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ENZYMATIC HYDROLYSIS OF PEPTIDES OF ASPARAGINE

C. E. CARTER

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ENZYMATIC HYDROLYSIS OF PEPTIDES OF ASPARAGINE

C. E. Carter*

The amino acid amides, asparagine and glutamine, though of widespread occurrence in nature as free amino acids and in certain proteins have not been studied in peptide linkage as substrates for intra-cellular proteolytic enzymes aside from the studies of the degradation of glutathione. An investigation of the enzymatic hydrolysis of the amide and peptide bonds of glycyl, chloroacetyl, and DL-leucyl-L-asparagine by extracts of mammalian tissues is reported in this paper.

Materials and Methods

The substrates used were synthesized according to the procedures described by Fischer (1). Hydrolysis of peptide bonds was followed by potentiometric formol titrations and by a manometric chloramine T reaction. The latter technique was applied for detection of free amino acids as described by Cohen (2) with the modification of using 0.2 ml aliquots of enzyme-substrate mixtures for assay and omitting preliminary protein precipitation. Under the conditions employed the enzyme blanks were negligible. Potentiometric formol titrations were performed as described by Doherty.[1] This method for following the liberation of free amino acids from peptide linkage is based on the increment of titratable acidity arising from the reaction of formaldehyde with free amino groups. Titrations were done at a glass electrode using 0.02 N NaOH in the micropipette assemply described by Schwert (3). The procedure consisted of adding a 0.2 ml aliquot of an enzyme digest containing 0.028 M peptide to 1 ml of neutralized formol (37 per cent), adding 1 ml of water and titrating to pH 8.1. Mixing during

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titration was accomplished by a magnetic stirrer. Titration values for complete hydrolysis of the peptide were derived from data on aliquots of mixtures of composite amino acids equimolar with the peptide. Because of the high velocity of the hydrolysis, titration blanks were determined on the enzyme-substrate solution by extrapolating to zero enzyme concentration from titration values on three different concentrations of enzyme.

Results

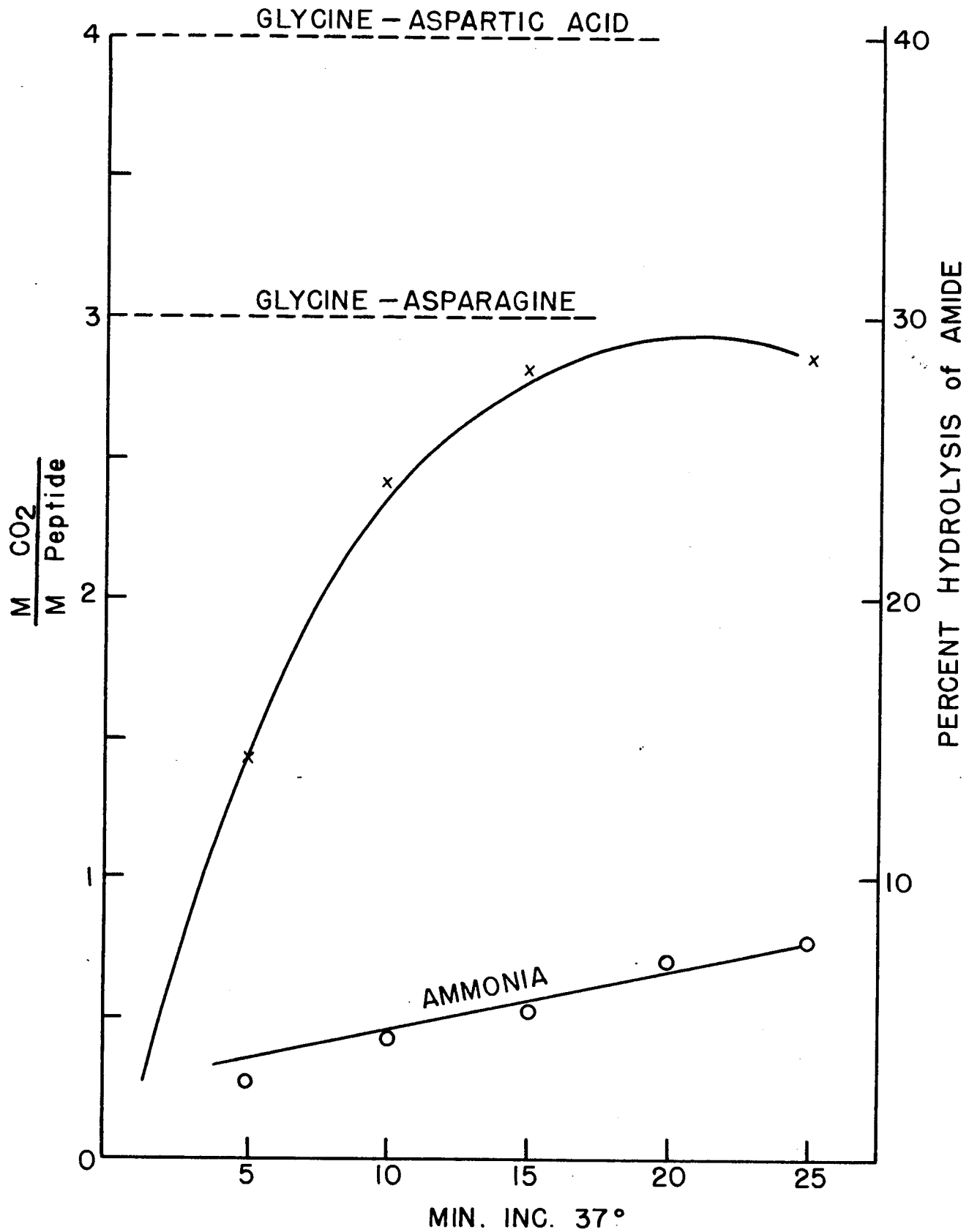
Glycyl-L-asparagine possesses both a peptide and an amide bond and both are hydrolyzed by enzymes in dilute aqueous extracts of several mammalian tissues, yielding on prolonged incubation glycine, aspartic acid, and ammonia. By employing the chloramine T reaction for detection of amino acids liberated by enzymatic hydrolysis of the peptide in conjunction with a determination of ammonia it was possible to show that the hydrolysis of the peptide bond precedes desamidation. This interpretation depends on the liberation of 2 moles of carbon dioxide per mole each of glycine and aspartic acid (2) and one mole of carbon dioxide per mole of asparagine. An analysis of the enzymatic degradation of glycyl-L-asparagine in a rat liver extract is illustrated in figure 1.

During the incubation period there is a rapid liberation of free amino acids as indicated by the chloramine T reaction and little formation of ammonia in the digest, indicating that hydrolysis of the peptide bond takes precedence over desamidation in the degradation of glycyl-L-asparagine. Since, in the chloramine T reaction glycine yields two and asparagine one mole of CO_2, the enzymatic hydrolysis of the peptide to these products should result, as the enzymatic reaction approaches complete hydrolysis, in a ratio equal to three of moles CO_2 produced in the chloramine T reaction to moles of peptide in the digest. The finding of this ratio as illustrated

Figure 1

Digests consisted of 0.5 ml rat liver extract prepared by homogenizing the tissue with six volumes of 0.06 M Na_2HPO_4 followed by light centrifugation to remove debris, plus 1 ml of 0.056 M glycyl-L-asparagine and 1 ml of 0.06 phosphate buffer to adjust the pH of the mixture to 8.0. Of this digest 0.2 ml aliquots were removed at 5-minute intervals during the incubation period, placed in the side arm of Warburg vessels and assayed for free amino acids according to the procedure described by Cohen. Simultaneously a duplicate series of digest were incubated and from these, following addition of alkali, ammonia was aerated, trapped in acid and determined by direct Nesslerization.

FIG. 1


GLYCINE – ASPARTIC ACID
GLYCINE – ASPARAGINE
AMMONIA
M CO2 / M Peptide
PERCENT HYDROLYSIS of AMIDE
MIN. INC. 37°
0
1
2
3
4
5
10
15
20
25
10
20
30
40

in figure 1, in conjunction with the low rate of desamidation, supports this interpretation. Under the conditions from which these data were obtained a longer incubation period (3 hours) results in about 80 per cent desamidation and a concomitant rise in the ratio of moles CO_2 produced by the chloramine T reaction to moles peptide to 3.7-3.9, a finding consistent with the production of aspartic acid from asparagine.

The pH optima of the peptidase which hydrolyzes glycyl-L-asparagine (pH 8.0) is the same as that for asparaginase in aqueous extracts of rat liver. (Figure 2).

Several attempts to separate the amidase and peptidase activity in extracts of liver and kidney were unsuccessful. However, glycyl-L-asparagine is hydrolyzed in several tissues (table I), and from calf thymus it was possible to prepare a fraction which was free of amidase activity but which rapidly hydrolyzed the peptide bond of glycyl-L-asparagine. The procedure effects a separation of activity but does not concentrate the peptidase activity sufficiently to constitute a purification. The method employed follows: 280 gms. of calf thymus were extracted with 1000 cc of 0.15 M NaCl in the cold in a Waring blender and centrifuged to remove insoluble desoxyribonucleoprotein. The supernatant was brought to pH 4.8 with 0.1 M acetate buffer and again centrifuged. The precipitate was dissolved in 100 cc of 0.06 M Na_2HPO_4 (Fraction T_2). In table II the amidase activity of the original tissue extract and the latter fraction (T_2), are compared. The tissue extract is not high in amidase activity, but,as shown in table II, that which is present is effectively removed by the fractionation procedure employed.

The results of assays of peptidase activity in this fraction by the potentiometric formol titration are shown in figure 3. It is apparent that the rate of hydrolysis of the peptide is proportional to the

Figure 2

Experimental conditions were the same as described in figure 1. By means of varying the composition of the phosphate buffer, pH was altered. Experiments with veronal buffers gave essentially the same results.

FIG. 2

Drawing # 6275

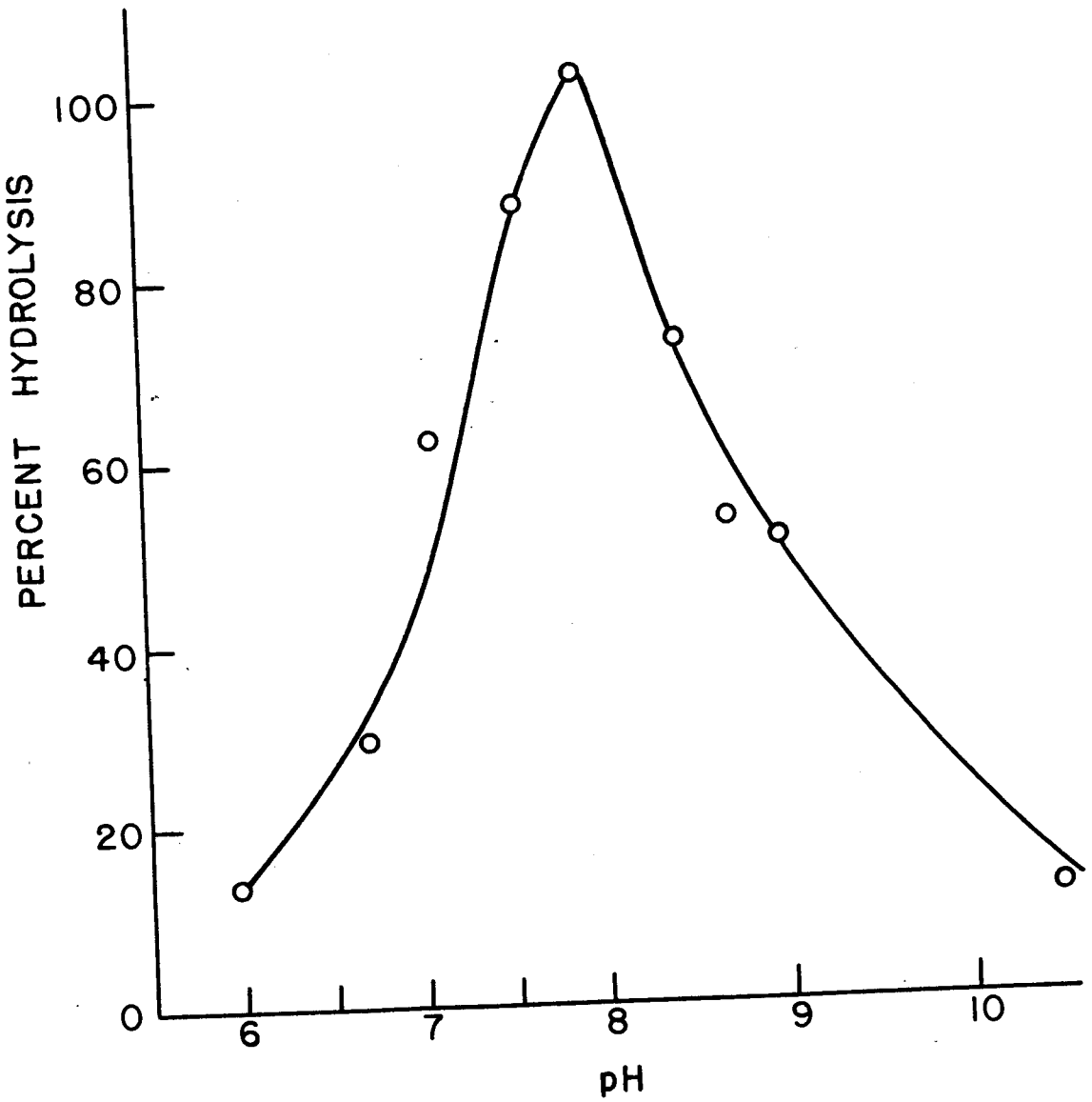


TABLE I

ENZYMATIC HYDROLYSIS OF GLYCYL-L-ASPARAGINE IN RAT TISSUE EXTRACTS

Tissue Extract[1]	Per Cent Hydrolysis of Peptide Bond in 20-Minute Incubation Period
Liver	98
Kidney	99
Spleen	90
Testis	60
Thymus	48

1. Tissue extracts were prepared as described in figure 1, and digests adjusted to pH 8. Hydrolysis of peptide bond was determined by the manometric chloramine T procedure.

TABLE II

ASPARAGINASE ACTIVITY IN THYMUS EXTRACT AND FRACTION T_2

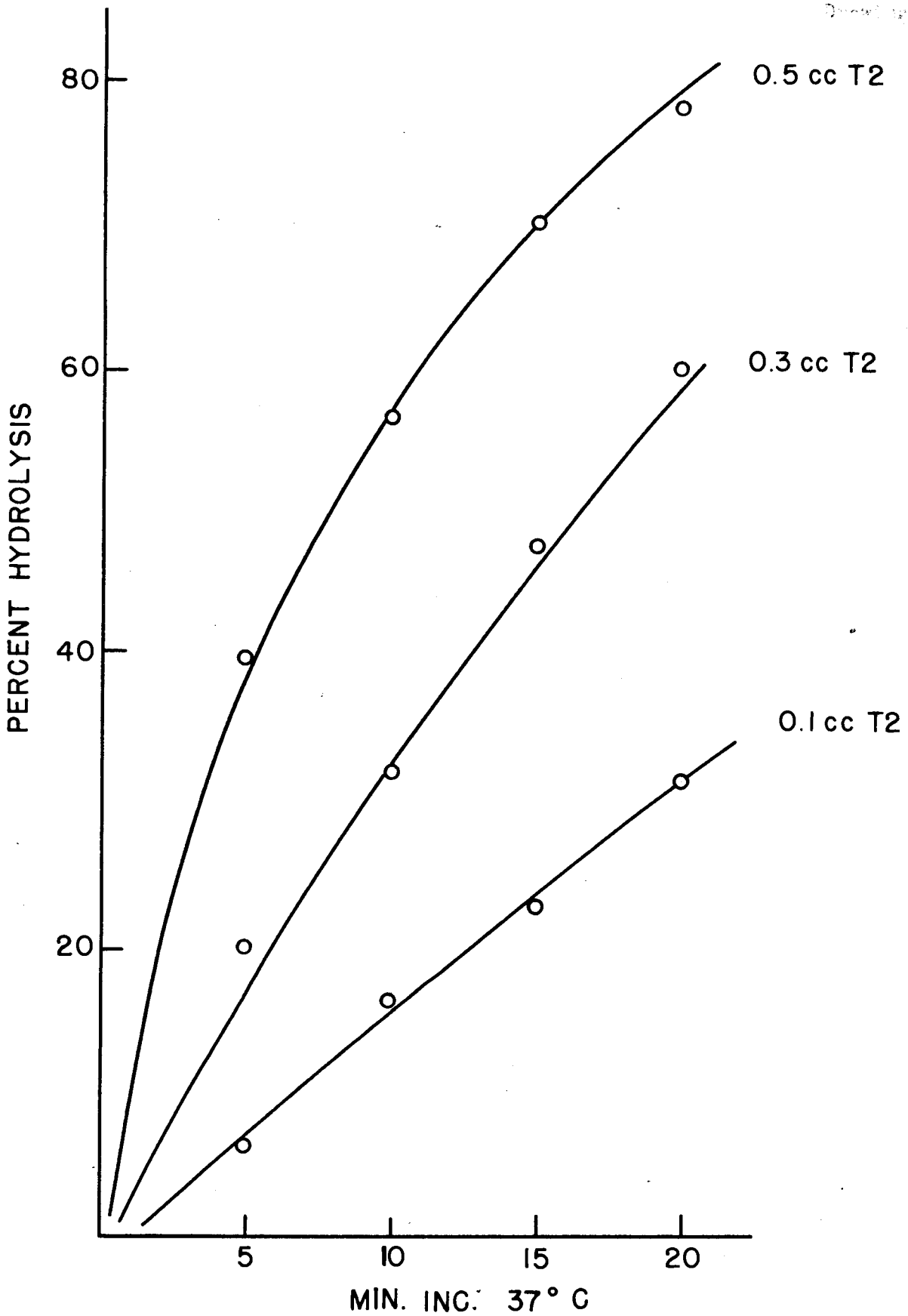
Digests consisted of 1 cc of neutralized substrate (5.6×10^{-2}M) plus 1 cc of enzyme solution and 1 cc of phosphate buffer to adjust the pH of the mixture to 8. The fresh thymus extract was prepared by homogenizing the tissue with 6 volumes of .06 M Na_2HPO_4 and centrifuging to remove debris. The incubation period was 2 hours at 37 C.

	SUBSTRATE	MICROGRAMS NH_3
Thymus Extract	asparagine	22
	glycyl-L-asparagine	26
	chloroacetyl-L-asparagine	0-3
Fraction T_2	asparagine	0
	glycyl-L-asparagine	0
	chloroacetyl-L-asparagine	0

Figure 3

Hydrolysis of glycyl-L-asparagine by a calf thymus fraction, determined by potentiometric formol titration. Substrate 0.028 M, and enzyme concentration varied as indicated.

FIG. 3


80
60
40
20
PERCENT HYDROLYSIS
0.5 cc T2
0.3 cc T2
0.1 cc T2
5
10
15
20
MIN. INC. 37° C

concentrations of enzyme. Chloroacetyl-L-asparagine was not enzymatically hydrolyzed at either the peptide or amide bond by this calf thymus fraction.

The amidase and peptidase activity may also be distinguished on the basis of cyanide inhibition. Concentrations of cyanide (0.04 M) that do not inhibit desamidation of asparaginase in liver extracts inhibit the enzymatic hydrolysis of the peptide bond of glycyl-L-asparagine about 80 per cent. (Figure 4).

Experiments with cyanide also indicate that glycyl-L-asparagine may be desamidated while the peptide linkage is intact but at a significantly slower rate.

The chloroacetyl peptide of L-asparagine is hydrolyzed at a slower rate in extracts of rat liver than the glycyl peptide (table III). However, the enzymatic desamidation of these two compounds occurs at approximately the same rate, and at a rate equal to that for the desamidation of asparagine. Price and Greenstein (4) reported higher rates of desamidation for glycyl-L-asparagine in liver extracts at pH 6.8.

The finding of cyanide inhibition of the enzymatic hydrolysis of glycyl-L-asparagine suggests metal activators for the reaction. However, there was no significant loss of activity of preparations which were dialyzed in the cold for several days against .01 M Na_2HPO_4 and only slight loss of activity followed dialysis against distilled water. Exposure to pH below 6 resulted in a loss of activity of preparations of hog kidney fractions obtained by three precipitations at 0.4 to 0.5 saturation with ammonium sulfate, followed by dialysis against .01 M Na_2HPO_4, but this activity could not be restored by addition of .005 M Mg^{++}, Zn^{++}, Mn^{++}, or Fe^{+++}.

A kinetic analysis of the hydrolysis of glycyl-L-asparagine by a fraction prepared from hog kidney extracts by two precipitations at 0.4

Figure 4

Cyanide inhibition of enzymatic hydrolysis of glycyl-L-asparagine. Peptidase activity determined by potentiometric formol titration. Digests consisted of 0.5 ml liver extracts prepared as described in legend to figure 1, 0.028 M peptide, 0.04 M cyanide and .06 M phosphate buffer to adjust pH to 8.0 in a total volume of 2.5 ml.

FIG. 4

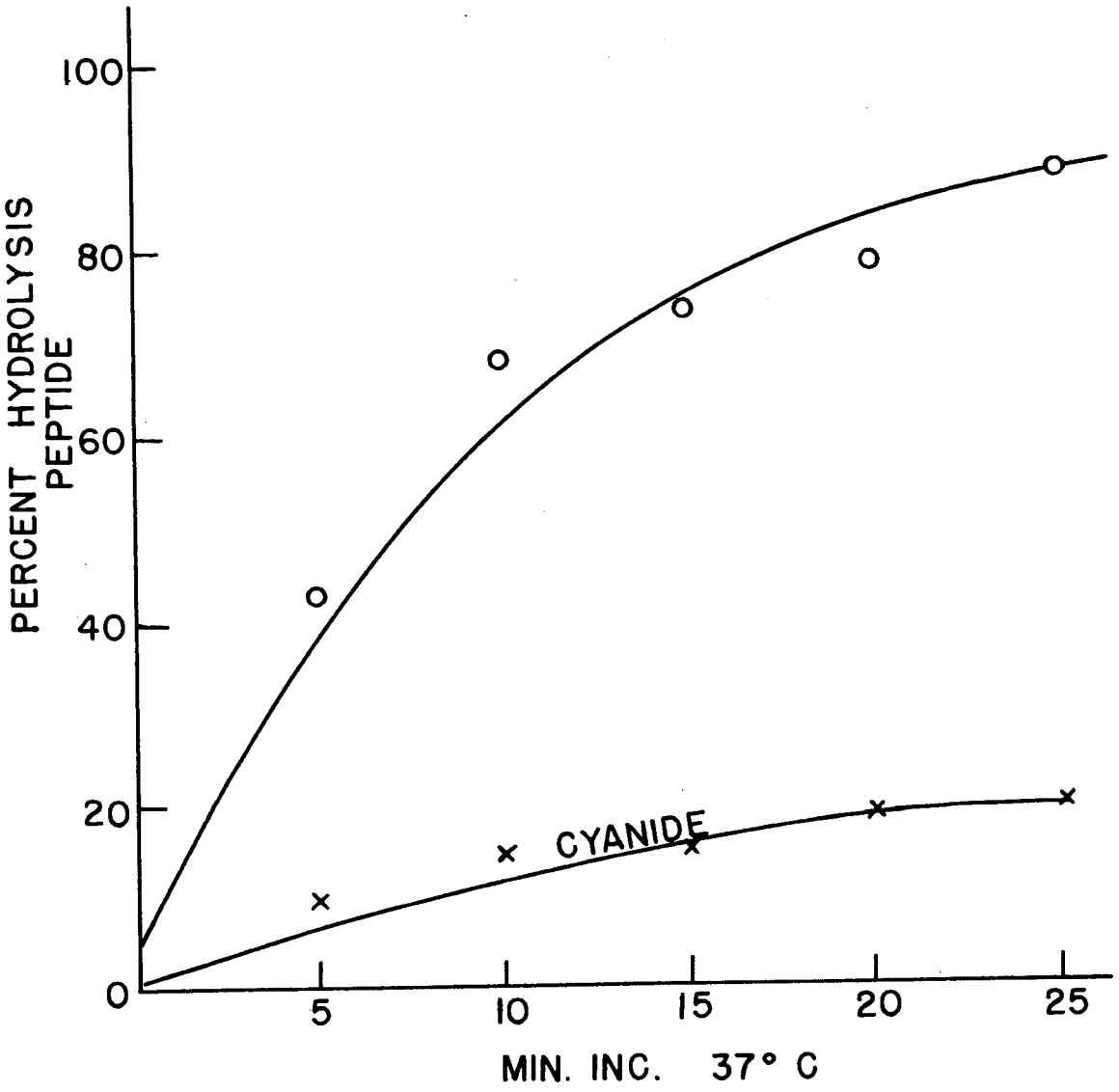

PERCENT HYDROLYSIS
PEPTIDE
100
80
60
40
20
0
5
10
15
20
25
MIN. INC. 37° C
CYANIDE

TABLE III

ENZYMATIC HYDROLYSIS OF CHLOROACETYL- AND GLYCYL-L-ASPARAGINE IN LIVER EXTRACT[1]

	INCUBATION PERIOD (MINUTES)	PER CENT HYDROLYSIS PEPTIDE	MICROGRAMS AMMONIA
Glycyl-L-asparagine	30	100	16
	60	100	30
	90	100	48
Chloroacetyl-L-asparagine	30	18	11
	60	35	25
	90	57	40

1. Experimental conditions were the same as described in legend to figure 1. Ammonia determined by aeration into acid and direct Nesslerization. Peptidase activity followed by potentiometric formol titration.

saturation with ammonium sulfate followed by dialysis against distilled H_2O shows that the reaction follows first order kinetics (figures 5a and 5b) and a plot of the first order velocity constant (figure 5b), against enzyme nitrogen for the three concentrations of enzyme solution employed demonstrated the linear proportionality of rate of reaction to enzyme concentration. Employing the purified hog kidney extract as a source of enzyme and glycyl-L-asparagine and glycyl-DL-asparagine as substrates, the antipodal specificity of the enzyme was shown (figure 6). It was also found that DL-leucyl-L-asparagine was hydrolyzed at a much slower rate than the glycyl peptide of L-asparagine (figure 6).

Glycyl-L-asparagine was not hydrolyzed at the peptide or amide bond by crystalline trypsin.

Summary

Glycyl-L-asparagine is hydrolyzed rapidly in aqueous extracts of several mammalian tissues. In aqueous extracts of rat liver the hydrolysis of the peptide bond precedes that of the amide. The pH optimum of the peptidase which splits glycyl-L-asparagine is at 8.0 and the kinetics of the reaction are first order. Glycyl-DL-asparagine and DL-leucyl-L-asparagine are hydrolyzed much more slowly than glycyl-L-asparagine in purified tissue extracts. A fraction prepared from calf thymus has high peptidase and no amidase activity for glycyl-L-asparagine.

Figure 5a and 5b

Hydrolysis of peptide bond determined by potentiometric formol titrations. Substrate was present in .028 M final concentration and the system was buffered with .06 M phosphate to pH 8. Titration values for zero reaction time were determined by extrapolating the values for 5-minute incubation periods with 3 different enzyme concentrations to zero enzyme concentrations. Velocity constants calculated from data on the first 15 minutes of incubation. After this period the reaction is not first order.

FIG. 5a

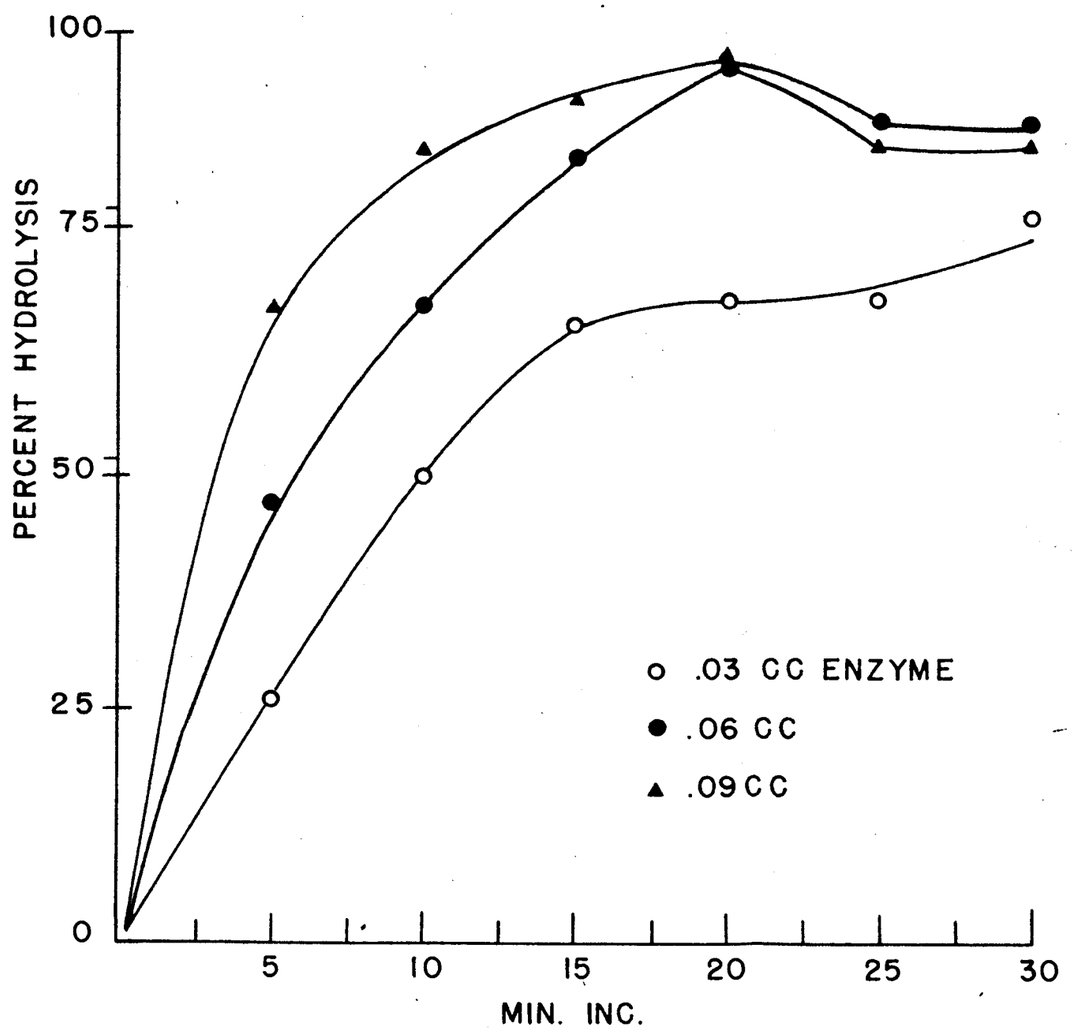

100
75
50
25
0
PERCENT HYDROLYSIS
5
10
15
20
25
30
MIN. INC.
o .03 CC ENZYME
● .06 CC
▲ .09 CC

FIG. 5b

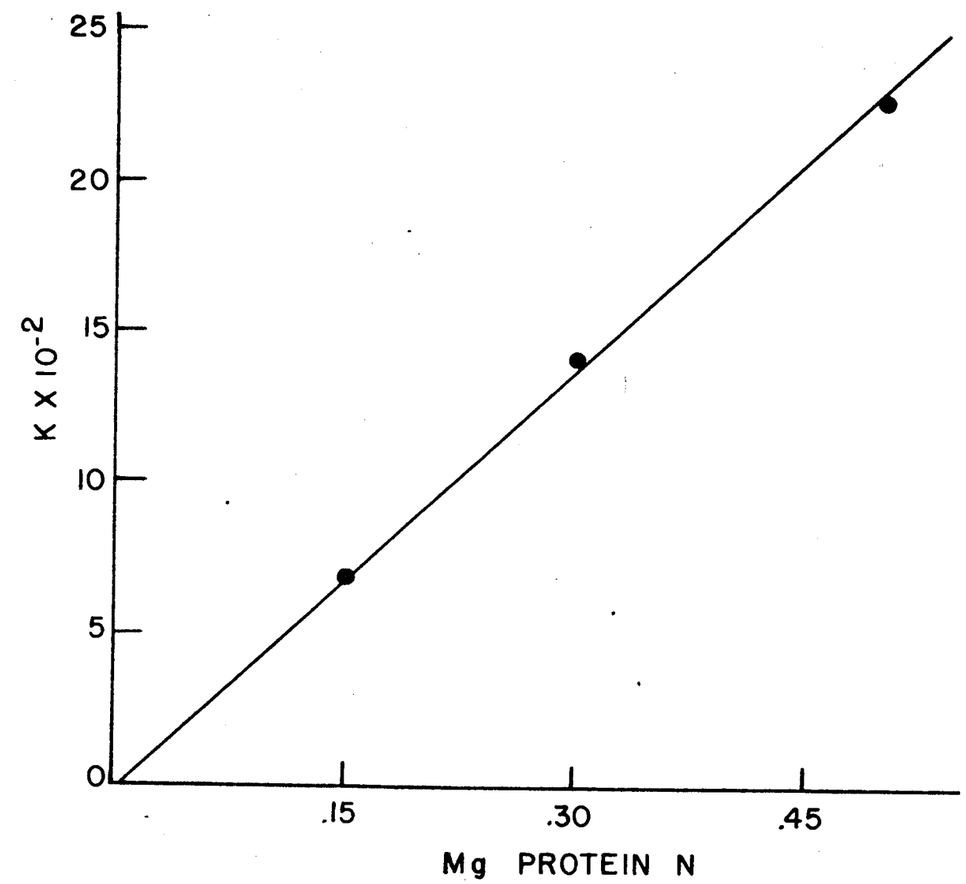

25
20
15
10
5
0
K X 10-2
.15
.30
.45
Mg PROTEIN N

Figure 6

Experimental conditions were those described in figures 5a and 5b.

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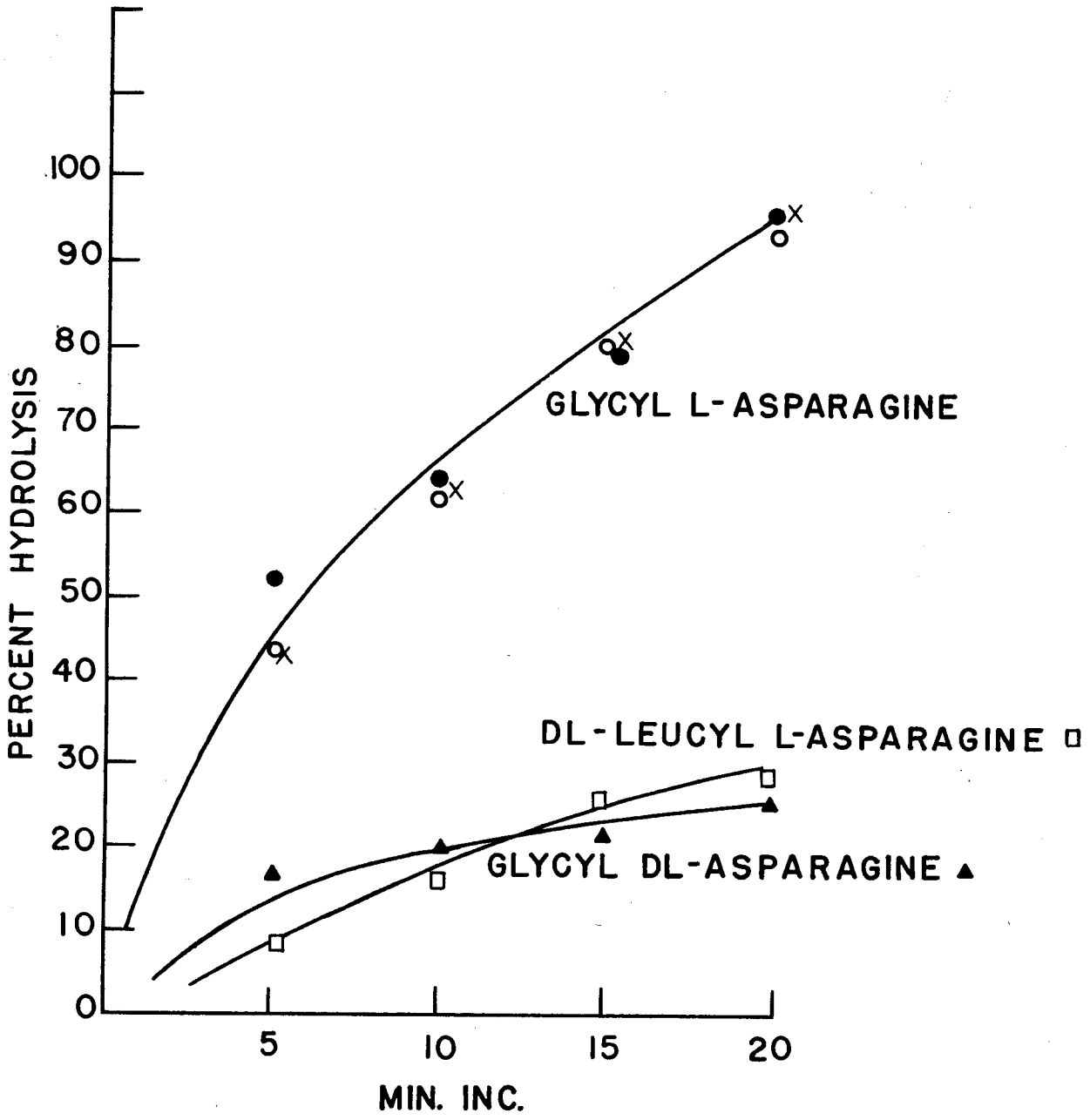
FIG. 6

Drawing # 6279



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