Short Communications

SC 11034

The purification and properties of glucose-6-phosphate dehydrogenase from human erythrocytes

Considerable interest has recently been shown in erythrocytic glucose-6-phosphate dehydrogenase (EC 1.1.1.49), since a deficiency of this enzyme occurs in hereditary primaquine sensitivity[1]. Reports of 80-fold[2], 500-fold[3] and recently of 10 000-fold purification[4] have appeared. This paper describes a modification and extension of the method of MARKS *et al.*[3] to yield 2000- to 3000-fold purification; on one occasion, a purification factor of 8300 was obtained.

The enzyme was assayed by following TPNH production at 340 mμ. Quartz cuvettes, 1 cm path, contained in 3 ml, 270 μmoles Tris–HCl buffer (pH 8.2), 5 μmoles disodium glucose 6-phosphate, 2.4 μmoles TPN^+ and 0.1 ml enzyme, suitably diluted to give a $\Delta A_{340\ m\mu}$ of 0.04–0.08/min in the Beckman DU Spectrophotometer fitted with a double thermospacer at 25°. 6-Phosphogluconate dehydrogenase (decarboxylating; EC 1.1.1.44) activity was measured by replacing the glucose 6-phosphate with 6-phosphogluconate. Protein was estimated by the method of WARBURG AND CHRISTIAN[5]. A unit of activity is the amount of enzyme giving a $\Delta A_{340\ m\mu}$ of 1.0 per min under the above conditions. Specific activity is the activity per mg protein. The Michaelis constant (K_m) for TPN^+ was measured using a Farrand fluorimeter[6], and plotting $1/v$ against $1/s$ (ref. 7).

Human bank blood, stored for less than 2 weeks, provided the enzyme source. All manipulations were carried out at 4°. The erythrocytes were washed, lysed and the preliminary enzyme purification carried out by the first ammonium sulphate fractionation (2-stage) of MARKS *et al.*[3]. The precipitate from the second stage was dissolved in 50 ml 0.1 M Tris. HCl (pH 7.4) and stored frozen with 10^{-5} M TPN^+ and 10^{-3} M cysteine until required. Storage of the enzyme at this stage sometimes caused up to 50% loss of activity, but at other times no losses occurred. Appreciable activity (30–50%) remained in the supernatant; this could not be recovered since addition of a further 2 g ammonium sulphate/100 ml precipitated the bulk of the haemoglobin. Yields could be improved somewhat by subdividing the second ammonium sulphate fraction, adding first 8 g ammonium sulphate/100 ml solution and then, after centrifuging, adding a further 4 g/100 ml. Most of the 6-phosphogluconate dehydrogenase activity (85%) remained in the supernatant.

Several batches were combined at this stage, thawed and dialysed for 2 h in Visking cellophane tubing (1 in) against several changes of deionized water. 5 successive 0.1-vol. portions of calcium phosphate gel[8] were added and collected by spinning at 500 × *g* after 10-min equilibration. The supernatants were assayed and the gel portions which had absorbed most activity were washed with 1 gel volume of water and eluted successively with 1 gel-volume portions of 0.2 M potassium phosphate buffer (pH 5.8) and 0.5 M buffer (pH 7.4). The eluates were assayed and those of highest specific activity combined and stored frozen overnight with 10^{-5} M TPN^+.

The next day the solutions were thawed and dialysed as above, and then allowed to drip at 1 drop/sec through a Whatman DEAE-cellulose (floc) column of exchange capacity of 1 mequiv./g, which had been equilibrated with 0.05 M phosphate buffer (pH 5.8). The column was washed with water until free of haemoglobin and eluted with 60 ml of 0.2 M phosphate buffer (pH 5.8) and 40 ml of 0.5 M phosphate buffer (pH 7.4). 10-ml fractions were collected. As storage of the eluates, even at −15°, resulted in considerable losses of activity, the fractions of highest specific activity were immediately adjusted to 60% ammonium sulphate saturation. The precipitate was collected by centrifugation at 10 000 × *g* and resuspended in a small volume of 60% satd. ammonium sulphate. This suspension was stable at 4° for at least a week and was redissolved for use by dialysing against water. The concentrated solution so obtained (1.25 mg protein/ml) was also stable on storage at 4°, but a 1 : 10 dilution lost 50% activity in 2 days. The suspension could be further purified by fractional elution from a cellulose column with gradually decreasing concentrations of ammonium sulphate solution. However, the additional purification obtained was only 1.2-fold and, since losses were fairly large, this step was subsequently omitted.

Ammonium sulphate fractionation of the calcium phosphate eluates, as recommended by MARKS *et al.*[3], gave no purification and fairly heavy losses were sustained. Heating of these eluates to 52–54° for 2 min, even in the presence of 10^{-5} M TPN^+, caused loss of activity with no further purification.

Table I shows the results of a typical purification procedure. The purified enzyme was free of 6-phosphogluconate dehydrogenase.

TABLE I

PURIFICATION PROCEDURE FOR PREPARATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Fraction	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification factor*
Haemolysate	200	0.0015	100	1
Ammonium sulphate Fraction 2	93	0.05	47	33
Calcium phosphate eluates	57	0.8	28	530
DEAE-cellulose Eluates 1	36	10.4	24	7000
DEAE-cellulose Eluates 2	12	4.6		3000
Fractional ammonium sulphate elution of precipitated Eluate 1	11	12.5	5.5	8300

The K_m for glucose 6-phosphate at pH 8.2 was $6.3 \cdot 10^{-5}$ M and for TPN^+ 1.3, 2.1 and $2.6 \cdot 10^{-6}$ M, as measured on 3 batches of purified enzyme. These figures are very similar to those described by KIRKMAN *et al.*[2] who found K_m values of $1.3 \cdot 10^{-5}$M and $2.1 \cdot 10^{-6}$ M for glucose 6-phosphate and TPN^+, respectively, at pH 8.0; and by MARKS *et al.*[3] who obtained $3.5 \cdot 10^{-5}$ M and $4.2 \cdot 10^{-6}$ M, respectively, at pH 7.6. The pH optimum was found to be a broad peak between pH 8 and 9 in Tris–HCl buffer; in glycine–NaOH and 2-amino-2-methylpropan-1,3-diol–HCl buffers, there was a slight peak at pH 9.6, the shape of the curve and the values obtained being very similar to those obtained by KIRKMAN *et al.*[2]. *p*-Chloromercuribenzoate (10^{-3} M) inhibits enzyme activity 44%, indicating the presence of –SH groups in the active centre. This inhibition was only slightly reversed by 10^{-2} M cysteine.

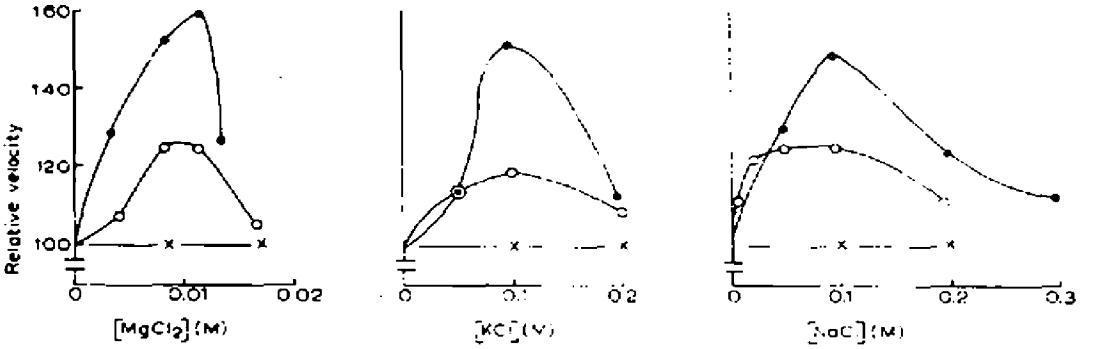

Fig. 1. Salt activation of glucose-6-phosphate dehydrogenase. ● ●, glycine–NaOH buffer (pH 9.5); ○—○, 2-amino-2-methylpropan-1,3-diol·HCl buffer (pH 9.0); × - ×, Tris·HCl buffer (pH 8.2).

Indications that yeast glucose-6-phosphate dehydrogenase is activated by various salts are given by GLASER AND BROWN[9] who showed that the enzyme had no absolute requirement for metal ions, but that these appeared to increase the affinity of the enzyme for the substrate. Fig. 1 shows that such activation also occurs with erythrocytic glucose-6-phosphate dehydrogenase, optimum activation being obtained with 0.01 M $MgCl_2$, 0.1 M NaCl and 0.1 M KCl. This activation was found to be pH dependent; at pH 8.2 no activation occurred, but the activation at the optimum salt concentration was 20 26% at pH 9.0 and 50 60% at pH 9.5. There was no inhibition of enzyme activity by 10 mM EDTA, indicating that the enzyme has no absolute requirement for divalent metal ions.

This work was supported in part by grant H-5448 of the National Institutes of Health (U.S. Public Health Service). The authors are indebted to Dr. J. H. S. GEAR for his continued interest in these studies and Dr. A. ZOUTENDYK for supplying the blood-bank samples.

Electrolyte and Metabolic Research Unit,
South African Institute for Medical Research,
Johannesburg (South Africa)

DORIS BALINSKY*
RALPH E. BERNSTEIN

1 E. BEUTLER, *Blood*, 14 (1959) 103.
2 H. N. KIRKMAN, H. D. RILEY, JR. AND B. B. CROWELL, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 938.
3 P. A. MARKS, A. SZEINBERG AND J. BANKS, *J. Biol. Chem.*, 236 (1961) 10.
4 H. N. KIRKMAN, *J. Biol. Chem.*, 237 (1962) 2364.
5 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1942) 354.
6 O. H. LOWRY, M. R. ROBERTS AND J. I. KAPPHAHN, *J. Biol. Chem.*, 224 (1957) 1047.
7 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
8 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc., London, Ser. B*, 124 (1938) 397.
9 L. GLASER AND D. H. BROWN, *J. Biol. Chem.*, 216 (1955) 67.

Received August 27th, 1962

* In receipt of a grant from the South African Council for Scientific and Industrial Research.