PROPERTIES AND CHARACTERISTICS OF PARTLY PURIFIED GLUTAMATE DEHYDROGENASE FROM SHEEP RUMEN MUCOSA

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The purification of glutamate dehydrogenase from sheep rumen mucosa on DEAE-cellulose afforded two enzyme fractions with glutamate dehydrogenase activity. The enzyme fraction II (tissue glutamate dehydrogenase) was freed of contaminating proteins in the subsequent purification step on Sephadex G-200. The approximate relative molecular weight (260 000) of tissue glutamate dehydrogenase (fraction II) was determined by gel filtration on Sephadex G-200 and the approximate relative molecular weight of its polypeptide chain (48 000) was established by polyacrylamide gel electrophoresis in SDS. The pH-optimum of fraction II was 7·9. The effect of substrate concentration on the rate of the enzymatic reaction was examined and the following apparent Michaelis' constants were found for the individual substrates: NADH $6{\cdot}25 \cdot 10^{-5}$ mol/l 2-oxoglutarate $4{\cdot}5 \cdot 10^{-3}$ mol/l, and NH_4^+ $77 \cdot 10^{-3}$ mol/l.

Glutamate dehydrogenase (EC 1.4.2—4) catalyzing the reaction L-glutamate + $NAD(P)^+$ + + $H_2O \rightleftarrows$ 2-oxoglutarate + NH_4^+ + NAD(P) + H^+ represents one of the key enzymes involved in the nitrogen metabolism. This enzyme, which has been isolated and characterized from various sources, is wide-spread in the tissues of living organisms[1]. The molecular, kinetic, regulatory, and physical characteristics of glutamate dehydrogenase (GDH) isolated from the individual sources widely differ. Generally it may be stated that animal GDH is extremely sensitive to the concentration of purine nucleotides and can catalyze the reaction in the presence of NAD^+ or $NADP^+$. It is easily reversibly polymerized. On the other hand, GDH isolated and purified from other than animal sources is specific either for NAD^+ or $NADP^+$; its activity is not affected by purine nucleotides and the enzyme is not reversibly polymerized[2].

Information on GDH of the digestive tract of ruminants is relatively meagre. As evidenced by the results of our experiments[3] GDH plays an important role in the fixation of ammonia in the whole digestive tract. We focused therefore our subsequent work on the determination of the homogeneity of purified GDH from rumen mucosa and on its detailed biochemical characterization.

EXPERIMENTAL

Material and chemicals: the rumen mucosa was obtained from sheep, var. Slovak Merino, 6 months old. The enzymatic determinations were carried out with the disodium salt of NADH, supplied by Koch-Light (England) and with 2-oxoglutaric acid purchased from Lachema. The purification of the enzyme was carried out on Sephadex G-200 (Pharmacia, Uppsala) and DEAE--cellulose (Balston, England). The polyacrylamide gel column was prepared from the following substances: acrylamide monomer (Lachema), N,N'-dimethyleneacrylamide (B.D.H., Lab. Chem. Div., England), tetramethylenediamine (Koch-Light, London); ammonium persulfate and riboflavin were of Czechoslovak origin. The staining was effected by Coomassie Brillant Blue R-250 (Serva, Heidelberg); bromophenol blue and sodium dodecyl sulfate used for the determination of the molecular weight of the polypeptide chain were from Koch-Light (England). The reference protein standards used in the molecular weight determination by gel filtration and polyacrylamide gel electrophoresis were human serum albumin (Koch-Light), cytochrome c (Reanal, Hungary), γ-globulin, ribonuclease A, and pepsin (Koch-Light). All the remaining chemicals were of Czechoslovak origin and analytical purity. The pH of the buffers was checked by a Radiometer pH-meter equipped with a glass electrode.

Preparation of tissue homogenate and supernatant: A 30% homogenate of the rumen mucosa in 0·01 mol/l phosphate buffer, pH 7·5, containing $4 . 10^{-3}$ mol/l EDTA and 0·25 mol/l sucrose was used to start with. Triton X-100 was added to the homogenate to a final concentration of 1%. The homogenate was set aside for 12 h and used for the preparation of the soluble cytoplasmic fraction. The preparation was carried out by ultracentrifugation at 105 000 *g* for 60 min at 2°C in model VAC 60 Janetzki preparative centrifuge. The supernatant was used for the subsequent isolation.

Determination of enzymatic activity: The glutamate dehydrogenase activity in terms of reducing amination was determined on the basis of NADH oxidation at 340 nm (ref.[4]). The decrease of NADH was measured at 340 nm in a Specord UV VIS recording spectrophotometer equipped with temperature control. The incubation was allowed to proceed in 0·01 mol/l triethanolamine buffer at pH 8 containing $4 . 10^{-3}$ mol/l EDTA in quartz cells (1 cm), accommodated in a water bath 30°C warm, for 10 min. The incubation mixture (total volume 3·2 ml) contained 2—2·9 ml of buffer, $1·5 . 10^{-4}$ mol/l NADH, 0·125 mol/l ammonium acetate, $5 . 10^{-3}$ mol/l 2-oxoglutarate, and the enzyme fraction. The enzymatic activity was expressed in micromoles of NADH oxidized per min per 1 g of protein. The NADH concentration was read off from a calibration curve.

The protein concentration during the purification process was determined by the Folin–Ciocalteau reagent using a modification of the method of Lowry[5]; the concentration was read off from a calibration curve prepared with human serum albumin.

Fractionation by ammonium sulfate: The precipitation of the supernatant fraction (105 000 *g*) of the rumen mucosa homogenate was carried out at 4°C with ammonium sulfate at pH 8 adjusted by concentrated ammonium hydroxide. The mixture was allowed to stabilize 24 h at 4°C and the proteins precipitated at 20—60% saturation with ammonium sulfate were filtered off. The sediment was dialyzed 24 h against 0·01 mol/l phosphate buffer at pH 7·5.

Chromatography on DEAE-*cellulose*: The enzyme fraction, obtained by precipitation by ammonium sulfate from the supernatant, was placed on a 2 × 23 cm column. The column was eluted by a linear gradient of sodium chloride (250 ml of 0·01 mol/l phosphate buffer, pH 7·5, $4 . 10^{-3}$ mol/l EDTA + 250 ml of 0·01 mol/l phosphate buffer, pH 7·5, $4 . 10^{-3}$ mol/l EDTA, 0·7M-NaCl). The flow rate was 18 ml/h and the fractions were collected at 10 min intervals by a fraction collector. The protein content was measured at 280 nm in a SF-4 spectrophotometer. All the purification procedures were performed at 4°C in a refrigerated room.

Gel filtration on Sephadex G-200: The enzyme fraction II from the rumen mucosa, which had been chromatographed on DEAE-cellulose, was subjected to gel filtration on a 2·5 × 90 cm column, equilibrated with 0·01 mol/l phosphate buffer at pH 7·5 and eluted at a rate of 14 ml/h. The fractions were collected at 15 min intervals at +4°C.

The relative molecular weight determinations were effected by gel filtration on a Sephadex G-200 column according to Determan[7]. The determination of the relative molecular weight of the polypeptide chain was performed according to Weber and Osborn[8] in a 10% gel.

Determination of apparent Michaelis constant: The effect of the concentration of substrates (NADH, 2-oxoglutarate, NH_4^+-) on the activity of partly purified glutamate dehydrogenase was studied under optimum conditions of the enzymatic reaction. The apparent Michaelis constant was determined graphically by the method of Lineweaver and Burk[9].

RESULTS

The dialyzed precipitate of the soluble cytoplasmic fraction after 20–60% saturation with ammonium sulfate, was applied onto a DEAE-cellulose column. The GDH activity was resolved into two fractions (I and II) by the chromatography. In view of the differences in the inhibition by Zn^{2+}-ions and the different sensitivity to purine nucleotides we assume that the glutamate dehydrogenase activity of fraction I is of bacterial origin whereas the activity of fraction II, showing an identical chromatographic behaviour with the analogically purified liver GDH, corresponds to tissue GDH localized in the rumen mucosa[10]. The most active parts of fraction II (tissue GDH) were pooled, dialyzed, concentrated, and applied onto a Sephadex G-200 column. When the sample was chromatographed on the Sephadex G-200 column the contaminating proteins were distinctly separated. The individual purification steps together with the specific activities and the corresponding purification factors are given in Table I. As can be seen from the Table the enzyme preparation isolated showed an activity many times higher than that of the starting material.

The approximate molecular weight of the enzyme (monomer) was determined by gel filtration on Sephadex G-200. The V_e/V_0 value as a function of the logarithm of molecular weight of the reference proteins with an extrapolation of the molecular weight of GDH is shown in Fig. 1. The approximate relative molecular weight of GDH read off from this graph is 260 000. The relative molecular weight of the polypeptide chain was calculated from the plot of the mobility on the logarithm of molecular weight shown in Fig. 2. The relative molecular weight of the polypeptide chain of GDH is 48 000.

The dependence of the enzymatic activity on pH is shown in Fig. 3. The pH-optimum lies at pH 7·9.

The effect of the increasing concentration of NADH, 2-oxoglutarate, and ammonium acetate on the activity of GDH in terms of reducing amination was studied and the apparent Michaelis constants for NADH, 2-oxoglutarate, and NH_4^+-were determined as $6{\cdot}25 \cdot 10^{-5}$ mol/l, $4{\cdot}5 \cdot 10^{-3}$ mol/l and $77 \cdot 10^{-3}$ mol/l, respectively (Figs 4–9).

DISCUSSION

In this study GDH from rumen mucosa was partly purified and its characteristics compared with the properties of purified GDH from other animal sources.

The animal glutamate dehydrogenase is characterized by two different dissociation types. The first type is reversible dissociation of the tetramer to the monomer which is affected by many factors such as enzyme concentration, purine and pyrimidine nucleotides, change in pH, *etc.*[11]. The other type is irreversible dissociation; the molecule (monomer) dissociates to smaller molecular forms lacking enzymatic activity[11]. Glutamate dehydrogenase dissociates in denaturing solutions (GM urea, guanidine–HCl, dodecyl sulfate) into six polypeptide chains. The relative molecular weight of GDH (monomer) from different sources was determined by many workers using different methods; this value varies between 250 000 and 450 000.

The approximate relative molecular weight of GDH from rumen mucosa was determined in this study by gel filtration on Sephadex G-200 at an enzyme concentration of 0·17 mg/ml as 260 000. Likewise Roger and coworkers[12] at low concentrations (0·46 – 117 μg/ml) of the enzyme from bovine liver found by gel filtration and ultracentrifugation a molecular weight of 270 000 which did not vary over the given concentration range. The relative molecular weight of the polypeptide chain of GDH from rumen mucosa determined by polyacrylamide gel electrophoresis in SDS was 48 000, in agreement with the data of other authors[1,2].

TABLE I
Purification of GDH from sheep rumen mucosa

Degree of purification	Enzyme activity μmol NADH/min/ml	Proteins mg/ml	Specific activity μmol NADH/min/mg prot.	Purification factor
1 30% Homogenate	0·0412	51·00	0·0008	1·0
2 105 00 *g* Supernatant	0·0592	37·70	0·0016	2·0
3 Salting out by 20–60% saturation with $(NH_4)_2SO_4$	0·3605	141·00	0·0025	3·25
4 DEAE-cellulose IInd peak	0·0618	3·20	0·0193	24·12
5 Gel filtration on Sephadex G-200	0·0309	0·170	0·1818	227·25

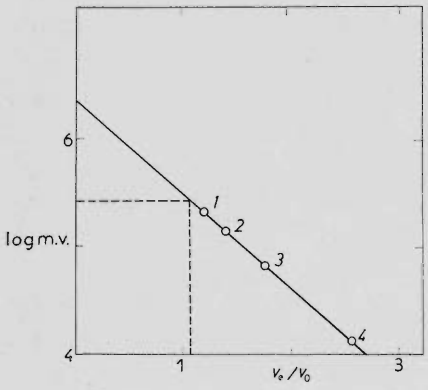

FIG. 1

Plot of V_e/V_0 *versus* logarithm of molecular weight of reference proteins. Markers: γ-globulin *2*, serum albumin *3*, ribonuclease A *4*. The extrapolated molecular weight of GDH *1* was 260 000

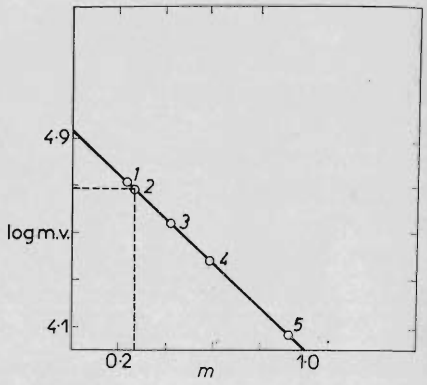

FIG. 2

Determination of relative molecular weight of polypeptide chain of GDH by polyacrylamide gel electrophoresis in SDS. Markers: γ-globulin H-chain *1*, pepsin *3*, γ-globulin L chain *4*, cytochrome C *5*, polypeptide chain of GDH 2 (48 000)

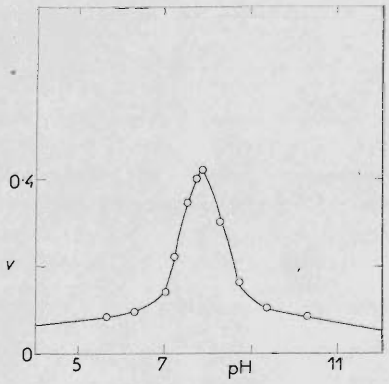

FIG. 3

pH-Profile. The pH-determination was carried out in Britton–Robinson buffer[6] in the range pH 2·1—10·3

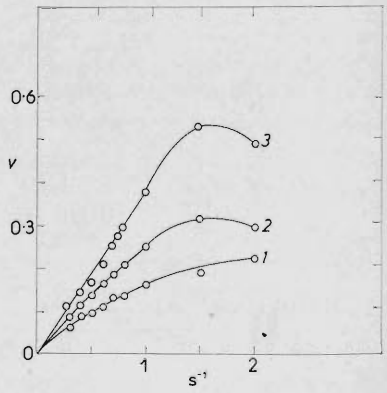

FIG. 4

Effect of increasing NADH concentration on activity of GDH in terms of reducing amination at constant ammonium acetate concentration (0·125 mol/l). Concentration of 2-oxoglutarate *1* $2{\cdot}5 \cdot 10^{-3}$ mol/l, *2* $5 \cdot 10^{-3}$ mol/l, *3* 10^{-3} mol/l

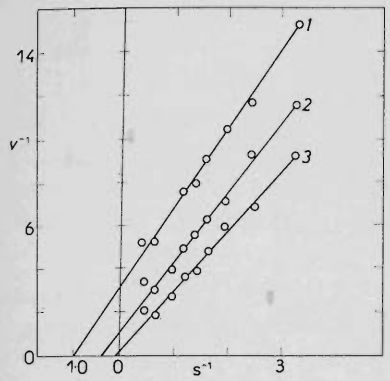

FIG. 5

Double reciprocal plot of rate *versus* NADH concentration at varying concentrations of 2-oxoglutarate and constant high concentration of ammonium acetate (0·125 mol/l). *s* concentration of $NADH^{-1}$ in mol/l . 10. Concentration of 2-oxoglutarate, *cf.* Fig. 4

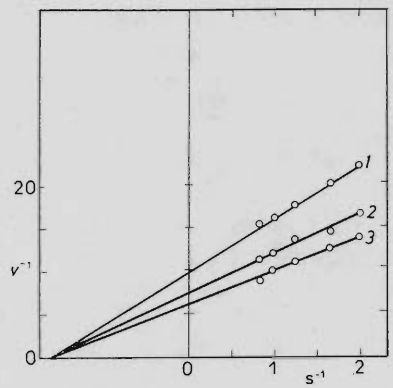

FIG. 6

Double reciprocal plot of rate *versus* NADH concentration at low concentration of ammonium acetate (0·0125 mol/l). Concentration of 2-oxoglutarate *1* 10^{-3} mol/l, *2* 2 . 10^{-3} mol/l, *3* 4 . 10^{-3} mol/l, *s* concentration of $NADH^{-1}$ in mol/l . 10^4

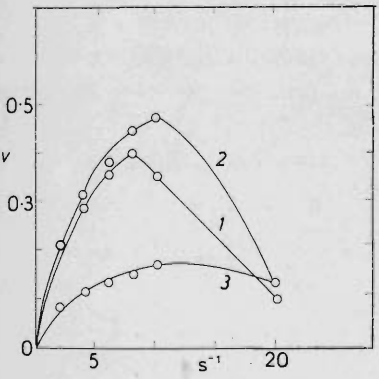

FIG. 7

Effect of increasing concentration of 2-oxoglutarate on activity of GDH at constant NADH concentration (1·54 . 10^{-4} mol/l). Concentration of ammonium acetate *1* 0·0625 mol/l, *2* 0·125 mol/l, *3* 0·250 mol/l, *s* concentration of 2-oxoglutarate in mol/l

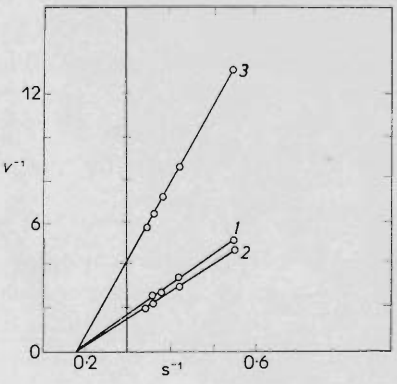

FIG. 8

Double reciprocal plot of rate *versus* 2-oxoglutarate concentration at varying ammonium acetate concentration and constant NADH concentration (1·54 . 10^{-4} mol/l). Concentration of ammonium acetate *cf.* Fig. 7. *s* concentration of 2-oxoglutarate in mol/l . . 10^3

The effect of the increasing concentration of NADH on the rate of the enzymatic reaction at a constant, high concentration of the ammonium ion and at various concentrations of 2-oxoglutaric acid (Fig. 4) points to the fact that the inhibition by an excess of NADH depends on the quantity of 2-oxoglutarate present in the incubation medium. The inhibition by NADH increases with the increasing concentration of 2-oxoglutarate; almost no inhibition was observed at low 2-oxoglutarate concentrations. Gilles[13] observed that the level of 2-oxoglutarate at which an excess of NADH shows inhibitory effect depends on the quantity of the ammonium ion present in the reaction mixture.

We found that at a constant, high concentration of the ammonium ion the plotted reciprocal value of the reaction rate *versus* NADH concentration almost paralleled the increasing concentration of 2-oxoglutarate (Fig. 5). This finding indicates that the K_m for NADH is dependent on the concentration of the ammonium ion. By contrast, the K_m for NADH at low concentrations of the ammonium ion is independent of the concentration of the remaining substrates (Fig. 6). According to Cleland's theory[14] such course of the kinetic curves points to the fact that the ammonium ion acts as a second substrate in the sequential order type of enzyme mechanism. A similar dependence was observed for GDH from bovine[15], frog[16], and dogfish[17] liver.

The determination of K_m for 2-oxoglutarate is complicated because of the inhibition observed at high concentrations of NADH and the ammonium ion. Corman and Kaplan[17] observed that at low concentrations of the ammonium ion and a high, constant concentration of NADH the K_m for 2-oxoglutarate is very low ($5 \cdot 10^{-4}$ mol/l). At higher concentrations (0·1 mol/l) of the ammonium ion, however, its inhibitory effect caused an apparent increase of the K_m for 2-oxoglutarate, an increase observed also in our experiments (Fig. 7, 8).

The Michaelis constant for the ammonium ion recorded in literature was de-

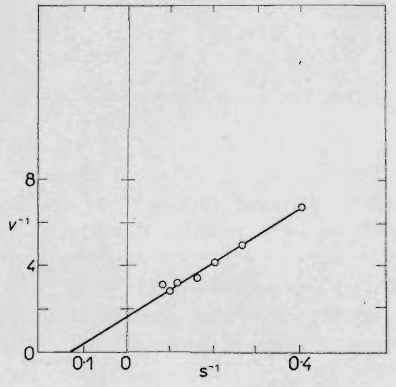

FIG. 9

Double reciprocal plot of rate *versus* ammonium acetate concentration. Concentration of NADH $1{\cdot}54 \cdot 10^{-4}$ mol/l, of 2-oxoglutarate 4 mol/l, *s* concentration of ammonium acetate in mol/l . 10^4

termined mostly with NH_4Cl as substrate. It has been reported that the Michaelis constants for various substrates apparently depend on the conditions of the reaction. Corman and Kaplan[17] reported a K_m of about $4{\cdot}5 \cdot 10^{-3}$ mol/l for 2-oxoglutarate and dogfish liver GDH when NH_4Cl served as a substrate; a value of $5 \cdot 10^{-4}$ mol/l was found for the same enzyme with ammonium acetate as substrate. Likewise the K_m for NADH with NH_4Cl as substrate is $15 \cdot 10^{-5}$ mol/l and $4{\cdot}5 \cdot 10^{-5}$ mol/l with ammonium acetate. This finding is in agreement with the conclusions of Schoffeniels[18] who pointed to the fact that the anion used as a component of the ammonium substrate can affect the behaviour of the enzyme. Schoffeniels[18] investigating the effect of various ions as a function of the increasing concentration of 2-oxoglutarate on the rate of the enzymatic reaction observed that the enzymatic reactions show a course varying with the anion present; the individual anions behave as competitive inhibitors of 2-oxoglutarate competing for a common binding site.

The apparent Michaelis constant for the ammonium ion determined by us with ammonium acetate as substrate (Fig. 9) is in agreement with the data reported by Corman and Kaplan[17] and Gilles[13].

The results show that rumen mucosa GDH resembles in its biochemical characteristics the enzyme isolated from the liver of other animal sources. It differs markedly, however, from GDH of microbial and vegetal origin[1].

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