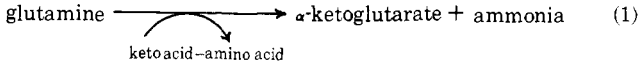
Peterson, D. L. (1970a), *J. Biol. Chem. 245*, 799.
Martinez-Carrion, M., and Tiemeier, D. (1967), *Biochemistry 6*, 1715.
Martinez-Carrion, M., Tiemeier, D. C., and Peterson, D. L. (1970b), *Biochemistry 9*, 2574.
Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., and Fasella, P. (1967), *J. Biol. Chem. 242*, 2397.
Matsuzawa, T., and Segal, H. L. (1968), *J. Biol. Chem. 243*, 5929.
Michuda, C. M., and Martinez-Carrion, M. (1969), *Biochemistry 8*, 1095.
Michuda, C., and Martinez-Carrion, M. (1970), *J. Biol. Chem. 245*, 262.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Morino, H., and Watanabe, T. (1969), *Biochemistry 8*, 3412.
Peterson, D. L., and Martinez-Carrion, M. (1970), *J. Biol. Chem. 245*, 806.
Polyanovsky, P. L. (1962), *Biochimia 27*, 734.
Torchinsky, Yu. M. (1964), *Biochimia 29*, 458.
Torchinsky, Yu. M., and Sinitsina, N. I. (1970), *Mol. Biol. USSR 4*, 458.
Turano, C., Giartosio, A., and Fasella, P. (1964), *Arch. Biochem. Biophys 104*, 524.
Turano, C., Giartosio, A., and Riva, F. (1963), *Enzymologia 25*, 196.
Vogle, R. H., and Kapac, M. J. (1960), *Biochim. Biophys. Acta 37*, 539.
Voratnitskaya, N. E., Lutovinova, G. F., and Polyanovsky, O. L. (1968), *in* Pyridoxal Catalysis: Enzyme and Model Systems, Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinsky, Y. M., Ed., New York, N. Y., Interscience Publishers, p 131.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 290.

Rat Liver ω-Amidase. Purification and Properties*

Louis B. Hersh

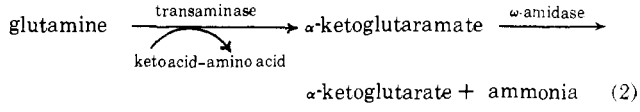
ABSTRACT: The ω-amidase from rat liver has been purified to homogeneity. The native enzyme, mol wt 58,000, can be dissociated into subunits of mol wt 27,000–28,000 by treatment with 7 M guanidine hydrochloride or 7 M urea. A study of the substrate specificity of the enzyme revealed that in addition to hydrolyzing α-ketoglutaramate, glutaramate, and succinamate, the enzyme also hydrolyzes the monomethyl and ethyl esters of α-ketoglutarate (δ esters), succinate, and glutarate. In addition to the hydrolytic reactions the enzyme catalyzes hydroxaminolysis and transamidation with esters and amides. Studies on the nature of the reaction of α-ketoglutaramate as a function of pH revealed that below pH 8, the rate-limiting step of the ω-amidase reaction could be the nonenzymatic conversion of 5-hydroxypyroglutamate into α-ketoglutaramate. This latter finding is discussed in terms of its possible physiological significance.

One of the pathways for the metabolism of glutamine in liver involves the transamination–deamidation of this compound to yield α-ketoglutarate and ammonia (reaction 1).



(1)

Although this system was initially described as a keto acid activated glutaminase (Greenstein and Price, 1949; Errera, 1949), subsequent studies by Meister and coworkers (Meister and Tice, 1950; Meister *et al.*, 1953, 1955; Meister, 1953, 1954) demonstrated that the reaction involves the transamination–deamidation of glutamine rather than a single hydrolytic reaction. Based on the observation that liver preparations would transaminate γ-methylglutamine, but would not deamidate *N*-methyl-α-ketoglutaramate, Meister (1954) proposed the involvement of two enzymes, a transaminase and an ω-amidase, in the overall conversion of glutamine into α-ketoglutarate and ammonia (reaction 2).



(2)

This proposal was supported by the observation that extracts of a mutant of *Neurospora crassa* catalyze the transamination of glutamine, but not the subsequent deamidation of α-ketoglutaramate (Monder and Meister, 1958).

Relatively little work has been done on the characterization of the two enzymes involved in the transamination–deamidation of glutamine. The glutamine transaminase has been purified by Yoshida (1967), and more recently by A. Cooper and A. Meister (in preparation).

The ω-amidase from rat liver has previously been purified 40-fold by Meister (1955). The enzyme was shown to deamidate α-ketosuccinamate, glutaramate, succinaminate, and α-ketoglutaramate. The present paper reports the preparation of rat liver ω-amidase in homogeneous form and a study of its physical and chemical properties.

Materials and Methods

Substrates. α-Ketoglutaramate, α-ketosuccinamate, δ-methyl α-ketoglutarate, and δ-ethyl α-ketoglutarate were pre-

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pared by oxidation of the corresponding amino acid with L-amino acid oxidase (Meister, 1953, 1954). Monoethyl succinate and monoethyl glutarate were prepared according to the procedure of Cohen and Crossley (1963, 1964). Monopropyl glutarate and monobutyl glutarate were prepared by the method of Friedlin *et al.* (1963). Succinanilic acid and glutaranilic acid were prepared as described by Fieser (1941). The latter compound was recrystallized from 20% ethanol. Glutaramic acid was prepared as previously described (Hersh, 1970). *N*-Methylsuccinamic acid was prepared from methylamine and succinic anhydride, according to the procedure for glutaramic acid synthesis. The free acid was crystallized from acetone: yield 65%, mp 114–115°. *Anal.* Calcd: C, 45.57; H, 6.68; N, 10.69; O, 37.69. Found: C, 45.80; H, 6.87; N, 10.69; O, 36.64. *N*-Ethylsuccinamate was prepared in a similar fashion, but was crystallized from water, mp 94–97° (lit. mp 95–96°) (Marrian, 1949).

Succinyl hydroxamate was prepared according to the procedure of Bernheim (1964). [^{14}C]Methylamine was obtained from New England Nuclear Corp. and was purified as previously described (Hersh, 1970).

Amines were redistilled or recrystallized before use, and all other compounds were used as obtained commercially.

Enzyme Assays. The standard assay used during enzyme purification is the determination of α-ketoglutaramate hydrolysis as measured with glutamate dehydrogenase (Hersh, 1970). Reaction mixtures contained 5 mM α-ketoglutaramate (barium or sodium salt), 50 mM Tris-HCl buffer (pH 8.5), 10 mM ammonium chloride, 0.128 μM DPNH, 50 μg of glutamate dehydrogenase, and enzyme in a final volume of 1.0 ml. The reaction is initiated by the addition of enzyme, and the decrease in DPNH absorbance at 340 nm is measured on a Gilford recording spectrophotometer at 30°. Enzyme dilutions are made in 20 mM Tris·HCl buffer (pH 8.5) containing 1 mM dithiothreitol. The enzyme is preincubated for 10 min in this buffer before being assayed.

The ability of the enzyme to hydrolyze various amides was initially screened by measuring ammonia release with Nessler's reagent (Su *et al.*, 1969). More precise kinetic measurements of amide hydrolysis were made at 30° using the continuous spectrophotometric method of Hersh and Srere (1970).

Ester hydrolysis was measured titrimetrically using a Sargent-Welch Model pHR recording titrimeter. These measurements were made at room temperature using 10 mM NaOH as the titrant. The hydrolysis of methyl and ethyl α-ketoglutarate were also measured by the glutamate dehydrogenase coupled assay. Hydroxamate formation was measured by the method of Lipmann and Tuttle (1945).

The hydrolysis of succinyl hydroxamate was followed by measuring the liberation of hydroxylamine (Csaky, 1948). Reaction mixtures containing 50 mM Tris-HCl buffer (pH 8.5), 1 mM dithiothreitol, potassium succinyl hydroxamate, and enzyme in a final volume of 0.5 ml were incubated for 30 min at 30°. The reaction was terminated by the addition of the reagents used for color development. Controls in which enzyme was deleted were used for each concentration of succinyl hydroxamate tested. Under the above assay conditions no significant oxidation of hydroxylamine was observed (Bernheim, 1964), nor did succinyl hydroxamate interfere with the determination of free hydroxylamine.

The ability of the enzyme to catalyze *N*-methylamide formation from esters and amides was determined by measuring the incorporation of [^{14}C]methylamine into the appropriate substrate (Hersh, 1970). Reaction mixtures containing [^{14}C]-methylamine, substrate, buffer, and enzyme were incubated at 30°. After termination of the reaction by the addition of 20% trichloroacetic acid to a final concentration of 3.3%, the reaction mixture was chromatographed on a small Dowex 50(H^+) column. The *N*-methylamides are washed from the column with water, whereas free methylamine is retained on the column.

Other Methods. Sedimentation equilibrium measurements were performed in a Spinco Model E ultracentrifuge by the high-speed method of Yphantis (1964). Protein concentrations of 200, 400, and 600 μg per ml were used. Fringe displacement was measured with a Nikon profile projector. Tryptic digestion and peptide mapping were performed according to the procedure of Chernoff and Lui (1961) as described by Cottam *et al.* (1969). After tryptic digestion of the enzyme for 3 hr a small amount of undigested protein remained. This "core" protein was subjected to a second 3 hr digestion by trypsin after which time no core protein remained. The peptide map of this core protein appeared to be the same as the original digest.

Amino acid analysis was performed in triplicate samples hydrolyzed in constant-boiling 6 N HCl for 24, 48, and 72 hr. Total cysteine plus cystine was determined after performic acid oxidation of the protein (Moore, 1963). Tryptophan was determined spectrophotometrically (Bencze and Schmid, 1957).

Analytical disc gel electrophoresis was performed according to the procedure of Davies (1964).

Results

Purification of ω-Amidase. The starting material for the preparation of the ω-amidase is the supernatant solution from rat liver microsomal preparations (Schenkman *et al.*, 1967) of phenobarbital-induced rats. Over 90% of the original activity in the crude homogenate is recovered in the supernatant with a three- to fourfold increase in specific activity. No significant changes in the amount or specific activity of the enzyme was observed in phenobarbital-treated rats, as compared to normal rats.

All steps are performed at 0–4°, unless otherwise noted.

To the supernatant solution is added 1 M Tris-HCl buffer (pH 8.5), to a final concentration of 50 mM, and mercaptoethanol and EDTA to 5 and 0.1 mM, respectively. Solid ammonium sulfate is added to approximately 50% saturation (287 g/l.). After stirring for 15 min, the solution is centrifuged and the precipitate discarded. The supernatant solution is adjusted to 70% ammonium sulfate by the addition of 126 g of ammonium sulfate/l. of supernatant. The precipitate is dissolved in 10 mM potassium phosphate buffer (pH 7.8), containing 20 mM mercaptoethanol and 0.1 mM EDTA, and dialyzed overnight against 18 l. of this buffer. The dialyzed enzyme is then placed on a DEAE-cellulose column (5 × 75 cm), previously equilibrated with the buffer described above. The enzyme eluted from the column with this same buffer is concentrated by ultrafiltration using an Amicon ultrafiltration apparatus with UM20E membrane.

The concentrated enzyme is dialyzed against 12 l. of 0.01 M sodium phosphate buffer (pH 6.5), containing 20 mM mercaptoethanol and 0.1 mM EDTA, and placed on a column of CM-Sephadex G-50 (4 × 45 cm), previously equilibrated with this same buffer. The enzyme eluted from the column with the starting buffer is concentrated as described above and then chromatographed on coupled Sephadex G-75 columns [downward flow in the first column (5.5 × 35 cm) and reverse flow in the second column (2.5 × 87 cm)], previously equilibrated

TABLE I: Purification of ω-Amidase.

Step	Vol (ml)	Total Protein (mg)	Total Units (μmoles/min)	Sp Act. (μmoles/min per mg)	% Recov
1. Rat liver supernatant	3500	50,000	4200	0.08	100
2. 50–70% ammonium sulfate	176	9,500	2500	0.26	60
3. DEAE	24.5	1,450	1800	1.24	43
4. CM-Sephadex	11.4	114	890	7.8	21
5. Sephadex G-75	9.4	69.5	734	10.6	17
6. Hydroxylapatite	4.0	34.8	408	11.7	10

with 50 mM potassium phosphate buffer (pH 7.4), containing 20 mM mercaptoethanol and 0.1 mM EDTA. The enzyme eluted from the column with this buffer is concentrated as previously described.

The concentrated enzyme is diluted one-to-five with cold distilled water, then washed hydroxylapatite equivalent to 50 times the amount of protein is added to the solution. The solution is intermittently stirred with a glass rod for 10 min and centrifuged and the supernatant discarded. Potassium phosphate (10 ml of 0.01 M, pH 7.4) is added to the gel and after 10-min stirring at 0° the gel is centrifuged and the supernatant discarded. This procedure is repeated two times with 0.02 M potassium phosphate (pH 7.4) and finally with 0.05 M potassium phosphate (pH 7.4). The enzyme is gradually washed from the gel with the latter buffer. The fractions containing enzyme are scanned for purity by disc gel electrophoresis. Those fractions showing a single protein band are pooled and concentrated. Over 75% of the enzymatic activity can be recovered from hydroxylapatite; however, generally only 25–50% of the enzyme is obtained in pure form. A typical purification is shown in Table I.

General Properties of the Enzyme. Disc gel electrophoresis at pH 8.5 in 4 and 7.5% polyacrylamide gels, followed by staining with Amido-Schwarz dye, reveals only one protein band. This band, when eluted from a parallel, nonstained gel, exhibits enzymatic activity.

The purified enzyme loses no activity when heated for 30 min at 50° in 0.02 M Tris-HCl buffer (pH 8.5). At 60°, the enzyme is rapidly denatured losing 70% of its activity in 5 min. No stabilization of the enzyme toward heat inactivation at 60° is observed in the presence of 10 mM α-ketoglutaramate.

Incubation of the enzyme over the pH range of 6–10 for 20 min at 30° results in no irreversible inactivation of the enzyme. At pH 5, the enzyme loses 32% activity in 20 min, while at pH 4, 90% of the activity is lost within 20 min.

The enzymatic hydrolysis of α-ketoglutaramate is not affected by dialysis of the enzyme against EDTA, or by the addition of 0.05 M NaCl, KCl, $MgCl_2$, $CaCl_2$, or $BaCl_2$ to the assay system. The ability of various amines and alcohols to inhibit the hydrolysis of α-ketoglutaramate was tested. At 0.05 M, only ammonia and methylamine were effective: 72 and 62% inhibition, respectively. Ineffective at 0.05 M were the alcohols: methanol, ethanol, and propanol; and the amines: ethylamine, propylamine, butylamine, dimethylamine, diethylamine, and triethylamine.

Kelly and Kornberg (1962) have found an inducible amidase from *Pseudomonas aeroginosa* which catalyzes hydrolysis and hydroxaminolysis of amides. The hydrolytic activity can be inhibited by low concentrations of urea (10^{-4}–10^{-2} M) with no effect on hydroxaminolysis. In the case of the ω-amidase, no effect on either the hydrolysis or hydroxaminolysis of succinamate or glutaramate was observed at 5×10^{-2} M urea.

Physical Properties. AMINO ACID COMPOSITION. The amino acid composition of the ω-amidase is given in Table II. The data were obtained according to the procedure of Moore *et al.* (1958). The partial specific volume of the enzyme is 0.735 ml/g, as calculated from the amino acid composition (Schachman, 1957).

TABLE II: Amino Acid Composition of Rat Liver ω-Amidase.

Amino Acid Residue	μg/mg of Protein	moles/58,000 g
Lysine	76.8	36
Histidine	32.7	14
Arginine	55.4	20
Aspartic acid	100.0	51
Threonine	42.6	24
Serine	61.3	41
Glutamic acid	124.7	56
Proline	53.9	32
Glycine	51.0	52
Alanine	39.9	33
Half-cystine	22.4	12
Valine	57.4	34
Methionine	20.6	9
Isoleucine	54.7	28
Leucine	98.0	50
Tyrosine	34.6	12
Phenylalanine	46.7	18
Tryptophan	26.5	8

MOLECULAR WEIGHT. The molecular weight of the native enzyme was determined by high-speed sedimentation equilibrium. Semilogarithmic plots of fringe displacement *vs.* [radial distance]^2 yielded straight lines indicating homogeneity of the enzyme. Based on the partial specific volume of 0.735 ml/g the molecular weight of the enzyme is 58,000.

The sedimentation coefficient of the enzyme was determined by sedimentation velocity in 0.02 M Tris-HCl buffer at pH 8.0. A single boundary was observed using absorption optics from which an $s_{20,w}$ of 4.7 was calculated at a protein concentration of 800 μg/ml.

In order to determine whether or not the enzyme is composed of subunits, high-speed sedimentation equilibrium runs were made in the presence of 7 M guanidine hydrochloride or 7 M urea containing 0.1 M 2-mercaptoethanol. Under these denaturing conditions the protein appears homogeneous as

TABLE III: Kinetic Constants for Reactions Catalyzed by ω-Amidase at pH 8.

	K_m (mM)	V_{max} (μmoles/min per mg)
A. Hydrolysis		
α-Ketoglutaramate	3.3	16.0
γ-Methyl α-ketoglutarate	0.03	23.0
γ-Ethyl α-ketoglutarate	0.06	21.0
Glutaramate	3.1	4.3
Methyl glutarate	1.8	3.7
Ethyl glutarate	9.9	1.5
Propyl glutarate	6.8	0.08
Succinamate	0 2	1.6
Methyl succinate	0.7	0.7
Ethyl succinate	8.3	0.3
Succinyl hydroxamate	0.8	0.04

	K_m NH_2OH (mM)	K_m Substrate (mM)	V_{max} (μmoles/min per mg)
B. Hydroxaminolysis			
Glutaramate	4.0	25	8.7
Methyl glutarate	3.6	52	7.6
Ethyl glutarate	Not determined	31[a]	1.2[a]
Propyl glutarate	Not determined	18[a]	0.2[a]
Succinamate	5.4	17	10.0
Methyl succinate	Not determined	8[a]	2.1[a]
Ethyl succinate	Not determined	17[a]	0.4[a]

[a] Determined in the presence of 20 mM hydroxylamine.

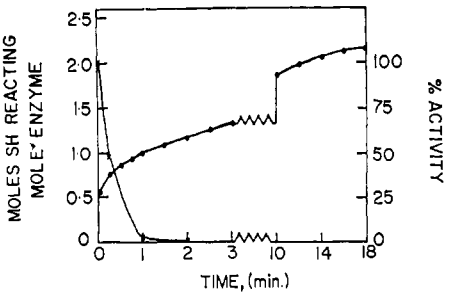


FIGURE 1: Titration of enzyme SH groups with Ellman's reagent. A 1-ml solution of enzyme in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA was incubated with 1 mM dithiothreitol for 1 hr at 0°. The enzyme was dialyzed overnight against 6 l. of the phosphate buffer to remove the dithiothreitol, and when assayed in the presence or absence of 1 mM dithiothreitol was found to contain full activity. An aliquot containing 490 μg of enzyme (0.1 ml) was diluted to 1 ml with the phosphate buffer and was reacted with 20 μl of 10 mM dithionitrobenzene. The number of sulfhydryl groups reacting per mole of enzyme was calculated on the basis of a molecular weight of 58,000 for the enzyme and a molar extinction coefficient of 14,000 for the dithionitrobenzene reaction product. Protein was determined by the microbiuret method (Zamenhof, 1957). To determine the rate of inactivation of the enzyme by dithionitrobenzene, an identical reaction mixture was prepared, from which 2-μl aliquots were withdrawn and assayed for enzyme activity at the time periods indicated.

judged by semilogarithmic plots of fringe displacement *vs.* [radial distance]^2. Utilizing the values for the density of urea and guanidine solutions, calculated by the method of Kawahara and Tanford (1966), the molecular weight is 27,000. These results suggest that the enzyme is composed of two subunits.

PEPTIDE MAP. The peptide map of trypsin-digested enzyme shows about 43 ninhydrin-positive spots. Based on the amino acid analysis the enzyme contains 55 arginine plus lysine residues per 58,000 molecular weight. If the enzyme was composed of identical subunits, one would expect to find 28 tryptic peptides, and if the subunits were nonidentical, 57 peptides would be expected. The finding of 43 peptides from the tryptic digest yields equivocal results, and no distinction between identical or nonidentical subunits can be made at the present time.

Sulfhydryl Reactivity. Storage of the enzyme at 3° results in a slow loss of enzymatic activity. During the first 2-weeks storage, the activity of these denatured enzyme preparations can be restored to over 90% of their original activity by incubation with 1 mM dithiothreitol for 5–10 min at room temperature. However, after approximately 2 weeks the enzyme becomes irreversibly denatured.

The above results suggest that the reversible denaturation of the enzyme is due to the oxidation of one or more sulfhydryl groups on the enzyme. To support this hypothesis, a number of sulfhydryl reagents were tested for their ability to inactivate the enzyme. Utilizing 0.12 μM enzyme (85% original activity) complete inactivation toward the hydrolysis of α-ketoglutaramate was observed within 30 sec upon incubation at room temperature with 20 μM *p*-mercuribenzoate or 20 μM Ellman's reagent. Complete inactivation also occurred within 10 min by incubation with 0.2 mM *N*-ethylmaleimide, and within 20 min by incubation with 0.2 mM iodoacetate or 0.2 mM iodoacetamide.

In order to determine the number of sulfhydryl groups required for enzymatic activity, the enzyme was reacted with Ellman's reagent and the number of sulfhydryl groups reacting was correlated with the loss in enzymatic activity. As shown in Figure 1, 1 sulfhydryl group/58,000 molecular weight rapidly reacts with Ellman's reagent, followed by a slower reaction of a second sulfhydryl group. Measurement of enzymatic activity in a parallel reaction mixture shows that the reaction of this first sulfhydryl group leads to complete loss of enzymatic activity. Reaction of the enzyme with Ellman's reagent in the presence of 6.7 M guanidine hydrochloride results in the reaction of 4 sulfhydryl groups/58,000 molecular weight.

Reactions Catalyzed by ω-Amidase. HYDROLYSIS OF AMIDES AND ESTERS. Meister has previously demonstrated that the partially purified enzyme hydrolyzes α-ketoglutaramate, α-ketosuccinamate, glutaramate, succinamate, and succinyl hydroxamate. These findings have been confirmed with the purified enzyme. The kinetic constants for the hydrolysis of these substrates (with the exception of α-ketosuccinamate) at pH.8 are shown in Table III. (In our hands α-ketosuccinamate is unstable and no quantitative studies were attempted with it.) The enzyme was found to have a rather broad specificity in that it also hydrolyzed the monomethyl and monoethyl esters of α-ketoglutarate (δ-ester), glutarate, and succinate (Table III), as well as the monopropyl ester of glutarate. The purified enzyme does not hydrolyze glutamine, asparagine, *N*-methyl-α-ketoglutaramate, dimethyl glutarate, or the anilides of succinate or glutarate (all at 20 mM). Of particular interest is the observation that the enzyme does

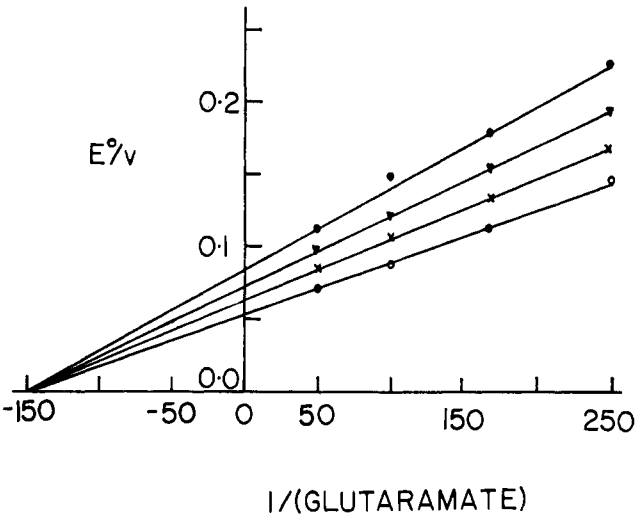


FIGURE 2: The effect of hydroxylamine on the K_m of glutaramate. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 8), 1 mM dithiothreitol, 20 μg of enzyme, glutaramate, and hydroxylamine at 20 (○), 8 (×), 6 (▽), and 4 mM (●), in a final volume of 0.5 ml. After incubation for 30 min at 30°, 1 ml of 15% ferric chloride in 1 N HCl was added, and the optical density at 540 nm was measured. E/V expressed as 1/μmoles of hydroxamate formed per min per mg of enzyme.

TABLE IV: Transamidation Reactions.[a]

Substrate	v (μmoles/min per mg)
α-Ketoglutaramate	0.40
γ-Methyl α-ketoglutarate	0.05
γ-Ethyl α-ketoglutarate	0.12
Glutaramate	1.11
Methyl glutarate	1.59
Ethyl glutarate	1.00
Succinamate	2.61
Methyl succinate	0.32
Ethyl succinate	0.36

[a] Reaction mixtures containing: 50 mM Tris-HCl buffer (pH 8.5), 1 mM dithiothreitol, 20 mM substrate (except γ-ethyl α-ketoglutarate, and γ-methyl α-ketoglutarate which were 2 mM), 60 mM [^{14}C]methylamine (sp act. 6.8 × 10^3 cpm/μmole), and 17.4 μg of enzyme, all in a final volume of 0.5 ml. After incubation for 1 hr at 30°, the reactions were terminated by the addition of 0.1 ml of 20% trichloroacetic acid. The reaction mixture (0.4 ml) was chromatographed on a Dowex 50 (H^+) column and the radioactive product was eluted with 3–4 ml of H_2O.

not hydrolyze *N*-methylsuccinamate or *N*-ethylsuccinamate, whereas methyl succinate and ethyl succinate are substrates for the enzymes.

HYDROXAMINOLYSIS OF ESTERS AND AMIDES. The enzyme has been shown to catalyze hydroxamate formation with glutaramate and succinamate as substrates (Meister *et al.*, 1955). Figure 2 shows a typical kinetic experiment in which the effect of hydroxylamine on the K_m of glutaramate was determined. The same kinetic pattern of intersecting lines was obtained for all combinations of all substrates. The maximal velocities and K_m's obtained from replots of the reciprocal of the rate *vs.* the reciprocal of the concentration of the fixed variable substrate at infinite concentrations of the variable substrate are shown in Table III. The ω-amidase also catalyzes hydroxamate formation from the monomethyl, ethyl, propyl, and butyl esters of glutarate (due to solubility problems accurate determinations of hydroxamate formation from butyl glutarate could not be determined), and the monomethyl and ethyl esters of succinate (Table III).

Under conditions in which hydroxamate formation could be readily detected, no reaction was observed between hydroxylamine and the following compounds: δ-methyl glutamate, δ-ethyl glutamate, malonate, glutamate, glutarate, aspartate, succinate, glutamine, asparagine, succinanilate, glutaranilate, α-ketoglutaramate *N*-methylsuccinamate, and *N*-ethylsuccinamate. (Meister *et al.* (1955) have reported hydroxamate formation with succinate and glutarate, pH optima about 5. The present studies were conducted at pH 8.)

TRANSFER REACTIONS WITH AMIDES AND ESTERS. The ability of the ω-amidase to catalyze amide formation was tested by measuring the incorporation of [^{14}C]methylamine into several of the amides and esters previously shown to be substrates for the hydrolytic reaction. As shown in Table IV, the enzyme catalyzes incorporation of [^{14}C]methylamine into the amide and monomethyl and monoethyl esters of α-ketoglutarate, succinate, and glutarate.

In order to provide evidence that both amidase activity and esterase activity are catalyzed by the same enzyme, we were able to demonstrate that the hydrolysis of both ethyl α-ketoglutarate and methyl α-ketoglutarate are inhibited by the substrates, glutaramate and succinamate, but not by glutamine, or asparagine. In a typical experiment utilizing 0.1 mM ester, 10 mM glutaramate inhibited the hydrolysis of the methyl ester 43%, and the ethyl ester 34%, whereas 5 mM succinamate inhibited the hydrolysis of the methyl and ethyl esters 57 and 42%, respectively. As expected, succinamate inhibition of ethyl α-ketoglutarate hydrolysis was competitive.

Further evidence that both esters and amides are substrates for the same enzyme was obtained by measuring the rate of inactivation of the enzyme by iodoacetamide as assayed by either the hydrolysis of α-ketoglutaramate or the hydroxaminolysis of methyl succinate. As shown in Figure 3, the

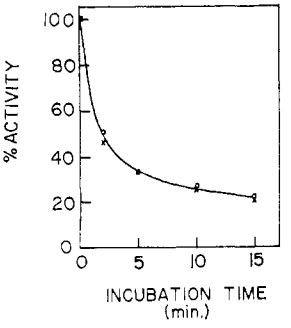
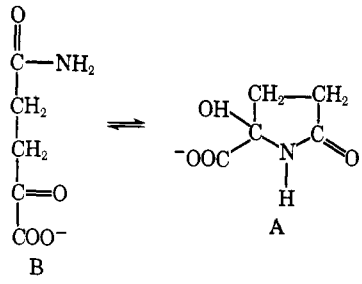


FIGURE 3: Comparison of iodoacetamide inactivation as measured by hydrolysis of α-ketoglutaramate, or hydroxaminolysis of methyl glutarate. Enzyme (120 μg) was incubated with 40 mμmoles of iodoacetamide in 20 mM Tris-HCl buffer (pH 8.0), at 30° in a final volume of 0.2 ml. At the time periods indicated, aliquots were withdrawn and the enzymatic activity, as assayed by the hydrolysis of α-ketoglutaramate (○) or the hydroxaminolysis of methyl glutarate (×), was measured.

rate of inactivation of the enzyme by iodoacetamide is identical when either of the two assays is used.

INABILITY OF α-KETOGLUTARATE ANALOGS TO FORM HYDROXAMATES. Since the amidase catalyzes hydroxamate formation from the esters and amides of both succinate and glutarate, it appeared surprising that no hydroxamate formation with the esters and amide of α-ketoglutarate was observed. The lack of reactivity of these α-keto acids appears to be due to the nonenzymatic reaction of hydroxylamine with the keto group to give the carbinolamine or oxime, neither of which are substrates for the enzyme. To support this hypothesis, we were able to demonstrate that mixing of 50 mM hydroxylamine with 5 mM α-ketoglutaramate in 0.02 M Tris-HCl at pH 8.5 results in the rapid disappearance of the carbonyl adsorption peak at 320 nm.

REACTIVE FORM OF α-KETOGLUTARAMATE. Meister has previously shown that α-ketoglutaramate exists in two forms, an A form which does not possess an active keto group, and a B form which does have an active keto group. That these forms are, in fact, α-ketoglutaramate and its cyclic isomer 5-hydroxypyroglutamate was proposed by Otani and Meister (1957) and supported by the finding that *N*-methyl-α-ketoglutaramate exists almost exclusively as 5-hydroxy-*N*-methylpyroglutamate (Otani and Meister, 1957; Hersh *et al.*, 1969).



B A

Based on the observation that succinamate, glutaramate, and the esters of α-ketoglutarate, succinate, and glutarate serve as substrates for the enzyme, the proposal by Meister that α-ketoglutaramate, rather than its cyclic isomer, is the true substrate for the ω-amidase seems reasonable (Meister, 1953). To confirm this hypothesis, and to learn about the rates of interconversion of the two forms, we studied the enzymatic hydrolysis of α-ketoglutaramate over the pH range of 6.5–8.0. As shown in Figure 4, addition of excess amidase to a reaction mixture containing an equilibrium mixture of α-ketoglutaramate and 5-hydroxypyroglutamate results in a biphasic curve below pH 8, in which the slower secondary rate decreases markedly with decreasing pH.

Since it is known that the equilibrium between α-ketoglutaramate and 5-hydroxypyroglutamate favors the latter compound, we interpret these biphasic curves in the following manner. The rapid rate reflects the hydrolysis of the α-ketoglutaramate which is present at equilibrium, while the slower secondary rate reflects a rate-determining conversion of 5-hydroxypyroglutamate into α-ketoglutaramate. To support this hypothesis, we have shown that at a given pH and a fixed level of α-ketoglutaramate, the secondary rate is invariable over a tenfold range of enzyme concentrations (Table V). (The initial rapid rate appears to show a dependence on protein concentration; however, it is too fast to make accurate measurements.) Also shown in Table V is that at a given pH, variations of α-ketoglutaramate over a fivefold concentration range yield identical values of the rate constant for conversion of 5-hydroxypyroglutamate into α-ketoglutaramate:

TABLE V: Rate Studies.

	Secondary Rate (mμmoles/min)	
μg of Enzyme	Tris-HCl (pH 7.3)	Potassium Phosphate (pH 6.8)
A. Independence of Secondary Rate on Enzyme Concentration		
5	4.0	2.2
10	4.1	2.1
20	4.0	2.1
50	4.3	2.1
	k_{obsd} ($min^{-1} \times 10^3$)	
[Substrate] (M)	Tris-HCl (pH 7.3)	Potassium Phosphate (pH 6.8)
B. Independence of Apparent Rate Constant for Ring Opening on Initial Substrate Concentration		
0.002	1.6	0.7
0.005	1.4	0.7
0.010	1.7	0.7

$k_{obsd\ (min^{-1})}$ = initial rate in molar per min/[5-hydroxypyroglutamate].

Figure 5 shows a plot of k_{obsd} *vs.* hydroxide ion concentration over the pH range of 6–8. These data were obtained by measuring the initial rate of the reaction after the initial burst had ceased. Rate constants were generally calculated using at least two different substrate concentrations and, at least, four buffer concentrations. The data, which are summarized in Table VI, fit the equation: $v = 7.1 \times 10^8\ M^{-1}\ min^{-1}$[5-hydroxypyroglutamate][OH^-]. Varying the buffer concentration from 0.02 to 0.2 M had little or no effect on the rate of the reaction, indicating the absence of general base catalysis. Also listed in Table VI are the equilibrium constants estimated from the initial phase of reaction. These equilibrium constants do not

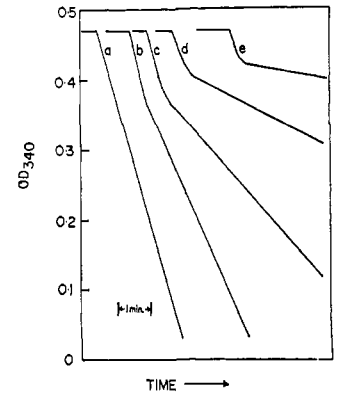


FIGURE 4: Enzymatic hydrolysis of α-ketoglutaramate as a function of pH. The ω-amidase-catalyzed hydrolysis of 5 mM α-ketoglutaramate in 50 mM Tris-HCl buffer at pH 8.3 (curve a), pH 7.8 (curve b), and in 50 mM potassium phosphate buffer at pH 7.4 (curve c), pH 6.9 (curve d), and pH 6.5 (curve e). The curves are direct tracings made from a Gilford recorder in which the absorbance at 340 nm was placed on scale by use of an off-set control. The hydrolysis of α-ketoglutaramate was measured by the glutamate dehydrogenase assay, using excess amidase (20 μg).

TABLE VI: Rate Constants for Conversion of 5-Hydroxypyroglutamate into α-Ketoglutaramate.[a]

Buffer	pH	$k_{obsd} \times 10^3$ (min^{-1})[b]	k_{obsd}/[OH^-] (min^{-1} M^{-1})	K_{eq}[c]
Imidazole (0.02–0.2 M)	7.10	0.95	7600	3.2×10^{-3}
	7.50	2.31	7290	3.0×10^{-3}
	8.20	9.88	6203	
Tris-HCl (0.02–0.2 M)	7.15	1.01	7160	3.0×10^{-3}
	7.42	1.91	7262	3.2×10^{-3}
	7.70	3.44	6850	
Potassium phosphate (0.02–0.2 M)	6.31	0.16	7804	2.9×10^{-3}
	6.61	0.30	7352	2.9×10^{-3}
	6.91	0.57	7100	3.0×10^{-3}
	7.21	1.31	8090	3.4×10^{-3}
	7.41	1.86	7240	3.3×10^{-3}
Morpholine (0.02–0.2 M)	7.77	4.20	6970	

[a] All reactions were measured at 30°, at an ionic strength of 0.25. [b] k_{obsd} calculated from initial velocities by equation: k_{obsd} (min^{-1}) = initial rate in molar per minute/[5-hydroxypyroglutamate]. [c] K_{eq} = [α-ketoglutaramate]/[5-hydroxypyroglutamate]. The initial concentration of α-ketoglutaramate was obtained by extrapolating the curve for the secondary rate to zero time.

vary over the pH range studied. Above pH 7.5 accurate determinations of this equilibrium constant could not be determined. Based on the equilibrium constant of $\sim 3 \times 10^{-3}$, one can calculate that the second-order rate constant for hydroxide ion catalyzed ring closure is on the order of 2×10^6 M^{-1} min^{-1}.

Discussion

The purification of rat liver ω-amidase to homegeneity provides additional evidence that the conversion of glutamine into α-ketoglutarate and ammonia is brought about by two distinct enzymes: glutamine transaminase and ω-amidase. Although the transaminase and amidase presumably operate in a sequential fashion, the product of the transaminase reaction, α-ketoglutaramate, rapidly cyclizes nonenzymatically to 5-hydroxypyroglutamate, thus decreasing the amount of substrate available to the amidase. At present we have no explanation for this apparent biological inefficiency; however, it is possible that the two enzymes exist associated *in vivo* and α-ketoglutaramate is transferred from the transaminase to the amidase without ever being "free" in solution.

Our studies on the structure of the ω-amidase indicate that the enzyme is composed of two sununits, mol wt 27,000–28,000. Unfortunately, the present data do not allow us to determine whether these subunits are identical or not. Experiments utilizing disc gel electrophoresis in the presence of sodium dodecyl sulfate result in incomplete dissociation of the enzyme. However, we do observe, in addition to the native enzyme, additional protein bands with mobilities which correspond to molecular weights of 30,000 and 27,000. This result suggests that the two subunits may be nonidentical.

As in the case of several other amidases, the ω-amidase has esterolytic activity. One notes that the K_m for the γ-methyl and γ-ethyl esters of α-ketoglutarate are two orders of magnitude less than the K_m for α-ketoglutaramate. The true K_m for α-ketoglutaramate is considerably less than what is observed experimentally due to the equilibrium between α-ketoglutaramate and 5-hydroxypyroglutamate. Based on an equilibrium constant (α-ketoglutaramate/5-hydroxypyroglutamate) of 3×10^{-3} for this reaction, one can calculate that the true K_m for α-ketoglutaramate is approximately 10 μM which is in the same order of magnitude as the γ-methyl and γ-ethyl esters.

The inability of the *N*-methylamide of α-ketoglutarate to serve as a substrate for the enzyme was presumed to be due to the fact that this compound exists almost completely cyclized. However, the observation that *N*-methylsuccinamate and *N*-ethylsuccinamate are not hydrolyzed by the enzyme may suggest that enzyme specificity rather than the nonavailability of the substrate accounts for the lack of reactivity of *N*-methyl-α-ketoglutaramate.

Previous studies by Meister on the hydrolysis of α-ketosuccinamate (Meister, 1953), glutaramate, and succinamate (Meister *et al.*, 1955) showed that there was little change in the reaction rate between pH 5 and 8 while α-ketoglutaramate (Meister, 1953) exhibits a sharp pH–rate profile with a maxima around pH 9. Our finding that the conversion of 5-hydroxypyroglutamate into α-ketoglutaramate is strongly pH dependent supports the suggestion of Meister (1953) that below pH 8 the observed dependence on pH reflects the nonenzymatic conversion of 5-hydroxypyroglutamate into α-ketoglutaramate, rather than a measure of the effect of pH on the enzymatic reaction.

The interconversions between 5-hydroxypyroglutamate and α-ketoglutaramate appear to be subject to specific base catalysis but not to general base catalysis. The mechanism for this reaction can thus be written as shown in Scheme I.

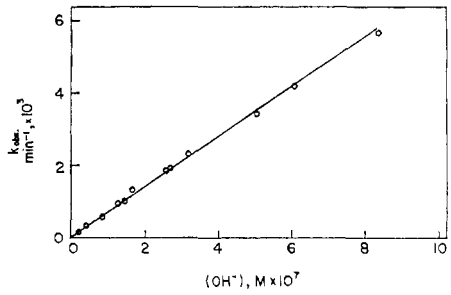
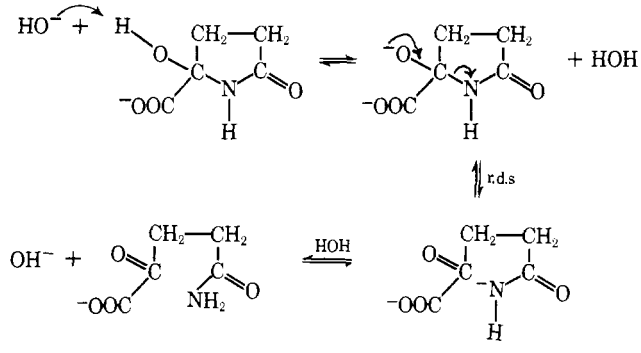


FIGURE 5: First-order rate constants for the conversion of 5-hydroxypyroglutamate into α-ketoglutaramate plotted against hydroxide ion concentration. The points were taken from the data in Table VI.

SCHEME I



These results can be compared to the studies of the reaction of amides and urea with aldehydes. In these reactions one can observe general base as well as specific base catalysis (Jencks, 1964). A recent study by Tarvainen and Koskikallio (1970) showed that although acetic acid–sodium acetate buffer gave general acid and general base catalysis for the reaction of benzamide with formaldehyde, only specific base catalysis could be seen in phosphate buffer. The interpretation that catalysis is not observed in phosphate buffers due to the strong hydroxide ion reaction might well apply to the present situation, in which general base catalysis cannot be demonstrated because of overwhelming catalysis by hydroxide ion.

It is interesting to compare the properties of the rat liver ω-amidase to a similar enzyme found in *Pseudomonas M.A.* (Hersh, 1970). The rat liver enzyme is relatively nonspecific for the acyl portion of the amide substrate (α-ketoglutaramate, glutaramate, α-ketosuccinamate, and succinamate are all substrates for the enzyme) whereas the bacterial enzyme will only hydrolyze α-ketoglutaramate derivatives. On the other hand, the rat liver enzyme does not hydrolyze the *N*-methylamide of α-ketoglutarate whereas this compound is a good substrate for the bacterial enzyme. The bacterial enzyme can synthesize N-substituted α-ketoglutaramates from free α-ketoglutarate plus alkylamine, whereas these same alkylamines are not even inhibitors of the rat liver enzyme. Thus, although both enzymes catalyze the hydrolysis of α-ketoglutaramate, the active sites have evolved specificities which differ in regard to the acyl group of the substrate and the leaving group of the substrate.

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References

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29*, 1193.
Bernheim, M. L. C. (1964), *Arch. Biochem. Biophys. 107*, 313.
Chernoff, A. J., and Lui, J. C. (1961), *Blood 17*, 54.
Cohen, S. G., and Crossley, J. (1963), *J. Amer. Chem. Soc. 86*, 1217.
Cohen, S. G., and Crossley, J. (1964), *J. Amer. Chem. Soc. 87*, 4999.
Cottam, G. L., Hollenberg, P. F., and Coon, M. J. (1969), *J. Biol. Chem. 244*, 1481.
Csaky, T. (1948), *Acta Chem. Scand. 2*, 450.
Davies, E. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Errera, M. (1949), *J. Biol. Chem. 178*, 483.
Fieser, L. F. (1941), Experiments in Organic Chemistry, Boston, Mass., D. C. Heath & Co., p 105.
Friedlin, G. N., Zhendodarova, S. M., Fomina, N. V., and Chukur, A. P. (1963), *J. Gen. Chem. USSR 33*, 919.
Greenstein, J. P., and Price, V. E. (1949), *J. Biol. Chem. 178*, 695.
Hersh, L. B. (1970), *J. Biol. Chem. 245*, 3526.
Hersh, L. B., and Srere, P. A. (1970), *Anal. Biochem. 37*, 353.
Hersh, L. B., Tsai, K., and Stadtman, E. R. (1969), *J. Biol. Chem. 244*, 4677.
Jencks, W. P. (1964), *Progr. Phys. Org. Chem. 2*, 63.
Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.
Kelly, M., and Kornberg, H. L. (1962), *Biochim. Biophys. Acta 64*, 190.
Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.
Marrian, D. H. (1949), *J. Chem. Soc.*, 1797.
Meister, A. (1953), *J. Biol. Chem. 200*, 573.
Meister, A. (1954), *J. Biol. Chem. 210*, 17.
Meister, A. (1965), *Methods Enzymol. 2*, 384.
Meister, A., Levintow, L., Greenfield, R. E., and Abendschein, P. A. (1955), *J. Biol. Chem. 215*, 441.
Meister, A., Sober, H. A., Tice, S. V., and Fraser, P. E. (1953), *J. Biol. Chem. 197*, 319.
Meister, A., and Tice, S. V. (1950), *J. Biol. Chem. 187*, 173.
Monder, C., and Meister, A. (1958), *Biochim. Biophys. Acta 28*, 202.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Otani, T. T., and Meister, A. (1957), *J. Biol. Chem. 224*, 137.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 70.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 113.
Su, S., Albizati, L., and Chaykin, S. (1969), *J. Biol. Chem. 244*, 2956.
Tarvainen, I., and Koskikallio, J. (1970), *Acta Chem. Scand.* 1129.
Yoshida, T. (1967), *Bitamin 35*, 227.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zamenhof, S. (1957), *Methods Enzymol. 3*, 702.