STUDIES OF PROTEOLYTIC SYSTEMS WITH SYNTHETIC DIPEPTIDES

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INTRODUCTION

The production of wheat for flour is the industry of the greatest importance to the economy of Western Canada. It is the high quality of the protein constituent, gluten, which allows it to command a premium on the world market. Although gluten has been the object of a great deal of study, the chemistry of it is not well understood. In view of the important role of this protein material, more complete knowledge of its chemical and physical properties is desirable. The study of gluten can be regarded as a general, long term problem for research which may be approached in many different ways. Since glutamic acid comprises about forty percent of the gluten, a study of the properties of various compounds involving that amino acid in a number of ways should be useful in the final elucidation of the gluten structure.

Previous work on this problem at the University of Saskatchewan has consisted of hydrolysis, dialysis, and analytical work on wheat gluten (1, 2). Studies of polymers of glutamic acid have been done and later copolymers of glutamic acid, lysine and cysteine were studied in the belief that the latter polypeptides had properties resembling more closely those of natural proteins (3). The observation that pepsin and pancreatin, in a consecutive reaction, did not hydrolyse the polymer of L-glutamic acid or the polymer of L-cysteine but did hydrolyze the copolymers of glutamic acid, cysteine and lysine is of

significance in relation to the present work.

In view of these findings and the large amount of related work that is reported in the literature, it was thought that valuable information could be gained from the study of synthetic dipeptides. A worthwhile contribution to the knowledge of wheat protein structure may be obtained by comparing the action of well characterized proteases on wheat gluten and on synthetic substrates of known structure.

This research was undertaken to obtain general basic information on the proteolytic constituents of some crude fungal enzyme preparations. A knowledge of these properties is necessary for studies involving fractionation of the crude material; for evaluating their usefulness in the study of gluten; and for assessing other possible uses for the enzymes.

The problem involved the synthesis of some dipeptides and derivatives and a study of the conditions under which the enzymes could be made to hydrolyze these substrates. Although it was not the object in the first phase of the work to try to establish new methods of synthesis, an attempt was made to critically appraise those which were used. From the work with the enzymes it was hoped to determine in what respects the fungal enzymes differ from each other and from other proteolytic enzymes that have been studied in detail.

PEPTIDE SYNTHESIS

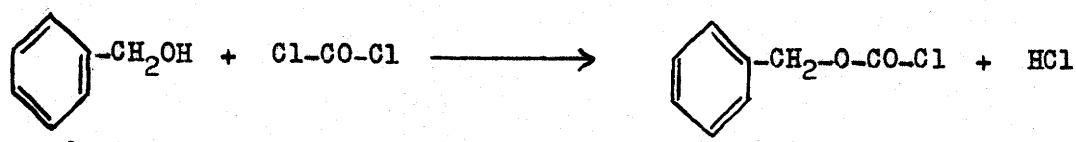
1. LITERATURE REVIEW

Synthetic dipeptides have been used extensively for the partial elucidation of protein structure and for the study of protease specificities. The first notable works in the field of synthesis were those of Fischer, who outlined the technique of suitably blocking the reactive groups of the amino acids in such a way that they could be coupled to yield dipeptide or polypeptide derivatives. No universal procedure has been established, but many suitable methods and modifications of these methods have been adapted to the different groups of amino acids. Today it is possible to link most of the amino acids into a synthetic peptide chain (4).

Fischer, in the preparation of the tripeptide of glycine, used carboethoxychloride (ethylchlorocarbonate) (5) to block the amino group.

$$C_2H_5\text{-}O\text{-}CO\text{-}Cl + NH_2\text{-}CH_2\text{-}COOH \longrightarrow C_2H_5\text{-}O\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH$$

Later Bergmann described the use of a somewhat better reagent, carbobenzyloxy chloride (6), as an amino group blocking agent. The reagent was made by the condensation of benzyl alcohol and phosgene in toluene as solvent at 0°C. (7).

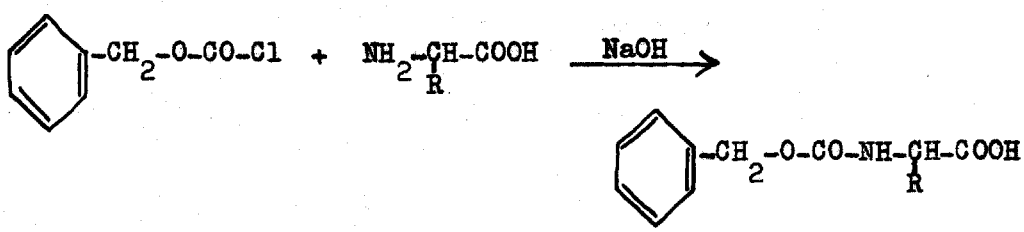


The product was a colorless liquid, if pure, and was obtained in 90%

yield. The compound was strongly lachrymatory. Estimation of the purity and of the yield was done by the formation of the amide by reacting an aliquot of the liquid with cold concentrated ammonia.

The amide was crystalline, m.p. 85 - 86°C. and was weighed.

The reaction of the carbobenzyloxy chloride is essentially the Schotten-Baumann reaction.



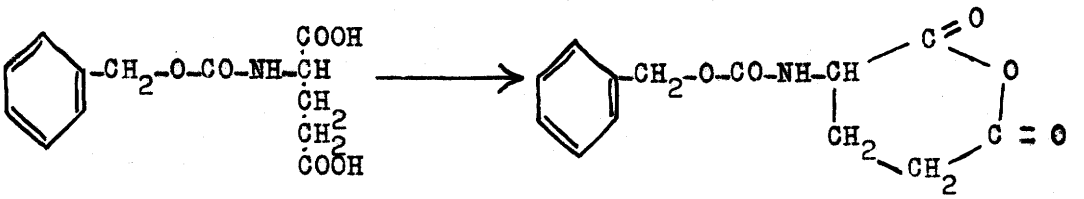
The alkali is present in an amount equivalent to the free carboxyl groups and the hydrochloric acid that is liberated by the reaction.

Recently a slight modification of this method has been described by Farthing (8) in which the phosgene is reacted directly with the benzyl alcohol in the absence of any other solvent at -20 to -30°C. Claims are made for a better product at this stage and purer, more easily crystallizable derivatives of the amino acids.

The conversion of N-cbzo-L-glutamic* acid to N-cbzo-L-glutamic acid anhydride was described by Bergmann (6). The product is reported to be readily crystallizable even though isolated initially as an oil. This anhydride has been shown to yield the α-carboxyl derivative

*The term "carbobenzyloxy" is abbreviated as "cbzo"

when the

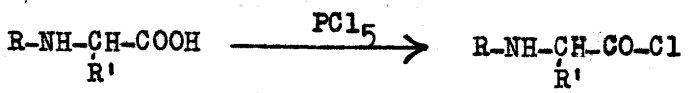


anhydride is decomposed with a free amine, alcohol, or ammonia to give the peptide, the mono ester, or the amide.

The removal of the carbobenzyloxy group was also described by Bergmann (6) who employed catalytic hydrogenation using hydrogen gas and platinum oxide catalyst. Many other workers have used the method (9, 10, 11, 12).

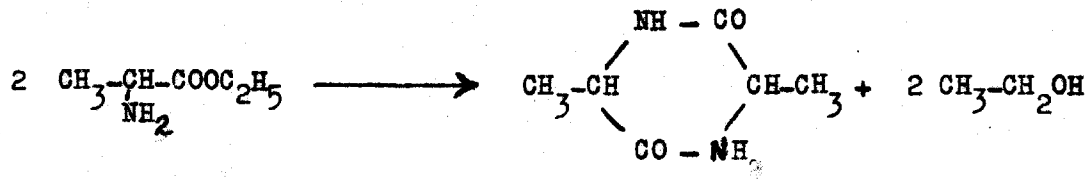
The reduction of cystine with sodium metal in liquid ammonia was described by du Vigneaud, Audrieth and Loring (13). The method has been applied to the removal of the carbobenzyloxy group in peptide synthesis (3, 14). Phosphonium iodide has also been used for the removal of this blocking group (15). All these methods have been found to be satisfactory under certain conditions.

The acyl halide method of Fischer (5) for coupling through the carboxyl group can be used where the acid anhydride is not possible or where the γ-derivatives of glutamic acid are desired. The method consisted essentially of suitably blocking the amino group followed by treatment with phosphorus penachloride.



In many cases the amino acid chloride was not isolated and purified but was used as the crude material for the coupling reactions to give the peptide derivatives.

The most generally used blocking group for the carboxyl group is the ester. When the ester is formed by the use of ethanol and hydrochloric acid the first product is the ester hydrochloride. Of most amino acids this product is a solid crystalline salt, readily prepared and easily purified, and usually obtained in good yield. In order to obtain the free ester, usually a liquid, it is necessary to neutralize the acid and extract the free amino acid ester with an organic solvent. Procedures for these derivatives have been described by various workers (16, 17). The free esters can be purified by distillation in vacuo, but they are difficult to store because of their instability. These esters readily decompose to form 2,5-diketopiperazines or the substituted diketopiperazines if the amino acid ester is other than that of glycine. The reaction for the condensation is shown for the alanine ester giving the 3,6-dimethyl-2,5-diketopiperazine.

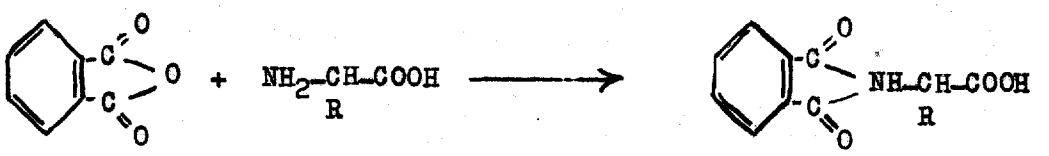


The preparation of the diketopiperazines from the amino acids by pyrolysis and subsequent partial hydrolysis of the diketopiperazines has been used by Sannié (18) to prepare several peptides. However, in

the use of an amino acid ester as an addend to a peptide chain, the formation of the diketopiperazine occurs as an undesirable side reaction. Sannié also states that the pyrolysis of the amino acid causes complete racemization of the optically active amino acids. In view of that the method is unsuitable for the preparation of peptides for use in enzymatic studies. Sannié did, however, prepare mixed anhydrides from which, by partial hydrolysis, he isolated dipeptides of more than one amino acid.

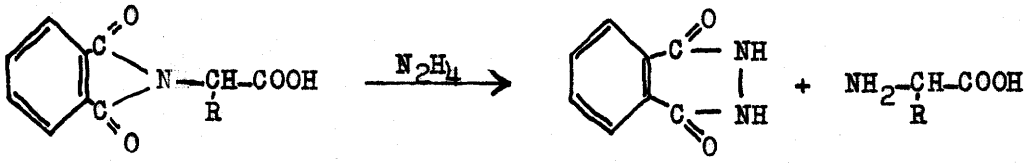
Another method for blocking the amino group has been described very recently by Wolfe and Seligman in which the carbonaphthoxy derivative was formed. The reagent was carbonaphthoxy chloride and was prepared similar to carbobenzyloxy chloride. The carbonaphthoxy derivatives were formed but were not used in the preparation of dipeptides. The procedure was described too recently to be used in this work. Its use as a blocking group in peptide synthesis is worth investigation.

In recent years some use has been made of the phthalyl group as a blocking agent for the amino group in peptide synthesis. Billman and Harting (20) have described a method for preparing the derivative in which the amino acid is pyrolyzed in the presence of molten phthalic anhydride. Such a reaction yields the phthalimido derivative:



Another procedure has been described by Kidd and King (21) in which the amino acid and phthalic anhydride are heated in boiling pyridine suspension. These latter authors have shown that both methods result

in racemization of the amino acids. They have used it, however, in the synthesis of dipeptides. Upon the formation of the N-phthalyl derivative, the carboxyl group was converted to the acyl halide and then it was coupled with an amino acid ester to give the dipeptide derivative. The phthalyl group was removed by treatment with hydrazine by the reaction:



Under the conditions of this reaction the peptide bond was unaffected.

The phthalimido derivative of the simple amino acids can be converted to the acyl halide by treatment with PCl_5 (22) or by the method of Grassmann et al (23) which employs thionyl chloride as the halogenating reagent.

The phthalimido derivative of glutamic acid has been of particular use in that the acid anhydride can be formed and decomposed to give derivatives similar to the derivatives of N-cbzo-glutamic acid. That is, upon reaction of the N-phthalyl-glutamic acid anhydride with an amino acid ester, the formation of an N-phthalyl-dipeptide ester linked through the α-carboxyl group of glutamic acid was obtained. It does differ from the N-cbzo-glutamic anhydride in that in the reaction with ammonia, the γ-amide is formed. This has been used as a route to the preparation of glutamine.

2. EXPERIMENTAL

(i) The following derivatives have been prepared:

N-phthalyl-glutamic acid.

Carbobenzyloxy chloride.

N-cbzo glycine.

N-cbzo-L-glutamic acid.

N-cbzo-L-glutamic acid anhydride.

N-cbzo-glycyl chloride.

N-cbzo-glycyl glycine.

Glycylglycine.

Glycine ethyl ester.

DL-alanine ethyl ester.

L-glutamic diethyl ester.

N-cbzo-α-L-glutamylglycine ethyl ester.

N-cbzo-α-L-glutamyl glycine.

N-cbzo-α-L-glutamyl-DL-alanine ethyl ester.

N-cbzo-α-L-glutamyl-DL-alanine.

N-cbzo-α-L-glutamyl-L-glutamic acid diethyl ester.

N-cbzo-α-L-glutamyl-L-glutamic acid.

α-L-glutamyl glutamic acid diethyl ester.

α-L-glutamyl-L-glutamic acid.

The methods of preparation and the results obtained are discussed below.

All melting points listed in the following section have been corrected.

(ii) Details and Discussion of the Preparations

a) N-Phthalyl-Glutamic Acid

The N-carbobenzyloxy derivatives of glutamic acid have been used at this University by Sumner and by Blakley. These derivatives have strong tendencies to oil and require long periods of time to crystallize. In view of this, the use of phthalyl derivatives was considered. Both the fusion method of preparation and the pyridine method were used.

The fusion method - (20)

A mixture of 0.5 gm. of the amino acid and 1.0 g. of phthalic anhydride was weighed into a pyrex test tube and placed in an oil bath at 185°C. for 15 minutes. During the first ten minutes the mixture was stirred and the phthalic anhydride which sublimed and deposited on the walls of the tube was pushed down. The mixture was left undisturbed during the last 5 minutes. After 15 minutes the tube was carefully removed and cooled until the liquid solidified. The tube was inverted and excess phthalic anhydride was scraped from the walls. The residue was recrystallized from 10% ethyl alcohol or from water. N-phthalyl-glutamic acid, m.p. 188 - 190°C., 45% yield.

The pyridine method - (21)

L-glutamic acid (32.6 g.) and phthalic anhydride (32.5 g.) in dry pyridine (120 cc.) was refluxed for 2 hours. After evaporation of the clear solution under reduced pressure, acetic anhydride was added and the mixture boiled for 2-3 minutes. The solvent was removed

under vacuum and ether added to complete precipitation of the product. Upon treatment with water the product, N-phthalyl-glutamic acid, was obtained., m.p. 189°C.

The N-phthalyl-glutamic acid was as difficult to crystallize as the carbobenzyloxy derivative, and, since both the methods of producing the N-phthalyl derivatives are known to cause racemization of the amino acid, these procedures were not pursued further. The synthesis of the N-phthalyl derivative was also tried by both methods using γ-methyl-L-glutamate in place of L-glutamic acid. In each case the product, an oil, did not crystallize.

b) Carbobenzyloxy Chloride

The carbobenzyloxy method of blocking the amino group has been used extensively for peptide synthesis, and the method for the preparation of Carbobenzyloxy Chloride has been described in detail in Organic Synthesis (7). That procedure was followed exactly in part of this work. If care was taken so that neither the toluene nor the liquid product came in contact with rubber stoppers, a clear liquid, as described, would be obtained.

The modification recently described by Farthing (8) in which the benzyl alcohol acts as its own solvent was also used. The procedure was as follows:

Benzyl alcohol (282 gm.) was placed in a 500 ml. round bottom, three necked flask. One neck allowed the use of a stirrer, the second

the gas inlet tube; and the third, the gas outlet tube to the fume hood or the gas trap. Provision was made so the flask could be lowered into an alcohol-dry ice bath. Phosgene gas was passed into the cooled benzyl alcohol at such a rate that the internal temperature remained between -20° and -30°C. At the end of 30 to 40 minutes, the internal temperature fell indicating the reaction was almost complete. The phosgene supply was shut off. It was necessary to remove solid benzyl alcohol from the sides of the flask to allow it to react with the excess phosgene that was present at that stage. That was done by removing the reaction flask from the cold bath and allowing it to warm to about -10°C. The solid broke away from the walls, floated to the surface, and reacted almost immediately. Care was taken not to use too great an excess of phosgene. Since it is also a liquid at the reaction temperature and is completely miscible, excessive waste may result. Allowing the volume of the liquid in the flask to increase by one half was found to give an adequate excess of phosgene. After complete disappearance of the solid benzyl alcohol, the reaction flask was allowed to attain room temperature, the stirrer was removed, and the gas lead was used to bubble air through the solution for 24 hours. The air was first passed through concentrated sulfuric acid and then over flake sodium hydroxide. The liquid was filtered through sintered glass and the last trace of phosgene was removed at the water pump. A clear, colorless, or only very slightly amber liquid was obtained. It was strongly lachrymatory and was removed from the glassware with ammonia in alcohol.

Farthing claims that this product was purer than that obtained from the unmodified Bergmann procedure. He indicated that an oil which crystallized upon standing was obtained by the reaction of the product with glycine, whereas with the product from the modified procedure, the crystalline N-cbzo-glycine was obtained at the outset. In this work it was found that crystalline N-cbzo-glycine was obtained from both preparations of carbobenzyloxy chloride. However, some differences were noticed in the preparation of N-cbzo-glutamic acid. The product from the Bergmann method was isolated as an oil and crystallized on standing. The first m.p. was usually 105 to 107°C., and three to four recrystallizations from water were necessary to reach the reported m.p. of 120°. From the Farthing modification the product did not crystallize with standing up to 2 weeks, but if the oil was dissolved in hot water then placed in the refrigerator for 2 to 3 days, the crystalline product was obtained with m.p. 118 to 119°C. and only one recrystallization was required to attain m.p. 120°C.

Farthing also stated that the reaction between benzyl alcohol and phosgene was complete in about 4 hours. It was found here that only 30 to 40 minutes were required for the reaction to go to completion, and further addition of phosgene was unnecessary. A considerable excess of phosgene was present by that time.

c) N-Cbzo-Glycine

For this product the procedure outlined in Organic Synthesis

(7) was followed. The crude product crystallized in the form of needles, 95.3% yield, m.p. 115 - 117°C. It was recrystallized from chloroform to give m.p. 119°C., yield 67.5% of theory. (Reported - yield pure 63.8%, m.p. 120°C.)

d) N-cbzo-L-Glutamic Acid

This derivative was prepared by the method of Hanby et al (24). The method is essentially a modification of the original Bergmann procedure (6).

L-glutamic acid (60 g.) was dissolved in 4 N. NaOH and stirred vigorously while carbobenzyloxy chloride (84 cc.) and 4 N. NaOH (120 cc.) were added over a period of 20 minutes. The mixture then had reached 60°C. A further 25 cc. of 4 N. NaOH was added and the mixture was stirred another 15 minutes. The solution was cooled and extracted twice with 40 ml. ether, and acidified to Congo Red with concentrated HCl. The aqueous solution was extracted four times with 50 ml. ethyl acetate and the ethyl acetate solution dried over anhydrous Na_2SO_4. The ethyl acetate solution was evaporated in vacuo leaving a viscous amber oil. The oil was covered with dry, light petroleum ether (b.p. 33°C.) and placed in the refrigerator to crystallize. Crystallization took place in two or three days. Whether the product crystallized or not, the petroleum ether was decanted and the product was dissolved in hot water and allowed to crystallize in the refrigerator over a period of 3 days. One to four crystallizations were required to obtain the product with a m.p. 120°C. The maximum

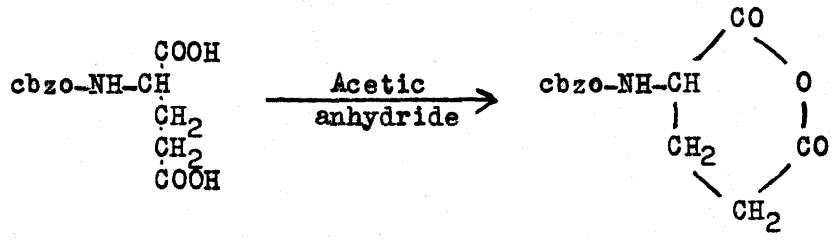
yield of the pure product was 52.3% of theory based on the glutamic acid used.

Some difficulty was encountered in the extraction with ethyl acetate due to the formation of a stable emulsion. The addition of either phase (water or ethyl acetate) was of some help, but gentle heating of the emulsion in the separatory funnel under the water tap was found to be a better way to break the emulsion.

e) N-cbzo-L-Glutamic Acid Anhydride

The conversion of the free acid derivative to the acid anhydride was first described by Bergmann (6). Most workers using this compound have used the procedure without any variation (11, 14, 25, 26). The method of Bergmann was described by Harrington and Mead in a little more detail and is as follows:

"N-cbzo-glutamic acid (45 gm.) was covered with freshly distilled acetic anhydride (120 ml.) and the mixture brought rapidly to a boil, boiled for 2 minutes, after which it was cooled quickly and evaporated as far as possible under reduced pressure on a boiling water bath. The residue was poured into a beaker, transferance with the aid of a little anhydrous Chloroform. With rubbing and cooling crystallization set in and was completed by the addition of 2 to 3 volumes of anhydrous ether. After a short time in the cold the product was collected and dried. Yield 88%, m.p. 94°C."



Upon several occasions a crystalline compound, which was neither the anhydride nor the original acid, was obtained. It was believed that it was a mixture of the anhydride and the free acid even though well-defined crystals were obtained.

Free acid - m.p. 120°C. - not well-defined crystals.

Anhydride - m.p. 94°C. - long prisms.

"Mixture" - sharp m.p. at temperatures between 102° and 109°C. The crystal form was minute needles.

It was found that the anhydride could be obtained pure if the N-cbzo-glutamic acid was completely anhydrous prior to the treatment with acetic anhydride. If the free acid derivative was stored without desiccation it was dried in vacuo at 60°C. for 2 hours immediately before treatment with acetic anhydride. When the above precautions were taken, the anhydride was obtained in a yield of 85% of theory, m.p. 93 - 94°C. Recrystallization from benzene was not found necessary.

f) N-cbzo-Glycyl Chloride

The preparation of this derivative was the same as described by Bergmann for the acyl chloride of N-cbzo-α-aminoisobutyric acid. The method as used for the derivative of glycine was as follows:

N-cbzo-glycine (3.0 gm.) was suspended in dry ether (25 cc.) and to it was added PCl_5 (3.0 gm.) with the flask in an ice-salt bath. The mixture was stirred vigorously for 15 minutes at the end of which time most of the solid PCl_5 had disappeared. The ether solution was

decanted from the excess PCl_5 and the ether was removed in vacuo in the cold. The remaining oil was washed several times with 10 ml. of light petroleum ether, the latter being decanted from the oil. The crude N-cbzo-glycyl chloride was redissolved in ethyl ether and used immediately for the next stage. No further attempt was made to purify this intermediate.

g) N-cbzo-glycylglycine

This dipeptide derivative was prepared by the method described for N-cbzo-glycyl-β-alanine by Hanson and Smith (27).

Glycine (4.75 gm.) was dissolved in 20 cc. of 2N. NaOH and to it was added the ethereal solution of N-cbzo-glycylchloride (from 12.0 gm. of N-cbzo-glycine) and an additional 25 cc. of 2N. NaOH. The mixture was cooled in an ice bath during the addition, then was left at room temperature for one hour with frequent shaking. The ether layer was discarded and the aqueous layer was acidified to Congo Red with 5N. HCl. The product crystallized and the flask was placed in the refrigerator for 2 hours to complete precipitation. The product was collected, dried in vacuo, and recrystallized from methanol.

The amount of crude material isolated was 4.8 gm., m.p. 172-174°C. It was recrystallized from methanol in order to obtain the reported m.p. 178°.

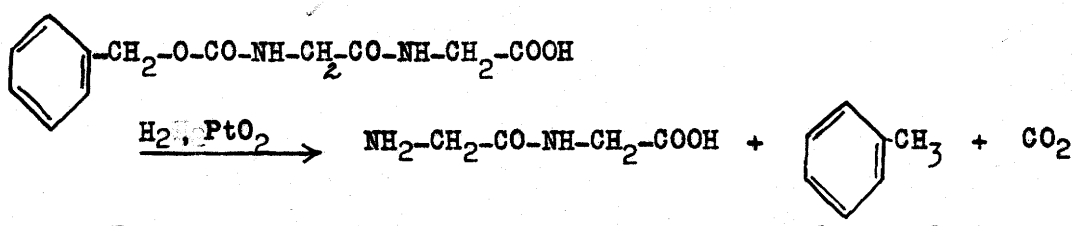
h) Glycylglycine

The reduction of N-cbzo-glycylglycine was attempted by the

use of the sodium in liquid ammonia method as previously used for the polymer derivatives by Blakley (3). No pure glycylglycine was obtained.

Hydrogenation of the carbobenzyloxy derivative at room temperature and atmospheric pressure using platinum oxide catalyst was found to be satisfactory. No products other than the original reactants and the free dipeptide were present at the end of the reaction, and, therefore, the glycylglycine was easily crystallized.

N-cbzo-glycylglycine (1.0 gm.) was suspended in glacial acetic acid (25 ml.) in a 125 ml. suction flask. To this was added 0.1 gm. PtO_2 catalyst and the mixture stirred with a magnetic stirrer. The top of the flask was fitted with a gas inlet through which a slow stream of hydrogen gas was passed. The side arm was used as the gas exit and a tube was fitted to it so that periodically the exit gas could be passed through a $Ba(OH)_2$ solution in a test tube. The passage of the exit gases through the barium hydroxide solution for 5 minutes without the formation of visible precipitate was taken to indicate that the reaction was complete.



The solution was filtered to remove the catalyst and the acetic acid was removed in vacuo at 60°C. The oily residue was

dissolved in as little water as possible and precipitated by the addition of absolute alcohol. The solid was then recrystallized from water-alcohol. Yield 200 mgm., 41% of theory. The dipeptide, with rapid heating, decomposed at 215°-220°C. without melting. (Reported m.p. 215°-220° d.)

i) Glycine Ethyl Ester

Glycine (15.0 g.) was added to absolute ethanol saturated with dry HCl (100 ml.) and to this was added 100 ml. absolute ethanol. The mixture was refluxed gently for 3 hours. The solvent was removed in vacuo during which time the glycine ethyl ester hydrochloride precipitated. The product was collected and washed four times with absolute alcohol. The washings and remaining mother liquors were placed in the refrigerator for a second crop. The product can be recrystallized from ethanol. Total yield 27.1 gm. (97%) m.p. 142°-143°C. (Reported m.p. 143°C.)

The free ester was extracted from the ester hydrochloride by the method described in Beilstein (28).

5.0 g. glycine ethyl ester hydrochloride was mixed with 2.5 cc. of water, the mixture covered with 40 cc. of ether, and with strong cooling was added a small amount of solid K_2CO_3 and 4.0 cc. of 33% NaOH to give a thick paste. After working for 2 minutes the ether was decanted and the paste was extracted twice more with ether. The

ether of the combined extracts was removed in vacuo. The crude, free ester was a slightly amber oil. Yield 2.5 g., 68% of theory.

The free ester was found to be extremely unstable even at refrigerator temperature. Visible precipitation occurred overnight at 0°C. and also over 48 hours at -40°C. This precipitate was presumed to be the diketopiperazine which is known to form by the spontaneous decomposition of amino acid esters.

The crude ester was used immediately after its preparation from the hydrochloride for the coupling reactions to form the dipeptide derivative.

j) DL-Alanine Ethyl Ester

The preparation of the ethyl ester hydrochloride of DL-alanine was done by the same procedure as for glycine. An equivalent amount of alanine was used. The product was isolated first as an oil and was crystallized from absolute ethanol overnight in the refrigerator. Yield 86.5%, m.p. 83°-84°C.

The free ester was extracted from the hydrochloride by the same method, using equivalent amounts, as for the glycine ethyl ester. The crude ester was a slightly amber oil. Yield 72% of theory. The free ester was more stable than the free ester of glycine. It did not precipitate the diketopiperazine during two days at 0°C., nor during four days at -40°. It was prepared immediately prior to use in the

coupling reactions. The crude ester was redissolved in ether and no further attempt was made to purify it.

The crude free ester was obtained in 86% yield from the hydrochloride.

k) L-Glutamic Acid Diethyl Ester

The procedure outlined by Chiles and Noyes (17) was used for the preparation of L-glutamic acid diethyl ester hydrochloride. Yield 87%, m.p. 98°-99°C. (Reported m.p. 96°-98°C.)

The extraction of the ester was done by the method described in Biochemisches Handlexikon (29) and, as applied, is as follows:

10 g. of the ester hydrochloride was added to 4.0 ml. of water, the mixture was covered with 40 ml. of ether and cooled in an ice-salt mixture. Solid K_2CO_3 was added in excess and the paste formed by the aqueous layer was rubbed for 3 minutes to allow extraction of the free ester. The ether layer was decanted and the paste extracted twice more with ether. The combined ether extracts were dried over anhydrous Na_2SO_4 and evaporated in vacuo in the cold. The crude product is a colorless oil and is stable for 2 days at 0°C. Yield 56.7%. The crude oil was used in ether solution without further purification.

l) N-cbzo-~~d~~-L-Glutamylglycine Ethyl Ester

The method for coupling N-cbzo-glutamic acid anhydride with

glycine ester to yield this dipeptide derivative was that described by Bergmann, Zervas and Fruton (30).

The crude product obtained was 43.2% of theory based on the amount of N-cbzo-L-glutamic acid anhydride used, m.p. 119°-120°C. (Reported m.p. 122°C.)

m) N-cbzo-α-L-Glutamylglycine

Saponification of the dipeptide ester derivative was done by dissolving the ester in 2 molar equivalents of N. NaOH and allowing to stand at room temperature for 1/2 hour. The solution was then acidified to Congo Red with concentrated HCl and extracted with ethyl acetate. The ethylacetate was evaporated in vacuo and the residue recrystallized from dry ethyl acetate. The yield was 45.5% of theory based on the weight of carbobenzyloxy peptide ester used, m.p. 141.5°C. (Reported m.p. 143°C.)

n) N-cbzo-α-L-Glutamyl-DL-alanine Ethyl Ester

The coupling of N-cbzo-L-glutamic acid anhydride with DL-alanine ethyl ester to form the dipeptide derivative was the same as for N-cbzo- -L-glutamylglycine ester. The yield obtained was 35.2% based on the anhydride; m.p. 135°. (Reported m.p. 135°C.)

o) N-cbzo-α-L-Glutamyl-DL-alanine

The saponification of the ester to the N-cbzo-dipeptide was

done by dissolving the cbzo-dipeptide ester in 2 molar equivalents of N. NaOH and leaving it at room temperature for 1/2 hour. The solution was then acidified to Congo Red with concentrated HCl and after cooling for one hour in the refrigerator the product had precipitated. Yield 51.0% of theory based on the anhydride, m.p. 149°-150°C. (Reported 153°C.) Recrystallization once from Ethyl-acetate gave m.p. 153°C.

p) N-cbzo-α-L-Glutamyl-L-Glutamic Acid Diethyl Ester

N-cbzo-L-glutamic acid anhydride was coupled with the diethyl ester of L-glutamic acid as described by Bergmann (6). The method was as follows:

"The solution of 3.0 gm. of N-cbzo-L-glutamic acid anhydride and 5.0 gm. of L-glutamic acid diethyl ester were mixed in dry chloroform, after 5 hours standing the solution was washed twice with dilute HCl, dried over Na_2SO_4 and evaporated in vacuo. By recrystallization of the residue from a very little alcohol 2.8 gms. of beautiful needles were obtained which had m.p. 137°C."

It was found that if the coupling took place in dry ethyl acetate and the solution left at room temperature for 3 hours, the product had started to precipitate. The precipitation of the product was completed by cooling the solution in the refrigerator for 2 hours. The product obtained was 39.7%, based on the anhydride, with m.p. 134°. (Reported 137°C.)

q) N-cbzo-α-L-Glutamyl-L-Glutamic Acid

The saponification of the carbobenzoxy dipeptide ester was done, as before, by dissolving the ester in 3 molar equivalents of N. NaOH and leaving it at room temperature for 3/4 hour. The solution was acidified with concentrated HCl and placed in the refrigerator for 1 hour. The product was collected and dried in vacuo.

It sintered at 140°-145°C. and melted at 174°C. (Reported sintered at 145° and m.p. 176°C.)

r) α-L-Glutamyl-L-Glutamic Acid Diethyl Ester

2.8 g. of the cbzo-dipeptide ester were hydrogenated with H_2 gas in 25 ml. of absolute alcohol and 2 ml. of glacial acetic acid in the presence of 0.1 gm. of platinum oxide catalyst at room temperature and atmospheric pressure. The catalyst was filtered from the solution and the latter was evaporated in vacuo, during which time the dipeptide precipitated. The product was recrystallized from absolute alcohol. Yield 1.4 gms., 70.5% of theory, m.p. 136°-137°C. (Reported m.p. 137°C.)

s) α-L-Glutamyl-L-Glutamic Acid

2.0 gms. of N-cbzo-α-L-glutamyl-L-glutamic acid were hydrogenated with hydrogen gas in glacial acetic acid in the presence of 0.4 gms. platinum oxide catalyst, at room temperature and atmospheric pressure. The catalyst was removed by filtration and the acetic acid

removed in vacuo. The residue was dissolved in as little water as possible, and 2 to 3 volumes of alcohol were added. The solution was placed in the refrigerator for two days. The product was collected and dried in vacuo. Yield 1.0 gms., 75.0% of theory, m.p. 190°C.

Bergmann (6) has reported α-glutamyl glutamic acid with m.p. 205°C. Boothe and coworkers (11) prepared the dipeptide with m.p. 184°-185°C. The other properties of the dipeptide and its precursors were reported to be the same as described by Bergmann.

ENZYME STUDIES

1. LITERATURE SURVEY

The use of synthetic peptides to study the action of the proteolytic enzymes has been used extensively in the last twenty years. These substrates have been extremely helpful in the identification and classification of the enzymes of crude mixtures and of crystalline enzymes.

There has been considerable controversy in the literature over the nomenclature of the peptidases. The general classification of the proteolytic enzymes as outlined by Bergmann (31) include:
Proteinases - this is a very general classification, but these are the enzymes which hydrolyze the whole proteins. The products of the reactions of proteinases are high molecular weight peptides.
Peptidases - this class is divided into carboxypeptidases, which require a free α-carboxyl group adjacent to the peptide bond that is to be split, and aminopeptidases which require the presence of a free α-amino group adjacent to the peptide bond that is to be split. Peptidases attack the terminal peptide bond of polypeptides and the products of the reaction are free amino acids.
Dipeptidases - the substrates for these enzymes are dipeptides which are unique in that they possess both a free α-amino group and a free α-carboxyl group.

A great deal of data has been accumulated which shows that

the susceptibility of dipeptides and polypeptides to enzymatic digestion is markedly dependent upon the amino acid residues that they contain. These observations suggest that there is a side chain specificity not indicated by the general terms peptidase and dipeptidase.

Bergmann, Zervas and Fruton (30) have shown that "Papain Peptidase 1" exhibits extremely high side chain specificity using the dipeptide derivatives of glycine and glutamic acid. They also showed that the enzyme required two peptide bonds, only one of which was hydrolyzed. On this basis they concluded there was no activity that could be classified as carboxypeptidase, aminopeptidase or dipeptidase.

Johnson and Peterson (32) studied the proteolytic system of Aspergillus parasiticus by measuring the rates of hydrolysis of certain proteins and selected synthetic peptides. They concluded that the crude enzymes isolated contained a proteinase, an aminopeptidase, a carboxypolypeptidase , and a dipeptidase. Subsequent studies of a large number of enzymes (33) showed that these microorganisms produced at least one proteinase and five peptidases.

Bergmann, Zervas and Fruton (30, 34) were able to demonstrate the antipodal effect of substrates on enzyme activity. They showed that where a peptide containing the L-amino acid was attacked, the same peptide containing the D-amino acid was also attacked under certain circumstances. They state that in such cases the L-peptide

was attacked much faster than the D-form. They believed that the susceptibility of the D-substrate to attack depended almost entirely upon the relative size of the R side group.

Many enzymes known to exhibit side group specificity have been named according to their substrate. Examples are tyrosine polypeptidase which hydrolyzes dipeptides of tyrosine linked through its NH_2 group to another amino acid, and leucine carboxypeptidase which hydrolyzed peptides with leucine in the terminal position. Neurath and Schwert in their reveiw state (35), "The range of specificities of the pancreatic proteases is much wider than inferred by the pioneer work of Bergmann and his collaborators."

The pH optimum at which the proteolytic enzymes catalyze cleavage of peptide bonds varies from the extremely low pH for pepsin to the high pH optimum for pancreatin. Most peptidase activity has been shown to be optimal at or above pH7.

Neurath and Schwert (35) have shown carboxypeptidase action to have the optimum at pH 7.5. However, Bergmann and his coworkers (30, 49) did their early studies on Papain Peptidase at pH 5.0. Later they observed that carboxypeptidase and aminopeptidase action was optimal at pH 7.4 (34). Fruton (36), studying the enzymatic hydrolysis of dipeptides of serine, leucine, and glycine found the activity to be optimal at pH 7.5 to 7.8. A leucylpeptidase and certain amino peptidases were shown to exhibit maximum rates at pH 8 to 9 (33).

The study of the kinetics of enzyme catalyzed reactions is often difficult because of simultaneous inactivation of the enzyme. If the enzyme inactivation proceeds at an appreciable rate, the decreasing catalyst concentration affects the course of the reaction and should be accounted for when attempting to assess the effect of substrate concentration.

Enzyme catalyzed reactions have been postulated to proceed by a cyclic process (37, 38). The substrate molecule combines with the enzyme, reacts, and after a time lag the products are ejected. The reactive position on the enzyme surface is again active and the process may be repeated. The rate of the reaction would be dependent on the number of cycles completed in a time interval.

At low concentration of substrate the time between cycles would be dependent on the number of substrate molecules, and, therefore, would be a function of the substrate concentration. As the concentration of the substrate is increased, relative to the enzyme concentration, a point will be reached where the time lag between cycles is negligible compared to the time required for the cycle. At that, or higher concentrations of substrate, the enzyme is working at "saturation" capacity and the rate of the reaction should be independent of substrate concentration.

If the rate of reaction is measured over a suitable length of time, during which the substrate concentration doesn't fall below "saturation" level, and there is no appreciable inactivation of the

enzyme or no inhibitory effect due to accumulation of end products, the reaction will be independent of concentration of substrate and will be zero order. A plot of the disappearance of substrate or the appearance of the end products vs. time will give a straight line as long as these conditions are met. The rate of the reaction will be directly proportional to the slope of the straight line.

The rate equation for the zero order reaction is

$$\text{Velocity} = \frac{dC}{dt} = k \qquad \text{(i)}$$

where C = concentration of products, t = time, and k = velocity constant.

If C is plotted against time, the slope of the line will be a direct measure of k.

The Arrhenius equation is

$$k = Ke^{-E/RT} \qquad \text{(ii)}$$

where k = the rate constant

E = the energy of activation for the reaction.

R = the gas constant = 1.987 cal. $deg.^{-1}$ $mole.^{-1}$

T = the absolute temperature

K = a constant, which in terms of the classical theory for reaction kinetics includes a factor for the collision frequency and a probability factor.

Taking the logarithm of equation (i) gives

$$\ln k = \frac{-E}{RT} + \ln K$$

When plotting lnk vs. 1/T a straight line is obtained with

slope $= \frac{-E}{R}$, and the intercept $= \ln K$

Considering the reaction at temperatures T_1 and T_2, and assuming E constant over the range of temperature, the integrated equations are:

$$\ln k_1 = \frac{-E}{RT_1} + \ln K \qquad \text{(iii)}$$

and $$\ln k_2 = \frac{-E}{RT_2} + \ln K \qquad \text{(iv)}$$

Subtracting equation (iv) from (iii),

$$\ln k_1 - \ln k_2 = \frac{E}{RT_2} - \frac{E}{RT_1} \qquad \text{(v)}$$

$$\ln \, k_1 / k_2 = \frac{E}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \qquad \text{(vi)}$$

$$E = \frac{R \ln \, k_1 / k_2}{\frac{1}{T_2} - \frac{1}{T_1}} \qquad \text{(vii)}$$

and since the ratio $k_1 / k_2 = \frac{\text{slope 1}}{\text{slope 2}}$

$$E = \frac{R \, (\ln \text{slope}_1 - \ln \text{slope}_2)}{(1/T_2 - 1/T_1)} \qquad \text{(viii)}$$

or $$E = \frac{2.303 \; R(\log \text{slope}_1 - \log \text{slope}_2)}{(1/T_2 - 1/T_1)} \qquad \text{(ix)}$$

Equation (ix) enables the simple calculation of the activation energy from the slopes of the lines obtained by plotting the percent hydrolysis vs. time at two different temperatures. The slope must be measured at the beginning of the reaction where the reaction is zero order.

Kavanau, in his review of enzyme kinetics (39) has shown that the plot of the Arrhenius equation for many biological reactions approaches but is not a perfect straight line. He concludes that the plot is probably rather a series of segments of straight lines with breaks at definite temperatures, and that over a short temperature range the straight line approximation is valid. This curved effect was observed to be more pronounced in enzyme reactions at extremely low temperatures, and was explained on the basis of a stepwise formation of reactive points on the surface of the enzyme.

2. METHODS OF ESTIMATING HYDROLYSIS

The formol titration has been used extensively to estimate the release of amino groups during the hydrolysis of proteins, and the method has been described in detail by Iselin and Niemann (40). The method was not used in this work because it would have been necessary to adapt it to micro amounts and because of the difficulty of obtaining a satisfactory acid-base titration in the strongly buffered systems. The method was used successfully by McConnell (41) to estimate the hydrolysis of proteins by enzymes. The use of the

alcohol titration to follow the release of carboxyl groups was described by Grassman and Hyde (42). This method presents the same difficulties as the formol titration.

Van Slyke and his coworkers (43, 44) have outlined a method whereby the carboxyl groups of free amino acids may be determined using the ninhydrin reaction. If the reaction is carried out at low pH (pH2.5), the reaction with ninhydrin (triketohydrindene hydrate) results in the decarboxylation of the amino acid with simultaneous formation of ammonia and an aldehyde.

$$R - \underset{NH_2}{CH}\text{-}COOH \xrightarrow[\text{pH 2.5, heat}]{\text{ninhydrin}} R\text{-}CHO + NH_3 + CO_2$$

These authors have shown that this method can be used to measure free amino acids quantitatively in the presence of dipeptides or higher peptides. Although they found that a few of the amino acids did not release carbon dioxide in strictly quantitative amounts, those used in the present work were found to give the theoretical yield. Either a manometric procedure or a titrimetric method could be used. For the latter, the carbon dioxide was distilled into $Ba(OH)_2$, and the excess base was back titrated with hydrochloric acid. The reactants were heated 8 minutes in a boiling water bath.

McConnell (45) has modified this procedure so that the reaction is carried out for one hour at 85°C. in an air oven, and has shown his modification to be satisfactory. This procedure is simpler

and less time per analysis is required. The only special apparatus required is the microdiffusion cells. This modification has been used in this work and the procedure is described in full in the section on experimental work.

3. EXPERIMENTAL

(i) Source and Preparation of the Enzymes

The mold enzymes* were produced by certain fungi and actinomycetes grown in submerged culture in a Klim-glucose medium (46, 47). At the end of the growth period, the culture medium was freed of the mycelium, the solutions were lyophilized, and the crude enzymes were stored dry. No attempt was made to fractionate or purify the crude material. A list of the culture numbers and the organisms which produced the enzymes are presented in table I.

Table I

Culture No.	Organism
P.R.L. 26	Mortierella sp.
P.R.L. 79	Gliocladium roseum.
P.R.L. 86	Gliocladium roseum.
P.R.L. 92	Trichoderma viride.
P.R.L. 232	Fusarium sp.
P.R.L. 319	Chaetomium sp.
P.R.L. 369	Alternaria tenuis.
P.R.L. 376	Streptomyces sp.

*The enzyme solutions were supplied by the courtesy of Dr. W.M. Dion, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.

Use is made of the culture number rather than the name of the organism for any subsequent reference to the enzymes. Unless otherwise indicated, all enzyme solutions used in this work were made up to give twice the concentration of solids that was present in the culture medium at the end of the growth period.

(ii) Method of Estimation of Hydrolysis

The method used here was McConnell's modification (45) of the ninhydrin-CO_2 procedure of Van Slyke and his coworkers (43, 44). Carbon dioxide is evolved from free amino acids only under the conditions of the reaction.

Reagents:

(i) Solution, 3.125% triketohydrindene hydrate (ninhydrin) and 3.125% citrate buffer pH 2.5.

(ii) 0.1 N. carbonate free sodium hydroxide with thymolphthalein indicator, and 0.25 N. with respect to sodium chloride.

(iii) H_2SO_4

(iv) 0.1 N. HCl.

Equipment - pipettes, microdiffusion cells with stopcock and rubber connection, 9 liter vacuum bottle at pressure 21 cm. Hg below atmosphere pressure, the syringe type with micrometer screw attachment, magmix and small magnetic stirrers 1/4" long.

Procedure:

0.2 ml. of the sample was pipetted into the outer chamber of the diffusion cell and to this was added 0.8 ml. of the citrate-ninhydrin solution and 2 drops of dilute sulfuric acid (to lower the pH to about

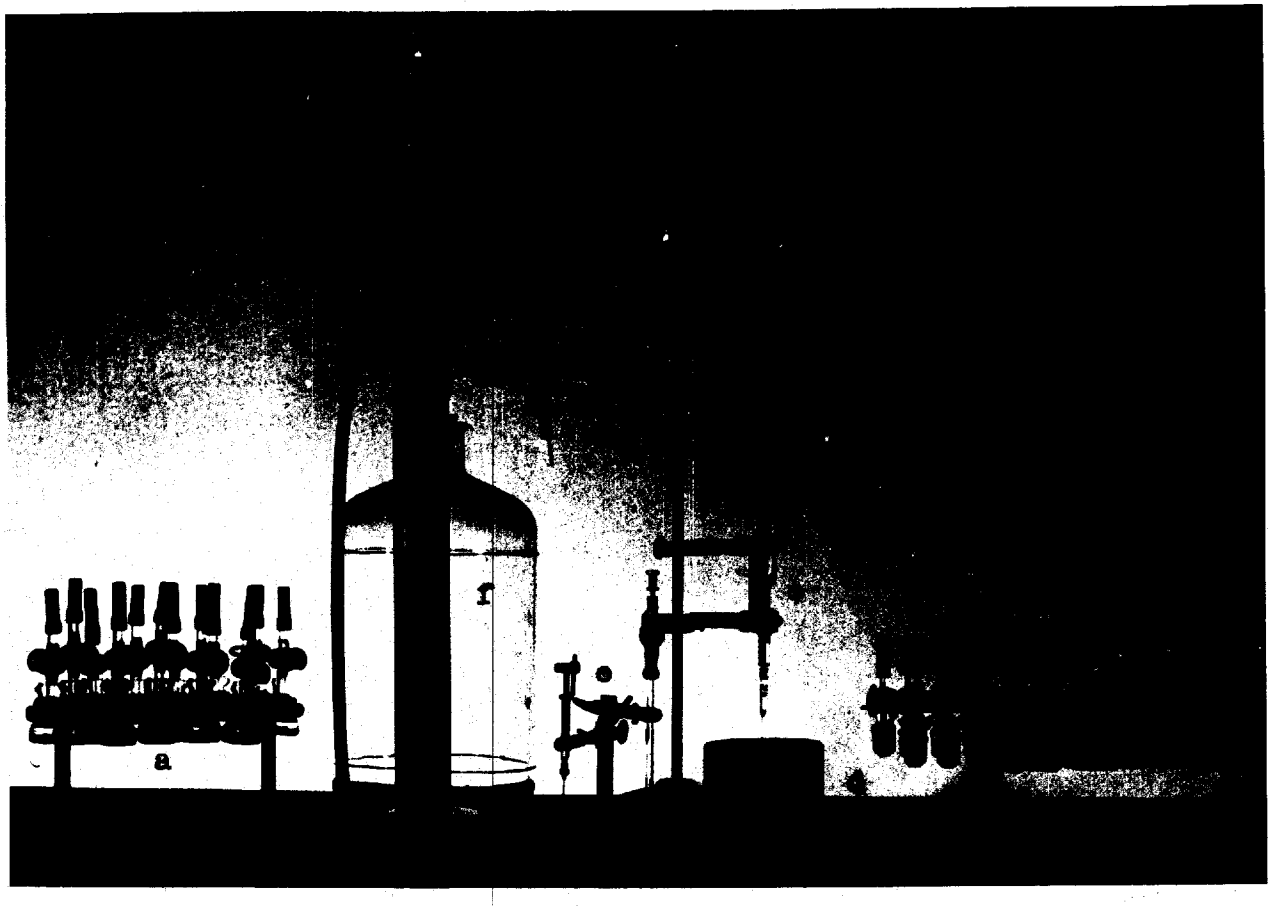


Fig. 1. The ninhydrin-carbon dioxide analysis apparatus

a. assembled diffusion flasks

b. unassembled diffusion flasks

c. microburette with micrometer screw

d. mag-mix

e. pipettes

f. vacuum flask.

2.5), 0.2 ml. of 0.1 N. NaOH was pipetted into the centre well. The stopcock attachment was placed on the cell and the cell evacuated at the vacuum bottle, and the stopcock closed. The diffusion cell was placed in an air oven at 85°C. for one hour. After cooling at room temperature for one half hour it was opened and 3 drops of 2% $BaCl_2$ solution were added to the centre well. The cell was placed on the magmix and the blue solution in the centre well was titrated with stirring to colorless with 0.1 N. hydrochloric acid. The concentration of amino acid can be obtained directly by calculation of the quantity of carbon dioxide collected in the alkali.

The hydrolysis of the substrates used resulted in the release of one or two free amino acids, depending upon whether the dipeptide was free or contained one substituted amino acid. The percentage of substrate hydrolyzed can be expressed directly in terms of the initial substrate concentration and the amount of carbon dioxide liberated by the ninhydrin. Since the same initial substrate concentration was used for all hydrolyses, a factor was calculated by which the titrations were converted to percent hydrolysis. The data on the calibration of the microburette and the calculation of the conversion factor are given below.

Calibration of the microburette:

The neutralization of 0.20 ml. of 0.0988 N. NaOH was found to require 0.100 N. HCl measured by 3.22 divisions on the microburette.

Volume per division $= \frac{1.00}{3.22} \times \frac{0.100}{0.0988} \times 0.20$ ml.

Carbon dioxide per division

$$= \frac{1.00}{3.22} \times \frac{0.100}{0.0988} \times 0.20 \times 22 \times 0.0988 = 0.1365 \text{ mgm.}$$

Carbon dioxide measured (mgm.) = 0.1365 x titration.

Calculation of the Factor:

Weight of carbon dioxide liberated per milliequivalent of free amino acid = 44 mgm.

Concentration of the substrate analyzed = 0.05 M.

Volume of the substrate analyzed = 0.20 ml.

Milliequivalents of substrate analyzed = 0.05 x 0.20 = 0.01

Weight of carbon dioxide released from the analysis of a sample yielding one mole of free amino acid per mole of substrate =

$$44 \times 0.01 = 0.44 \text{ mgm.}$$

Titration obtained for 100 percent hydrolysis =

$$\frac{0.44}{0.1365} = 3.22 \text{ divisions}$$

Percent hydrolysis $= \frac{100}{3.22} \times$ titration

Equation - Percent hydrolysis = 31.1 x titration.

Sample Calculation:

Determination no. 3, table no. VIII

Burette reading at zero time = 21.97, 21.94
mean = 21.96

Burette reading at 24 hours = 23.52, 23.50
mean = 23.51

Increase in titration = 1.55

Percent hydrolysis = 1.55 x 31.1 = 48.2 percent.

(iii) Preliminary Survey of the Enzymes

For the hydrolysis studies the digestion tubes contained*

substrate (0.125 M.)	1.00 ml.
buffer solution	0.50 ml.
$CoCl_2$ solution (0.01 M.)	0.25 ml.
enzyme solution	0.75 ml.

The first trial hydrolyses were made using P.R.L. 79, phosphate buffer pH 7.6. The results are shown in table II.

Table II

Tube no.	Substrate	Percent Hydrolysis		
		Zero time	5 hours	20 hours
1	N-cbzo-α-L-glutamyl glycine	5.6	2.6	6.32
2	N-cbzo-glycyl glycine	0.0	10.7	30.1
3	N-cbzo-α-L-glutamyl DL-alanine	1.7	58.7	56.6
4	glycyl glycine	0.7	3.34	4.3

A further preliminary survey of the enzymes with the substrates available was done. These hydrolyses were done for a period of 24 hours at 37°C. using phosphate buffer pH 7.6. A duplicate sample was analyzed at the beginning of the hydrolysis and at the end

*Each digestion tube was covered with toluene to prevent growth of the microorganisms.

of 24 hours. The amount of hydrolysis indicated in table III was based on the increased titer of the 0.2 ml. aliquot after 24 hours.

Table III

Preliminary Hydrolysis Studies

Substrate	Approx. pH	Percent Hydrolysis							
		Enzyme							
		PRL 26	PRL 79	PRL 86	PRL 92	PRL 316	PRL 319	PRL 369	PRL 376
α-L-glutamyl-L-glutamic Acid	3.5	0	0	0	0	0	0	0	0
N-cbzo-α-L-glutamyl-L-glutamic Acid	5.5	9.6	76	97	45	3.0	40	51	1.0
N-cbzo-α-L-glutamyl-DL-alanine	7.5	16	23	40	9.6	18	0	0	7.6
α-L-glutamyl-L-glutamic acid diethyl Ester	10.5	32	7.8	21	10	19	7.1	25	6.7
N-cbzo-α-L-glutamyl-glycine	11.0	33	18	2.1	10	28	28	24	10

The pH listed in the first column is indicated as approximate because it was found that the buffer was not concentrated enough to hold the pH of the mixture. This pH was determined by making up new mixtures and measuring the pH with the pH meter.

By considering the action of the enzymes on N-cbzo-α-L-glutamyl -L-glutamic acid at pH 5.5, the enzymes were divided into three groups:-

(a) those that gave very little or no hydrolysis of the substrate in 24 hours - P.R.L. 26, 316 and 376.

(b) those that gave intermediate hydrolysis in 24 hours - P.R.L. 92, 319, and 369.

(c) those that gave very high hydrolysis - P.R.L. 79 and 86.

The enzymes P.R.L. 26, P.R.L. 86, and P.R.L. 369 were chosen on which to do further study.

Hydrolysis of some synthetic substrates by the enzymes P.R.L. 26 and P.R.L. 86 were attempted using phosphate buffer pH 6.0, and duplicate aliquots of the hydrolysates were analyzed at various time intervals. The results are shown in table IV.

Table IV

The Effect of Time on the Hydrolysis of Various Substrates with P.R.L. 86

Digest No.	Substrate	Enzyme	Percent Hydrolysis					
			1 hr	2½ hr.	3½ hr.	24 hr.	48 hr.	72 hr.
1	N-cbzo-α-L-glutamyl-DL-alanine	26	0	0		0		
2	N-cbzo-α-L-glutamyl-DL-alanine	86			12.4	56	65.5	69.5
3	N-cbzo-α-L-glutamyl-glycine	26	0	0		0		
4	N-cbzo-α-L-glutamyl-glycine	86			1.9	11.5	18.3	23.8
5	α-L-glutamyl-L-glutamic acid diethyl ester	26	0	0		0		
6	α-L-glutamyl-L-glutamic acid diethyl ester	86			10.95	57.5	77	85.3

The curves for the three mixtures which gave hydrolysis are shown on Figure 2.

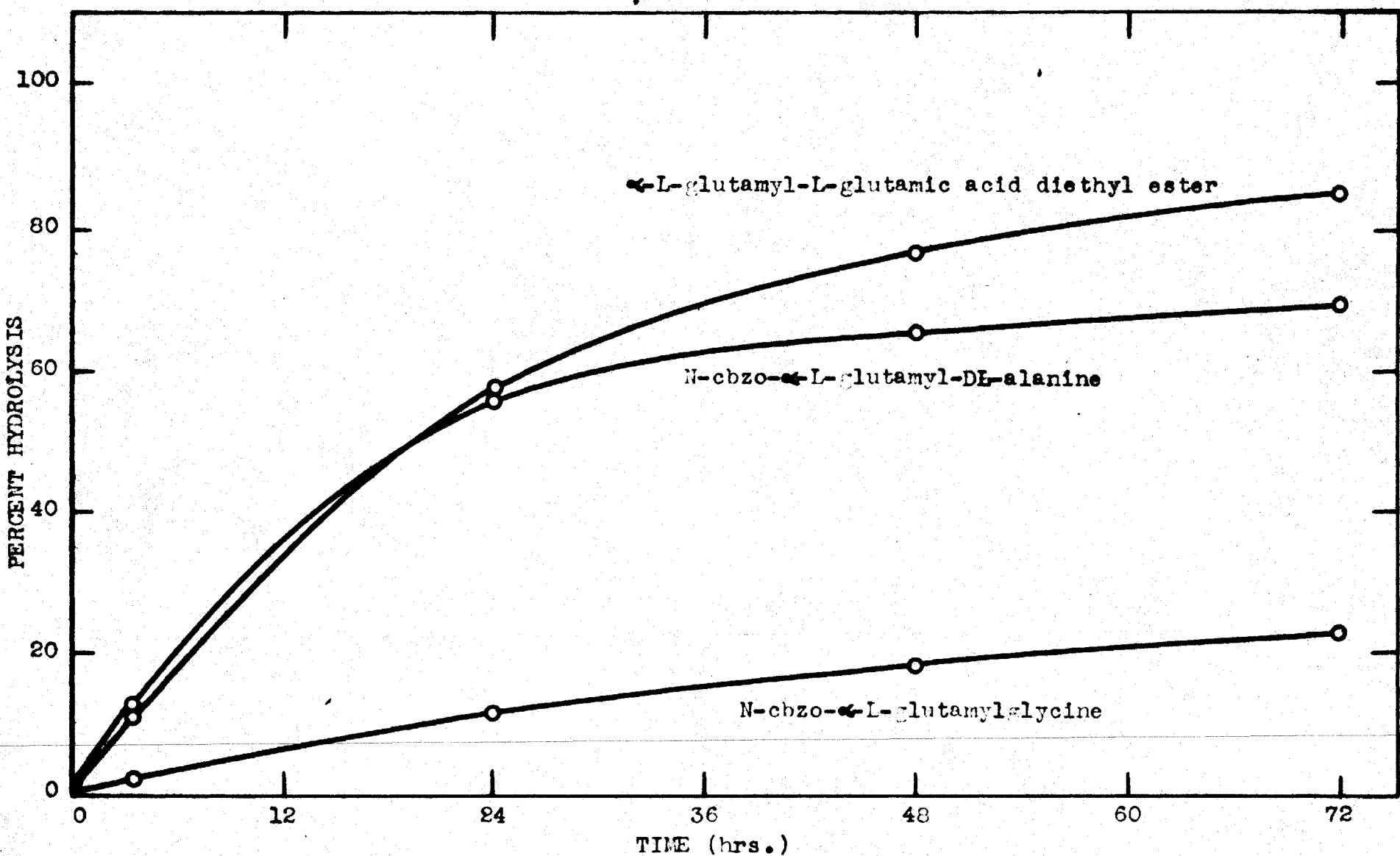


Fig. 2. The effect of time on the hydrolysis of various substrates by P.R.L. 86.

The activity of the three enzymes P.R.L. 26, 86, and 369 was checked again against the substrate ∝-L-glutamyl-L-glutamic Acid.

Table V

Enzymes at Different pH against ∝-L-glutamyl-L-glutamic Acid

pH of hydrolysate	Enzyme	Percent Hydrolysis in 24 hours
3.65	P.R.L.26	2.6
4.18	26	3.6
6.30	26	8.1
7.23	26	5.6
3.50	P.R.L.86	2.3
4.80	86	10.9
6.50	86	8.2
7.40	86	9.5
3.80	P.R.L.369	4.7
4.90	369	0.0
6.45	369	6.5
7.30	369	6.5

(iv) Effect of Buffer Systems on Enzyme Activity

In order to get a longer range of buffer systems, McIlvaine's phosphate-citrate buffer at pH 6 was used and compared with the phosphate buffer at pH 6. The results are shown in table VI.

Table VI

The Effect of Buffer on Enzyme Activity

Substrate	Enzyme	Percent Hydrolysis in 24 hours	
		Phosphate buffer	Phosphate-citrate buffer
N-cbzo-α-L-glutamyl-DL-alanine	PRL 86	56%	37%
N-cbzo-α-L-glutamyl-glycine	PRL 86	11%	0%
α-L-glutamyl-L-glutamic acid diethyl ester	PRL 86	57%	33%

A phosphate-acetate buffer was also tried but satisfactory duplicates could not be obtained. Therefore a check was made on the different constituents of the digest solutions. For this aliquots of the stock solutions were added to the outer chamber of the cell and the ninhydrin analysis carried out as usual. The results are shown in table VII.

Table VII

Sample	Burette reading	Back titration of excess base
0.1 ml. N-cbzo-α-L-glutamyl	22.58	2.42
-L-glutamic acid	22.42	2.58
0.1 ml. phosphate-acetate	24.62	0.38
buffer pH 7.0	25.00	___
0.2 ml. H_2O	21.93	3.07
	22.07	2.93

It was concluded that some acid other than carbon dioxide was being collected in the alkali. Therefore the results using that buffer solution were discarded.

It was decided to use phosphate buffers for the range that could be obtained with them and to use phthalate buffers to obtain lower pHs.

(v) Effect of pH on Enzyme Activity

The pH optimum for the action of P.R.L. 86 on N-cbzo-α-L-glutamyl-L-glutamic Acid was determined. The data is given in table VII, and the curves are shown on Figure 3. The solutions were made up as previously described and incubated at 37°C. for 24 hours. The pH of the individual hydrolysates was measured. Each point is the mean value of duplicate determinations. The data is shown in table VIII and on Figure 3.

The pH optimum was found to be 4.85

Table VIII

The Effect of pH on the Activity of P.R.L. 86 on N-chzo-α-L-glutamyl-L-glutamic Acid

Buffer	pH	Percent Hydrolysis in 24 hours
phthalate	3.47	0
	4.03	15.2
	4.30	48.2
	4.50	67.2
	4.85	74.4
	5.00	71.5
	5.25	61.3
	5.30	61.5
	5.50	57.2
	5.65	48.8
phosphate	4.85	84.1
	5.35	81.5
	5.90	70.4
	6.0	65.4
	6.5	52.2
	7.0	50.5
	7.5	40.2
	8.0	38.0

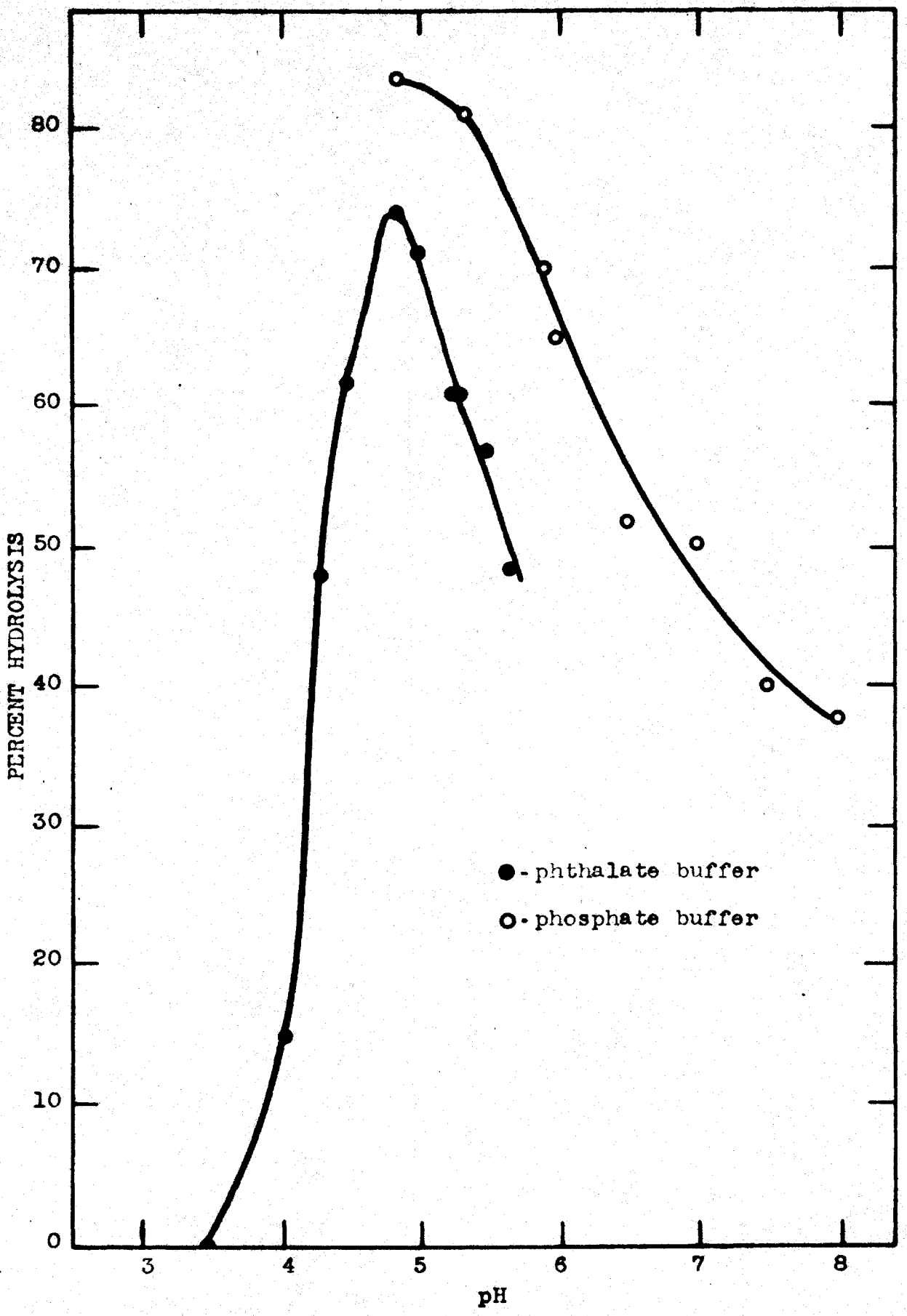


Fig. 3. The effect of pH on the activity of P.R.L.86 against N-cbzo-α-L-glutamyl-L-glutamic acid.

The pH optimum for P.R.L. 86 on N-cbzo-α-L-glutamyl-DL-alanine was determined. The data is given in table IX and presented graphically on Figure 4.

Table IX

The Effect of pH on the Activity of P.R.L. 86 on N-cbzo-α-L-glutamyl-DL-alanine

Buffer	pH	Percent Hydrolysis in 24 hours
phthalate	2.80	1.0
	3.75	2.5
	4.12	20.2
	4.45	52.2
	4.83	55.0
	5.15	54.5
	5.25	53.5
	5.50	49.7
	5.70	52.2
	5.80	45.0
phosphate	5.40	69.0
	5.80	59.1
	6.20	52.9
	6.25	56.5
	6.70	46.3
	7.02	41.6
	7.25	36.0
	7.45	32.6

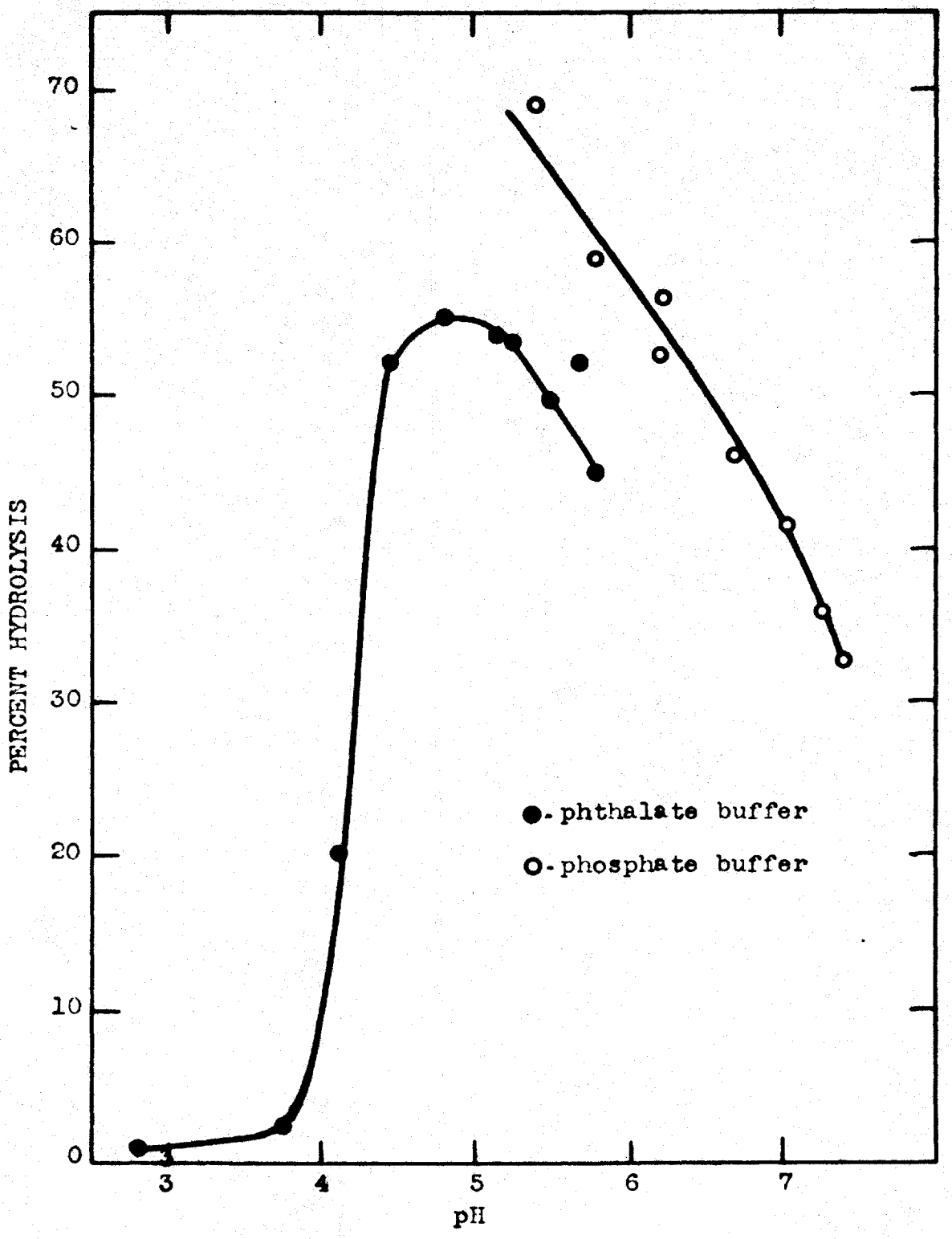


Fig. 4. The effect of pH on the activity of P.R.L.86 against N-cbzo-α-L-glutamyl-DL-alanine.

The pH optimum was found to be 4.85.

The pH optimum was also determined for the action of P.R.L. 369 on N-cbzo-α-L-glutamyl-DL-alanine. The data is presented on table X and the graph is shown on Figure 5.

Table X

The Effect of pH on the Activity of P.R.L. 369 against N-cbzo-α-L-glutamyl-DL-alanine

Buffer	pH	Percent Hydrolysis in 24 hours
phthalate	2.90	1.0
	3.97	5.0
	4.23	57.0
	4.50	58.5
	4.68	59.2
	5.18	53.2
	5.50	46.4
phosphate	5.90	17.8
	6.25	14.3
	6.55	11.9
	6.75	12.1
	6.90	10.3

The pH optimum was found to be 4.70.

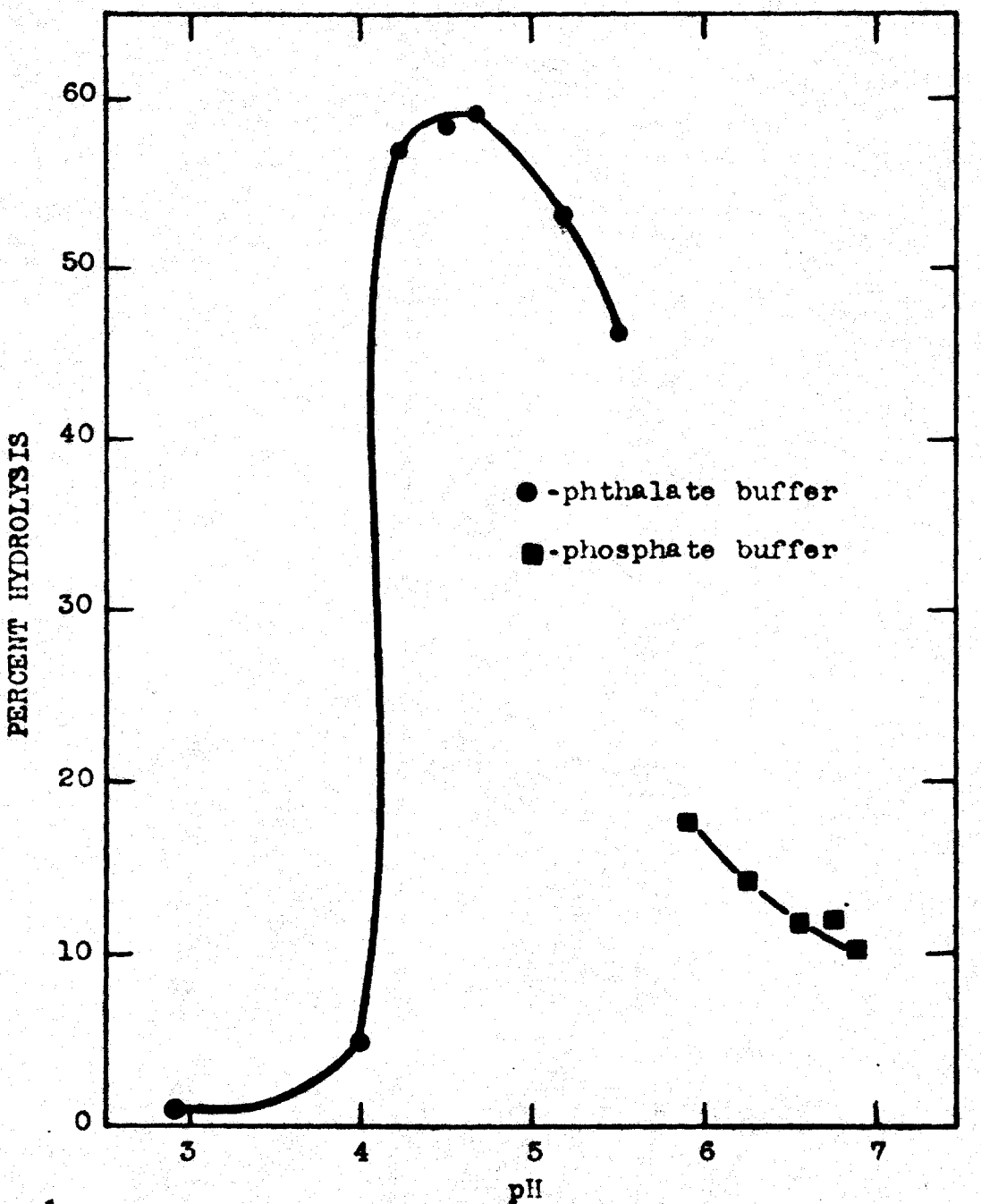


Fig. 5. The effect of pH on the activity of P.R.L.369 against N-cbzo-α-L-glutamyl-DL-alanine.

(vi) Effect of Temperature on the Enzyme Activity

The action of P.R.L. 86 on N-cbzo-α-L-glutamyl-L-glutamic Acid was studied at three different temperatures. Duplicate samples were taken at zero time and at suitable time intervals thereafter. In order to obtain the best possible evaluation of the straight line region all determinations were plotted rather than the mean of duplicate samples as previously described. The best possible straight line was drawn through the points at the shorter time intervals to give the slope used in the later calculations. The data obtained from the hydrolysis is presented in table XI and the curves are shown on Figure 6.

The study of the rate of reaction of P.R.L. 86 against N-cbzo-α-L-glutamyl-DL-alanine was done at three different temperatures. The data obtained was plotted in the same way as described for the first temperature study. The data is presented in table XII and the graph is shown on figure 7.

The results of a study at different temperatures of the rate of reaction of P.R.L. 369 against N-cbzo-α-L-glutamyl-L-glutamic Acid are shown in table XIII and on Figure 8.

Table XI

P.R.L. 86 against N-cbzo-α-L-glutamyl-L-glutamic acid.

Time	Percent Hydrolysis at different temperatures		
	36.6°C.	25.0°C.	9.5°C.
½ hr.	5.0 4.7		
1 hr.	10.2 8.7	1.2 0.9	
1½ hrs.	12.0 11.0		4.7 2.8
2 hrs.	16.8 15.5	10.5 6.9	
2½ hrs.	20.6 19.9		
3 hrs.	24.2 27.4	12.4 12.4	5.9 4.7
3½ hrs.	27.4 26.1		
4 hrs.	32.0 28.6	12.1 12.4	
4½ hrs.	32.0 29.8		8.7 6.9
5 hrs.	32.0 ——	—— 17.4	
6 hrs.	38.9 ——	20.6 19.0	6.9 5.3
14½ hrs.	62.0 ——	32.2 33.6	11.5 10.0
Slope	7.92	3.37	1.40

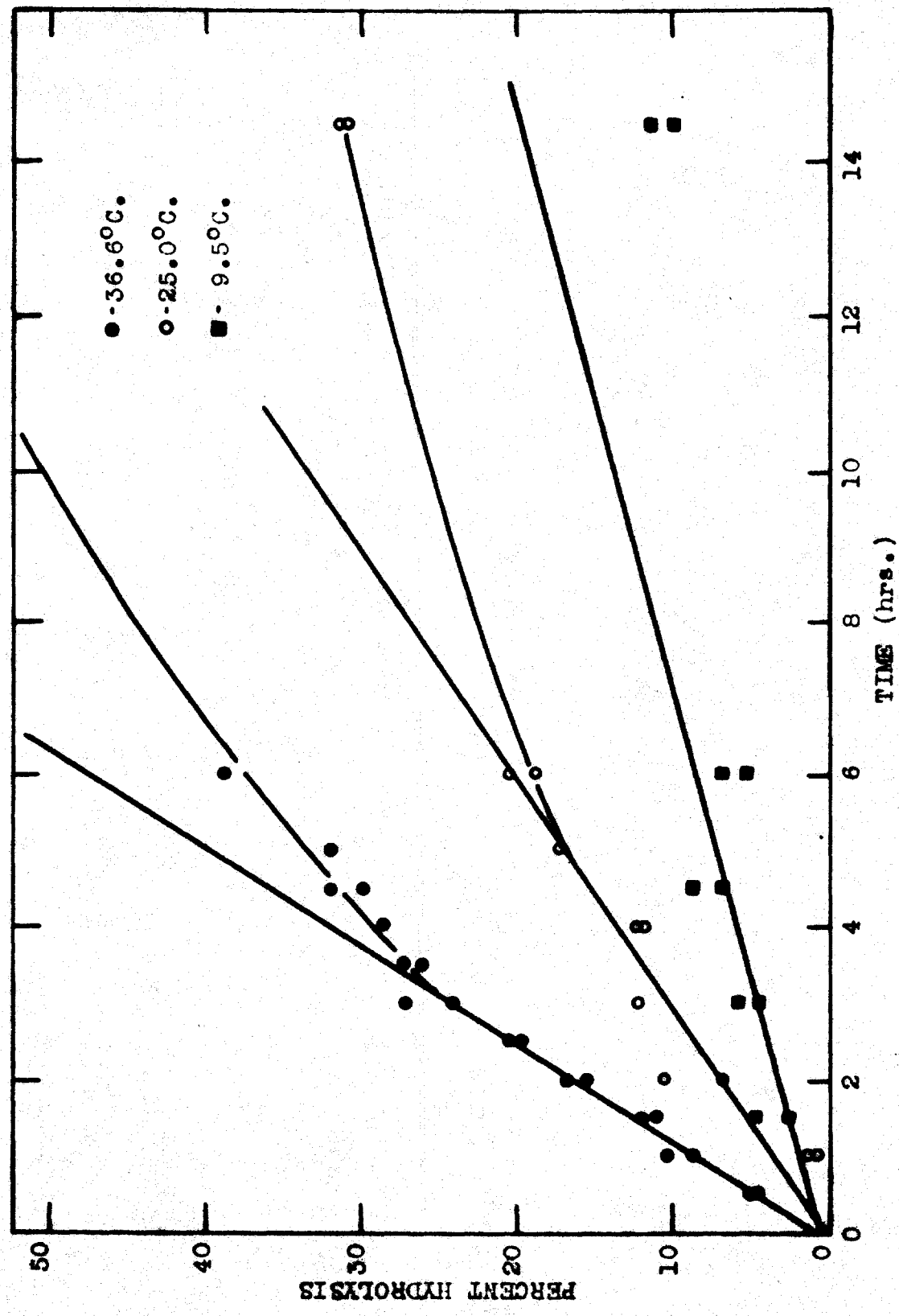


Fig. 6. The effect of temperature on the action of P.R.L.86 against N-cbzo-α-L-glutamyl-L-glutamic acid.

Table XII

P.R.L. 86 against N-cbzo-α-L-glutamyl-DL-alanine

Time	Percent Hydrolysis at the different temperatures		
	36.6°C.	25.0°C.	14.5°C.
1 hr.	—— 6.7	2.2 1.2	
2 hrs.	10.9 10.6	—— 5.6	1.9 1.2
3 hrs.	14.6 14.0	6.9 6.2	
4 hrs.	20.2 19.0	9.0 ——	3.1 2.5
5 hrs.	23.3 22.7	9.3 10.6	
6 hrs.	24.9 27.8	15.2 14.0	6.7 5.6
8 hrs.	31.1 32.4	16.5 15.9	6.5 7.8
13 hrs.	42.2 42.6	25.3 25.3	11.8 13.4
Slope	4.35	2.20	1.1

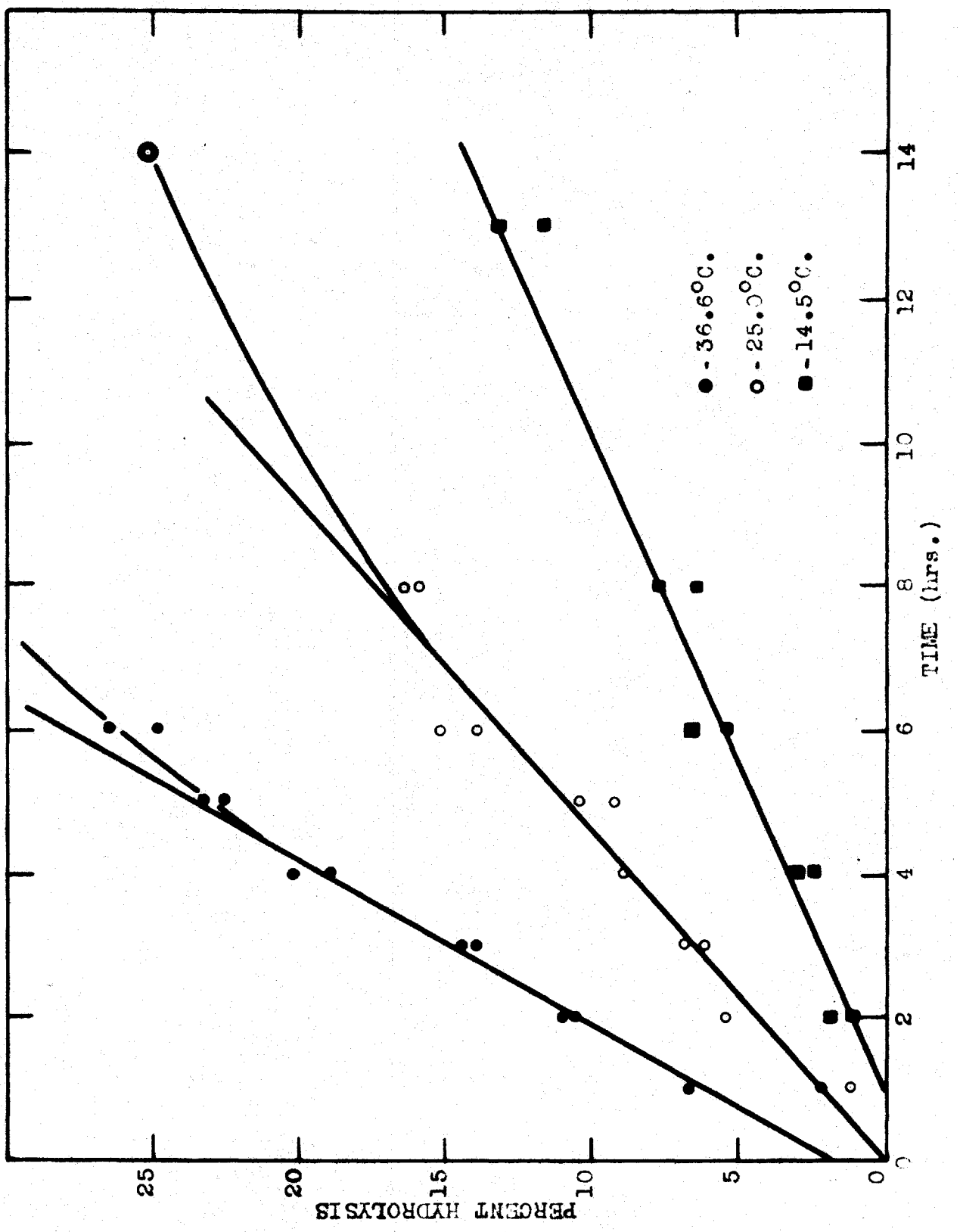


Fig. 7. The effect of temperature on the action of P.R.I. 86 against N-cbzo-◄L-glutamyl-DL-alanine.

Table XIII

P.R.L. 369 against N-cbzo-α-L-glutamyl-L-glutamic Acid

Time	Percent Hydrolysis at different temperatures		
	36.6°C.	25.0°C.	14.5°C.
1 hr.	7.5 10.0	3.1 3.4	
2 hrs.	9.3 10.2	3.1 3.1	2.8 3.4
3 hrs.	11.2 10.8	5.6 6.2	
4 hrs.	14.0 14.6	8.7 10.0	4.4 —
5 hrs.	18.6 17.4	9.3 8.7	
6 hrs.	20.8 20.2	10.9 10.9	5.9 5.3
8 hrs.	25.5 24.0	11.2 12.1	5.0 4.3
13 hrs.	35.5 34.1	18.0 17.4	6.9 6.9
Slope	3.55	1.40	0.55

The data from the rates of reaction at different temperatures is shown summarized in tables XIV, XV, and XVI with the calculations of the energy of activation.

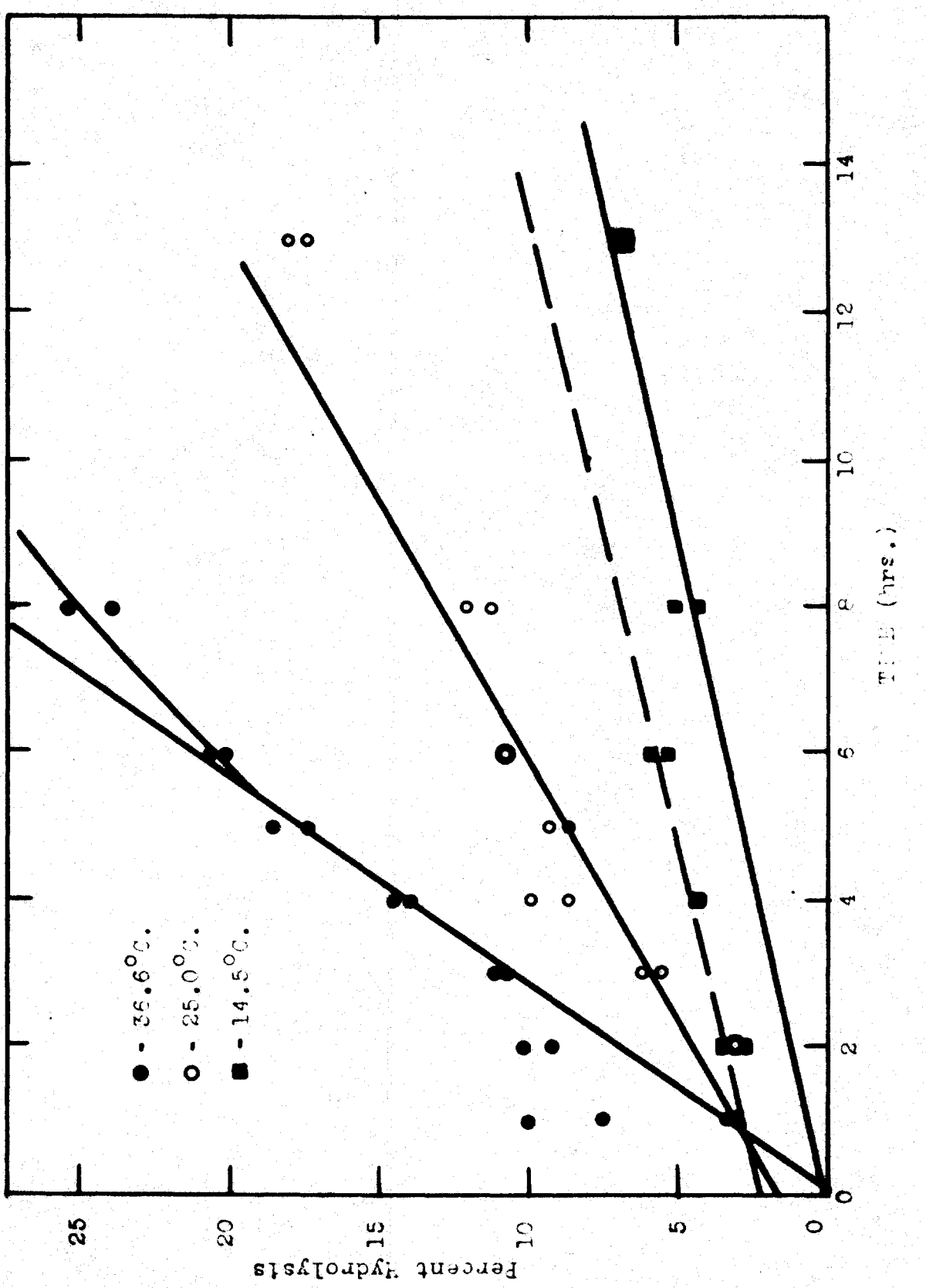


Fig. 8. The effect of temperature on the action of P.F.L 369 against N-cbzo-α-L-glutamyl-L-glutamic acid.

Table XIV

P.R.L. 86 against N-cbzo-α-L-glutamyl-L-glutamic Acid

	T_1 = 36.6°C.	T_2 = 25.0°C.	T_3 = 9.5°C.
Slope	7.92	3.37	1.40
Log Slope	0.899	0.527	0.146
1/T	32.3×10^{-4}	33.6×10^{-4}	35.4×10^{-4}
ln Slope	2.07	1.215	0.336

$$E = \frac{4.56 \times (\log \text{slope}_1 - \log \text{slope}_2)}{(1/T_2 - 1/T_1)}$$

$$E_{(T_1,T_2)} = \frac{4.56 \times 0.372}{1.3 \times 10^{-4}} = 13,050 \text{ calories}$$

$$E_{(T_2,T_3)} = \frac{4.56 \times 0.381}{1.8 \times 10^{-4}} = 9,650 \text{ calories}$$

Table XV

P.R.L. 86 against N-cbzo-α-L-glutamyl-DL-alanine

	T_1 = 36.6°C.	T_2 = 25.0°C.	T_3 = 14.5°C.
Slope	4.35	2.20	1.1
Log slope	0.639	0.343	0.042
1/T	32.3×10^{-4}	33.6×10^{-4}	34.8×10^{-4}
ln slope	1.47	0.788	0.0965

$$E_{(T_1,T_2)} = \frac{4.56 \times 0.296}{1.3 \times 10^{-4}} = 10{,}400 \text{ calories}$$

$$E_{(T_2,T_3)} = \frac{4.56 \times 0.301}{1.2 \times 10^{-4}} = 11{,}450 \text{ calories}$$

Table XVI

P.R.L. 369 against N-cbzo-α-L-glutamyl-L-glutamic Acid

	T_1 = 36.6°C.	T_2 = 25.0°C.	T_3 = 14.5°C.
Slope	3.55	1.40	0.55
Log slope	0.550	0.146	-0.260
1/T	32.3×10^{-4}	33.6×10^{-4}	34.8×10^{-4}
ln slope	1.27	0.336	-0.600

$$E_{(T_1,T_2)} = \frac{4.56 \times 0.404}{1.3 \times 10^{-4}} = 14{,}200 \text{ calories}$$

$$E_{(T_2,T_3)} = \frac{4.56 \times 0.406}{1.2 \times 10^{-4}} = 15{,}400 \text{ calories}$$

In Figure 9 values of ln slope are plotted vs. 1/T to give the Arrhenius line.

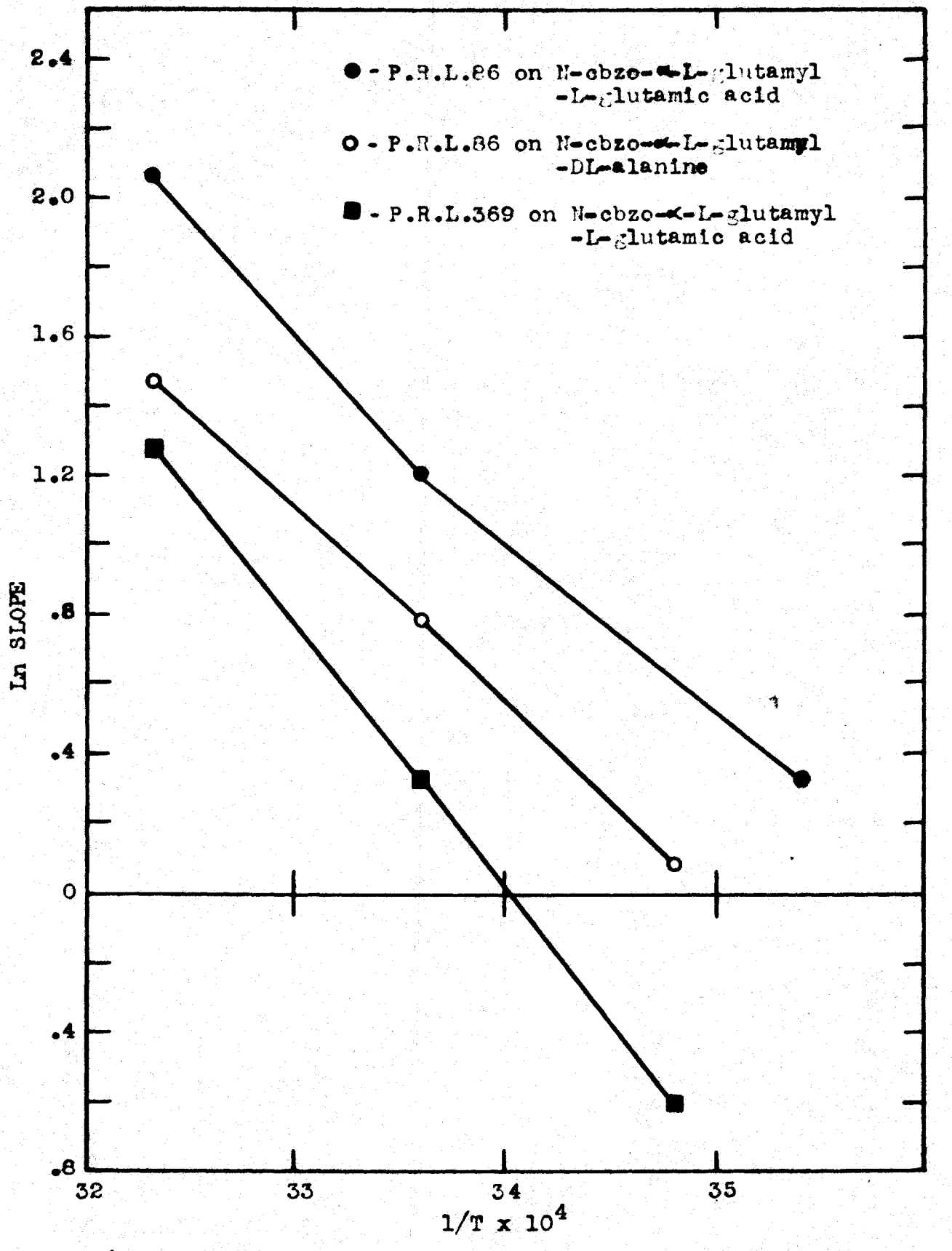


Fig. 9. The Arrhenius plot for the action of two enzymes.

(vii) Apparent Self-Hydrolysis of the Enzymes

Previous work with the fungal enzymes indicated that no self digestion had occurred. Since some work with pepsin and pancreatin was planned, it was thought desirable to check that possibility with all of the enzymes. In order to do this, digestion tubes were incubated for 24 hours using water in place of the substrate solutions. Duplicate samples were analyzed at zero time and 24 hours. The data obtained is shown in table XVII.

Table XVII

Self-Hydrolysis of the Enzymes

Enzyme	Buffer pH	Increase in titer in 24 hours	Mean of duplicate	Equivalent percent Hydrolysis
pepsin	3.0	0.07 0.17	0.12	37%
pancreatin	8.0	0.59 0.57	0.58	14.9%
P.R.L. 86	5.0	-0.08 -0.06	-0.07	0
P.R.L. 369	5.0	-0.10 -0.05	-0.08	0

(viii) Activity of the Enzymes on Poly-γ-Methyl-L-Glutamate

It was of interest to test the activity of the enzymes used here against a poly-L-glutamic acid derivative*. The average chain length of the polymer was estimated by end group analysis

*The sample of poly-γ-methyl-L-glutamate was supplied by Mr. L. Wiseblatt, University of Saskatchewan.

to be eight units. The results of the action of the enzymes on this polymer are shown in table XVIII.

Table XVIII

Action of the Enzymes on Poly-γ-Methyl-L-Glutamate

Enzyme	pH	Increase in titer from 2 - 22 hours	Apparent percent Hydrolysis
pancreatin	8.5	0.36 0.43	12.4
pepsin	3.0	-0.01 -0.06	0.9
P.R.L. 86	5.7	-0.02 -0.04	0.0
P.R.L. 369	5.7	0.23 0.26	7.8

(ix) Activity of Pepsin and Pancreatin on Dipeptide Derivatives

It was also of interest to find out whether the enzymes, pepsin, or pancreatin would attack the dipeptide derivatives that were studied with the other enzymes. Pepsin (Nutritional Biochemicals Corporation, Cleveland, Ohio), and pancreatin (Takamine Laboratory, Inc., Clifton, N.J.) were each made up to a concentration of 200 mg. per 10 ml. of solution and used for hydrolysis studies as previously described. The results are shown in table XIX.

Table XIX

The Action of Pepsin and Pancreatin on some Dipeptide Derivatives

Substrate	Enzyme	pH of digest	Apparent percent Hydrolysis
N-cbzo-α-L-glutamyl-L-glutamic acid	pepsin	2.7	0
	pepsin	4.6	0
	pancreatin	7.5	17.9
	pancreatin	4.4	0
α-L-glutamyl-L-glutamic acid diethyl ester	pepsin	2.3	0
	pepsin	4.6	0
	pancreatin	4.7	0
	pancreatin	8.0	-
N-cbzo-α-L-glutamyl-DL-alanine	pepsin	2.5	0
	pepsin	4.6	0
	pancreatin	7.5	13.7
	pancreatin	4.7	0

(x) The Effect of Cobalt as a Metal Activator

Cobalt is a known metal activator and has been used in some of the previous work that has been done with these fungal enzymes. Since it was necessary to determine the effect of several other variables, first it was decided to do all the hydrolyses in the presence of a standard amount of $CoCl_2$ (0.001 M.) However, it

was desirable to determine whether the cobalt was actually necessary. Table XX shows the comparison of the action of P.R.L. 86 on N-cbzo-α-L-glutamyl-L-glutamic acid in the presence and absence of cobalt. The data was obtained by duplicate samples of duplicate parallel hydrolysates. The hydrolyses were done at 37°C. and pH 5.0 for 24 hours.

Table XX

The Effect of Cobalt on Hydrolysis by P.R.L. 86

Hydrolysate number	Cobalt added or absent	Percent Hydrolysis
1	added	81.3
2	added	84.3
3	absent	84.2
4	absent	84.5

4. DISCUSSION and CONCLUSIONS

(i) Preliminary Survey of the Enzymes

Table II shows the results of the first hydrolysis experiments. It is noteworthy that the enzyme P.R.L. 79 attacks N-cbzo-glycyl-glycine more readily than it does N-cbzo-α-L-glutamyl glycine. Glycylglycine was attacked only very slightly. This is in agreement with hydrolysis data reported in the literature for the non-fungal proteolytic enzymes.

N-cbzo-α-L-glutamyl-DL-alanine was hydrolyzed more than the 50% that might be expected if steriospecificity was an absolute requirement for any enzymatic action. This observation was confirmed by results shown later. It is also in accord with conclusions Bergmann drew from his studies on the specificity of proteolytic enzymes (30, 34).

Table III shows the results of the more complete survey of activity of several of the enzymes against the various substrates involving L-glutamic acid. As mentioned earlier, the pH control was found to be quite inadequate because the base that was added to dissolve the substrate apparently was too strong for the capacity of the buffer. For later work the buffer concentration was increased five fold so that in the hydrolysis mixture it was 0.2 M. The grouping of the enzymes on the basis of these results has already been discussed. The table only served to group the enzymes in order to pick out a few which gave characteristic activities against the substrates.

The action of P.R.L. 26 and P.R.L. 86 on the three substrates, N-cbzo-α-L-glutamyl glycine, N-cbzo-α-L-glutamyl-DL-alanine, and α-L-glutamyl-L-glutamic acid diethyl ester, was compared. P.R.L. 26 has shown no peptidase action against either the cbzo-dipeptides or the dipeptide ester. This may be compared with the relatively high protease activity of this enzyme as reported by McConnell (41).

P.R.L. 86, which was shown previously to have high protease activity, is also shown here to have high peptidase activity against the substrates used. P.R.L. 86 has shown somewhat higher activity toward N-cbzo-α-L-glutamyl glycine than it did before, but only reached 23.8% hydrolysis after three days.

A comparison of the action of this enzyme against α-L-glutamyl-L-glutamic acid diethyl ester and N-cbzo-α-L-glutamyl-DL-alanine is shown on the graph in Figure 2. It was observed that they are very nearly the same up to just over 50% hydrolysis of the substrates. After that, the action on N-cbzo-α-L-glutamyl-DL-alanine decreases at somewhat greater rate than the action on the dipeptide ester. There appears to be a break in the curve for the former substrate which may be due to a marked difference in the rates of hydrolysis of the peptides that contain the D and the L forms of alanine, while the curve for the action on the dipeptide ester shows only a gradual decrease in the rate. This is suggestive of two separate phases of the reaction on the dipeptide in which the DL-alanine is present.

Table V shows the results of the action of the enzymes P.R.L. 26, 86, and 369 against α-L-glutamyl-L-glutamate at different pH's. In the earlier work this substrate showed no hydrolysis with any of the enzymes used. It was desirable to show that such was the case over a range of pH's. The three enzymes gave very low hydrolysis over a suitable pH range. This indicates that a dipeptidase

which will attack this substrate was present in only very low concentration, if at all. These low hydrolyses may also be compared with conclusions drawn in the literature that peptides containing only one amino acid are hydrolyzed less readily than peptides of more than one amino acid (3).

(ii) Effect of Buffers on the Enzyme Activity

When phosphate-citrate buffer mixture was used less hydrolysis was observed than was expected. Therefore, the action of P.R.L. 86 was compared using the two buffer systems at the same pH. Table VI shows that the citrate exhibited an inhibitory effect upon the hydrolysis reaction. It is possible that the presence of the citrate, in the concentration used, effectively formed complexes with the metals in the solutions that might otherwise have acted as trace metal activators. These metals would be supplied either by the salts added for the growth of the organism, or in the nutrients supplied at the time of growth. $CoCl_2$ was added for these hydrolyses, but it was shown later that it was not essential to the reaction with P.R.L. 86.

A phosphate acetate buffer was also tried, but as shown in table VII the buffer was unsatisfactory. From the results it was concluded that an acid other than carbon dioxide was being collected in the alkali during the ninhydrin reaction. Since it was shown that the interfering acid was coming from the buffer, it is highly

probable that it was free acetic acid. Because the reaction is not reproduceable, under these conditions, it is unlikely that a satisfactory correction could be applied for it.

(iii) The Effect of pH on the Enzyme Activity

The determination of pH optimum for the action of P.R.L. 86 on two of the substrates and for P.R.L. 369 on one substrate are shown in tables VIII, IX, and X, and on Figures 3, 4, and 5. The pH optimum was found to be pH 4.85 for P.R.L. 86 on N-cbzo-α-L-glutamyl-DL-alanine and for the action of P.R.L. on N-cbzo-α-L-glutamyl-L-glutamic acid. It was found to be pH 4.70 for the action of P.R.L. 369 on N-cbzo-α-L-glutamyl-L-glutamic acid. There is no significant difference in the optimum found for the three combinations. The fact that the pH optimum is so low was of particular interest. The optimum for the action of peptidases reported in the literature has in many cases been at a pH from 7.0 to 7.5 (32, 33, 34, 35, 36, 48).

The effect of the different buffer systems has also been demonstrated in Figures 3 and 4. In general, it appears that the enzymatic activity is greater in the presence of the phosphate buffer. The observed effect of changing the buffer system does not, however, affect the conclusions about the pH optimum. Where sufficient data is available, it appears that the activity would be optimal at the same pH with both buffer systems. The data presented in Figure 5

is such that no inference can be drawn regarding the relative effects of the buffer used.

(iv) Effect of temperature on Enzyme Activity

On examination of the tables XI, XII, and XIII, and the curves on Figures 6, 7, and 8 it was considered that the data for the rate of reaction at the lowest temperature was not accurate enough to be used. However, the straight lines from which the slopes are calculated do fit the data somewhat better for the temperatures 36.6°C. and 25°C. The activation energies based on measurements at these two temperatures show a difference of 2600 calories using the same enzyme with two different substrates. Using the two enzymes on the same substrate, a difference of 1100 calories in the activation energy was observed. It would appear that the activation energy is more characteristic of the substrate than the enzyme, or that the same enzyme is present in both crude mixtures. The latter is in agreement with the observation that the pH optimums were to be found almost the same.

(v) Apparent Self-Hydrolysis of the Enzymes

Since the proteases are proteins, it was thought that in the complex mixtures obtained from the culture media, there might be a tendency for them to hydrolyze themselves or each other. It would be quite reasonable to suppose that, if a proteinase were present with a peptidase, the former might attack the latter. Table XVII has

shown that hydrolysis to free amino acids did not occur for the enzymes P.R.L. 86 and P.R.L. 369. This may be explained by either of two alternatives. The crude mixture contained a single enzyme which acts either as a proteinase or a peptidase, depending on the pH of the medium. Alternatively, the proteinase did not exhibit appreciable activity at pH where the peptidase activity was measured. The optimum activity for the digestion of proteins by these two enzymes was shown by McConnell (41) to be about pH 7.

Pepsin showed a very small increase in the presence of free amino acids, and pancreatin showed considerable increase. It is not known whether this was auto digestion of the enzyme or hydrolysis of a protein impurity in the enzyme. The increase was reported in the table as calculated on the basis of a substrate present. However, on the basis of the solid enzymes being one hundred percent protein, this represents about two percent of the protein hydrolyzed to amino acids.

(vi) Activity of the Enzymes on the Poly-γ-methyl-L-glutamate

On the basis of the above observations and after examination of table XVIII, it was concluded that no significant hydrolysis of poly-γ-methyl-L-glutamate to free amino acids was observed with pepsin at pH 3.0 or with pancreatin at pH 8.5. This is in accord with the work of Summer (1) and Blakley (3). Similarly P.R.L. 86, the enzyme showing the highest peptidase activity of those enzymes

studied, did not attack that substrate. P.R.L. 369 did liberate about 8% of the glutamic acid in the form of the monomer. Since this method does not take account of any dipeptides or polypeptides produced in the hydrolysis, it is possible that there was more degradation of the polymer than is indicated by this percentage.

(vii) Activity of Pepsin and Pancreatin on Dipeptide Derivatives

The action of pepsin and pancreatin was tested against three of the dipeptide derivatives. Each enzyme was tested near the pH known to be its optimum and at the pH found to be the optimum for the fungal enzymes studied here. Pepsin was found to have no effect on the substrates used. That was expected since pepsin is known to exhibit mainly proteinase activity (50).

In the experiments where pancreatin was used in the presence of a substrate, the increase of free amino acids was small compared to the blank. For some unknown reason, the results obtained with pancreatin were always more erratic than with the other enzymes. Therefore, it was impossible to conclude that there was any hydrolysis of these substrates by pancreatin, even though it is known to exhibit high peptidase activity (51).

(viii) The Effect of Cobalt as a Metal Activator

The data in table XX shows that the addition of cobalt does not affect the peptidase activity of the crude enzyme P.R.L. 86

(ix) Summary

A group of seven dipeptide derivatives have been synthesized using known methods, and some conclusions have been drawn regarding the relative merits of certain alternative procedures.

Several proteases were observed to exhibit high peptidase activity, and the rates of digestion of the dipeptide derivatives by a particular enzyme were found to be quite different from each other. Another significant observation was that a given substrate was hydrolyzed at different rates by different enzymes. Similarities in the action of the enzymes were shown by the determination of the pH optimum and the effect of temperature on the rates of hydrolysis. Information has also been obtained about the effect of the various buffer systems and the use of cobalt as a metal activator.

Comparing the action of the mold proteases with two well-known animal proteases has shown marked differences in the pH optimum and in the specificity requirements.

A modified ninhydrin analysis procedure was tested for use in following the course of the enzymatic hydrolysis of synthetic dipeptide derivatives and was shown to be satisfactory.

SUGGESTIONS FOR FUTURE WORK

It would be of interest to test the activity of the various mold proteases on polymers of amino acids other than glutamic acid, and on copolymers. The observation that P.R.L. 369 hydrolyzed 8% of the polymer to the monomer suggests that it has some unique properties that would make it useful in the further study of gluten.

An extension of the work on the pH optimum of the various enzymes would be useful, especially with a wider range of dipeptide derivatives as substrates. Such information would also be required before results of the pH optimum studies could be used to draw conclusions regarding the number and nature of the peptidases that were present in a particular crude enzyme. The observations that even at a high pH some of the substrates were hydrolyzed and that the proteinase activity is optimal at higher pH may indicate that other pH optimums may be found and related to the substrate being attacked, and, therefore, correlated to a specific enzyme.

The fractionation, purification, and identification of the components of the proteolytic systems produced by the molds is another phase of the work which would be greatly accelerated by the use of a wider variety of synthetic substrates. In that way, it might be possible to isolate and identify the proteinases, the carboxypeptidases, the aminopeptidases, and the dipeptidases.

It would be of interest to clarify the effect that has been observed between the buffer systems. If the citrate and phthalate buffers were shown to give different inhibitory effects with different enzymes, it would become possible to infer the presence of different enzymes and assist in the classification.

No study has been done on the temperature stability of these peptidases. The inclusion of that with further study on the effect of temperature on the rates of reaction and with a study of activators and inhibitors would help in the ultimate identification of the enzymes. It is probable that the various proteases would react differently to at least some of these factors.

The partial hydrolysis and the identification of the peptides and the amino acids in the hydrolysate by paper chromatography has not been applied to wheat gluten. That approach would require the synthesis of a greater number of dipeptides in order to identify the products of the hydrolytic action. Both acid and enzymatic hydrolysis would be of use for the study. Information regarding the glutamyl linkages in the protein may be obtained in that way.

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