and aggregation has been investigated by several workers. Hellem (1960) described a factor in erythrocytes; later identified as ADP by Gaarder, Jonsen, Laland, Hellem & Owren (1961) which caused platelet aggregation. Hellem, Borchgrevink & Ames (1961) showed that platelet adhesiveness was decreased in anaemia, a close relationship existing between the haematocrit values and the number of glass adhesive platelets suggesting that erythrocytes increased platelet adhesiveness by virtue of their contents. Caspary (1965) observed that platelet adhesiveness measured in platelet-rich plasma was far less than that seen in whole blood. This difference was associated with the presence of erythrocytes and since it could be prevented by the addition of adenosine the author concluded that the increased adhesiveness could be due to ADP released from erythrocytes. Further evidence that ADP released from erythrocytes causes increased platelet adhesiveness to glass has been provided by Harrison & Mitchell (1966). They showed that if ADP released from erythrocytes is removed by enzymatic phosphorylation the level of whole blood platelet adhesiveness is reduced to that seen in platelet rich plasma.

These observations on the effects of erythrocyte ADP on platelet adhesiveness agree with the results presented in this paper. Since between 98 and 99% of whole blood adenine nucleotides is contained within erythrocytes, the results in Table 2 show essentially changes in erythrocyte adenine nucleotides during aspirin administration. The ATP:ADP ratio is significantly shifted towards ATP (see Table 3 and Fig. 4) which could play a part in delaying platelet aggregation. This view is supported by the significant correlations that exist between the delay in platelet aggregation and the relative decrease in ADP; the relative increase in ATP and the increase in the whole blood ATP:ADP ratio during aspirin administration. Using the methods described for the enzymatic determination of adenine nucleotides we were unable to detect any of these substances in plasma. We are, therefore, unable to say whether the small amounts of adenine nucleotides that may leak from erythrocytes do so in similar proportions to their intracellular content. However, Rørvik, Holmsen & Stormokren (1968) showed that adenine nucleotides released from citrated whole blood during passage through a column of glass beads was in the same proportion as the whole blood contents of these substances. Further support derives from the present results which show a close correlation between the

delay in platelet clumping and the changes in red cell adenine nucleotides achieved by salicylate administration. Rørvik, Holmsen & Stormokren (1968) also showed that the release of adenine nucleotides from erythrocytes was related to the degree of haemolysis as adenine nucleotides and haemoglobin were released in the same proportion as their intracellular contents. Inglot & Wolna (1968) have shown that salicylate inhibits the hypotonic haemolysis of erythrocytes; whether this phenomenon of erythrocyte membrane stabilization by salicylate is of any significance in whole blood remains to be determined. However, in considering the effect of salicylate upon the blood only, it seems possible that part of its effect in delaying platelet clumping may be mediated through shifting the adenine nucleotide away from ADP towards ATP, especially as ADP is so active in clumping platelets in minute concentrations in plasma.

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