Biochem. J. (1984) **221**, 197–202
Printed in Great Britain

Glyceraldehyde-3-phosphate dehydrogenase release from erythrocytes during haemolysis

No evidence for substantial binding of the enzyme to the membrane in the intact cell

Gillian T. RICH,* Alan P. DAWSON and Jack S. PRYOR
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

(*Received 7 February 1984/Accepted 3 April 1984*)

Glyceraldehyde-3-phosphate dehydrogenase (G3PDH, EC 1.2.1.12) release from haemolysing erythrocytes, and its redistribution between free and membrane-bound states, were studied with a new type of rapid-mixing filtration apparatus. The apparatus is described. The results indicate that the rate of G3PDH appearance in filtrates is determined not only by the enzyme redistribution but also by the kinetics of haemolysis. We have quantified the extent of haemolysis as a function of time, by measuring the amounts of filterable K^+ and lactate dehydrogenase. These are cytoplasmic components that are not membrane-bound. When we correct for incomplete haemolysis, extrapolation to zero time indicates that very little G3PDH is membrane-bound in the intact cell.

For some years there has been interest in binding of glycolytic enzymes to the cytoplasmic surface of erythrocyte membranes. It has been proposed (Schrier, 1966; Mercer & Dunham, 1981) that the bound enzymes might form supramolecular complexes which also compartmentalize a fraction of the cell ATP at the membrane. G3PDH (EC 1.2.1.12) (Yu & Steck, 1975), fructose-bisphosphate aldolase (EC 4.1.2.13) (Strapazon & Steck, 1977) and phosphofructokinase (EC 2.7.1.11) (Higashi *et al.*, 1979) bind to the cytoplasmic portion of the Band-3 membrane protein in isolated membranes. However, the association of these enzymes involves electrostatic interactions. Experiments on isolated membranes indicate that at physiological ionic strength all these enzymes would be eluted from the Band-3 sites.

It is difficult to extrapolate results from such experiments *in vitro* to the intact cell, where local surface concentrations of ions and metabolites are not known. Kliman & Steck (1980) obtained evidence for binding of G3PDH *in situ* by studying the kinetics of the enzyme release when cells were rapidly haemolysed in hypo-osmotic solutions containing saponin. Extrapolation to zero time indicated that about 65% of the total G3PDH was membrane-bound at the time of haemolysis. This result is supported by studies on intact cells, which include cross-linking of G3PDH to the membrane with glutaraldehyde (Keokitichai & Wrigglesworth, 1980) and an investigation by electron microscopy of the distribution of ^{3}H-labelled enzyme (Solti *et al.*, 1981). However, when G3PDH is bound to Band 3, its activity is inhibited (McDaniel *et al.*, 1974; Tsai *et al.*, 1982). A ^{1}H-n.m.r. study (Brindle *et al.*, 1982) of the kinetic properties of G3PDH in the intact cell indicated that its activity was not inhibited, and so it is unlikely that a substantial fraction of the enzyme is membrane-bound in the intact cell.

To resolve this discrepancy, we have re-examined the kinetics of G3PDH release from haemolysing erythrocytes. In our hands the rapid filtration technique of Kliman & Steck (1980), whereby free enzyme is separated from membrane-bound enzyme by rapid filtration, was not always successful. Although our initial observations were similar to those by Kliman & Steck (1980), it was apparent that there was incomplete mixing of the cells into the haemolysing solution, and less than 100% haemolysis. To study the fast kinetics a new rapid-mixing filtration apparatus was designed. For fast filtration the filter was incorporated into the mixing chamber. Corrections for incomplete

Abbreviations used: G3PDH, glyceraldehyde-3-phosphate dehydrogenase. LDH, lactate dehydrogenase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* To whom reprint requests should be addressed.

haemolysis were made by measuring the kinetics of release of K^+ and LDH (EC 1.1.1.27). The results of our studies, described here, indicate that very little G3PDH is membrane-bound in the intact cell.

Experimental

Materials and general methods

Saponin was obtained as a mixture of saponins from Sigma (London) Chemical Co. K^+ (and other cations) in the saponin were exchanged for Na^+ by passage of a 10% (w/v) saponin solution down a column of cation-exchange resin (BioRad AG 50W-X8, Na^+ form), followed by freeze-drying. Enzymes and substrates were obtained from Boehringer Corp. Other chemicals were of analytical grade or better.

Blood was obtained from healthy human donors by venipuncture, with heparin (10–20 units/ml of blood) as anticoagulant. Packed cells were washed with 3 × 10 vol. of 0.15 M-NaCl, either with or without 5 mM-glucose, at 0–4°C. The packed cells were then resuspended in 0.15 M-NaCl (with or without 5 mM-glucose) at a haematocrit of 0.47 ± 0.03, and kept at 0–4°C until used (within 3 h of the venipuncture).

The haemolysing solutions contained 0.1% saponin, 1 mM-dithiothreitol, 0.1 mM-NaEDTA, 10 mM-Hepes (pH 7.4 at 37°C) and NaCl as required. The filtrates and total haemolysates were analysed for G3PDH, LDH, acetylcholinesterase (EC 3.1.1.7) and K^+. To prevent inactivation of G3PDH by aggregation of the enzyme in solutions of low ionic strength, haemolysates and filtrates containing less than 30 mM-NaCl were diluted into 0.1 M-Tris/HCl (pH 8.2) containing 1 mM-EDTA, 2 mM-dithiothreitol and 0.1% saponin. Enzymes were assayed at 25°C on a Pye–Unicam SP.8-400 spectrophotometer in 1 cm cells; K^+ was measured by flame photometry on a Corning 400 flame photometer, with appropriate corrections for Na^+ interference where necessary.

LDH and acetylcholinesterase were assayed by the methods of Beutler (1975) and Steck (1974) respectively.

G3DPH was assayed by a modification of the Warburg & Christian (1941) method. The assay medium contained 4.3 mM-fructose 1,6-bisphosphate plus 0.02 mg of aldolase/ml to generate glyceraldehyde 3-phosphate, 17 mM-arsenate, 0.72 mM-NAD^+, 2.5 mM-EDTA and 2.0 mM-dithiothreitol in 0.1 M-Tris/HCl buffer (pH 8.2). The reaction was started by the addition of NAD^+. Two estimates were made of the G3PDH activity: firstly from the initial rate of reduction of NAD^+, and secondly from the rate between 1 and 2 min. When the filtrate activity was expressed as a fraction of the total haemolysate activity, the two estimates usually agreed within 3%. However, in some cases there was a larger discrepancy, and the initial rate of reduction of NAD^+ by higher concentrations of total haemolysate appeared to underestimate the G3PDH activity. We have attributed this to restricted accessibility of some of the enzyme.

In analysing the total haemolysates for G3PDH and LDH we had to be sure that all the enzymes were accessible to substrates and not sequestered in unhaemolysed cells or resealed vesicles. Since saponin was present in the haemolysates, one might assume this was the case, and it is in principle easy to check for complete accessibility of enzymes by the usual procedure (Steck, 1974) of adding a detergent, such as Triton X-100, to make the intact membranes 'leaky'. However, we found it difficult to adjust the detergent concentration to the optimum concentration without inhibiting some of the unsequestered enzyme. Therefore we adopted another strategy. Samples of the total haemolysates were routinely frozen twice in liquid N_2 and thawed at 25°C. This procedure either enhanced ($\leqslant$10%) or had no effect on enzyme activities. Freezing and thawing the filtrates was without effect. The true activities of the total haemolysates were taken as the highest measured. When these values were used, the calibration of activity against volume of haemolysate was linear, and the two estimates of filtrate G3PDH activity expressed as a fraction of total haemolysate activity agreed within 3%

The rapid-mixing filtration apparatus

To separate free G3PDH from haemolysing cells, a suspension of cells is injected rapidly into a stirred mixing vessel containing the haemolysing solution. At different time intervals thereafter filtrates are collected in four compartments through a filter which forms the base of the mixing vessel. Filtration is achieved by applying a vacuum to the relevant compartment. Filtration is stopped by gas pressure exceeding the hydrostatic pressure tending to drive fluid through the filter.

Fig. 1 shows the details of the apparatus. The three sections (glass mixing vessel, filter and filter support, and glass filtrate-collecting chamber) are clamped together with a Quickfit JC29 clip. The mixing vessel is stirred by an overhead stirrer normally rotating at 5500 rev./min. Temperature is controlled by water circulating through the jacket of the vessel. The filter (Sartorius; 0.8 μm pore size; SM 111 04) is supported on a sectored Perspex disc, the four quarters of which have 1 mm slits and holes through which the filtrate passes to the bottom glass collection chamber. The latter comprises four separate compartments, each compartment having a single inlet tube. The Perspex disc and top surfaces of the collection chamber are

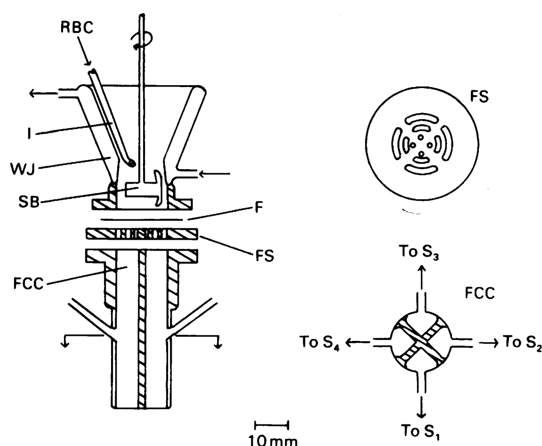


Fig. 1. *The rapid-mixing filtration apparatus*
On the left it is shown in vertical cross-section; horizontal cross-sections of the filter support and filtrate-collection chamber are shown on the right. Key: F, filter; FS, filter support; FCC, filtrate-collection chamber; I, injection tube connected to a 2ml plastic syringe (not shown) containing the erythrocyte suspension (RBC); S_1–S_4, solenoid valves; SB, stirrer bar; WJ, thermostatically controlled water jacket of the mixing vessel.

ground to flatness and a thin layer of silicone vacuum grease is smeared over the collection-chamber top surfaces, so that each collection compartment is essentially isolated from pressure changes in neighbouring compartments.

The pressures controlling filtration are applied via three-way solenoid valves connected by short lengths of pressure tubing to the inlet tubes of the collection compartments. (The valves were either Bürkett type 330, circuit function E or Webber Electro Components 17034700.) In the de-energized state each valve connects a pressure of 10 ± 1 mmHg above atmospheric pressure to a collection compartment. When energized, this port is closed and a vacuum of 700mmHg is applied. The positive pressure is supplied from an oxygen cylinder via a constant-pressure device, and the vacuum from a Millipore (XX60) vacuum pump. The solenoids are energized manually by double-pole switches: one pole connected to the solenoid, the other to the event marker of a Lectromed high-speed recorder. Thus, by energizing the valves sequentially, four filtrates are obtained together with a record of when they were collected.

Before injection the cell suspension is held in a 2ml plastic syringe with a spring-loaded plunger. The top of the plunger carries a printed circuit board switch connected to the event marker of the recorder. The syringe is held in a brass holder heated by water circulating in copper tubes. Injection is via a pure polythene 280 tube with five to six 0.6mm holes drilled round its sealed end. Thus the cell suspension enters the haemolysing solution just above the stirrer bar as a 'spray'.

Results and discussion

Performance of the filtration apparatus

(i) *Injection of the blood suspension.* Erythrocyte suspension (0.33 ± 0.04 ml) was injected into 3.8ml of haemolysing solution at 37°C. Before injection the suspension was kept for 4min in the thermostatically controlled syringe. At this time the temperature of the suspension was 37 ± 0.5°C and its pH between 7.2 and 7.3. Injection time was less than 200ms. The spring-loaded plunger prevented unhaemolysed cells being swept into the haemolysate after the injection.

(ii) *Filtration.* To collect filtrates, the solenoids were energized for 300–500ms. (A metronome was used as an aid for timing the switching). When the solenoids are switched between two gas pressures, 90% of the pressure change is achieved in 20–25ms (manufacturers' information).

The nature of the filter (0.8 μm pore size) was determined by the requirements of good membrane retention and flow rates for sufficient filtrate volumes. The membrane concentration in the filtrates, measured by acetylcholinesterase assays, was 0.5–4.5% of that in the total haemolysates; the filtrate volumes were 30–60 μl. Thus after four filtrates had been collected the membrane concentration in the haemolysate increased by less than 6%.

(iii) *Efficiency of mixing and haemolysis.* Ideally, cell contents that are not membrane-bound should be completely recovered in the filtrates. Initially, we chose K^+ as a marker for the free cytoplasm. Fig. 2(*a*) shows some examples of the time course of K^+ release from haemolysing cell suspensions. The K^+ concentration in the filtrate is expressed as a fraction, f_K, of the K^+ concentration in the total haemolysate. It is clear that complete recovery of K^+ is not reliably obtained and the actual time course is rather erratic. Two factors could be responsible for this: incomplete mixing and less than 100% haemolysis. Experiments on dye recovery in filtrates when dye solutions were injected into sucrose solutions, giving final viscosities comparable with those in haemolysates, indicated that mixing was complete to within 5% in 0.5s. Therefore, although we cannot entirely exclude slow mixing, incomplete haemolysis would appear to be an important factor. This view is supported by stopped-flow experiments (C. Greenwood & G. T. Rich, unpublished work) on erythrocytes from a single donor haemolysing in 0.1% saponin solutions containing 75mM-NaCl. The time to

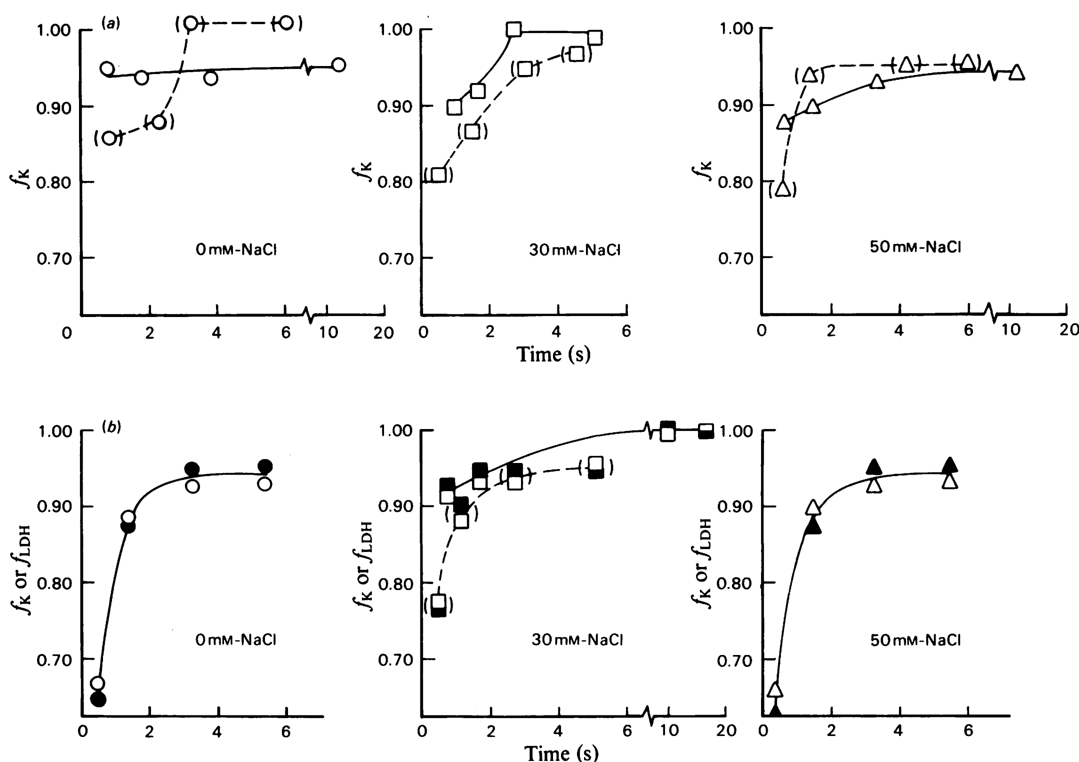


Fig. 2. *Kinetics of release of K^+ and LDH during haemolysis*

f_K or f_{LDH} = (concentration of K^+ or LDH in filtrate)/(concentration of K^+ or LDH in total haemolysate). Cells were injected into haemolysing solutions containing the indicated concentrations of NaCl. The points in parentheses relate to the broken curves, ----; open symbols, f_K; filled symbols, f_{LDH}. In this Figure and Figs. 3 and 4 the injection of the blood suspension took up to 0.2s. Therefore the origin of the time axis is uncertain by this time period.

reach 50% haemolysis as judged by the fall in light-scattering (with white light) was 450ms, and 95% of the total fall was not achieved until 560ms. Increasing the saponin concentration above 0.1% had no detectable effect on K^+ release and the kinetics of haemolysis.

In studying the kinetics of G3PDH release, we have calculated the percentage of free G3PDH as

$$\frac{\text{\% of G3PDH filtered}}{f_K}$$

which corrects for incomplete mixing and haemolysis. The kinetics of LDH release closely paralleled K^+ release (see Fig. 2*b*). This suggests that LDH does not bind to the membranes, and we may also use the fraction of LDH filtered, f_{LDH}, as a correction for incomplete haemolysis. (The K^+ and LDH in the extracellular fluid of the unhaemolysed cell suspension contributed less than 1% to the total haemolysate K^+ and LDH.)

We were concerned that the high-speed stirring of the haemolysate might be causing membrane fragmentation. Phase-contrast microscopy indicated that this was not the case. For some experiments we arranged that energizing the solenoid for collection of the first filtrate also activated a relay switch to decrease the stirring rate to 550rev./min. The equilibrium distribution of G3PDH was unaffected by this procedure.

Kinetics of G3PDH release

Assuming that membrane rupture does not alter the association of G3PDH with the membrane, a kinetic curve of G3PDH release from haemolysing cells should show the approach to a new equilibrium distribution of the enzyme between free and bound states, from the initial distribution in the intact cell. Fig. 3(*a*) shows the results of an experiment when cells washed and suspended in 0.15M-NaCl without glucose were injected into a haemolysing solution containing 50mM-NaCl. This kinetic curve of G3PDH release indicates that more than 30% of the enzyme does not pass

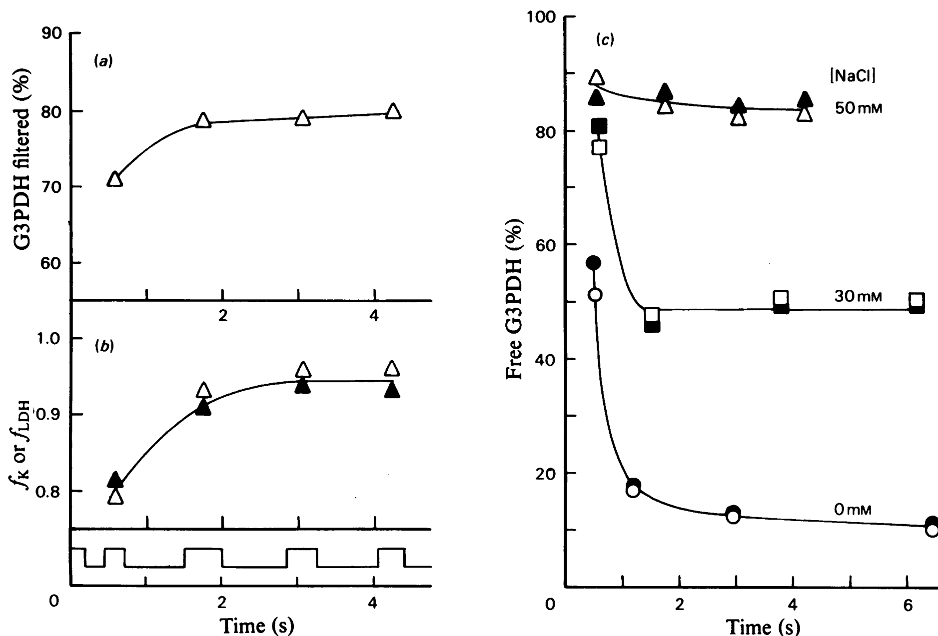
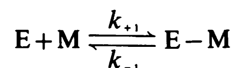


Fig. 3. *Kinetics of G3PDH recovery in filtrates during haemolysis, and redistribution between free and bound states*
Cells were washed and suspended in 0.15M-NaCl without glucose. (*a*) Percentage of G3PDH filtered from cells haemolysing in 50mM-NaCl. (*b*) Release of K^+ and LDH in the same experiment; △, f_K; ▲, f_{LDH}. The bottom trace shows the event markings for injection and filtrate collection. The data shown in (*b*) were used to calculate the percentage of free G3PDH in the top curve of (*c*). (*c*) Kinetics of G3PDH redistribution during haemolysis in solutions containing (△, ▲) 50mM, (□, ■) 30mM and (○, ●) 0mM-NaCl. Open and filled symbols refer to how the percentage of free G3PDH was calculated from the percentage of G3PDH filtered: open symbols, using f_K; filled symbols, using f_{LDH}.

through the filter at times less than 0.5s. Therefore, on a simple interpretation we might conclude that at least 30% of the enzyme is membrane-bound in the intact cell. However, when we correct for incomplete haemolysis by using the K^+ and LDH released (Fig. 3*b*), the amount of bound enzyme is substantially decreased, so that extrapolation of the corrected amount of free G3PDH to zero time (top curve of Fig. 3*c*) indicates that less than 10% of the enzyme is membrane-bound in the intact cell. Fig. 3(*c*) also shows the results from the same experiment when cells were haemolysed in lower-ionic-strength solutions. It is clear that very little G3PDH is membrane-bound at the moment of haemolysis.

The same conclusion is reached when cells suspended in 0.15mM-NaCl with 5mM-glucose are haemolysed. Fig. 4 shows the results of several experiments under these conditions, including one where saponin was omitted from a haemolysing solution, which contained no added NaCl. The kinetics of redistribution of G3PDH under these conditions are indistinguishable from those when saponin was present. This observation contrasts with the finding by Kliman & Steck (1980) that the presence of saponin in their system more than doubled the apparent association rate constant; We attribute this difference to the fact that we have corrected for the membrane acting as a diffusion barrier, whereas Kliman & Steck (1980) considered that the saponin-treated membrane did not impose a barrier.

We do not have sufficient points on our kinetic curves to do a rigorous analysis of the G3PDH redistribution. However, we have shown that the model used by Kliman & Steck (1980) of an approach to a new equilibrium of the reaction



$$E + M \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E - M$$

(E = enzyme, M = Band-3 binding site), with invariant rate constants for a given haemolysing solution, does not fit our data. This is not surprising: the rate constants are dependent on ionic strength and metabolite concentration. Both of these will be changing at the cytoplasmic membrane surface at a rate comparable with the apparent rate of G3PDH redistribution. In fact, the time scale of the redistribution does not

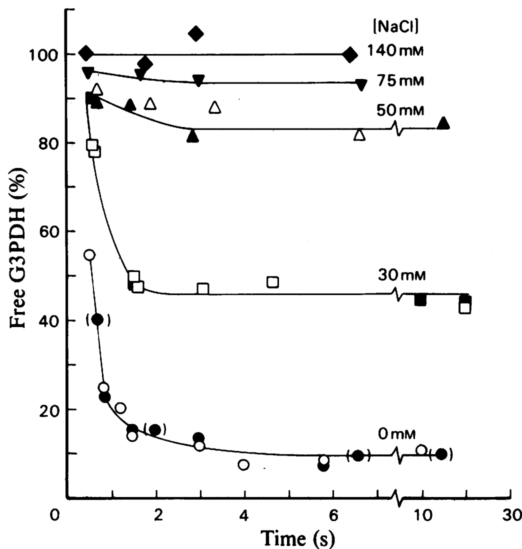


Fig. 4. *Kinetics of G3PDH redistribution during haemolysis*

Erythrocytes were washed and suspended in 0.15M-NaCl+5mM-glucose, and haemolysed in solutions containing the indicated concentrations of NaCl. Symbols are the same as in Fig. 3(*c*), plus: ♦, 140mM-NaCl; ▼, 75mM-NaCl. The points in parentheses (●) for the 0mM-NaCl curve are from an experiment where saponin was omitted from the haemolysing solution.

preclude the process having a diffusion-limiting component.

Conclusions

Our results indicate that less than 10% of the total erythrocyte G3PDH is membrane-bound in the intact cell. However, it would be premature to conclude this is generally true. Erythrocytes at the point of haemolysis in buffered NaCl solutions equilibrated with the atmosphere, and after cooling, followed by only a short time at 37°C, are not exactly comparable with cells *in vivo*. Furthermore, a rapid redistribution of bound enzyme when the membrane is stretched cannot be excluded, and we have yet to establish whether the oxygen-linked binding of haemoglobin to Band 3 (Shaklai & Abrahami, 1980) affects G3PDH binding.

We thank the staff and students of the University of East Anglia who donated blood for the experiments, Mr. J. Preston for glass blowing, and Mr. D. Fulton for technical assistance. The work was supported by the East Anglian Regional Health Authority.

References

Beutler, E. (1975) *Red Cell Metabolism*, 2nd edn., Grune and Stratton, New York, San Francisco and London
Brindle, K. M., Campbell, I. D. & Simpson, R. J. (1982) *Biochem. J.* **208**, 583–592
Higashi, T., Richards, C. S. & Uyeda, K. (1979) *J. Biol. Chem.* **254**, 9542–9550
Keokitichai, S. & Wrigglesworth, J. M. (1980) *Biochem. J.* **187**, 837–841
Kliman, H. J. & Steck, T. L. (1980) *J. Biol. Chem.* **255**, 6314–6321
McDaniel, C. F., Kirtley, M. E. & Tanner, M. J. A. (1974) *J. Biol. Chem.* **249**, 6478–6485
Mercer, R. W. & Dunham, P. B. (1981) *J. Gen. Physiol.* **78**, 547–568
Schrier, S. L. (1966) *Am. J. Physiol.* **210**, 139–145
Shaklai, N. & Abrahami, H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1105–1112
Solti, M., Bartha, F., Halasz, N., Toth, G., Sirokman, F. & Friedrich, P. (1981) *J. Biol. Chem.* **256**, 9260–9265
Steck, T. L. (1974) *Methods Membr. Biol.* **2**, 245–281
Strapazon, E. & Steck, T. L. (1977) *Biochemistry* **16**, 2966–2971
Tsai, I. H., Murthy, S. N. P. & Steck, T. L. (1982) *J. Biol. Chem.* **257**, 1438–1442
Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–397
Yu, J. & Steck, T. L. (1975) *J. Biol. Chem.* **250**, 9176–9184