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The Site at which 4-Iodoacetamidosalicylate Reacts with Glutamate Dehydrogenases

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1. Bovine, porcine and chicken liver glutamate dehydrogenases were irreversibly inhibited by a tenfold excess of radioactive 4-iodoacetamidosalicylic acid at pH7.5. 2. Inhibition was accompanied by the covalent incorporation of 1.1 mol of labelled inhibitor/mol of polypeptide chain. Acid hydrolysis yielded N^{ϵ}-carboxymethyl-lysine as the sole labelled amino acid. No labelled *S*-carboxymethylcysteine was recovered from the bovine or porcine enzymes. 3. The labelled bovine enzyme was hydrolysed with trypsin. The radioactivity was found at lysine-126 in a peptide comprising residues 119–130 of the sequence. 4. The amino acid compositions of the tryptic peptides containing labelled lysine from the porcine and chicken enzymes were similar to that of the bovine peptide.

Glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] from bovine liver is irreversibly inactivated by many alkylating and acylating agents [acetic anhydride (Colman & Frieden, 1966); 1-fluoro-2,4-dinitrobenzene (di Prisco *et al.*, 1970); 2,4,6-trinitrobenzenesulphonate (Coffee *et al.*, 1971); *N*-(*N'*-acetylsulphamoylphenyl)-maleimide (Holbrook & Jeckel, 1969); and also by pyridoxal 5'-phosphate (Piszkiewicz *et al.*, 1970; Goldin & Frieden, 1972)]. All of these reagents have been shown to label lysine amino groups. Baker *et al.* (1962*a*) showed that 4-iodoacetamidosalicylic acid competes with the substrate 2-oxoglutarate (K_i = 0.7mM) in assay media containing NADH and NH_3. At 37°C excess of the reagent irreversibly inactivated the enzyme in a pseudo-first-order reaction, and at high increasing concentrations of inhibitor the rate of inhibition no longer increased. The concentration of inhibitor required for one-half the maximum rate of inhibition was 0.7mM. This evidence suggests that 4-iodoacetamidosalicylic acid is reversibly bound to the enzyme before irreversible inactivation occurs.

The nature of the amino acids which react with 4-iodoacetamidosalicylic acid in this protein is as yet not fully clarified. Malcolm & Radda (1970) found *S*-carboxymethylcysteine in acid hydrolysates of the inhibited enzyme but failed to show the absence of other modified amino acids. There are reports that the reagent also reacts with lysine (Holbrook *et al.*, 1970) and methionine (Rosen *et al.*, 1972). By using a radioactive preparation of 4-iodoacetamidosalicylate, we show in the present paper that the sole amino acid labelled under certain conditions is lysine-126 of the amino acid sequence (Moon *et al.*, 1972).

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Materials and Methods

Glutamate dehydrogenases were purified by steps involving heat treatment, absorption on DEAE-cellulose, and fractionation with Na_2SO_4 and $(NH_4)_2SO_4$ in a method based on that of Kubo *et al.* (1959) and Corman *et al.* (1967). Specific activities of 6.2 units/mg for bovine enzyme, 5.1 units/mg for the pig enzyme and 3.8 units/mg for the chicken enzyme were found. The chicken enzyme was not a pure preparation. A subunit relative molecular mass of 56100 was used (Moon *et al.*, 1972). Trypsin was obtained from Serva, Heidelberg, Germany; Carboxypeptidase B was from Sigma Chemical Co., St. Louis, Mo., U.S.A. NAD^+ and 2-oxoglutarate were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. *S*-Carboxymethylcysteine and other L-amino acids, 1,4-bis-(5-phenyloxazol-2-yl)benzene and general laboratory reagents were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and were of the highest purity available. 2,5-Diphenyloxazole was purchased from Thorne Electronics, Tolworth, Surrey, U.K.

4-Iodoacetamidosalicylic acid was prepared by the method of Baker *et al.* (1962*b*).

Radioactive 4-iodoacetamidosalicylic acid was prepared as follows. Iodoacetyl chloride was freshly prepared before use by warming iodoacetic acid with a threefold molar excess of thionyl chloride on an oil bath at 50–60°C until evolution of HCl had ceased (approximately 1 h). Moisture was excluded by solid NaOH. Thionyl chloride was removed by rotary

evaporation at 2kPa and 25°C for 15min. Then 8.5mmol of the resulting iodoacetyl chloride was added to 23.8μmol of iodo[2-^{14}C]acetic acid (The Radiochemical Centre, Amersham, Bucks., U.K.; specific radioactivity 21mCi/mmol). Exchange was allowed to take place in the dark over a period of 5h at 25°C. The exchange mixture was added dropwise to a stirred ice-cold aqueous solution of 8.5mmol of sodium 4-aminosalicylate and 53.2mmol of $NaHCO_3$ (in 45ml). With periodic shaking the reaction was allowed to proceed for 1h. The labelled 4-iodoacetamidosalicylic acid was then precipitated by acidifying the solution with 0.1M-HCl and was washed with ice-cold water. It was recrystallized twice from aqueous ethanol (1:1, v/v). The crystals were dried overnight under vacuum over P_2O_5. The melting point of the non-radioactive material prepared by this method was 224°C (decomp.) [Baker *et al.* (1962*b*) found 225°C]. The specific radioactivity was about 0.05Ci/mol indicating that equilibration of radioactivity between iodoacetyl chloride and iodoacetic acid was almost complete. Chromatography of freshly prepared iodoacetamidosalicylic acid (either labelled or unlabelled) gave only one spot. However, owing to the instability of the reagent on storage over 2 years, the purity was determined after completion of the experiments. The melting point had fallen to 198°C. T.l.c. of the radioactive material on silica gel S in butanol–acetic acid–pyridine–water (68:14:40:25, by vol.) separated it into two radioactive components of which only one reacted with excess of thioglycollic acid. This component contained 57% of the radioactivity. All the results quoted in this paper have therefore been corrected for the impurity of 43%.

3-Carboxymethylhistidine was prepared by a modification of the method of Crestfield *et al.* (1963), in which the products of carboxymethylation were separated by thin-layer electrophoresis at pH6.5 on silica gel S as described by Holbrook *et al.* (1966). Products were detected by staining a guide strip with ninhydrin–collidine reagent. 3-Carboxymethylhistidine was eluted with 0.5% NH_3 solution and dried at 25°C over NaOH.

N^{ϵ}-Carboxymethyl-lysine was synthesized by a modification of the method of Gundlach *et al.* (1959) in which polylysine was replaced by N^{α}-tosyl-L-lysine (Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.).

Glutamate dehydrogenase activity was measured at 25°C by the initial rate of reduction of NAD^+ (1mg/ml) by sodium L-glutamate (10mg/ml) after addition of enzyme in a small volume (2–10μl) to a cuvette containing 3ml of assay medium. This was made up in 0.067M-NaH_2PO_4 buffer adjusted to pH7.5 with NaOH. The reaction was monitored at 340nm with a Hilger–Gilford recording spectrophotometer with a full-scale recorder deflexion of 0.1.

Enzymes were prepared for experiments by dialysis of the $(NH_4)_2SO_4$ suspension against the phosphate buffer described above (two changes of 20vol. each) at 4°C.

Protein concentrations were measured by the E_{280} of a solution after suitable known dilution: 1mg of glutamate dehydrogenase/ml (bovine, porcine or chicken) was assumed to have an E_{280} of 0.95 (Sund & Åkeson, 1964).

Radioactive samples were counted with a Nuclear–Chicago Unilux II liquid-scintillation counter. A fluid containing 60g of naphthalene, 37.5mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 2g of 2,5-diphenyloxazole in 500ml of dioxan was used. All samples were left for 24h before radioactivity counting.

High-voltage electrophoresis was carried out as described by Ambler (1963) on Whatman no. 3 paper. Paper chromatography was on Whatman no. 3 paper in a butanol–acetic acid–water solvent as described by Katz *et al.* (1959). Peptides were eluted from paper with 2% acetic acid.

Peptides were hydrolysed in 6M-HCl in sealed evacuated tubes at 105°C for 24h unless otherwise stated. The acid was removed under vacuum and the residue analysed on a Technicon TSM1 Auto-Analyser. Carboxymethylcysteine was eluted before aspartic acid and carboxymethyl-lysine was usually eluted immediately after valine and before methionine. However, occasionally it was eluted in the same place as valine.

Other techniques were as described by Holbrook & Jeckel (1969).

Labelling of bovine liver enzyme

Bovine liver glutamate dehydrogenase (600mg; 2.1mg/ml) was incubated with 0.4mM-iodo[^{14}C]-acetamidosalicylic acid at 37°C in 0.067M-NaH_2PO_4 buffer, adjusted to pH7.5 with 5M-NaOH. Samples (2μl) of this and a control containing no inhibitor were assayed at known time-intervals. The reaction was terminated by the addition of 2-mercaptoethanol (to a final concentration of 8mM) after 120min when 82% of the enzymic activity had been lost. The 330ml of enzyme was separated from unchanged inhibitor by gel filtration on a column (9cm×40cm) of coarse-grade Sephadex G-50 which had been equilibrated with 0.01M-NH_4HCO_3. The protein peak was detected by its u.v. absorption. Samples were taken for determination of the radioactivity incorporated, protein concentration, and for acid hydrolysis. The labelled protein was recovered from the remainder (560ml) by addition of 25ml of 4M-trichloroacetic acid followed by centrifugation.

The washed precipitate was dissolved in deionized 10M-urea (equilibrated in N_2 by bubbling the gas through it) containing 30mM-2-mercaptoethanol to a final concentration of about 20mg of protein/ml. The pH was adjusted to 9.0 by the addition of 5M-

NaOH. The whole mixture was then enclosed in an Erlenmeyer flask containing a glass electrode and a tube through which N_2 was continuously passed. After 30min incubation, solid iodoacetic acid (freshly recrystallized from *n*-hexane) was added anaerobically to the protein to a final concentration of 135mM. The pH value was readjusted to 9.0 with 5M-NaOH and this was maintained for a further 45min. The solution was then dialysed overnight against two changes of 20vol. of water. The labelled carboxymethylated protein precipitated as the urea dialysed away.

Preparation of the radioactively labelled peptide

To obtain ideal conditions for proteolytic digestion the precipitated protein from the previous step was recovered by centrifugation and was dissolved in 3mM-HCl (to a final concn. of approx. 25mg/ml) by heating it to 85°C in a water bath. The pH was kept at approx. 3.5 by further addition of dilute HCl. After cooling solid NH_4HCO_3 was added to the solution to give a final concentration of 0.2M. A flocculent precipitate appeared at this stage. Trypsin was then added to be one-thirtieth of the weight of protein. After 17½h digestion at 25°C it was estimated from the decrease in turbidity that 98% of the protein had redissolved. The trypsin was then precipitated in a boiling-water bath and removed by centrifugation. The supernatant was concentrated to 5ml by rotary evaporation at 40°C. Some material precipitated at this stage and was removed by centrifugation. It contained about 5% of the total radioactivity but the site of attachment of the label was not determined.

The soluble peptides were applied to a column (2.0cm×145cm) of fine-grade Sephadex G-25 which had been previously equilibrated in 10mM-NH_4HCO_3. The radioactivity was found in one peak accounting for 82% of the radioactivity applied to the column. The contents of the tubes containing the radioactive peak were concentrated to 5ml by rotary evaporation as described above. The labelled peptide was further purified by high-voltage paper electrophoresis at pH6.5. After drying at 60°C, the paper was examined under u.v. light (approx. 254nm). Peptides labelled by 4-iodoacetamidosalicylic acid have a characteristic pale-blue fluorescence under these conditions (Malcolm & Radda, 1970). A separate experiment showed that all spots detected by radioautography (6 weeks exposure, with Kodak Blue Brand X-ray film) exhibited this fluorescence. All spots detected during this work by fluorescence have been found to contain radioactivity. The single fluorescent band was cut out, sewn on to another sheet of chromatography paper and further purified by paper electrophoresis at pH3.5. As a final purification the peptide was subjected to descending paper chromatography. The recovery of radioactivity in the labelled peptide was 4% based on the radioactivity in the labelled enzyme.

Samples containing 3.9nmol of radioactive label were separately hydrolysed with 6M-HCl for 0, 12, 24, 48 and 72h. Two control samples were set up. Sample A contained no acid and was analysed immediately. Sample B contained no peptide but the same volume of acid as used in the other samples. The amino acid analyses from samples A and B were added together to calculate the zero value.

Inhibition of the pig liver enzyme and isolation of labelled peptide

This enzyme was inhibited in an analogous manner to the bovine enzyme. However, since the purity of the enzyme preparation was (erroneously) not estimated to be very high an attempt was made to block non-specific nucleophiles by incubating the enzyme with unlabelled alkylating agent while protecting the active-centre groups with oxoglutarate. The enzyme (900mg of protein) was incubated with 4.0mM-unlabelled 4-iodoacetamidosalicylic acid in the presence of 30mM-2-oxoglutarate for 15min in 67mM-sodium phosphate buffer, pH7.5. The possible reaction was stopped by the addition of 0.1ml of 2-mercaptoethanol to the 420ml of incubation mixture. Only 10% of the enzyme activity was lost during the incubation with unlabelled alkylating agent. The enzyme was recovered by filtration through a column (10cm×40cm) of coarse-grade Sephadex G-50, which removed the protecting 2-oxoglutarate, and was then inhibited to 22% residual activity by 0.4mM-4-iodoacetamidosalicylic acid as described for the bovine enzyme.

The enzyme was carboxymethylated and digested with trypsin [trypsin/protein 1:15 (w/w)] for 9h. The trypsin was removed as described above and the peptides were concentrated by rotary evaporation to 5ml. The labelled peptide was purified in the same manner as that described above for the bovine peptide. The overall recovery of labelled peptide was 2% of the radioactivity in the labelled enzyme.

Inhibition of the chicken enzyme and isolation of a labelled peptide

Chicken liver enzyme (525mg of protein) was labelled in a manner identical with that described for the porcine enzyme. However, only about 50% of the radioactivity in the tryptic digest applied to the Sephadex column was recovered in a sharp peak at an elution volume similar to that found for the other two enzymes. The remaining radioactivity was smeared throughout the whole void volume and was not further purified. In contrast with the other two enzymes the electrophoresis at pH3.5 resolved two radioactive

bands; these were labelled Ct1 and Ct2 and were present in the ratio 9:1. Both were eluted, hydrolysed with 6M-HCl and analysed. Band Ct2 was still clearly impure but insufficient was recovered after further attempts to purify it for analysis. Band Ct1 contained 9.5% of the radioactivity in the labelled enzyme. A sample of Ct1 was digested with 0.075mg of carboxypeptidase B in 20μl of 10mM-NH_4HCO_3. Samples (9nmol) were taken after 0, 30, 60 and 120min, dried down and analysed to find the *C*-terminal amino acid.

Results

Bovine enzyme

The inhibition of the bovine liver enzyme followed pseudo-first-order kinetics as far as 70% inhibition (Fig. 1). Enzyme that had been 82% inhibited had incorporated 0.99mol of labelled inhibitor/mol of polypeptide chain.

The carboxymethyl derivatives of amino acids are the expected products of hydrolysis of side chains which have reacted with 4-iodoacetamidosalicylate. Acid hydrolysis of a sample of the inhibited enzyme and subsequent analysis by thin-layer electrophoresis demonstrated the presence of N^{ϵ}-carboxymethyllysine as the major radioactive component (Fig. 2). A second radioactive component was also present and from a visual estimation of the blackening of the film in a radioautograph this corresponded to 5–15% of the total radioactivity on the thin-layer plate. The structure of the minor component is not known. The release of amino acids from the bovine peptide after hydrolysis in 6M-HCl for various periods was investigated. Unchanging ratios of amino acids were obtained after 24h. The composition was calculated after correction of the molar ratios to zero time except for valine where the maximum molar ratio was taken. The bovine peptide has the molar composition shown in Table 1.

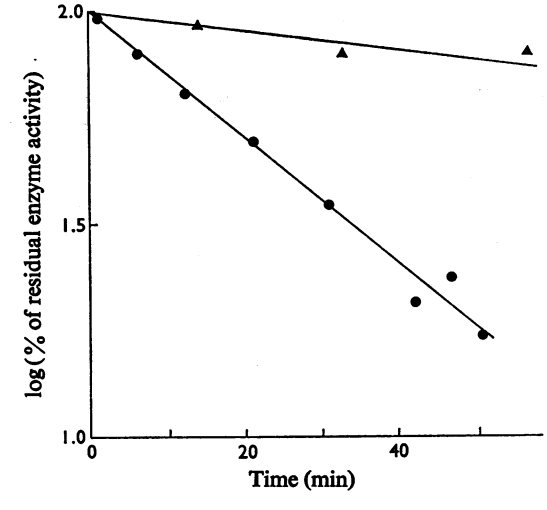


Fig. 1. *First-order inactivation of bovine liver glutamate dehydrogenase by 4-iodoacetamidosalicylate*

Bovine enzyme (2.5mg/ml) was incubated in 67mM-NaH_2PO_4 buffer adjusted to pH7.5 with 5M-NaOH either alone (▲) or with 0.4mM-4-iodoacetamidosalicylate (●). Samples (2μl) were taken at the times indicated and assayed as described in the text. The results are expressed as the logarithm of % of residual enzyme activity.

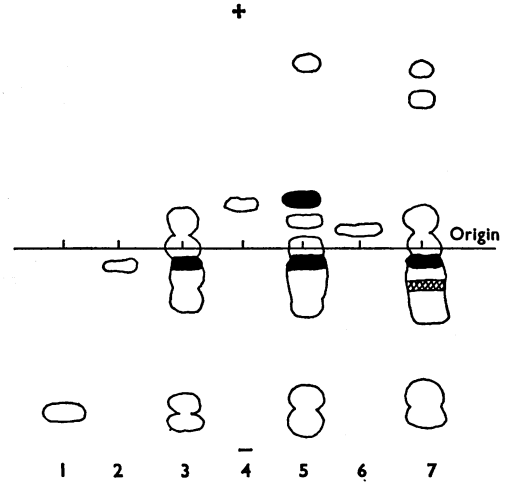


Fig. 2. *Identification of the radioactively labelled amino acids from glutamate dehydrogenases inhibited with 4-iodoacetamidosalicylate at pH7.5*

Sample 1 was lysine, sample 2 was N^{ϵ}-carboxymethyl-lysine, sample 4 was *S*-carboxymethylcysteine and sample 6 was 3-carboxymethylhistidine. Samples 3, 5 and 7 were respectively the acid hydrolysates (6M-HCl for 24h) of 4-iodoacetamidosalicylate-labelled porcine, chicken and bovine liver glutamate dehydrogenases. Electrophoresis was on silica gel S on 20cm long glass plates in a pH3.1 buffer (pyridine–acetic acid–water; 3:52:130, by vol.) for 1h at 25°C and 1kV. The open symbols were spots detected by ninhydrin. The closed symbols were spots detected by radioautography. The minor component in sample 7, shown cross-hatched, was estimated from the blackening of the film to represent 5–15% of the radioactivity in the spot corresponding to carboxymethyl-lysine.

Table 1. *Comparison of the amino acid compositions of radioactively labelled peptides purified from glutamate dehydrogenases inhibited by radioactive 4-iodoacetamidosalicylate*

The values of the valine and carboxymethyl-lysine content of the pig and chicken peptides are given together since these two amino acids did not separate cleanly in these two experiments. Assumed integral values are given in parentheses. Abbreviation: -CH_2CO_2H, carboxymethyl-.

	Amino acid composition after 24h hydrolysis (mol/mol of polypeptide)		
	Ox	Pig	Chicken
Cys-CH_2CO_2H	0	0	0
Asp	1.1 (1)	0.99	1.1
Thr	0.24	0.40	0.4
Ser	0.31	0.46	0.3
Glu	0.14	0.68	0.2
Pro	0.86 (1)	1.01	0.8
Gly	2.2 (3)	2.00	1.9
Ala	2.0 (2)	1.83	1.3
Val	1.7 (2)	1.43	2.3
Lys-CH_2CO_2H	0.71 (1)		
Met	0	0	0
Ile	0	0.24	0.2
Leu	0	0.27	0
Tyr	0	0.42	0.2
Phe	0.90 (1)	0.95	1 0
Lys	1.1 (1)	0.60	0.7
His	0	0.09	0.05
Arg	0	0	0
Pseudo-first-order rate constant of inactivation by 0.4mM-4-iodoacetamidosalicylate	0.023 min^{-1}	0.017 min^{-1}	0.016 min^{-1}

Porcine enzyme

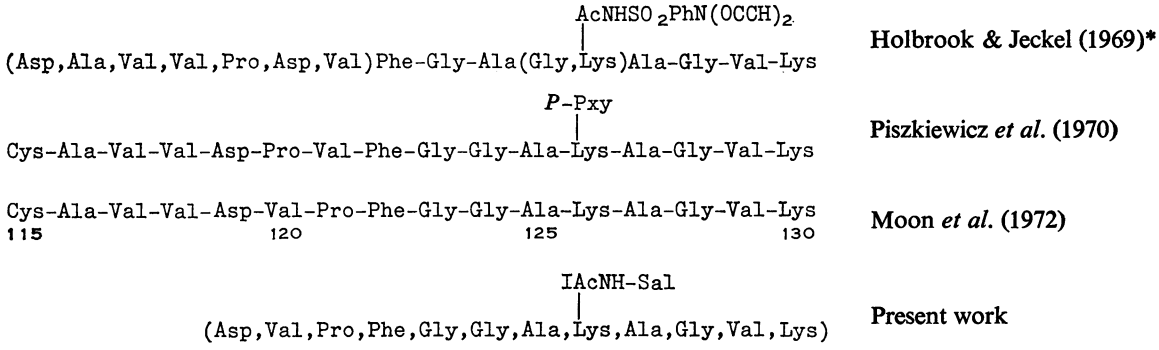
Inhibition of the porcine enzyme also follows first-order kinetics and proceeds at about the same rate as with the bovine enzyme. Enzyme that had been inhibited to 78% contained 1.0mol of inhibitor/mol of polypeptide. The label was found after acid hydrolysis as N^{ϵ}-carboxymethyl-lysine (Fig. 2). No minor component was found similar to that from the bovine enzyme. There was no *S*-carboxymethylcysteine present. The amino acid composition of the purified peptide after hydrolysis for 24h is shown in Table 1 and may be compared with the composition of the bovine peptide also given after 24h hydrolysis.

Chicken enzyme

Inhibition of the chicken enzyme follows first-order kinetics at about the same rate as the bovine enzyme. Enzyme which had been 74% inhibited contained 0.58mol of inhibitor/mol of polypeptide chain. The label was present in both lysine and cysteine in about equal amounts (Fig. 2). However, only the peptide containing labelled lysine was eluted from the Sephadex column as a sharp peak. It contained 50% of the radioactivity applied to the column and was further purified and gave the amino acid composition shown in Table 1. The remaining 50% of the radioactivity was eluted from the Sephadex column as a smear throughout the whole elution pattern and has not been further characterized. Carboxypeptidase B released lysine in stoicheiometric quantity from the peptide containing labelled lysine.

Discussion

The results in the present paper demonstrate that modification of a little more than one lysine residue per polypeptide chain destroys the enzymic activity of bovine liver glutamate dehydrogenase. No labelled cysteine was obtained, although in preliminary experiments with early preparations of the enzyme both labelled lysine and cysteine were found. Malcolm & Radda (1970) reported that cysteine was a labelled amino acid. However, these authors failed to exclude



* This composition has subsequently (J. J. Holbrook, unpublished work) been corrected by the detection of proline.

Fig. 3. *Sequences of tryptic peptides containing labelled lysine isolated by various labelling methods from bovine liver glutamate dehydrogenases*

$AcNHSO_2PhN(OCCH)_2$, *P*-Pxy and IAcNH-Sal are used to represent the products of reaction of *N*-(*N'*-acetylsulphamoylphenyl)maleimide, pyridoxal 5'-phosphate and 4-iodoacetamidosalicylate respectively with lysine.

the possibility that lysine is labelled, since labelled lysine (N^{ϵ}-carboxymethyl-lysine) is eluted together with valine in the amino acid analysis system that they used and it is noteworthy that the apparent valine content of their enzyme increases after modification by 4-iodoacetamidosalicylic acid. The peptide containing the labelled lysine residue compares reasonably with the peptide comprising residues 119–130 of the sequence (Moon *et al.*, 1972) of this protein and cannot be derived from any other portion of the sequence. This peptide contains two lysine residues and consequently two possible labelling sites. However, since the peptide is the product of tryptic digestion and this proteinase would be expected to hydrolyse all bonds involving the carboxyl group of lysine, this amino acid must be the *C*-terminus. Thus, lysine-126 is the residue that is labelled, preventing tryptic hydrolysis of peptide bond 126–127. Fig. 3 compares this peptide with a peptide containing lysine labelled with *N*-(*N'*-acetylsulphamoylphenyl)maleimide (Holbrook & Jeckel, 1969) and also a peptide containing lysine labelled by pyridoxal 5'-phosphate (Piszkiewicz *et al.*, 1970). These peptides, despite small discrepancies, are clearly the same.

A reason for the unusual tryptic cleavage of the bond between valine-118 and aspartic acid-119 is difficult to suggest but is possibly due to contamination of the trypsin with other proteases and the conditions used for the proteolytic digestion.

Examination of the 4-iodoacetamidosalicylic acid-labelled peptides from pig and chicken liver glutamate dehydrogenase (Table 1) reveals that these probably have very similar compositions to the bovine peptide. The high glutamic acid content in the porcine peptide is probably an impurity, since it actually increased during the final purification procedure. Similarly the serine, threonine, isoleucine, leucine and tyrosine content of these peptides is probably due to contamination.

Thus the amino acid sequence containing residues 118–130 of glutamate dehydrogenase is probably highly conserved, at least in the three vertebrates studied, and may have an important role in the function of the enzyme. 4-Iodoacetamidosalicylic acid, by all known criteria, is active-site-directed, so lysine-126 is likely to be very near to the active site (Baker *et al.*, 1962*a*; Malcolm & Radda, 1970).

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