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Complete Amino Acid Analysis of Peptides and Proteins after Hydrolysis by a Mixture of Sepharose-Bound Peptidases

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Incubation with a mixture of Sepharose-bound peptidases was shown to result in the quantitative release of amino acids from certain peptides and *S*-aminoethylated proteins. Subtraction of the low background values of amino acids generated by the enzymes enables amino acid ratios of corticotrophin-(1–24)-tetracosapeptide to be determined with a standard deviation on repeat digestions of 3–5%. Good values were obtained for amino acids that are completely or partially destroyed on acid hydrolysis, i.e. tryptophan, tyrosine, serine, asparagine and glutamine. Experiments with peptides containing D-amino acids showed that the enzyme mixture is stereospecific and could therefore be used to detect the presence of D-residues in peptides. The enzyme mixture completely hydrolyses peptide fragments obtained after Edman degradation and should therefore be useful for determining sequences of peptides containing acid-labile amino acid residues. The activities of the bound enzymes were unaltered over a period of 7 months and they provide a simple, reproducible procedure for the quantitative determination of amino acids in peptides and proteins containing L-amino acids.

Hill & Schmidt (1962) reported that incubation of adrenocorticotrophin and a number of proteins with papain followed by a mixture of leucine aminopeptidase and prolidase resulted in quantitative hydrolysis to the constituent amino acids. Because this treatment did not modify the amino acids it offered advantages over acid hydrolysis, where the partial destruction of serine, tyrosine and tryptophan occurs, the amide groups of glutamine and asparagine are hydrolysed and residues modified with acid-labile groups are converted into the parent amino acid. In addition interconversion of methionine and its sulphoxide occurs under conditions of acid hydrolysis. The obvious practical drawbacks of this enzyme method are the complex incubation procedure and the lack of stability of prolidase and leucine aminopeptidase when stored in a condition suitable for addition to an incubation.

To simplify the procedure to a single incubation that would be suitable for routine use we investigated the properties of a number of Sepharose-bound peptidases. Many enzymes have been reported to be stabilized when bound to a solid support (Silman & Katchalski, 1966) and we have shown that a mixture of peptidases rendered insoluble in this manner completely hydrolysed peptides and could also be stored as a mixture (Bennett *et al.*, 1971). The present paper reports the extension of this study to include the hydrolysis of proteins and the detection of acid-labile amino acids during subtractive Edman degradation.

Materials and Methods

Trypsin (2×crystallized; TRL 6263) and α-chymotrypsin (3×crystallized; CDI 6150-1) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Aminopeptidase M (50units/mg) was supplied by Rohm G.m.b.H., Darmstadt, Germany. Cyanogen bromide was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

Corticotrophin-(1–24)-tetracosapeptide (SYNACTHEN), [D-Ser^1, Lys^{17,18}]-corticotrophin-(1–18)-octadecapeptide amide (Riniker & Rittel, 1970), [Asn^1, Val^5]-angiotensin II, [Asn^1, Val^5, D-His^6]-angiotensin II and [Asn^1, D-Arg^2, Val^5]-angiotensin II were kindly supplied by Dr. W. Rittel and Dr. B. Riniker, CIBA–GEIGY, Basle, Switzerland, and bradykinin was given by Dr. R. Wade of these laboratories Ribonuclease (type IIIA), lysozyme (grade I) and Sepharose 4B were obtained from Sigma (London) Chemical Co., London, S.W.6, U.K. Oxford samplers were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Polystyrene tubes (3ml) with polythene stoppers were obtained from C. E. Payne, London S.W.4, U.K.

Prolidase was prepared from 500g of pig kidney by using the first two steps of the method of Davis & Smith (1957) suitably scaled down.

The enzymes were coupled to Sepharose by the cyanogen bromide method under the conditions of Hurwitz *et al.* (1970). The appropriate enzyme (50mg)

or 50% of the prolidase obtained from 500g of pig kidney was coupled to 5g of Sepharose as follows.

Sepharose 4B (5g wet wt.) was washed on a coarse-sintered filter funnel with 5×20ml portions of water and kept in suspension in 25ml of water by means of a magnetic stirrer. Cyanogen bromide (0.5g) was added and the pH was immediately adjusted to and maintained at pH11 by dropwise addition of 5M-NaOH over a period of 8min. The activated Sepharose was collected on a sintered funnel and washed with 7×20ml portions of ice-cold water and finally with 100ml of ice-cold 0.1M-$NaHCO_3$. The Sepharose was then resuspended in 25ml of ice-cold 0.1M-$NaHCO_3$ containing the enzyme to be coupled and the mixture was stirred for 16h at 4°C. Then the Sepharose–enzyme complex was packed into a chromatographic column (1.2cm×5cm) and washed with a steady flow of 1 litre of 0.05M-tris–HCl buffer, pH7.4, containing 0.03% toluene over a period of 24h. With Sepharose–prolidase this washing buffer also contained 10mM-$MnCl_2$. The Sepharose–enzymes were stored as damp swollen gels at 4°C.

The buffer used for incubations was 0.05M-tris–HCl [Sigma (London) Chemical Co.], pH7.4, containing 0.03% toluene (to inhibit bacterial growth), 0.5% thiodiglycol (to prevent methionine oxidation) and 10mM-$MnCl_2$ (for prolidase activation). For convenience a mixture of Sepharose–enzymes was prepared in sufficient quantity for many hydrolyses. Thus 200mg of Sepharose–trypsin, 200mg of Sepharose–chymotrypsin, 200mg of Sepharose–prolidase and 400mg of Sepharose–aminopeptidase M were mixed, washed with incubation buffer for 24h at 20°C and finally stored in 10ml of buffer at 4°C. Before use the peptidase mixture was always shaken gently and a portion (50–500μl) (1ml/mg of peptide or protein) was immediately dispensed into a 3ml polystyrene tube containing the substrate by using an Oxford sampler with 3mm of the tip removed to give a wider orifice. The tube was stoppered and incubated at 25°C with agitation for the appropriate time (24h for simple peptides).

Oxidized ribonuclease was prepared by the method of Hirs (1956). *S*-Aminoethylated lysozyme and *S*-aminoethylated ribonuclease were prepared by the method of Raftery & Cole (1963). With oxidized ribonuclease and *S*-aminoethylated lysozyme and ribonuclease, typically 0.5mg of the substrate and 500μl of the enzyme slurry were incubated for 48h. Products resulting from Edman degradation were incubated with 100μl of enzyme slurry/10μg of substrate for 2h.

Enzyme activities were assayed spectrophotometrically in the incubation buffer. In the present study 'units' involved were defined as follows.

The trypsin 'unit' is that amount of enzyme causing an increase in $E^{1\,cm}_{253}$ of 0.001/min at 25°C, in hydrolysing 1mM-*N*-benzoyl-L-arginine ethyl ester in 3ml of buffer.

The α-chymotrypsin 'unit' is that amount of enzyme causing a decrease in $E^{1\,cm}_{237}$ of 0.001/min at 25°C in hydrolysing 0.25mM-*N*-acetyl-L-tyrosine ethyl ester in 3ml of buffer.

The aminopeptidase M 'unit' is that amount of enzyme hydrolysing 1μmol of 16.6mM-leucine *p*-nitroanilide/min at 25°C (reaction followed by increase in $E^{1\,cm}_{405}$).

The prolidase 'unit' is that amount of enzyme catalysing the hydrolysis of 1μmol of 39mM-glycyl-L-proline/min at 25°C (reaction followed by decrease in $E^{1\,cm}_{242}$).

Activities were determined by measuring the increase in product (for trypsin and aminopeptidase M) or initial rate of decrease in substrate (for chymotrypsin and prolidase) resulting from substrate cleavage, at the appropriate wavelength.

The free enzyme incubations (25°C) required no special treatment and the reaction progress was recorded automatically. The bound enzymes were maintained in homogeneous suspension by shaking in polythene tubes in a water bath (25°C) and extinction changes of samples were measured at intervals. Only with prolidase was the Sepharose concentration high enough to warrant centrifugation before extinction measurement. This was because higher prolidase concentrations were used on account of the relative insensitivity of the assay.

Acid hydrolysis of peptides was performed in 1ml of 6M-HCl containing a small crystal of phenol (to minimize tyrosine breakdown) *in vacuo*, at 115°C for 18h.

Quantitative amino acid determinations were carried out by using a Beckman 120C amino acid analyser. Glutamine and asparagine were determined by using the lithium citrate buffer described by Benson *et al.* (1967). The modifications of the Edman procedure used in this study have been described previously (Lowry & Chadwick, 1970).

Results

Activities of Sepharose-bound peptidases towards synthetic substrates were determined 1 month after binding to Sepharose and are shown in Table 1. The bound enzymes were stable at room temperature for 24h and prolidase and aminopeptidase M were stable at 4°C for at least 7 months. The stability of bound trypsin and chymotrypsin has been reviewed by Silman & Katchalski (1966). Although free trypsin is partially inhibited by 0.5% thiodiglycol and free aminopeptidase M is partially inhibited by 10mM-Mn^{2+}, the activities of the bound enzymes were much less sensitive to these substances. Free prolidase was unstable and had probably lost 50% of its activity

Table 1. *Activities of the stated amounts of free and Sepharose-bound enzymes in different buffers all at 25°C*

Units are defined in the Materials and Methods section. Weight refers to moist Sepharose + enzyme. Values marked N.D. were not determined. Values marked * represent the yield from 5g of pig kidney.

Preparation	Trypsin (units)	α-Chymotrypsin (units)	Amino-peptidase M (m-units)	Prolidase (units)
Soluble enzyme (1mg) in incubation buffer	9200	3400	1600	23*
Soluble enzyme (1mg) in incubation buffer without 0.5% thiodiglycol	16000	3200	1600	23*
Soluble enzyme (1mg) in incubation buffer with 1mM-Mn^{2+}	8000	3500	2500	22*
Sepharose-bound enzyme (100mg) in incubation buffer	1950	850	470	20
Sepharose-bound enzyme (100mg) in incubation buffer without 0.5% thiodiglycol	1800	780	500	20
Sepharose-bound enzyme (100mg) in incubation buffer with 1mM-Mn^{2+}	N.D.	N.D.	560	21
Sepharose-bound enzyme (100mg) in incubation buffer after 7 months at 4°C	N.D.	N.D.	540	19

in 10 weeks when the assay shown in Table 1 was performed.

In pilot experiments the binding of leucine aminopeptidase was unsuccessful and the Sepharose retained no activity. Also a mixture (2mg of each of Sepharose–aminopeptidase M, Sepharose–trypsin and Sepharose–chymotrypsin) only released small amounts of proline from 100μg of corticotrophin-(1–24)-tetracosapeptide in 72h at 25°C. The addition of Sepharose–prolidase (2mg) to the other peptidases (2mg each) permitted quantitative recovery of the free amino acids from corticotrophin-(1–24)-tetracosapeptide (100μg) after 48h digestion at 25°C. Complete digestion could be obtained in 24h by doubling the quantity of Sepharose–aminopeptidase M (4mg).

At this stage a 10ml slurry of mixed enzymes was prepared as described in the Materials and Methods section and portions of this mixture were used for the experiments described below.

Table 2 shows results obtained by using bound enzymes for repeated test hydrolyses of corticotrophin-(1–24)-tetracosapeptide during the course of 10 weeks after their preparation. The blank values of amino acids released when the required amount of enzyme mixture was incubated in the absence of the substrate were of the order of 3% of a single residue. After storage for several weeks the blank value rose slightly, but by decanting and replacing the supernatant buffer it could be decreased to a value even lower than the initial one. After prolonged storage and decantation blank values were decreased from 3 to 1% of a single residue. Amino acid values obtained after enzyme digestion were always corrected by the subtraction of the appropriate amino acid blank. The standard deviations obtained (Table 2) were in every case less than 5% of a single residue, a value slightly higher than the error introduced by the autoanalyser. The enzymes even 18 months after preparation gave a quantitative release of the amino acids in corticotrophin-(1–24)-tetracosapeptide.

Table 2. *Comparison of the amino acid analyses obtained from corticotrophin-(1–24)-tetracosapeptide after acid hydrolysis and enzyme digestion*

The amino acid ratios are calculated by taking arginine to be exactly 3. The means ±S.D. of the results from five enzyme digestions corrected for background amino acids are given.

	Amino acid content (mol/mol of peptide)		
	Theoretical	Acid hydrolysis	Enzyme digest
Trp	1	0.65	0.95±0.03
Lys	4	4.06	3.91±0.07
His	1	1.05	0.98±0.03
Arg	3	3.00	3.00
Ser	2	1.79	2.01±0.08
Glu	1	1.04	1.00±0.04
Pro	3	2.96	3.05±0.13
Gly	2	2.04	1.93±0.09
Val	3	3.07	3.04±0.12
Met	1	0.97	0.97±0.03
Tyr	2	2.07	1.98±0.06
Phe	1	0.99	0.98±0.04

Table 3. *Analyses obtained from acid hydrolysis and enzyme digestion of* [D-*Ser*[1], *Lys*[17,18]]-*corticotrophin*-(1–18)-*octadecapeptide amide and bradykinin*

For experimental details see the text.

	[D-Ser[1], Lys[17,18]]-Corticotrophin-(1–18)-octadecapeptide amide			Bradykinin		
	Acid hydrolysis	Enzyme digest	Theoretical	Acid hydrolysis	Enzyme digest	Theoretical
Trp	0.34	0.87	1	—	—	—
Lys	5.2	4.98	5	—	—	—
His	1.00	1.00	1	—	—	—
Arg	1.06	1.00	1	1.99	2.05	2
Ser	1.70	1.02	2	0.90	0.95	1
Glu	1.06	1.00	1	—	—	—
Pro	1.00	0.97	1	3.00	3.05	3
Gly	2.1	1.99	2	1.03	0.95	1
Val	1.06	1.02	1	—	—	—
Met	1.01	1.02	1	—	—	—
Tyr	0.93	0.02	1	—	—	—
Phe	0.97	1.05	1	2.03	2.05	2

Table 4. *Amino acid analyses for ribonuclease and lysozyme*

For experimental details see the text. N.D., not determined, either because of tryptophan or deamidation of asparagine and glutamine

	Ribonuclease				Lysozyme		
			Enzyme hydrolysis				
	Accepted whole-number ratios	Acid hydrolysis	After performic acid oxidation	After *S*-amino-ethylation	Accepted whole-number ratios	Acid hydrolysis	*S*-Amino-ethylated and enzyme-hydrolysed
Lys	10	10.1	10.2	9.6	6	6.1	6.1
His	4	3.7	3.9	3.8	1	1.0	1.0
Arg	4	3.7	4.0	3.8	11	11.2	11.2
Asp	5	16.9	5.4	5.0	8	21.5	6.9
Thr	10	10.0	10.5	10.1	7	6.5	7.3
Ser	15	13.5	14.4	14.8	10	8.7	10.4
Asn	10	N.D.	9.2	8.6	13	N.D.	12.9
Glu	5	13.1	5.4	5.5	2	4.9	2.2
Gln	7	N.D.	5.5	6.3	3	N.D.	3.0
Pro	4	4.2	4.4	4.0	2	2.1	1.9
Gly	3	3.2	2.9	3.1	12	11.9	11.6
Ala	12	12.9	12.6	12.0	12	12.0	12.0
Cys	8	7.7	7.6*	7.2†	8	7.4	7.7†
Val	9	9.3	8.8	9.3	6	5.8	6.0
Met	4	3.9	2.4‡	4.0	2	1.4	1.8
Ile	3	2.5	3.1	3.1	6	5.6	5.9
Leu	2	2	2.1	2.2	8	7.8	7.9
Tyr	6	5.4	5.0	5.7	3	2.9	2.7
Phe	3	3.0	3.0	3.0	3	2.9	3.0
Trp	0	0	0	0	6	N.D.	5.9

* Calculated as cysteic acid. † Calculated as *S*-aminoethylcysteine. ‡ Calculated as methionine sulphone.

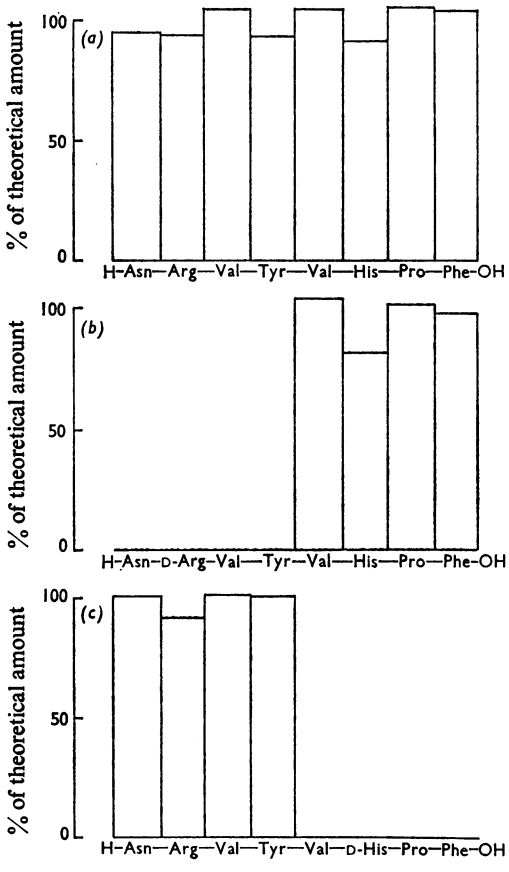

Fig. 1. *Histograms representing the amino acids released from (a) [Asn¹, Val⁵]-angiotensin II, (b) [Asn¹, D-Arg², Val⁵]-angiotensin II and (c) [Asn¹, Val⁵, D-His⁶]-angiotensin II after incubation with Sepharose-bound enzymes*

For experimental details see the text. The peptides had theoretical amino acid compositions after acid hydrolysis.

Table 3 and Fig. 1 shows the amino acids released from a variety of peptides. Amino acid analysis revealed that pure asparagine and glutamine suffered no deamidation on incubation with the enzyme mixture; thus the digestion method should enable the glutamine and asparagine content of peptides and proteins to be determined directly. When enzyme hydrolysis was applied to performic acid-oxidized ribonuclease (Table 4) methionine sulphone and glutamine were incompletely released and the quantity of these amino acids did not increase when the incubation time was extended by 50%.

Amino acid analyses of enzyme-hydrolysed *S*-aminoethylated lysozyme and ribonuclease along with the results obtained after acid hydrolysis of these proteins are also shown in Table 4.

The absence of certain amino acids from the enzyme hydrolysates of peptides containing specific D-residues ([D-Ser¹, Lys¹⁷,¹⁸]-corticotrophin-(1–18)-octadecapeptide amide, Table 3, and angiotensin analogues, Fig. 1) indicates that bonds adjacent to D-amino acids are not cleaved. A comparison of the amounts of amino acids liberated after acid hydrolysis and after enzyme digestion of corticotrophin-(1–24)-tetracosapeptide which had been racemized by heating in 0.1M-NaOH for 10min (Lee & Buettner-Janusch, 1963) is presented in the histogram in Fig. 2. A certain amount of destruction of side-chain substituents, particularly of arginine, occurred during the alkali treatment.

Table 5 demonstrates the use of the peptidase mixture in the subtractive Edman degradation of the *N*-terminal sequence of 50μg of [Asn¹, Val⁵]-angiotensin II. In initial experiments inhibition of the enzymes occurred probably as a result of residual substances accumulating in the course of the degradation. This was overcome by using a large excess of enzyme mixture as described in the Materials and Methods section. Hydrolyses were then complete in 2h.

Although Mn^{2+} was used both in the preparation and coupling of prolidase to Sepharose, recently, because of the instability of this metal ion in solution it has been omitted from the final incubation and storage buffer without any noticeable decrease in prolidase activity in the bound state.

Discussion

Our results show that it is possible to prepare a mixture of stable Sepharose-bound enzymes that can catalyse the complete hydrolysis of a number of polypeptides and proteins. The mixture used contains trypsin and chymotrypsin, which will cleave most peptides to small fragments, and aminopeptidase M, which will hydrolyse *N*-terminal peptide bonds linking L-amino acids. Imidopeptide bonds (i.e. when proline is penultimate) were resistant to the action of aminopeptidases and prolidase was therefore included in the peptidase mixtures. Although this enzyme is an imidodipeptidase and should only cleave X–Pro dipeptides, the mixture was successful in releasing proline quantitatively from every substrate tested, including bradykinin which contains a Pro–Pro bond.

The slightly low values of glutamine and methionine sulphone obtained from performic acid-oxidized ribonuclease even after prolonged enzymic digestion suggest that either a certain amount of racemization had occurred during oxidation or that

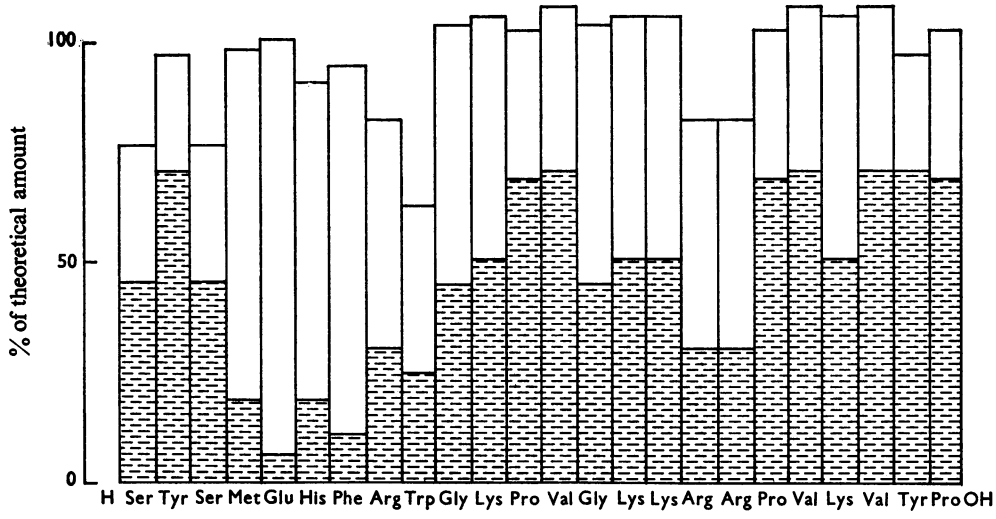

Fig. 2. *Histogram representing the free amino acids found after acid hydrolysis (total area) and after enzyme digestion (shaded area) of corticotrophin-(1–24)-tetracosapeptide which had been heated in* 0.1 M-*NaOH at* 100°C *for* 10 *min*

For experimental details see the text. Where amino acids occur at more than one site the total amount found has been distributed equally.

Table 5. *Amino acid analyses, after enzymic hydrolysis, at four stages of the subtractive Edman degradation of [Asn*[1]*, Val*[5]*]-angiotensin II*

For experimental details see the text.

	Asn	Pro	Val	Tyr	Phe	His	Arg
Original material	1.2	0.9	1.9	1.1	1.0	1.1	0.9
1st. step	0.0	1.0	2.1	1.1	1.0	1.0	0.8
2nd. step	0.0	1.0	2.0	1.0	1.0	1.0	0.0
3rd. step	0.0	1.0	1.0	1.0	1.0	1.0	0.0
4th. step	0.0	1.0	0.9	0.1	1.0	1.0	0.0

the polar tripeptide Gln(28)-Met(O_2)(29)-Met(O_2)-(30) was resistant to hydrolysis. This result should be contrasted with the observation that the hydrophobic sequence Ile(106)-Ile(107), which was incompletely hydrolysed in 18h by acid hydrolysis at 115°C, was completely hydrolysed by the enzyme mixture.

S-Aminoethylation of both lysozyme and ribonuclease resulted in substrates which were completely hydrolysed. For the enzyme method of hydrolysis *S*-aminoethylation is probably the most advantageous pretreatment since it provides extra trypsin-cleavage sites within the molecule. Although the method gives close to quantitative recoveries of most amino acids, there remain a few small discrepancies which are so far unexplained.

Enzyme hydrolysis of peptide analogues with specific D residues showed that adjacent amino acids were not released and that the extent of degradation of the chain linked to the carboxyl group of D residues probably depends on the availability of endopeptidase cleavage sites. This method could therefore be used to detect small amounts (down to 10%) of racemization. The results obtained with partially racemized corticotrophin-(1–24)-tetracosapeptide illustrate this type of application. The low values of methionine, glutamate, histidine, phenylalanine, lysine, glycine and arginine released by the enzyme mixture indicate that racemization has occurred most extensively in regions 5–7 and 15–17. These results complement those obtained with alkali racemization of α-melanocyte-stimulating hormone (Lande & Lerner, 1971) with respect to the 5–7 region.

Recently (Sieber *et al.*, 1972) this method of enzyme hydrolysis has been used as supporting evidence for the identity and purity of human adrenocorticotrophin synthesized according to the revised sequence as reported by Riniker *et al.* (1972). In particular, the correct asparagine and serine content, as well as the absence of methionine sulphoxide, were demonstrated.

In conclusion, we have found that incubation with a mixture of Sepharose-bound enzymes can provide a simple procedure for the complete amino acid analysis of a number of peptides and proteins. Since the enzymes do not hydrolyse peptide linkages adjacent to D-amino acids, enzyme hydrolysis can be used in conjunction with acid hydrolysis for the detection of the presence of D-residues. Our findings suggest that this method of hydrolysis has many of the properties which a general method requires.

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