Biochimica et Biophysica Acta, 396 (1975) 1–10

BBA 46948

EFFECT OF HYDROGEN ION CONCENTRATION ON ENERGY METABOLISM IN PIG PLATELETS

JUNJI NISHIMURA and SHIGEKI MINAKAMI

Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812 (Japan)

(Received December 30th, 1974)

SUMMARY

Respiration and glycolysis of pig platelets suspended in a dialyzed plasma were studied at various hydrogen ion concentrations. Respiration of platelets was high at acidic pH and decreased at physiological pH. This pH profile may not be attributed to properties of mitochondria, since the respiratory rate of mitochondria prepared from platelets was maximal at physiological pH. A low respiratory rate at physiological pH seemed to be attributable to depression of respiration by glycolysis, since the addition of glucose further depressed the rate. The Crabtree effect was more prominent at alkaline pH. Glycolysis increased with an increase in the pH of the plasma, contrary to oxygen consumption. The Pasteur effect was less prominent at alkaline pH. The effect of pH on lactate production by the cytosol fraction of platelets was similar to that of whole platelets. The glycolytic intermediate pattern showed that phosphofructokinase was the committed step. Both ATP concentration and ATP formation calculated from respiratory and glycolytic rates were constant at various pH values. These observations may indicate that the pH primarily affects platelet glycolysis at the phosphofructokinase step and the respiration is secondarily controlled by glycolysis.

INTRODUCTION

The metabolic energy of platelets is supposed to be provided by both glycolysis and oxidative phosphorylation [1, 2, 3]. Platelet metabolism and function are influenced by various factors such as temperature, washing procedure and suspending media [4, 5]. Although pH has been known to be one of the most influential factors in controlling metabolism, few studies have so far been carried out on the effect of pH on platelet metabolism [6, 7, 8].

Use of blood cells for study of cellular control mechanism has the advantage that cells can be obtained in quantity and can be studied as an homogeneous suspension. The effect of pH on glycolysis in red cells [9] and in leucocytes [10, 11] has been studied.

Since platelets have both glycolytic and respiratory systems and since their structure is relatively simple compared with other eukaryotic cells, platelets seemed to

be useful for the study on the intracellular interaction of glycolysis and respiration. Furthermore, the study can be related to their function, the aggregation process.

In this report, respiration and glycolysis of pig platelets as well as those of disintegrated cells were studied at various pH values. The glycolysis was primarily affected at the phosphofructokinase step and the respiration was secondarily affected by glycolysis.

EXPERIMENTAL PROCEDURES

Isolation of pig platelets

All procedures were carried out at room temperature with plastic vessels and pipettes. Pig blood was collected into an acid citrate solution (0.085 M sodium citrate, 0.075 M citric acid) in a ratio of 1 part to 7 parts blood. The blood was centrifuged at $250 \times g$ for 15 min and the supernatant was centrifuged again to obtain platelet-rich plasma. Platelets were separated from plasma by centrifugation at $1200 \times g$ for 15 min, resuspended in a small volume of the plasma and centrifuged at $120 \times g$ for 10 min to remove any contaminating erythrocytes and leucocytes. The platelets were collected by centrifugation at $1000 \times g$ for 15 min.

In order to reduce any injurious effects of artificial media, dialyzed plasma was used as a suspending medium. Plasma was dialyzed with 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 5 mM sodium phosphate buffer, pH 7.4 for 24 h and stored in small aliquots at -20 °C. The concentrations of citrate, glucose, pyruvate and lactate were 2, 0.09, 0.008 and 0.2 mM, respectively. Platelets were washed twice with the dialyzed plasma by centrifugation at $1000 \times g$ for 15 min and were then suspended in the dialyzed plasma. Numbers of platelets were counted microscopically. Contamination of erythrocytes and leucocytes was less than one cell per 5000 platelets. Citrate in plasma was determined colorimetrically after conversion to pentabromoacetone [12].

Measurement of respiratory rate

Oxygen uptake was measured by a Clark-type oxygen electrode (Yellow Spring Instrument Co., Ohio) in a closed vessel of 3 ml at 37 °C. Platelets were suspended in the dialyzed plasma. The pH was initially adjusted by the addition of 0.2 M NaOH or 0.2 M HCl.

Studies of glycolysis

The platelets suspended in the dialyzed plasma were incubated in 30-ml plastic centrifuge tubes under gentle stirring with a magnetic bar at 37 °C. The incubation was carried out under a pH meter and the pH was kept constant by the addition of 0.2 M NaOH with a tuberculin syringe. The total volume of added alkali was not more than 1/20 vol. of the platelet suspension. At intervals, a 2- or 3-ml aliquot of the suspension was taken out, deproteinized with an equal volume of 6 % perchloric acid solution and centrifuged at $10000 \times g$ for 10 min.

The deproteinized supernatant was neutralized with 5 M potassium bicarbonate solution and was kept at 4 °C. Pyruvate, fructose diphosphate and triosephosphates were measured immediately after the neutralization and other intermediates within 2 days. Intermediates and nucleotides were measured enzymatically with a recording spectrophotometer Hitachi 124 [13]. For the measurement of glycogen, a 1-

ml aliquot of the platelet suspension was added to 2 ml of 30 % KOH. The mixture was heated for 15 min at 100 °C and was supplemented with 2 ml of 30 % ethanol. Succeeding steps of extraction and hydrolysis was carried out according to Pfleiderer [14]. Glucose was measured spectrophotometrically with hexokinase and glucose-6-phosphate dehydrogenase.

Reagents

Enzymes, coenzymes and glycolytic intermediates were obtained from Böhringer und Söhne, Mannheim. Other reagents were of analytical reagent grade.

RESULTS

Respiratory activity of pig platelets

Oxygen electrode traces of pig-platelet respiration are shown in Fig. 1. The respiratory activity in the dialyzed plasma at pH 7.4 without addition of glucose was about 300 nmol/min per 10^{11} platelets, which was inhibited completely by KCN and partially by rutamycin. The respiration inhibited by rutamycin was relieved by 2,4-dinitrophenol, indicating that the respiration was coupled to phosphorylation. The respiration was inhibited by 30 % when glucose was added to the medium (Crabtree effect). A further influence of glycolysis on the respiration was shown by the addition of monoiodoacetate or deoxyglucose, which transiently accelerated the respiration and then depressed it.

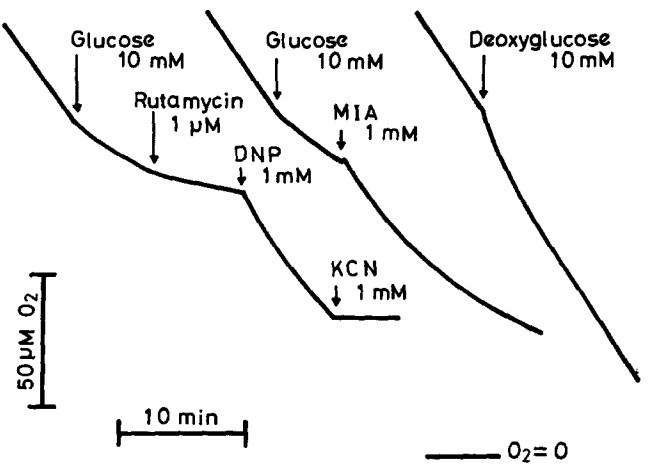

Fig. 1. Oxygen electrode traces of pig-platelet respiration. The platelets were suspended in 3 ml of a dialyzed plasma at pH 7.4 (1.9×10^9 platelets/ml) and the decrease of oxygen concentration in a closed vessel was measured by a Clark oxygen electrode at 37 °C. The final concentrations of reagents added were shown in the figure. Abbreviations: DNP, 2,4-dinitrophenol; MIA, monoiodoacetate.

Effect of pH on respiration

An unexpected observation was that the respiratory activity increased when the suspension was acidified from pH 8.2 to 6.2. This was especially conspicuous when the cells were incubated with glucose. The Crabtree effect became distinct from

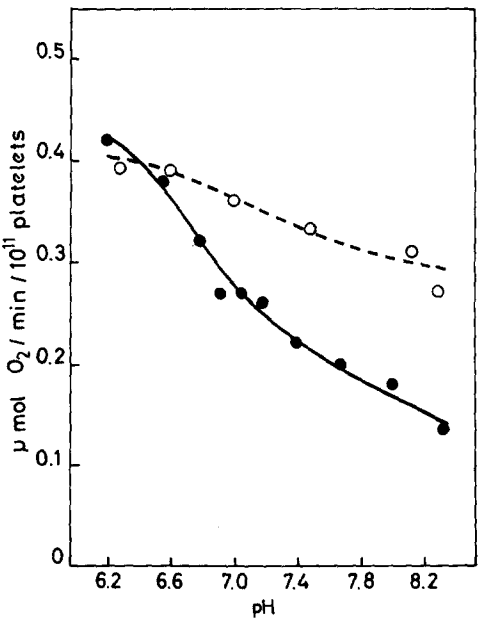

Fig. 2. Effect of pH on oxygen consumption by the platelets. Oxygen uptake by the platelet suspension (2.2×10^9 platelets/ml) was measured either with or without glucose for about 10 min as described in Fig. 1. The final pH values are given. ● — ●, with 10 mM glucose; ○- - -○, without glucose.

0 % to 50 % as the pH value increased from 6.2 to 8.2 (Fig. 2).

The indication that the acidic pH optimum of platelet respiration cannot be ascribed to any property of the mitochondria was obtained from the pH curve of respiration by the mitochondrial fraction of platelets prepared by sonication. The respiratory rate was maximal at physiological pH when succinate was used as a substrate. This conclusion was further confirmed from the respiration of intact platelets which was uncoupled with 2,4-dinitrophenol, though accurate analysis of the pH effect was difficult due to the difference in permeability of dinitrophenol at various pH. The optimal dose of the reagent for acceleration of the respiration differed with pH and the respiration was inhibited at higher concentrations. Concentrations of about 0.1, 1 and 5 mM of dinitrophenol in the plasma were most effective in accelerating respiration at pH 6.2, 7.4 and 8.2, respectively. The maximal respiration with dinitrophenol had no tendency to increase with decrease in pH (not shown).

Effect of pH on glycolysis

In the presence of glucose, both glucose consumption and lactate production proceeded over the period of 100 min and they were essentially stoichiometric at pH 6.6, 7.4 or 8.2, increasing as the pH of the suspension increased (Fig. 3). Glycogen content of platelets was about 20 μmol/10^{11} platelets, and decreased by about half at pH 8.2 in 100 min, changed a little at pH 7.4 and increased slightly at pH 6.6. Lactate production of the platelets studied at various pH values from 6.2 to 8.2 increased as the pH of the suspension increased (Fig. 4), which was essentially a mirror image of the

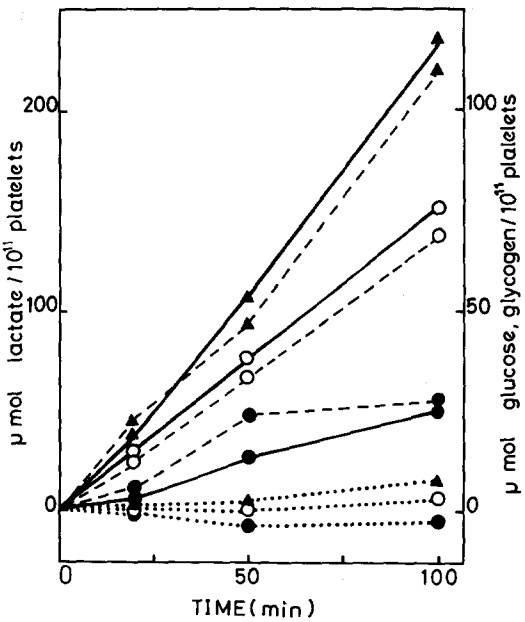

Fig. 3. Glucose consumption, glycogen utilization and lactate formation of the platelets at various pH. The platelet suspension (1.4×10^9 platelets/ml) was incubated with 4 mM glucose at 37 °C. The pH of the medium was monitored and kept constant throughout the incubation. —, glucose consumption, · · ·, glycogen utilization; - - -, lactate formation; ●, pH 6.6; ○, pH 7.4; ▲, pH 8.2.

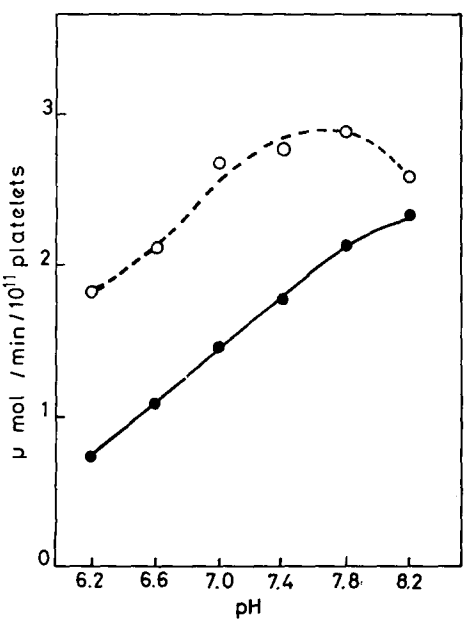

Fig. 4. Effect of pH on lactate formation by the platelets. The platelet suspension (1.0×10^9 platelets/ml) was incubated as described in Fig. 3 with 10 mM glucose and with or without 1 mM KCN for 50 min. ●—●, without KCN, ○- - -○, with KCN.

pH curve of the respiration shown in Fig. 2. In the presence of KCN, a similar tendency was observed but the Pasteur effect was less prominent at alkaline pH. An essentially similar pH profile of lactate production was observed when platelets were incubated without glucose, though the rate of lactate production was low.

This effect of pH on glycolysis can be ascribed to the property of the glycolytic system itself, since the cytosol fraction of the sonicated platelets showed a similar pH curve when it was incubated with glucose, NAD and ATP for 60 min at various pH values. The optimum fell at around pH 7.8.

Effect of pH on concentrations of glycolytic intermediates

In order to obtain information on the control mechanism of platelet glycolysis, steady-state concentrations of glycolytic intermediates at various pH values were determined. Fig. 5 indicates a cross-over plot of platelets incubated with 10 mM glucose for 20 min with the pH kept constant during incubation. The apparent effect of the increase in pH was a decrease of glucose 6-phosphate and fructose 6-phosphate and an increase of fructose diphosphate, triosephosphates, monophosphoglycerates and phosphoenolpyruvate, which indicates that phosphofructokinase is the main

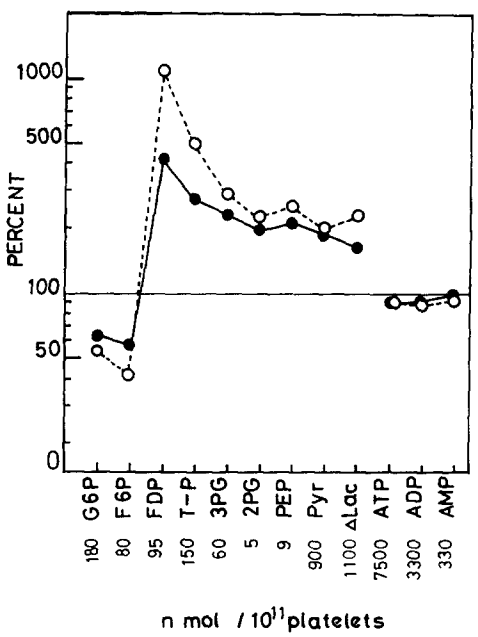

Fig. 5. Cross-over plot of glycolytic intermediates in the platelets incubated at various pH. The platelet suspension (5.8×10^9 platelets/ml) in a dialyzed plasma was incubated as described in Fig. 3 with 10 mM glucose for 20 min. Concentrations of the intermediates at pH 7.4 and 8.2 were expressed as a percentage of those at pH 6.6. Concentrations of intermediates at pH 6.6 are given under the names of the intermediates (nmol/10^{11} platelets). Lactate formation is expressed as nmol/10^{11} platelets per min. ●—●, pH 7.4; ○- - -○, pH 8.2. Abbreviations: G6P: glucose 6-phosphate. F6P: fructose 6-phosphate. FDP: fructose 1,6-diphosphate. T-P: triose phosphate. 3PG: 3-phosphoglycerate. 2PG: 2-phosphoglycerate. PEP: phosphoenolpyruvate. Pyr: pyruvate. ΔLac: lactate formation.

control step. A similar cross-over plot was obtained when platelets were incubated for 10 min. The concentration of pyruvate increased with the increase in pH from 6.2 to 7.0 but stayed essentially constant between pH 7.0–8.2. The ratio NAD/NADH calculated from the lactate/pyruvate ratio did not show any definite tendency.

Effect of pH on the energy state of platelets

The levels of ATP, ADP and AMP were about 8, 3 and 0.3 μmol per 10^{11} platelets and no marked change was observed by change in pH (Fig. 5). The levels were essentially constant with glucose even during 60 min incubation.

TABLE I

CALCULATION OF ATP FORMATION FROM RESPIRATORY AND GLYCOLYTIC RATES

Calculations were made from the experiments shown in Figs 2 and 4, based on 1 ATP formation for each lactate formed and 6 ATP for each O_2 consumed. Values expressed as μmol ATP formed per min per 10^{11} platelets.

	pH					
	6.2	6.6	7.0	7.4	7.8	8.2
Glycolysis	0.74	1.08	1.47	1.77	2.13	2.33
Respiration	2.52	2.16	1.68	1.32	1.14	0.90
Total	3.26	3.24	3.15	3.09	3.27	3.23

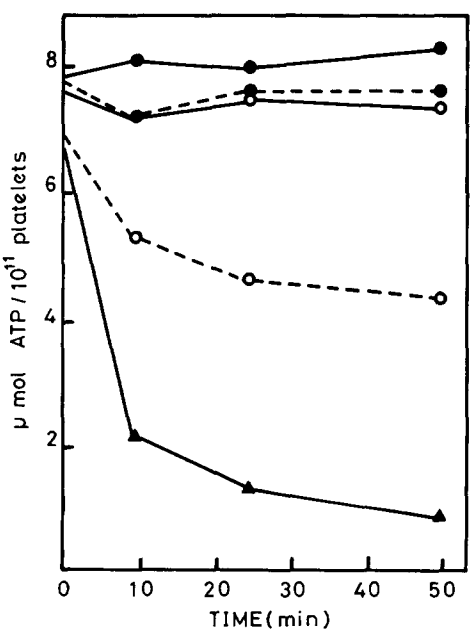

Fig. 6. ATP concentrations of the platelets under various conditions. Platelet suspension (1.2×10^9 platelets/ml) was incubated at pH 7.4 for 50 min as described in Fig. 3 —, with 10 mM glucose; - - -, without glucose; ●, aerobic condition; ○, with 1 mM KCN; ▲, with 1 mM monoiodoacetate.

The total ATP production calculated by glycolysis and oxidative phosphorylation was constant at various pH values, as shown in Table I, while ATP formation by glycolysis played a major part and that by oxidative phosphorylation a minor part as the PH increased. The strong compensation for each other was ascertained by the following experiment (Fig. 6). Aerobic platelets were able to maintain the ATP level during 50 min incubation even without glucose as did the anaerobic platelets with glucose (treated with KCN). However, when the platelets were incubated both without glucose and under anaerobic conditions, the ATP level declined gradually over a period of 20 min, and thereafter remained low. Monoiodoacetate, a potent inhibitor of glycolysis, decreased the ATP level more rapidly.

DISCUSSION

Erythrocytes, polymorphonuclear leucocytes and platelets, three types of blood cells of bone marrow origin, have a common property of high aerobic glycolytic activity. The influence of H^+ concentrations on glycolysis has been studied in erythrocytes [9] and in polymorphonuclear leucocytes [10, 11]. High glycolytic activity at alkaline pH and a similar cross-over plot were obtained for pig platelets as those reported for erythrocytes and polymorphonuclear leucocytes. The cross-over point fell at the phosphofructokinase step. The platelets also belong to the group of exceptional normal cells to show the Crabtree effect, which can be expected from their high aerobic glycolytic activity; this property is also shown by polymorphonuclear leucocytes.

Dependence of the respiratory rate on pH has been studied by Ibsen et al. [15] for Ehrlich ascites tumor cells, typical neoplasmic cells which show the Crabtree effect. The respiratory rate is maximal at physiological pH. The pH curve of the respiration in the platelets differed from those in ascites tumor cells: the lower the pH, the higher the respiratory rate. These observations, together with high glycolytic rate and prominent Crabtree effect at alkaline pH, strongly suggest a marked influence of glycolysis on the platelet respiration compared with ascites tumor cells.

The contribution of glycolysis to the energy metabolism of pig platelets was also shown from the arithmetic calculation of ATP formation by respiration and glycolysis. About 60 % of ATP formation could be ascribed to the glycolysis at pH 7.4 when the platelets were incubated aerobically with glucose, which was apparently higher than that calculated by Detwiler and Zivkovic [2] in rat platelets (45 %) and that by Gross et al. [1] in human platelets (20 %). It was also higher than that in guinea pig polymorphonuclear leucocytes (42 %) calculated by Quastel and his colleagues [16]. This difference may be in accord with the strong influence of glycolysis on respiration observed in pig platelets. High glycolytic activity in pig platelets was supported by the relatively weak Pasteur effect observed (60 % at pH 7.4) as compared with those reported by Karpatkin [17], Detwiler and Zivkovic [2] and Doery et al. [3] who found glycolysis under anaerobic condition was doubled.

Another point to be noted was that the calculated rate of ATP formation was essentially constant over a pH range of more than 2 units, pH 6.2–8.2, though both glycolysis and respiration were affected markedly by pH. Glycolysis and respiration by the platelets seemed to be strongly compensated by each other, since the ATP level was constant either under anaerobic conditions or without glucose, which was con-

sistent with the result of Detwiler and Zivkovic [2].

Metabolism and function of platelets are known to be greatly influenced by experimental conditions: selection of anticoagulants, temperature during isolation and composition of suspending medium [18]. In the present experiment, the following precautions were made for handling platelets: acidic citrate solution was used as an anticoagulant, since it was confirmed that EDTA exerted an effect on the platelets both morphologically and functionally. Chilling of platelet suspension was avoided, since it was known from the experience on red cell suspensions that the chilling caused an unexpected increase of the intracellular pH and thereby caused breakdown of ATP [19]. Plasma was used for washing and suspending platelets, instead of saline solutions, since plasma seemed to be a better medium for maintaining normal metabolism and functions of platelets. For studies of respiration and glycolysis, a pooled dialyzed plasma was used to give definite incubation conditions with regard to concentrations of citrate, glucose, pyruvate and lactate. Of special importance for measurement of the glycolytic intermediates was the concentration of pyruvate in the medium. Pyruvate caused the decrease in fructose diphosphate and triosephosphates. The above-mentioned precaution seemed to be essential not only for study of aggregation but also for the study of metabolism. The platelet suspension used in the present study had essentially the same functional property as platelet-rich plasma and investigation of the metabolic process in connection with aggregation is under study.

ACKNOWLEDGEMENTS

This investigation was supported in part by a Scientific Research Grant from the Ministry of Education.

REFERENCES

1 Gross, R. and Schneider, W. (1968) in Metabolism and Membrane Permeability of Erythrocyte and Thrombocyte (Deutsch, E., Gerlach, E. and Moser, K., eds), pp. 206–214, Georg Thieme, Stuttgart
2 Detwiler, T. C. and Zivkovic, R. V. (1970) Biochim. Biophys. Acta 197, 117–126
3 Doery, J. C. G., Hirsh, J. and Cooper, I. (1970) Blood 36, 159–168
4 Rossi, E. C. (1967) Blood 30, 758–766
5 Rossi, E. C. (1967) J. Lab. Clin. Med. 69, 204–216
6 Campbell, E. W., Small, W. J. and Dameshek, W. (1956) J. Lab. Clin. Med. 47, 835–843
7 Engel, P. F. (1966) Acta Physiol. Scand. 66, 282–289
8 Kim, B. K. and Baldini, M. G. (1972) Haematologia, 6, 437–447
9 Minakami, S. and Yoshikawa, H. (1966) J. Biochem. 59, 145–150
10 Halperin, M. L., Connors, H. P., Relman, A. S. and Karnovsky, M. L. (1969) J. Biol. Chem. 244, 384–390
11 Kakinuma, K. (1970) J. Biochem. 68, 177–185
12 Stern, J. R. (1957) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. III, pp. 425–431, Academic Press, New York
13 Minakami, S., Suzuki, C., Saito, T. and Yoshikawa, H. (1965) J. Biochem. 58, 543–550
14 Pfleiderer, G. (1963) in Method of Enzymatic Analysis (Bergmeyer, H. U., eds), pp. 59–62, Academic Press, New York
15 Ibsen, K. H., Coe, E. L. and McKee, R. W. (1960) Cancer Research 20, 1399–1407
16 Iyer, G. Y. N., Islam, M. F. and Quastel, J. H. (1961) Nature 192, 535–541
17 Karpartkin, S. (1967) J. Clin. Invest. 46, 409–417

18 Mannucci, P. M. (1972) Adv. Exp. Med. Biol. 34, 57–78
19 Minakami, S., Tsuda, S. and Tomoda, A. (1974) VII Internat. Symp. Über Struktur und Funktion der Erythrozyten, 1973, in Abhandlung der Akademie der Wissenschaften der DDR, Berlin, in press