Amino Acid Sequence in Lysozyme

1. DISPLACEMENT CHROMATOGRAPHY OF PEPTIDES FROM A PARTIAL HYDROLYSATE ON ION-EXCHANGE RESINS

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It has been shown (Fraenkel-Conrat, 1950) that the enzymic activity of lysozyme depends upon the presence of some of its amino, carboxyl and hydroxyl groups. A study of the structure of the lysozyme molecule as a whole has been undertaken in an approach to the problem of defining that portion of the molecule which is concerned in its activity.

The lysozyme molecule probably consists of a single polypeptide chain (Fraenkel-Conrat, Mohammad, Ducay & Mecham, 1951) internally cross-linked by disulphide bridges. The *N*-terminal residue has been shown to be lysine (Green & Schroeder, 1951; Thompson, 1951*a*) by the fluorodinitrobenzene method (Sanger, 1945), and the *C*-terminal residue leucine with the enzyme carboxypeptidase (Thompson, 1952*a*). A sequence of four residues at the *N*-terminal end of the chain (Lys.Val.Phe.Gly) has been established by Schroeder (1952) by a study of the 2:4-dinitrophenyl-(DNP-)peptides liberated on hydrolysis of DNP-lysozyme. This sequence has been confirmed by Landmann, Drake & Dillaha (1953), who reported that the fifth residue is serine, and by R. Acher & J. Thaureaux, whose results indicate, however, that arginine is the fifth residue (private communication from Professor C. Fromageot, 1953). A tripeptide sequence Arg.His.Lys and two dipeptides containing tyrosine have also been identified in partial hydrolysates by Acher, Thaureaux, Crocker & Fromageot (1952).

The successful investigation of the sequence of amino acids in fractions *A* and *B* of oxidized insulin (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*) suggested that a considerable portion of the sequence in lysozyme could be elucidated by similar methods.

The most generally useful method for degrading proteins to smaller peptides is partial hydrolysis with concentrated acid at low temperatures (Synge, 1943). Since the lysozyme molecule contains approximately 130 amino acid residues, the mixture of peptides produced on partial hydrolysis would be expected to be much more complicated than that from either of the insulin chains. Obviously a method for the preliminary separation of the peptides into fractions as well defined as possible was necessary. A preliminary experiment in which the peptides were separated into groups by ionophoresis on filter paper had indicated the complexity of the fractions so obtained.

The successful separation of amino acids by means of displacement chromatography on ion-exchange resins (Partridge & Westall, 1949; Partridge, 1949; Partridge, Brimley & Pepper, 1950; Partridge & Brimley, 1951*a*, *b*, 1952) suggested the use of such columns for fractionation of the complex mixture of peptides in a protein hydrolysate. The high capacity of these columns is an obvious advantage.

The behaviour of peptides on ion-exchange columns had not previously been studied. But on the assumptions that the order of displacement of peptides is governed by the p*K* value of the ionizing group as is the case with amino acids (Partridge & Brimley, 1951*b*), and that other factors are not much involved, it was envisaged that effluent fractions from the column would be considerably simpler than the original peptide mixture.

The two experiments described in this paper involving displacement chromatography on ion-exchange resins are exploratory and do not necessarily give the best conditions for obtaining final fractions for subsequent identification of the peptides. Nevertheless, a large number of peptides was isolated from these experiments. It was possible to determine the structure of a number of them and finally deduce certain of the amino acid sequences in lysozyme. These experiments showed, however, that a method of much higher resolving power is required for preliminary fractionation of the peptides in partial hydrolysates of proteins. Further experiments have shown that elution chromatography on ion-exchange resins (cf. Moore & Stein, 1951; Dowmont & Fruton, 1952) is very successful for this purpose and will be described in a subsequent paper.

A preliminary account of the present work was presented at the 2nd International Congress of Biochemistry in Paris (Thompson, 1952*b*).

METHODS

The lysozyme was purchased from Armour and Co. The ash content was 1·5% and the nitrogen content was 18·6% on a moisture-free and ash-free basis. The moisture content was determined by the method of Chibnall, Rees & Williams (1943). It was electrophoretically homogeneous in the Tiselius apparatus (Thompson, 1951*a*) and at least 96% pure with respect to other proteins, as shown by Dr K. Boardman of this laboratory, by elution chromatography on ion-exchange resins (Tallan & Stein, 1953).

Experiment I

Hydrolysis of lysozyme. Lysozyme (500 mg.) was partially hydrolysed with 12N-HCl (4 ml.) at 37° for 7 days. HCl was removed by repeated evaporation *in vacuo*. The solution was then oxidized with performic acid (Sanger, 1947) to break the disulphide bonds between half-cystine residues, converting these into cysteic acid residues. The methionine residues were converted into methionine sulphone (abbreviated $MetO_2$) by this treatment. H_2O_2 (0·5 ml., 30%, w/v) and formic acid (4·5 ml.) were added and the mixture allowed to stand 15 min. Water (5 ml.) was then added and the mixture repeatedly evaporated *in vacuo*.

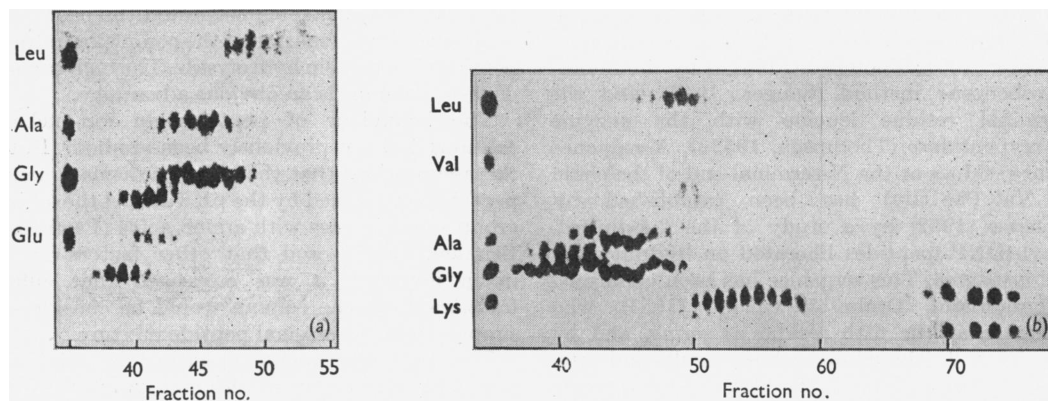

Fig. 1. Experiment I. Fractionation of peptides on sulphonated-polystyrene columns. Paper chromatograms of fractions developed: (*a*) with phenol–water–NH_3; (*b*) with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figures.

Treatment with activated charcoal. An attempt was made to remove selectively the peptides containing aromatic amino acids by adsorption on activated charcoal as described by Sanger & Tuppy (1951*a*). However, on subsequent examination of this fraction, it contained little material.

Displacement development of peptides from lysozyme hydrolysate. A column of sulphonated polystyrene (diam. 0·95 cm., height 8·2 cm.) and a smaller column (diam. 4 mm., height 5 cm.) mounted in series below it, were packed and regenerated as described by Partridge *et al.* (1950). A small amount of ammonia was added to the material not adsorbed on charcoal to act as 'carrier' to increase the separation between lysine and arginine. This solution was then added to the top of the column and 0·075N-NaOH used as displacement developer. Fractions (2 ml.) were collected every 15 min. with an automatic fraction collector and tested afterwards for the presence of amino acids and peptides with ninhydrin.

Fractions 35–75 were run on paper chromatograms in phenol–water–NH_3 and butanol–acetic acid–water (Partridge, 1949); these are shown in Fig. 1. (The figures give preponderantly the distribution of the amino acids, as peptides give weak colours in comparison.) The absence of spots in fractions 59–68 indicates the ammonia band. Fractions 70–73, following the ammonia band and corresponding to arginine in the displacement chromatogram of the amino acids (Partridge & Brimley, 1952), were clearly differentiated from the rest of the material and were bulked as 'arginine peptides'. The distribution of the remainder of the amino acids and peptides was fairly satisfactory and approached the limit of resolution for the amount of hydrolysate, the size of column and the rather large particle size of the resin (100–120 mesh/in., which was the finest sulphonated polystyrene available).

The fractions were pooled as follows: 37–39; 40–43; 44–47; 48–51; 52–58. This was done on the basis of the distribution of amino acids as shown in Fig. 1.

Separation of peptides by paper chromatography. Each of the fractions obtained from the ion-exchange columns was evaporated to dryness and run on duplicate two-dimensional filter-paper chromatograms (Whatman no. 3). The solvents were phenol–water–NH_3 (Consden, Gordon & Martin, 1944) and butanol–acetic acid–water (40:10:50 by vol.; Partridge, 1948). The spots were detected by spraying with 0·025% ninhydrin in ethanol, cut out and eluted (cf. Sanger & Tuppy, 1951*a*).

Identification of amino acid residues in peptides. About one-third of each peptide was hydrolysed with 6N-HCl for 24 hr. at 100° and the resulting amino acids identified by chromatography. Phenol–water–NH_3 was used in the first instance and was the solvent used throughout this work for one-dimensional chromatograms. When confusion arose from amino acids having the same R_F, either *n*-butanol–acetic acid–water or *tert.*-pentanol–water (Consden *et al.* 1944) was used. It is an advantage to add a trace of 8-hydroxyquinoline to the last solvent to avoid 'shadow'

spots caused by metal impurities in the filter paper (cf. Thompson, 1951*b*).

In certain cases colour tests for histidine (Sanger & Tuppy, 1951*a*) and for proline (Acher, Fromageot & Jutisz, 1950) have proved valuable.

N-*Terminal residues of peptides with fluorodinitrobenzene.* The method of Sanger (1945) was used to identify the *N*-terminal amino acid in the remainder of the peptide material. Trimethylamine was used in place of $NaHCO_3$ for the condensation reaction (Sanger & Thompson, 1953*a*), thus avoiding the desalting procedure. After hydrolysis for 8 hr. in 6N-HCl the DNP-amino acids were recovered by ether extraction and identified on paper chromatograms with *tert.*-pentanol buffer (pH 6·0) on buffered paper (Blackburn & Lowther, 1951). DNP-amino acids were always run as controls on each chromatogram. It was found essential to run these chromatograms in the dark and at constant temperature to achieve satisfactory results (cf. Biserte & Osteaux, 1951). DNP-Arginine and ε-DNP-lysine were always identified by similar chromatography of the yellow aqueous layer after ether extraction. The amino acids in the aqueous phase were also identified on ordinary paper chromatograms, generally with phenol as solvent.

C-*Terminal residues with carboxypeptidase.* Towards the end of this work it was found possible to use the enzyme carboxypeptidase to hydrolyse the *C*-terminal amino acid from isolated peptides in the same way as with lysozyme itself (Thompson, 1952*a*) and thus identify the *C*-terminal residue. The method is only suitable when the peptide has been shown to be pure by the DNP procedure. The peptide was dissolved in 0·2 ml. 0·05M ammonium acetate, the pH of which (8·0 in these experiments) was such as to give final pH approx. 7·5 after addition of 0·02 ml. carboxypeptidase suspension (10 mg./ml. water, purchased from Armour and Co.). After incubation at 37° for 16 hr. the enzyme was inactivated and precipitated by boiling, the supernatant solution evaporated to dryness and the liberated amino acid identified by paper chromatography. In certain cases the appearance of a second amino acid in less intensity than the first suggests that its position in the chain is next to the *C*-terminal residue.

In other cases, the terminal amino acid is not easily liberated, e.g. glycine, lysine, proline and, probably, cysteic acid and arginine. In such cases the method fails, but the amount of material and labour thus expended is small.

Experiment II

Lysozyme (500 mg.) was partially hydrolysed for 4 days. The conditions for hydrolysis, oxidation of the disulphide bonds and adsorption of the aromatic peptides on charcoal were the same as in Expt. I.

The fraction not adsorbed on charcoal was evaporated to dryness twice to remove acid and put first through sulphonated-polystyrene columns. It was proposed to separate a fraction corresponding in position to the amino acid arginine after the ammonia band as in Expt. I and also a fraction corresponding to lysine. The remainder of the material was to be eluted and run on a Zeo-Karb 215 column.

This procedure was chosen since one of the possible limiting factors in the degree of resolution of the peptides on sulphonated-polystyrene columns was the rather large particle size of the sample available. Very finely powdered Zeo-Karb 215 was available and, moreover, this resin gives rather more satisfactory separations of the non-basic amino acids than does sulphonated polystyrene (Partridge & Brimley, 1952). However, arginine cannot be displaced from Zeo-Karb 215 and lysine is incompletely displaced. Hence peptide fractions corresponding to these two amino acids were first cut from the sulphonated-polystyrene columns. Four columns (diam. 1·2, 0·9, 0·6, 0·4 cm. and heights 14, 6·3, 5·0 and 4·0 cm. respectively) were used in series. There was ample capacity to allow adequate turnover in one run. The eluate from the column, after the material had been applied and washed through with water, was collected, and, on concentration, gave a weakly positive ninhydrin reaction. This fraction, termed 'cysteic peptides' corresponds in behaviour to cysteic acid, which is not adsorbed by sulphonated-polystyrene resin.

After removal of the cysteic acid fraction, the adsorbed material was displaced with 0·075N-NaOH, flow rate 7 ml./hr. Fractions of approx. 1·2 ml. were collected. Paper chromatography of successive fractions (as in Expt. I) is shown in Fig. 2.

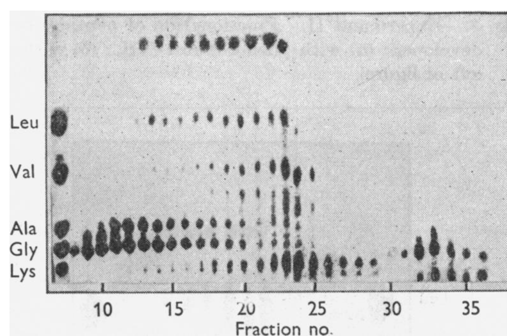

Fig. 2. Experiment II. Fractionation of peptides on sulphonated-polystyrene columns. Paper chromatogram developed with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figure.

The fraction corresponding to arginine was clearly defined in fractions 30–36. The ammonia band was not quite as sharp as in Expt. I as no additional ammonia was added as carrier. The position of the cut for the 'lysine' fraction was less clearly defined and was made between fractions 21 and 22. Accordingly, fractions 22–29 ('lysine peptides') were set aside for an attempt at further fractionation with the anion exchanger Dowex-2. The remaining fractions 8–21 were pooled and run on a series of three Zeo-Karb 215 columns (diam. 1·05, 0·7 and 0·4 cm. and height 10·6, 6·0 and 4·0 cm. respectively). The material was applied to the column and displaced with 0·075N-NH_3. Fractions (0·6 ml.) were collected at 5 min. intervals.

Fig. 3 shows results of paper chromatography of the fractions emerging from the column.

The pattern for fractions 52–69 (Fig. 3*a*) indicates that the chromatogram had been successfully run from the point of view of the separation of the amino acids, which appear in the typical displacement chromatogram pattern (cf. Partridge, 1949). The later fractions consisted largely of peptides. For instance, fractions 77–90 contained only small quantities of leucine and some lysine; the rest of the

material appeared to be of peptide nature. The fractions were pooled as follows: 52–55, 56–61, 62–66, 67–71, 72–76, 77–82, 83–91. 2 N-NH_3 was then put through the column to remove any very strongly adsorbed peptides. These and the fractions after 91 were combined with the 'lysine peptides' fraction from sulphonated polystyrene.

There was some resolution on the column as shown by paper chromatography of the fractions (Fig. 4) but the degree of separation was disappointing. Fractions were pooled in three groups (5–7, 8–10, 11–14 of 4·5, 5·5 and 4·0 ml. respectively) which were examined separately.

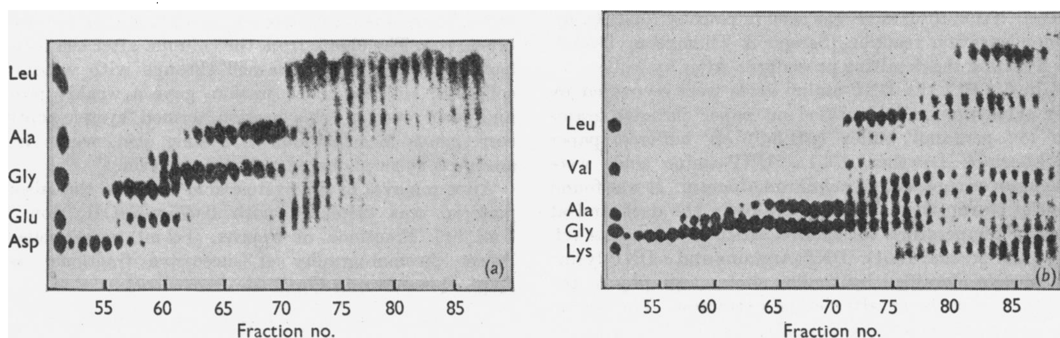

Fig. 3. Experiment II. Fractionation of peptides on columns of Zeo-Karb 215. Paper chromatograms of fractions developed: (*a*) with phenol–water–NH_3; (*b*) with *n*-butanol–acetic acid–water. Control spots of amino acids on left of figure.

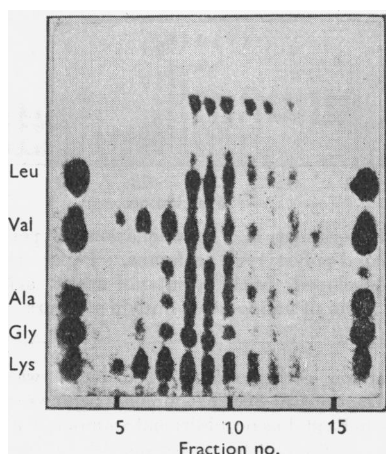

Fig. 4. Experiment II. Fractionation of 'lysine peptides' on columns of Dowex-2. Paper chromatogram developed with *n*-butanol–acetic acid–water. Control spots of amino acids on left and right of figure.

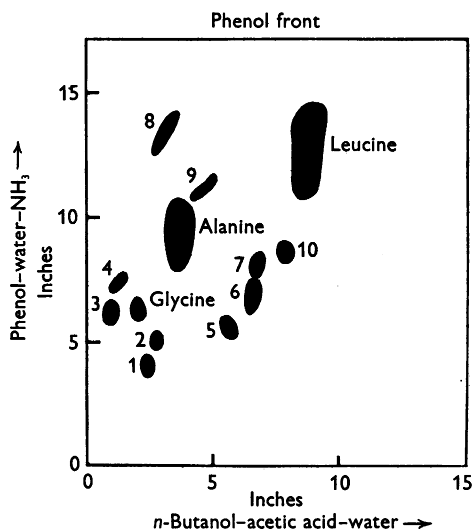

Fig. 5. Chromatogram of fractions 44–47, Expt. I. The butanol–acetic acid solvent was allowed to drip from the bottom of the paper. R_F for a leucine spot in the butanol direction is approx. 0·6.

The considerable overlap of peptides in the lysine fraction indicated that there was a large number of peptides which behaved similarly on cation-exchange resins.

It was hoped that, by using another property of the molecule (the ionization of the amino rather than of the carboxyl group) a fractionation of the peptides on columns of the anion exchanger (Dowex-2) would prove useful.

Two columns (diam. 0·6 and 0·4 cm.; height, 6·7 and 5·0 cm., respectively) were prepared and operated in series as described by Partridge & Brimley (1951*b*). Precautions were taken to exclude CO_2. The material was displaced from the column with 0·075 N-HCl.

RESULTS

The results of the analysis of a typical fraction, 44–47 of Expt. I, are shown in Table 1 and a diagram of the corresponding two-dimensional chromatogram in Fig. 5. The amino acids and peptide structures are abbreviated as by Brand & Edsall (1947) and Sanger & Tuppy (1951*a*). Table 2 con-

tains a list of peptides from both experiments for whose structure the evidence is considered definite.

Tables 3 lists a few peptides of which sufficient was available for examination of the *C*-terminal residue with carboxypeptidase, and the results of such tests.

columns by the method of Moore & Stein (1951), which is the most reliable general method for amino acid analysis now available. The results of such an analysis, kindly performed by Mr D. F. Elsden, are also listed in Table 4. It is worth noting the close correspondence of these values with those of Lewis, Snell, Hirschmann & Fraenkel-Conrat (1950) obtained mostly by microbiological assay.

Table 1. *Peptides from fraction* 44–47, *Expt. I*

Spot relative intensities are indicated roughly by +signs.

Spot no. (Fig. 5)	Spot intensity	Amino acids after hydrolysis		DNP treatment and hydrolysis: Amino acids		DNP treatment and hydrolysis: DNP-amino acids		Suggested structure
1	+ +	Asp	+ + +	Asp	+ + + +	Ser	(+ + + +)	Ser.Asp
		Ser	+ + +	Ser	+ +			
2	+ + + +	Asp	+ + +	Asp	+ + + + +	Thr	(+ + + +)	Thr.Asp
		Thr	+ + +	Thr	+ + +			
3	+	$CySO_3H$	+ +	$CySO_3H$	+	Arg*		Arg.($CySO_3H$, Lys)
		Lys	+ +			ε-DNP-Lysine		
		Arg	+					
4	+	$CySO_3H$	+	$CySO_3H$	+ + +	Arg*		Probably contains Arg.$CySO_3H$
		Ala	+					
		Arg	Trace					
5	+	Asp	+ + +	Asp	+	Asp	(+ +)	Probably Asp.(Glu, Val)
		Glu	+ + +	Glu	+			
		Val	+ +	Val	Trace			
6	+ +	Asp	+ +	Asp	+	Glu	(+ +)	Glu.(Asp, Ileu)
		Glu	+ +	Glu	Trace			
		Ileu†	+ +	Ileu	+			
7	+	As no. 6						
8	+	Asp	+ +					(Asp, Gly)
		Gly	+ +					
9	+	Thr	+ + + +			Thr	(+ +)	Probably Thr.Ala and free proline
		Ala	+ + + +	Ala	+ + +	Pro	(Trace)	
		Pro	‡					
10		Asp	+ + +	Asp	+	Asp	(+ + +)	Asp.Ileu
	+	Ileu	+ + + +	Ileu	+ + + +			

* Identified by running the yellow aqueous layer on *tert.*-pentanol-buffer chromatograms. The ether extract yielded insignificant amounts of DNP-amino acids.

† Identified as Ileu on *tert.*-pentanol–water filter-paper chromatogram.

‡ Hydrolysate gave a positive test for proline with isatin but the spot was close to the free-proline position.

Amino acid composition of lysozyme

Since a knowledge of the amino acid composition of lysozyme and thus the number of residues of each amino acid present in the molecule is essential for assembling sequences from the available information on the structure of isolated peptides, the analyses of lysozyme published by various workers are tabulated in Table 4. They are expressed as number of residues in lysozyme, assuming a molecular weight of 14 900 for the sake of comparison. The considerable discrepancies in the values of different workers for certain of the amino acids, and evidence from the peptides containing glutamic acid that the value for this amino acid was low, emphasized the need for an analysis on ion-exchange

The analytical value for glutamic acid cannot be regarded as definite. Hydrolysis for 24 hr. will not split all the valyl and isoleucyl bonds in a protein (cf. Harfenist & Craig, 1952) and both Ileu.Glu and Val.Glu bonds have been established in lysozyme (Table 5). There must, therefore, be at least five residues of glutamic acid and the figure may well be higher. In fact, the peptides so far isolated suggest six residues at least.

DISCUSSION

Examination of the results of the ion-exchange experiments shows that the displacement technique is unsuitable for separating the peptides from

Table 2. *Peptides recognized in effluent fractions from ion-exchange chromatograms*

Italicized residues were judged *C*-terminal from experiments with carboxypeptidase.

Peptide	Expt.	Fraction
Asp.Gly	I	37–39
	II	67–71
	II	83–91
Asp.Ala	I	37–39
	II	72–76
Asp.Ileu	I	37–39
	I	40–43
	I	44–47
	I	48–51
	II	62–66
	II	72–76
Asp.(Gly, MetO$_2$)	II	56–61
Asp.(Glu, *Ala*)	II	77–82
	II	Lysine 8–10
Asp.(Val, *Glu*)	I	44–47
	II	56–61
Asp.(Glu, Ala, Val)	II	72–76
Ala.Leu	II	Lysine 11–14
Ala.MetO$_2$	I	48–51
	II	72–76
	II	77–82
Arg.CySO$_3$H	I	44–47
	II	77–82
	II	83–91
Arg.Asp	I	48–51
	I	52–58
Arg.(CySO$_3$H, Glu)	II	77–82
Arg.(CySO$_3$H, Asp)	I	37–39
	I	52–58
Arg.(CySO$_3$H, Lys)	I	44–47
CySO$_3$H.(Gly, Lys)	II	77–82
CySO$_3$H.(Ala, Lys)	II	77–82
Gly.MetO$_2$	II	77–82
Gly.Lys	I	52–58
	II	Lysine residues
Glu.Ala	I	48–51
Glu.Leu	I	52–58
	II	83–91
Glu.(Asp, Ileu)	I	44–47
	I	48–51
	I	52–58
	II	72–76
	II	77–82
	II	Lysine 8–10
Glu.(Ala, Leu)	II	77–82
	II	83–91
	II	Lysine 8–10
Leu.Ala	I	52–58
	II	83–91
Ileu.Asp	I	48–51
Leu.Glu or Ileu.Glu	II	Lysine 8–10
Leu.(Thr, Ala)	I	48–51
	II	77–82
	II	83–91
Leu or Ileu.(Glu, Ala, Leu or Ileu, Leu or Ileu)	II	48–51
Lys.(Val, Phe)	II	Lysine residues
Phe.Glu	II	83–91
Ser.Asp	I	44–47
	I	48–51
	II	72–76
Ser.Ala	I	48–51
	II	77–82
Ser.Leu	I	48–51
Ser.(Asp, Val)	I	48–51
	II	77–82
	II	Lysine 11–14
Ser.(Asp, Gly)	II	67–71
	II	72–76
	II	Lysine 8–10
	II	Lysine 11–14
Ser.(Asp, Leu)	II	83–91
Ser.(Leu, Arg)	II	83–91
Ser.(Gly, Leu)	II	83–91
	II	Lysine 8–10
	II	Lysine 11–14
Ser.(CySO$_3$H, Asp, Val)	II	Cysteic
Ser.(Gly, Ala, Leu?, *Leu*)	II	83–91
Ser.(Asp, Gly, MetO$_2$)	II	72–76
Ser.(Asp, Gly, Ala)	II	72–76
	II	Lysine 8–10
Thr.Asp	I	40–43
	I	44–47
	I	48–51
	II	67–71
	II	72–76
	II	Lysine 11–14
Thr.Glu	I	48–51
	II	72–76
Thr.Ala	I	48–51
Thr.(Asp, Gly)	II	Lysine 8–10
	II	Lysine 11–14
Thr.(Glu, Ala)	II	77–82
	II	Lysine 11–14
Thr.(Asp, *Glu*, Val)	II	67–71
	II	72–76
	II	Lysine 8–10
Thr.(Asp, Arg, Arg?)	II	83–91
Val.Asp	I	40–43
Val.CySO$_3$H	II	Cysteic
Val.(CySO$_3$H, Ala)	II	Cysteic

Table 3. *C-Terminal residues of peptides determined with carboxypeptidase*

Fraction of origin	Peptide structure judged from DNP treatment	Amino acids liberated by carboxypeptidase		Structure inferred
I, 44–47	Asp.(Glu, Val)	Glu Val	+ + + +	Asp.Val.Glu
I, 44–47	Glu.(Asp, Ileu)	Ileu	+	Glu.Asp.Ileu
I, 48–51	Glu.(Asp, Leu or Ileu)	Leu or Ileu Asp	+ + +	Glu.Asp.Leu or Ileu
I, 48–51	Leu.(Thr, Ala)	Ala	+ + +	Leu.Thr.Ala
I, 48–51	Leu or Ileu.(Glu, Ala, Leu or Ileu, Leu or Ileu)	Leu* Ala	+ + + + + + + +	Leu or Ileu (Glu, Ala, Leu or Ileu).Leu
II, 72–76	Thr.(Asp, Glu, Val)	Glu Val Asp	+ + + + + + +	Thr.(Asp, Val).Glu†
II, 77–82	Asp.(Glu, Ala)	Ala Glu	+ + + +	Asp.Glu.Ala
II, 85–91	Ser.(Gly, Ala, Leu)‡	Leu§ Ala	+ + + + + + + +	Ser.(Gly, Ala, Leu?).Leu

* This spot from a butanol–acetic acid chromatogram was cut out, eluted, and, run with *tert.*-pentanol–water, showed Leu (+ +) Ileu (+). This is suggestive that the *C*-terminal residue is leucine and not isoleucine. In the same peptide the liberation of alanine in next highest intensity to leucine is strong presumptive evidence for its being next to the *C*-terminal residue and hence for the sequence Leu or Ileu.(Glu, Leu or Ileu).Ala.Leu in the peptide.

† Liberation of valine in next highest intensity to glutamic acid strongly suggests the sequence Thr.Asp.Val.Glu.

‡ Possibly two leucine residues.

§ Run in butanol–acetic acid. If, as seems likely, there are two leucine residues in the peptide, the results could be interpreted by either Ser.Gly.Ala.Leu.Leu or Ser.(Gly, Leu).Ala.Leu.

Table 4. *Amino acid composition of lysozyme*

The more firmly established figures in the last column are in black.

	moles/14900 g. lysozyme				
	Present work*	Lewis *et al.* (1950)	Fromageot & de Garilhe (1950)	Mills† (1952)	Preferred integer
Alanine	12·4	9·7	10·2	10·1	10–12
Arginine	11·0	10·9	11·2‡	12·5	**11**
Aspartic acid	20·4	20·4	17·9§	13·4	**20**
Cystine	—	4·96	5·0	11·4	5
Glutamic acid	5·1	4·36	3·4	4·35	5
Glycine	11·8	11·3	10·6	9·5	11–12
Histidine	1·0	1·00	1·0	3·75	**1**
Isoleucine	5·9	5·91	6·1§	5·55	**6**
Leucine	8·2	7·84	9·6§	7·3	**8**
Lysine	5·8	5·81	6·2‡	6·23	**6**
Methionine	1·9	2·06	2·3	2·37	**2**
Phenylalanine	3·4	3·82	2·08	2·57	**3**
Proline	1·9	1·81	1·7	2·17	**2**
Serine	8·6	9·51	10·2	8·9	**9**
Threonine	6·7	6·89	6·7	5·75	**7**
Tryptophan	—	7·73	6·1	—	—
Tyrosine	3·16	2·92	2·9	2·76	**3**
Valine	5·3	6·11	6·0§	8·3	5–6

* Determined after hydrolysis by refluxing for 24 hr. in 6N-HCl. Analysis by Mr D. F. Elsden.

† Determined as dinitrophenylamino acids.

‡ Monier *et al.* (1952).

§ Personal communication quoted by Fevold (1950).

partial hydrolysates of proteins. This is not due to the difficulties involved in scaling down the size of the columns, as is shown by the comparatively successful separation of amino acids in the mixture (cf. Fig. 3, where the strong spots represent amino acids), but rather to the behaviour of the peptides themselves. The amino acids are preferentially displaced from the columns in their normal sequence but the proportion of longer-chain peptides increases gradually throughout the chromatogram till the final fractions consist entirely of peptides, especially those of highest molecular weight. It is clear that the behaviour of the peptides is governed less by the p*K* values of their ionizing groups than by van der Waals adsorptive forces between the resin and the peptide molecules (Partridge & Brimley, 1951*b*) which are regulated, as a first approximation, by molecular weight. This adsorption is far more serious than is indicated in Table 2, which omits many peptides altogether since it was not possible to determine their structure. This is because, in general, the larger the peptide molecule, the more difficult it is to determine its composition and the less there is of it in the hydrolysate. The importance of adsorption might have been predicted from a consideration of the behaviour of aromatic amino acids, which are displaced from ion-exchange chromatograms in a different order from that which would be expected on the basis of p*K* values alone.

As these adsorptive forces are relatively unspecific, it is not surprising that, when they preponderate over the ion-exchange reactions, the columns should fail to differentiate between large numbers of peptides of similar molecular size. This helps to explain why, to take two examples from Expt. I where the amino acids themselves were reasonably differentiated, Asp.Ileu should occur in fractions 37–39, 40–43, 44–47 and 48–51 and Glu.(Asp.Ileu) in fractions 44–47, 48–51 and 52–58. Unfortunately, the distribution of any one peptide between several (ion-exchange) fractions not only complicated the fractions but also furnished less of the peptide to work with. This lack of material sometimes made it impossible to obtain confirmatory evidence in doubtful cases, hence with several peptides no definite result was obtained. It is possible that, on ion-exchange columns operated at higher temperatures, a reduction of the effect of the adsorptive forces might permit a more typical ion-exchange behaviour and give more useful separations (cf. Partridge & Brimley, 1951*a*), but this involves the risk that some decomposition of the peptide material might occur.

Despite the fact that displacement chromatography on ion-exchange columns proved disappointing as a method for the preliminary separation of peptides resulting from the hydrolysis of lysozyme, it was possible in these two experiments to determine the structure of a considerable number of peptides and, from these, to deduce several probable sequences. These are listed in Table 5, and beneath them the structures of the peptides from which the evidence for them is derived.

Table 5. *Probable peptide sequences in lysozyme*

1. **Ser.Asp.Gly.$MetO_2$**
Ser.(Asp, Gly, $MetO_2$)
Ser.(Asp.Gly)
Asp.(Gly, $MetO_2$)
Ser. Asp Gly.$MetO_2$
Asp. Gly

2. **Thr.Glu.Ala**
Thr.(Glu, Ala)
Thr. Glu
Glu.Ala

3. **Thr.Asp.Val.Glu.Ala**
Thr.(Asp, Val, Glu, Ala)*
Thr.(Asp, Val, *Glu*)
Asp, (Val, Glu, Ala)
Thr.(Asp, Val)*
Asp.(Val, *Glu*)
Thr. Asp Glu.Ala
Val.Glu*

4. **Leu.Thr.Ala**
Leu.(Thr, *Ala*)
Thr.Ala

5. **Ileu.Glu.Leu.Ala.Leu**
Leu or Ileu.(Glu, Leu or Ileu, Ala, *Leu*)
Ileu. (Glu, Leu)*
Glu.(Leu, *Ala*)
Leu or Ileu. Glu. Leu. Ala
Glu. Leu Ala.Leu

6. **Asp.Glu.Ala**
Asp.(Glu, *Ala*)
Glu.Ala

7. **Glu.Asp.Ileu**
Glu.(Asp, *Ileu*)
Asp.Ileu

* Evidence for the presence of these peptides was obtained by using elution chromatography on ion-exchange resins in recent experiments which will be described in a later paper. The *C*-terminal residue of Glu.(Leu, *Ala*) was also shown to be alanine using carboxypeptidase.

Sequence (1) Ser.Asp.Gly.$MetO_2$ follows directly from the fact that lysozyme contains only two methionine residues (see analysis in Table 4) and since peptide Ala.$MetO_2$ has been well established, any peptide containing $MetO_2$ and not preceded by Ala must belong to the other $MetO_2$ sequence.

Sequence (2) Thr.Glu.Ala. On partial hydrolysis of the peptide Thr.(Glu, Ala) and chromatography, a spot appeared which was slow to develop a ninhydrin colour, consisted of Thr (trace) and Glu (+ +), and corresponded in position to Thr.Glu. This establishes the sequence Thr.Glu.Ala.

Sequence (3) Thr.Asp.Val.Glu.Ala. The sequence Thr.Asp.Val.Glu was well established by two peptides where carboxypeptidase was used to determine the *C*-terminal residue. Alanine is established as the next residue by the pentapeptide Thr.(Asp, Val, Glu, Ala).

Sequence (4) Leu.Thr.Ala. This sequence is definitely established despite the fact that the bond involving the amino group of the threonine is unbroken, which is unusual as such bonds, involving serine and threonine, are generally the most labile to acid hydrolysis. The occurrence of the peptide Leu.(Thr, Ala) on at least three occasions leaves no doubt that it is genuinely present in the hydrolysate. It is, however, the only case encountered in these experiments of a peptide containing serine or threonine bound through its amino group.

It was clear from the results of hydrolysis that the peptide represented by sequence (5) had one or two leucine residues in addition to an isoleucine residue. One of these three residues was *N*-terminal and one of the leucines *C*-terminal, with a suggestion that alanine preceded it. The peptide Ileu.(Glu, Leu) showed that Ileu was *N*-terminal and confirmed that the second and third residues were Glu and Leu (order unknown). The correct order was actually Glu.Leu as shown by the peptide Glu.(Leu, *Ala*) which also confirmed the position of the alanine residue. All possible dipeptides consistent with this pentapeptide sequence have been identified.

The tripeptide sequences (6) and (7) were established by identification of both *N*- and *C*-terminal residues.

SUMMARY

1. An attempt has been made to simplify the mixture of peptides produced on partial acid hydrolysis of lysozyme through fractionation by displacement chromatography on ion-exchange resins.

2. The peptides in the fractions so obtained have been separated by two-dimensional paper chromatography. The resulting peptide spots have been eluted and analysed for the constituent amino acids, the *N*-terminal residue and, in certain cases, the *C*-terminal residue.

3. The peptides whose structure is known are listed. From this information it has been possible to assemble a number of probable amino acid sequences as they occur in the lysozyme molecule.

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Mr D. F. Elsden carried out the analysis of lysozyme, Miss J. E. Dixon assisted in the experiments and Mr D. P. Gatherum prepared the photographs.

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