# Characterization of Peptides and Proteins with Enzymes

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Since the fundamental work of *Fred Sanger* on the structure of insulin[1-8], an increasing amount of research has been undertaken to determine the amino acid sequences in proteins and peptides. It is useful to degrade the proteins to be analysed with proteolytic enzymes, since the specificity of enzymes permits the isolation of uniform fragments. The "spectrum" of the peptides obtained in this way is sometimes characteristic of a certain protein. The action of peptidases on individual peptides allows some determination of amino acid sequences.

## A. Hydrolysis of Proteins to Peptides

### 1. Method with Trypsin

#### Principle

The high specificity of this enzyme permits the isolation of defined fragments[9,10]. Trypsin splits peptide bonds wherever the basic amino acids, lysine and arginine, occur; thus the carboxyl group of these amino acids is liberated. In this way peptides with a basic amino acid at the carboxyl end are obtained. Only two exceptions are known: trypsin does not hydrolyse peptide bonds, if 1. the α-amino group of lysine or arginine is free or 2. lysine is followed by proline in the peptide chain[11,12].

If a protein cannot be degraded directly or completely by enzymatic means, then it must be prepared for the enzymatic hydrolysis. This is accomplished by denaturing the protein in solutions of 6–8 M urea[13] or 2 M guanidine hydrochloride[14], and by splitting the disulphide bonds. The latter is accomplished 1. oxidatively with performic acid[15,16] (tryptophan is destroyed in the oxidation), 2. reductively with thiols, such as thioglycollic acid[17], with sodium borohydride[18] (followed by reaction of the sulphydryl groups formed with the carboxymethyl residues) or with sulphite[19,20] (formation of thiosulphonic acids).

The proteins are usually hydrolysed at room temperature in a buffer solution at the pH optimum of trypsin (pH 7–8) or if it is desired to work in salt-free conditions, in an autotitrator. The peptides formed are separated either on ion exchange columns (*e.g.* Dowex 50×2)[11] or chromatographically

1) *F. Sanger et al.*, Biochem. J. *44*, 126 [1949].
2) *F. Sanger et al.*, Biochem. J. *45*, 563 [1949].
3) *F. Sanger et al.*, Biochem. J. *53*, 353 [1953].
4) *F. Sanger et al.*, Biochem. J. *53*, 366 [1953].
5) *F. Sanger et al.*, Biochem. J. *59*, 509 [1955].
6) *F. Sanger et al.*, Biochem. J. *49*, 463 [1951].
7) *F. Sanger et al.*, Biochem. J. *49*, 481 [1951].
8) *F. Sanger et al.*, Biochem. J. *60*, 541 [1955].
9) *P. Jolles* and *J. Thaureau*, C. R. Séances hebd. Acad. Sci. *243*, 1685 [1956].
10) *G. H. Dixon, D. L. Kauffman* and *H. Neurath*, J. biol. Chemistry *233*, 1373 [1958].
11) *C. H. W. Hirs, S. Moore* and *W. H. Stein*, J. biol. Chemistry *219*, 623 [1959].
12) *C. H. W. Hirs, S. Moore* and *W. H. Stein*, J. biol. Chemistry *235*, 633 [1960].
13) *G. Braunitzer*, Hoppe-Seylers Z. physiol. Chem. *315*, 271 [1959].
14) *D. H. Spackman, W. H. Stein* and *S. Moore*, J. biol. Chemistry *235*, 648 [1960].
15) *C. H. W. Hirs*, J. biol. Chemistry *219*, 611 [1956].
16) *V. Du Vigneaud et al.*, J. biol. Chemistry *191*, 309 [1951].
17) *M. Sela, F. H. White* and *C. B. Anfinsen*, Biochim. biophysica Acta *31*, 417 [1959].
18) *S. Moore, R. D. Cole, H. G. Gundlach* and *W. H. Stein:* IVth International Symposium on Proteins. Pergamon Press, London 1960, Vol. VIII, p. 52.
19) *J. N. Swan*, Nature [London] *180*, 643 [1957].
20) *J. F. Pechère, G. H. Dixon, H. Neurath* and *R. H. Maybury*, J. biol. Chemistry *233*, 1664 [1958].

or electrophoretically on paper. The most elegant method is two dimensional separation on paper, in which one dimension is run electrophoretically and the other dimension chromatographically ("Fingerprint")[21,22].

### a) Hydrolysis in Buffer Solution[11]

#### Reagents

1. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$, A. R.
2. Disodium hydrogen phosphate, $Na_2HPO_4$, anhydrous, A. R.
3. Hydrochloric acid, A. R., 2 N
4. Ninhydrin
5. Sodium acetate·3 $H_2O$, A. R.
6. Hydrindantin (preparation, see[23])
7. Acetic acid, A. R.
8. Ethyleneglycol monomethyl ether (Cellosolve)
9. Ethanol, *ca.* 50% (w/v)
10. Trypsin
    recrystallized repeatedly, salt-free or containing 50% magnesium sulphate. Commercial preparation, see p. 999.

**Purity of the enzyme preparation**

Commercially available trypsin usually contains small amounts of chymotrypsin and elastase. It is therefore necessary in special cases to start with trypsinogen, which is activated with a small amount of trypsin before the experiment and recrystallized[24]. Part of the chymotryptic activity can be eliminated by the addition of sufficient diisopropylfluorophosphate so that 50% of the tryptic activity is inhibited, or the trypsin is incubated with N/16 HCl for 24 hours at 37°C. Another possibility is to dissolve the trypsin in 8 M urea solution[25], allow to stand for several hours and then dilute the mixture to give a urea concentration of 2 M (the denaturation of trypsin is more easily reversed than that of the contaminants). This method is particularly suitable if the protein to be hydrolysed has been treated with urea. Trypsin is still active in 2 M urea solution.

#### Preparation of Solutions

I. Phosphate buffer (0.2 M; pH 7.0):
Dissolve 8.28 g. $NaH_2PO_4 \cdot H_2O$ and 19.88 g. $Na_2HPO_4$ (anhydrous) in distilled water and make up to 1000 ml.

II. Ninhydrin reagent[23]
Dissolve 20 g. ninhydrin and 3 g. hydrindantin in 750 ml. peroxide-free ethyleneglycol monomethyl ether and add 250 ml. 4 M sodium acetate buffer pH 5.5 (544.0 g. sodium acetate·3 $H_2O$ and 100 ml. acetic acid in distilled water and make up to 1000 ml.). The reddish solution is stable under nitrogen in the dark. Do not prepare more than a week's supply.

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[21] *V. M. Ingram*, Nature [London] *178*, 792 [1956].
[22] *A. M. Katz*, *W. J. Dreyer* and *C. B. Anfinsen*, J. biol. Chemistry *234*, 2897 [1959].
[23] *S. Moore* and *W. H. Stein*, J. biol. Chemistry *211*, 907 [1954].
[24] *J. H. Northrop*, *M. Kunitz* and *R. M. Herriott:* Crystalline Enzymes. Columbia University Press, New York 1948, p. 263, 267.
[25] *J. I. Harris*, Nature [London] *177*, 471 [1956].

III. Trypsin (0.05% protein):

Dissolve 50 mg. salt-free trypsin in phosphate buffer (solution I) and make up to 100 ml. Prepare the solution freshly for each hydrolysis.

## Procedure

### Experimental material

Dissolve the material to be hydrolysed in phosphate buffer (solution I) to give a final concentration of 1% (w/v).

### Enzymatic hydrolysis

For the ninhydrin reaction and determination of the autolysis products of trypsin, prepare a blank containing phosphate buffer (solution I) instead of the sample.
Pipette into a 50 ml. round-bottomed flask (in a 37° C water bath):

10 ml. sample
10 ml. trypsin solution (III) *).

Mix and remove 0.1 ml. samples at 0, 20, 40, 60, 120, 240 and 360 min. Carry out the ninhydrin reaction on these samples **). When the ninhydrin-positive material no longer increases stop the enzymatic reaction by the addition of

*ca.* 2 ml. 2 N HCl.

The pH should be 2.2. The mixture can be directly transferred to an ion exchange column for the separation of the peptides[11].

### Ninhydrin reaction

Wavelength: 570 mμ; light path: 1 cm. Measure against the blank (see above).
Pipette into stoppered test tubes:

0.1 ml. sample from the enzymatic reaction mixture
1.0 ml. ninhydrin reagent (solution II).

Heat for exactly 15 min. in a boiling water bath, add

5.0 ml. 50% ethanol,

mix and allow to cool to room temperature. Measure the optical density. If the optical densities are very high, the solutions can be diluted with 50% ethanol.

The enzymatic hydrolysis is followed by the formation of ninhydrin-positive substances. When these no longer increase the hydrolysis is complete (plot the colour intensity against the time).

## b) Hydrolysis in "Salt-free" Solution

If the peptides must be worked up by methods in which non-volatile salts interfere and if an automatic titration apparatus is available, then the hydrolysis is carried out in a solution which contains only volatile salts[10]. The tryptic hydrolysis is followed by the consumption of alkali with time.

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*) The "digestion mixture" is 0.5% with respect to substrate and 0.025% with respect to trypsin (weight ratio of substrate to enzyme 20 : 1). In many cases, the enzyme concentration in the mixture can be reduced to 0.0025%.

**) The enzymatic reaction is stopped on mixing with the ninhydrin reagent.

## Reagents

1. Triethylamine, redistilled, b. p. 89.4°C/760 mm.
2. Formic acid, A. R.
3. Trypsin

   see p. 999

## Preparation of Solutions

I. Triethylamine (0.1 M):
Mix 1.01 g. triethylamine with distilled water and make up to 100 ml.

II. Trypsin (0.05% protein):
Dissolve 50 mg. salt-free trypsin in distilled water, adjust to pH 8 with 0.1 M triethylamine solution (I) and dilute to 100 ml. with distilled water. Prepare freshly for each experiment.

## Procedure

### Experimental material

Dissolve the salt-free protein to be hydrolysed in distilled water to give a final concentration of 0.5 to 1% (w/v), adjust the pH of the solution to 8.0 with formic acid or 0.1 M triethylamine solution (I).

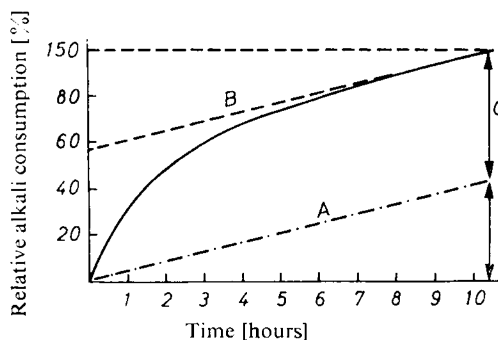


Fig. 1. Hydrolysis of a protein with trypsin (in an autotitrator)
Curve A: Blank ($CO_2$ absorption from the atmosphere)
Curve B: Extrapolation to t = 0
Portion C: Alkali consumption equivalent to the hydrolysis of the protein (net alkali consumption)

### Enzymatic hydrolysis

Prepare a blank containing water at pH 8.0 (adjusted with triethylamine) instead of the sample. It gives the alkali consumption due to the absorption of $CO_2$ from the atmosphere during the experiment. In the titration vessel of the autotitrator (equilibrated at 37°C) mix

10 ml. sample
10 ml. trypsin solution (II) *)

and maintain the pH automatically at 8.0 with

0.1 M triethylamine solution (I).

Record the amount of solution I consumed per 30 min. Stop the titration when the rate of alkali consumption corresponds to that of the blank (due to absorption of $CO_2$ from the atmosphere) (Fig. 1).

After freeze-drying, the peptides formed can be separated directly by paper chromatography; the salts contained in the reaction mixture are volatile.

*) The "digestion mixture" is 0.25 to 1% with respect to the substrate to be hydrolysed and about 0.025% with respect to the trypsin.

## 2. Method with Chymotrypsin

### Principle

The specificity of chymotrypsin is not quite so high as that of trypsin. Generally, chymotrypsin hydrolyses proteins at the carboxyl group of aromatic amino acids such as phenylalanine, tyrosine and tryptophan. However, the enzyme is also capable of hydrolysing amino acid residues which have a similar configuration to that of the aromatic amino acids. This means that peptides have been obtained with leucine, valine, methionine, glutamic acid, asparagine, glutamine or histidine as carboxyl end group. Although the aromatic residues are always hydrolysed, providing they are not terminal amino residues, the other group of residues are only hydrolysed occasionally[10,26,27]. The pH optimum of chymotrypsin is at 8.0, but the hydrolysis can also be carried out at pH 7 and 9.

The technique of hydrolysis with chymotrypsin is the same as that with trypsin. The reaction can be carried out in buffer or "salt-free" solution. In addition to phosphate buffer, 0.2 M ammonium acetate-ammonia buffer[27] (pH 8.5) has been used successfully. This buffer has the advantage that its components can be sublimed off *in vacuo* and so a salt-free peptide mixture is obtained. In this case, the hydrolysis cannot be followed by the ninhydrin method because of the presence of ammonia.

### Reagents

See under "Trypsin", p. 351. Use chymotrypsin instead of trypsin: recrystallized repeatedly, salt-free. Commercial preparation, see p. 972.

#### Purity of the enzyme preparation

Chymotrypsin usually contains a small amount of tryptic activity. The occurrence of "tryptic" peptides must therefore be reckoned with. For special cases, the chromatographic purification of chymotrypsin[28] or chymotrypsinogen[29] (followed by activation to chymotrypsin[24]) is recommended.

### Preparation of Solutions

See under "Trypsin", p. 351 or p. 353.

### Procedure

See under "Trypsin", p. 352 or p. 353.

## 3. Method with Pepsin

### Principle

Compared with trypsin the specificity of pepsin is not very high. It hydrolyses synthetic substrates at aromatic amino acids, so that the amino group is liberated. Most of the proteins so far studied are not completely hydrolysed by pepsin. Often only peptides from the amino or carboxyl end are hydrolysed. The reaction is usually not quantitative. Very often "unexpected" peptide bonds, which contain no aromatic amino acids, are rapidly and quantitatively hydrolysed, while peptide bonds containing aromatic amino acids are not attacked at all. In spite of this, pepsin can be used for structural studies[30,31], since every new peptide which clarifies an amino acid sequence is of value. The pH optimum for the hydrolysis of proteins is at pH 2, while for many synthetic substrates it is at pH 4[32].

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26) *C. H. W. Hirs*, *W. H. Stein* and *S. Moore*, J. biol. Chemistry *221*, 151 [1956].
27) *J. Leonis*, *C. H. Li* and *D. Chung*, J. Amer. chem. Soc. *81*, 419 [1959].
28) *C. H. W. Hirs*, J. Amer. chem. Soc. *77*, 5743 [1955].
29) *C. H. W. Hirs*, J. biol. Chemistry *205*, 93 [1953].
30) *J. L. Bailey*, *S. Moore* and *W. H. Stein*, J. biol. Chemistry *221*, 143 [1956].
31) *R. D. Cole*, *C. H. Li*, *J. I. Harris* and *N. G. Pon*, J. biol. Chemistry *219*, 903 [1956].
32) *N. M. Green* and *H. Neurath* in *H. Neurath* and *K. Bailey:* The Proteins. Academic Press, New York 1954, Vol. II, p. 1126.

## Reagents

1. Citric acid, A. R.
2. Sodium hydroxide, A. R.
3. Hydrochloric acid, conc., 37% (w/v), A. R.
4. Ninhydrin
5. Hydrindantin
6. Sodium acetate·3 $H_2O$, A. R.
7. Acetic acid, A. R.
8. Ethyleneglycol monomethyl ether (Cellosolve)
9. Propanol
10. Pepsin

    crystalline, salt-free. Commercial preparation, see p. 990.

#### Purity of the enzyme preparation

The purity of the crystalline commercial preparations is generally satisfactory. The specific activity should be about 0.33 units *)/mg.

## Preparation of Solutions

I. Citrate buffer (0.2 M; pH 2.2):
Dissolve 21.0 g. citric acid and 8.4 g. NaOH in distilled water, add 16.0 ml. conc. HCl and dilute to 1000 ml. with distilled water. If necessary, adjust the pH to 2.2 (glass electrode).

II. Sodium hydroxide (0.1 N):
Dissolve 4 g. NaOH in distilled water and make up to 1000 ml.

III. Ninhydrin reagent:
Preparation, see p. 351.

IV. Hydrochloric acid (0.01 N):
Dilute 1 ml. conc. HCl to 1000 ml. with distilled water, titrate against solution II diluted 1:10 and, if necessary, adjust the HCl concentration.

V. Pepsin (*ca.* 0.1% w/v protein):
Dissolve 50 mg. pepsin in citrate buffer (solution I) and make up to 50 ml. Prepare freshly before each experiment.

## Procedure

### Experimental material

Dissolve the material to be hydrolysed in citrate buffer (solution I) or in 0.01 N HCl (solution IV) to give a final concentration of 1% (w/v).

### Enzymatic hydrolysis

Incubate the blank and the "digestion mixture" at 37°C as described for trypsin, p. 352. At the end of the hydrolysis adjust the mixture to pH 7.0 with 0.1 N NaOH (solution II).

## 4. Methods with other Proteinases

Other proteolytic enzymes are used for special purposes. For example, when subtilisin is allowed to act for a short time on ribonuclease it hydrolyses only one peptide bond[33]. Papain hydrolyses nume-

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*) According to *Anson*. For definition and conditions of measurements, see p. 821.

[33] *F. M. Richards*, Proc. nat. Acad. Sci. *44*, 162 [1958].

rous bonds (benzoylarginine amide is used as assay substrate[34]). In the elucidation of the structure of ribonuclease papain was used to hydrolyse a peptide[35], which was not attacked by trypsin, chymotrypsin or pepsin. Elastase has been used to study the B chain of insulin[36].

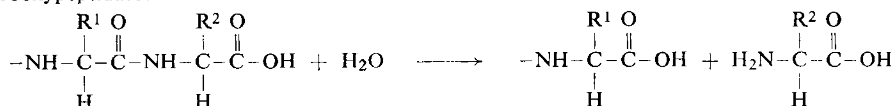
## B. Stepwise Degradation of Proteins with Carboxypeptidase and Leucine Aminopeptidase

Peptides (*e.g.* obtained by the tryptic hydrolysis of a protein), as well as proteins can be degraded enzymatically in a stepwise manner from either the carboxyl or amino end. Carboxypeptidase was introduced into protein chemistry by *Waldschmidt-Leitz*[37], *Grassmann*[38], *Lens*[39] and others. Leucine aminopeptidase has only been used more recently, particularly by *Bergmann*[40], *Smith*[41], *Hill*[42] and *Spackmann*[43].

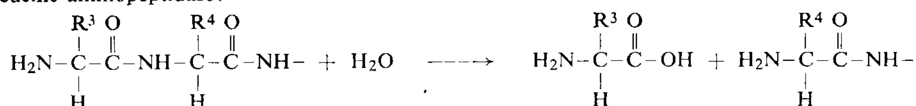
### Principle

As these two enzymes are typical exopeptidases, they hydrolyse successive amino acids from the carboxyl or amino end of the protein.

Carboxypeptidase:



Leucine aminopeptidase:



Conclusions about the amino acid sequence of a peptide or protein can be made, if the stepwise enzymatic hydrolysis is followed with time. Samples are removed from the reaction mixture and the amino acids are determined qualitatively and semi-quantitatively. The terminal amino acid appears first, followed sequentially by the second and third, *etc.* The semi-quantitative determination of the concentration of the amino acids at different times confirms the results.

### Reagents

*For the enzymatic reaction:*

1. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 12\ H_2O$, A. R.
2. Sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$, A. R.

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34) *E. L. Smith* and *M. J. Parker*, J. biol. Chemistry *233*, 1387 [1958].
35) *C. H. W. Hirs*, J. biol. Chemistry *235*, 625 [1960].
36) *M. A. Naughton* and *F. Sanger*, Biochem. J. *70*, 4P [1958].
37) *E. Waldschmidt-Leitz:* Chemie der Eiweißkörper. Ferd. Enke, Stuttgart 1950, p. 67.
38) *W. Grassmann, H. Dycherhoff* and *H. Eibeler*, Hoppe-Seylers Z. physiol. Chem. *189*, 112 [1930].
39) *J. Lens*, Biochim. biophysica Acta *3*, 367 [1949].
40) *E. L. Smith* and *M. Bergmann*, J. biol. Chemistry *138*, 789 [1941].
41) *D. H. Spackman, E. L. Smith* and *D. M. Brow*, J. biol. Chemistry *212*, 255 [1955].
42) *E. L. Smith* and *R. L. Hill:* 3. Congress international biochimique, Résumes des communications. Pergamon Press, London 1955, p. 9.
43) *D. H. Spackman, E. L. Smith* and *W. J. Polglase*, J. biol. Chemistry *199*, 801 [1952].

3. Tris-hydroxymethyl-aminomethane, tris
4. Hydrochloric acid, conc., *ca.* 36% (w/w), A. R.
5. Hydrochloric acid, A. R., 0.1 N
6. Sodium hydroxide, A. R., 0.1 N
7. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$, A. R.
8. Manganous chloride, $MnCl_2 \cdot 4\,H_2O$, A. R.
9. Sodium hydrogen carbonate, $NaHCO_3$, A. R., 1% solution
10. Lithium chloride, LiCl, A. R.
11. Trichloroacetic acid, A. R., 10% solution
12. Diisopropylfluorophosphate, DFP
13. Isopropanol, A. R., absolute
14. Carboxypeptidase
    crystalline, from pancreas[44,45], suspension in water; commercial preparation, see p. 970.
15. Leucine aminopeptidase, LAP
    isolated from pig kidney according to[46]. The enzyme solution is usually about $3 \times 10^{-5}$ M (estimated molecular weight about 300000). Commercial preparation, see p. 987.

*For the ninhydrin reaction and paper chromatography:*

16. Polystyrene sulphonic acid ion exchange resin
    $H^+$-form, particle size 30 mesh or smaller, 8–12% cross-linked, *e.g.* Dowex 50×8 or Nalcite HCR[47].
17. Acetic acid, *ca.* 96%, A. R.
18. Formic acid, A. R.
19. Ammonia solution, A. R., *ca.* 15 N, sp. gr. 0.88
20. Ninhydrin, pure
21. n-Butanol, redistilled
22. Collidine, redistilled
23. Chromatography paper
    *e.g.* Schleicher & Schüll 2043 b; Whatman No. 1 or 4; Machery & Nagel 621.

**Purity of the enzyme preparations**

Carboxypeptidase: Carbobenzoxy-glycyl-phenylalanine is used as substrate to assay the activity; the proteolytic coefficient $C_1$ *) should be at least 10. Before starting the experiment the enzyme crystals should be washed four times with distilled water (at the centrifuge) to remove free amino acids and resuspended in 1% $NaHCO_3$ solution to give a final concentration of 1 mg. protein/ml. Contaminants such as trypsin, chymotrypsin, *etc.*, are inhibited with diisopropylphosphate (DFP).

Leucine aminopeptidase: Leucinamide is used for the assay of the activity[46].

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*) $C_1 = \frac{k_1}{E}$ ; where E = amount of enzyme in mg. and $k_1$ = reaction constant (first order). Refer to "Peptidases", p. 827. The substrate concentration should be 0.05 M. The initial rate of the reaction is used for the calculations.

44) *M. L. Anson*, J. gen. Physiol. *20*, 663 [1937].

45) *H. Neurath, E. Elkins* and *S. Kaufman*, J. biol. Chemistry *138*, 789 [1941].

46) *D. H. Spackman, R. L. Hill, D. M. Brown* and *E. L. Smith:* Biochemical Preparations. Wiley, New York 1958, Vol. 6, p. 35.

47) *J. A. Gladner* and *H. Neurath*, J. biol. Chemistry *206*, 911 [1954].

## Preparation of Solutions

I. Phosphate buffer (1 M; pH 8.0):
Dissolve 358 g. $Na_2HPO_4 \cdot 12\ H_2O$ in doubly distilled water and make up to 1000 ml. Adjust this solution to pH 8.0 (glass electrode) with 60 ml. of a solution of 138 g. $NaH_2PO_4 \cdot H_2O$ in 1000 ml. distilled water.

II. Tris buffer (0.2 M; pH 8.3):
Dissolve 24.3 g. tris-hydroxymethyl-aminomethane in 800 ml. doubly distilled water, adjust to pH 8.3 (glass electrode) with *ca.* 8 ml. conc. HCl and dilute to 1000 ml. with doubly distilled water.

III. Phosphate buffer (0.1 M; pH 8.3):
Dissolve 35.8 g. $Na_2HPO_4 \cdot 12\ H_2O$ in 800 ml. doubly distilled water, adjust to pH 8.3 with *ca.* 2 ml. 0.1 N HCl and dilute to 1000 ml. with doubly distilled water.

IV. Ammonia (5 N):
Dilute *ca.* 28 ml. conc. ammonia (sp. gr. 0.88) to 100 ml. with doubly distilled water; titrate with ·1 N HCl.

V. Tris buffer (*ca.* 0.005 M; pH 8.0; 0.005 M $MgCl_2$):
Dissolve 607 mg. tris-hydroxymethyl-aminomethane and 1.016 g. $MgCl_2 \cdot 6\ H_2O$ in 900 ml. boiled, doubly distilled water, adjust to pH 8.0 with 0.1 N HCl and dilute to 1000 ml. with doubly distilled water.

VI. Diisopropylfluorophosphate, DFP (0.1 M):
Beware of inhaling even small amounts of the vapour. Antidote: atropine. Add 1 g. DFP to 54.5 ml. absolute isopropanol with an automatic pipette and mix.

VII. Manganous chloride (1 M):
Dissolve 19.8 g. $MnCl_2 \cdot 4\ H_2O$ in doubly distilled water and make up to 100 ml.

VIII. Tris buffer (0.5 M; pH 8.5):
Dissolve 6.05 g. tris-hydroxymethyl-aminomethane in 50 ml. doubly distilled water, adjust to pH 8.5 (glass electrode) with *ca.* 1.5 ml. conc. HCl and dilute to 100 ml. with doubly distilled water.

IX. Spray reagent:
Dissolve 250 mg. ninhydrin in 95 ml. water-saturated n-butanol and add 5 ml. collidine.

X. Carboxypeptidase (*ca.* 3 mg. protein/ml.):
Dissolve the crystals from 1 ml. (suspension in $NaHCO_3$ solution) by the addition of 0.05–0.1 ml. 0.1 N NaOH (pH 10) and gentle stirring[10]. Add 0.1 ml. phosphate buffer (solution III) or 0.1 N HCl to give pH 7.8–8.3 (pH-meter). For each mg. enzyme add 0.015 ml. DFP solution (VI), and after allowing to stand for one hour at 25°C the solution is ready for use[48].
Stir larger amounts of crystals with cold 10% LiCl solution; arrange the volume so that the LiCl concentration in the experiments is 0.5–1%. Add $10^{-8}$ moles DFP (undiluted) for each ml. of the clear solution and store at 0°C[49].

XI. Leucine aminopeptidase, LAP (*ca.* 6 mg. protein/ml.):
Add 0.015 ml. DFP solution (VI) per ml. enzyme solution (50-fold molar excess). Contaminating proteinases are inactivated. Dialyse against tris buffer (solution V)

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48) *D. Glick:* Methods of Biochemical Analysis. Interscience, New York 1958, Vol. 2, p. 406.
49) *J. A. Gladner* and *H. Neurath*, J. biol. Chemistry *205*, 345 [1953].

to remove free amino acids which have been hydrolysed from contaminating proteins. Activation of the enzyme: to 0.1 – 1.5 ml. of the dialysed solution (usually 1 to 3% protein) add 0.025 ml. $MnCl_2$ solution (VII) and 0.5 ml. tris buffer (solution VIII), dilute to 2.5 ml. with doubly distilled water and incubate for 30 min. at 40° C.

**Stability of the solutions**

Carboxypeptidase which is dissolved in $NaHCO_3$ solution or buffer should be used within a few days. The solution in 10% LiCl solution is stable for several weeks.

Leucine aminopeptidase in 0.005 M tris-$MgCl_2$ buffer loses none of its activity over several years, if stored in the cold under toluene. As the enzyme is very unstable at pH $<7$, it is recommended that the pH of the stock solution should be occasionally checked.

## Procedure

### Experimental material

Protein is dissolved in 0.001 N HCl and dialysed for 24 hours at 4° C against the solvent in order to remove adsorbed amino acids, peptides or contaminating salts. Freeze-dry the dialysed solution. For the preliminary experiments, dissolve weighed amounts in phosphate buffer (solution III) or tris buffer (solution II), while for the main experiment use 1% $NaHCO_3$ solution as the solvent.

### Preliminary experiments

As the rate of the enzymatic hydrolysis varies from substrate to substrate, the optimum conditions must be determined by trial and error. The substrate and enzyme concentrations, temperature of the reaction and time of sampling are varied in preliminary experiments. The amino groups liberated are determined on small amounts of the hydrolysis mixture with ninhydrin or by paper chromatography. The latter also gives a general idea of the amino acids formed.

*Enzymatic hydrolysis:*

Pipette into a 10 ml. centrifuge tube (in a 25° C water bath):

1.0 ml. sample (0.2 μmoles protein*))
0.05 ml. enzyme solution (X or XI) (150 μg. carboxypeptidase or 300 μg. LAP).

Mix, and stopper the tube. Over a period of 8 hours, remove 0.1 ml. samples for the ninhydrin reaction and 0.01 – 0.03 ml. samples for paper chromatography. Times: 5, 10, 30, 60, 120, 300, 420 min., *etc.*

Variations:

| | |
|---|---|
| Carboxypeptidase solution (X): | 0.01 – 0.1 ml. (30 – 300 μg. protein) |
| LAP solution (XI): | 0.01 – 0.1 ml. (60 – 600 μg. protein) |
| Temperature: | 8 – 40° C. |
| Duration of reaction: | 4 hours to 3 days. |

With experiments lasting more than 12 hours cover the reaction solution with a 5 mm. layer of toluene. With reaction volumes over 1.5 ml. take 0.2 ml. samples instead of 0.1 ml.[23,50].

*) *i.e.* 14 mg. for a protein with a molecular weight of 70000.

50) *W. Troll* and *R. K. Cannan*, J. biol. Chemistry *200*, 803 [1953].

*Ninhydrin reaction:*

To deproteinize, add to a 10 ml. centrifuge tube:

0.1 ml. 10% trichloroacetic acid solution*)
0.1 ml. sample from the enzymatic reaction.

Mix, centrifuge in the cold and carry out the ninhydrin reaction (according to p. 352) on 0.1 ml. of the supernatant. Plot the μmoles amino acid (ordinate) against the time of taking the sample (abscissa).

*Preparation for paper chromatography:*

Pipette into a small test tube:

0.01 – 0.03 ml. sample after the enzymatic hydrolysis
0.05 ml. 0.1 N HCl (ice-cold)
or 0.10 ml. conc. formic acid (ice-cold).

Freeze the mixture until ready for paper chromatography (see "Main Experiment").

The supernatant after trichloroacetic acid deproteinization can also be used for paper chromatography. However, the trichloroacetic acid must first be removed with an anion exchange resin (*e.g.* Amberlite IR 4 B)[51].

**Main experiment**

*Enzymatic hydrolysis:* Use the optimum reaction temperature, amount of enzyme and time of sampling (0.2 ml.) determined in the preliminary experiments. Total volume of the reaction mixture: 1 – 5 ml.; 0.5 – 2 μmoles protein sample dissolved in 1% bicarbonate solution. Otherwise the mixture is as for the preliminary experiments. pH 8.0 – 8.3.

Check the pH of the mixture (glass electrode) when each sample is removed and adjust with 0.1 N NaOH from a micro-burette.

*Preparation of the samples for paper chromatography:*

Pipette into 20 ml. test tubes with ground-glass stoppers:

suspension of Dowex 50 × 8**) ($H^+$-form)
(25 mg. of ion exchange resin per 0.1 μmole amino acid calculated according to the preliminary experiments)
0.2 ml. sample after the enzymatic hydrolysis (main experiment)
or 0.1 ml sample after the enzymatic hydrolysis (preliminary experiment).

The peptidase reaction is stopped by lowering the pH to *ca.* 3. Stopper the tubes and shake mechanically for 1 hour. Decant the supernatant and discard. Wash the resin 4 times with

5 ml. water per gram resin

and discard the washings. To elute the amino acids shake for 10 min. with

4 ml. 5 N $NH_4OH$ per gram resin,

leave the combined supernatants overnight in desiccator over conc. $H_2SO_4$ and then lyophilize or dry completely over $H_2SO_4$.

*) Same volume as the sample, *i. e.* 0.2 ml. for 0.2 ml. sample.
**) Or Nalcite HCR or other suitable polystrene sulphonic acid ion exchange resin.
51) *J. I. Harris,* J. Amer. chem. Soc. *74,* 2944 [1952].

*Paper chromatography* *):

Ascending, length of run 30–45 cm. Solvent systems: a) n-butanol : acetic acid : water 4 : 1 : 5; b) sec-butanol : formic acid : water 70 : 15 : 5; c) methyl ethyl ketone: pyridine: water 70 : 15 : 5.

For the rapid selection of a suitable system, prepare trial two dimensional chromatograms according to[55] or [56]. For the semi-quantitative determination **) chromatograph 1–15 μg. of each amino acid in the best solvent system. Spot the treated samples from the main and preliminary experiments on the paper at 2 cm. intervals (5 mm. diameter spots). Also spot test mixtures containing known amino acids in various concentrations (serial dilutions).

Dry the chromatograms which have been developed with solvent, spray with the spray reagent (solution IX) until the paper shows a "silky lustre" and then develop in a drying oven at 100° C for 6 min.+).

**Evaluation**

Cut the chromatogram of the main experiment into strips. Classify the amino acid spots of the experiment by comparison with the corresponding dilution of the known amino acids. This approximation is liable to an error of 10%. The strips can also be evaluated colorimetrically. The chromatograms of the serial dilutions of the known amino acids serve as the standards.

Plot the μg. amino acid (ordinate) against the time of taking the samples (abscissa). The type of curve obtained with each amino acid indicates the amino sequence (Fig. 2).

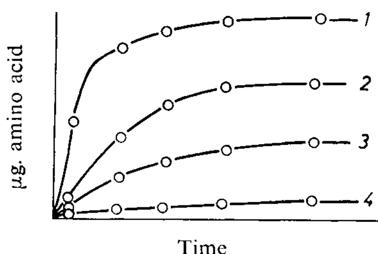
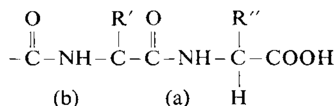



Fig. 2. Schematic representation of the action of carboxypeptidase on a protein. Semi-quantitative paper chromatographic determination of the amino acids 1–4. It follows from the figure that the sequence at the carboxyl end of the protein is 1, 2, 3 and 4.

## Specificity and Limits

*Carboxypeptidase:* For the action of carboxypeptidase the free carboxyl group and the peptide bond on the amino group of the second amino acid (b) are essential.




*) High voltage electrophoresis at pH 6.5[52], 3.5[53] or 1.8[54] can be used to identify the hydrolysis products, especially in studies on peptides. This can be followed by paper chromatography for the second dimension.

**) The carboxyl end group of a protein can be determined quantitatively after the reaction with carboxypeptidase (pH 9; 16 hours) by means of the dinitrofluorobenzene method[57].

+) The addition of collidine to the spray reagent facilitates the identification of the amino acids by the formation of different colours.

52) *H. Michl*, Mh. Chemie *82*, 489 [1951].
53) *F. Rodbell* and *R. Frederickson*, J. biol. Chemistry *234*, 562 [1959].
54) *G. N. Atfield* and *C. J. O. R. Morris*, Biochem. J. *74*, 37 P [1960].
55) *W. Hausmann*, *J. R. Weisinger* and *L. Craig*, J. Amer. chem. Soc. *77*, 721 [1955].
56) *F. Turba:* Chromatographische Methoden in der Proteinchemie. Springer, Heidelberg 1954, p. 174.
57) *A. L. Levy*, Nature [London] *174*, 126 [1954].

The residues R″ and R′ determine the rate of the hydrolysis. From the action of carboxypeptidase on synthetic peptides it follows that the aromatic amino acids are hydrolysed most easily, and then amino acids with aliphatic R″. The long chain amino acids rank before the short chain. An amino acid at the carboxyl end carrying an ionized group in the side chain (*e.g.* arginine or aspartic acid) considerably retards the enzymatic reaction, while proline and hydroxyproline are not attacked at all. Therefore at these points in a peptide chain the hydrolysis comes to a stop[58,59]. The residue R′ also exerts an influence: a glutamyl or prolyl residue in the adjacent position retards the hydrolysis of the bond[60,61].

*Leucine aminopeptidase:* The action of this enzyme has been tested with the amides of the common amino acids[62]. The activity with leucinamide, which is hydrolysed best, serves as a point of reference (100%). Apart from leucine, n-valine, isoleucine, phenylalanine, tryptophan, tyrosine, histidine and valine (84−16%) provide the best substrates. Amides of amino acids with charged side chains are hydrolysed more slowly (7−2%), similarly amides of alanine and glycine (3 and 0.13%), and also amides of proline and hydroxyproline (0.7 and 0.5%). However, an imino acid obviously forms no obstacle to the hydrolysis of a peptide chain. In general, the hydrolysis with leucine aminopeptidase does not come to a stop so rapidly as that with carboxypeptidase; the hydrolysis of 24 amino acid residues has been described[42,63].

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58) *E. L. Smith* in *G. E. W. Wolstenholme* and *M. P. Cameron:* Chemical Structure of Proteins. Little Brown, Boston 1954, p. 109.

59) *E. L. Smith, N. C. Davis, E. Adams* and *D. H. Spackman* in *W. D. McElroy* and *B. Glass:* Mechanism of Enzyme Action. John Hopkins Press, Baltimore 1954, p. 191.

60) *M. Bergmann* and *J. S. Fruton,* J. biol. Chemistry *145,* 247 [1942].

61) *F. Sanger* and *E. O. P. Thompson,* Biochem. J. *53,* 366 [1953].

62) *E. L. Smith* and *D. H. Spackman,* J. biol. Chemistry *212,* 271 [1955].

63) *E. L. Smith,* Fed. Proc. *16,* 801 [1957].