Characterization of *Peptostreptococcus asaccharolyticus* Glutamate Dehydrogenase Purified by Dye-ligand Chromatography

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Glutamate dehydrogenase (L-glutamate:NAD⁺ oxidoreductase (deaminating); EC 1.4.1.2) has been purified from *Peptostreptococcus asaccharolyticus* in a single step using dye-ligand chromatography. The enzyme (GDH) was present in high yields and was stabilized in crude extracts. A subunit molecular weight of 49000 ± 500 was determined by SDS polyacrylamide gel electrophoresis and six bands were obtained after cross-linking the subunits with dimethyl suberimidate. This bacterial GDH was predominantly NAD⁺-linked, but was able to utilize both NADP⁺ and NADPH at 4% of the rates with NAD⁺ and NADH, respectively. An investigation of the amino acid specificity revealed some similarities with GDH from mammalian sources and some clear differences. The values of apparent K_m for the substrates ammonia, 2-oxoglutarate, NADH, NAD⁺ and glutamate were 18.4, 0.82, 0.066, 0.031 and 6 mM, respectively. The *P. asaccharolyticus* GDH was not regulated by purine nucleotides, but was subject to strong inhibition with increasing ionic strength.

INTRODUCTION

Glutamate dehydrogenase (GDH) has been purified from *Peptostreptococcus asaccharolyticus* (formerly called *Peptococcus aerogenes* and *Micrococcus aerogenes*) by three independent groups (Kew & Woolfolk, 1970; Johnson & Westlake, 1972*b*; Lerud & Whiteley, 1971). In all three cases the enzyme was reported to be present in high levels in cell-free extracts, but yields of pure enzyme were relatively low. The enzyme catalyses the first step in the fermentation of glutamate via the hydroxyglutarate pathway (Johnson & Westlake, 1972*a*). It operates in the direction of oxidative deamination, providing 2-oxoglutarate for subsequent reduction by 2-oxoglutarate reductase. Buckel & Barker (1974) have subsequently identified the pathway in a number of other anaerobes; all are characterized by the presence of these two enzymes.

The hydroxyglutarate pathway is one of two main routes for glutamate fermentation in anaerobic bacteria (see Barker, 1981); the other is the citramalate pathway (e.g. Barker, 1961) characterized by the presence of the enzyme β -methyl aspartase (Buckel & Barker, 1974), and a different distribution in the fermentation product, butyrate, of the carbon atoms derived from glutamate (Horler *et al.*, 1966*a, b*; Buckel, 1980). Studies of *P. asaccharolyticus* and *Acidaminococcus fermentans* (Buckel, 1980) have led to the elucidation of the hydroxyglutarate pathway.

This particular GDH has been partially characterized (Johnson & Westlake, 1972*b*; Kew & Woolfolk, 1970) and seems to be predominantly NAD⁺-linked. It has, however, been claimed to be able also to utilize NADPH but not NADP⁺ (Kew & Woolfolk, 1970). This unusual finding requires clarification. In this paper we report the purification of *P. asaccharolyticus* GDH by a single-step method employing dye-ligand chromatography, and the characterization of the

Abbreviation: GDH, glutamate dehydrogenase.

enzyme with respect to its subunit structure and basic kinetic properties. We have also overcome the problems of GDH instability in crude extracts reported by Kew & Woolfolk (1970).

METHODS

Materials. The reagents and media used are given below with the appropriate supplier in parentheses. Yeast extract, agar and bacterial peptone (Oxoid); monosodium L-glutamate, acrylamide and imidazole (Koch-Light); biotin, dimethyl suberimide, norvaline, ovalbumin, 2-oxoglutaric acid, bovine serum albumin, hen egg-white lysozyme, β -lactoglobulin and trypsinogen (Sigma); all other enzymes and proteins (Biozyme); cofactors and nucleotides (Boehringer); with the exception of Biogel P2 (Bio-Rad) all column materials were obtained from Pharmacia.

The triazine dyes used in screening experiments were gifts from Dr C. V. Stead (ICI Dyestuffs Division, Manchester, UK) and were linked to Sepharose 6B according to the method of Dean & Watson (1979). DL- β -methylglutamate was a gift from Dr S. Ghisla (University of Konstanz, FRG) whilst all other glutamate analogues were gifts from Dr S.-I. Hatanaka (College of General Education, University of Tokyo, Japan). All other reagents were AnalaR grade from BDH.

Growth and extraction of bacteria. Cultures of *P. asaccharolyticus* were obtained from the American Type Culture Collection (ATCC 14963) and as a gift from Dr W. Buckel (University of Regensburg, FRG). The growth medium contained 0.4% yeast extract, 0.1% NaCl, 0.4% potassium diphosphate, 0.02% ascorbic acid, 1% monosodium L-glutamate (all w/v) and 100 μ g biotin l^{-1} . Peptone (0.1%, w/v) and agar (0.2%, w/v) were only included in tube cultures. This 'Biotin' medium differs from that of Whiteley (1957), which is normally used to cultivate *P. asaccharolyticus*, and gave higher cell yields. The 'Biotin' medium induced high levels of GDH and, when peptone was excluded (in large-volume cultures) activity in crude extracts frozen in 20 mM-potassium phosphate, pH 7, remained stable for a year. These modifications to the medium, notably the presence of biotin (W. Buckel, personal communication) and the exclusion of peptone, alleviated earlier problems of instability. Peptone, however, was necessary in subcultures, for long-term maintenance of GDH activity.

Cells were grown in 40 litre batches at 37 °C and pH 7, and were harvested and washed in 0.1 M-potassium phosphate buffer, pH 7, containing 0.02% (w/v) ascorbic acid. A typical yield from this volume was 100 g (wet wt) of packed cells. Cell breakage was effected either by 30 min sonication of a 1:1 cell suspension in 20 mM-potassium phosphate buffer, pH 7, using a Heat Systems sonifier, or by grinding mechanically in a steel ball-mill, manufactured in the departmental workshop. The cell-free extract was obtained after centrifugation for 1 h at 27000 g on a Sorvall RC5 centrifuge.

Purification of *P. asaccharolyticus* GDH. GDH activity was monitored by the method of Johnson & Westlake (1972b), and the units (U) are expressed as μ mol NADH min^{-1} . The routine assay mixture contained 10 mM-L-glutamate and 1 mM-NAD⁺ in 40 mM-Tris/HCl, pH 8.8. Protein was determined by the method of Bradford (1976), with ox-liver GDH as a standard. All assays were performed on a recording fluorimeter similar to that described by Engel & Dalziel (1969). This instrument is designed to give high sensitivity and stability, together with a facility for 'backing off' a large part of the signal by using a variable voltage in opposition. Since reduced coenzyme is the only compound that changes in concentration during reaction, the instrument has a wide band-pass filter (maximum transmission at 450 nm) rather than any more sophisticated means of obtaining monochromatic light. The high sensitivity of such instruments, even when compared with modern spectrophotometers, makes it possible to follow accurately very small NAD(P)H concentration changes, so that rate measurements can be made confidently from the early, linear part of the progress curve.

All commercial column materials were prepared according to the manufacturers' instructions and were run under the conditions specified. Triazine dye columns were equilibrated in 20 mM-potassium phosphate, pH 7, containing 0.02% (w/v) sodium azide and were developed at room temperature. After each run the columns were washed with a column volume of 6 M-urea and re-equilibrated in the starting buffer.

The general procedure for screening was that used by Hey & Dean (1981). However, no negative step, in which the desired product passes through the column, whilst impurities are retained, was found with the dyes tested. For some experiments, notably the determination of coenzyme specificity, GDH was purified by the method of Hornby & Engel (1983), which includes a heat step similar to that of Kew & Woolfolk (1970).

Gel electrophoresis. Non-SDS gels (7.5%, w/v) were prepared according to the method of Davies (1964) and were used for activity and protein staining. The GDH activity stain comprised: 1 mM-NAD⁺, 40 mM-glutamate, 1 mg nitro violet tetrazolium ml^{-1} (Sigma) and 2 μ g Meldolabue ml^{-1} in 40 mM-Tris/HCl, pH 8.8, at 37 °C. The same mixture, but with 2-hydroxyglutarate in place of glutamate, could be used for detecting 2-oxoglutarate reductase in crude cell extracts.

The subunit molecular weight of the bacterial GDH was determined from a series of SDS gels, prepared according to the method of Laemmli (1970). Each gel was calibrated with the following proteins: ovalbumin (mol. wt 45000), bovine serum albumin (66000), alkaline phosphatase (67000), ox-liver GDH (56000), yeast glucose-6-phosphate dehydrogenase (51000), hog renal acylase (48000), yeast alcohol dehydrogenase (40000), bacterial

butyryl-CoA dehydrogenase (39000, a gift from Dr G. Williamson of this Department), pig heart lactate dehydrogenase (35000), trypsinogen (24000), β -lactoglobulin (18400) and hen egg-white lysozyme (14400). Gels were made up to 10, 12, and 15% and the mean result of three experiments was taken as the best estimate.

The subunit structure of *P. asaccharolyticus* GDH was determined by cross-linking with the bifunctional reagent dimethyl suberimidate. In two parallel experiments the bacterial enzyme and ox-liver GDH were cross-linked simultaneously in order to compare structures. The procedure which gave the best banding pattern was as follows. Both GDHs at a concentration of 1 mg ml⁻¹ were dialysed against 1 litre of 200 mM-sodium borate pH 9; 0.1 ml of a freshly prepared solution of 20 mg dimethyl suberimidate ml⁻¹ was then added to each sample and the two mixtures were incubated at room temperature for 3 h. Excess dimethyl suberimidate was subsequently removed by dialysis against distilled water, and the samples were freeze-dried. The residual white powders were re-dissolved in the SDS sample additive containing 1% SDS, 2% mercaptoethanol, 0.1% bromophenol blue and 20% sucrose (all w/v), and were boiled for 5 min. The final protein concentration was 2 mg ml⁻¹ in each case, and 30 μ l of each sample was loaded and run on 3% rod gels as described by Thomas (1978). The gels were subsequently stained with Coomassie blue in a mixture of methanol/water/acetic acid (5:5:1, by vol.) and destained in water/methanol/acetic acid (16:2:1, by vol.) in the presence of activated charcoal.

RESULTS

Growth of P. asaccharolyticus

During growth of this anaerobe over 26 h, stationary phase was reached after about 10 h and GDH levels reached a sharp maximum after 24 h (Fig. 1) – this was somewhat surprising in view of the dependence of energy production upon glutamate fermentation. Cells were therefore harvested routinely after 24 h growth.

Purification of GDH from P. asaccharolyticus

Initially the low yields obtained by earlier workers were improved upon by combining the most successful of the published purification steps and incorporating the use of a Blue Sepharose column. The resulting four-step procedure, hereafter referred to as Method I, gave a 55% yield of pure enzyme (Hornby & Engel, 1983). Subsequently we examined the possibility of developing a single-step purification by using dye-ligand chromatography. An overall purification of around 10-fold is required in view of the very high starting levels of GDH in the crude extract. Dyes linked to Sepharose 6B were used to screen the crude extract (Table 1).

With the exception of Procion Brown MX-5BR, all of the dyes showed some affinity for the GDH. The three dyes chosen for further study were Procion Blue PGR, Orange MX-G and Red MX-5B; all gave approximately six-fold purification by simple salt elution in a single 1 M-NaCl

Fig. 1. Growth curve for *P. asaccharolyticus* grown on the 'Biotin' medium and the specific activity of glutamate dehydrogenase. ●, Growth (OD₅₈₀); ○, log OD₅₈₀; □, activity of GDH (arbitrary units).

Fig. 2. Elution of GDH from Procion Blue PGR-linked Sepharose 6B. Activity ($\blacktriangle$; arbitrary units) was detected after the column (7×7 cm) was developed with a 100 mM-NaCl wash and a pH gradient from 6 to 9 followed by a salt gradient (broken line) up to 1 M-NaCl. The A_{280} measurements ($\circ$) are also shown. The inset shows an SDS gel of (1) crude extract and (2) the purified product; in both cases 10 μ g protein was loaded.

Table 1. *Purification of P. asaccharolyticus GDH on dye-linked columns*

A 5 ml sample of crude extract of *P. asaccharolyticus* was loaded onto a 10 ml column of the composition indicated. The columns were washed with 2 vols 20 mM-potassium phosphate, pH 7, followed by elution with 20 ml 1 M-NaCl. The activity was determined as described in Methods, and protein was estimated by the method of Bradford (1976).

Sepharose-linked Procion dye	Specific activity after elution ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Purification factor
Blue MX-3G	3.8	2.7
Blue MX-R	5.2	3.7
Blue PGR	8.4	6
Orange MX-2R	2.9	2.1
Orange MX-G	7.9	5.6
Red MX-5B	8.2	5.9
Red HE-3B	3.7	2.6
Green HE-4BD	4.1	3
Brown MX-5BR	—	—

wash. The elution profile in Fig. 2 shows how first a pH gradient, 6–9, and then a salt gradient, 0–1 M-NaCl in 20 mM-potassium phosphate, pH 7, resolved GDH from the other proteins on Procion Blue PGR-linked Sepharose 6B. The pH gradient was created using two buffers of constant ionic strength containing 0.2 M-potassium phosphate/0.1 M-glycine, adjusted to the appropriate pH with either 1 M-HCl or 1 M-KOH. This procedure is referred to as Method II (see Fig. 2). The GDH purified in this way had a specific activity of 13.8 U mg^{-1} and gave a single band on SDS-PAGE. Similar results were obtained with Orange MX-G and Red MX-5B columns, and compare favourably with those from Method I, but with yields of more than 80%.

Characterization of P. asaccharolyticus GDH

Kew & Woolfolk (1970) and Johnson & Westlake (1972*b*) have both reported that this GDH can utilize NAD^+ , NADH and NADPH but surprisingly not NADP^+ . The coenzyme specificity of *P. asaccharolyticus* GDH was therefore examined using highly purified coenzyme preparations. The four coenzymes were purified on DEAE-Sephadex A25, essentially according to the method of Dickinson & Engel (1977) and used within a few weeks of purification.

At pH 7 in 0.1 M-potassium phosphate, GDH was able to utilize both NADP^+ and NADPH at 4% of the rate obtained when NAD^+ and NADH were the coenzymes, respectively. In the

Fig. 3. Subunit molecular weight of *P. asaccharolyticus* GDH (●) as determined by SDS-PAGE on a 10% gel using the molecular weight standards (○) listed in Methods. The mean estimate from three such gels (at different acrylamide concentrations) was 49000 ± 500 .

assays with oxidized coenzymes (at $82 \mu\text{M}$) the glutamate concentration was 40 mM , whilst with the reduced coenzymes (at $64 \mu\text{M}$) the substrate concentrations were: 2-oxoglutarate, 5 mM and NH_4Cl , 50 mM . The demonstration of activity with NADP^+ is in contrast to the results of Kew & Woolfolk (1970) who could detect no NADP^+ -linked activity at both pH 7.0 and pH 8.5. The possibility of NADP^+ being contaminated with NAD^+ in these experiments was ruled out since the two coenzymes are readily separated by the above purification procedure.

Kew & Woolfolk (1970) also reported the presence of a separate NADP^+ -linked GDH which differed from the NAD^+ -linked enzyme in terms of both molecular weight and thermostability. The enzyme used in our specificity study was prepared by Method I, which includes a heat step that would be expected to remove the separate NADP^+ -linked enzyme (Kew & Woolfolk, 1970). Nevertheless, to test further whether the NADP^+ -linked activity in our preparation was an intrinsic property of the NAD^+ -linked GDH or due to a contaminant, the enzyme was rechromatographed on Sephacryl S-400, and the specific activity of eluted fractions was determined with both NAD^+ and NADP^+ . The ratio of activities remained essentially constant across the peak, implying that a single GDH species was present.

When non-SDS gels were run with the purified GDH, and stained for both NAD^+ - and NADP^+ -linked activity at pH 8.8, no NADP^+ -linked GDH activity could be detected. Since Kew & Woolfolk (1970) were unable to detect NADP^+ -linked activity in 20 mM -Tris at pH 8.5, the observations made here would seem to imply that in phosphate at pH 7 the activity towards NADP^+ is measurable whilst in Tris it is not. Hence the differences in assay conditions may explain the discrepancy between these two investigations. Under the appropriate assay conditions, the predominantly NAD^+ -linked GDH of *P. asaccharolyticus* can utilize NADP^+ as coenzyme.

Quaternary structure and subunit molecular weight of P. asaccharolyticus GDH

With the exception of the tetrameric, fungal GDHs which are NAD^+ -linked and have subunit molecular weights around 116000 (Smith *et al.*, 1975), GDHs generally have subunit molecular weights of between 45000 and 60000 , with the bacterial enzymes falling at the bottom end of this range.

The subunit molecular weight of *P. asaccharolyticus* GDH has not previously been determined, but the native enzyme has a molecular weight of about 266000 , estimated from gel filtration and analytical ultracentrifugation studies (Johnson & Westlake, 1972*b*). Comparison of the mobility of GDH and various marker proteins on SDS gels yielded a subunit molecular weight of 49000 ± 500 (Fig. 3), suggesting that this GDH is either a pentamer or a hexamer. The latter result seems more plausible in view of the common occurrence of hexameric GDHs.

Fig. 4 shows the banding pattern for GDH, cross-linked with dimethyl suberimidate, as a densitometric gel-scan. There are clearly six major bands, together with a higher molecular

Fig. 4

Fig. 5

Fig. 4. Densitometric scan of a stained gel after electrophoresis of cross-linked *P. asaccharolyticus* GDH on a 3% gel in the presence of SDS.

Fig. 5. Plot of the mobilities of the six GDH species from Fig. 4, showing the linear dependence of mobility on log molecular weight, assuming a subunit molecular weight of 49000 and a hexameric structure (●). The open circles are the analogous results obtained with ox-liver GDH.

Table 2. Preliminary kinetic data for *P. asaccharolyticus* GDH at pH 7 in 0.1 M-potassium phosphate buffer at 25 °C

The fixed concentrations of other reactants were, where appropriate, 40 mM-L-glutamate or 1 mM-NAD⁺ for the oxidative deamination reaction and 400 μM-2-oxoglutarate, 50 mM-NH₄Cl and 200 μM-NADH for the reductive amination.

Substrate	Concn range tested (μM)	<i>K_m</i>
NAD ⁺	1–4 × 10 ³	31 μM
Glutamate	10 ³ –200 × 10 ³	6.6 mM
NADH	5–2 × 10 ³	66 μM
2-Oxoglutarate	150–450	0.82 mM
NH ₄ ⁺	10 ⁴ –10 ⁶	18.4 mM

weight species. This confirms the conclusion that the bacterial GDH is hexameric. Under the conditions of the experiment shown in Fig. 4 the trimer was the least prominent of the cross-linked species, with both the bacterial and the ox-liver enzymes, a finding in contrast to the results of Hucho & Janda (1974), who found the trimer to be a major component of the cross-linked set.

The plot of *R_e* vs log molecular weight of the cross-linked species (assuming a subunit molecular weight of 49000 for the bacterial enzyme) was completely linear for both enzymes (Fig. 5). The plot only deviated from linearity when the acrylamide concentration was raised significantly above 3%.

Kinetic properties of *P. asaccharolyticus* GDH

The apparent *K_m* values for all five substrates were determined in 0.1 M-potassium phosphate, pH 7, at 25 °C (Table 2). The NAD⁺ primary plot (Fig. 6) was investigated with saturating glutamate concentrations (40 mM) over a wide range of coenzyme concentration (1–4000 μM). This detailed investigation was undertaken in order to establish whether *P. asaccharolyticus* GDH shows any of the kinetic complexities seen with the mammalian GDHs. Clearly, no 'abrupt transitions' (Engel & Dalziel, 1969) are seen over this range: the Eadie plot is perfectly linear within error.

Fig. 6

Fig. 7

Fig. 6. Eadie plot for *P. asaccharolyticus* GDH with NAD^+ concentration varied between 1 and $4000 \mu\text{M}$ at pH 7 in 0.1 M -potassium phosphate with the concentration of glutamate fixed at 40 mM .

Fig. 7. Inhibition of *P. asaccharolyticus* GDH by a variety of ions and DL-aspartate. Main figure: X, MgCl_2 ; ●, potassium phosphate; Δ, NaCl; ○, KCl; ■, KNO_3 ; ▼, K_2SO_4 . The inset shows the competitive nature of the inhibition by KCl (▲, ■; $\mu = 0.5$ and 0.3 , respectively) and DL-aspartate (●, ○; 10 mM and 50 mM , respectively) as compared with the uninhibited enzyme (Δ). The KCl data in the inset are extrapolated from more detailed plots for clarity.

Similarly the plots of primary data for NADH ($5\text{--}2000 \mu\text{M}$), glutamate ($1\text{--}200 \text{ mM}$) and NH_4Cl ($10\text{--}1000 \text{ mM}$) were linear at saturating concentrations of 2-oxoglutarate ($400 \mu\text{M}$), NAD^+ (1 mM), NADH ($200 \mu\text{M}$), glutamate (40 mM) and NH_4Cl (50 mM) where appropriate. The plot for 2-oxoglutarate under these conditions was linear up to 0.45 mM , but non-linear above this concentration.

The amino acid specificity of GDHs has attracted considerable interest, especially that of the ox-liver enzyme (e.g. Struck & Sizer, 1960; Hornby & Engel, 1983). The mammalian enzymes seem to exhibit a dual specificity: thus at pH 8.5, glutamate is oxidized optimally, whilst there is a second pH optimum (around 10) for the oxidation of a variety of monocarboxylic amino acids, including alanine and norvaline (Struck & Sizer, 1960; Markau & Steinhübel, 1972). The prokaryotic GDHs, although generally poorly characterized, seem not to exhibit this 'alanine' activity: a possible exception is the NADP-linked GDH from *Mycobacterium smegmatis* (Savada *et al.*, 1980).

Several monocarboxylic amino acids were tested for activity together with a series of glutamate analogues retaining the correct dicarboxylate spacing (see Hornby *et al.*, 1983). With NAD^+ at 1 mM and the amino acids all at 20 mM , initial rates were measured at pH 7, pH 8 and pH 9 (Table 3). The relative rates of turnover indicate that there is no counterpart to the 'alanine' activity observed with ox-liver GDH, but that there is a similar discrimination among the glutamate analogues (Hornby *et al.*, 1983). Replacement of the hydrogen atoms attached to the γ and β carbons with $-\text{CH}_3$ groups results in a marked decrease in turnover. Only L- α -amino- γ -nitraminobutyrate gave a rate of turnover greater than 1% of that with glutamate. This analogue is also utilized more efficiently than the methylated analogues by ox-liver GDH (D. P. Hornby, P. C. Engel & S.-I. Hatanaka, unpublished results).

The purine nucleotides AMP, ADP, ATP and GTP, at concentrations up to 1 mM , only slightly inhibited the activity of *P. asaccharolyticus* GDH. This inhibition was probably due to weak competition with the coenzyme. Thus *P. asaccharolyticus* GDH does not exhibit the same type of allosteric regulation as shown by ox-liver GDH and several bacterial GDHs, but could still exhibit the same type of regulation by other effectors.

Table 3. *Substrate specificity of P. asaccharolyticus GDH*

Relative rates of turnover of the indicated amino acids by *P. asaccharolyticus* GDH are shown. The 100% value, for L-glutamate at pH 7, corresponds to a rate of 3.45 s^{-1} . The NAD^+ concentration was $250 \mu\text{M}$ in each case, amino acids were fixed at 20 mM (or 40 mM for racemic mixtures) and the buffer system comprised 50 mM-potassium phosphate/50 mM-glycine at the appropriate pH. All rates were measured at 25°C with the enzyme concentration varied between 0.2 and $200 \mu\text{g ml}^{-1}$.

Substrate	Relative rate at pH 7	Relative rate at pH 8	Relative rate at pH 9
L-Glutamate	100	209	965
L-threo- γ -Methylglutamate	0.61	3.8	3.5
L-erythro- γ -Methylglutamate	0.45	—	1.13
DL- β -Methylglutamate	0.10	—	0.02
L- γ -Methyleneglutamate	0.04	—	—
L- α -Amino- γ -nitraminobutyrate	4.2	18.6	29.3
L-Norvaline	0.04	0.21	1.22
L-Alanine	0.013	0.021	0.027
L-Leucine	0.017	0.021	0.027
β -Nitraminoalanine	0.03	0.038	0.042
DL-Glutamate	42.4	98.5	501
L-Aspartate	—	—	—

The ionic strength dependence of initial rates

The most detailed kinetic studies of GDHs have been performed with phosphate buffers (Engel & Dalziel, 1969; Electricwala & Dickinson, 1979). However, phosphate buffer is known to exert specific effects upon initial rates with mammalian GDHs (e.g. Engel & Dalziel, 1969) and so the effects of various ions on the kinetic behaviour of the bacterial GDH were investigated in the hope of finding suitable conditions for detailed kinetic studies. A series of mono-, di- and trivalent ions was used, with all rates being measured in 50 mM-imidazole buffer, pH 7. DL-Aspartate, which is not a substrate for *P. asaccharolyticus* GDH, was also included in some initial rate measurements to determine whether, in assessing its contribution to the ionic strength, glutamate should be considered as mono- or trivalent at neutral pH.

The standard assay mixture comprised $200 \mu\text{M-NAD}^+$ and 50 mM-glutamate in 50 mM-imidazole, pH 7. Fig. 7 shows the inhibitory effects of potassium phosphate upon the reaction, together with the effects of K^+Cl^- and several other ions. All ionic strengths were calculated from the relationship $\mu = 1/2\sum cz^2$, where μ is ionic strength, c is molarity and z is charge. Most of the salts seemed to exert a non-specific inhibition which depended on the value of μ and not on the chemical nature of the ions. However, phosphate was less inhibitory than the other ions tested. Enzyme activity declined rapidly with increasing μ up to a μ value of about 0.2, at which it was about 10% of the control activity. Above $\mu = 0.2$ there was a more gradual decline in activity.

Inhibition by both DL-aspartate and K^+Cl^- was competitive with respect to glutamate (see inset to Fig. 7). The dependence of DL-aspartate inhibition on the concentration of DL-aspartate suggested that the aspartate should be regarded as effectively singly-charged. Consequently, the net charge, and not the sum of the charges, on glutamate seems to be the most appropriate quantity to use in calculating its contribution to the ionic strength.

DISCUSSION

The modifications of the Whiteley (1957) growth medium adopted here resulted in high levels of a stable GDH in crude extracts of *P. asaccharolyticus*, provided that harvesting was done at the correct time (Fig. 1). The single-step purification of GDH on Procion Orange MXG (and Blue PGR and Red MX-5B) linked to Sepharose 6B represents a major improvement over the methods already available. Furthermore, yields of more than 80% (and often 100%) are obtained from these dye-columns.

The mode of adsorption of GDH onto the series of dye-columns was not highly specific (eight out of nine columns retained the enzyme); the three columns chosen were those that bound and

released the fewest proteins in the same way as the GDH. The use of pH elution to resolve the GDH component further proved very successful.

The *P. asaccharolyticus* GDH is apparently hexameric, like several other bacterial GDHs (Smith *et al.*, 1975). The coenzyme specificity has been resolved: both NADP⁺ and NADPH are utilized at 4% of the rate for NAD⁺ and NADH, respectively, under the conditions used here. The *P. asaccharolyticus* GDH exhibits a much narrower amino acid specificity than ox-liver GDH in that there is no counterpart to the 'alanine' activity. The utilization of several methylated analogues of glutamate by *P. asaccharolyticus* GDH is, however, similar to that of ox-liver GDH. Both enzymes seem to be unable to accommodate lateral substitutions as bulky as methyl groups, even when the dicarboxylate spacing is conserved. The rates of enzyme activity with L- γ -methyleneglutamate are very low (0.004% of the rate with glutamate at pH 7), implying that the presence of a double bond decreases the dicarboxylate spacing, rendering the substrate virtually inactive. However, L- α -amino- γ -nitraminobutyrate is a reasonable replacement for glutamate, giving 4% of the glutamate activity at pH 7, which suggests that the resultant distance between the two negative charges in this analogue must be close to 0.74 nm, the correct dicarboxylate spacing (Caughey *et al.*, 1957).

Potassium phosphate buffer (0.2 M) minimized ionic interference and permitted easy estimation of kinetic parameters, without introducing any non-linearity. This buffer saturated the ionic effects of high glutamate (or 2-oxoglutarate and NH₄Cl) concentrations and thus proved suitable for kinetic studies.

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