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GLYCOLYSIS IN HUMAN BLOOD IN THE PRESENCE OF SODIUM SALICYLATE AND THE IMPORTANCE OF THE INCUBATION MEDIUM

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Abstract—Sodium salicylate in final concentrations of 1 to 5 × 10^{-3} M increases glucose utilization and lactate production in heparinized human whole blood and in separated washed human erythrocytes suspended in Tyrode–Locke's solution or in potassium phosphate buffer with added glucose when incubated at 37°. The stimulant action of salicylate varies considerably with the incubation medium, being extremely active on erythrocytes suspended in Tyrode–Locke's solution (61 per cent increase in glucose utilization) and showing only a marginal activity (4 per cent increase in glucose utilization) on those suspended in the phosphate buffer with added glucose. Its effect on whole blood was quite marked (43 per cent increase in glucose utilization). The rates of anaerobic glycolysis in each of these three different types of cell suspensions are also quite different, one from another. These differences are discussed as are the differences in the effectiveness of salicylate in these media. The mode of action of salicylate on erythrocytes is considered.

A SIGNIFICANT positive correlation exists between the delay in platelet clumping and the increase in whole blood ATP:ADP ratio seen in human subjects taking aspirin.[1,2] The increase in whole blood ATP to ADP ratio induced by aspirin administration may be mediated by one of two mechanisms; a decreased utilisation or an increased synthesis of high energy phosphate bonds. Since it is well known that phosphorylation is coupled to glycolysis in erythrocytes the effect of salicylate on glucose consumption and lactate production was measured in heparinized human whole blood. It is frequently convenient to perform metabolic studies on red cells washed free from most of the other formed elements of the blood, from clottable and other proteins and from the influence of anticoagulants. This work has therefore been extended to determine whether the type of artificial incubation medium used can influence the effectiveness of a drug on cell metabolism. Thus, additionally, the effect of sodium salicylate on glucose uptake and lactate production in erythrocytes has been studied using washed human erythrocytes incubated in two different media, Tyrode–Locke's solution or a potassium phosphate buffer with added glucose (after Sturman and Smith[3]).

MATERIALS

"Analar" quality reagents were used for the incubation media and solutions were

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prepared in glass distilled deionized water. Tyrode–Locke's solution had the following composition: NaCl 6·36 g, KCl 0·2014 g, $CaCl_2$ 0·2 g, $NaHCO_3$ 1 g, NaH_2PO_4 0·066 g, $MgCl_2$ 0·2134 g, Glucose 2 g, Water to 1 l. Sodium salicylate B.P. was obtained from British Drug Houses Ltd. Glucose oxidase and lactate test packs were supplied by C. F. Boehringer and Soehne, Mannheim, Germany. Lithium heparin plastic tubes from Stayne Laboratories Ltd., High Wycombe, Bucks, U.K.

METHODS

Incubation of whole blood

Human venous blood from the antecubital vein of healthy individuals or convalescing hospital patients was added to lithium heparin plastic tubes. The blood was immediately incubated at 37° prior to distribution into plastic tubes containing 1/20th of its volume of 0·9 per cent sodium chloride solution or isotonic solutions of sodium salicylate in 20-fold concentrates of the final concentrations desired, which in these studies was in the range $1 \times$ to 5×10^{-3} M. The tubes were then incubated at 37° with constant agitation, samples being removed for glucose and lactate determinations immediately after mixing the blood sample with saline or salicylate and at 90-min intervals thereafter.

Preparation of erythrocyte suspensions

Freshly withdrawn heparinized venous blood was centrifuged at 3000 *g* for 10 min in a swing-bucket centrifuge and the supernatant plasma and buffy layer discarded. Erythrocytes were washed three times in the Tyrode–Locke's solution before the final resuspension and a haematocrit adjustment to 50 per cent. The suspension was incubated for a few minutes at 37° before aliquots were removed and mixed with 1/20th of their volume of either 0·9 per cent sodium chloride solution or isotonic solutions of sodium salicylate, as above, to give final concentrations of 1, 2 or 5×10^{-3} M salicylate. Incubation at 37° with constant agitation was then continued, with samples being removed (for glucose and lactate estimations) immediately after mixing the erythrocyte suspension with saline or salicylate and at 90 min intervals thereafter.

In determining the influence of incubation media on the response of cell metabolism to salicylate a direct comparison was made on each individual sample of erythrocytes. Freshly withdrawn heparinized human venous blood was divided into two samples, and the erythrocytes were separated from the plasma and buffy layers as described above. One sample was washed and resuspended to a 50 per cent haematocrit in Tyrode–Locke's solution as outlined above and the other sample washed three times in 0·1 M potassium phosphate buffer pH 7·4 with 0·01 M glucose (PPBG) before the final resuspension and haematocrit adjustment. By trial a much lower haematocrit—28 per cent—was used with PPBG because of the high rate of red cell glycolysis in PPBG. The two erythrocyte samples in their differing suspending media were brought to 37° before aliquots of each were mixed with 1/20th of their volume of 0·9 per cent sodium chloride solution or an isotonic sodium salicylate solution to give a final concentration of 5×10^{-3} M. Samples were then removed immediately for glucose and lactate determinations and again after 180 min incubation at 37° with constant agitation.

Glucose levels in plasma or incubation medium were determined by the glucose

oxidase method[4] and lactate by the enzymatic method of Hohorst.[5] Spectrophotometric readings were made at 366 mμ to avoid possible interference by salicylate at lower wavelengths. Concentrations of glucose and lactate are expressed as a μmole/100 ml packed cells. pH values of whole blood and erythrocyte suspensions were determined on the first and last samples withdrawn for glucose and lactate estimations, using an E.I.L. blood pH meter with standard phosphate buffers of pH 8·839 and 7·412.

RESULTS

The marked rate of glucose utilization in the control samples of whole blood at 37° compared with those of erythrocytes in Tyrode–Locke's solution is clearly evident in Fig. 1. It is approximately three times faster in whole blood and significantly faster

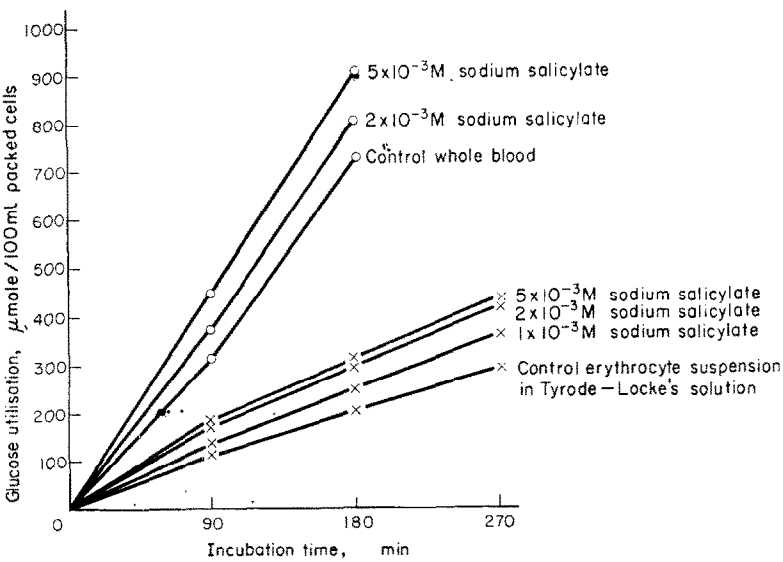

FIG. 1. Glucose utilization in heparinized human whole blood (○) and in washed human erythrocytes in Tyrode–Locke's solution (×) incubated at 37° alone and in the presence of increasing concentrations of sodium salicylate. Each point the mean of sixteen determinations for whole blood (○) and nineteen to twenty-three determinations for the erythrocyte suspensions (×).

($P < 0{\cdot}001$) in the second 90-min period of incubation than in the first 90 min. After 180 min of incubation at 37° over 60 per cent of the initial *plasma* glucose level had been exhausted with the control whole blood samples (100 μmole glucose = 18 mg glucose). Tyrode–Locke's solution with nearly twice the glucose level of normal plasma (i.e. 180 mg/100 ml) supports glucose utilization over a 270-min period with an almost constant rate of utilization, which is in agreement with Ponder.[6] Figure 1 also demonstrates the stimulant action of sodium salicylate on glucose utilization in whole blood and in erythrocytes in Tyrode–Locke's solution. With a final concentration of 5×10^{-3} M salicylate the residual plasma glucose is almost completely exhausted after 180 min incubation in the whole blood samples. When the stimulant action of sodium

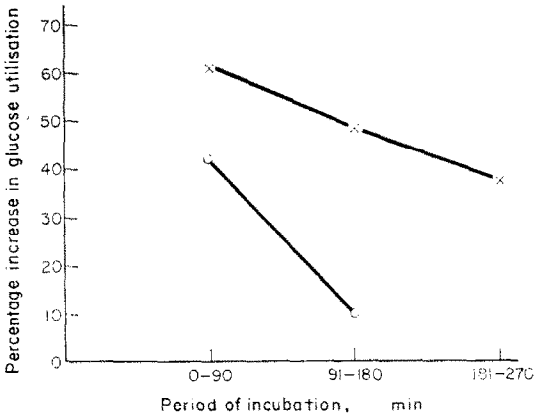

FIG. 2. Decline in the stimulating action of sodium salicylate (5×10^{-3} M) on glucose utilization in heparinized human whole blood (○) and in washed human erythrocytes suspended in Tyrode–Locke's solution (×) during succeeding 90-min periods of incubation at 37°. The effect of salicylate is expressed as a percentage of the glucose utilization of the control samples during each 90-min period. Each point is the mean of sixteen observations with whole blood (⊙) and sixteen observations with erythrocyte suspensions (×).

salicylate on glucose utilization is estimated in whole blood or in erythrocytes in Tyrode–Locke's solution for each 90-min period of incubation, a decline in its stimulant action appears to occur after the first 90 min. This is seen in Fig. 2 where the stimulant effect of salicylate (5×10^{-3} M) is expressed as a percentage increase of glucose utilization compared with the control in each period of 90 min incubation at 37°. It can also be seen that salicylate has a greater stimulant action on the utilization of glucose by erythrocytes suspended in Tyrode–Locke's solution than in whole blood.

Tables 1 and 2 record the rate of glucose utilization and lactate production in heparinized human whole blood and in separated washed human red cells suspended in Tyrode–Locke's solution, respectively, during the first 90 min of incubation (100 μmoles glucose = 18 mg glucose; 100 μmoles lactate = 8·9 mg lactate). Sodium salicylate significantly increases both the utilization of glucose and lactate production

TABLE 1. INFLUENCE OF SODIUM SALICYLATE ON GLUCOSE UTILIZATION AND LACTATE PRODUCTION IN HEPARINIZED HUMAN WHOLE BLOOD INCUBATED AT 37°

	Glucose utilization and lactate production in whole blood expressed as μmole (±S.E.M.)/100 ml packed cells after 90-min incubation at 37°			
	Control	Sodium salicylate		
		2×10^{-3} M	5×10^{-3} M	*n*
Glucose utilization	315·9 ± 15·68	374 ± 21·73 *P < 0·05	449·3 ± 27·68 *P < 0·001	16
Lactate production	548·2 ± 5·3	682 ± 58 *P < 0·05	735 ± 52 *P < 0·01	8

* Where P is the significance of the difference from the control samples.

in whole blood (Table 1) and in erythrocytes in Tyrode–Locke's solution (with the exception of 1×10^{-3} M salicylate on glucose utilization—Table 2). Sodium salicylate in a final concentration of 5×10^{-3} M increases the utilization of glucose in whole blood by 43 per cent and the production of lactate by 34 per cent, whereas in erythrocytes suspended in Tyrode–Locke's solution this concentration of salicylate stimulates glucose utilization by 61 per cent and lactate production by 40 per cent.

In the course of anaerobic glycolysis two molecules of lactate are produced for each

TABLE 2. INFLUENCE OF SODIUM SALICYLATE ON GLUCOSE UTILIZATION AND LACTATE PRODUCTION IN WASHED HUMAN RED CELLS SUSPENDED IN TYRODE–LOCKE'S SOLUTION AND INCUBATED AT 37°

	Glucose utilization and lactate production in erythrocytes suspended in Tyrode–Locke's solution expressed as μmoles (±S.E.M.)/100 ml packed cells after 90 min incubation at 37°				
	Control	Sodium salicylate			
		1×10^{-3} M	2×10^{-3} M	5×10^{-3} M	*n*
Glucose utilization	116·8 ± 11·6	143·5 ± 21·1 *P > 0·2	177·9 ± 19·9 *P < 0·02	187·9 ± 18·9 ($n = 19$) *P < 0·01	23
Lactate production	232·5 ± 7·5	261 ± 7·5 *P < 0·01	291 ± 9 *P < 0·001	346·5 ± 12 *P < 0·001	22

* Where P is the significance of the difference from the control samples.

molecule of glucose consumed. The ratio of lactate production to glucose utilisation in the whole blood control sample is 1·74 and in the sample with 5×10^{-3} M salicylate it is 1·64 (from Table 1). In separated washed erythrocytes in Tyrode–Locke's solution this ratio for the control sample is 1·99 and for the sample with 5×10^{-3} M salicylate it is 1·84 (from Table 2).

A striking difference in the metabolism of aliquot samples of the same erythrocytes suspended and incubated in different media is seen in Table 3. In this experiment glucose utilization and lactate production was estimated in samples of separated human erythrocytes that had been divided, washed and suspended in either Tyrode–Locke's solution or 0·1 M potassium phosphate buffer with 0·01 M glucose, and incubated for 3 hr at 37°. Thus enabling a direct within-sample comparison of the effect of these two incubation media on erythrocyte metabolism and on the responses produced by salicylate. The results are expressed as an average for the two consecutive 90-min incubation periods. Potassium phosphate buffer increases glucose utilization in the control sample by 3·5-fold and lactate production 2·5 times compared with the erythrocytes in Tyrode–Locke's solution. The phosphate buffer also reduces, remarkably, the stimulant action of sodium salicylate on glucose utilization (only an increase of 3·9% which is not statistically significant) and lactate production (17·2% increase) compared with its effect on erythrocytes in Tyrode–Locke's solution (see Table 3).

The mean pH of the three different suspensions, whole blood, erythrocytes in Tyrode–Locke's and erythrocytes in phosphate buffer were virtually identical at 7·42, 7·41 and 7·42 respectively at zero time at the start of the incubation period, for the

TABLE 3. A COMPARISON OF THE EFFECT OF SODIUM SALICYLATE ON GLUCOSE UTILIZATION AND LACTATE PRODUCTION IN ALIQUOTS OF THE SAME SAMPLES OF WASHED HUMAN ERYTHROCYTES SUSPENDED IN TYRODE–LOCKE'S SOLUTION OR 0·1 M POTASSIUM PHOSPHATE BUFFER pH 7·4 WITH 0·01 M GLUCOSE AND INCUBATED FOR 3 hr AT 37°

	Incubation medium	Glucose utilization and lactate production in erythrocytes suspended in Tyrode–Locke's solution or potassium phosphate buffer with added glucose, expressed as μmoles (±S.E.M.)/100 ml packed cells/90 min incubation at 37°			
		Control	Sodium salicylate 5×10^{-3} M	Percentage increase	*n*
Glucose utilization	Tyrode–Locke's solution	124·5 ± 24	183 ± 24 *P = 0·06	46·9	7
	Potassium phosphate buffer with added glucose	417 ± 42	433·5 ± 37·5 *P > 0·35	3·9	
Lactate production	Tyrode–Locke's solution	268·5 ± 9	396 ± 13·5 *P < 0·001	47·5	7
	Potassium phosphate buffer with added glucose	603 ± 30	703 ± 30 *P < 0·025	17·2	

* Where P is the significance of the difference from the control sample.

control samples and for those with 5×10^{-3} M sodium salicylate. After 180 min incubation the whole blood control sample pH had increased by 0·06 and the salicylate by 0·03 pH. In the Tyrode–Locke's suspension the control sample pH was down by 0·11 units after 270 min incubation and the sample with 5×10^{-3} M salicylate dropped by 0·14 pH. There was no change in the mean pH of the control samples of erythrocytes in phosphate buffer after 180 min incubation but the sample containing salicylate was 0·02 pH less at that time. Thus the real differences in pH between the control samples and those containing 5×10^{-3} M sodium salicylate at the termination of incubation is a lower pH with salicylate of from 0·02 to 0·04 pH units.

DISCUSSION

Sodium salicylate in the concentration range $1–5 \times 10^{-3}$ M stimulates glucose utilization and lactate production in whole blood, and in erythrocytes suspended in Tyrode–Locke's solution or in potassium phosphate buffer with added glucose. However, its effectiveness as a stimulant of these metabolic processes differs considerably in erythrocytes in artificial incubation media from that in whole blood. In trying to compare the metabolic response to salicylate in whole blood and in washed erythrocytes suspended in artificial electrolyte solutions the fundamental differences between the two systems must be appreciated. Binding sites for salicylates on plasma proteins[7] are absent from the artificial incubation media used here allowing more salicylate in an active form for distribution between extra and intracellular fluid. Whole blood also contains leucocytes and platelets which have relatively large stores

of glycogen[8, 9] while erythrocytes contain very little[10] or none.[11] The glycogenolytic effect of salicylate[12-14] could thus increase intracellular glucose levels and reduce glucose uptake in whole blood. If this action of salicylate was significant in these experiments the ratio of lactate production to glucose uptake in whole blood with salicylate would be higher than that of the whole blood control sample. In fact at 5×10^{-3} M salicylate it is less. This ratio is also lower in the erythrocyte suspension in Tyrode–Locke's solution with salicylate, which suggests a degree of inhibition of glycolysis, possibly of dehydrogenase enzymes (using rabbit erythrocytes we have found an increasing glucose utilization and lactate production with increasing concentrations of salicylate up to 10×10^{-3} M. Between 10×10^{-3} M to 20×10^{-3} M salicylate there is a progressive fall in glucose consumption and lactate production.) Finally, the rate of leucocyte glycolysis is such that their glucose consumption is about a thousand times that of erythrocytes on a cell to cell basis,[15] there are, of course, considerably fewer leucocytes than erythrocytes in whole blood.

The marked increase in glucose utilization and lactate production in erythrocytes in potassium phosphate buffer with added glucose compared with those in Tyrode–Locke's solution was to a large extent expected. From the work of Minakami, Kakinuma and Yoshikawa[16] and others[17-20] inorganic phosphate (P_i) appears to exert some influence over the rate of erythrocyte glycolysis, which increases as the concentration of P_i in the incubation medium rises. The normal P_i value for whole blood plasma is in the range 8×10^{-4} M to $1{\cdot}25 \times 10^{-3}$ M, in Tyrode–Locke's solution it is 5×10^{-4} M and 10^{-1} M in the phosphate buffer. We have found that anaerobic glycolysis in whole blood does not increase until the plasma P_i level exceeds $7{\cdot}5 \times 10^{-3}$ M. This increased rate of glycolysis has been attributed to an antagonism by phosphate of inhibitors of the first two irreversible steps of glycolysis–glucose-6-phosphate inhibition of hexokinase[21] and ATP inhibition of phosphofructokinase.[17, 18] The high potassium content of the phosphate buffer is also of importance as increased extracellular potassium increases the conversion of sugar phosphate to lactate.[22]

The diminished effectiveness of salicylate in stimulating glucose utilization in erythrocytes suspended in phosphate buffer with added glucose compared to its pronounced activity on erythrocytes in Tyrode–Locke's solution may be due to the erythrocytes in phosphate buffer functioning at or near maximal rates without salicylate. Erythrocyte metabolism is pH dependent,[23] with a fall of 0·4 pH units from pH 7·5 reducing glucose utilization by 53 per cent, whereas a rise in pH from 7·5 to 7·8 results in a 43 per cent increase. It is unlikely that the minor differences in pH observed between the control and salicylate-containing erythrocyte suspensions in the media at the end of incubation would account for these differences in the responses to salicylate by these cells. This is not the first report of excess inorganic phosphate reducing the effect of a drug. Increasing concentrations of inorganic phosphate in the incubation medium progressively reduces the inhibition of erythrocyte glycolysis by ouabain.[16] Furthermore, salicylate failed to increase glucose uptake in rat diaphragms when these were suspended in a phosphate buffered medium.[24, 25]

What determines the level of the response to salicylate in erythrocyte suspension and whole blood? Is it the rate at which glucose utilization and anaerobic glycolysis proceed during incubation in a particular medium, as suggested above for the erythrocytes in phosphate buffer and their poor response to salicylate? Figure 3 shows the relationship that appears to exist between the percentage increase in glucose utilization

and lactate production in whole blood and erythrocytes exposed to 5×10^{-3} M salicylate and the rate of these two processes in the control samples during the first 90 min of incubation in the various media.

The choice of an incubation medium is obviously of great importance if the activity of a drug on cell metabolism is not to be overlooked or considered to be insignificant.

In considering the mode of action of sodium salicylate in producing these increases in anaerobic glycolysis in erythrocytes, the pentose phosphate pathway (occasionally referred to as the hexose monophosphate oxidative pathway) may appear as a possible

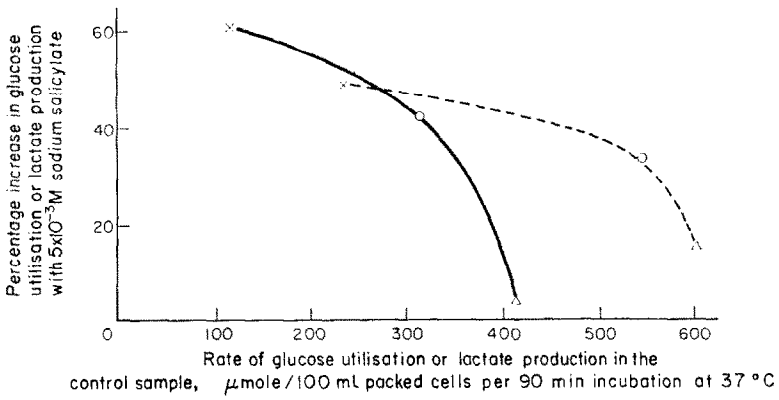

FIG. 3. The relation between the percentage increase in the response produced by sodium salicylate (5×10^{-3} M) on glucose utilization (——) and lactate production (- - - - -) in heparinized human whole blood (○), and in washed human erythrocytes suspended in Tyrode–Locke's solution (×) or 0·1 M potassium phosphate buffer pH 7·4 with 0·01 M glucose (Δ) and the rate of glucose utilization and lactate production in the respective control samples during the first 90 min of incubation at 37°.

site for salicylate activity. The consumption of glucose by this catabolic route is relatively small in the erythrocyte, about 14 per cent.[26] Although the precise effect of salicylate on this pathway remains to be established it is unlikely that the large increase in lactate production induced in the erythrocyte by salicylate could be accounted for entirely by an inhibition of the pentose phosphate pathway. We believe that the increase in anaerobic glycolysis in erythrocytes is secondary to a primary action of sodium salicylate elsewhere in the cell. Charnock, Opit and Hetzel,[27] using rat liver mitochondria suggest that potassium loss precedes or may even cause the changes in phosphorylation observed with salicylate. It also increases the passive efflux of potassium from erythrocytes[28, 29] and recently we reported[30] an increased formation of inorganic phosphorus by erythrocytes in the presence of sodium salicylate. The reason for the decline in the activity of salicylate with time in the experiments reported here is not known. A firmer binding to cell or plasma protein with time is possible or it might reflect the residual glucose concentration in the incubation media at that time. This decline in the activity was particularly evident in whole blood.

Meanwhile, the relationship between salicylate stimulated inorganic phosphorus (P_i) formation and anaerobic glycolysis in erythrocytes is under investigation as is the site of action of salicylate on P_i formation in the erythrocyte.

REFERENCES

1. D. T. P. Davies, A. Hughes and R. S. Tonks, *Arch. Pharmak. exp. Path.* **259**, 163 (1968).
2. D. T. P. Davies, A. Hughes and R. S. Tonks, *Br. J. Pharmac.* **36**, 437 (1969).
3. J. R. Sturman and M. J. H. Smith, *Biochem. Pharmac.* **15**, 1857 (1966).
4. H.-U. Bergmeyer and E. Bernt, *Methods of Enzymatic Analysis*, p. 123. Academic Press, New York (1965).
5. H. J. Hohorst, *Methods of Enzymatic Analysis*, p. 266. Academic Press, New York (1965).
6. E. Ponder, *Haemolysis and Related Phenomena*, p. 365. Churchill, London (1952).
7. W. L. Stafford, *Salicylates*, p. 74. Churchill, London (1963).
8. V. Esmann, *Scand. J. clin. Lab. Invest.* **13**, 134 (1961).
9. R. Gross, *Blood Platelets*, p. 407. Churchill, London (1961).
10. J. B. Sidbury, M. Cornblath, J. Fisher and E. House, *Pediatrics* **27**, 103 (1961).
11. R. Wagner, *Archs Biochem.* **11**, 249 (1946).
12. C. Lutwak-Mann, *Biochem. J.* **36**, 706 (1942).
13. M. J. H. Smith, *Biochem. J.* **57**, 349 (1954).
14. O. A. Zaki, *Am. J. Physiol.* **199**, 1056 (1960).
15. G. M. Guest, B. Mackler, H. Graubarth and P. A. Ammentrop, *Am. J. Physiol.* **172**, 295 (1953).
16. S. Minakami, K. Kakinuma and H. Yoshikawa, *Biochim. biophys. Acta* **90**, 434 (1964).
17. S. Minakami and H. Yoshikawa, *Biochim. biophys. Acta* **99**, 175 (1965).
18. K. K. Tsuboi and K. Fukunaga, *J. biol. Chem.* **240**, 2806 (1965).
19. I. A. Rose and J. V. B. Warms, *J. biol. Chem.* **241**, 4848 (1966).
20. S. C. Rizzo and R. E. Eckel, *Am. J. Physiol.* **211**, 429 (1966).
21. I. A. Rose, J. V. B. Warms and E. L. O'Conell, *Biochem. biophys. Res. Commun.* **15**, 33 (1964).
22. R. E. Eckel, S. C. Rizzo, H. Lodish and A. B. Berggren, *Am. J. Physiol.* **210**, 737 (1966).
23. J. R. Murphy, *J. Lab. clin. Med.* **55**, 286 (1960).
24. K. L. Manchester, P. J. Randle and G. H. Smith, *Br. med. J.* **i**, 1028 (1958).
25. P. J. Randle and G. H. Smith, *Biochem. J.* **70**, 490 (1958).
26. P. A. Marks, *The Red Blood Cell*, p. 214. Academic Press, New York (1964).
27. J. S. Charnock, L. J. Opit and B. S. Hetzel, *Metabolism* **10**, 874 (1961).
28. K. Waltner, Jr., M. Csernovsky and E. Kelemen, *Experientia* **14**, 277 (1958).
29. K. Waltner, Jr., M. Csernovsky and E. Kelemen, *Biochem. Pharmac.* **2**, 50 (1959).
30. D. T. P. Davies, R. S. Tonks and A. Hughes, *Experientia* **25**, 366 (1969).