BBA 66470

PURIFICATION AND PROPERTIES OF AN NADP+-DEPENDENT GLYCEROL DEHYDROGENASE FROM RABBIT SKELETAL MUSCLE

ALFRED W. KORMANN, ROBERT O. HURST AND T. G. FLYNN
Department of Biochemistry, Queen's University, Kingston, Ontario (Canada)

(Received August 2nd, 1971)

SUMMARY

1. Glycerol dehydrogenase from rabbit skeletal muscle has been isolated and some properties of the enzyme have been determined to gain information about its identity and its biological significance.

2. The enzyme has been purified 300-fold by $(NH_4)_2SO_4$ precipitation, gel filtration on Sephadex G-100 and chromatography on DEAE-Sephadex A-50.

3. Formation of glycerol and NADP+ is strongly favoured resulting in an apparent thermodynamic equilibrium constant of $1.7 \cdot 10^{-6}$ for the enzyme at pH 7.0 and 37°.

4. Molecular weight for the enzyme as determined by gel filtration was 34 000 ± 1000.

5. Using the glycerol dehydrogenase prepared in this study a method has been determined for assaying the amount of D-glyceraldehyde in solution.

6. D-Glyceraldehyde was found to be the preferred substrate but the enzyme also catalyzed the reduction of other aldehydes with close structural similarity. Dihydroxyacetone, aldoses and ketoses with 5 or 6 carbon atoms were poor substrates. The activity of the enzyme with NADH as cofactor was 10–12% of the activity with NADPH as cofactor.

7. The affinity of the enzyme towards reduced compounds was demonstrated on acrylamide gels by applying a technique of combining zymogram and protein stain. The results confirmed the findings of the assays with the corresponding aldehydes.

8. Enzyme activity was almost completely inhibited by 1 mM *p*-chloromercuribenzoate and to 84% by 4 M urea at 25°. The presence of 0.5 M $(NH_4)_2SO_4$ decreased enzyme activity by one-third.

9. Comparison with other enzymes led to the conclusion that glycerol dehydrogenase from rabbit skeletal muscle has its own identity and that this enzyme should be classified as "glycerol:NADP+ oxidoreductase (D-glyceraldehyde forming)".

INTRODUCTION

Several mammalian tissues contain enzymes which catalyze the interconversion

Abbreviation: PCMB, *p*-chloromercuribenzoate.

of glyceraldehyde and glycerol. However, the nomenclature of these enzymes is not clearly established yet and has caused considerable confusion in the literature. For example, glycerol dehydrogenase activity has been found in rat tissues such as liver[1–4,6], skeletal muscle[4–6] and other organs[4,5]. Aldose reductases (EC 1.1.1.21) with reducing activity towards glyceraldehyde have been demonstrated in several rabbit tissues[7], calf eye lens[8] and seminal vesicle and placenta of sheep[9,10]. A polyol dehydrogenase (EC 1.1.1.21) which can utilize D-glyceraldehyde as substrate[11] has been isolated from bull seminal vesicle tissue and alcohol dehydrogenases (EC 1.1.1.1, EC 1.1.1.2) from horse liver[12] or from the liver of other vertebrates[13] are also capable of catalyzing the reduction of glyceraldehyde.

Our interest in glycerol dehydrogenase from rabbit skeletal muscle was attracted by the following findings. To our knowledge, no triokinase (EC 2.7.1.28) or aldehyde dehydrogenases (EC 1.2.1.3, EC 1.2.1.4) with activity towards glyceraldehyde have been described yet to occur in muscle tissues. In addition, only low glycerokinase (EC 2.7.1.30) activity has been demonstrated in rat and rabbit skeletal muscle[14]. Therefore glycerol dehydrogenase might provide a link of some importance for the metabolism of glyceraldehyde and glycerol in muscle. Secondly, it was intended to examine some characteristic properties of the purified enzyme, particularly with regard to substrate specificity. The latter was felt to be valuable in order to gain a basis for an appropriate nomenclature of the enzyme.

The present paper deals with the separation and identification of glycerol dehydrogenase from rabbit skeletal muscle. The enzyme is compared to other dehydrogenases with similar functions.

This work has been presented in part at the 14th annual meeting of the Canadian Federation of Biological Societies, Toronto, June 1971.

MATERIALS AND METHODS

Reagents

NADPH, $NADP^+$, NADH, NAD^+, glycerokinase (*Candida mycoderma*), ATP (Sigma grade) and the aldehydes and sugars used for the substrate specificity studies were obtained from Sigma, St. Louis, Mo., U.S.A., except L-glyceraldehyde which was purchased from Fluka, Buchs SG, Switzerland.

Alcohols for the substrate specificity studies were products from British Drug House, Poole, England.

$(NH_4)_2SO_4$ (enzyme grade), urea (ultra pure) and non-enzymic molecular weight standards were supplied by Mann, Orangeburg, N.Y., U.S.A.

Reagents for disc gel electrophoresis were obtained from Eastman, Rochester, N.Y., U.S.A.

Extraction and purification of the enzyme

Mature rabbits were killed by a blow on the neck and the muscle from the back and hind legs removed and chilled immediately. The following procedures were all carried out at 0–4°. Muscle was cut into small pieces and homogenized for 3 min in a Waring Blendor (1 g of muscle per 4 ml of distilled water). Crude extracts were obtained by centrifugation of the homogenates at 20 000 × *g* for 1 h. The supernatant fluid was brought to 50% $(NH_4)_2SO_4$ saturation by addition of the solid salt followed

by centrifugation. The precipitate was discarded and the retained supernatant brought to 80% $(NH_4)_2SO_4$ saturation. Centrifugation was repeated and the resulting precipitate was dissolved in a small volume of 0.05 M phosphate buffer (pH 7.0). This fraction was either subjected to further treatment at once or stored at −20°.

The enzyme solution obtained by the previous step was applied to a column of Sephadex G-100 (10 cm × 88 cm) prepared according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Equilibration of the column and elution of the enzyme was achieved with 0.02 M phosphate buffer (pH 7.0). The flow rate was 185–195 ml/h and fractions of 20 ml were collected. Fractions with high specific enzyme activity were pooled, concentrated and adjusted to a phosphate concentration of 0.01 M in a Diaflo Ultrafilter with filter type PM-10 (Amicon, Lexington, Mass., U.S.A.).

Sephadex G-100 eluates were chromatographed on a DEAE-Sephadex A-50 column (initial size 5 cm × 90 cm) prepared according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The gel was equilibrated with 0.01 M phosphate buffer (pH 7.0) prior to the application of the sample. The column was developed with a continuous salt gradient from equilibration buffer to 1.0 M NaCl in 0.01 M phosphate (pH 7.0). The elution rate was 30 ml/h and the fraction volume was 4.3 ml. Fractions with high specific activity were pooled and concentrated as described above.

Assay of enzyme activity and protein

Enzyme activity was measured spectrophotometrically at 340 nm in quartz cuvettes in 1-cm light path. The standard assay mixture contained 125 μmoles of phosphate (pH 7.0), 0.5 μmole of NADPH and 1 μmole of D-glyceraldehyde in a total volume of 2.5 ml. The reaction, carried out at 25°, was started by addition of enzyme and absorbance was measured at 1-min intervals for 10 min. Control cuvettes contained all reagents except D-glyceraldehyde. One unit of enzyme activity is defined as the amount of enzyme which converts 1 μmole of substrate per min at 25° under the conditions as specified above.

The same assay method as described above was employed for the substrate specificity studies except the amount of substrate used was 25–75 μmoles per assay and the amount of NADPH (or NADH) was increased to 5 μmoles.

Protein concentrations were measured according to the method of LAYNE[15] applying the following equation:

$$\text{Protein concentration (mg/ml)} = 1.55 \times A_{280\text{ nm}} - 0.76 \times A_{260\text{ nm}} \quad (1)$$

Assay of D-glyceraldehyde

The syrupy D-glyceraldehyde was kept over "Drierite" ($CaSO_4$) at 2–4°. An aqueous stock solution of about 10 mg of D-glyceraldehyde per ml was prepared and stirred at room temperature under light protection for 1 h. The D-glyceraldehyde concentration of this solution was determined by the following method. The assay cuvette contained 125 μmoles of sodium phosphate (pH 7.0), 1 μmole of NADPH, 4 μmoles of $MgCl_2$, 3 mg of ATP (disodium salt), a known volume of D-glyceraldehyde stock solution and 15 μl of glycerokinase in a total volume of 2.50 ml. The reaction, carried out at 37°, was started by addition of an excessive amount of glycerol dehydrogenase. The decrease in absorbance at 340 nm was recorded for 90 min after addition

of glycerol dehydrogenase. Remaining glycerol dehydrogenase activity was measured after 90 min by adding D-glyceraldehyde and recording the change in absorbance per min at 340 nm. Control cuvettes contained the same reagents as the assay mixture except no D-glyceraldehyde was added.

Apparent thermodynamic equilibrium constant K'

K' for the enzyme was determined with an assay system containing 125 μmoles of phosphate (pH 7.0), 0.5 μmoles of $NADP^+$ and 0.25 mmoles of glycerol in a total volume of 2.50 ml. The reaction was carried out at 37° and absorbance at 340 nm was recorded for 2.5 h.

Molecular weight determination

The molecular weight was estimated by the method of ANDREWS[17] except that a Sephadex G-100 column (1.5 cm × 82 cm) was utilized for this purpose.

Disc gel electrophoresis

Disc gel electrophoresis on polyacrylamide was performed according to the method of ZWEIG AND WHITAKER[18] with gels containing 7% acrylamide and an acrylamide/bisacrylamide ratio of 38:1. Both acrylamide and bisacrylamide (*N,N'*-methylenebisacrylamide) were purified by the method of LOENING[19]. No stacking or spacing gels were used. The samples were run at pH 9.5 and at a current of 2.5 mA/gel (250–400 V). Electrophoresis, carried out at 2–5°, was completed after 2–3 h. The gels were then stained in 1% Buffalo Black NBR (Allied Chemical, Morristown, N.Y., U.S.A.) in 7% acetic acid for 1 h after which time excess stain was removed electrophoretically.

Enzyme activity was located on gels by adapting the zymogram method of SMITH[20] for lactate dehydrogenase to glycerol dehydrogenase. Electrophoresis was carried out as outlined above and then each gel was placed in a solution containing 6.0 mg of $NADP^+$, 3.5 mg 2-(*p*-iodophenyl)3-*p*-nitrophenyl-5-phenyl tetrazolium chloride, 0.2 mg of phenazine methosulfate and 0.9 g of glycerol made up to a total volume of 10 ml with 0.063 M phosphate buffer (pH 7.0). The gels were stained in tightly stoppered tubes which were protected from light with aluminum foil at 37° for 45–120 min.

A similar staining technique was utilized for the gels of the substrate specificity studies except that the staining solution contained 10 mmoles of substrate and the staining time was exactly 1 h.

RESULTS

Purification of glycerol dehydrogenase

Table I summarizes the purification procedures carried out as described above.

Chromatography on DEAE-Sephadex A-50 revealed the presence of two enzymes with reducing activity towards D-glyceraldehyde. Fig. 1 shows a typical elution pattern. The main peak of enzyme activity (I) was eluted at an ionic strength of about 0.9 and was identified, following assay of the fractions using D-glyceraldehyde as substrate, as a glycerol dehydrogenase. The small peak of enzyme activity (II), eluted ahead of I, was found to contain a dehydrogenase which was not identifiable

TABLE I

PURIFICATION OF GLYCEROL DEHYDROGENASE FROM 400 g OF RABBIT SKELETAL MUSCLE

Purification step	*Specific activity**	*Purification factor (-fold)*	*Total activity***	*Yield (%)*
Crude extract	approx. 0.001		approx. 55	100
50–80% $(NH_4)_2SO_4$ satn. ppt.	0.0074	7.5	29	53
Sephadex G-100 eluates pooled/conc.	0.038	38	8.9	16
DEAE-Sephadex eluates***				
I pooled/conc.	0.20	200	4.3	8
I Fraction 410	0.30	300		
II pooled/conc.	0.005 (approx.)			

* Units/mg protein.
** Specific activity × mg of total protein.
*** I = glycerol dehydrogenase, II = non-specific aldehyde reductase (see Fig. 1).

with glycerol dehydrogenase. The provisional name non-specific aldehyde reductase was given to enzyme II.

Disc gel electrophoresis showed the presence of two major protein bands in the pooled concentrated fractions of enzyme I (Fig. 2). One of these bands gave a strong zymogram reaction for glycerol dehydrogenase.

Unless otherwise indicated, the enzyme properties described below are those of the pooled concentrated fractions which eluted as the main peak (I) of enzyme activity from DEAE-Sephadex A-50.

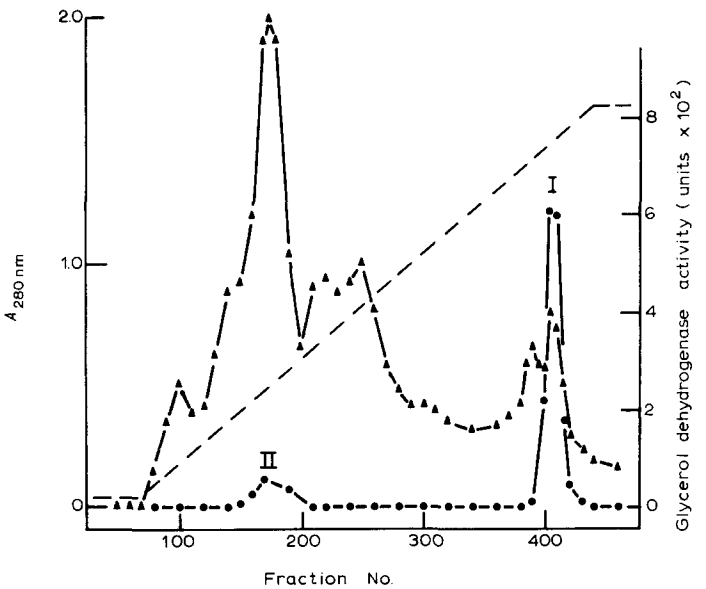

Fig. 1. Chromatography of glycerol dehydrogenase on DEAE-Sephadex A-50. Glycerol dehydrogenase activity (●), absorbance at 280 nm (▲), salt gradient from I = 0.022 (Fraction 70) to I = 1.022 (Fraction 440), (— — —).

Fig. 2. Analytical disc gel electrophoresis of glycerol dehydrogenase. 1, pooled and concentrated eluate from Sephadex G-100 column; 2, pooled and concentrated fractions corresponding to Enzyme I from DEAE-Sephadex A-50 column. The lower band on the gel stained for protein (P) corresponds to glycerol dehydrogenase. A denotes gel was stained for enzymic acitivity. P denotes gel was stained for protein.

Stability of the enzyme

Enzyme preparations obtained by $(NH_4)_2SO_4$ fractionation retained their glycerol dehydrogenase activity for about 1 year at −20°. Purified enzyme preparations did not lose their activity for several weeks if they were stored as concentrates (about 10 mg/ml) at pH 6–7 and at 0°.

Heat treatment of the enzyme at 55 or 60° resulted in a loss of 95–100%

TABLE II

INHIBITION OF GLYCEROL DEHYDROGENASE BY *p*-CHLOROMERCURIBENZOATE (PCMB), 4 M UREA AND $(NH_4)_2SO_4$

Standard error (3 assays) did not exceed ± 3% in any case except at PCMB concn. of 1.0 M and 2.0 M.

*PCMB concn. (mM)**	*% Initial activity*	*Exposure time to 4 M urea (h)***	*% Initial activity*	*$(NH_4)_2SO_4$ concn. (M)**	*% Initial activity*
0	100	0	100	0	100
0.004	82	0.5	26	0.05	96
0.01	74	1.0	16	0.10	93
0.04	67	1.5	14	0.20	83
0.1	54	2.0	14	0.50	67
0.2	41	2.0***	47	1.0	57
0.3	30				
0.4	20				
1.0	1				
2.0	1				

* Assays were performed as described in METHODS except varying amounts of PCMB or $(NH_4)_2SO_4$ were added to the reaction mixture.

** At 25°, enzyme assays were performed as described in METHODS. In the absence of urea at 25° less than 10% of the initial activity was lost after 2 h.

*** At 0°.

activity after 3 min. The presence or absence of substrates did not influence this property of the enzyme. Enzyme activity was relatively well preserved at 37°: 87% after 30 min, 79% after 1 h, 71% after 2 h, 65% after 3 h and 55% after 5 h. No measurable protein precipitation occurred during this time period.

Inhibition experiments

Glycerol dehydrogenase was inhibited to various degrees by *p*-chloromercuribenzoate (PCMB), 4 M urea and $(NH_4)_2SO_4$. Table II summarizes the results of the inhibition experiments with these reagents.

pH optimum

As shown in Fig. 3 the enzyme displayed the highest activity over a pH range of 5.0 to 7.0. Glycerol dehydrogenase denatured rapidly at pH values below 4 or above 9.

Apparent thermodynamic equilibrium constant K'

K' for glycerol dehydrogenase was determined in 0.05 M phosphate buffer (pH 7.0) at 37°. Equilibrium was reached after 1 h and 50 min to 2 h. K' was calculated for the reaction

$$\text{Glycerol} + \text{NADP}^+ \rightleftharpoons \text{D-glyceraldehyde} + \text{NADPH} + \text{H}^+ \qquad (2)$$

It was assumed that $[H^+]$ remains constant at the pH of the buffer throughout the reaction. This led to a value of $1.67\ (\pm 0.23) \cdot 10^{-6}$ which indicates that the formation of $NADP^+$ and glycerol is strongly favoured under these conditions.

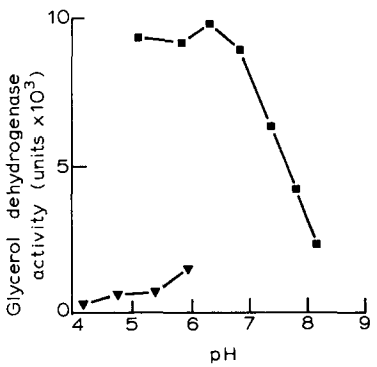

Fig. 3. pH–activity curve for rabbit muscle glycerol dehydrogenase. Activity determined in 0.05 M phosphate buffer (■), in 0.05 M citrate buffer (▲).

Determination of K_m

The K_m values for D-glyceraldehyde and NADPH were determined and found to be $1.5 \cdot 10^{-4}$ M for D-glyceraldehyde and $1.6 \cdot 10^{-5}$ M for NADPH.

Molecular weight

The molecular weight of the enzyme was found to be 34 000 ± 1000. Fig. 4 shows a plot of elution volume *versus* log (molecular weight) and the determination of the molecular weight for glycerol dehydrogenase.

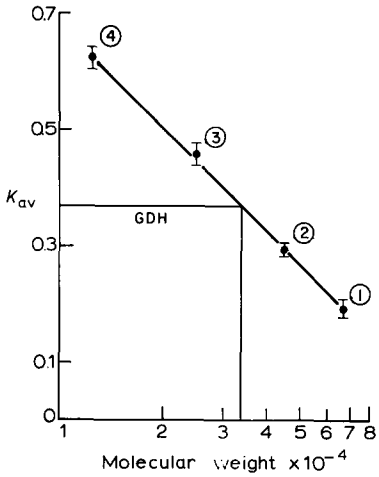

Fig. 4. Semi-logarithmic plot of molecular weights of standard marker proteins and glycerol dehydrogenase (GDH) as a function of the partition coefficient, K_{av}. 1, albumin; 2, ovalbumin; 3, chymotrypsinogen A; 4, cytochrome *c*. Vertical bars represent standard error of the mean.

Assay of D-*glyceraldehyde*

D-Glyceraldehyde is commercially available as a syrupy liquid. The percentage of D-glyceraldehyde in an aqueous solution was determined to evaluate the amount of substrate actually available to the enzyme. This was achieved by assaying a known amount of syrupy D-glyceraldehyde with an excess of glycerol dehydrogenase and NADPH.

It was assumed that 1 μmole of oxidized NADPH is equivalent to 1 μmole of D-glyceraldehyde converted. The molar extinction coefficient for NADPH was taken as $6.22 \cdot 10^{-6}$ cm^2/mole at 340 nm and a light path of 1 cm (ref. 16). The commercial D-glyceraldehyde used in these studies was shown to be free of dihydroxyacetone by incubating a large excess of D-glyceraldehyde with glycerokinase, α-glycerophosphate dehydrogenase, triose phosphate isomerase and NADH when no detectable reaction occurred.

It was found that syrupy D-glyceraldehyde (kept over "Drierite" at 2–4°) contained 57–61% of D-glyceraldehyde which is in good agreement with the values of 58–63% reported by CHARLTON AND VAN HEYNINGEN[21]. Slight differences between different commercial batches were observed. For example one batch contained 58 $\pm$ 1% of D-glyceraldehyde, another batch 59 $\pm$ 2%.

The remaining glycerol dehydrogenase activity after 90 min was found to be 70–75% of the initial activity.

The possible influence of depolymerisation of D-glyceraldehyde prior to the assay was determined. A stock solution was prepared and the solution was stored at room temperature in the dark for 2–3 days prior to the assay of D-glyceraldehyde. The values for D-glyceraldehyde content obtained by this method did not differ significantly from the values for freshly prepared D-glyceraldehyde solutions.

The use of the method according to CHARLTON AND VAN HEYNINGEN[21] by us for the assay of D-glyceraldehyde yielded the same results as were determined with glycerol dehydrogenase.

TABLE III

SUBSTRATE SPECIFICITY OF GLYCEROL DEHYDROGENASE

Initial velocity of substrates at optimum substrate concentration expressed relative to the initial velocity of D-glyceraldehyde. For experimental details see text.

Substrate	*Relative activity with NADPH*	*Relative activity with NADH*
D-Glyceraldehyde	1.00	0.10
DL-Glyceraldehyde	0.84	
L-Glyceraldehyde	0.36	
DL-Glyceraldehyde-3-*P*	0.24	
Dihydroxyacetone	0.04	0.008
Dihydroxyacetone-*P*	<0.002	
Formaldehyde	0.04	<0.0001
Acetaldehyde	0.07	0.003
Glycolaldehyde	0.44	0.046
Methylglyoxal	0.80	0.081
Propionaldehyde	0.45	0.020
Crotonaldehyde	0.13	
n-Butyraldehyde	0.68	0.052
Isobutyraldehyde	0.59	0.029
n-Valeraldehyde	0.74	0.076
Isovaleraldehyde	0.47	0.071
n-Hexanal	0.69	0.056
n-Heptanal	0.53	0.052
D-Erythrose	0.74	0.078
D-Ribose	0.05	
D-Lyxose	0.09	
D-(−)-Arabinose	0.02	
L-(+)-Arabinose	0.02	
D-(+)-Glucose	<0.004	0
D-(−)-Fructose	0.0	
D-Fructose-1-*P*	<0.005	
Sodium glucuronate	<0.01	
Glucuronolactone	0.18	

Substrate specificity

Glycerol dehydrogenase (enzyme I, see Fig. 1) was assayed with a number of different substrates. The initial velocity obtained with D-glyceraldehyde was taken as a standard of unit activity and initial velocities for other substrates, estimated under the same conditions, were expressed relative to this standard. The optimum substrate concentration for each of the substrates was determined beforehand. Table III summarizes the results obtained. Each of these reaction rates was within $\pm 5\%$ of its value given in Table III (S.E., 3 assays per substrate).

When NADPH was replaced by equivalent amounts of NADH the activity exhibited by the enzyme was 10–12% of the activity shown in the presence of NADPH.

Attempts to apply the spectrophotometric assay method for the reverse reaction (alcohol→aldehyde) were not successful. Therefore, the affinity of glycerol dehydrogenase towards reduced substrates was analyzed on polyacrylamide disc gels by using the zymogram method. Fig. 5 shows the results of these experiments with several substrates and $NADP^+$ as cofactor. The enzyme exhibited strong activity with 1,2-propane diol, glycerol, *n*-butanol, and *n*-pentanol. 1,3-Propane diol caused considerably less staining and no reaction was observed with methanol, ethanol, 1,2-ethane

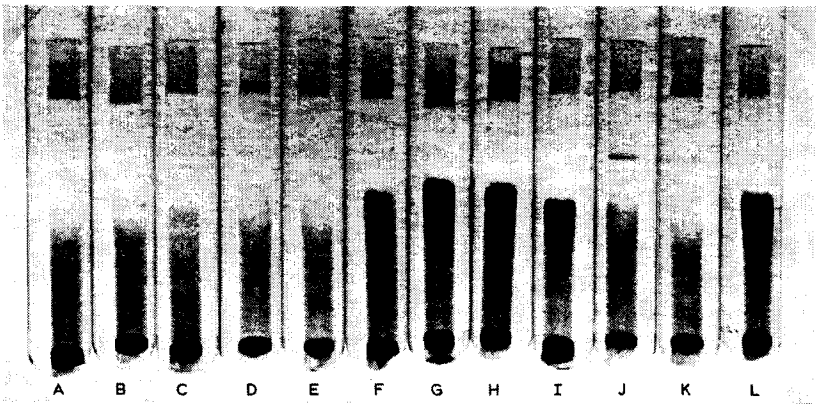

Fig. 5. Analytical disc gel electrophoresis patterns showing the substrate specificity of glycerol dehydrogenase with NADP+ as cofactor. The gels were stained for enzyme activity with the zymogram procedure described in the text. The heavy bands at the lower positive end of the gels are due to the tracking dye. A, methanol; B, ethanol; C, 1,2-ethane diol; D, *n*-propanol; E, 2-propanol; F, 1,3-propane diol; G, 1,2-propane diol; H, glycerol; I, *n*-butanol; J, 2-butanol; K, *tert.*-butanol; L, *n*-pentanol.

diol, *n*-propanol, 2-propanol, 2-butanol or *tert.*-butanol as substrate. The gels were knowingly overloaded with enzyme to ensure zymogram stains from poor substrates as well. It was established that the zymogram stains were caused by equivalent bands on the gels by subsequent staining for protein with Buffalo Black NBR (not shown here). The pink zymogram stain superimposed on the dark blue protein stain could easily be distinguished. Therefore the position of the enzyme band within the whole protein pattern of the gel could be evaluated by this technique.

TABLE IV

SUBSTRATE SPECIFICITY OF NON-SPECIFIC ALDEHYDE REDUCTASE WITH NADPH AS COFACTOR

Initial velocity of substrates at optimum substrate concentration expressed relative to the initial velocity of D-glyceraldehyde. For experimental details see text.

Substrate	*Relative activity*
D-Glyceraldehyde	1.00
Dihydroxyacetone	0.07
Formaldehyde	0.19
Acetaldehyde	0.12
Glycolaldehyde	0.20
Methylglyoxal	1.15
Propionaldehyde	0.58
n-Butyraldehyde	1.75
Isobutyraldehyde	1.04
n-Valeraldehyde	2.50
Isovaleraldehyde	2.10
n-Hexanal	3.05
n-Heptanal	1.90
D-Erythrose	0.41
D-(+)-Glucose	<0.001

Non-specific aldehyde reductase (enzyme II, see Fig. 1) was also assayed with a number of different substrates. Table IV shows the results of spectrophotometric assays with NADPH as cofactor. When NADH was used as cofactor no significant enzyme activity could be detected indicating that enzyme II is essentially NADPH specific. Zymogram reactions on disc gels (done similarly as shown for glycerol dehydrogenase, Fig. 5) confirmed the findings of the spectrophotometric assays.

Comparison of Tables III and IV reveals that glycerol dehydrogenase and non-specific aldehyde reductase have distinctly different properties. In addition, the elution pattern on DEAE-Sephadex A-50 indicates a considerable difference in net charge of the two enzymes. This was confirmed by the observation that the two enzymes displayed different mobility ratios (R_m, migration of protein band/migration of tracking dye) on disc gel electrophoresis. R_m for glycerol dehydrogenase (I) was found to be 0.4 to 0.5 and R_m for enzyme II was 0.2.

DISCUSSION

Glycerol dehydrogenase from rabbit skeletal muscle has been purified about 300-fold. The presence of interfering enzymes prevented an accurate determination of glycerol dehydrogenase activity in the crude homogenate. Most of these enzymes could be separated from glycerol dehydrogenase by $(NH_4)_2SO_4$ precipitation. LIN AND MAGASANIK[22] encountered similar problems during the purification of glycerol dehydrogenase from *Aerobacter aerogenes*. Gel filtration on Sephadex G-100 as an intermediate step was essential to ensure a considerable purification of the enzyme by subsequent chromatography on DEAE-Sephadex as indicated in Table I.

Treatment at 55° or 60° denatured the enzyme quite rapidly. Similar results have been obtained with purified aldose reductases from placenta and seminal vesicles of sheep[10] and beef[11,23] although HERS[9] found that crude aldose reductase from sheep seminal vesicle was stable at 55° if $NADP^+$ was present. It appears that the pH dependence of these aldose reductases[8,10,11,23] and glycerol dehydrogenase from rat skeletal muscle[4,5] and rabbit skeletal muscle is quite similar. Maximum reduction rates for aldehydes were found in a pH range from 5.5 to 7.0 for these enzymes.

The glycerol dehydrogenase reaction at pH 7.0 strongly favours the formation of glycerol and $NADP^+$ as demonstrated by the apparent equilibrium constant K' ($1.7 \cdot 10^{-6}$). This explains why the reverse reaction, *i.e.* the formation of glyceraldehyde and NADPH, could not be studied with the spectrophotometric assay method. However, the conditions of the zymogram technique enabled the thermodynamically unfavoured reaction to take place. NADPH formed is quickly oxidized back to $NADP^+$ by the reaction mixture[20] and a large excess of glycerol and $NADP^+$ is present thus facilitating the formation of glyceraldehyde and NADPH. The equilibrium for rat muscle glycerol dehydrogenase[5] has also been found to be in the direction of glycerol formation ($K = 1.6 \cdot 10^{-3}$ at pH 7.0).

The molecular weight for the enzyme from rabbit skeletal muscle was estimated to be 34 000 ± 1000 which is similar to the values of 28 000 to 33 000 reported by TOEWS[4] for glycerol dehydrogenase from different rat tissues.

The observations of RIDDLE AND LORENZ[24] that DL-glyceraldehyde is converted quantitatively to dihydroxyacetone during paper chromatography in the presence of

phosphate had little relevance for the enzymic assay of D-glyceraldehyde with glycerol dehydrogenase. This was demonstrated by the low reaction rate of dihydroxyacetone with this enzyme (4% of the rate of D-glyceraldehyde) and by the good agreement of our results with those of CHARLTON AND VAN HEYNINGEN[21]. RIDDLE AND LORENZ[24] also measured non-enzymic methylglyoxal formation from DL-glyceraldehyde when phosphate was present but this phenomena seems to be negligible as well since only about 2% of DL-glyceraldehyde is transferred to methylglyoxal at 40° and pH 7.3 after 2 h of incubation[24]. In addition to that, methylglyoxal proved to be a very good substrate for glycerol dehydrogenase (Table III).

The lack of knowledge of the molecular forms of D-glyceraldehyde in solution requires special techniques to assay the amount of D-glyceraldehyde that is actually available to the enzyme. Aqueous D-glyceraldehyde might be present in polymerized forms[25,26] which would not be distinguished from the monomer by a chemical assay method. CHARLTON AND VAN HEYNINGEN[21] have discussed this problem and presented a specific enzymic assay for dihydroxyacetone and D-glyceraldehyde. This assay includes four different enzymes of which triokinase has to be purified by a time-consuming procedure[27]. The other enzymes are commercially available in purified form.

Other methods have been developed for the determination of glycerol, glyceraldehyde and dihydroxyacetone[28,29] but they all have certain shortcomings with regard to specificity or convenience. A simpler assay for D-glyceraldehyde is reported in this paper.

Our studies indicated that glycerol dehydrogenase from rabbit skeletal muscle reduced dihydroxyacetone but at a much slower rate than D-glyceraldehyde. Furthermore, the apparent thermodynamic equilibrium constant indicated that the formation of glycerol and $NADP^+$ was strongly favoured under these conditions. Therefore, the enzymic reaction of glycerol dehydrogenase was suitable for the determination of D-glyceraldehyde provided this substrate was not contaminated with dihydroxyacetone which can be formed from glyceraldehyde under certain conditions[24]. Glycerokinase from *Candida mycoderma* phosphorylates dihydroxyacetone as well as glycerol and it is also very heat-stable[30]. On this basis a system was developed for the assay of D-glyceraldehyde involving only glycerol dehydrogenase from rabbit muscle and glycerokinase from *C. mycoderma*. Glycerokinase was used in this assay to remove glycerol from the assay system and therefore to reduce any possible product inhibition.

The substrate specificity studies with the rabbit muscle enzyme showed that NADPH is the major cofactor for this enzyme. These results also gave evidence that D-glyceraldehyde is the preferred substrate being reduced about 3 times faster than the L-form. The intermediate reduction rate of DL-glyceraldehyde confirmed these findings. Examination of the data in Table III reveals that other substrates with a reaction rate of 50% or more of the rate measured with D-glyceraldehyde exhibit a considerable degree of close structural similarity. This is particularly evident for methylglyoxal, D-erythrose and to some extent for glycolaldehyde and the aliphatic aldehydes with 3 to 7 carbon atoms. The low reaction rates for other aldoses such as D-ribose, D-lyxose and the arabinoses emphasize that the enzyme differentiates between D-erythrose (C_4) and aldoses with 5 or more carbon atoms even if the latter have otherwise similar molecular properties.

The zymogram reaction on disc gels with different alcohols as substrates (Fig. 5)

TABLE V

Enzyme:	*GDH Rabbit muscle*	*Ald. red. Rabbit muscle*	*GDH Rat*		*Aldose reductase*					*ADH Horse liver*	*Polyol DH C. utilis*
			muscle	*heart*	*Sheep s. ves.*	*Calf lens*	*Rabbit*				
							Lens	*Muscle*	*Heart*		
Cofactor:	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADPH*	*NADH*	*NADPH*
Standard:	D-*GA*	D-*GA*	D-*GA*	D-*GA*	D-*GA*	DL-*GA*	DL-*GA*	DL-*GA*	DL-*GA*	DL-*GA*	D-*GA*
Reference:	*This paper*		*4*	*4*	*9*	*8*	*7*	*7*	*7*	*12*	*33*
1 D-GA	1.00	1.00	1.00	1.00	1.00						1.00
2 DL-GA	0.84					1.00	1.00	1.00	1.00	1.00	
3 L-GA	0.36		0.77	0.82							
4 DHA	0.04	0.07	0.29	0.31							0
5 Formaldehyde	0.04	0.19								3.50	
6 Acetaldehyde	0.07	0.12				0.17				15.00	
7 Glycolaldehyde	0.44	0.20				0.85					
8 Methylglyoxal	0.80	1.15									
9 Propionaldehyde	0.45	0.58				0.89					
10 *n*-Butyraldehyde	0.68	1.75				0.80					
11 Isobutyraldehyde	0.59	1.04				0.90				260.00	
12 *n*-Valeraldehyde	0.74	2.50									
13 Isovaleraldehyde	0.47	2.10				0.10				100.00	
14 Hexanal	0.69	3.05									
15 Heptanal	0.53	1.90									
16 D-Erythrose	0.74	0.41				0.96					0.90
17 D-Ribose	0.05				0.50	0.57					0.21
18 D-Lyxose	0.09					0.25					0
19 D-Arabinose	0.02					0.33					0
20 L-Arabinose	0.02	0.00	0.41	0.37	0.51	0.58					0.68
21 D-Glucose	<0.004	<0.01	0.05	0.035	0.46	0.10					0.07
22 D-Fructose	0.00		0.02	0.02							0
23 Sodium glucuronate	<0.01				0.64	0.43	0.87	2.20	8.10		
24 Glucuronolactone	0.18				0.64	0.83	1.02	0.88	1.15		

Abbreviations: D-GA, D-glyceraldehyde; DL-GA, DL-glyceraldehyde; L-GA, L-glyceraldehyde; DHA, dihydroxyacetone; GDH, glycerol dehydrogenase; Ald. red., aldehyde reductase; s. ves., seminal vesicle; ADH, alcohol dehydrogenase; polyol DH, polyol dehydrogenase.

generally confirmed the results as obtained by the spectrophotometric studies with oxidized substrates (Table III). The method of combining the zymogram and protein stain techniques proved to be very useful for the evaluation of the purity of the enzyme and for the planning of further purification steps. The usefulness of this staining technique could be further expanded by combining it with size and charge isomer studies on disc gels with different acrylamide concentrations.

Comparison of the properties of glycerol dehydrogenase from rabbit skeletal muscle with similar enzymes such as other glycerol dehydrogenases, alcohol dehydrogenases and aldose reductases gave strong evidence that the enzyme described in the present paper is not identifiable with any of these enzymes (Table V). All these enzymes display reducing activity towards glyceraldehyde and a number of other substrates. However, the substrate specificity patterns of glycerol dehydrogenase from rabbit skeletal muscle and the other enzymes differ significantly. Glycerol dehydrogenase (I) and the non-specific aldehyde reductase (enzyme II) from rabbit skeletal muscle display differences in the reduction rates towards aliphatic aldehydes (Tables III and IV), *e.g.* for *n*-hexanal 69% with enzyme I and 305% with enzyme II. The different electrophoretic behaviour for these two enzymes gave additional evidence that they are not identical. Substantial differences were also found between enzyme I and glycerol dehydrogenase from rat skeletal muscle and heart[4]. The latter enzymes reduced dihydroxyacetone at rates of 29% and 31%, respectively, and L-arabinose at rates of 41% and 37%, respectively, of the rate of D-glyceraldehyde. Aldose reductase from sheep seminal vesicle[9] has a much higher affinity for D-ribose, L-arabinose, D-glucose, sodium glucuronate and glucuronolactone than enzyme I. Propionaldehyde is reduced 2.5 times faster and D-ribose twice as fast as D-glyceraldehyde by polyol dehydrogenase from bull seminal vesicle tissue[11]. HAYMAN *et al.*[7] measured reduction rates for aldose reductases from several rabbit tissues and found very high values for sodium glucuronate and glucuronolactone. A wide variety of substrates is reduced by aldose reductase from the eye lens of calf[8] but these reaction rates differ significantly from those of enzyme I. Glycerol dehydrogenase from *Aerobacter aerogenes*[31] prefers NADH as cofactor and it reduces dihydroxyacetone at a faster rate than glyceraldehyde. Similar properties have been found for alcohol dehydrogenase from horse liver[12]. *Leuconostoc mesenteroides* contains a NADP+ dependent alcohol dehydrogenase but this enzyme does not act on glyceraldehyde or dihydroxyacetone[32]. Polyol dehydrogenase from *Candida utilis*[33] has a much higher affinity towards L-arabinose and D-ribose than enzyme I. In summary, the substrate specificities of all these enzymes compared with glycerol dehydrogenase from rabbit skeletal muscle appear to exclude that these enzymes can be identified with the rabbit muscle enzyme.

There are some similarities, however, between glycerol dehydrogenase from rabbit muscle and aldose reductases from several sources. Aldose reductases from sheep seminal vesicles[10] and from cow placenta[23] are inhibited by urea and PCMB in a similar fashion to that reported in this study for glycerol dehydrogenase from rabbit skeletal muscle. In contrast to these results $(NH_4)_2SO_4$ has a stimulatory effect on aldose reductase from sheep[10] and bovine tissues[8,23], and also on bacterial glycerol dehydrogenase[22]. However, glycerol dehydrogenase from rabbit skeletal muscle is inhibited by $(NH_4)_2SO_4$ (Table II).

At present, the only glycerol dehydrogenase systematically classified in the List

of Enzymes of the IUB Enzyme Commission[34] is NAD^+-dependent glycerol dehydrogenase (EC 1.1.1.6), an enzyme which catalyzes the reaction, glycerol + $NAD^+ \rightleftarrows$ dihydroxyacetone + NADH. This classification obviously does not describe the glycerol dehydrogenase from rabbit skeletal muscle.

On the basis of the results reported in this paper, we propose that the enzyme known only under the trivial name NADP dependent glycerol dehydrogenase from rabbit skeletal muscle, also be given the systematic classification glycerol:$NADP^+$ oxidoreductase (D-glyceraldehyde forming) according to the recommendation of the International Union of Biochemistry[34] and be incorporated as such in their List of Enzymes[34].

ACKNOWLEDGEMENTS

This work has been supported by the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada.

REFERENCES

1 H. P. WOLF AND F. LEUTHARDT, *Helv. Chim. Acta*, 36 (1953) 1463.
2 F. LEUTHARDT AND H. P. WOLF, *Helv. Chim. Acta*, 37 (1954) 1732.
3 B. W. MOORE, *J. Am. Chem. Soc.*, 81 (1959) 5837.
4 C. J. TOEWS, Ph. D. Thesis, Queen's University, Kingston, Ontario, Canada, 1967.
5 C. J. TOEWS, *Biochem. J.*, 98 (1966) 27C.
6 C. J. TOEWS, *Biochem. J.*, 105 (1967) 1067.
7 S. HAYMAN, M. F. LOU, L. O. MEROLA AND J. H. KINOSHITA, *Biochim. Biophys. Acta*, 128 (1966) 474.
8 S. HAYMAN AND J. H. KINOSHITA, *J. Biol. Chem.*, 240 (1965) 877.
9 H. G. HERS, *Biochim. Biophys. Acta*, 37 (1960) 120.
10 T. HÄSTEIN AND W. VELLE, *Biochim. Biophys. Acta*, 178 (1969) 1.
11 R. L. HANCOCK, *Physiol. Chem. Phys.*, 2 (1970) 581.
12 A. D. WINER, *Acta Chem. Scand.*, 12 (1958) 1695.
13 F. HEINZ AND F. WEINER, *Comp. Biochem. Physiol.*, 31 (1969) 283.
14 E. A. NEWSHOLME AND K. TAYLOR, *Biochem. J.*, 112 (1969) 465.
15 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1st ed., 1957, p. 447.
16 H.-U. BERGMEYER, *Methods of Enzymatic Analysis*, Verlag Chemie, Weinheim/Bergstr., 1st ed., 1965, p. 1030.
17 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
18 G. ZWEIG AND J. R. WHITAKER, *Paper Chromatography and Electrophoresis*, Vol. I, Academic Press, New York, 1st ed., 1967, p. 159.
19 U. E. LOENING, *Biochem. J.* 102 (1967) 251.
20 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. II, Interscience, New York, 2nd ed., 1968, p. 345.
21 J. M. CHARLTON AND R. VAN HEYNINGEN, *Anal. Biochem.*, 30 (1969) 313.
22 E. C. C. LIN AND B. MAGASANIK, *J. Biol. Chem.*, 235 (1960) 1820.
23 W. VELLE AND L. L. ENGEL, *Endocrinology*, 74 (1964) 429.
24 V. RIDDLE AND F. W. LORENZ, *J. Biol. Chem.*, 243 (1968) 2718.
25 W. S. BECK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 201.
26 R. M. C. DAWSON, D. C. ELLIOTT, W. H. ELLIOTT AND K. M. JONES, Data for Biochemical Research, Oxford, 1959, p. 34.
27 F. HEINZ AND W. LAMPRECHT, *Z. Physiol. Chem.*, 324 (1961) 88.
28 J. K. PINTER, J. A. HAYASHI AND J. A. WATSON, *Arch. Biochem. Biophys.*, 121 (1967) 404.
29 T. NAKAI, M. KOYAMA AND H. DEMURA, *J. Chromatogr.*, 50 (1970) 338.
30 H.-U. BERGMEYER, G. HOLZ, E. M. KAUDER, H. MOLLERING AND O. WEILAND, *Biochem. Z.*, 333 (1961) 471.
31 R. M. BURTON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1st ed., 1955, p. 397.

32 R. D. DE MOSS, in S. P. COLOWICK ADN N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1st ed., 1955, p. 504.
33 B. M. SCHER AND B. L. HORECKER, *Arch. Biochem. Biophys.*, 116 (1966) 117.
34 Enzyme Nomenclature—Recommendations (1964) of the International Union of Biochemistry, Elsevier, Amsterdam, 1965.