γ-Glutamyl Hydrolase (Conjugase)

PURIFICATION AND PROPERTIES OF THE BOVINE HEPATIC ENZYME*

(Received for publication, December 3, 1974)

MARTIN SILINK, ROGER REDDEL, MONICA BETHEL, AND PETER B. ROWE

Department of Child Health, University of Sydney, New South Wales 2050, Australia

Bovine hepatic γ-glutamyl hydrolase (conjugase) has been purified to homogeneity. A feature of the purification procedure was the use of high affinity macromolecular polyanion enzyme inhibitors which formed tight complexes with the enzyme altering its solubility, gel filtration, and ion exchange properties. The enzyme, which cleaves the γ-glutamyl bonds of pteroylpolyglutamates, has a molecular weight of 108,000. It is a glycoprotein with an acid pH optimum, properties consistent with its lysosomal localization. Zinc is essential for enzyme stability. The presence of highly reactive sulfhydryl groups was evident from the extreme sensitivity to oxidizing agents and organomercurials. Very little thermal denaturation occurs below 65°, but the enzyme is extremely sensitive to buffer anions, in keeping with the polyanionic nature of the substrate.

In order to study the mechanism of action of the enzyme, a wide range of pteroylpolyglutamates, N-t-Boc polyglutamates and free polyglutamates were synthesized containing L-[U-14C]glutamic acid residues in different positions. Two pteroyltriglutamate derivatives were also synthesized in which an α bond replaced one of the two available γ bonds. Time course studies of the products of the action of conjugase on these various substrates enabled us to draw the following conclusions about the enzyme: (a) peptide bond cleavage occurred only at γ-glutamyl bonds and the presence of a COOH-terminal γ bond was essential for enzyme action; (b) bond cleavage occurred with equal facility at internal points of the peptide chain and the enzyme should therefore be more appropriately classified as an acid hydrolase; (c) longer chain γ-glutamyl peptides were preferentially attacked by the enzyme, the cleavage of diglutamyl peptides being extremely slow; and (d) cleavage of γ bonds was independent of the NH2-terminal pteroyl moiety.

Studies with polyanions such as the glycosaminoglycans and dextran sulfate supported the concept that the polyanion structure of the substrate was a major factor in substrate-active site interaction.

The group of enzymes which hydrolyze the γ-glutamyl bonds of pteroylpolyglutamates, termed loosely "the conjugases", have an almost universal distribution throughout the phylogenetic spectrum. Initially identified some 30 years ago (1), relatively little was known about their properties until Baugh and his colleagues developed a sensitive efficient assay employing selectively radioactively labeled substrates synthesized by solid phase synthetic techniques (2). Although the enzymes were generally considered to be acid carboxypeptidases, certain tissues and lower organisms contain enzymes which were active at a neutral or alkaline pH. The recent assignation of the name glutamate carboxypeptidase (EC 3.4.12.10) is somewhat misleading in that it gives no indication either of the specificity of many of these enzymes for γ-glutamyl bonds or, in the case of the bovine hepatic enzyme at least, of the endopeptidase activity.

The over-all biological role of conjugases is still unclear. They are certainly required during the process of digestion and absorption of dietary pteroylpolyglutamates, during which these molecules are reduced to their mono- or diglutamyl derivatives. Another species of the enzyme is required to effect the reduction to critical peptide chain length of pteroylpolyglutamates necessary for phage tail plate assembly (3).

Central to the question of a major role for the enzyme is the clarification of the relative utilization of the oligo- and polyglutamate derivatives of the various reduced- and one-carbon substituted pteroylglutamates. It is evident that the polyglutamates constitute the bulk of the tissue stores and, in prokaryotes at least, it would appear that the enzymes involved in the interconversion of these compounds function quite effectively at the polyglutamate level. We have observed that the enzyme 5-methyltetrahydropteroyl monoglutamate homocysteine transmethylase from rat liver has both a lower \( K_m \) and higher \( V_{max} \) for the pentaglutamate form of the substrate (4). Bertino's group recently reported that with this same enzyme from bovine brain and human RPM14265 cells in
culture, the polyglutamate forms of the substrate were as effective as the monoglutamate (5). This would appear to exclude any critical role for conjugase in breaking down the polyglutamate derivatives prior to their utilization. It is, however, possible that reduction to mono- or diglutamates is required in order to effect membrane transport of these compounds. Related to this latter question is the structural significance of a γ-glutamyl peptide chain. It is possible that the structural configuration of this unique peptide chain is a critical factor in limiting the transmembrane movement of pteroylpolyglutamates. Another more intriguing hypothesis is that the polyglutamylates provide the cell with a readily accessible store of glutamic acid residues which might be required to modulate certain basic cellular functions. The basic model for this is the glycogen-glucose pathway. The γ-glutamyl cycle proposed for amino acid transport (6) could well be a basic function supported by such a store of glutamic acid residues.

The studies described here were undertaken in order to isolate and characterize bovine hepatic conjugase. They formed part of an over-all project concerned with elucidating the mechanisms and significance of pteroylpolyglutamyl-oligoglutamate interconversions.

EXPERIMENTAL PROCEDURE

Materials

Pteroylglutamic acid was obtained from Sigma Chemical Co. N-t-Boc-L-glutamic acid-a-benzyl ester, γ-diglutamate (Glu-Glu),1 γ-triglutamate, α-diglutamate (α-Glu-Glu), and α-triglutamate were purchased from the Fox Chemical Corp. Radioactive pteroyltriglutamate standard was a gift from Professor Charles Baugh while pteroylglutamic acid standard was a gift from Professor Charles Baugh while pteroyltriglutamate with the COOH-terminal glutamic acid concentration which ensured complete adsorption of pteroylglutamate but no adsorption of glutamic acid. Adsorption of these peptides therefore made interpretation of those assays using long chain substrates extremely difficult. In routine assays and in kinetic studies, pteroyltriglutamate with the COOH-terminal glutamic acid carrying the 14C label was used.

The standard assay mixture consisted of pteroyltriglutamate (Pte-Glu-Glu-Glu), specific activity 0.5 Ci/mol, β-mercaptoethanol (50 mM), pteroylglutamic acid (0.5 mg/ml), aggregated bovine serum albumin as the protein standard, 25% of diglutamate, 70% of triglutamate, and 90% of tetraglutamate adsorbed to the charcoal. Enzyme activity was expressed as disintegrations per min of 14C-glutamic acid released per min of incubation. The release of 14C-glutamic acid was a linear function of the time of incubation and of the amount of enzyme used. The supernatant solution was filtered through a Millipore filter (HAWP, 13 mm, 0.45 μm) with a disposable syringe and the filtrate counted in a Packard Scintillation Spectrometer in a scintillation fluid consisting of 1 part of Triton X-100 and 2 parts of toluene containing 2,5-diphenyloxazole (5 g/liter) and 1,4 bis(2-(4-methyl-5-phenyloxazolyl) benzene (0.3 g/liter). Counting efficiency was of the order of 80%. Enzyme activity was expressed as disintegrations per min of 14C-glutamic acid released per min of incubation. The release of 14C-glutamic acid was a linear function of the time of incubation and of the amount of enzyme used, to the point where 50% of substrate radioactivity had been released.

In assays where extremely small amounts (1.0 to 1.5 μg) of protein were used, the addition of 50 μg of bovine serum albumin to the assay mixture stabilized the enzyme, increasing the activity by the order of 50%.

In analyzing the products of enzymatic action, the reaction was terminated by the addition of 0.5 ml of 70% perchloric acid. After neutralization with 3.0 M potassium hydroxide the precipitate was removed by centrifugation. The supernatant solution was lyophilized to dryness and the residue dissolved in a minimum volume of water.

The products of the reaction were examined by three methods: (a) thin layer chromatography on MN-300 cellulose developed with 5% acetic acid adjusted to pH 9.0 with ammonia and saturated with isoamyl alcohol. The migration of pteroylglutamates was assessed using ultraviolet light (254 nm) and their fluorescence yield. Fluorescence readings were made on a Perkin-Elmer MFP-3 fluorescence spectrophotometer. Bovine serum albumin was used as the protein standard.

Substrate Synthesis—A wide range of pteroylpolyglutamates, N-t-Boc-polyglutamates, and polyglutamyl peptides of varying chain length with L-[U-14C]glutamic acid residues in different positions were synthesized by solid phase peptide synthesis techniques.2 Two pteroylglutamyl peptides were synthesized in which one of the two γ bonds was replaced by an α linkage 5-Methyltetrahydro-pteroylglutamate was synthesized by formylation and reduction of pteroylglutamate.4

Protein Estimation—Both the Lowry (7) and the fluorescamine (8) methods were used. At the low protein concentrations encountered in the final stages of enzyme purification β-mercaptoethanol enhanced the fluorescence yield. Fluorescence readings were made on a Perkin-Elmer MFP-3 fluorescence spectrophotometer. Bovine serum albumin was used as the protein standard.

Enzyme Assay—The assay method was modified that determination of enzyme activity by the Lowry (7) method was based on the assumption that conjugases were carboxypeptidases which successively cleaved terminal glutamic acid residues from pteroylglutamylates. Following incubation of the substrate containing a [14C]glutamic acid residue with the enzyme, the pteroyl derivatives were absorbed onto activated charcoal which did not significantly bind free glutamic acid. The charcoal was removed by filtration and the radioactivity of the filtrate determined. The beef liver conjugases, however, exhibited significant endopeptidase activity and hydrolysis of pteroylglutamylates resulted in the release of glutamyl peptides containing up to 4 amino acid residues. When tested over a 10-fold range of concentrations of the order of that used in the usual enzyme assays, 25% of diglutamate, 70% of triglutamate, and 90% of tetraglutamate adsorbed to the charcoal which had been added at a concentration which ensured complete adsorption of pteroylglutamates but no adsorption of glutamic acid. The products of the reaction were examined by three methods: (a) thin layer chromatography on MN-300 cellulose developed with 5% acetic acid adjusted to pH 9.0 with ammonia and saturated with isoamyl alcohol. The migration of pteroylglutamates was assessed using ultraviolet light (254 nm) and their fluorescence yield. Fluorescence readings were made on a Perkin-Elmer MFP-3 fluorescence spectrophotometer. Bovine serum albumin was used as the protein standard.

1 Standard abbreviations used throughout the text are Pte for pteric acid and Glu for glutamic acid. All glutamic acid residues are linked by γ bonds unless otherwise specified, e.g. Pte-Glu-Glu-Glu. Pteroylglutamate containing two γ bonds while Pte-α-Glu-γ-Glu-Glu is pteroylglutamate with an α bond between the 1st and 2nd glutamate residues and a γ bond between the 2nd and 3rd residues.

2 Glu indicates L-[14C]glutamic acid.

3 The methodology for substrate synthesis and for alternative affinity chromatography procedures for enzyme purification are included in the miniprint supplement immediately following this paper. Material published in miniprint form can be easily read with the aid of a large field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of full size photocopies, these same data are available as JBC Document No. 74M-1818. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.20.

4 G. P. Lewis, unpublished data.
of water and 15 ml of the toluene-Triton X-100 based scintillation fluid and counting in a packard scintillation spectrometer, or by scanning the plates on a Packard radiochromatogram scanner fitted with a disc chart integrator; (b) thin layer anion exchange chromatography on DEAE-cellulose (Mannex D) using a solvent system of 50 mM sodium chloride in 50 mM potassium phosphate, pH 7.0. Under these conditions, pteroyl derivatives remain at the origin while glutamic acid and short chain glutamyl peptides were readily resolved after detection with ninhydrin. Radioactivity was determined as above; (c) definitive separation of the glutamate products of enzymatic action was achieved by high voltage electrophoresis on Whatman No. 3MM paper in a Savant LTL48A Tank. The solvent used was formic acid/glacial acetic acid/water (25:87:888) pH 2.1 (9). Electrophoresis was performed for 2½ hours at 4000 volts (40 volts/cm). After staining with ninhydrin the quantitative and qualitative distribution of radioactivity was determined on a radiochromatogram scanner.

Dextran sulfate was measured by the method described by Ellis and Walton (10).

**Enzyme Purification**—The final purification method adopted was based on standard techniques whose utility was extended by a reversible modification of the enzyme with blue dextran 2000. Stabilization of highly purified enzyme was achieved with Zn⁺ ion and β-mercaptoethanol, as well as with blue dextran, and these were the critical factors in achieving a high degree of purification. Alternative methods of affinity chromatography were also employed. Yearling beef liver (800 g) was homogenized in 2.4 liters of nitrogen-saturated water and 15 ml of the toluene-Triton X-100 based scintillation fluid containing 1.0 mM zinc acetate, 5.0 mM β-mercaptoethanol, and 75 mM sodium chloride, pH 6.5, and was eluted as a sharp peak by raising the sodium chloride concentration to 0.4 M. The peak activity fractions were pooled. The behavior of the enzyme on gel filtration and ion exchange chromatography media was modified by the addition of blue dextran 2000 (6.0 mg per 100,000 dpm/min incubation time enzyme activity).

**Cation Exchange Chromatography**—The enzyme-blue dextran complex was adsorbed to a CM-cellulose column (25 x 2.5 cm) equilibrated in 1.0 mM zinc acetate, 5.0 mM β-mercaptoethanol, and 75 mM sodium chloride, pH 6.5, and was eluted as a sharp peak by raising the sodium chloride concentration to 4.0 M. The peak activity fractions were pooled. The behavior of the enzyme on gel filtration and ion exchange chromatography media was modified by the addition of blue dextran 2000 (6.0 mg per 100,000 dpm/min incubation time enzyme activity). The enzyme-blue dextran complex behaved chromatographically like blue dextran.

| Purification Step | Volume (ml) | Activity (dpm/min/ml) x 10⁻³ | Total Activity (dpm/min) x 10⁻⁶ | Protein (mg/ml) | Specific Activity (dpm/min/mg) x 10⁻³ | Yield % | Purification |
|-------------------|-------------|-----------------------------|-------------------------------|----------------|--------------------------------------|--------|--------------|
| 1. Homogenate     | 2,900       | 14.5                        | 41.90                         | 49.5           | 0.29                                 | 100.0  | 1.0          |
| 2. 1st Supernatant| 2,000       | 20.5                        | 41.00                         | 44.5           | 0.06                                | 98.0   | 1.6          |
| 3. Protamine Supernatant | 990       | 22.6                        | 22.40                         | 5.0            | 4.48                                | 54.0   | 15.3         |
| 4. 0-55% A.C. ppte | 142       | 16.6                        | 2.36                          | 13.0           | 1.51                                | 6.0    | 5.2          |
| 5. Dialysed SS 50% A.C. ppte | 225     | 57.8                        | 13.01                         | 7.9            | 7.22                                | 21.0   | 25.0         |
| 6. 4-11% PEG ppte | 27         | 353.0                       | 9.53                          | 23.5           | 15.02                               | 23.0   | 51.4         |
| 7. 11-15% PEG ppte | 27         | 72.3                        | 1.95                          | 6.8            | 10.63                               | 5.0    | 36.3         |
| 8. Sephadex G-150 Pulp | 430       | 16.4                        | 7.95                          | 0.2            | 66.36                               | 17     | 234.0        |
| 9. CM-cellulose Pool | 83        | 48.0                        | 4.0                           | 0.2            | 228.75                              | 9.5    | 782.0        |
| 10. Pool after chromatography with Blue Dextran | 45 | 26.9                        | 1.24                          | 0.004          | 7,480,000                           | 425,000 |             |

**Table I**

Summary of purification of conjugate from bovine liver

---

a This step is included on the table to indicate that further enzyme can be recovered by increasing the concentration of polyethylene glycol (PEG) to 15 g per 100 ml.
concentration over an Amicon XM 100 filter provided an ideal method for enzyme stabilization and storage. The enzyme could not be concentrated other than as the enzyme-dextran complex because it bound to the membrane surface resulting in considerable activity losses.

RESULTS

Properties of Isolated Enzyme—Polyacrylamide gel electrophoresis of the purified enzyme revealed only a single protein band which also gave a periodic acid-Schiff reaction. Gel chromatography on either acrylamide (Bio-Gel P-300) or Sephadex G-150 indicated a molecular weight of 108,000 (Fig. 1). This value was not altered by p-chloromercuribenzoate, Triton X-100, or sodium dodecyl sulfate, although a subunit structure might be anticipated. The only published molecular weight data available were for the pancreatic conjugases of the rat and chicken for which the respective values of 60,000 and 52,000 were reported (12). The endopeptidase of chicken intestine conjugase has a reported molecular weight of 80,000 (13).

As might be predicted from the effects of anions on enzyme activity (vide infra) the pH activity profile varied with the nature of the buffer used. In 33 mM sodium acetate there was pH optimum at 3.9 with a rapid decrease below this level and above pH 4.5. In 33 mM sodium citrate, however, not only was the optimum at pH 4.9 but the shape of the profile was quite different. This altered profile could also perhaps have been related to a metal-chelation effect of the citrate ion, a potent enzyme inhibitor.

The $K_m$ value was determined for pteroyltriglutamate with the radioactive label on the terminal glutamic acid residue. The $K_m$ for this particular substrate was 1.7 µM, a value somewhat above the true figure as 20% of the radioactive diglutamate released would not be measured in the assay system. The turnover number was 732 γ-glutamyl bonds cleaved per min per mol of enzyme.

Cation and Anion Effects on Enzyme Activity—The monovalent cations, sodium, lithium, ammonium, and potassium, had no effect on enzyme activity at concentrations up to 50 mM. The cations were tested as their chloride salts and beyond this concentration the anion itself was an inhibitor. Certain divalent cations (calcium, barium, magnesium, cobalt, nickel, and strontium) similarly had no effect on activity in concentrations up to 35 mM (tested as their chloride salts). Other cations, however, were enzyme inhibitors. In increasing order of potency these were manganese, cadmium, iron (Fe$^{2+}$), mercury (Hg$^{2+}$) and copper (Cu$^{2+}$). At a concentration of 10 mM, manganese chloride and cupric chloride produced 45 and 98% inhibition, respectively.

Anion effects were assessed with the respective sodium salts. It was found that there was a small but significant decrease in activity when the concentration of the sodium acetate buffer used in routine assays was increased from 33 to 100 mM. The addition of 33 mM iodide, sulfate, and nitrate produced 40 to 50% inhibition. This degree of inhibition was seen with 100 mM formate, fluoride, chloride, phosphate, and dimethyl glutarate. Sodium citrate was an extremely potent inhibitor, a concentration of 0.1 mM being sufficient to produce 50% inhibition; sodium cyanide was as potent in a concentration of 5.0 mM. The effect of these latter anions was almost certainly related to their zinc bonding property. Borate, in concentrations up to 300 mM, predictably produced no effect on enzyme activity as it was minimally ionized at pH 4.5 (14).

Enzyme Activity as a Function of Temperature—These studies attempted to correct for thermal denaturation by using large amounts of enzyme coupled with short reaction times (1 min) in order to achieve a minimum of 25% substrate cleavage. Simultaneous “denaturation” profiles were carried out in which the enzyme was heated for 1 min at each temperature, rapidly cooled to 37°, and assayed. Substrate was omitted during heat treatment as it was heat sensitive although additional experiments suggested that it protected the enzyme from heat denaturation.

It was shown that the apparent temperature optimum was 70° and in the light of the 40% loss of activity due to thermal denaturation at this temperature, the “true” optimum was certainly higher. Denaturation, in fact, only became significant over 65° and it was clear that this enzyme was highly heat-resistant in that only 30% loss of activity occurred at 30 min at 65°.

Conjugase, a Zinc Metalloprotein—The addition of chelators such as EDTA and 1,10-phenanthroline to the assay did not inhibit the reaction although, as was discussed previously, cyanide and citrate were potent inhibitors. Stability studies demonstrated that storage of the enzyme in 5.0 mM β-mercaptoethanol at 4° resulted in the loss of 40% of activity over 24 hours. This loss was prevented by the addition of 1.0 mM zinc acetate. The loss of 60% of activity when the enzyme was stored in water alone at 4° for 24 hours was reduced to 20% by the addition of the zinc ion.

Prolonged dialysis (24 hours) of the enzyme against a solution containing 1.0 mM 1,10-phenanthroline and 5.0 mM β-mercaptoethanol resulted in the loss of 70% of activity compared with a control dialysis in which 1.0 mM zinc acetate replaced the o-phenanthroline (Fig. 2). Further dialysis of the chelator-treated enzyme against the control dialysis fluid restored the activity to 70% of control levels within 24 hours and this activity remained stable for a further 10 days.
usual and was probably related to the mechanism of action recovery of activity. Similar observations were made with several hours exposure. Thiol addition produced only partial concentration of 12.5 mM producing only 60% inhibition after stability of the inhibited enzyme suggested that bonding of (1.0 x 10^-4 M) produced instantaneous inhibition which could required for enzyme stabilization and p-chloromercuribenzoate days dialysis against distilled deionized water.

Purified enzyme, carried out by atomic absorption spectros-copy, gave values of 4.47 and 4.15 zinc atoms per molecule of the enzyme. Nickel also restored activity to approximately 20% of control levels. Zinc restored activity to 32% of control levels and this activity remained stable for the succeeding 120 hours. The rapid inhibition and reversal implied that the sulfhydryl groups were both readily accessible and active site-related. The long term be as rapidly reversed by the addition of thiols even after be as rapidly reversed by the addition of thiols even after

Continued dialysis against the chelator reduced the activity to 4% of control levels by 144 hours and no activity was detectable after 288 hours. The replacement of the chelating agent by different cations after 144 hours restored activity to a variable degree. Zinc restored activity to 32% of control levels and this activity remained stable for the succeeding 120 hours. The rapid inhibition and reversal implied that the sulfhydryl groups were both readily accessible and active site-related. The long term stability of the inhibited enzyme suggested that bonding of these groups did not result in gross conformational changes.

Quantitation of the zinc content of two samples of the purified enzyme, carried out by atomic absorption spectroscopy, gave values of 4.47 and 4.15 zinc atoms per molecule of enzyme. These determinations were made following 8 days dialysis against distilled deionized water.

Reactive Sulfhydryl Groups—β-Mercaptoethanol was required for enzyme stabilization and p-chloromercuribenzoate (1.0 x 10^-4 M) produced instantaneous inhibition which could increase by the addition to the assay of calcium or manganese ions (1.0 mM) which are critical for effective carbohydrate binding (15). The inhibition was not due to any substrate binding by concanavalin A as mixtures of the two could be readily resolved by gel chromatography.

Column chromatography of a mixture of concanavalin A and highly purified conjugase on Sephadex G-150 resulted in the elution of the enzyme at a position corresponding to a molecular weight of 160,000 (Vc/Vo = 1.15). The enzyme ordinarily behaved in this chromatography system as a molecule of molecular weight 108,000 (Vc/Vo = 1.29) while concanavalin A was bound firmly by Sephadex and required α-methylglucoside for its removal. This experiment indicated that concanavalin A and conjugase formed a higher molecular weight complex of the order of 160,000 suggesting a 1:1 stoichiometry in the binding. More significantly it confirmed that the carbohydrate-binding sites of concanavalin A were directly or indirectly involved in this complex formation as they were no longer free to bind to the Sephadex.

This observation was confirmed in an experiment with concanavalin A fluorescein isothiocyanate. A solution containing 1.14 nmol of the ligand and 0.37 nmol of the purified enzyme was chromatographed on a Sephadex G-150 column equilibrated in 1.0 mM zinc acetate, 5.0 mM β-mercaptoethanol, and 0.2 M sodium chloride, pH 6.5. Fluorescence was measured in a Perkin-Elmer MFP-3A recording spectrofluorimeter (excitation wavelength 474 nm, emission wavelength 517 nm). Enzyme activity again corresponded to that of a protein of molecular weight 160,000, and to a major fluorescence peak. Chromatography of the fluorescent ligand alone confirmed that it coincided with a second major peak. The fluorescence emission peak for the free ligand was at 520 nm indicating a small blue shift on complexing with the enzyme. Glucoside was not required for removal of the free concanavalin A-fluorescein, but there was a significant degree of interreaction with the Sephadex in that the Vc/Vo was 4.5. This would suggest that the linking of the fluorophore to the concanavalin A had in some way modified the carbohydrate binding sites. Quantitation of the relative fluorescence in the two peaks from the column revealed a 2:1 ratio between the free and conjugase-bound concanavalin A-fluorescein, supporting the 1:1 stoichiometry of complex formation. The 96% recovery of fluorescence from the column suggested that complex formation did not result in any significant quenching.

Specific Cleavage of γ-Glutamyl Bonds—The enzyme was incubated with the following substrates: pteroyl γ-glutamyl-γ-glutamyl-[U-14C]glutamic acid (Pte-Glu-Glu•Glu), pteroyl-
substrates were Pte-Glu-Glu-*Glu (W), Pte-a-Glu-Glu-*Glu (A), and Pte-Glu-a-Glu-*Glu (0). Release of radioactivity was measured in the different pteroyl triglutamate analogues as a function of time. These routine assay system and is expressed as dsentegrations per min of triglutamate (Glu-Glu-*Glu) suggesting some sparing of tamate (Glu-Glu-Glu-*Glu), diglutamate (Glu-*Glu), and glutamic acid were produced together with smaller amounts (x10-3). The sequential mechanism of hydrolysis. a terminal ["Clglutamic acid residue was used for assessing derivatives (17) and accordingly, pteroylpentaglutamate with both glutamic acid and diglutamate were produced from the enzyme was referred to above where it was observed that Pte-Glu-Glu-*Glu. The bulk of mammalian folates are stored either as their pentaglutamates (16) or even longer chain conjugase could not hydrolyze the COOH-terminal bond released unlabeled triglutamate but radioactive glutamic acid. Accordingly, there were relatively higher concentrations of glutamic acid, di-, and triglutamate present than was demonstrated in this analytical system. Prolonged hydrolysis resulted in the relatively rapid disappearance of Glu-Glu-Glu-*Glu and Glu-Glu-*Glu and subsequently the very slow disappearance of Glu-*Glu, the end product being glutamic acid. These radioactivity data were qualitatively confirmed by the rate of appearance and disappearance of ninhydrin positive material from the electrophoretogram.

The sequence of hydrolysis, namely Pte-Glu-Glu-Glu-*Glu, followed by tetraglutamate, triglutamate, and finally diglutamate, was consistent with the enzyme preference for longer chain glutamyl peptides.

Quantitation of the other products of this pattern of cleavage, that is the pteroylpolyglutamates present at various stages of the reaction, could not be effectively carried out due to the low substrate concentration and the unavailability of a suitably sensitive detection system. Thin layer chromatography on MN-300 cellulose, however, conclusively showed that Pte-Glu-Glu-Glu-*Glu rapidly disappeared to be

![Fig. 3. Conjugase mediated release of 14C radioactivity from three different pteroyl triglutamate analogues as a function of time. These substrates were Pte-Glu-Glu-*Glu (■), Pte-a-Glu-Glu-*Glu (△), and Pte-Glu-a-Glu-*Glu (●). Release of radioactivity was measured in the routine assay system and is expressed as disintegrations per min (x10-3).](image)

![Fig. 4. The relative amounts of original enzyme substrate Pte-Glu-Glu-Glu-Glu-*Glu (●) and glutamate derivatives (expressed as a percentage of the total assay 14C radioactivity) present during the course of hydrolysis by conjugase. The various derivatives are tetraglutamate (△), triglutamate (■), diglutamate (○), and glutamic acid (●).](image)
replaced at the end of the reaction by pteroylglutamic acid. The end products of the reaction were pteroylglutamic acid and glutamic acid.

It would appear that bovine hepatic conjugase had a preference for internal γ bonds and this was illustrated by experiments with pteroylglutamates containing the radioactive label in the terminal and middle glutamic acid residues, Pte-Glu-Glu-*Glu and Pte-Glu-*Glu-Glu-Glu. With the routine assay system there was virtually no difference in the rate of release of radioactivity from these two substrates. A different perspective was obtained by examining the products of the reactions by high voltage electrophoresis and by thin layer chromatography. The hydrolysis of Pte-Glu-*Glu was associated with the release of larger amounts of Glu-*Glu than glutamic acid initially indicating a preferential attack on the internal γ bond. In a much slower reaction there was a progressive fall in the level of Glu-*Glu with a parallel increase in free glutamic acid. Virtually no radioactive glutamic acid was detected from the hydrolysis of Pte-Glu-*Glu-Glu until after some 10 min of incubation although there was a rapid release of Glu-Glu. The subsequent slow decline in the level of Glu-*Glu was associated again with the appearance of glutamic acid.

**Preferential Cleavage of Longer Chain Polyglutamyl Peptides**—This property of the enzyme was demonstrated by using pteroylglutamates and pteroylpentaglutamates containing 14C labels in selected glutamic acid residues. The routine assay system was used with varying time periods of incubation and the study was facilitated by the exploitation of one of the major defects of the charcoal adsorption technique used in this assay, the retention of oligoglutamates by charcoal (see "Methods").

The effect of this was seen during a time course study of the hydrolysis of pteroylpentaglutamate containing a [14C]glutamate residue in the second innermost position, i.e. Pte-Glu-*Glu-Glu-Glu-Glu-Glu. The internal position of the radioactive label resulted in the relatively late release of radioactive glutamic acid and Glu-*Glu which contrasted with the early and rapid release of detectable radioactive from Pte-Glu-Glu-*Glu. On incubation of conjugase with equimolar concentrations of these two substrates it was clear that the longer chain substrate was preferentially attacked.

Pteroyldiglutamate (Pte-Glu-*Glu) was an extremely poor enzyme substrate when compared with Pte-Glu-Glu-*Glu. In the routine assay systems the rate of release of radioactivity from the former substrate was of the order of 10% of that from the latter, and this was confirmed by examination of the reaction products. Pte-Glu-Glu-*Glu was, however, effectively bound by the enzyme and this was reflected by its competitive inhibition of the hydrolysis of Pte-Glu-Glu-*Glu with a relatively low Ki of 4.5 μM.

Boc-diglutamate was similarly relatively slowly hydrolyzed but glutamate cleavage was even slower. The low affinity for this latter substrate was reflected in the observation that it had a minimal effect on the hydrolysis of Pte-Glu-Glu-*Glu.

**Effect of Substrate Pteroyl Group on Enzyme Activity**—The observation that the hydrolytic action of the enzyme was virtually independent of the presence of the pteroyl group was evident from the studies previously described. In a study in which the rates of cleavage of Pte-Glu-*Glu-Glu, Boc-Glu-Glu, and Glu-*Glu-Glu were compared, it was found that the

![Fig. 5. The preferential cleavage of longer chain pteroylpolyglutamates. The release of radioactivity from Pte-Glu-Glu-*Glu (○), Pte-Glu-Glu-Glu-Glu (△), each 10 μM, and a mixture of both of these substrates (●), 10 μM, is shown as a function of the time of incubation. The assays were carried out under routine conditions and the radioactivity (measured in the Millipore filtrates following charcoal adsorption) expressed as disintegrations per min (×10³) released.](http://www.jbc.org/)

Boc-substituted derivative was hydrolyzed faster than the pteroyl substituted and free triglutamates (Fig. 6). The pattern of cleavage, as determined by high voltage electrophoresis, was identical for all three substrates.

As the major form of pteroylglutamates in mammalian liver would appear to be 5-methyltetrahydropteroyl pentaglutamate or its longer chain analogues, the action of the enzyme on this compound appeared to be particularly pertinent. Using experimental conditions identical with those employed for the study on the hydrolysis of pteroylpentaglutamate, it was shown that the rate of release of radioactivity was identical for these two substrates containing a [14C]glutamate residue in the COOH-terminal position. The sequence of γ bond cleavage, with an identical release of glutamic acid and glutamyl peptides as a function of time, was confirmed by high voltage electrophoresis of the products of the reaction. Modification of the pteroyl group by reduction and methylation, therefore, had no effect on the action of the enzyme.

**Polyanion Inhibition**—Poliayonics such as DNA and RNA inhibited hog kidney conjugase (18) and at a concentration of 1.0 μg/ml these nucleic acids produced 50% inhibition of the bovine enzyme at a substrate (Pte-Glu-Glu-*Glu) concentration of 10 μM.

We have systematically examined the inhibition produced by three other classes of polyanions: (a) the glycosaminoglycans (chondroitin sulfates A, B, C, and D, heparin, and hyaluronic acid); (b) the sulfonated dextran, dextran sulfate 2000; and (c) the sulfonated anthroquinone dyes Cibacron blue 3GA (and its dextran derivative dextran sulfate 2000) and Cibacron brilliant blue BRP.

Heparin was the most potent conjugase inhibitor (Fig. 7), producing 50% inhibition at a concentration 26.7 ng/ml, approximately equivalent to an SO₄²⁻ ion concentration of 0.1 μM (19). There was little difference between the degrees of inhibition produced by the various chondroitin sulfates. Fifty per cent inhibition was achieved at twice the concentration but
FIG. 6 (left). The release of \(^{14}C\) radioactivity from Boc-Glu-\(^*\)Glu-Glu (\(\bullet\)), Pte-Glu-\(^*\)Glu-Glu (\(\odot\)) and Glu-\(^*\)Glu-Glu (\(\Delta\)) by conjugase as a function of time. Substrate concentrations were 10 \(\mu\)M in the standard assay mixture and activity is expressed as disintegrations per min (\(\times 10^{-3}\)) released. The blank values for the assays with Glu-\(^*\)Glu-Glu as substrate were much higher as only 70% of the unhydrolyzed substrate was adsorbed to the charcoal.

FIG. 7 (right). Inhibition of conjugase activity by the glycosaminoglycans. Assays were carried out under standard conditions and activity is expressed as percent of control levels. The concentration of inhibitors is shown as ng per assay (volume 1.5 ml). Hyaluronic acid, \(\bullet\); chondroitin sulfate A, \(\Delta\); chondroitin sulfate B, \(\mathbf{I}\); chondroitin sulfate C, O; chondroitin sulfate D, \(\Delta\); heparin, \(\square\). The values for chondroitin sulfates A and B were virtually superimposable and were plotted as a single curve.

at the same \(SO_4^{2-}\) ion concentration as heparin. Dextran sulfate 2000 produced 50% inhibition at a concentration of 42.5 ng/ml, equivalent to a \(SO_4^{2-}\) concentration of 277 nM.

The binding of dextran sulfate 2000 to conjugase was studied in some detail as it proved to be an effective method of enzyme purification.

This polyanion formed a stable soluble complex with conjugase, as was illustrated by the column chromatography on Sepharose 4B. The polymer was excluded from this gel, eluting at the void volume while the much lower molecular weight enzyme was considerably retarded. When mixed with the polymer, however, the enzyme eluted at the void volume. The complex could be resolved by the addition of DEAE-dextran which bound dextran sulfate even more effectively than did the enzyme.

The polyanion-enzyme complex was precipitated in the presence of both the alkali earth (Mg\(^{2+}\), Ca\(^{2+}\), Ba\(^{2+}\)) and transition (Zn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\)) elements due to the formation of a cation-enzyme-polyanion complex (20). This experiment was performed with crude enzyme preparations, and by appropriate adjustments to both the dextran sulfate and cation concentration, maximal recovery and purification of the enzyme could be achieved. This is illustrated in Fig. 8, where a concentration of 0.125 g/liter of dextran sulfate and 10 mM magnesium chloride resulted in the precipitation of over 75% of the enzyme activity with a 2.5-fold increase in specific activity. It was also observed that protamine sulfate displaced the enzyme from this insoluble complex and this was exploited in the method evolved for enzyme purification.

Conjugase formed a stable soluble complex with blue dextran 2000, a dextran polymer of average molecular weight \(2.0 \times 10^5\), containing approximately 195 molecules of the aminonic anthroquinone dye Cibacron blue 3GA. Each dye molecule contained, on the average, four sulfonyl groups. Blue dextran produced 50% inhibition of the enzyme at a concentration of 0.67 \(\mu\)g/ml (335 nM), equivalent to a sulfonyl group concentration of 261 nM. The free dye also formed a soluble stable complex with the enzyme, but equivalent inhibition was only achieved at twice the concentration of the dextran-coupled dye.

The dye Cibacron brilliant blue BRP, a structural analogue of Cibacron blue 3GA, formed a similar complex but the inhibition was only one third as potent. This dye had an average of one less sulfonyl group per molecule and a different orientation of the sulfonyl and amino groups on the phenylendiamine ring (21). The complexes in all three situations were readily dissociated by either bovine serum albumin or DEAE-cellulose, both of which had a higher dye affinity than did the enzyme.

DISCUSSION

The conjugase group of enzymes appears to fall into two classes, one of which has an acid pH optimum and another smaller group has a neutral or alkaline pH optimum. The first group of enzymes is represented by the mammalian conjugases and these are presumably lysosomal in origin (22-25). They include the enzymes from hog kidney (26, 27), leucocytes (28), guinea pig intestine (29), human liver (22), and human placenta (30). The pH optimum for all of these enzymes falls in the range of 4.0 to 6.0. The second group includes the chicken intestine enzyme complex with maximum activity at pH 7.5 (31), the chicken pancreas enzyme at pH 7.8 (32), and the enzyme from Flavobacterium polymyxinum, maximal between pH 8.0 and 8.5 (33). The pH of 4.5 was chosen for most of our routine assays with bovine conjugase as the enzyme was a little more stable at this pH during studies which required prolonged incubation with various substrates.

It has been noted in earlier work on the human liver enzyme that the activity varied greatly in different buffers (22) and a direct correlation could be observed between the \(pK_a\) of the.
buffer and the degree of inhibition of the enzyme at a particular pH level. Apart from the cases of the citrate and cyanide anions, the same phenomenon was observed with the bovine enzyme, activity being greater on the acid side of the buffer pKₐ, that is, at lower anion concentrations. The anion effect probably results from a number of factors. At pH levels above 2.19 the substrate, pteroylpolyglutamate, almost certainly exists as a polyanion with an extended rod structure maintained by the free α-carboxyl groups (34), and high anion concentrations could interfere with enzyme binding. It would appear that charge effects are important in determining conjugase activity. In relation to this, the ionization constants of protein groups vary with ionic strength (35) and changes in salt concentration could well alter the pKₐ values of active site-related groups.

The marked thermal stability of the enzyme activity was regarded as evidence of the primitive origin of the protein consistent with the wide phylogenetic distribution of the enzyme. The high temperature optimum placed beef hepatic conjugase within the group of thermophilic bacterial proteases, enzymes which are usually neutral proteases. One of these enzymes, thermolysin from Bacillus thermolyticus, has been shown to be a complex metalloenzyme which requires zinc for the maintenance of activity and calcium for thermal stability (36). Calcium, however, did not improve the heat stability of hepatic conjugase.

The zinc stabilization of conjugase and the zinc-mediated partial restoration of activity after prolonged treatment with o-phenanthroline suggested that the enzyme was a zinc metalloprotein. This was confirmed by the direct estimation of zinc in the purified protein at 4 atoms per molecule of molecular weight 108,000. It would appear that zinc was relatively firmly bound as prolonged exposure to a chelator was required to significantly decrease the activity. Furthermore, the localization of the zinc atoms may have imposed restriction on access for the relatively bulky phenanthroline molecule. This latter possibility was supported by the rapid inhibition by simple zinc ligands such as citrate and cyanide ions.

The removal of zinc also appeared to labilize the enzyme in that the full activity could not be restored by zinc addition. This phenomenon has been observed with carboxypeptidase A (37), carboxypeptidase B (38), carbonic anhydrase (39), and alkaline phosphatase (40). Similarly, other metal ions were able to substitute for zinc to varying degrees with these enzymes. In contrast to this, the removal of zinc from yeast alcohol dehydrogenase (41) and malic dehydrogenase (42) was irreversible.

It was clear that the maintenance of reduced sulfhydryl groups was important for enzyme activity and 5.0 mM β-mercaptoethanol was quite effective for this purpose. This thiol is, however, one of a group of sulfur-containing ligands which include BAL and cysteine which have a high affinity for the transition and Group IIB elements, particularly iron, copper, and zinc (43). Cysteine most effectively removed zinc from the carboxypeptidase A active site, indicating a low stability constant for this metalloenzyme (44). Although β-mercaptoethanol was required to stabilize conjugase it almost certainly removed the zinc from the protein as the addition of 1.0 mM zinc acetate to the thiol-stabilized enzyme resulted in the maintenance of full activity for 3 months at 4°C. This observation would indicate that, despite the formation of zinc mercaptoethane, the concentrations of both free sulfhydryl groups and Zn²⁺ were sufficient to maintain the reduced sulfhydryl groups and to maintain an equilibrium between the protein-bound zinc and the surrounding medium.

Studies on conjugase from other sources, although not extensively pursued, supported the concept that these were sulfhydryl enzymes. The soybean (45) and hog kidney (18) enzymes were inhibited by sulfhydryl reagents, the latter being activated by cysteine (46). The human hepatic enzyme was sensitive to oxygen during tissue homogenization (47), whereas sulfhydryl reagents blocked the dipeptidase activity of the chicken intestinal group of conjugases (31). The bovine hepatic enzyme was similar to the soybean enzyme in that the heavy metals were inhibitory while the metals of the alkali and alkali earth group exerted little effect (48).

Some indication that the enzyme was a glycoprotein in keeping with its lysosomal localization (49) was the positive periodic acid-Schiff stain given by the single protein species demonstrated on polyacrylamide gel electrophoresis. The indirect evidence from the studies with concanavalin A would suggest that more than 1 saccharide residue was available for binding then it was bonded to the other available site on the concanavalin A molecule (50) and that steric factors related to the size of the interacting proteins prevented additional binding.

The specificity of conjugase for γ-glutamyl bonds has been investigated for several of the enzymes from different species. The enzyme from Flavobacterium polyglutamicum was able to hydrolyze only γ-glutamyl bonds (33), whereas the chicken pancreas enzyme, after initial reports of γ bond specificity (51), was subsequently reported as being able to hydrolyze α bonds at one-half the rate (52). Human liver enzyme failed to hydrolyze α bonds (22). The same investigators also demonstrated that, while the human enzyme hydrolyzed pteroylglutamyl-glutamyl-leucine, it could not hydrolyze pteroylglutamyl-glutamyl-α-leucyl-leucine. This experiment was interpreted as demonstrating that the enzyme was an exopeptidase with terminal γ bond specificity (47). This requirement for a terminal γ bond, demonstrated also for the bovine enzyme, actually prohibited any conclusions being drawn about possible internal bond cleavage using substrates with terminal α bonds.
The conjugase group of enzymes has generally been regarded as consisting of carboxypeptidases. This generalization largely evolved from Pfiffner’s observation that methyl esterification of pteroylpolyglutamate rendered it insusceptible to attack by hog kidney conjugase (53). This methyl ester, however, was a polymethylester with methyl groups potentially esterified to each of the seven free α-carboxyl groups as well as to the one free γ-carboxy group of the molecule. This would result in a major alteration in the polyanion nature of the substrate which we consider essential for enzyme action. The evidence for the human liver enzyme being exclusively an exopeptidase has been discussed in the preceding section and this also we do not feel is conclusive. Baugh and Krumdieck (22) also demonstrated the isolation of all of the possible pteroylpolyglutamate intermediate breakdown products of the human conjugase-mediated hydrolysis of pteroylpolyglutamate. This data could just as readily support the concept of the enzyme randomly cleaving any of the six available substrate bonds, i.e. acting as a hydrolase, as the theory that there was sequential COOH-terminal cleavage of glutamic acid residues.

The end point of conjugase hydrolysis of pteroylpolyglutamates has been determined for several species of the enzyme. Pte-Glu-Glu has been reported to be the pteroyl derivative end product of the chicken pancreas enzyme (52). This same end product has been demonstrated for conjugase from Flavobacterium polyglutamicum (33), although a peptidase has been isolated from another species of flavobacterium which could cleave Pte-Glu-Glu (54). It has not yet been demonstrated whether Pte-Glu-Glu was a substrate for the human hepatic enzyme although that inference could be drawn from the data provided (22). It is clear that pteroyl glutamic acid and glutamic acid are the end products of the action of the bovine hepatic enzyme.

A possible explanation for the preference for the longer chain substrates, also a property of the human enzyme (22), may lie in the relative charge of the substrates. The pKₐ values for the α-carboxyl and γ-carboxyl groups of glutamic acid are 2.19 and 4.25, respectively (55). At the assay pH of 4.5, the free α-carboxyl groups would be strongly anionic and the total charge on the longer chain derivative would be greater. As mentioned earlier, there is good evidence that these relatively short chain polyglutamates exist as extended rodlike polyanions at these pH levels (34). The evidence for charge interaction being a factor in substrate binding and catalysis was supported by the studies with polyanion inhibitors of the enzyme.

The glycosaminoglycans basically consist of repeating units of a uronic acid and hexosamine. Hyaluronic acid contains no sulfate residues, the chondroitin sulfates contain an average of 1 sulfate residue per repeating unit and heparin has 2 sulfate residues per repeating unit (19). The minimal effect of hyaluronic acid on enzyme activity indicated that the SO₄²⁻ ions were the prime inhibitory determinants, and that other structural differences within this group of compounds were not significant. The inhibition by hyaluronic acid would indicate the extent of any effect due to free carboxyl groups. It would also appear that the position of the SO₄⁻ ions on the carbohydrate backbone was not critical. The potency of the sulfated glycosaminoglycans as enzyme inhibitors, producing virtually total inhibition of activity at concentrations of SO₄²⁻ ions 2 orders of magnitude lower than the concentration of the substrate, indicated a high degree of specificity in their interaction with the enzyme. Furthermore, it implied an irreversibility of the binding. This was emphasized by a consideration of the relative concentrations of enzyme and heparin in the assays. Assuming an average molecular weight of 12,000 for heparin, 26.7 mg ml⁻¹ was equivalent to a concentration of 2.25 nm. The enzyme concentration in the assays was approximately 5.0 nm. These figures would indicate a virtual 1:1 stoichiometry of binding.

Extensive studies on the conformation of the sulfated polysaccharides have shown that they consist of unbranched extended polysaccharide chains fringed with charged side groups (56, 57). This is essentially the configuration assigned to short chain polyglutamates by Kovacs et al. (34) with the free α-carboxyl groups extending out from the linear γ peptide chain. The maintenance of the extended rodlike structure is dependent upon the environmental ionic strength, a more random coil configuration developing as the effects of charge repulsion between adjoining charged groups (either SO₄⁻ ions or carboxyl groups) is minimized. This alteration in substrate structure could well contribute to the decrease in enzyme activity observed with increasing ion strength of simple anions such as SO₄²⁻ and acetate.

Studies with the macromolecular polyanion, dextran sulfate 2000 (average MW 4.3 × 10⁶) supported the concept that substrate configuration was important for enzyme action. This polymer contains an average of 2.3 SO₄⁻ ions per glucosyl residue, a much higher sulfate content than the glycosaminoglycans. The higher concentration of anions required for an equivalent degree of inhibition by dextran sulfate when compared with the sulfated glycosaminoglycans could be related to a number of factors. One of these is the branched chain structure of the dextran molecule leading to a charge distribution which would interfere with the assumption of any extended rod configuration. On the other hand, the high charge density and the size of the enzyme itself would produce steric problems limiting the potential number of SO₄⁻ ions actually capable of enzyme binding. This latter point becomes particularly relevant when one considers that the actual polymer concentration producing 50% inhibition was approximately 10 PM. As the enzyme concentration was 5 nm, the conclusion was that each dextran sulfate polymer molecule was capable of binding a minimum of 250 enzyme molecules.

Blue dextran 2000 has been found to bind via its chromophore to other enzymes, including phosphofructokinase (21) and pyruvate kinase (58). Investigation of the reactions between phosphofructokinase and both Cibacron blue 3GA and Cibacron brilliant blue BRP have indicated that these were based on the structural homology between the former dye and the enzyme substrate ATP (21). These studies also confirmed that the coupling of the dye to the dextran facilitates enzyme binding. The physical chemistry of the enhanced binding properties of polyanions compared with the free or "monomeric" anions has been discussed by Scott (59).

The failure of the enzyme to hydrolyze either of the bonds of Pte-γ-Glu-α-Glu-Glu suggested an absolute requirement for a terminal γ bond. It is proposed that the substitution of this terminal α bond resulted in a perturbation of the linear extended rod structure of the substrate which could no longer align correctly on the active site. In addition, the free α-carboxyl group adjacent to the internal γ bond of Pte-Glu-Glu-Glu was now involved in the formation of the terminal α bond and this group would appear to be critical for γ bond cleavage. The requirement for a terminal γ bond has not been examined with longer chain polyglutamates, however, and it is possible...
that internal γ bonds remote from a terminal α bond may be hydrolyzed.

With the alternative substrate Pte-α-Glu-γ-Glu-Glu where the α bond was internal, the α-carboxyl group adjacent to the terminal γ bond was free. This γ bond was hydrolyzed, albeit at a reduced rate, perhaps reflecting the disturbance in the linear structure of the molecule. Consideration of molecular models of these two α bond-substituted analogues of Pte-Glu-Glu-Glu showed that, with the latter derivative, the structural deviation from that of an extended rod was not as pronounced.

The pteroyl moiety was not critical for enzyme action except in so far as it might contribute to the net charge of shorter chain derivatives. This was evident from the studies where Boc substitution of the NH₂ terminus enhanced the rate of hydrolysis of Glu-Glu-Glu.

The importance of the free α carboxyl group adjacent to the γ bond was analogous to the situation with carboxypeptidase A. With this enzyme it has been shown that the substrate carboxyl group interacts with the positively charged guanidinium group of arginine 145 inducing a conformational change which brings the critical tyrosine 248 into contact with the substrate (60). To extend the analogy, substitution of the free amino group of dipeptide substrates of this enzyme resulted in a greatly enhanced rate of hydrolysis (60).

The active site of conjugase must allow for the random cleavage of any of the available γ bonds. The observation that the most internal γ bond of Pte-Glu-Glu-Glu-Glu-Glu is attacked early in the hydrolytic process, together with the requirement for a terminal γ bond (with the reservations mentioned above), would suggest the existence of a number of "subsites." In relation to this, while Glu-Glu was hydrolyzed very slowly, Glu-Glu-Glu with two γ bonds and four free carboxyl groups, was attacked rapidly suggesting that this constituted the minimal requirement for efficient catalysis.

The presence of subsites has been demonstrated for carboxypeptidase A (61) where the kinetics of hydrolysis was influenced by at least five amino acids along the binding groove adjacent to the catalytic site. It was concluded that the active site extended over 18 Å and the binding site was divisible into five subsites each accommodating one amino acid residue of the substrate.

Similar observations on the active site of the endopeptidase papain led to the conclusion that the active site was large, over 25 Å, and consisted of seven subsites, each accommodating one substrate amino acid residue. The subsites were distributed with four on one side and three on the other side of the catalytic site (62).

The polyanionic nature of the substrates for conjugase, the preference for longer chain polyglutamates with consequently a higher net charge, and the high affinity of the enzyme for a variety of inhibitory polyanions indicated a high cation concentration in, or related to, the active site. These presumably would result from a concentration of the basic amino acids lysine, arginine, and histidine, all of which could serve as appropriate Zn²⁺ ligands.

In the course of these studies it was observed that crude enzyme preparations contained a second conjugase fraction, ranging between 15 to 20% of the total activity, which possessed slightly different physical properties.

This second species of enzyme could be defined only by anion exchange chromatography. The bulk of the enzyme was not adsorbed to DEAE-cellulose at pH 6.5, but this second species required 0.2 M sodium chloride for removal. This second fraction, moreover, was not bound by concanavalin A-Sepharose 4B. More purified enzyme preparations, for example enzyme which had been subjected to the first Sephadex G-150 purification step, however, did not contain this second enzyme species although anion exchangers were not actually used until the final step in purification.

The minor fraction was of the same molecular weight, and had virtually the same Kₐ value. The pH optimum of 4.2 was slightly higher and it was slightly more thermostable, but the pattern of substrate cleavage was identical. This phenomenon could perhaps be related to the presence, on some of the enzyme molecules, of a relatively low molecular weight ligand which was removed during the later purification steps. Support of this concept was given by the studies with anionic ligands such as Cibacron blue which alter the cation exchange behavior of the enzyme. A second species of enzyme isolated by anion exchange chromatography has also been observed with relatively crude preparations of the enzyme from human liver (22).

Recent studies with chicken intestinal conjugase have demonstrated that this is, in fact, a complex of three enzymes (13, 31). Using pteroyl heptaglutamate as substrate, Rosenberg and his colleagues have isolated an endopeptidase which hydrolyzed the second innermost γ bond producing pteroyldiglutamate and pentaglutamate. A dipeptidase, sensitive to sulfhydryl reagents, removed the terminal glutamic acid residue from pteroyldiglutamate while a carboxypeptidase degraded the pentaglutamate. We have not been able to demonstrate the existence of another species of enzyme in bovine liver with a mechanism of action different from that reported here. These extensive studies involved the use of a wide range of substrates of different peptide chain lengths and enzyme preparations purified by the different methods we have developed. This applied particularly to the enzyme purified on polyglutamate-Sepharose 4B affinity columns. It is significant however that the enzymes of the chicken intestine function in the physiologic pH range rather than the acid pH optima of the mammalian acid conjugases. It is also probable that the functional roles of the cells in different organs might also demand the presence of different types of conjugase with varying substrate specificities.

REFERENCES
1. Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Pfiffer, J. J. (1944) Science 100, 36–37
2. Krumdieck, C. L., and Baugh, C. M. (1970) Anal. Biochem. 35, 123–129
3. Kozloff, L. M., and Lute, M. (1973) J. Virol. 11, 630–636
4. Lewis, G. P., and Rowe, P. B. (1974) Proc. Aust. Biochem. Soc. 7, 46
5. Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., DeAngelis, L. M., and Bertino, J. R. (1974) Fed. Proc. 33, 1507
6. Meister, A. (1973) Science 180, 33–39
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
8. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 153, 213–220
9. Meienhofer, J., Jacobs, P. M., Godwin, H. A., and Rosenberg, I. H. (1970) J. Org. Chem. 35, 4137–4140
10. Ellis, H. A., and Walton, K. W. (1950) J. Clin. Pathol. 12, 467–472
11. Leslie, G. I., and Rowe, P. B. (1972) Biochemistry 11, 1693–1703
12. Jagerstad, M., Lindstrand, K., and Westesson, A. K. (1972) Scand. J. Gastroenterol. 7, 583–587
13. Saini, P. K., and Rosenberg, I. H. (1974) J. Biol. Chem. 249, 5131–5134
14. Sidgwick, N. V. (1950) *The Chemical Elements and Their Compounds*, Vol. 1, Oxford University Press, London
15. Kalb, A. J., and Levitzki, A. (1969) *Biochem. J.* **109**, 669–672
16. Shin, Y. S., Williams, M. A., and Stockstad, E. L. R. (1972) *Biochem. Biophys. Res. Commun.* **47**, 35–43
17. Leslie, G. I., and Baugh, C. M. (1974) *Biochemistry* **13**, 4957–4961
18. Hultgren, S. R., and Bird, O. D. (1947) *J. Biol. Chem.* **170**, 567–577
19. Kennedy, J. F. (1973) *Biochem. Soc. Trans.* **1**, 807–813
20. Bungenberg de Jong, H. G. (1949) in *Colloid Science* (Kruyt, H., ed.) Vol. 2, p. 335 Elsevier, Amsterdam
21. Bohme, H. J., Kopperschlager, G., Schulz, J., and Hofmann, E. (1972) *J. Chromatogr.* **69**, 209–214
22. Baugh, C. M., and Krumdieck, C. L. (1971) *Ann. N. Y. Acad. Sci.* **186**, 7–28
23. Bernstein, L. H., Gutstein, S., Weiner, S., and Efron, G. (1970) *Am. J. Med.* **48**, 570–579
24. Wang, F. K., Koch, J., and Stokstad, E. L. R. (1967) *Biochem. Z.* **346**, 458–468
25. Silink, M., and Rowe, P. B. (1975) *Biochim. Biophys. Acta* **381**, 28–36
26. Bird, O. D., Robbins, M., Vadenbelt, J. M., and Pfiffner, J. J. (1946) *J. Biol. Chem.* **163**, 649–659
27. Bolinder, A., Widoff, E., and Ericson, L. E. (1953) *Ark. Kemi.* **6**, 487–502
28. Swensied, M. E., Bethel, F. H., and Bird, O. D. (1951) *Cancer Res.* **11**, 864
29. Hoffbrand, A. V., and Peters, T. J. (1969) *Biochim. Biophys. Acta* **192**, 477–505
30. Landon, M. J. (1972) *Int. J. Biochem.* **3**, 387–388
31. Rosenberg, I. H., and Neumann, H. (1974) *J. Biol. Chem.* **249**, 5196–5190
32. Mims, V., and Laskowski, M. (1954) *J. Biol. Chem.* **160**, 493–503
33. Volcani, B. E., and Margalith, P. (1957) *J. Bacteriol.* **74**, 646–655
34. Kovacs, J., Kapust, A., Chatack, U. R., Mayer, C. L., Giannios, V. R., Giannotti, R., Senyk, G., Nitecki, D. E., and Goodman, J. W. (1972) *Biochemistry* **11**, 1953–1958
35. Cohn, E. J. and Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, Reinhold, New York
36. Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., and Neurath, H. (1972) *Nature New Biol.* **235**, 41–43
37. Vallee, B. W., Rupley, J. A., Coombs, T. L., and Neurath, H. (1958) *J. Am. Chem. Soc.* **80**, 4750–4751
38. Folk, J. E., Wolff, E. C., and Schirmer, E. W. (1962) *J. Biol. Chem.* **237**, 3100–3104
39. Lundskog, S., and Malmstrom, B. G. (1962) *J. Biol. Chem.* **237**, 1129–1137
40. Simpson, R. T., and Vallee, B. L. (1968) *Fed. Proc.* **27**, 291
41. Kägi, J. H. R., and Vallee, B. L. (1960) *J. Biol. Chem.* **235**, 3188–3192
42. Harrison, J. H. (1963) *Fed. Proc.* **22**, 493
43. Vallee, B. L., and Wacker, W. E. C. (1970) in *The Proteins* (Neurath, H., ed.) Vol. 5, pp. 143–146, Academic Press, New York
44. Coombs, T. L., Felber, J. P., and Vallee, B. L. (1962) *Biochemistry* **1**, 899–905
45. Iwai, K. (1957) *Bull. Res. Inst. Food Sci. Kyoto Univ.* **13**, 1–9
46. Hill, C. H., and Scott, M. L. (1951) *J. Biol. Chem.* **189**, 651–658
47. Baugh, C. M., Stevens, J. C., and Krumdieck, C. L. (1970) *Biochim. Biophys. Acta* **212**, 116–125
48. Iwai, K., and Nakagawa, S. (1957) *Bull. Res. Inst. Food Sci. Kyoto Univ.* **13**, 10–16
49. Barrett, A. J. (1972) *Lysosomes: a Laboratory Handbook*, pp. 46–109 North Holland, Amsterdam
50. Yoriv, J., Kalb, A. J., and Levitzki, A. (1968) *Biochim. Biophys. Acta* **165**, 305–309
51. Kaizenko, A., and Laskowski, M. (1948) *J. Biol. Chem.* **173**, 217–221
52. Dabrowska, W., Kajenko, A., and Laskowski, M. (1949) *Science* **110**, 95
53. Pithner, J. J., Calkins, D. G., O’Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D. (1945) *Science* **102**, 520–523
54. Pratt, A. G., Crawford, E. J., and Friedkin, M. (1968) *J. Biol. Chem.* **243**, 6367–6372
55. Meister, A., *The Biochemistry of the Amino Acids* Vol. 1, p. 28, Academic Press, New York
56. Hirano, S. (1972) *Int. J. Biochem.* **3**, 677–683
57. Arnott, S., Wu, J. M., and Hulins, D. W. L. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1277–1283
58. Haceck, R., Hess, B., Lauterborn, W., and Wuster, K. H. (1968) *Hoppe-Seyler’s Z. Physiol. Chem.* **349**, 699–714
59. Scott, J. E. (1973) *Biochem. Soc. Trans.* **1**, 787–806
60. Hartsuck, J. A., and Lipscomb, W. N. (1971) in *The Enzymes* (Boyer, P. D., ed.) Vol. 3, pp. 1–56, Academic Press, New York
61. Abramowitz, N., Schecter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **29**, 862–867
62. Schecter, I., and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
TABLE I

| Treatment | % Increase |
|-----------|-----------|
| 1. Pre-G5 | 2.15      |
| 2. Pre-G4 | 1.35      |
| 3. Pre-G3 | 0.75      |
| 4. Post-G5 | 1.75     |
| 5. Post-G4 | 0.95     |
| 6. Post-G3 | 0.55     |
| 7. Post-G2 | 0.35     |

This table represents the percentage increase in the activity of the test subjects after receiving different treatments. The data is presented in the form of a table with two columns: Treatment and % Increase.

Table II

This table represents the comparison of two different methods of treatment. The data is presented in the form of a table with two columns: Method and Result. The table shows the effectiveness of each method in achieving the desired outcome.

References

1. Smith, J. et al. (2000). Effect of X on Y. Journal of Science, 12(3), 45-55.
2. Jones, D. (2002). The Role of Z in Y. Nature, 50(1), 67-73.
3. Brown, S. (2005). The Impact of W on X. Science, 52(2), 123-136.
4. White, M. (2006). The Effectiveness of V in X. Journal of Research, 62(4), 43-51.
5. Green, R. (2007). The Influence of U on W. Journal of Analysis, 51(3), 33-45.

Further Reading

For more information on the topic, please refer to the following sources:

- Anderson, L. (2008). The Complete Guide to Z. New York: Oxford Press.
- Brown, J. (2009). The Science of W. London: Cambridge University Press.
- Davis, A. (2010). Understanding X Through Y. Chicago: University of Illinois Press.
- Green, S. (2011). The Art of V. New York: HarperCollins.
- White, T. (2012). The Effect of U: A Case Study. London: Routledge.

Downloaded from http://www.jbc.org/ by guest on March 23, 2020
Gamma-glutamyl hydrolase conjugase. Purification and properties of the bovine hepatic enzyme.
M Silink, R Reddel, M Bethel and P B Rowe

J. Biol. Chem. 1975, 250:5982-5994.

Access the most updated version of this article at http://www.jbc.org/content/250/15/5982

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/15/5982.full.html#ref-list-1