GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM HUMAN ERYTHROCYTES: MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION.

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In the last few years several authors have reported contrasting data on the molecular weight of glucose 6-phosphate dehydrogenase (G6PD) from human erythrocytes. Kirkman and Hendrickson (1962) and Tsutsui and Marks (1962) obtained a value of 105.000 by gradient centrifugation; Chung and Langdon (1963a) obtained a value of 190.000 by the moving boundary method; these values were found to be reduced to about one half upon treatment of the enzyme with acid ammonium sulphate. More recently Yoshida (1966) by the sedimentation equilibrium method obtained a value of about 240.000 for the crystalline G6PD, a m.w. of about 120.000 for the acid ammonium sulphate treated enzyme, and a value of (about) 40.000 for the urea-guanidine HCl treated enzyme, and suggested that this protein may consist of six subunits of similar size. The data presented in this paper indicate that the molecular weight of human erythrocytes G6PD, estimated by gel filtration on Sephadex G200, is 104.000 ± 3,000 (S.D.) and that the molecular weight of the subunits obtained by the acid ammonium sulphate treatment is 52.000 ± 1,000 (S.D.).

Materials and methods.

G6PD was partially purified from human and rat red cells by the procedure of Yoshida (1966); DEAE-cellulose treatment and ammonium sulphate precipitation gave preparations with a specific activity of

about 0.3 I.U./mg. The enzyme activity was assayed according to Zinkham (1958) (Beckman DU spectrophotometer with Gilford accessory and recorder), and expressed in International Units (WHO, 1967). Protein concentration was determined spectrophotometrically at 280 mμ. Sephadex G100 or G200 (Pharmacia) was treated according to the manufacturer's recommendations. The chromatography columns (LKB 4901A, cm 105 x3.2 i.d.) were packed at room temperature and stabilized for three days at + 4°C. Upwards flow was used. The columns were calibrated with Blue Dextran 2000 (Pharmacia; m.w. > 1.000.000; 10% sol., 25 μl), human haptoglobin type 1.1 (apparent m.w. 154.000; 10 mg), human serum albumin dimer (m.w. 135.000; 5 mg) *), bovine serum albumin (BDH; m.w. 67.000; 10 mg), egg albumin (Sigma; m.w. 45.000; 10 mg) and α-chymotrypsinogen (Sigma; m.w. 25.000; 5 mg). Although the same two columns were used in all experiments, Blue Dextran and at least two protein markers were always applied to the columns together with the enzyme preparations being studied. These consisted of aliquots of ammonium sulphate precipitates dissolved in buffer to give a total of 3 to 9 units in a sample volume of 2,5 ml. In the experiments with "native" G6PD, 2,5 ml of fresh haemolysate (about 2,8 I.U.) were directly applied to the columns. Yeast purified G6PD (Boehringer; ammonium sulphate suspension, 25 μl) was dissolved in buffer and applied to the column as such. The buffer used was Na-phosphate 0,1 M, pH 6,8, containing EDTA (10^{-3}M), ß-mercaptoethanol (10^{-3}M) and, for the G200 column, NADP (2×10^{-5}M). The flow rate was kept at 18 ml/h (G100) and 10 ml/h (G200) by means of a peristaltic pump (LKB 4912A). Fractions of 3,0 ml were collected.

*) Although haptoglobin 1.1 has a m.w. of about 100.000, owing to its carbohydrate content it is eluted from Sephadex columns with an apparent m.w. of 154.000 (Rattazzi and Bernini, unpublished data). Haptoglobin 1.1 prepared in our laboratory (Connell and Shaw, 1961), was usually preferred to human albumin dimer, which is present in albumin preparations (Debro et al., 1957) only in small quantities and unstable in the presence of ß-mercaptoethanol.

All experiments were performed at + 4°C. Elution volumes (Ve) were calculated from elution diagrams by measuring the volume corresponding to the maximum protein concentration (or max. G6PD activity) extrapolated to the nearest 0,5 ml. Although remarkably constant, the volume of the fractions was always measured. Calibration lines were obtained by the method of Whitaker (1963). Dissociation of human G6PD was obtained according to Warburg (1938), using 0,1 M phosphate buffer pH 6,8 (containing 10^{-3}M ß-mercaptoethanol and EDTA) to resuspend the enzyme. The partially inactive enzyme eluted from the G100 column was reactivated by incubation at 36,5°C for 1 hour in the presence of NADP (10^{-4}M). (Yoshida, pers. communication).

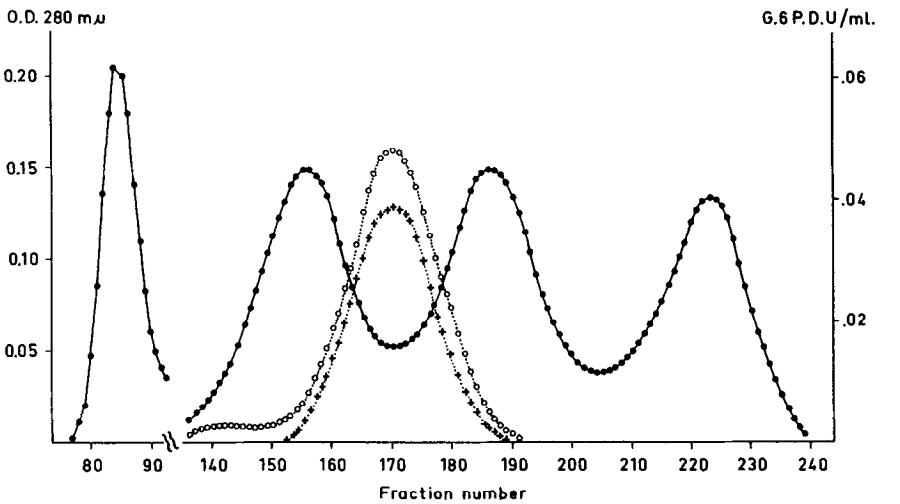
Results.

A) Partially purified human G6PD was eluted from the G200 column between the haptoglobin and the albumin peaks in six experiments. The elution volume was found to be consistent with a molecular weight of 104.000 ± 3.000 (S.D.) (Fig. 1a, Fig. 2). A small peak of enzyme activity (about 2% of the main enzyme peak) was always present; its elution volume suggested a m.w. of about 210.000.

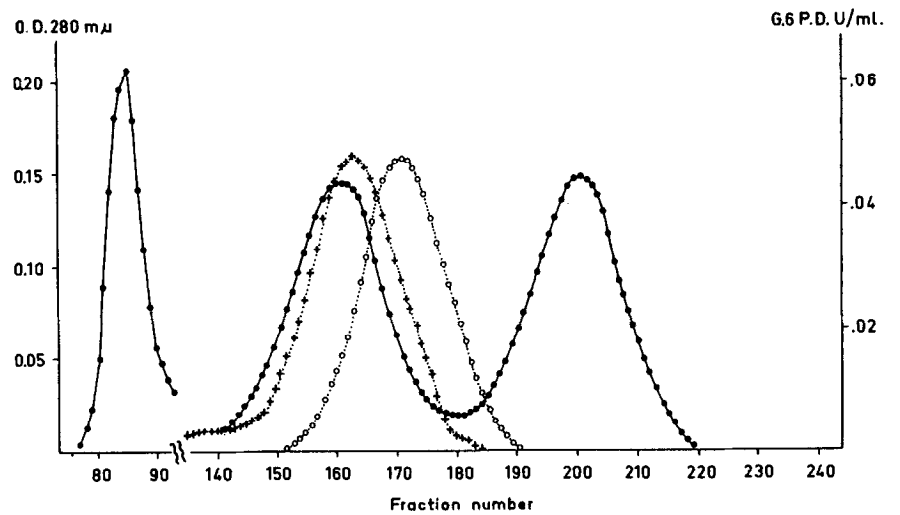
B) "Native" human G6PD from untreated haemolysates was eluted from the same G200 column with an elution volume coincident with that of the partially purified samples, indicating the same m.w. of 104.000. (Fig. 1a, Fig. 2).

C) Yeast purified G6PD was eluted in three experiments between haptoglobin and albumin, its elution volume indicating a m.w. of 101.000 ± 3.000 (S.D.), in good agreement with the value of 102.000 obtained by Yue, Noltmann and Kuby (1967). (Fig. 1b, Fig. 2).

D) Rat erythrocyte G6PD was eluted from the column in three experiments with an elution volume slightly larger than that of human serum



(a)



(b)

Fig. 1 - Elution diagram of Sephadex G200 column, cm. 100 x 3,2 (0, 1M phosphate buffer pH 6,8, containing ß-mercaptoethanol 10^{-3}M, EDTA 10^{-3}M, NADP 2 x 10^{-5}M. Flow rate 10 ml/h, fraction volume 3,0 ml.).

a) ●———● (l. to r.): Blue dextran, human haptoglobin 1.1, bovine serum albumin, α-chymotrypsinogen. o--------o : partially purified human erythrocyte G6PD. +---------+: "native" human G6PD.

b) ●———● (l. to r.): Blue dextran, human serum albumin dimer, egg albumin. +--------+: partially purified rat erythrocyte G6PD. o--------o : purified yeast G6PD.

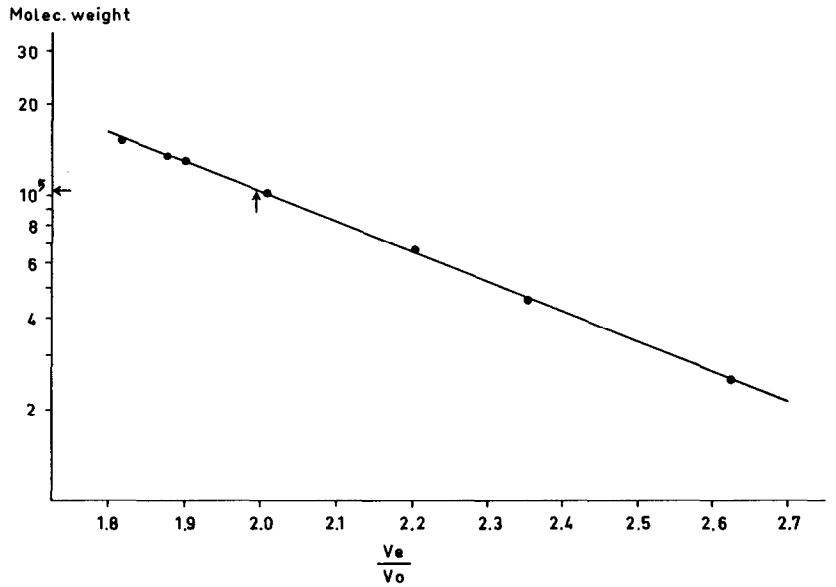
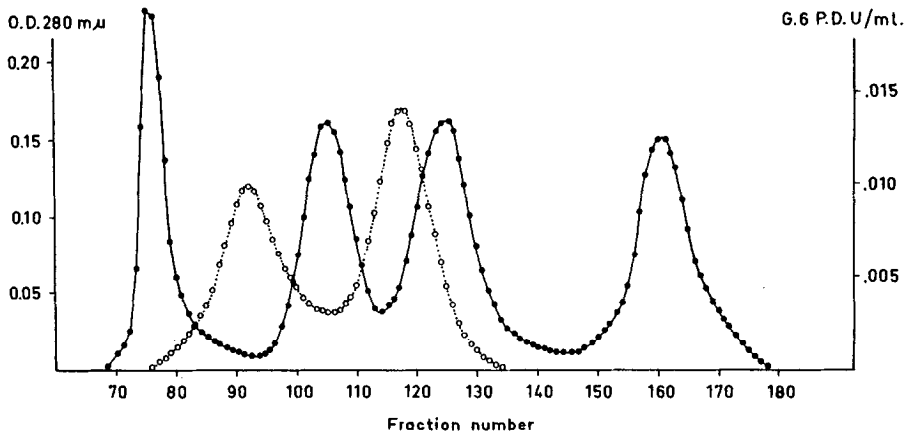


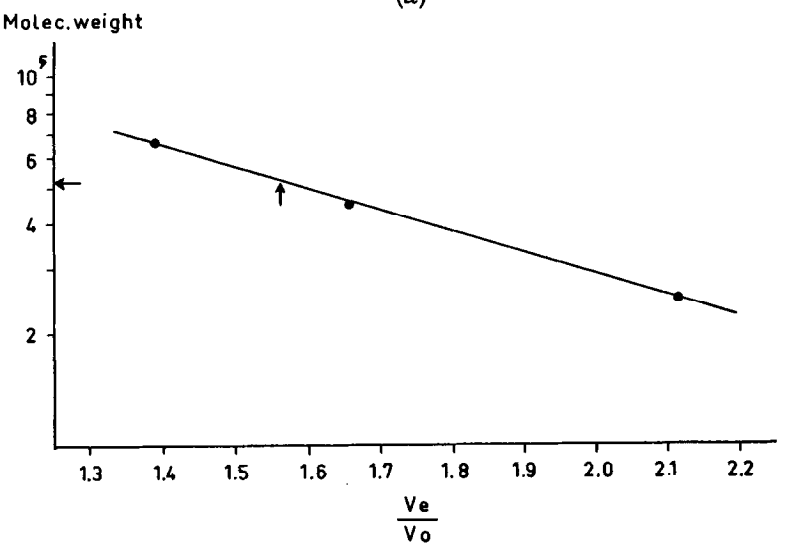
Fig. 2 - - Calibration line of the Sephadex G200 column cm.100 x 3,2. Points represent average Ve/Vo values for (1. to r.): human haptoglobin 1.1, human serum albumin dimer, rat erythrocyte G6PD, yeast G6PD, bovine serum albumine, Egg albumin, α-chymotrypsinogen, from fifteen experiments. Arrows: Ve/Vo and estimated molecular weight of human erythrocyte G6PD (average of seven experiments).

albumin dimer, which indicated a m.w. of 131.000 ± 3.000 (S.D.). This value agrees well with that of 130.000 found by Nevaldine and Levy (1965) for the rat mammary gland enzyme (Fig. 1b, Fig. 2).

E) Acid ammonium sulphate treated, partially purified human G6PD was applied to a column of Sephadex G100 in the absence of NADP, to obtain an independent check on the size of the intact enzyme, by determining the molecular weight of its subunits (Nevaldine and Levy, 1965). Upon reactivation, two peaks of activity were present in the eluate. The first one having an elution volume coincident with that of yeast G6PD under the same conditions, was taken to represent the reassociated form of the enzyme. The second peak, eluted between albumin and egg albumin, had an elution volume indicating a molecular weight of 52.000 ± 1.000 (S.D.). (Fig. 3a, 3b).



(a)



(b)

Fig. 3

a) Elution diagram of a Sephadex G100 column, cm. 100 x 3,2 (0, 1M phosphate buffer pH 6,8, containing β-mercaptoethanol 10^{-3}M and EDTA 10^{-3}M. Flow rate 18 ml/h, fraction volume 3,0 ml). Fractions between tubes 80 and 140 containing the partially inactive enzyme were incubated for reactivation at 36.5° for 1 hour in the presence of NADP (10^{-4}M) before the assay.

●——● (l. to r.): Blue dextran, bovine serum albumin, egg albumin, α-chymotrypsinogen. o--------o (l. to r.): reassociated form and dissociated form of partially purified human G6PD treated with acid ammonium sulphate.

b) Calibration line of the Sephadex G100 column, cm. 100 x 3,2. Points represent average Ve/Vo values for (l. to r.): bovine serum albumin, egg albumin, α-chymotrypsinogen, from nine experiments. Arrows: Ve/Vo value and extimated molecular weight of dissociated form of human G6PD (average of three experiments).

Discussion and conclusions.

Although the mechanism of gel filtration has not been completely explained, the correlation between elution volume and molecular weight holds true for a great number of proteins including several enzymes. Exceptions are represented by non globular proteins with extreme asymmetry and some glycoproteins (Andrews, 1965; Determann, 1967). The use of large columns and of internal standards greatly reduce the chances of ambiguous results.

The agreement between the molecular weight value of yeast G6PD obtained by gel filtration with that obtained by ultracentrifugation seems to rule out the possibility that a peculiar shape of the enzyme molecule would affect its behaviour on Sephadex columns, resulting in misleading elution volumes. The use of this enzyme as a marker, however, rests on the assumption that no major conformational differences exist between human and yeast G6PD. The results of the experiments with rat erythrocyte G6PD prove that the method is sensitive enough in discriminating between G6PD types having a relatively small difference in molecular weight. It seems therefore fair to conclude that the value of 104.000 found for human erythrocyte G6PD is reasonably accurate, and not due to inherent fallacies of the method. The results of the experiments with the dissociated human enzyme indicate that human erythrocyte G6PD consists of two reassociable subunits of (approximately) the same molecular weight (52.000). The dimeric structure suggested by the present data seem to offer a simpler explanation than the trimeric one put forward by Yoshida (1966) for the hybridization experiments between human and rat G6PD (Beutler and Collins, 1965), where only one electrophoretic "hybrid" band was found in addition to the "parental" ones. In view of the recent results of Matsuda and Yugari (1967) who could further dissociate the reassociable subunits of rat liver G6PD into smaller

units, the possibility that the same situation may exist in human G6PD is to be considered.

While being in good agreement with the data of Tsutsui and Marks (1962) and Kirkman and Hendrickson (1962), the data presented here are at variance with those of Chung and Langdon (1963 a,b) and Yoshida (1966). The latter authors, however, used for their determination far more concentrated enzyme solutions than those used in the present work (0.3-0.2 and 0.2-0.08 mg/ml against 0.005-0.002 mg/ml) furthermore, one cannot exclude the possibility that their extensive purification, including several ammonium sulphate precipitations, might have resulted in the formation of aggregates of the G6PD molecules, not dissociating upon moderate dilution. The presence of a small amount of G6PD having a m.w. of about 210.000 in the eluates of the Sephadex G200 columns, when partially purified preparations were used, and its absence in the case of untreated enzyme, although not conclusive, might give some support to this possibility. In view of the fact that dissociated G6PD reassociates rapidly at neutral pH and high concentrations even in the absence of NADP (Kirkman and Hendrickson, 1962), the values of about 100.000 found by Chung and Langdon (1963 b) and Yoshida (1966) for the acid ammonium sulphate treated enzyme need not necessarily contradict this hypothesis

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References

Andrews, P., Biochem. J., 96, 595 (1965)
Beutler, E., Collins, Z., Science, 150, 1306 (1965)

Chung, A. E., Langdon, R. G., J. Biol. Chem., 238, 2309 (1963a)
Chung, A. E., Langdon, R. G., J. Biol. Chem., 238, 2317 (1963b)
Connell, G. E., Shaw, R. W., Canad. J. Biochem. Physiol., 39, 1013 (196]
Debro, J. R., et al., J. Lab. Clin. Med., 50, 728 (1957)
Determann, H., in Protides of the biological fluids, 14, 563,
ed. M. Peeters, Elsevier, Amsterdam (1966)
Kirkman, H. N., Hendrickson, E. M., J. Biol. Chem., 237, 2371 (1962)
Matsuda, T., Yugari, Y., J. Biochem., 61, 535 (1967)
Nevaldine, B. H., Levy, M. R., Biochem. Biophys. Res. Comm., 21,28 (196
Tsutsui, E. A., Marks, P. A., Biochem. Biophys. Res. Comm. 8, 338 (1962)
Warburg, O., Christian, W., Biochem. J., 298, 150 (1938)
Whitaker, J. R., Anal. Chem., 35, 1950 (1963)
World Health Organization, Rechn. Rep. Ser., 366, 31 (1967)
Yoshida, A., J. Biol. Chem., 241, 4966 (1966)
Yue, R. H., Noltmann, E. A., Kuby, S. A., Biochemistry, 6.1174 (1967)
Zinkham, W. H. et al., Bull. Johns Hopkins Hosp., 102, 169 (1958)