BBA 65945

THE ASSOCIATION OF SOME OXIDOREDUCTASES WITH THE RED CELL MEMBRANE

P WINS* AND E SCHOFFENIELS**
Department of Biochemistry, University of Liège, Liège (Belgium)

(Received April 1st, 1969)

SUMMARY

1 The presence of several oxidoreductases has been investigated in various fractions of pig red-cell stroma The degree of firmness of their association with the membrane structure was in the sequence Glyoxylate reductase > NADH dehydrogenase > malate dehydrogenase > NADPH dehydrogenase > lactate dehydrogenase > triosephosphate dehydrogenase

2 After partial solubilization of the stroma with Triton X-100 and subsequent $(NH_4)_2SO_4$ fractionation, most oxidoreductase activities were recovered in the fraction precipitating at more than 50% saturation

3 The membrane-bound glyoxylate reductase required a pH lower than 7 0 for full activity, the activity was fairly stable and relatively insensitive to most inhibitors The enzyme–substrate interactions seem to be complex, and rather high amounts of glyoxylate are required for maximal velocity

INTRODUCTION

During the last few years, it has been gradually substantiated that many enzymes were bound to the membrane in the mammalian erythrocyte This was shown to be true for enzyme systems involved in ion transport[1] as well as for glycolytic enzymes[2]

In this paper, we shall mainly consider the case of some NAD(P)-linked oxidoreductases not involved in glycolysis, namely malate dehydrogenase (L-malate NAD^+ oxidoreductase, EC 1 1 1 37), glyoxylate reductase (glycollate NAD^+ oxidoreductase, EC 1 1 1 26), NADH dehydrogenase (reduced-NADH (acceptor) oxidoreductase, EC 1 6 99 3) and NADPH dehydrogenase (NADPH·(acceptor) oxidoreductase, EC 1 6 99 1) There is some uncertainty in the characterization of the enzymes catalyzing the reduction of dyes by NADH or NADPH, especially in erythrocytes In the present study, NADH dehydrogenase will refer to the enzyme catalyzing reduction of ferricyanide by NADH, and NADPH dehydrogenase will refer to the

* Abbreviation DCIP = 2,6-dichlorophenolindophenol

enzyme catalyzing reduction of 2,6-dichlorophenolindophenol (DCIP) by NADPH.

So far as we know, glyoxylate reductase has never been described in erythrocytes The presence of large amounts of malate dehydrogenase in human cells has been reported[3], but this enzyme was considered as being "soluble" On the other hand, NADH dehydrogenase has been recognized as a membrane-bound enzyme system[4] At any rate, the physiological role of all these dehydrogenase activities is far from being clearly understood in the case of erythrocytes Some properties of these enzymes are described below, this will perhaps help us to state the problem on a sounder basis

EXPERIMENTAL

Preparation and fractionation of ghosts

First method Fresh pig blood, obtained by exsanguination, was diluted with 3 vol of isotonic NaCl, buffered with 5 mM Tris–HCl buffer (pH 7 2) The red cells were centrifuged down and were washed twice with 6 vol of 0 2 M sucrose solution, adjusted to pH 7 2 with a trace of histidine The packed cells were resuspended in 4 vol of 0 25 M sucrose, and the suspension was frozen at $-25°$ in order to lyse the cells

After thawing, the stroma was centrifuged down at 0–4° and was washed once with about 10 vol of 0 25 M sucrose Finally, the packed ghosts were resuspended in 0 5 M sucrose so that the suspension contained about 20 mg protein/ml If the suspension was kept more than 1 week at $-20°$, the ghosts tended to clump, but this did not affect the activity of the membrane-bound enzymes

In several experiments, ghosts obtained by this method were resuspended in 5 vol of distilled water at pH 7 5, and Triton X-100 was added to the suspension (final concn 0 2% (v/v)) The detergent was allowed to act during 10 min at ordinary temperature, a marked clearing of the suspension was observed Upon centrifugation of this preparation at 125 000 $\times$ *g* for 2 h, 20–40% of total protein was sedimented, but enzyme activities were mainly recovered in the supernatant, indicating that most enzyme molecules were in solution The preparation could then be fractionated with $(NH_4)_2SO_4$ The precipitate obtained at 30% satn exhibited rather low specific enzyme activities, except that malate dehydrogenase was relatively abundant This fraction was centrifuged down and usually was discarded The precipitate obtained when the supernatant solution was brought to 60% satn could generally be recovered as a floating layer after 1 h centrifugation at 15 000 $\times$ *g* This fraction contained all the oxidoreductases described here, with relatively high specific activities The precipitate was resuspended in dilute Tris–HCl buffer (3 mM, pH 7 2) so that the protein concentration was about 5 mg/ml

Second method Erythrocytes were washed 3 times with isotonic NaCl, adjusted to pH 7 2 with 3 mM Tris–HCl buffer The cells were lysed by adding them quickly to 10 vol of chilled distilled water The hemolysate was frozen and kept overnight at $-20°$ After thawing, the pH was adjusted to 6 2 with acetic acid in order to facilitate the sedimentation of ghosts The hemolysate was centrifuged at 5000 $\times$ *g* during 15 min The packed ghosts were washed once with distilled water The sediment obtained at this stage is referred to as "S_1 fraction"

This fraction could be washed 2–3 times with 3 mM Tris–acetate (pH 8 1), so

that in most cases practically all visible hemoglobin was removed The pellet obtained at this stage is referred to as "S_2 fraction"

Assay of glyoxylate reductase, malate and lactate dehydrogenase

NADH or NADPH oxidation could, in principle, be followed in the spectrophotometer by measuring the decrease in absorbance of the incubation mixture at 340 mμ However, a correction was found to be necessary in view of the relatively weak specific activity of many preparations This compelled us to measure the NADH level in media of relatively high turbidity and hemoglobin content, thus highly absorbing at 340 mμ However, this absorbance in the absence of NADH was not very different at 340 and 380 mμ, the difference of absorbance between 340 and 380 mμ of the incubation mixture could thus be taken as a satisfactory estimation of NADH or NADPH concentration

The reaction was usually started by adding glyoxylate, oxaloacetate or pyruvate to a medium containing 200 μM NADH, 50 mM Tris–maleate buffer at various pH values and various amounts of the enzyme preparation The initial rate of disappearance of NADH was taken as a measurement of enzyme activity, except when this activity did not follow the classical kinetic patterns (see RESULTS)

NADH dehydrogenase activity was determined by measuring ferricyanide oxidation, essentially as described by ZAMUDIO AND CANESSA[4]

NADPH dehydrogenase activity was measured in the same way, DCIP reduction being followed at 600 mμ

The concentrations of NADPH and DCIP in the medium were 100 and 40 μM, respectively

Protein was determined by the method of LOWRY *et al* [5]

Organic acids were always added as their sodium salts

RESULTS

Enzyme pattern in different particulate fractions

A comparison of the specific activities of several oxidoreductases in different preparations is presented in Table I Lactate dehydrogenase (L-lactate·NAD^+ oxidoreductase, EC 1 1 1 27), malate dehydrogenase, glyoxylate reductase, NADH dehydrogenase and NADPH dehydrogenase were found in appreciable amounts in ghosts obtained by the first method For all enzymes tested, it can be seen that the specific activities were much higher in this preparation than in the hemoglobin-free stroma (Fraction S_2) From data obtained with Fraction S_1, it may be concluded that the very hypotonic hemolysis involved in the preparation of this fraction results in the release of most lactate dehydrogenase and NADPH dehydrogenase activities However, appreciable amounts of NADH dehydrogenase, glyoxylate reductase and malate dehydrogenase were retained in this fraction

In the extensively washed S_2 fraction, the specific activities of glyoxylate reductase, NADH dehydrogenase and malate dehydrogenase amounted respectively to 22, 12 and 10% of the activities found in ghosts prepared by the first method. Lactate dehydrogenase and NADPH dehydrogenase were practically absent

The presence of triosephosphate dehydrogenase (D-glyceraldehyde-3-phosphate NAD^+ oxidoreductase, EC 1 2 1 11) was investigated in various fractions but

TABLE I

SPECIFIC ACTIVITIES OF LACTATE DEHYDROGENASE, MALATE DEHYDROGENASE, GLYOXYLATE REDUCTASE, NADH DEHYDROGENASE AND NADPH DEHYDROGENASE IN VARIOUS FRACTIONS OBTAINED FROM RED CELL STROMA

Lactate dehydrogenase, malate dehydrogenase and glyoxylate reductase activities are expressed as nmoles NADH oxidized per ml of incubation mixture per mg protein per min NADH dehydrogenase activity is given in nmoles $K_3Fe(CN)_6$ reduced per ml of incubation mixture per mg protein per min NADPH dehydrogenase activity is given in nmoles DCIP reduced per ml of incubation mixture per mg protein per min Conditions of experiment see EXPERIMENTAL The pH of the medium was 6 2 for glyoxylate reductase and 7 2 for other assays The concentrations of pyruvate, oxaloacetate and glyoxylate were 0 5, 2 0 and 7 0 mM, respectively

Preparation used	*Specific activity*				
	Lactate dehydrogenase	*Malate dehydrogenase*	*Glyoxylate reductase*	*NADH dehydrogenase*	*NADPH dehydrogenase*
Ghosts obtained by the first method	29 2	59	23 1	31 6	2 05
S_1 fraction	3 2	11 7	8 8	12 3	0 33
S_2 fraction	0 3	6 1	5 2	3 8	0 05
Fraction obtained by $(NH_4)_2SO_4$ precipitation					
Ppt at 30% satn	0 2	43 4	10 8	—	—
Ppt at 55% satn	87	73	123	73	8 23

practically no activity could be detected On the other hand, a rather weak glutathione reductase activity (NAD(P)H oxidized-glutathione oxidoreductase, EC 1 6 4 2) was found in ghosts prepared by the first method (data not presented)

The preparation obtained by $(NH_4)_2SO_4$ fractionation exhibited a particularly high activity of glyoxylate reductase (Table I) Lactate dehydrogenase, NADH dehydrogenase and NADPH dehydrogenase were also concentrated in this fraction Malate dehydrogenase, however, was not much more abundant than in the crude stroma

Properties of the membrane-bound oxidoreductases

Lactate dehydrogenase From data presented in Table II, it can be seen that membrane-bound lactate dehydrogenase was active over a wide range of pH values (6 5–9 0) and exhibited the well-known phenomenon of inhibition by excess substrate—but only to a small extent The activity was markedly inhibited by sodium sulfite As shown in Table V, relatively high concentrations of NaCl (0 05–0 5 M) stimulated the activity at high pyruvate concentration (2 mM), at low pyruvate concentration, however, lower concentrations of NaCl (0 05 M) had little effect, while higher amounts (0 5 M) were definitely inhibitory In other words, NaCl increased the apparent K_m value for pyruvate

Malate dehydrogenase This enzyme was also active over a wide range of pH values (6 0–8 5) As shown in Table II, it was inhibited by sulfite as well as by relatively high amounts of oxalate and malonate

The effects of NaCl were analogous to those observed with lactate dehydrogenase, except that lower amounts of NaCl were sufficient to induce an appreciable activation (Table V)

TABLE II

LACTATE AND MALATE DEHYDROGENASE ACTIVITY OF GHOSTS PREPARED BY THE FIRST METHOD

The oxaloacetate concentration in malate dehydrogenase assays was 2 mM pH of the medium in Expts. 2 and 3 is 7 2 The pyruvate concentration in lactate dehydrogenase assays was 0 5 mM in Expts 1 and 3 The specific activities are expressed as in Table I

Expt No	*Conditions*	*Specific activity*	
		Lactate dehydrogenase	*Malate dehydrogenase*
1	pH of the medium		
	6 0	23 4	49 5
	7 5	31 6	66 7
	9 0	27 1	52 6
2	Pyruvate concn (mM)		
	0 08	25 2	—
	0 4	29 3	—
	2	24 3	—
3	Inhibitor added (mM)		
	None	27 8	55 8
	Sulfite, 0 1	10 4	4 3
	Oxalate, 20	—	23 3
	Malonate, 20	—	35

Glyoxylate reductase. When ghosts prepared by the first method were used, it was generally observed that the initial rate of NADH oxidation was very low at low substrate concentration and that the steady-state rate was reached after several minutes only On the other hand, at high glyoxylate concentration the absorbance at 340 mμ dropped markedly on glyoxylate addition, and this drop did not appear to be due to NADH oxidation In any case, however, the rate of disappearance of NADH remained linear between 5 and 10 min after glyoxylate addition, and this steady-state rate was taken as a measurement of enzyme activity.

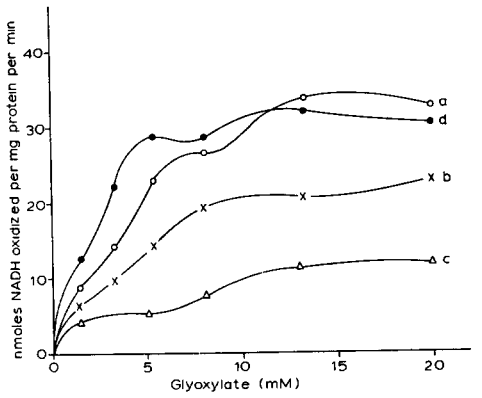

Fig 1 Effect of glyoxylate concentration on the glyoxylate reductase activity of ghosts obtained by the first method Curve a, the composition of the incubation medium was 50 mM Tris–maleate buffer (pH 6 2), 7 mM sodium glyoxylate, 200 μM NADH, 0 2 mg protein/ml Curve b, 0 5 M sucrose added, Curve c, 0 5 M KCl added, Curve d, 0 2% Triton X-100 added

The effects of increasing concentrations of sodium glyoxylate under different experimental conditions are reported in Fig 1 First it must be pointed out that the kinetics of the reaction apparently did not follow the classical Michaelis–Menten pattern Indeed, when the experiment was reproduced several times, we always found that the experimental points obtained could not be made to yield a hyperbola The curves actually appeared to exhibit two inflection points, thus suggesting that the enzyme–substrate interaction is complex On the other hand, the glyoxylate concentration necessary for half-maximal activity was unusually high, generally 2–3 mM or more This value was generally smaller when ghosts were partially solubilized with Triton X-100 (Curve b) and higher when the tonicity of the mdeium was increased (Curves c and d) High concentrations of KCl (Curve d) as well as of NaCl (Table V) were inhibitory at any glyoxylate concentration

Maximum reductase activity was found at pH around 6 0 (Fig 2) Only a small activity was detected above pH 7 3

In the fraction obtained by $(NH_4)_2SO_4$ fractionation at 60% satn , the properties of glyoxylate reductase were essentially the same, except that the initial rate of disappearance of NADH always corresponded to the maximal activity Fig 3 shows the effects of increasing concentrations of glyoxylate on the activity of this fraction at various pH values It can be seen that the substrate concentration necessary for half-maximal activity markedly increased with the pH of the medium

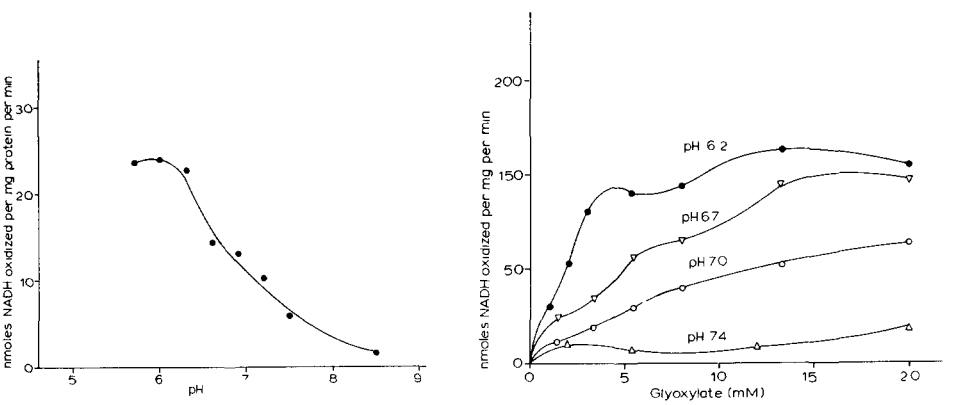

Fig 2 Effect of pH on the glyoxylate reductase activity of ghosts obtained by the first method The glyoxylate concentration was 7 mM

Fig 3 Effect of glyoxylate concentration on the glyoxylate reductase activity of a preparation obtained by $(NH_4)_2SO_4$ fractionation (60% satn) at various pH values The composition of the medium was 50 mM Tris–maleate buffer at various pH's, 7 mM sodium glyoxylate, 200 μM NADH, 0 2 mg protein per ml

From data shown in Table III, it appears that the enzyme also catalyzed NADPH oxidation, although at a slower rate than NADH On the other hand, the activity was inhibited by relatively high concentrations of mercurials (mersalyl, *p*-chloromercuribenzoate), oxalate and malonate Sulfite (0 1 mM) and *n*-ethylmaleimide (1 mM) were practically uneffective

NADH and NADPH dehydrogenases As shown in Table IV, both activities

TABLE III

GLYOXYLATE REDUCTASE ACTIVITY OF GHOSTS OBTAINED BY THE FIRST METHOD

pH of the medium, 6 2 Glyoxylate concn , 7 mM In Expt 2, NADH was used as the coenzyme Specific activity is expressed as in Table I

Expt No	*Conditions*	*Specific activity of glyoxylate reductase*
1	Coenzyme used	
	NADH	24 5
	NADPH	8 9
2	Inhibitor added (mM)	
	None	27
	Mersalyl, 1	13 1
	p-Chloromercuriben-zoate, 0 1	14 9
	Oxalate, 20	7 2
	Malonate, 20	9 1

were markedly inhibited by mercurials, but no specific inhibitor of these activities could be found

The two types of activities can therefore be distinguished only on the basis of coenzyme specificity, in fact, NADPH was oxidized in the presence of DCIP (Table IV) but not in the presence of $K_3Fe(CN)_6$, whereas NADH was oxidized by both electron acceptors This suggests the presence of two different enzymes but does not indicate which of them is responsible for NADH oxidation by DCIP

TABLE IV

ACTIVITY OF NADH DEHYDROGENASE AND NADPH DEHYDROGENASE IN DIFFERENT ENZYME PREPARATIONS

Preparation used	*Conditions*	*Specific activity*		
		NADH dehydrogenase (nmoles electron acceptor per ml per mg protein per min)		*NADPH dehydro-genase (nmoles DCIP per ml per mg protein per min)*
		With $K_3Fe(CN)_6$ as electron acceptor	*With DCIP as electron acceptor*	
Ghosts prepared by the first method	Control	45 5	8 41	2 08
	1 mM mersalyl added	8 9	2 01	0 19
	10 mM mersalyl added	0 3	0 07	None
	0 1 mM *p*-chloro-mercuribenzoate added	10 1	1 98	0 14
Fraction obtained by $(NH_4)_2SO_4$ precipitation at 55% satn	Fresh preparation	93	18 2	8 62
	Aged preparation (15 days at −15°)	41	9 1	7 38

TABLE V

EFFECTS OF NaCl ON LACTATE DEHYDROGENASE, MALATE DEHYDROGENASE AND GLYOXYLATE REDUCTASE ACTIVITY OF GHOSTS PREPARED BY THE FIRST METHOD

The pH of the medium was 6 2 for glyoxylate reductase and 7 2 for other assays Specific activities expressed as in Table I

Enzyme tested	*Substrate concn (mM)*	*Specific activity*		
		Control	*0 05 M NaCl*	*0 5 M NaCl*
Lactate dehydrogenase	Pyruvate, 0 2	24 5	23 3	17 1
	Pyruvate, 2	15 8	16 9	27 5
Malate dehydrogenase	Oxaloacetate, 0 5	36 6	41 5	30 0
	Oxaloacetate, 5	41 0	50 8	62 4
Glyoxylate reductase	Glyoxylate, 1	11 5	9 9	3 8
	Glyoxylate, 10	28 7	28 4	12 0

However, using preparations obtained by $(NH_4)_2SO_4$ fractionation, it was observed that the NADH dehydrogenase activity was occasionally rather low, especially after aging This was true not only with $K_3Fe(CN)_6$ but also with DCIP as electron acceptor (Table IV) On the other hand, NADPH dehydrogenase activity appeared to be more stable This suggests that DCIP reduction is catalyzed by the same enzyme as $K_3Fe(CN)_6$ reduction, namely NADH dehydrogenase NADPH dehydrogenase would be a distinct enzyme

DISCUSSION

The present data suggest that the conclusions reached by GREEN *et al* [2] about glycolytic enzymes may be extended to most oxidoreductases of the red cell These enzymes appear, indeed, to be more or less firmly bound to the membrane, the milder the conditions of hemolysis, the higher are the specific activities of most enzymes in the ghost fraction It would be tempting to extrapolate and to assume that in the intact cell very few enzyme molecules are really free in the intracellular fluid

However, the absence of bound triosephosphate dehydrogenase in pig red cell ghosts is worth noticing HARKNESS *et al* [6] reported that appreciable amounts of this enzyme were present in whole hemolysates of pig erythrocytes, thus, we must consider this enzyme as soluble or, at least, very weakly bound in porcine erythrocytes This may be in relation with the very low rate of glycolytic activity in the pig red cell[7]

As to the physiological significance of the enzyme activities described here, conclusive evidence is still lacking in most cases The presence of large quantities of malate dehydrogenase in the particulate as well as in the soluble[8] fraction is puzzling, since no pathway leading to the synthesis of malate or oxaloacetate is known in mammalian erythrocytes Anyway, the presence of a large excess of an enzyme is often indicative of a regulatory function, for instance, malate dehydrogenase might compete with lactate dehydrogenase for NADH binding

Concerning the role of NADH dehydrogenase, no indication can be drawn from the present data Methemoglobin reduction may, in fact, be its only function in the mature erythrocyte[9,10].

On the other hand, our data suggest that this enzyme is distinct from NADPH dehydrogenase Similar conclusions, based on electrophoresis of hemolysates, were reached by KAPLAN AND BEUTLER[10] The membrane-bound NADH dehydrogenase described here may be identical with the "NAD^+-diaphorase" purified by SCOTT AND MCGRAW[11], whereas NADPH dehydrogenase more probably corresponds to the "methemoglobin reductase" purified by HUENNEKENS *et al* [12]

The presence of firmly bound glyoxylate reductase in the erythrocyte remains puzzling as well Indeed, no pathway for glyoxylate synthesis has been shown to exist in red cells, moreover, FISHER AND WATTS[13] have shown that in the living erythrocyte, glyoxylate was oxidized to oxalate rather than reduced to glycollate As a matter of fact, the unusually high K_m for glyoxylate reduction might indicate that we are not dealing with the physiological substrate Maybe the enzyme described here is by no means involved in glyoxylate metabolism

Recent data on transport processes in the erythrocyte membrane may help us to propose an alternative explanation We have been able to find several indications that some link might exist between the Ca^{2+}-dependent ATP splitting by ghosts and an hypothetical electron transfer reaction[14], the overall process possibly being associated with Ca^{2+} translocation. Since DCIP as well as glyoxylate, but not $K_3Fe(CN)_6$, were found to be potent inhibitors of the ATPase reaction (P WINS, unpublished data)[14], NADPH dehydrogenase and glyoxylate reductase may appear as possible candidates for association with the ATPase system Evidence supporting this hypothesis will be reported elsewhere

The effects of NaCl on lactate dehydrogenase, malate dehydrogenase and glyoxylate reductase have already been investigated in extracts of Crustacean tissues[14,15] They have been interpreted as indicative of the fact that the fate of the reducing power is partially under the control of the ionic composition of the intracellular fluid, thus linking the activity of the cellular membrane and that of crucial metabolic sequences The present findings, if they do not demonstrate the significance of the inorganic ions in the case of red cells, nevertheless are suggestive of a possible role of inorganic ions in the integration of the metabolic sequences of the cell membrane

ACKNOWLEDGMENTS

P W was an aspirant of the Fonds National de la Recherche Scientifique E S was aided by a "Crédit aux Chercheurs" from the Fonds National de la Recherche Scientifique

REFERENCES

1 R L POST, C R MERRIT, C R KINSOLVING AND C D ALBRIGHT *J Biol Chem*, 235 (1960) 1796
2 D E GREEN, E MURER, H O HULTIN, S H RICHARDSON, B SALMON, G P BRIERLEY AND H BAUM, *Arch Biochem Biophys*, 112 (1965) 635
3 R G DAVIDSON AND J A CORTNER, *Nature*, 215 (1967) 761
4 I ZAMUDIO AND M CANESSA, *Biochim Biophys Acta*, 120 (1966) 165

5 O H LOWRY, N J ROSEBROUGH, A L FARR AND R J RANDALL, *J Biol Chem*, 193 (1951) 265
6 D R HARKNESS, J PONCE AND V GRAYSON, *Comp Biochem Physiol*, 28 (1969) 129
7 P C LARIS, *J Cellular Comp Physiol*, 51 (1958) 273
8 G W LOHR AND H D WALLER, *Folia Haematol*, 78 (1961) 384
9 E R JAFFE, in CH BISHOP AND D M SURGENOR, *The Red Blood Cell*, Academic Press, New York, 1964, p 397
10 J C KAPLAN AND E BEUTLER, *Biochem Biophys Res Commun*, 29 (1967) 605
11 E M SCOTT AND J C MCGRAW, *J Biol Chem*, 237 (1962) 249
12 F M HUENNEKENS, R W CAFFREY, R E BASFORD AND B W GABRIO, *J Biol Chem*, 227 (1957) 261
13 V FISHER AND R W E WATTS, *Biochem J*, 104 (1967) 42P
14 P WINS AND E SCHOFFENIELS, *Life Sci*, 7 (1968) 673
15 E SCHOFFENIELS, *Arch Intern Physiol Biochem*, 76 (1968) 319