The Amino Acid Sequence in a Hypertensin

By D. F. ELLIOTT
National Institute for Medical Research, Mill Hill, London, N.W. 7

and W. S. PEART
Medical Unit, St Mary's Hospital, London, W. 2

(*Received* 29 *June* 1956)

The isolation of a hypertensin peptide, obtained by the action of rabbit renin on ox serum, was described recently (Peart, 1956). The amino acid analysis was consistent with a decapeptide structure involving eight different amino acids, which were leucine (Leu) (1), aspartic acid (Asp) (1), arginine (Arg) (1), phenylalanine (Phe) (1), proline (Pro) (1), tyrosine (Tyr) (1), histidine (His) (2) and valine (Val) (2).

The present communication describes experiments which have led to the elucidation of the sequence of amino acid residues. A number of different techniques were applied simultaneously; they included partial acid hydrolysis, end-group determination and digestion by various enzymes; these can now be regarded as standard procedures in this type of work (see, for example, Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). In addition, the stepwise method of degradation devised by Edman (1950) was employed. We were unable to utilize aminopeptidase (Smith & Spackman, 1955) for stepwise degradation, but, as shown below, this enzyme proved to be useful in other directions. A preliminary account of this work has already been published (Elliott & Peart, 1956).

METHODS

Hypertensin

Prepared as previously described by Peart (1956), this was contaminated with inorganic material in spite of very careful washing of the supporting media used in the columns. These inorganic impurities interfered with the electrophoretic separations carried out on enzymic digests. They were removed as follows. An amount of hypertensin equivalent to 1 mg. of active peptide was dissolved in 0·5% trichloroacetic acid solution (2 ml.; prepared from redistilled trichloroacetic acid) and extracted twice with peroxide-free *n*-butanol (2 ml.). The combined butanol layers were diluted with peroxide-free ether (8 ml.) and the solution was extracted twice with water (4 ml.). The combined aqueous layers were acidified with 6N-HCl (0·05 ml.) and extracted continuously with ether (250 ml.) in the apparatus described by Elliott & Crawhall (1955). The aqueous solution was then evaporated to dryness in a vacuum desiccator. The recovery of hypertensin was quantitative.

Paper chromatography

For most work Whatman no. 3 paper was used. Paper used for isolation of peptides was washed for several days in a chromatography tank with 4N acetic acid and then with water. Phenol–water was prepared by adding 5 vol. of liquid phenol to 2 vol. of water. It was used in a tank containing NH_3 vapour and a small quantity of KCN was dissolved in it immediately before use. The butanol–acetic acid mixture was prepared by shaking together *n*-butanol, water and acetic acid (63:27:10, by vol.) and keeping for several days before using the upper layer. Chromatograms were dried in a current of air at 40°, sprayed with a 0·1% ninhydrin solution in *n*-butanol containing 2% of acetic acid, moistened by steaming and heated at 90°. Chromatograms of peptides from which spots were to be cut for subsequent end-group determination or hydrolysis were sprayed with 0·025% ninhydrin solution, moistened by steaming and heated at 40° until colour development had reached a maximum. The isatin reaction described by Acher, Fromageot & Jutisz (1950) was used for the detection of proline.

Chromatography of dinitrophenyl amino acids (DNP amino acids) was carried out on Whatman no. 1 paper with the *tert.*-pentanol system of Blackburn & Lowther (1951). This one-dimensional system was adequate for the separation of the DNP derivatives of the amino acids in hypertensin.

Electrophoresis

This was carried out on strips of Whatman no. 3 MM paper, 45 cm. × 12·5 cm., in an uncooled apparatus similar to that described by Durrum (1950). The paper was washed with 4N acetic acid before use. Acetic acid (2N) was used throughout as the buffer; the potential gradient was 15 v/cm. and the current was approximately 10 mA. Substances were applied to the centre of the dry paper in the form of a narrow band varying from 0·5 to 2·5 cm. in length, according to the quantity of sample (0·25–1·0 mg.). At the end of the run the paper was dried in a current of warm air. A strip of approximately 2 mm. width was cut from the centre and along the length of the paper and stained by one of several methods. These included the usual ninhydrin method, the Pauly reagent of Ames & Mitchell (1952) and the chlorine method of Rydon & Smith (1952) as modified by Reindel & Hoppe (1954). The two unstained portions of the paper were marked accordingly and the parts containing the desired material cut and eluted by the technique described by Sanger & Thompson (1953*a*); 2–3 ml. of eluate was generally collected from these strips.

Determination of end groups by the fluoro-2:4-dinitrobenzene method

In most cases the method of Sanger & Thompson (1953*a*) was used, except that the amounts of trimethylamine and ethanol were increased to 0·2 and 0·4 ml. respectively/0·01 ml. of fluoro-2:4-dinitrobenzene (FDNB). These quantities of reagent were considered satisfactory for 0·5 mg. or less of peptide. With hypertensin itself, however, this technique had previously given poor yields of DNP-aspartic acid (Peart, 1956), and it was necessary to use $NaHCO_3$ as a buffer (Sanger, 1945) to improve the yield. The DNP peptides were hydrolysed with 6N-HCl at 105° for 12 hr. The usual ether extractions of the hydrolysate were replaced by those of ethyl acetate; this resulted in the extraction of bis-DNP-histidine which remains in the acid layer (Sanger, 1945) unless continuous ether extraction is used (Mills, 1952). The DNP amino acids were identified by paper chromatography. In quantitative experiments the spots of DNP amino acids were eluted with 1% $NaHCO_3$ and estimated at 350 mμ. in the Hilger Uvispek spectrophotometer. To correct for losses a known quantity of the DNP amino acid was subjected to chromatography and eluted under the same conditions.

Enzymic hydrolyses

Carboxypeptidase was an aqueous suspension obtained from Armour Laboratories. Trypsin and chymotrypsin were recrystallized samples kindly supplied by Mr J. Lightbown of this Institute. Aminopeptidase was kindly prepared by Dr R. R. Porter, by the method of Smith & Spackman (1955). Final purification was by zone electrophoresis on a column (40 cm. × 2·6 cm.) with treated cotton wool as supporting medium (Flodin & Porath, 1954; Porath, 1954; Campbell & Stone, 1956). Buffer solutions for carboxypeptidase, chymotrypsin and trypsin digestions were prepared by mixing M solutions of ammonium acetate and NH_4HCO_3 to give the required pH as shown by the glass electrode, and diluting to 0·05M. Carboxypeptidase digestions were carried out at pH 8 at room temperature or 37°. The enzyme:substrate molar ratio was generally about 1:80, but in some cases a ratio of 1:40 was used. Immediately before use any proteolytic impurities which may have been present were poisoned by the addition of di*iso*propyl phosphorofluoridate (DFP) (cf. Sanger & Thompson, 1953*b*). For example, 0·028 ml. of a suspension of carboxypeptidase (13 mg./ml.) and 0·5 ml. of 0·002M DFP were added to 3·5 ml. of 0·05M buffer at pH 8. The mixture was incubated at 37° for 30 min., cooled and divided into two equal portions. One portion was used as a control and was treated identically with the second portion, to which was added 0·6 mg. of hypertensin. The mixture was incubated at 37° and 0·5 ml. samples were removed at intervals. To each sample was added 0·05 ml. of acetic acid and the solution evaporated to dryness in a vacuum desiccator over KOH and H_2SO_4. The residue was dissolved in water and applied directly to Whatman no. 3 paper. Chromatograms were run in butanol–acetic acid, a mixture of all the amino acids present in hypertensin being used as markers.

Chymotrypsin digestion was carried out as follows: hypertensin (0·25 mg.) was dissolved in buffer at pH 7·5 (1·5 ml.) and to the solution was added a solution of chymotrypsin (0·016 ml. of a solution containing 4 mg./ml.) in the same buffer. The mixture was incubated for 16–20 hr. at 37°, acidified with 0·1 ml. of acetic acid and evaporated as in the carboxypeptidase digestion. The residue was redissolved in water and evaporated a second time. The products were separated by ionophoresis on 3 MM paper. Quantities of hypertensin up to 1 mg. were handled in a similar way.

Trypsin digestion was carried out in a similar fashion to the chymotrypsin digestion at pH 7·5 and 37° for 18 hr. The products were separated by paper chromatography with butanol–acetic acid.

The amount of aminopeptidase required for digestion was discovered by a preliminary trial. For example, 0·01 ml. of a solution of aminopeptidase was activated by incubation for 40 min. at 37° with 0·1 ml. of 0·1M ammonium acetate–NH_3 soln. buffer at pH 8, made 2·5 mM with respect to $MnCl_2$. This was then added to 0·5 ml. of the same buffer system at pH 8·6, made 2 mM with respect to $MnCl_2$ and containing 0·5 mg. of hypertensin. Such a mixture was incubated at either room temperature or 37° and samples were withdrawn at intervals. A control solution omitted only the hypertensin. The samples were acidified with a drop of acetic acid and dried in a desiccator over NaOH and H_2SO_4. The products of digestion were separated by paper chromatography in butanol–acetic acid.

Partial acid hydrolyses

The peptide was dissolved in HCl (36%, w/w) and incubated at 37° for 3–4 days. The product was evaporated to dryness in a vacuum desiccator and submitted to paper chromatography or electrophoresis.

Edman stepwise-degradation method

For the reaction with phenyl *iso*thiocyanate and subsequent cyclization to give a thiohydantoin, the technique worked out by Fraenkel-Conrat, Harris & Levy (1955) was adapted as follows: 1·2 mg. of hypertensin was dissolved in 50% aqueous dioxan (6 ml.) to which was added freshly distilled phenyl *iso*thiocyanate (0·1 ml.). The mixture was heated to 40° in a thermostat bath, stirred with a stream of N_2 and 0·01N-NaOH added from an Agla microburette to maintain a pH of 9 with the glass electrode. The rate of addition of alkali was plotted graphically and the experiment was stopped when a constant 'end-slope' was reached (see Fig. 1). The solution was evaporated to small bulk under reduced pressure at about 35° to remove most of the dioxan and extracted seven times with redistilled benzene. The aqueous layer was evaporated to dryness in a vacuum desiccator.

The residue was dissolved in 3N-HCl (10 ml.) (prepared from redistilled constant-boiling HCl) and the optical density of the solution measured as rapidly as possible over the range 230–270 mμ. in the Hilger Uvispek instrument at intervals of 5 mμ. The cyclization was considered to be complete when no further change in optical density occurred at 265 mμ. The solution was then extracted five times with ethyl acetate (Spectrosol brand from Hopkin and Williams Ltd., London). The ethyl acetate extracts were combined, evaporated under reduced pressure and the residue was hydrolysed by heating with 0·1 ml. of HI (redistilled; *d* 1·7) at 140° for 2 hr. in a sealed tube. The hydrolysate was evaporated to dryness in a desiccator over KOH and the amino acid identified by chromatography in butanol–acetic acid. The acid solution remaining after ethyl acetate extraction was evaporated to dryness over KOH. The above cycle of operations was then repeated on the residue. After

the third series of reactions, the volume of 3N-HCl used for cyclization was reduced to 5 ml. and the acid solution remaining after ethyl acetate extraction of the phenylthiohydantoin was evaporated to dryness. The residue was redissolved in a small volume of dilute $NaHCO_3$ and submitted to a second extraction with ethyl acetate. This ensured that the phenylthiohydantoin of histidine, which would otherwise remain in the acid solution (Fraenkel-Conrat *et al.* 1955), would be extracted. The ethyl acetate extracts were combined and treated exactly as those from the acid solution. The aqueous solution was made weakly acid with HCl (pH 4) and evaporated to dryness. Reaction with phenyl *iso*thiocyanate could then be carried out on the residue.

Stepwise degradation of the peptide HC2 was performed in a similar fashion, the double-extraction procedure being performed at each stage.

RESULTS

N-*Terminal residue of hypertensin*

Although a number of attempts were made it was not possible to obtain more than 50% of the theoretical yield of DNP-aspartic acid. Other workers have obtained low yields of DNP-aspartic acid from aspartyl peptides (Schroeder & Le Gette, 1953). It seemed probable, therefore, that an *N*-terminal residue of aspartic acid or asparagine was present in hypertensin.

Hydrolysis of hypertensin by aminopeptidase

Digestion with aminopeptidase for 14 hr. at 37° released all the amino acids present in hypertensin and there was no evidence of peptides remaining undigested. At room temperature, when samples were taken at 0·5, 2 and 6 hr. respectively, all the amino acids appeared simultaneously and seemed to increase in amount at the same rate. The quantities were approximately gauged by visual comparison with serial dilutions of an equimolar solution of the amino acids present in hypertensin, run in parallel on the chromatogram. Although small differences in the rate of release of the amino acids would not be detected, the method was sufficiently sensitive to show that there were not big differences in the relative rates. It was decided that with these methods aminopeptidase was not suitable for determination of the amino acid sequence in hypertensin. However, it was useful in showing that aspartic acid and not asparagine was present in hypertensin since the acid was always liberated by the action of this enzyme. This is consonant with the finding that the quantity of alkali consumed in the reaction of hypertensin with phenyl *iso*thiocyanate was in agreement with the presence of two free carboxyl groups (see Edman procedure below).

Edman degradation of hypertensin

Fig. 1 illustrates the uptake of alkali by 1·2 mg. of hypertensin in the presence of phenyl *iso*thiocyanate. Curves obtained at subsequent stages of the degradation were similar in shape, although the total alkali uptake was variable, often being greater than in the first step. In Fig. 2 are shown the initial

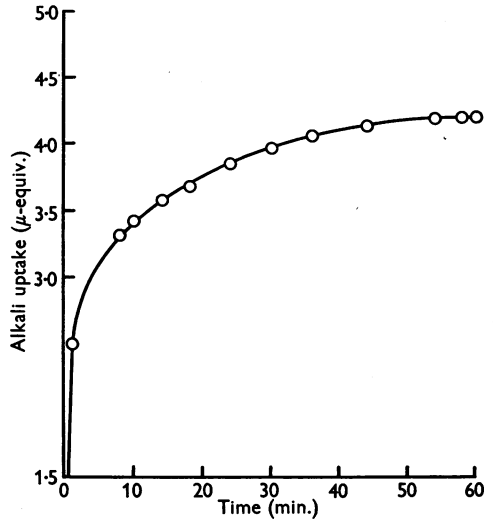

Fig. 1. Titration with 0·01N-NaOH of 1·2 mg. of hypertensin dissolved in 6 ml. of 50% aqueous dioxan at pH 9 and 40° and in the presence of 0·1 ml. of phenyl *iso*thiocyanate.

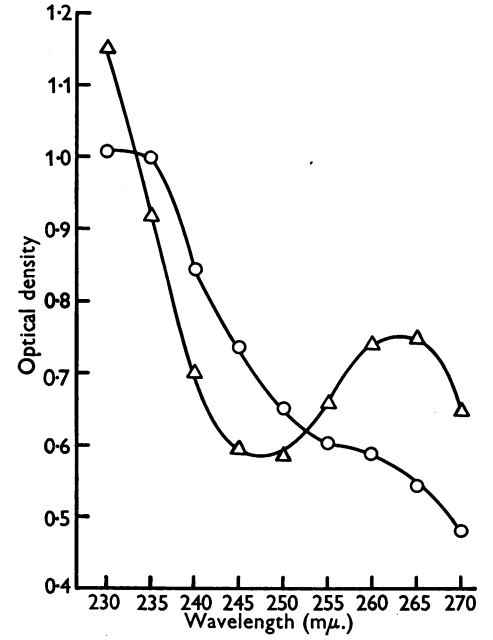

Fig. 2. Light-absorption curves obtained during treatment of the phenylthiocarbamyl derivative from 1·2 mg. of hypertensin with 10 ml. of 3N-HCl. ○, Curve obtained after 8–13 min.; △, curve obtained after 3·3 hr.

Table 1. *Amino acids liberated from hypertensin during various periods of digestion by carboxypeptidase*

Expt. no.	Time (hr.)	Temp.	Relative amounts of amino acids liberated
1	0·25	22°	Leu X
	1		Leu X ? His (trace)
2	2	37	Leu XX Phe X ? His X
	4		Leu XX Phe X ? His X
	8		Leu XXX Phe X ? His X
3	14	37	Leu XXX Phe XX ? His X
	25		Leu XXX Phe XX ? His XX

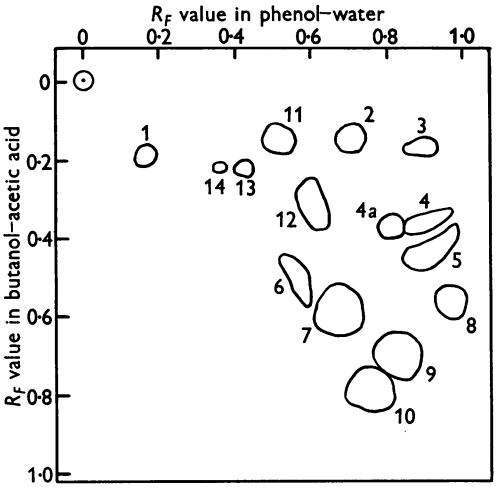

Fig. 3. Chromatogram obtained after partial hydrolysis of 1·1 mg. of hypertensin with HCl (36%, w/w) at 37° for 3–4 days.

and the final light-absorption curves obtained during the first cyclization step. In the subsequent cyclization steps, this characteristic change of shape of the absorption curve did not appear to take place; the only effect observed was a small change in the absorption at 265 mμ. The solution was extracted when no further change occurred. In the first step aspartic acid was obtained, whereas in the second step no recognizable amino acid was detected; the third and fourth steps yielded valine and tyrosine respectively. A total of seven degradations was carried out, but in the fifth, sixth and seventh steps it was not possible to identify any amino acid with certainty owing to the weakness of the spots.

It seemed probable therefore that the *N*-terminal sequence in hypertensin was Asp.Arg.Val.Tyr.... Arginine was placed with less confidence than the other amino acids owing to the fact that its phenylthiohydantoin cannot be extracted from aqueous solution by solvents and its presence is assumed when a thiohydantoin is not found in the ethyl acetate extract after cyclization (Fraenkel-Conrat *et al.* 1955).

C-Terminal sequence in hypertensin

Carboxypeptidase. The results of carboxypeptidase digestion are shown in Table 1. In this and subsequent tables X signifies a readily identifiable spot on a chromatogram, XX has twice the intensity of X, XXX three times the intensity of X, and so on. It was clear that the *C*-terminal amino acid was leucine. The next amino acid in the chain appeared to be histidine, followed by phenylalanine. Histidine was not identified with certainty; in view of its low R_F value in butanol–acetic acid the spot regarded as histidine might have been a peptide consisting of the hypertensin molecule minus one or two *C*-terminal residues.

Partial hydrolysis of hypertensin with acid. Fig. 3 shows the chromatogram obtained from a 3 days' hydrolysate of 1·1 mg. of hypertensin. The results obtained on examination of the spots by total hydrolysis and the FDNB technique are given in Table 2. Spots 11–14 gave on hydrolysis very weak spots of amino acids not present in hypertensin and were probably derived from impurities. The main result was that one dipeptide was isolated in about 60% of the theoretical yield as determined by visual examination of the spots produced by a total hydrolysate. Although this peptide was contaminated with small amounts of other peptides or amino acids (Table 2, spot no. 5) there seemed no doubt that its structure was His.Leu. It then follows that the *C*-terminal sequence in hypertensin is ...Phe.His.Leu. The fact that no other amino acids were liberated from hypertensin after digestion for 25 hr. with carboxypeptidase indicated that proline was connected to phenylalanine (see Smith, 1953), but further proof of this was needed.

Hydrolysis of hypertensin by trypsin

The FDNB technique revealed the presence of two *N*-terminal residues in the trypsin digest of 0·25 mg. of hypertensin. These were aspartic acid and valine. Paper chromatography of a digest of 0·25 mg. of hypertensin run in butanol–acetic acid revealed the presence of a substance of lower R_F value (0·08) than histidine. This was eluted and hydrolysed. Aspartic acid and arginine were the only two amino acids present. The other product of digestion proved

Table 2. *Amino acids and peptides obtained by partial acid hydrolysis of hypertensin*

Spot no.	Relative strengths of amino acids in total hydrolysate			*N*-Terminal amino acid (FDNB method)	Relative strengths of amino acids remaining after removal of *N*-terminal amino acid	Structure
1		Asp XX		Asp	—	Aspartic acid
2		His XX		His	—	Histidine
3	Arg XX	Val X		Arg	Val X	Arg.Val
4	His XX Tyr X	Val XX Phe X	Pro X Leu X	Not found	—	—
4a	Pro XXX	His X	Val X	Not found	—	Peptide + proline
5	His XXXX Pro X	Leu XXXX Val X		His	Leu XXXX	His.Leu
6		Tyr X		Tyr	—	Tyrosine
7		Val XXXX		Val	—	Valine
8	His XX Val XX	Pro XX Phe XX	Tyr XX Leu XX	Not found	—	—
9	Phe X Val (trace)	Leu (trace)		Not found	—	? Phenylalanine
10	Pro XX Leu XXX	Phe XX	Val X	Leu	—	Mixture of peptides + leucine

impossible to identify satisfactorily by paper chromatography. These results, however, indicate the sequence Asp.Arg.Val.... and confirm the *N*-terminal sequence. The sequence Arg.Val had been demonstrated by partial hydrolysis with acid (spot no. 3, Table 2).

Hydrolysis of hypertensin by chymotrypsin

Application of the FDNB technique to a chymotrypsin digest from 0·2 mg. of hypertensin revealed the presence of three *N*-terminal residues. These were aspartic acid, valine and histidine. The peptides in a similar digest could not be separated by paper chromatography. Fig. 4 illustrates the result obtained on paper electrophoresis of a digest of 0·25 mg. of hypertensin. The paper was cut into three sections and stained by different techniques. The experiment was then repeated on the same scale and the bands HC1, HC2, HC3 were eluted and hydrolysed completely. The results are shown in Table 3. In a separate experiment the *N*-terminal residues of HC3 and HC2 were determined by the FDNB method.

*Peptide HC*1. This contained aspartic acid, arginine, valine and tyrosine, and as the *N*-terminal sequence Asp.Arg.Val.Tyr had been previously established for hypertensin, it was clearly the *N*-terminal tetrapeptide. Further confirmation was obtained from the action of carboxypeptidase under various conditions, which revealed the *C*-terminal sequence Val.Tyr (Table 4).

*Peptide HC*2. This contained valine, histidine, proline and phenylalanine, and the *N*-terminal amino acid was shown to be valine by application of the FDNB technique to one-quarter of a batch of

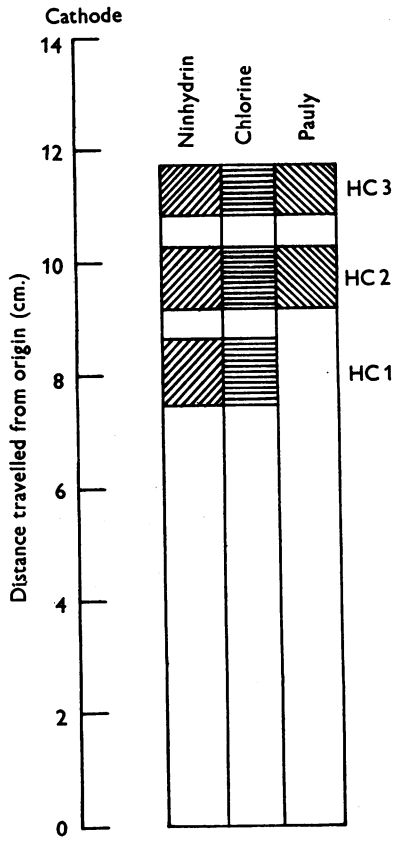

Fig. 4. Result of electrophoresis, for 1·5 hr. in 2N acetic acid at 15 v/cm., of a chymotrypsin digest of hypertensin. Bands stained by various techniques. Scale, ×4 in horizontal direction.

Table 3. *Peptides isolated by electrophoresis of a chymotryptic digest of hypertensin*

Band no.	Relative strengths of amino acids in the hydrolysate	*N*-Terminal residue	Structure
HC1	Asp XX Arg XX Tyr X Val XX	Not determined	Asp.Arg.Val.Tyr
HC2	His XX Pro XX Val XX Phe XX	Val	See Tables 4 & 5
HC3	His XX Leu XX	His	His.Leu

Table 4. *Amino acids liberated from peptides HC1 and HC2 during various periods of digestion by carboxypeptidase*

Expt. no.	Peptide	Time (min.)	Temp.	Enzyme:substrate (molar ratio)	Relative amounts of amino acids liberated
1	HC1	2	20°	1:160	Tyr X
		5	20		Tyr XX
2		15	20	1:160	Tyr XXX Val X
		60	20		Tyr XXX Val XX
3		1080	39	1:40	Tyr XXXX Val XXXX
4	HC2	1080	39	1:40	Phe XXXX

HC2 isolated from 1 mg. of hypertensin. The hydrolysate remaining after removal of the DNP amino acid contained proline, phenylalanine and (iminazole)-DNP-histidine.

Edman degradation carried out on HC2 isolated from 1 mg. of hypertensin indicated the *N*-terminal sequence Val.His.Pro and the action of carboxypeptidase for 18 hr. at an enzyme:substrate ratio of 1:40 showed only phenylalanine (Table 4), which strongly suggested that the adjacent residue was proline (see Smith, 1953).

Partial acid hydrolysis of HC2 derived from 0·75 mg. of hypertensin was carried out for 3 days. Fig. 5 shows the results obtained on electrophoresis of one-half of the product. The remaining material was separated as before, and, after staining a marker strip by the chlorine method, the peptides HC2A1, HC2A2 and HC2A3 were eluted. Table 5 gives the results of hydrolysis of these peptides and of an end-group determination on HC2A3. For the latter purpose, another partial hydrolysis of HC2 was carried out.

Peptide HC2A1 gave only proline and phenylalanine on hydrolysis and was, therefore, Pro.Phe; peptide HC2A2 was clearly unchanged tetrapeptide, and HC2A3 must have been Val.His. The latter sequence was confirmed by the FDNB method. The complete sequence of HC2 was therefore confirmed as Val.His.Pro.Phe.

Peptide HC3. This contained histidine and leucine and the FDNB technique showed histidine to be *N*-terminal. This helped to confirm the *C*-terminal sequence of hypertensin, previously shown to be Phe.His.Leu by other techniques.

Consideration of all these results showed that the amino acid sequence in hypertensin was

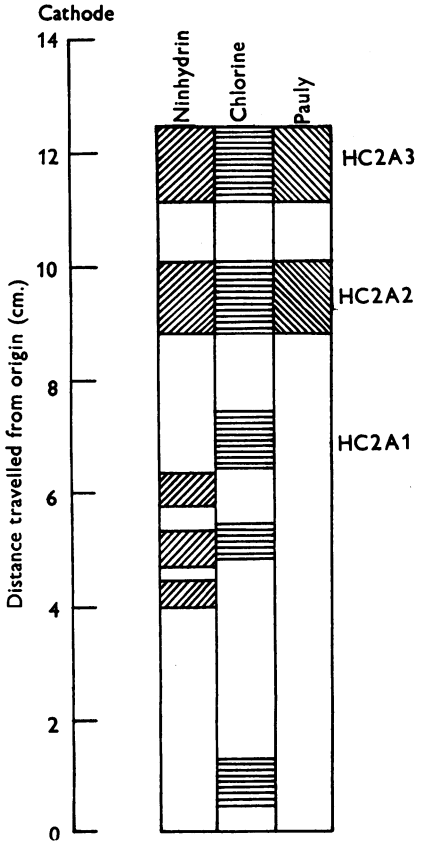

Fig. 5. Result of electrophoresis, for 1·25 hr. in 2N acetic acid at 15v/cm., of a partial hydrolysate of peptide HC2. Bands stained by various techniques. Scale, ×4 in horizontal direction.

Table 5. *Peptides isolated by electrophoresis of a partial acid hydrolysate of HC2*

Peptide	Amino acids present	*N*-Terminal residue	Structure
HC2A1	Pro, Phe	Not determined	Pro.Phe
HC2A2	His, Pro, Val, Phe	Not determined	Unchanged HC2
HC2A3	His, Val	Val	Val.His

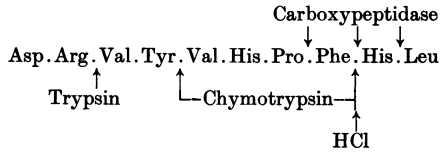

Fig. 6. Fission of hypertensin by various reagents.

Asp . Arg . Val . Tyr . Val . His. Pro . Phe . His . Leu. The principal linkages in hypertensin which are broken by the techniques described above are shown in Fig. 6.

DISCUSSION

N-Terminal sequence in hypertensin. When this work was commenced it was not certain that hypertensin had an open-chain structure. Although it seemed probable that it possessed an *N*-terminal residue consisting of aspartic acid,* the yield of DNP-aspartic acid obtained on hydrolysis of DNP-hypertensin was not good. Furthermore, this was obtained only when bicarbonate was used in the reaction with FDNB; with trimethylamine, the yield was extremely small. On the other hand, a simple cyclic structure seemed unlikely when it was found that the molecule was attacked by carboxypeptidase in the presence of di*iso*propyl phosphorofluoridate. In the course of the structural studies described in this paper, the presence of an *N*-terminal residue of aspartic acid has been demonstrated in several ways. The Edman degradation revealed aspartic acid as the *N*-terminal residue and, furthermore, both chymotrypsin and trypsin digests contained peptides possessing *N*-terminal aspartic acid, the DNP derivative of which was obtained in good yield by the FDNB method with trimethylamine as buffer. It seems unlikely that both these enzymes could break the same bond in view of their widely different specificities, and it thus follows that the amino group of aspartic acid must have been free in the original hypertensin molecule. It was also found that aminopeptidase, which is known to require a free *N*-terminal residue in its peptide substrate (Smith, Spackman & Brown, 1955), rapidly attacked hypertensin with liberation of aspartic acid and all the remaining amino acids. Although the work of Schroeder & Le Gette (1953) has shown that aspartyl peptides may give rather poor yields in the FDNB method, it seems possible that the chemical reactivity of the amino group in hypertensin is hindered in some way. This is indicated by the marked difference in the yield obtained when trimethylamine was replaced by sodium bicarbonate. A low yield of the *N*-terminal serine residue of corticotrophin (ACTH) was obtained by Levy & Li (1955), using the FDNB technique; this was due to destruction during acid hydrolysis. Owing to the scarcity of material, this interesting point has not been examined further.

Considering in more detail the results of the Edman degradation of hypertensin, Fig. 1 shows that 4·2 micro-equivalents of alkali were consumed in the reaction with phenyl *iso*thiocyanate. Hypertensin prepared by the method of Peart (1956) and freed from inorganic impurities as described in this paper could contain a maximum of four molecules of bound hydrochloric acid. Of these, the two molecules bound to the histidine residues would be completely titrated at pH 9, whereas the one bound to arginine would not. The two carboxyl groups would also be completely titrated. Thus a maximum of four titratable groups would be present and in addition one molecule of alkali would be consumed in the reaction:

$$R.NH_3^+ + PhN{:}C{:}S + OH^- \rightarrow R.NH.CS.NH.Ph + H_2O.$$

The molecular weight of a tetrahydrochloride of hypertensin would be 1427. The weight of hypertensin actually taken, 1·2 mg., should therefore consume $(1{\cdot}2/1{\cdot}43) \times 5 = 4{\cdot}2$ micro-equivalents. This consumption of the maximum possible quantity of alkali is satisfactory evidence that hypertensin contains one free amino group per ten amino acid residues. The change that occurred in the absorption when the phenylthiocarbamyl derivative of hypertensin was treated with 3N hydrochloric acid (Fig. 2) is characteristic of the formation of a phenylthiohydantoin derivative of the *N*-terminal amino acid (Fraenkel-Conrat *et al.* 1955). With the value of 16 000 for the molecular extinction coefficient (Fraenkel-Conrat *et al.* 1955) the yield of phenylthiohydantoin was calculated to be 55·9 %. The fact that this characteristic change in the

* In this discussion aspartic acid is used in a somewhat vague sense. None of the techniques used, with the exception of aminopeptidase digestion, would have distinguished between aspartic acid and asparagine.

absorption spectrum was not observed in the subsequent stages is believed to be due to the accumulation of artifacts which absorb light in the region of minimum absorption of the thiohydantoin. The curve would thus be flattened out. It seemed unlikely that the absorption at 265 mμ. represented a true measure of the yields of the thiohydantoins in these cases and no attempt was made to calculate them. The idea that artifacts accumulated was upheld by the results of alkali titration in the reactions with phenyl *iso*thiocyanate; the uptake was always greater than theoretical from step 2 onwards. Fortunately the constant end slope, signifying the end of the reaction, was still clearly visible on the graphs plotted during these experiments.

Although it was found possible to identify thiohydantoins of amino acids by the various techniques which have been described (see Fraenkel-Conrat *et al.* 1955) these were not sufficiently reproducible to warrant their use with hypertensin. Various methods of hydrolysis of the thiohydantoins to amino acids have been investigated (see Fraenkel-Conrat *et al.* 1955); most recently Schramm, Braunitzer & Schneider (1955) have used hydriodic acid for the hydrolysis of the thiohydantoin derivative of proline. It was found that this method gave better results than other methods with the thiohydantoins of all the amino acids present in hypertensin. The thiohydantoin derivative of arginine was not investigated. This method was therefore used for the identification of the thiohydantoins produced during the Edman degradation of hypertensin and peptide HC2. It was found that all chromatograms contained ninhydrin-positive artifact spots, often as strong as the *N*-terminal amino acid in the peptide; these were not a result of the method of hydrolysis of the thiohydantoins, which had already been found to give amino acids substantially free from other substances. Fortunately, these artifact spots were produced consistently in every step of the degradation and the R_F values were different from the amino acids in hypertensin. It is evident from a recent preliminary publication (Edman, 1956) that the cleavage of phenylthiocarbamyl derivatives of peptides is more complicated than was previously believed, but until a full account appears it is not possible to decide whether the difficulties encountered in the present work could be explained in the light of this knowledge.

In view of the difficulty which was sometimes experienced in obtaining a satisfactory yield of tyrosine on acid hydrolysis of hypertensin, it was interesting to find similar results on hydrolysis of peptide HC1 (Table 3). The tyrosine appeared to be present in only one-half of the theoretical amount, but it was obtained in good yield when liberated by long digestion with carboxypeptidase (Table 4). Although tyrosine has been shown to be one of the less-stable amino acids (Hirs, Stein & Moore, 1954), destruction to this extent is abnormal and is perhaps due to the presence of carbohydrate extracted from the filter paper or to oxidation, in view of the very small quantities of material being handled.

All the amino acids present in hypertensin could be assumed to have the L-configuration since they were freely liberated by aminopeptidase (see Smith *et al.* 1955, for evidence of optical specificity).

SUMMARY

1. The amino acid sequence in a hypertensin is shown to be aspartic acid.arginine.valine.tyrosine.valine.histidine.proline.phenylalanine.histidine.leucine.

2. On the basis of digestion by aminopeptidase, it is believed that all the amino acids have the L-configuration and that the *N*-terminal residue is aspartic acid, not asparagine.

The authors wish to thank Mr D. R. Lind for assistance with the degradative work, Mr C. F. O'Neill for assistance with the preparation of hypertensin, and various colleagues for their help and advice.

REFERENCES

Acher, R., Fromageot, C. & Jutisz, M. (1950). *Biochim. biophys. Acta*, **5**, 81.
Ames, B. N. & Mitchell, H. K. (1952). *J. Amer. chem. Soc.* **74**, 252.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Campbell, P. N. & Stone, N. E. (1956). *Biochem. J.* **62**, 9P.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Edman, P. (1950). *Acta chem. scand.* **4**, 283.
Edman, P. (1956). *Nature, Lond.*, **177**, 667.
Elliott, D. F. & Crawhall, J. C. (1955). *Biochem. J.* **61**, 264.
Elliott, D. F. & Peart, W. S. (1956). *Nature, Lond.*, **177**, 527.
Flodin, P. & Porath, J. (1954). *Biochim. biophys. Acta*, **13**, 175.
Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 359. Ed. by Glick, D. New York: Interscience Publishers, Inc.
Hirs, C. H. W., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 941.
Levy, A. L. & Li, C. H. (1955). *J. biol. Chem.* **213**, 487.
Mills, G. L. (1952). *Biochem. J.* **50**, 707.
Peart, W. S. (1956). *Biochem. J.* **62**, 520.
Porath, J. (1954). *Acta chem. scand.* **8**, 1813.
Reindel, F. & Hoppe, W. (1954). *Chem. Ber.* **87**, 1103.
Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
Sanger, F. (1945). *Biochem. J.* **39**, 507.

Sanger, F. & Thompson, E. O. P. (1953*a*). *Biochem. J.* **53**, 353.
Sanger, F. & Thompson, E. O. P. (1953*b*). *Biochem. J.* **53**, 366.
Sanger, F. & Tuppy, H. (1951*a*). *Biochem. J.* **49**, 463.
Sanger, F. & Tuppy, H. (1951*b*). *Biochem. J.* **49**, 481.
Schramm, G., Braunitzer, G. & Schneider, J. W. (1955). *Nature, Lond.*, **176**, 456.
Schroeder, W. A. & Le Gette, J. (1953). *J. Amer. chem. Soc.* **75**, 4612.
Smith, E. L. (1953). In *The Chemical Structure of Proteins*, p. 109. Ed. by Wolstenholme, G. E. W. & Cameron, M. P. London: J. and A. Churchill Ltd.
Smith, E. L. & Spackman, D. H. (1955). *J. biol. Chem.* **212**, 271.
Smith, E. L., Spackman, D. H. & Brown, D. M. (1955). *J. biol. Chem.* **212**, 255.

Steroid Utilization and Fatty Acid Synthesis by the Larva of the Housefly *Musca vicina* Macq.

By Z. H. LEVINSON
Medical Research Laboratories, Medical Corps, Israel Defence Forces
and E. D. BERGMANN
Scientific Department, Israel Ministry of Defence

(*Received* 22 *November* 1955)

Recent studies on the lipid requirements (Silverman & Levinson, 1954) of, and on the specificity of steroids as growth factors (Bergmann & Levinson, 1954) for, housefly larvae, have shown that a sterol (phytosterol or zoosterol) is the only lipid essential for growth and metamorphosis. The growing larva consumes the sterols of the wheat bran of the medium together with considerable amounts of unsaturated fatty acids; however, whilst the latter are metabolized during pupation, the sterol content remains relatively constant throughout this period (Levinson & Silverman, 1954). The importance of steroids in insect physiology has been known for some time, but relatively little work appears to have been done on the housefly.

In the present study, therefore, the utilization of different steroids by *Musca vicina* larvae has been investigated. A preliminary account of this work has been given by Levinson & Bergmann (1955).

MATERIALS AND METHODS

Musca vicina *Macq.* The strain of houseflies was the same as that used in previous investigations; the method of its maintenance has been described by Silverman & Silverman (1953).

Preparation of newly hatched larvae. Adult houseflies, 2 days of age, were fed for 2 days on a water suspension of 20% (w/v) powdered milk and subsequently sucrose and water. Six- to seven-day-old females were allowed to oviposit within 3 hr. on a pad of damp cotton wool. Approximately 750 eggs were collected, carefully washed with water, placed on damp blotting paper in a covered Petri dish (diam. 9 cm.) and incubated at 30°. Usually 80–90% of these eggs were fertile, and larvae hatched from them $11 \pm 1{\cdot}5$ hr. after oviposition. Soon after hatching, the larvae congregated inside the cover of the Petri dish.

Since up to the present time no satisfactory method for the sterile culture of houseflies is known, the experiments have been carried out in the presence of a controlled bacterial flora. Although this flora failed to provide utilizable steroids for larvae reared on a lipid-free diet, it is possible that the bacteria cause changes in the steroid structure before its ingestion.

Rearing method. The medium (approximately 1 g.) was placed in a test tube (150 mm. × 15 mm.), and 0·15 ml. of a suspension of stock bacterial flora (Silverman & Silverman, 1953; $1{\cdot}5\text{–}2{\cdot}5 \times 10^8$ bacteria/ml., 70% of which were *Escherichia coli*, the balance *Sarcina*, *Lactobacillus* and *Bacillus subtilis*) was added together with one larva. The tubes were then incubated at 35°. The internal temperature of the medium was $36 \pm 0{\cdot}5°$ during the period of larval growth. At an age of 72 hr., the larvae were removed from their diets in the same sequence as they had been inoculated, cleaned of adhering medium, and weighed to the nearest 0·1 mg. Immediately after weighing, they were returned to their media. For convenient observation of the appearance of pupae, a flock of cotton wool was loosely plugged into each test tube (Fig. 1). The larvae were reincubated (35°) and inspected daily at a constant time until pupation or death. Records were kept of the number and average weight of the larvae, as well as of the number of larvae pupating or perishing on successive days and the number of adults emerging from the pupae. In a few instances the fecundity of the females was determined.

Composition of the experimental media. No. 1. Wheat-bran diet: a quantity (25 g.) of sieved wheat bran (Golan-Flour Mills, Tel-Aviv) was covered with 50 ml. of pure ether and incubated at 35° for 2 days, at which time the ether had completely evaporated. Before use, 25 ml. of water was mixed uniformly with the bran. The ether treatment was applied in order to make the conditions as similar as possible for all three diets used.

No. 2. Casein diet: the chemicals listed in Table 1 were intimately mixed and the pH was adjusted to 7·0 by dropwise addition of 5N-NaOH solution. Pieces of chloroform-