THE ACTIVATION OF CHYMOTRYPSINOGEN

ISOLATION AND IDENTIFICATION OF A PEPTIDE LIBERATED DURING ACTIVATION*

BY WILLIAM J. DREYER AND HANS NEURATH

(From the Department of Biochemistry, University of Washington, Seattle, Washington)

(Received for publication, May 12, 1955)

Reactions attending the conversion of precursors of proteolytic enzymes to the active form are distinguished by several features of unusual interest: (*a*) they are enzyme-catalyzed; (*b*) they are irreversible; (*c*) they involve the opening of a limited number of peptide bonds; (*d*) they provide an experimental system for the study of the final steps in formation of a biologically specific protein and of the relation of chemical structure to biological activity of proteins (3).

In recent studies in this laboratory (4), including the present work, several intermediates formed during the activation of chymotrypsinogen have been recognized by a combination of methods. These have included electrophoresis, end-group analysis, enzymatic activity, peptide analysis, and sedimentation. As a result of the present experimental study, which dealt in particular with the nature of the peptide released during the rapid activation of chymotrypsinogen, it has been possible to identify the nature of the limited number of peptide bonds hydrolyzed during the activation process.

EXPERIMENTAL

Materials

Chymotrypsinogen was prepared (5) from freshly frozen beef pancreas glands.[1] One of the preparations employed was recrystallized six times with ammonium sulfate, whereas the other was recrystallized seven times with ammonium sulfate and twice with ethanol (6).

α-Chymotrypsin was a twice crystallized preparation, made as previously described (5).

Trypsin was a twice crystallized sample, containing approximately 50 per

* Presented in part before the Forty-sixth annual meeting of the American Society of Biological Chemists at San Francisco, California, April 11–15, 1955 (1). A preliminary report has been published (2).

[1] We are indebted to The Wilson Laboratories, Chicago, Illinois, for a crude protein precipitate, obtained from freshly collected beef pancreas glands, which served as starting material for the isolation of chymotrypsinogen and α-chymotrypsin.

cent magnesium sulfate, obtained from the Worthington Biochemical Sales Company, Freehold, New Jersey. Prior to use, the protein was dialyzed against 0.001 N HCl and lyophilized.

β-Phenylpropionic acid (reagent grade) was obtained from the Eastman Kodak Company, Rochester, New York.

Ion exchange resins (Dowex 50 and XE-64) were obtained from The Dow Chemical Company and from Rohm and Haas.

DNP[2] derivatives of twenty-two amino acids were prepared by Mr. H. L. Pan according to published methods.

Methods

Moving boundary electrophoresis was carried out in the Spinco, model H, electrophoresis apparatus. Sodium acetate buffer, ionic strength 0.1, pH 4.97 (at 25°), was used. For adequate resolution of the components, electrophoresis for extended periods of time, up to 24 hours, was necessary; electrolytic compensation (7) was employed to maintain the boundaries within view at all times during the electrophoretic experiment. In order to minimize proteolytic changes during the electrophoretic analysis, DFP was added to the activation mixtures to convert the active enzymes into the inactive diisopropylphosphoryl derivatives.

Paper electrophoresis was carried out with an apparatus similar to that described by Durrum (8); strips of Whatman No. 1 filter paper, 1.5 inches wide, were used.

Chymotrypsin activity was determined under conditions similar to those described in recent publications (9) with acetyl-L-tyrosine ethyl ester as substrate.

Activation—The composition of rapid activation mixtures was as follows: chymotrypsinogen 40 mg. per ml., trypsin 1.2 mg. per ml., sodium phosphate buffer, pH 7.8, 0.05 M. Activation was carried out at 0°. In certain cases the activation mixtures also contained 0.1 M sodium β-phenyl propionate. Prior to activation, chymotrypsinogen was dialyzed for 30 hours against 0.001 N HCl in order to remove all traces of peptides or amino acids. The solutions were then passed slowly through a column containing Dowex 50 ion exchange resin, X4, 20 to 50 mesh, in the hydrogen form.

Protein concentrations were determined spectrophotometrically; extinction coefficients were $E^{1\%}_{282} = 20.6$ for chymotrypsinogen (10) and $E^{1\%}_{280} = 14.4$ for trypsin (11).

[2] The following abbreviations are used: DNP, dinitrophenyl; DFP, diisopropyl phosphofluoridate; TCA, trichloroacetic acid.

Results

Preliminary Identification of Activation Peptide

Rapid activation mixtures were prepared, and activation was terminated by addition to a suspension of Dowex 50 ion exchange resin, X4, 20 to 50 mesh, in the hydrogen cycle (12). Material adsorbed on the resin was eluted with 8 N ammonium hydroxide and subjected to two-dimensional paper chromatography on Whatman No. 1 filter paper, with butanol-acetic acid-water (4:1:5) as solvent in one dimension and 2-butanone-*tert*-butanol-diethylamine-water (40:40:4:10) (13) in the second dimension. After development with ninhydrin in butanol and collidine (14), a pale yellow spot appeared which, upon steaming for 5 to 10 minutes, became purple. Several very faint purple spots also appeared, probably denoting the presence of minor amounts of other peptides arising from non-specific, secondary proteolytic degradations. The R_F value of the major peptide was in both solvent systems intermediate between those of arginine and lysine.

Amino Acid Analysis of Peptide Fraction of Activation Mixtures

In order to relate the appearance of a peptide to the activation process proper, it was necessary to demonstrate that the constituent amino acid residues of the peptide bear stoichiometric relationships to the amount of chymotrypsin formed. Experiments were, therefore, carried out to determine which amino acids were present in stoichiometric amounts in an acid hydrolysate of the peptide fraction. Such an analysis includes the constituents of all peptides, ninhydrin-positive or negative, and the results are independent of secondary degradations of the primary peptide products. The method thus places an upper limit to the size and composition of the peptide being determined.

In a typical experiment, a rapid activation mixture (65 minutes of activation at 0°) was treated with a 50-fold molar excess of 1 M DFP in isopropanol for 30 minutes and then added to an equal volume of 20 per cent TCA at 0°. After centrifugation, the supernatant fluid was decanted and the precipitate washed with cold 10 per cent TCA. The combined supernatant solutions were then extracted five times with ethyl ether and evaporated to dryness. After 24 hours of hydrolysis in 5.7 N HCl at 110° in sealed tubes, amino acid analysis was carried out by the procedure of Moore and Stein (15). A small amount of all amino acids known to be present in chymotrypsinogen (3) was found, but only two amino acids were present in quantities approximately equivalent to the amount of δ-chymotrypsin formed. These were serine and arginine. A similar

amino acid pattern was obtained when analysis was carried out on the peptides isolated from the activation mixture by the use of Dowex 50 ion exchange resin; however, only approximately 60 per cent of the theoretical yield of serine and arginine was obtained in this case.

Purification of Peptide on XE-64 Resin

In view of the success attending the use of XE-64 resin for the separation of basic peptides and proteins (16, 17), this resin was employed for the purification and quantitative estimation of the activation peptide.

The columns (0.9 × 15 cm.) were prepared according to the procedure of Hirs *et al.* (17). The method of gradient elution was used, by feeding 0.3 M citrate buffer, pH 5.3, into a 50 ml. mixing chamber containing the same buffer in 0.01 M concentration. Magnetic stirring was employed throughout the operation. In some experiments a 0.2 or 0.3 M buffer was applied directly to the column. The detergent BRIJ35 was added to all buffers prior to use (15). 1 ml. samples were collected in a fraction collector, and aliquots of the fractions were analyzed quantitatively with the ninhydrin reagent of Moore and Stein (18).

An activation mixture was prepared identical to that described in the preceding section dealing with the amino acid analysis. The supernatant solutions resulting from TCA precipitation were extracted five times with ethyl ether and then adjusted to pH 5.3 prior to application to the column. Fig. 1 is a typical chromatogram of peptides derived from the activation of 20 μmoles of chymotrypsinogen. The first major peak appearing was found to consist of a mixture of acidic and neutral peptides which were subsequently resolved on a Dowex 50 column, X2, 200 to 400 mesh, in the sodium cycle (11, 15). The lower chromatogram of Fig. 1 represents the resolution of the fraction collected in tubes 4 to 12 from the experiment represented by the upper chromatogram. It is clear that none of the acidic or neutral peptides occurs in amounts comparable to the peak representing the activation peptide (see below). It is likely that these peptides are the products of secondary degradation of the protein components present in the activation mixture.

The second major peak appearing on the XE-64 chromatogram was found to consist primarily of ammonia. This was ascertained by experiments in which the ninhydrin color of aliquots was determined before and after removal of ammonia by evacuation of samples treated with sodium hydroxide (19). Approximately 75 per cent of the material under this peak was lost by this treatment. It is likely that the ammonia was introduced during experimentation, since much less ammonia was seen in the chromatograms when the entire activation mixture was applied directly to the column (precipitation with TCA omitted).

The major peak appearing with an effluent volume of 50 to 60 ml. represents the peptide liberated during activation. It appears at the same effluent volume as free arginine. The true color yield of the peptide was determined on a molar basis by relating the differences in optical density of aliquots of various tubes within the peptide peak to the corresponding differences in the amount of Kjeldahl nitrogen. Assuming the

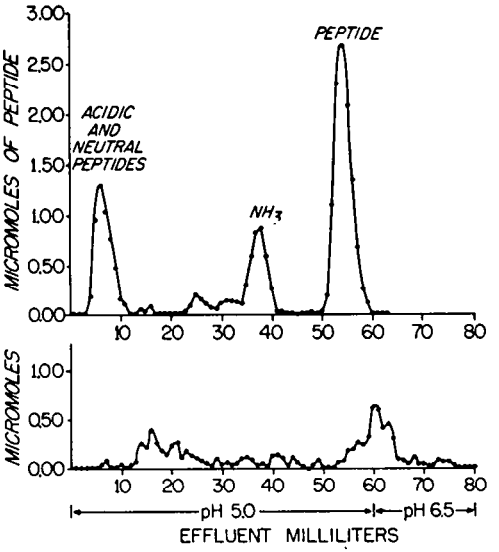

FIG. 1. Chromatogram of peptide fraction derived from the activation of 20 μmoles of chymotrypsinogen. In the upper chromatogram, XE-64 ion exchange resin was used, and in the lower chromatogram Dowex 50, X2, 200 to 400 mesh. The lower chromatogram represents the resolution of the fraction collected from aliquots of tubes 4 to 12 of the upper chromatogram. Since aliquots were taken, volume corrections were applied so as to render the ordinate values of the two chromatograms strictly comparable. The ninhydrin color value used for all components was that determined for the major peptide.

peptide to be serylarginine, the usual ninhydrin procedure yielded an optical density of 2.36 for a solution containing 1 μmole of peptide in a final volume of 8 ml. This corresponds to a color value of 0.91 on a molar basis relative to leucine.

Identification of Peptide

Paper chromatograms of an acid hydrolysate of the purified peptide (24 hours hydrolysis in 5.7 N HCl in sealed tubes) revealed only two ninhydrin-positive spots corresponding in R_F values (butanol-acetic acid-water) and color to serine and arginine. A quantitative analysis of the

acid hydrolysate on XE-64 columns, as previously described, yielded approximately 1 mole of each, serine and arginine, per mole of peptide.

Following periodate oxidation, the peptide gave a positive Nessler test for ammonia, thus indicating that serine occupied the *N*-terminal position in the dipeptide. The presence of arginine was confirmed by a positive Sakaguchi test in the intact peptide. In view of the destruction of tryptophan during acid hydrolysis, it was important to note that the unhydrolyzed peptide gave a negative test for tryptophan (20).

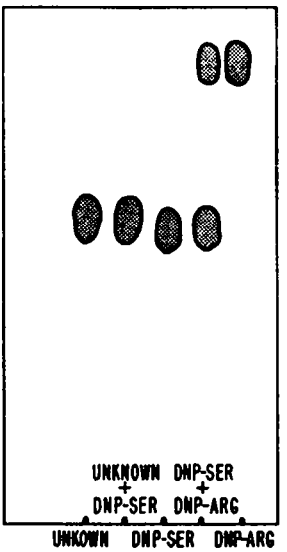

FIG. 2. Artist's representation of a paper chromatogram of an acid hydrolysate of DNP peptide (see the text). The solvent was *tert*-amyl alcohol saturated with 0.05 M phthalate buffer, pH 6.5. The papers were sprayed with phthalate buffer prior to use.

When the peptide was allowed to react with 2,4-dinitrofluorobenzene and subsequently hydrolyzed, only one ether-soluble DNP amino acid was found on paper chromatograms, namely DNP-serine. The results are shown in the chromatogram represented by Fig. 2. The aqueous layer was colorless, indicating the absence of DNP-arginine. It is evident, therefore, that the dipeptide has the structure serylarginine.

Paper Electrophoresis

In accordance with its basic properties, the purified peptide was found to migrate in paper electrophoresis toward the anode. In these and the following experiments, a sodium acetate buffer, pH 5.0, 0.1 ionic strength,

was employed. Following spraying with ninhydrin reagent, the peptide appeared as a faintly yellow spot which could be readily visualized by its fluorescence under an ultraviolet lamp. The purified peptide appeared homogeneous.

In the experiments described below, the peptide fraction was isolated from the activation mixtures by the resin method, and an amount approximately equivalent to 0.15 μmole of chymotrypsin formed was placed on the paper strip. When this method was applied to a rapid activation mixture in which approximately 90 per cent conversion to δ-chymotrypsin had occurred, a major spot, corresponding to the peptide, was observed in addition to a minor, ninhydrin-positive, spot which remained near the point of application. However, when rapid activation was carried out in the presence of β-phenyl propionate and DFP was added in excess after approximately 90 per cent conversion to π-chymotrypsin had occurred, the spot corresponding to the dipeptide was entirely absent and only a very faint spot near the point of application could be seen. It was of interest to note that the spot corresponding to the dipeptide could also be found in the peptide fraction obtained after slow activation of chymotrypsinogen (24 hours at 0°, chymotrypsinogen-trypsin ratio 5000:1); there did appear, however, in addition, several other ninhydrin-positive basic, neutral, and acidic peptides. The release of the dipeptide appears to correlate with the large shift in mobility of the protein constituents occurring during activation (see below).

Quantitative Studies of Activation Process

It has been found in this work that the intermediate products arising during the rapid activation of chymotrypsinogen could be distinguished from one another by prolonged moving boundary electrophoresis in an acetate buffer of pH 4.97, ionic strength 0.1. With the aid of these findings it has been possible to describe the kinetics of the formation and disappearance of the protein components during activation and to relate to these the amounts of peptide formed. In these experiments rapid activation mixtures were prepared in the usual manner, and, at predetermined time intervals, aliquots were removed and pipetted into a 50-fold molar excess of a 1 M solution of DFP in isopropanol. After 30 minutes, the solutions were adjusted to pH 5 and dialyzed against large volumes of acetate buffer, pH 4.97. All of these operations were carried out in the cold room (0–4°).

Representative electrophoretic diagrams are presented in Fig. 3. The electrophoretic pattern of chymotrypsinogen (not shown) indicated that the protein was at least 97 per cent homogeneous. It will be noted that during activation the boundary corresponding to chymotrypsinogen was replaced by components of decreasing electrophoretic mobility. The mo-

bility difference between chymotrypsinogen and the first activation product, π-chymotrypsin, is so small that it could not be observed after 3 hours of electrophoresis (4), but it became clearly evident after prolonged electrophoresis. π-Chymotrypsin has been obtained in nearly pure form, as judged by electrophoresis, when activation was carried out in the presence of β-phenyl propionate, as previously described (4). Much larger mobility

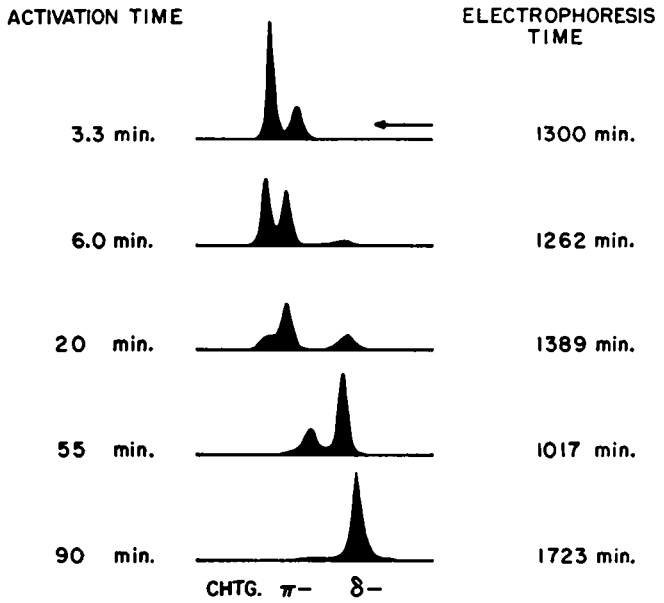

FIG. 3. Electrophoretic diagrams (ascending) of activation mixtures. The time of activation is given on the left and the time of electrophoresis on the right-hand margin. The patterns have been aligned horizontally to facilitate comparison of the three components marked at the bottom of the figure. Sodium acetate buffer, pH 4.97, ionic strength 0.1, was used. The potential gradient was 5.8 volts cm.$^{-1}$. The ascending mobilities (10^{-5} sq. cm., volt^{-1}, sec.$^{-1}$) of the components were as follows: chymotrypsinogen 3.8, π-chymotrypsin 3.6$_5$, δ-chymotrypsin 3.2$_6$.

changes occur as π-chymotrypsin is converted to the δ form. The patterns in Fig. 3 have been aligned in a horizontal direction to facilitate comparison of the three components; *i.e.*, chymotrypsinogen, π-, and δ-chymotrypsin. It is evident that after 90 minutes of activation, under the conditions employed herein, at least 90 per cent of the protein had an electrophoretic mobility characteristic of δ-chymotrypsin. The relative concentrations of the components corresponding in electrophoretic mobility to chymotrypsinogen, π-, and δ-chymotrypsin are plotted in Fig. 4 against the time of activation. Relative component distribution was calculated from the

Rayleigh interference fringes (21) which were recorded simultaneously with their derivative patterns on the photographic plates.[3]

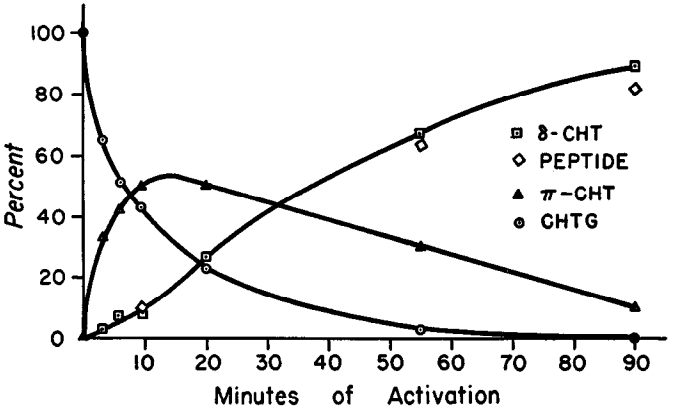

FIG. 4. Distribution of protein and peptide components of activation mixtures as a function of the time of activation. The relative distribution of the protein components was calculated from the electrophoretic patterns, the total protein concentration being taken as 100 per cent (see the text). The amount of the peptide is the ratio, in per cent, of micromoles of peptide to the micromoles of chymotrypsinogen (mol. wt. 23,000) initially present.

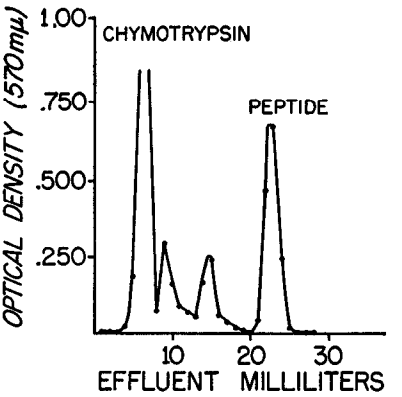

FIG. 5. Chromatogram of a DFP-inactivated activation mixture applied directly to an XE-64 column and eluted with 0.3 M sodium citrate buffer.

Activity measurements on similar activation mixtures indicated that the per cent of maximally attainable esterase activity corresponded approxi-

[3] It is recognized that these calculations yielded only approximate values, since no corrections were applied to account for deviations from "ideal conditions" (22).

mately to the amount of chymotrypsinogen disappearing. Furthermore, when an activation mixture retaining approximately 40 per cent chymotrypsinogen (similar to the 6.0 minute pattern in Fig. 3) was freed of excess DFP by dialysis against 0.001 N HCl and then activated under the usual conditions, 30 per cent of maximal activity could be obtained.

Also shown in Fig. 4 are the amounts of peptide liberated during activation. These followed closely the curve describing the rate of appearance of δ-chymotrypsin. The data were obtained in the following manner.

Samples of the DFP-inactivated activation mixtures were adjusted to pH 5.3 and applied directly to the XE-64 column. Elution was carried out with 0.2 or 0.3 M sodium citrate buffer, pH 5.3; a representative chromatogram obtained by elution with 0.3 M buffer is shown in Fig. 5. It is clear that the elution of the protein components precedes that of the peptide material. Even sharper separation occurred when elution was carried out with 0.2 M citrate buffer or when gradient elution was applied. The concentration of the peptide was calculated in terms of the previously determined color value of the peptide.

DISCUSSION

The present experimental data show conclusively that during the rapid activation of chymotrypsinogen a peptide is liberated, having the structure serylarginine. Since the concentration of the peptide follows closely the time curve describing the appearance of δ-chymotrypsin, it is apparent that it must have arisen during the conversion of π-chymotrypsin to the δ form. This conclusion is also in accord with the much larger differences in electrophoretic mobility between π- and δ-chymotrypsins, compared to the difference between chymotrypsinogen and π-chymotrypsin. Indeed direct experimentation failed to provide any evidence for the release of a peptide during the conversion of chymotrypsinogen to π-chymotrypsin.

On the basis of these findings and others previously reported (4, 23–25), it now appears possible to identify the amino acid sequence in chymotrypsinogen which is primarily involved in the activation process. The following considerations summarize the pertinent points of evidence: (1) π-chymotrypsin differs from chymotrypsinogen in possessing an *N*-terminal isoleucyl-valine sequence, both proteins being devoid of a *C*-terminal group reactive toward carboxypeptidase; (2) the conversion of π- to δ-chymotrypsin yields a *C*-terminal leucine group, no new *N*-terminal group, and the dipeptide serylarginine; (3) the action of α-chymotrypsin on chymotrypsinogen yields neither enzymatic activity nor the dipeptide,[4] suggesting that serylarginine is not a *C*-terminal sequence in chymotrypsinogen.

If we omit from consideration factors arising from the presence of the

[4] Unpublished experiments.

N-terminal half cystine group found in chymotrypsinogen (26) and consider, in addition to the data already presented, the specificity requirements of the activating enzymes (25), it seems most likely that the amino acid sequence involved in the activation process is as shown in Fig. 6. According to this scheme, the arginyl-ioleucine bond would be opened in the trypsin-catalyzed formation of π-chymotrypsin; the subsequent chymotrypsin-catalyzed conversion of π- to δ-chymotrypsin would involve the hydrolysis of the leucyl-serine bond, giving rise to the dipeptide, and a protein having a *C*-terminal leucine group and an *N*-terminal isoleucyl-valine sequence. When the present results are viewed together with those previously reported for the activation of trypsinogen (11), it becomes abundantly clear that the splitting of a single bond suffices to produce enzymatic activity. The liberation of a peptide is not an obligatory event, and all active forms

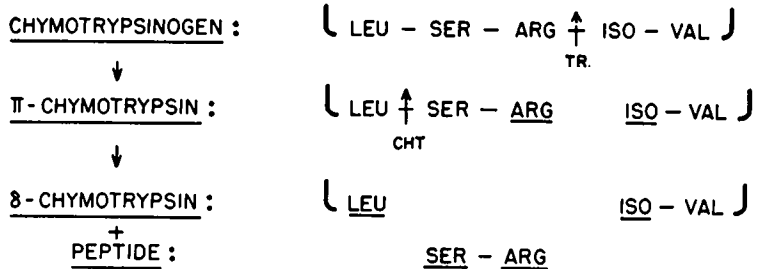

FIG. 6. Proposed sequence of reactions in the rapid activation of chymotrypsinogen.

of these two proteolytic enzymes have the same *N*-terminal dipeptide sequence of isoleucyl-valine (4, 27).

The question remains as to how the opening of a single peptide bond is related to the appearance of enzymatic activity.[5] While an answer to this question lies mainly in the realm of speculation, the following considerations are advanced to indicate the direction of future investigation of this problem. Cleavage of a strategically located peptide bond may simply unmask a preexisting structural region of the enzyme molecule which is associated with catalytic activity. Such unmasking would involve a certain degree of reorientation in the protein molecule. Alternatively, an

[5] The question may, in fact, be raised whether the opening of a peptide bond is a necessary or an incidental accompaniment of activation. All that can be said is that it is the only chemical event known to occur. While the well known denaturing effect of proteolytic enzymes could conceivably produce an intramolecular rearrangement of the substrate molecule, the high degree of specificity of the activating enzymes renders it extremely unlikely that activation can occur without peptide bond hydrolysis.

enzymatically active site may be created by molecular rearrangement. Either of these physical changes may lead to significant differences in the x-ray diffraction pattern of the zymogen and of the active enzymes. From a chemical point of view it may be suggested that the difference in reactive groups between zymogen and active enzyme is responsible for activation. The only common feature which distinguishes the active enzymes from the zymogen is the *N*-terminal isoleucyl-valine sequence. Hence, it would be of interest to determine whether chemical modification or removal of components of this sequence might lead to the abolition of enzymatic activity.

Our thanks are due to Miss Elaine Cohen and Miss Anne Cederquist for performing the amino acid analysis and to Mr. Roger D. Wade for his assistance in electrophoretic measurements.

This work has been supported by the United States Public Health Service, research grant C-2286, by contract No. Nonr-477-04 between the University of Washington and the Office of Naval Research, Department of the Navy, and by funds made available by the people of the State of Washington, Initiative 171.

SUMMARY

In the course of the rapid activation of chymotrypsinogen a single peptide is liberated which has been isolated and identified as serylarginine. The appearance of this peptide is exclusively associated with the conversion of π-chymotrypsin to the δ form. The present findings, together with those previously reported, have been interpreted to indicate that the splitting of a single peptide bond between arginine and isoleucine, in the sequence leucyl-seryl-arginyl-isoleucyl-valine, suffices to produce enzymatic activity when chymotrypsinogen is converted to π-chymotrypsin. The subsequent chymotrypsin-catalyzed reaction involves the liberation of the dipeptide from the *C*-terminal portion of π-chymotrypsin.

BIBLIOGRAPHY

1. Neurath, H., and Dreyer, W. J., *Federation Proc.*, **14**, 260 (1955).
2. Dreyer, W. J., and Neurath, H., *J. Am. Chem. Soc.*, **77**, 814 (1955).
3. Green, N. M., and Neurath, H., in Neurath, H., and Bailey, K., The proteins, New York, **2**, chapter 25 (1954).
4. Bettelheim, F. R., and Neurath, H., *J. Biol. Chem.*, **212**, 241 (1955).
5. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline enzymes, New York, 2nd edition (1948).
6. Kunitz, M., *J. Gen. Physiol.*, **32**, 265 (1948).
7. Johnson, P., and Shooter, E. M., *Science*, **109**, 39 (1949).
8. Durrum, E. L., *J. Am. Chem. Soc.*, **72**, 2943 (1950); **73**, 4875 (1951).
9. Cunningham, L. W., Jr., *J. Biol. Chem.*, **207**, 443 (1954).
10. Schwert, G. W., *J. Biol. Chem.*, **190**, 799 (1951).

11. Davie, E. W., and Neurath, H., *J. Biol. Chem.*, **212,** 507 (1955).
12. Gladner, J. A., and Neurath, H., *J. Biol. Chem.*, **205,** 345 (1953).
13. Redfield, R. R., *Biochim. et biophys. acta*, **10,** 344 (1953).
14. Levy, A. L., and Chung, D., *Anal. Chem.*, **25,** 396 (1953).
15. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **192,** 663 (1951).
16. Neilands, J. B., *J. Biol. Chem.*, **197,** 701 (1952).
17. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.*, **200,** 493 (1953).
18. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176,** 367 (1948).
19. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211,** 893 (1954).
20. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **155,** 441 (1944).
21. Longsworth, L. G., *Anal. Chem.*, **23,** 346 (1951).
22. Alberty, R. A., in Neurath, H., and Bailey, K., The proteins, New York, **1,** chapter 6 (1953).
23. Rovery, M., Poilroux, M., and Desnuelle, P., *Biochim. et biophys. acta*, **14,** 145 (1954).
24. Rovery, M., and Desnuelle, P., *Biochim. et biophys. acta*, **13,** 300 (1954).
25. Neurath, H., Gladner, J. A., and Davie, E. W., in McElroy, W. D., and Glass, B., The mechanism of enzyme action, Baltimore (1954).
26. Bettelheim, F. R., *J. Biol. Chem.*, **212,** 235 (1955).
27. Rovery, M., Fabre, C., and Desnuelle, P., *Biochim. et biophys. acta*, **9,** 702 (1952); **10,** 481 (1953).