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THE SEDIMENTATION PATTERN OF D-GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM BOVINE FETAL AND ADULT ERYTHROCYTES*

By

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STEENSGAARD, JENS and LARS FUNDING: *The sedimentation pattern of D-glucose-6-phosphate dehydrogenase from bovine fetal and adult erythrocytes.* Acta vet. scand. 1972, *13*, 305—318. — Erythrocytes from bovine fetuses contain about 2.4 times higher D-glucose-6-phosphate dehydrogenase activities than erythrocytes from adult cows and bulls. Studying whether this is due to the existence of a special fetal type of enzyme or an increased amount of enzyme in fetal erythrocytes, the sedimentation coefficients of the enzymes have been estimated by s-zonal ultracentrifugation, and compared to normal and deficient human erythrocyte D-glucose-6-phosphate dehydrogenase. s-zonal ultracentrifugations have been performed with a computer optimized isokinetic sucrose gradient. The mainlines in the program used for calculation of sedimentation coefficients are described.

Bovine fetal and adult erythrocyte D-glucose-6-phosphate dehydrogenase was found to have the same sedimentation coefficient of 7.4 S which is different from the sedimentation coefficient of 6.4 S of both human types of the enzyme. The sedimentation coefficients of 6-phospho-D-gluconate dehydrogenase from bovine fetal, bovine adult and human erythrocytes were 6 S for all three types of this enzyme.

By cellulose acetate electrophoresis bovine fetal and adult D-glucose-6-phosphate dehydrogenase show the same mobility, again differing from the normal and deficient human type.

The results of these experiments show that bovine fetal and adult erythrocytic D-glucose-6-phosphate dehydrogenase with respects to molecular parameters are closely related and perhaps identical enzymes.

D-glucose-6-phosphate dehydrogenase; glucose-6-phosphate dehydrogenase deficiency; bovine erythrocytes; fetal erythrocytes; human erythrocytes; s-zonal ultracentrifugation.

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Bovine fetal erythrocytes contain about 2.4 times higher activities of D-glucose-6-phosphate dehydrogenase than bovine adult erythrocytes (9), and bovine fetal erythrocytes show corresponding higher glutathione stabilities (12). These findings raise an important question of whether the high D-glucose-6-phosphate dehydrogenase activities in fetal erythrocytes are due to an increased concentration of the enzyme or to the existence of a special fetal type of enzyme. As part of some investigations on this subject the present paper deals with a determination of the equivalent sedimentation coefficient of D-glucose-6-phosphate dehydrogenase from bovine fetal and adult erythrocytes, and for the sake of comparison with the enzyme from normal and deficient human erythrocytes. The equivalent sedimentation coefficients are determined by s-zonal ultracentrifugation, allowing use of slightly purified samples and small amounts of starting material. In addition the sedimentation coefficient for 6-phospho-D-gluconate dehydrogenase is given. A description of the program used for the calculation of sedimentation coefficients is included.

MATERIALS AND METHODS

Blood samples from adult cows were collected during bleeding of newly slaughtered animals at the public slaughterhouse in Aarhus. All cows were of Danish red or Danish black and white dairy breeds. Blood samples from fetuses were taken by venous puncture shortly after evisceration of the maternal cow. Blood samples from men were drawn from healthy donors, and from a man with inborn deficiency of D-glucose-6-phosphate dehydrogenase in the erythrocytes (6). Blood samples were collected in tubes containing in substance 3.8 mg sodium citrate and 5.0 mg glucose per ml blood.

Two types of samples were used for s-zonal ultracentrifugation, namely crude hemolysate and DEAE-cellulose chromatographied D-glucose-6-phosphate dehydrogenase. This chromatography was performed as follows: The erythrocytes from 5 ml of blood were washed four times in 0.15 M-KCl in 5 mM phosphate buffer at pH 7.0 and 0.1 mM EDTA. After the last centrifugation the supernatant was removed, and an equal volume of 0.1 mM EDTA, 0.02 mM NADP and 1 mM mercaptoethanol in demineralized water was added. Complete hemolysis was provoked by sonication. 2.5 ml hemolysate was chromatographied

on a 6 × 60 mm DEAE-cellulose column (Whatman DE 11). Hemoglobin was eluted with 5 ml of 5 mM potassium phosphate buffer pH 7.0 containing 0.1 mM EDTA, 1 mM mercaptoethanol, and 0.02 mM NADP. D-glucose-6-phosphate dehydrogenase was eluted with 0.3 M-KCl in the same buffer. The first 0.75 ml was discarded and the following 2.5 ml containing about 95 % of the total D-glucose-6-phosphate dehydrogenase activity was collected and used directly for s-zonal ultracentrifugation. By this procedure D-glucose-6-phosphate dehydrogenase is practically freed from hemoglobin (4).

The activity of D-glucose-6-phosphate dehydrogenase was determined by the difference method of Glock & McLean using the previously described concentrations of reagents (9). D-glucose-6-phosphate dehydrogenase activity is by this method measured as the difference between the combined activity of D-glucose-6-phosphate dehydrogenase and 6-phospho-D-gluconate dehydrogenase activity and the pure 6-phospho-D-gluconate dehydrogenase activity, both estimated by increase with time in optical density at 340 nm at 37°C and pH 7.3. Hemoglobin was estimated as cyanmethemoglobin (9).

Electrophoresis was performed as described previously on cellulose acetate strips using the Beckman MicroZone apparatus (4).

s-zonal ultracentrifugation is a specially devised form of gradient centrifugation, performed in a hollow zonal rotor (1). The principle is illustrated in Fig. 1. For these experiments an M.S.E. (Measuring and Scientific Company, London) Mk. II ultracentrifuge with a B-XIV titanium zonal rotor and integrator was used. Computer optimized isokinetic gradients (10) constructed for separation and analysis of particles having sedimentation coefficients between 0 and 35 S were formed in an automatic variable gradient former. For s-zonal ultracentrifugation of DEAE-chromatographied enzyme, the stock solutions for the gradient former consisted of 2.99 % (w/w) and 22.86 % (w/w) sucrose in 0.05 M acetate buffer pH 6.0, containing 1 mM EDTA. The overlay was 100 ml of the acetate buffer. The rotor was unloaded by pumping in 700 ml of a 28 % (w/w) sucrose through the edge feed channels. The temperature in the rotor was 8°C. The centrifugation was continued at 47000 rev./min., until a predetermined force time integral of $4.545\ 10^{11}$ rad.^{2} sec was achieved. The fraction volume collected on a time schedule was 10 ml.

For s-zonal ultracentrifugation of crude hemolysate a steeper

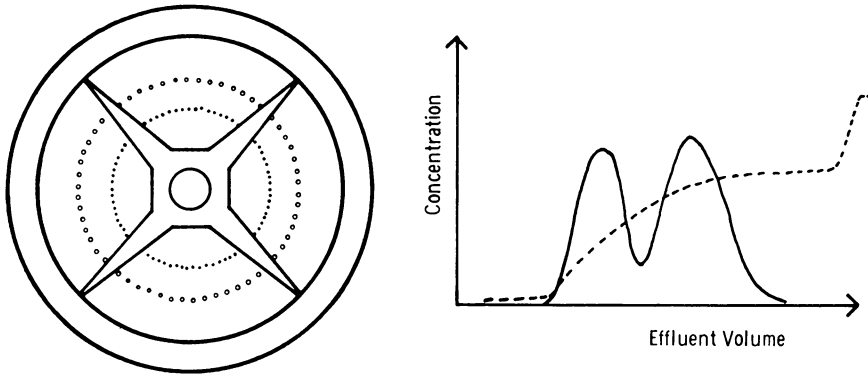


Figure 1. Horizontal section through a spinning zonal rotor, and a schematic effluent diagram.

A zonal rotor is a hollow titanium cylinder, whose inner chamber is divided in four identical compartments. The zonal rotor can whilst spinning be loaded and unloaded through a stationary feed head. Initially the rotor is loaded with gradient through the edge feed channels. Sample and overlay, which move the sample mass center to its starting position in the rotor, are loaded in through the centre feed channels. After centrifugation at maximum speed, the rotor is unloaded through the centre feed channels by pumping in a very heavy solution through the edge feed channels. The rotor effluent is monitored by passage through a spectrophotometer giving an effluent pattern as shown. The dots and circles in the rotor represent particles of different sizes.

and more concentrated computer optimized isokinetic gradient was used. The stock solutions consisted of 8.45 % (w/w) and 22.86 % (w/w) sucrose in the medium described above. The amount of overlay was 150 ml. In all other respects the experimental conditions were as described above.

During unloading of the rotor the effluent was monitored at 220 and 280 nm by a Gilford 2400 spectrophotometer.

The refractive index of each fraction was measured in a Bellingham & Stanley refractometer thermostated at 20°C.

The results of s-zonal ultracentrifugations can provide the basis for calculation of equivalent sedimentation coefficients for the particles of interest. The theoretical background for these calculations has been thoroughly described (3, 10). The program, however, for the practical performance of the calculations has not previously been published (11), and the main sequences of operations will be described shortly in the following:

The equivalent sedimentation coefficient, i.e. the sedimentation coefficient ($s_{20.w}$) recalculated to a standard medium (water at 20°C) is formally defined by:

$$s_{20.w} = \frac{(\rho_p - \rho_{20.w})}{\omega^2 t \; \eta_{20.w}} \cdot \int_i^r \frac{\eta_{T.m}}{(\rho_p - \rho_{T.m})} \cdot \frac{dr}{r}$$

where $\omega^2 t$ is the force time integral, i the initial sample mass center, ρ the density, η the viscosity and r radius in the rotor. The indices p, T and m refer to the particle, the temperature and a sucrose containing medium, respectively. Data to be read in are sample volume, overlay volume, rotor temperature, the force time integral or the rotor speed, duration of centrifugation, period of acceleration and deceleration, the particle densities, and for every single fraction, the weights of the empty and the filled fraction tube, the refractometer reading or the sucrose concentration, and the measured activities.

While reading in the data these are checked against inserted standard limits, making immediate manual correction of faultily coded data possible.

Refractometer readings are converted to sucrose concentrations, if these themselves are not provided as data. The conversion is performed by lineary interpolation between standard table values (5). Sucrose concentrations are converted to densities at the stated temperature by means of an approximated polynomium (2). The volume of every single fraction is then calculated as the weight difference divided by the calculated density. The radius corresponding to every single fraction is calculated from the accumulated fraction volume (10).

Viscosities of the medium in every single fraction tube are computed from the sucrose concentration and the temperature by means of a set of polynomiums (2). The density and viscosity of water at 20°C are stated as constants in the program. The integration is performed as a trapezoid integration with a step length of one fraction.

The activities given as data are converted to per cent of the maximum value. For the single activities the mean of the main peak is calculated and the corresponding sedimentation coefficient is found. In experiments giving a simple effluent pattern, the effluent pattern is converted to a function of the rotor radius and by means of a least square procedure fitted to a series of gaussian peaks believed to contain one component each. For one

component activities this gives a valuable check of the shape of the zone.

The results are printed in tables containing input data, description of the experiment, important intermediate results, converted data and sedimentation coefficients. Finally plots of the activities versus accumulated fraction volumes and of activities and sedimentation coefficients versus calculated rotor radii are drawn. An example of one of the plots is shown in Fig. 2.

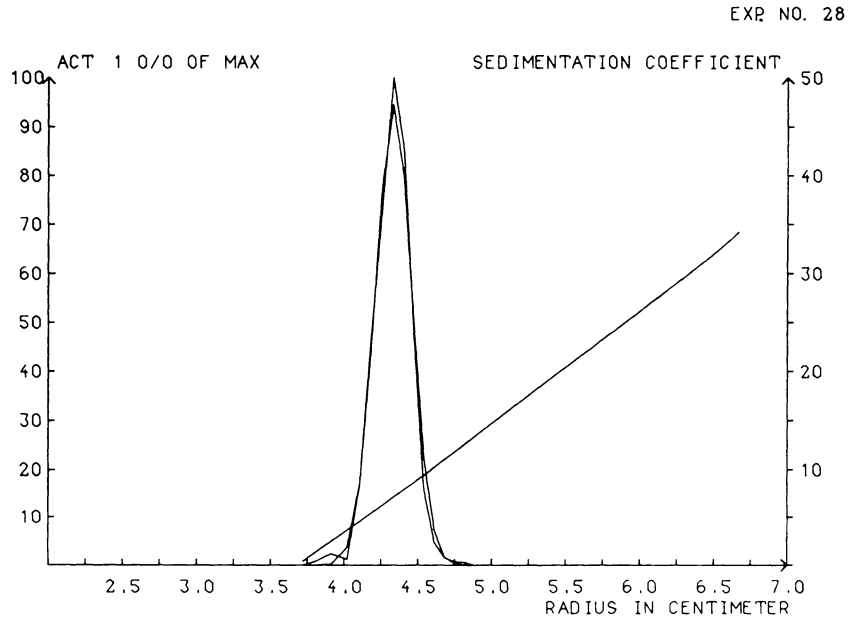


Figure 2. Computer drawn plot from an s-zonal ultracentrifugation experiment with bovine hemolysate as sample. The double peak shows D-glucose-6-phosphate dehydrogenase activity and the straight line sedimentation coefficients versus rotor radius, demonstrating the isokinetic character of the gradient.

The highest peak represents the measured activities in the fractions. The lower peak shows the computed best fitting curve drawn with the experimental step length. The curves are drawn through 49 experimentally obtained points.

RESULTS AND DISCUSSION

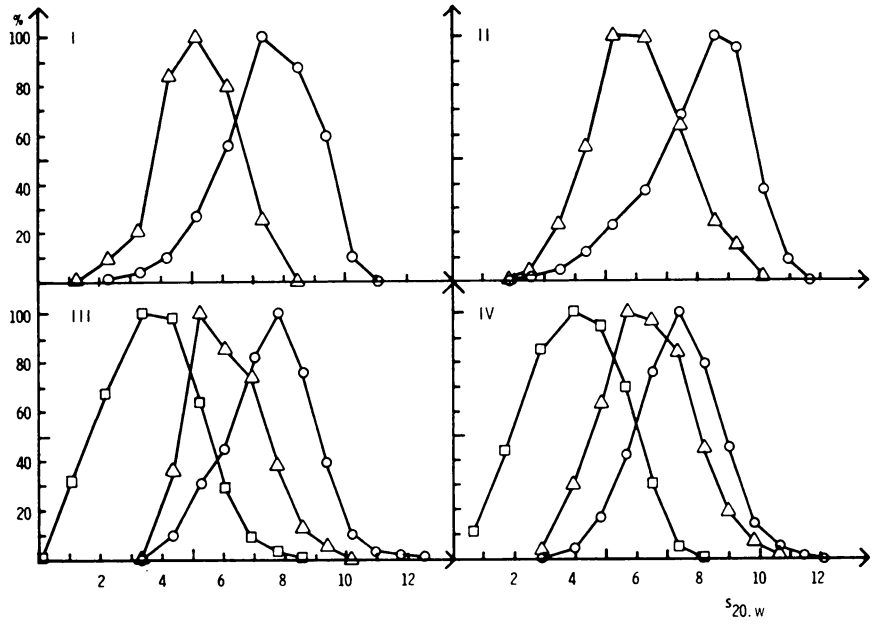
Diagrams of the measured activities versus the calculated sedimentation coefficients are instructive plots to present the results of s-zonal ultracentrifugations. Using isokinetic gradients as in these experiments the sedimentation coefficient axis will

bear a linear relation to the rotor radius. Such diagrams from s-zonal ultracentrifugations of DEAE-cellulose chromatographied D-glucose-6-phosphate dehydrogenase and of the same enzyme in a crude hemolysate of erythrocytes from adult cows, bovine fetuses, normal men and from a case of inborn D-glucose-6-phosphate dehydrogenase deficiency are shown in Figs. 3 a, b and c. Table 1 summarizes the results of all experiments in this series of s-zonal ultracentrifugations.

Table 1. Equivalent sedimentation coefficients ($s_{20.w}$) ± standard error of mean of D-glucose-6-phosphate dehydrogenase (E.C.1.1.1.49), 6-phospho-D-gluconate dehydrogenase (E.C.1.1.1.44) and hemoglobin from bovine fetal, bovine adult, normal human and D-glucose-6-phosphate dehydrogenase deficient erythrocytes estimated by s-zonal ultracentrifugation. The number in brackets denotes the number of samples.

	$s_{20.w}$ ± s.e.m.	(n)
Bovine fetal		
D-glucose-6-phosphate dehydrogenase	7.5 ± 0.1	(10)
6-phospho-D-gluconate dehydrogenase	5.7 ± 0.1	(9)
Hemoglobin	3.6 ± 0.1	(5)
Bovine adult		
D-glucose-6-phosphate dehydrogenase	7.2 ± 0.2	(8)
6-phospho-D-gluconate dehydrogenase	6.1 ± 0.1	(6)
Hemoglobin	3.8 ± 0.1	(7)
Normal human		
D-glucose-6-phosphate dehydrogenase	6.4 ± 0.1	(17)
6-phospho-D-gluconate dehydrogenase	6.1 ± 0.2	(8)
Hemoglobin	4.3 ± 0.1	(3)
Deficient human		
D-glucose-6-phosphate dehydrogenase	6.4 ± 0.3	(4)
6-phospho-D-gluconate dehydrogenase	6.2 ± 0.1	(4)
Hemoglobin	4.0 ± 0.2	(4)

The results show that D-glucose-6-phosphate dehydrogenase from adult and from fetal bovine erythrocytes has an equivalent sedimentation coefficient of 7.5 S and 7.2 S, respectively, where 1 S denotes a Svedberg unit equivalent of 1 cm sec^{-1} $dyn^{-1} \times 10^{13}$. Adult and fetal bovine erythrocyte D-glucose-6-phosphate dehydrogenases hence seem to be closely similar with respect to the molecular size of the active form. Adult and fetal bovine D-glucose-6-phosphate dehydrogenases, however, have a higher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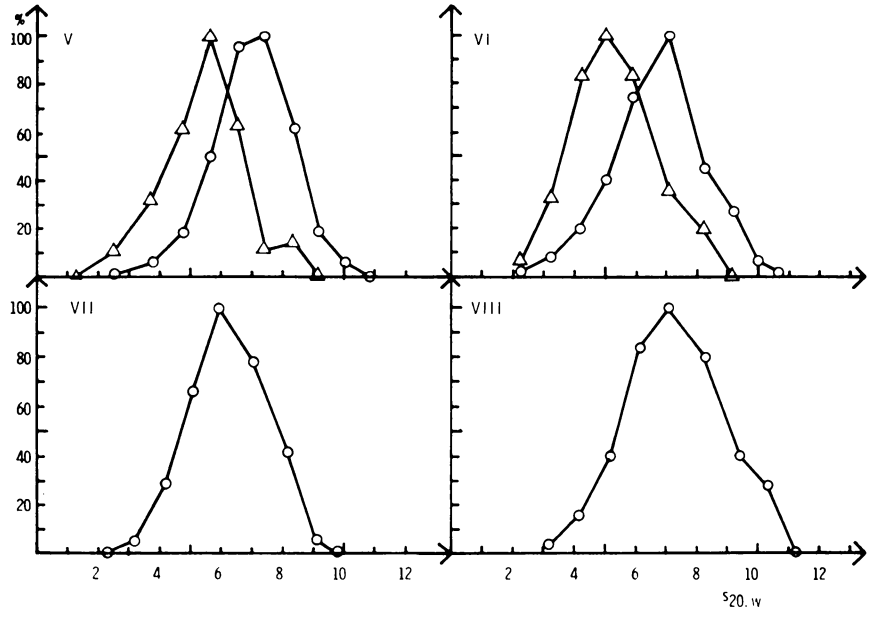
a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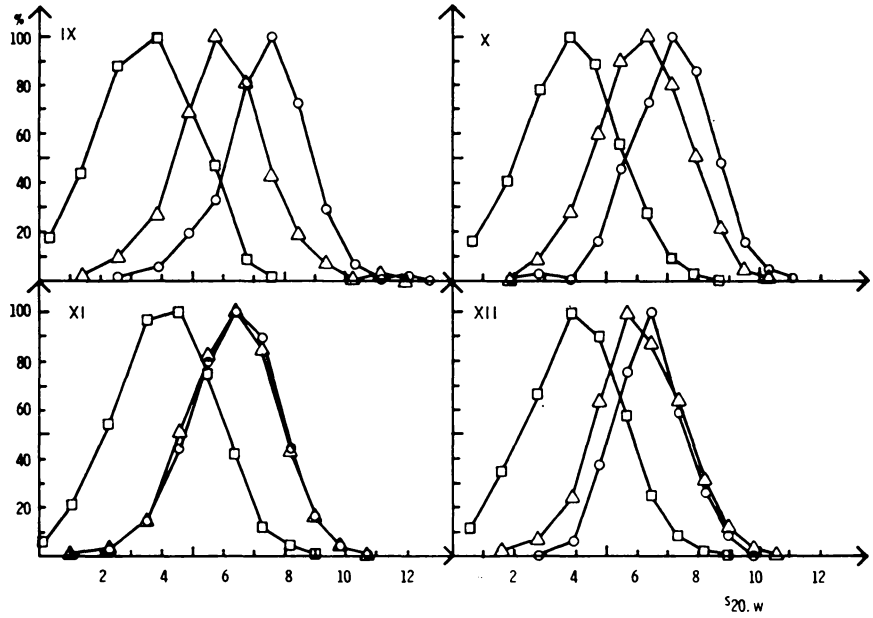

c

Figure 3. Diagrams of characteristic individual zonal experiments showing activities of the fractions as per cent of maximum values (%) as a function of sedimentation coefficients in Svedberg units ($s_{20.w}$).

○——○ D-glucose-6-phosphate dehydrogenase (G6PD)

△——△ 6-phospho-D-gluconate dehydrogenase

□——□ hemoglobin

In Fig. 3a bovine fetal DEAE-chromatographied (I) and bovine adult DEAE-chromatographied (II) samples are compared with bovine fetal crude hemolysates (III) and bovine adult crude hemolysates (IV).

In Fig. 3b bovine fetal DEAE-chromatographied (V) and bovine adult DEAE-chromatographied (VI) are compared with normal human DEAE-chromatographied (VII) and G6DP deficient human samples (VIII).

In Fig. 3c bovine fetal crude hemolysates (IX) and bovine adult crude hemolysates (X) are compared with normal human crude hemolysates (XI) and G6PD deficient crude hemolysates (XII).

sedimentation coefficient than the enzyme from the two human types of erythrocytes, having sedimentation coefficients of 6.4 S. The molecular weight of normal human erythrocyte D-glucose-6-phosphate dehydrogenase has by means of sedimentation equilibrium analytical ultracentrifugation been estimated to 120000 for the enzymatic active dimeric form (15). Assuming that human and bovine erythrocyte D-glucose-6-phosphate dehydrogenases have partial specific volumes, degrees of solvation and frictional ratios of the same order of magnitude, the molecular weight of bovine D-glucose-6-phosphate dehydrogenase can be calculated by comparison to the human type to 150000.

The mathematical relationship between the molecular parameters mentioned above can be derived as follows: The Svedberg equation states that (13)

$$s = \frac{M(1-\bar{v}\rho)}{Nf}$$

where M is the molecular weight, $\bar{v}$ the partial specific volume, ρ the density of the medium, N Avogadros number and f the frictional coefficient; f can be defined (14) by

$$f = 6\pi\eta\,(f/f_o)\left[\frac{3M(\bar{v}-\delta v_o)}{4\pi N}\right]^{1/3}$$

where η is the viscosity of the medium, δ the degree of solvation of the molecule, v_o is the specific volume of the solvent, and (f/f_o) the fricitonal ratio. Assuming that the parameters related to the hydration of the molecules, i.e. the partial specific volume, the degree of solvation and the specific volume of the solvent are identical, the sedimentation coefficients and molecular weights for two different molecules will be related by:

$$\frac{s_1}{s_2} = \left(\frac{M_1}{M_2}\right)^{2/3} \cdot \frac{(f/f_o)_2}{(f/f_o)_1}$$

This expression can be further reduced by assuming the shapes of the molecules as defined by their frictional ratios to be identical.

The sedimentation coefficient of bovine fetal 6-phospho-D-gluconate dehydrogenase is found to be 5.7 S and for the bovine adult and the human type 6.1 S. Bovine and human types of this enzyme then appear to have about identical molecular sizes. Although the precise molecular weight of this enzyme is un-

known, measurements of the sedimentation coefficient in these experiments provide a standard with nearly common sedimentation behaviour for the four types of erythrocytes studied here.

In s-zonal ultracentrifugation experiments overloading of the gradient is the most important source of experimental error. Overloading will cause the particles to move in the gradient by hydrodynamic instability of the zones (8, 4). However, by including in the sample a substance with a known sedimentation coefficient, overloading can be detected as dislocation of the sample mass center of the standard (4). As seen in Table 1 human and bovine adult hemoglobin show sedimentation coefficients of the same size as those arising from the analytical ultracentrifuge (7). The experiments with crude hemolysates can therefore safely be regarded as not-overloaded. In the experiments with DEAE-cellulose chromatographied enzymes the samples are identical with respect to enzyme content but are freed from hemoglobin so that the protein content is about 20 times lower, and no risk for overloading exists. Experimentally this is proved by the finding of no difference between the results of the two techniques. On the other hand as the DEAE-chromatography procedure provides samples with a low protein concentration and hence creates more ideal sedimentation conditions, this technique is regarded as the standard technique.

It should be noted that determination of sedimentation coefficients for D-glucose-6-phosphate dehydrogenase by analytical ultracentrifugation with optical registration would require a highly purified enzyme preparation.

Although the analytical ultracentrifuge yields results with a higher accuracy, s-zonal ultracentrifugation is far the simplest way to determine sedimentation coefficients for enzymes especially when the amount of starting material is small. Furthermore it allows determination on several samples as it only requires a one-step purification before centrifugation.

During the work with DEAE-chromatography of hemolysates it appeared that the elution pattern of 6-phospho-D-gluconate dehydrogenase for bovine and humane types differed. The bovine fetal and adult enzyme was eluted with the D-glucose-6-phosphate dehydrogenase while only about 15 % of human 6-phospho-D-gluconate dehydrogenase was eluted with the D-glucose-6-phosphate dehydrogenase.

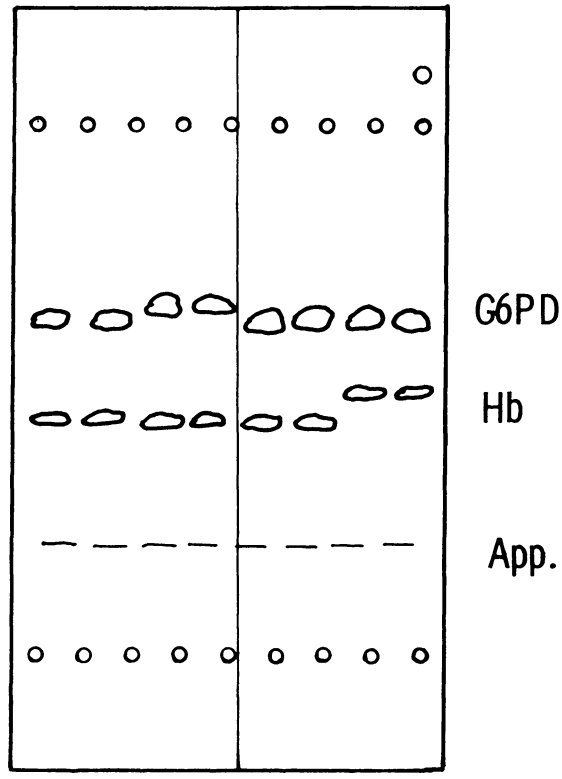

Figure 4. The electrophoretic pattern of two runs with hemolysates, comparing bovine adult (1 & 2) with normal human (3 & 4) samples and bovine adult (5 & 6) with bovine fetal (7 & 8) samples. G6PD: D-glucose-6-phosphate dehydrogenase. Hb: hemoglobin. App.: application line.

The electrophoretic experiments shown in Fig. 4 show no differences between bovine fetal and adult D-glucose-6-phosphate dehydrogenase, although the technique is able to distinguish between normal and deficient human erythrocytic D-glucose-6-phosphate dehydrogenase.

The results described here suggest that bovine adult and fetal erythrocytic D-glucose-6-phosphate dehydrogenases are very similar enzymes, and perhaps identical with respects to molecular parameters. The hypothesis is supported by the comparisons with human normal and deficient enzyme where several

distinct differences were found, demonstrating the sensitivities of the techniques used. Thus the high D-glucose-6-phosphate dehydrogenase activity in bovine fetal erythrocytes as compared to bovine adult erythrocytes is probably due to an increased amount of enzyme in each fetal erythrocyte.

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SAMMENDRAG

Sedimentationsmønstret for D-glucose-6-phosphat dehydrogenase fra bovine føtale og adulte erythrocyter.

Kalvefostre har ca. 2,4 gange højere D-glucose-6-phosphat dehydrogenase aktiviteter i erythrocyterne end voksne køer og tyre. I en undersøgelse af, om dette skyldes forekomsten af en speciel føtal type af enzymet eller en forøget mængde, er enzymets sedimentationskoefficient bestemt ved s-zoneultracentrifugering. Endvidere er det bovine enzym sammenlignet med to typer af humant erythrocyt dehydrogenase. s-zoneultracentrifugeringen er udført med en computeroptimeret isokinetisk sucrose gradient, og sedimentationskoefficienterne er beregnet med et program, hvis principielle opbygning beskrives.

Bovin føtal og adult D-glucose-6-phosphat dehydrogenase fandtes at have samme sedimentationskoefficient på ca. 7,4 S. De bovine typer adskiller sig dermed fra normal og deficient human D-glucose-6-phosphat dehydrogenase, der har en sedimentationskoefficient på 6,4 S. Sedimentationskoefficienten for 6-phospho-D-gluconat dehydrogenase fandtes for bovine føtale og adulte, samt for begge humane typer at være 6 S.

Ved elektroforese på celluloseacetat strips viste bovin føtal og adult D-glucose-6-phosphat dehydrogenase samme mobilitet, der var forskellig fra mobiliteten for begge humane typer af enzymet.

Resultaterne af disse undersøgelser viser, at bovin føtal og adult D-glucose-6-phosphat dehydrogenase med hensyn til molekylære parametre er meget nærtstående, måske identiske enzymer.

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