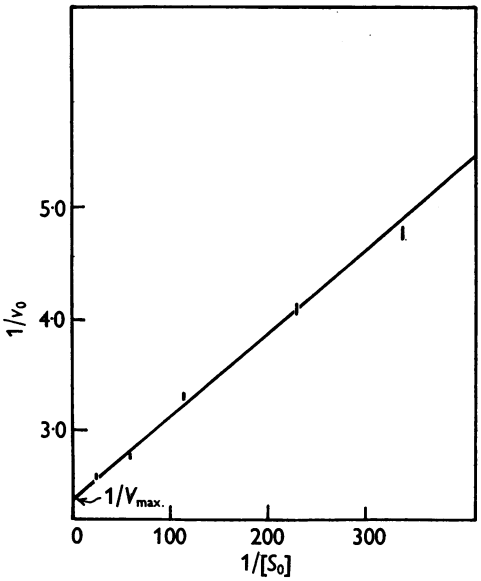

Fig. 4. Determination of K_m of benzoyl-L-arginineamide at pH 7·60 in 0·01 M phosphate buffer, 0·2 M-NaCl and 2% (w/v) formaldehyde. Each reaction mixture contained 3·3 mg. of enzyme. [S_0] and v_0 are the respective initial substrate concentrations and rates. K_m was calculated from the slope by means of the equation

$$\frac{(V_{max.})_{[S]\to\infty}}{v_0} = \frac{K_m}{[S_0]} + 1.$$

(Neurath & Schwert, 1950). It would be unlikely, therefore, that a free amine group (ammonium ion at pH 7·6) would be found in the region of the active site. It is of interest to note that acetylation of trypsin results in no loss of esterase activity (Jansen, Nutting, Jang & Balls, 1949).

SUMMARY

1. Although formaldehyde alters the chemical structure of trypsin it has no effect on the activity of the enzyme at the pH optimum.

2. A method for following the trypsin-catalysed hydrolysis of amides based on the above observation has been described.

3. The enzyme–substrate dissociation constant for benzoyl-L-arginineamide has been determined ($K_m = 3·1 \times 10^{-3}$ M) as well as the first-order specific rate of decomposition of the enzyme–substrate complex ($k_3 = 0·043$ sec.$^{-1}$).

4. The pronounced deceleration of trypsin-catalysed hydrolysis with time has been explained in terms of the self-digestion of the enzyme (a process which is inhibited by the substrate).

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REFERENCES

Bergmann, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
Gutfreund, H. (1954). *Trans. Faraday Soc.* **50**, 624.
Harmon, K. M. & Niemann, C. (1949). *J. biol. Chem.* **178**, 743.
Huang, H. T. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 475.
Iselin, B. M. & Niemann, C. (1950). *J. biol. Chem.* **183**, 403.
Jansen, E. F., Nutting, M.-D. F., Jang, R. & Balls, A. K. (1949). *J. biol. Chem.* **179**, 189.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Neurath, H. & Schwert, G. W. (1950). *Chem. Rev.* **46**, 69.
Northrop, J. H., Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes.* Columbia University Press.
Schwert, G. W. & Eisenberg, M. A. (1949). *J. biol. Chem.* **179**, 665.
Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.

The Amide Groups of Insulin

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Previous work has shown that insulin consists of two types of polypeptide chains the amino acid sequences of which have been determined by partial hydrolysis experiments (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). For an assumed molecular weight of approximately 6000, which is the simplest chemical unit consisting of one phenylalanyl and one glycyl chain, there are six amide groups present (Rees, 1946). From a quantitative study of the products of reduction of insulin ester with lithium borohydride Chibnall & Rees (1952) showed that the three aspartic acids, one of which is

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the *C*-terminal residue of the glycyl chain, are all present as amides, whereas three glutamic acid residues are amidized and the other four have free carboxyl groups. The following paper describes experiments to locate these amide groups on the individual dicarboxylic acid residues. To achieve this, use has been made of the fact that amide groups are split rapidly by the action of acid, but are not in general attacked by proteolytic enzymes. Thus peptides obtained from enzymic digests contain the original amide groups intact, whereas those obtained from acid hydrolysates do not. Two methods are thus available for identifying amide groups. In the first, the ionophoretic rates of the peptides from enzymic digests are compared with the rates of other similar peptides obtained from acid and enzymic hydrolysates. From these results it is possible to calculate the number of charged groups on the peptides and hence the number of carboxyl groups that are masked as amides. In the second method the number of amide groups present in the peptides from enzymic digests is determined directly by an estimation of the ammonia produced by hydrolysis with acid.

A number of peptides were identified from peptic and chymotryptic hydrolysates of fraction *A* (Sanger & Thompson, 1953*b*) and these were used to locate most of the amide groups. However, other peptides were required, especially those containing only one of the two glutamic acid residues in positions 4 and 5 (see Table 7), and so hydrolysates obtained by the action of two other enzymes (papain and mould protease) were investigated and the results are reported. The mould protease used was first isolated in a crystalline form by Crewther & Lennox (1950), but was not considered to be a pure substance. It is extremely active though rather unspecific in its attack.

The abbreviations and reference numbers to the peptides are those reported in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). Pyr is used to denote a residue of pyrrolidone carboxylic acid (5-oxopyrrolidine-2-carboxylic acid).

EXPERIMENTAL

Action of mould protease on fraction A (*Expt. A*m). The mould protease used was a crystalline sample obtained through Dr R. R. Porter of the National Institute for Medical Research, Mill Hill, London.

A solution of fraction *A* (30 mg.) in 5 ml. water was brought to pH 8 and 1·5 mg. mould protease were added. After incubating at 37° for 24 hr. the mixture was boiled to inactivate the enzyme and taken to dryness *in vacuo*. Samples (5 or 10 mg.), after centrifuging to remove insoluble matter, were fractionated on Whatman no. 4 or no. 3 paper chromatograms, using phenol–0·3% NH_3 followed by butanol–acetic acid as solvents and the peptide spots eluted and subjected to hydrolysis and end-group determination as described in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*).

Action of papain on fraction A (*Expt. A*pa). The papain used was a commercial preparation supplied by British Drug Houses Ltd. A sample (16 mg.) was activated before use by incubation for 1 hr. with 1·6 ml. 1% NaCN solution at 37°. The activated enzyme (0·5 ml.) was then added to a solution of 50 mg. fraction *A* in 2 ml. water. The pH was adjusted to 7 with dilute NH_3 and the mixture incubated at 37° for 24 hr. Subsequent working up was as described for mould protease.

Preparation of peptide Ap5 by ionophoresis. Peptide *A*p5, which consists of the first thirteen residues of the glycyl chain (Sanger & Thompson, 1953*b*) was prepared from insulin by making use of its high mobility as an acid in 0·2 N acetic acid, owing to its content of three cysteic acid residues.

Insulin (50 mg.) in 10 ml. 0·01 N-HCl was treated with 1·0 mg. pepsin and incubated at 37° for 24 hr. After being taken to dryness, the residue was oxidized with performic acid (Sanger, 1949*a*). The formic acid was removed *in vacuo*, the residue dissolved in 1·5 ml. water and applied along the centre line of a sheet of Whatman no. 3 filter paper. It was then subjected to ionophoresis in an apparatus similar to that of Durrum (1950). A potential of 220 v was applied for 21 hr. A strip was cut from the paper and tested with ninhydrin. The *A*p5 was present in a band which stained rather weakly with ninhydrin and had moved 12·5 cm. towards the anode. From a ninhydrin determination on a hydrolysate of the eluted material it appeared that the yield of *A*p5 was about 30% of the theoretical yield from insulin. In several experiments a faint slower moving band could be distinguished, which appears to be peptide *A*p9 (the first ten residues of the glycyl chain). For further purification the *A*p5 was subjected to ionophoresis at pH 3·7 in the apparatus of Michl (1951). The material obtained from 50 mg. insulin was put on a 18 cm.-wide strip of no. 3 filter paper; the ionophoresis was continued for 3·5 hr. at 1500 v. The main *A*p5 band had moved 19 cm. towards the anode. Several fainter slow moving bands were also present.

Ionophoresis of peptides. The ionophoretic rates of peptides have been compared using the apparatus of Durrum (1950). The electrolyte used was 0·05 M ammonium acetate (pH 6·8). The peptides obtained from paper chromatograms were applied to a sheet of Whatman no. 1 filter paper either directly by the 'strip transfer' method, or after elution from the paper and drying in a desiccator. The potential used was 220 v; since the rates were not reproducible from experiment to experiment, only those done on the same paper were compared. If the original peptide spot had been treated with ninhydrin, the purple colouring matter usually migrated slightly towards the anode and was distinguished from the colour given by neutral spots on subsequent spraying, since they moved towards the cathode with the electroendosmotic flow.

Micro amide determinations on peptides. Estimations of amide N on the peptides eluted from paper chromatograms were carried out by the standard procedure (see Bailey, 1937), the ammonia being estimated by the micro-distillation method of Conway (1950). In order to determine the amount of peptide present in the samples, portions were subjected to hydrolysis, and the total amino acids estimated by the ninhydrin method of Moore & Stein (1948).

Samples of the peptides required for analysis were collected from several chromatograms on Whatman no. 3 filter paper, or from ionophoresis experiments. If the spots had been located with dilute ninhydrin, the 'cuts' were thoroughly washed in acetone before elution. The peptide sample was dissolved in a small amount of water and divided into portions which were transferred to small flat glass dishes or Polythene strips. In general, one-sixth was taken for estimation of the amount of peptide present and two one-third samples for amide estimation.

The smaller portion for estimation of total peptide was taken to dryness in a desiccator, dissolved in 5·7N-HCl, transferred to a capillary tube, and incubated at 105° for 24 hr. The hydrolysate was then transferred to Polythene and the excess HCl removed by repeated evaporation in a desiccator. In order to remove any NH_3, which would react with the ninhydrin reagent, 0·1 ml. 0·1M-K_2CO_3 was added to the residue on the Polythene strip, which was placed *in vacuo* for 2 hr. The NH_3-free hydrolysate was then dissolved in 5 ml. water and portions taken for estimation by the ninhydrin method using 1 ml. of ninhydrin reagent and a 30 min. reaction period (Moore & Stein, 1951).

The larger samples of the eluted peptides for amide estimations were taken to dryness, 0·1 ml. 0·1M-K_2CO_3 added and free NH_3 was removed by evaporation in a desiccator for 2 hr. The dry samples were then dissolved in 0·2 ml. 2N-HCl, transferred to capillary tubes and incubated at 105° for 3 hr. Reagent blanks were carried out simultaneously. The hydrolysed samples were divided into portions, each containing *ca.* 5μg. NH_3-N, which was determined by the standard Conway method. 0·0005N-HCl was used in the centre compartment and back titration was carried out with 0·0025N-$Ba(OH)_2$.

RESULTS

Ionophoresis of peptic hydrolysate of fraction B (*B*p). In order to obtain samples of peptides from fraction *B* for amide estimations, 50 mg. of a peptic hydrolysate were fractionated by ionophoresis in 0·05M ammonium acetate using a 25 cm.-wide sheet of Whatman no. 3 paper. A number of clear bands were obtained and were cut out as shown in Fig. 1. Paper chromatography of hydrolysates of samples of the fractions indicated the composition shown and with the exception of band γ seemed to be reasonably pure.

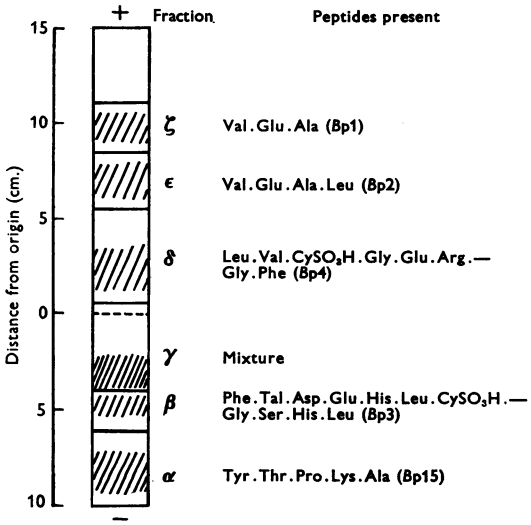


Fig. 1. Ionophoresis of peptic hydrolysate of fraction *B* in 0·05M ammonium acetate. 220v, 20 hr.

Action of mould protease on fraction A. The results obtained with the mould protease hydrolysates of fraction *A* are summarized in Fig. 2 and Table 1. All peptides were hydrolysed to identify the amino acids. In Tables 1–4 the approximate relative amount (strength) of the peptide spots is indicated in the second columns. This was determined by the strength of the ninhydrin colour reaction of the amino acids obtained on hydrolysis of the peptide. The × 's have the same significance as in previous papers (Sanger & Tuppy, 1951*a*, *b*; Sanger & Thompson, 1953*a*, *b*). The *N*-terminal residues of some of the peptides were determined by hydrolysis of their DNP-derivatives. The experimental results are summarized in column 3 (composition) and in column 4 are shown the most probable structures. These are deduced from a consideration of the data in column 3, the structure of fraction *A* and the known points of splitting by the enzyme, as determined from the structure of the other peptides present. Also the structures of some of the peptides have been inferred by comparison of their R_F values and ionophoretic rates with those of peptides of known structure.

Action of papain on fraction A (*A*pa). The results obtained with the papain hydrolysate are summarized in Fig. 3 and Table 2. Better resolution of the slower-moving peptides (spot nos. 1–9) was obtained by running both solvents two lengths of the paper. The results in Table 2 have the same significance as those in Table 1 and the probable structures were deduced in the same manner.

Peptide *A*pa1, which contained no free α-amino group, was detected by examination of the chromatogram in ultraviolet light. It was subjected to partial hydrolysis with 12N-HCl at 37° for 3 days and the peptides listed in Table 3 were identified. There was also present a spot (R_F in phenol, 0·7; R_F in butanol–acetic acid, 0·68) which gave no colour with ninhydrin, but gave glutamic acid on hydrolysis. This was most probably pyrrolidone carboxylic acid, which was shown to have these R_F values. This and the fact that peptide *A*pa1 contains no free amino group indicate that it is a pyrrolidone carboxylic acid derivative, and the partial hydrolysis experiments show that its structure is that given in Table 2.

No satisfactory explanation can be offered for the presence of spot *A*pa9, which does not fit into the structure of fraction *A*. On ionophoresis it moved

toward the anode, suggesting the glutamic acid residue is not present as an amide. The yellow colour with ninhydrin suggests that serine is *N*-terminal. It is tentatively suggested that synthesis may have occurred under the influence of the papain, but it is hoped to investigate this effect further.

Action of papain on Ap5. Peptide *Ap5* purified by a single ionophoresis, was hydrolysed with cyanide-activated papain as described above and the hydrolysate subjected to ionophoresis in 0·2M acetic acid overnight at 220v. Test strips were coloured with ninhydrin, and by the chlorination procedure of Rydon & Smith (1952). The distribution of the bands is shown in Fig. 4, and their composition in Table 4. Since band 5 gave no colour with ninhydrin but a strong colour by the chlorination method and contained glutamic acid, it is presumed to be a pyrrolidonoyl peptide. The presence of free tyrosine (band 2) is probably due to some impurity in the *Ap5*.

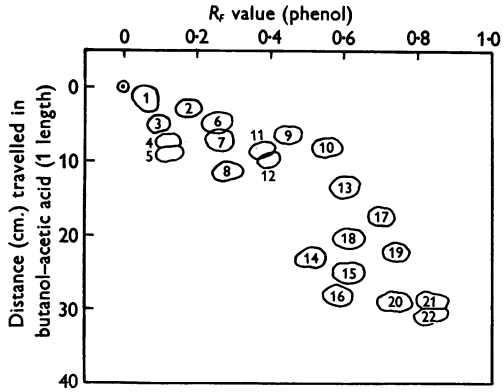


Fig. 2. Chromatogram of mould protease hydrolysate of fraction *A* (Expt. *Am*) (see Table 1).

Total amides of fraction A. Sanger (1949*a*) obtained a value of 15·15% for the amide-N of fraction *A* as percentage of the total N. This approximates to four residues per chain (theoretical 16·0%). Since it was essential to be certain of this value, it has been confirmed in the present work.

Bound NH_3 was removed before amide estimation by dissolving the fraction *A* (15–20 mg.) in 1 ml. 0·1M-K_2CO_3 and leaving in a vacuum desiccator over H_2SO_4 for 4 hr. The value obtained was 16·7% amide-N as percentage of total N. The theoretical value for five residues would be 19·2%.

Further evidence that there are four amide groups in fraction *A* was obtained from plotting a titration curve. Since fraction *A* contains no histidine, the only groups titrating in the range pH 2·5–7 are the

Table 1. *Peptides from mould protease hydrolysates of fraction A* (*Am*)

Spot no. (Fig. 2)	Approx. strength of peptide	Composition*	Probable structure
1	× × ×	Glu.[$CySO_3H$, Ser, Ala, Val]	Glu.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$
2	× × ×	[$CySO_3H$, Ser, Ala, Val]	$CySO_3H$.Ala.Ser.Val.$CySO_3H$
3	×	[$CySO_3H$, Glu, Ser, Ala]	Mixture
4	× ×	[$CySO_3H$, Asp, Tyr]	Tyr.$CySO_3H$.Asp
5	×	[$CySO_3H$, Asp, Tyr]	—
6	× × × ×	[$CySO_3H$, Asp, Tyr]	Asp.Tyr.$CySO_3H$.Asp
7	×	[$CySO_3H$, Asp, Tyr]	—
8	×	—	Glutamic acid
9	× ×	[Asp]	Asparagine
10	× ×	[Glu]	Glutamine
11	× × ×	[Ser]	Serine
	×	[$CySO_3H$, Val]	Val.$CySO_3H$ and/or Ser.Val.$CySO_3H$
12	× ×	[$CySO_3H$, Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu.Glu.$CySO_3H$
13	×	—	Alanine
14	× × ×	Val.Glu	Val.Glu
15	× ×	Glu.[Glu, Leu]	Glu.Leu.Glu
16	× × ×	Leu.Glu	Leu.Glu
17	× ×	[Glu, Tyr]	Tyr.Glu
18	× ×	—	Tyrosine
19	×	[Glu, Val]	?
20	× ×	[Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu
21	× × ×	—	Leucine
22	× × ×	[Ser, Tyr, Leu]	Ser.Leu.Tyr

* Leucine and isoleucine were not distinguished. Both are recorded as Leu.

Spot 1. Faint colour with ninhydrin. *Spots* 1 *and* 2. May have an extra serine in the *C*-terminal position. *Spot* 3. Contained both glutamic acid and cysteic acid as *N*-terminal residues, so it was clearly a mixture. *Spot* 4. Grey colour with ninhydrin similar to tyrosine, suggesting tyrosine is *N*-terminal. *Spot* 6. Similar R_F value and colour with ninhydrin (yellow) to *Ap3* and *Apa3* (Asp.Tyr.$CySO_3H$.Asp). *Spots* 5 *and* 7. May be deamidated forms of *Am4* and *Am6* or one could be Asp.Tyr.$CySO_3H$. *Spot* 9. Yellow-brown with ninhydrin. *Spots* 12 *and* 22. Yellow with ninhydrin. *Spot* 20. On the chromatogram was a mixture with a tyrosine-containing peptide. It was purified by ionophoresis, yellow colour with ninhydrin. Same R_F value as peptide *A*1η3 (Gly.Ileu.Val.Glu).

Table 2. *Peptides from papain hydrolysate of fraction A (Apa)*

Spot no. (Fig. 3)	Approx. strength of peptide	Composition*	Probable structure
1	× × ×	[$CySO_3H$, Glu, Ser, Ala, Val]	Pyr.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.$CySO_3H$
2	× ×	[$CySO_3H$, Asp, Glu, Tyr]	Glu.Asp.Tyr.$CySO_3H$.Asp
3	× ×	[$CySO_3H$, Asp, Tyr]	Asp.Tyr.$CySO_3H$.Asp
4	×	[$CySO_3H$, Asp, Tyr]	—
5	× × ×	[$CySO_3H$, Asp, Glu, Tyr, Leu]	Leu.Glu.Asp.Tyr.$CySO_3H$.Asp
6	×	[$CySO_3H$, Asp, Glu, Tyr, Leu]	—
7	×	—	Glutamic acid
8	× ×	—	Glycine
9	×	[Glu, Ser, Tyr]	?
10	× ×	—	Tyrosine
11	× × ×	[Glu, Tyr]	Tyr.Glu
12	×	[Glu, Tyr, Leu]	Tyr.Glu.Leu.Glu
13	×	[Glu, Ser, Tyr, Leu]	Ser.Leu.Tyr.Glu
14	×	[Glu, Tyr, Leu]	Tyr.Glu.Leu
15	× × × ×	[Glu, Gly, Val, Leu]	Gly.Ileu.Val.Glu
16	× × ×	Leu, Glu	Leu.Glu
17	× ×	[Glu, Val, Leu]	Ileu.Val.Glu
18	× ×	[Ser, Leu]	Ser.Leu
19	× ×	[Glu, Leu]	Pyr.Leu

* Leucine and isoleucine were not distinguished. Both are recorded as Leu.

Spot 1. No colour with ninhydrin. Located by slight fluorescence in ultraviolet light. It gave no DNP derivative, indicating no free —NH_2 group. *Spot* 2. R_F values and ionophoresis rates identical with those of peptide *A*p1. *Spot* 3. Yellow with ninhydrin. R_F values and ionophoretic rates identical with those of peptide *A*p3. *Spots* 4 *and* 6. Only detected when solvents run 2 lengths. May be deamidated forms of 3 and 5 or may have other structures. *Spot* 5. R_F values and ionophoretic rates identical with those of peptide *A*p6. *Spot* 8. Gives traces of serine, valine and cysteic acid after hydrolysis. *Spots* 9, 13 *and* 18. Yellow with ninhydrin. *Spot* 10. Corresponds in composition to spots *A*p12 and *A*m18 while spot 11 corresponds to spots *A*p11 and *A*m17, but the rates of movement in phenol and butanol–acetic acid were reversed. *Spot* 12. Obtained from the chromatogram was contaminated with spot 15, from which it could be separated by ionophoresis. Is probably identical with *A*p14. *Spot* 14. Probably identical with *A*p15. *Spot* 15. Strong grey colour with ninhydrin. *Spot* 19. No visible colour with ninhydrin. Located by fluorescence. Its R_F value in phenol is greater than the known peptides of leucine and glutamic acid. It is probably a pyrrolidone carboxylic acid derivative formed from an *N*-terminal glutamine residue.

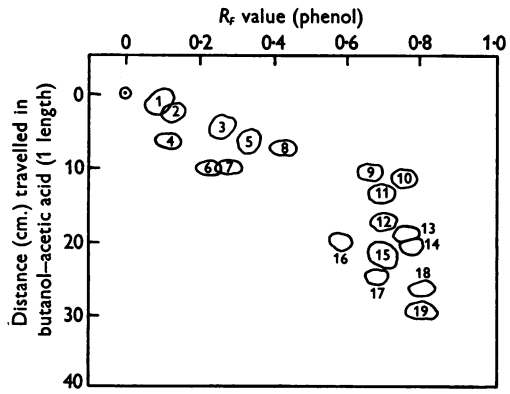


Fig. 3. Chromatogram of papain hydrolysate of fraction *A* (Expt. *A*pa) (see Table 2).

Table 3. *Partial hydrolysis products of peptide A*pa1

Peptide	Strength
$CySO_3H$.$CySO_3H$.Ala (*A*2δ1)	× ×
Cysteic acid	× ×
Glu.$CySO_3H$ (*A*1α1)	×
$CySO_3H$.Ala (*A*1γ3)	× ×
Glutamic acid	× ×
Ser.Val.$CySO_3H$ (*A*1ζ2)	× × × ×
Serine	×
Alanine	× × ×

carboxyl groups. The —SO_3H groups are considerably stronger and are probably completely ionized in this range. Fraction *A* (20·1 mg.) from which the bound acetate had been removed by washing well with 0·1 N-HCl in acetone, was titrated to pH 2·27 with 0·025 N-HCl. It was then titrated back to pH 7 with 0·025 N-NaOH, taking the usual precautions. The results (Fig. 5) are corrected for the activity of HCl in water. Although there was considerable scatter at the lower pH values, it is evident that there are three free —COOH groups on fraction *A*. One of these is the free α-COOH group, so that two of the six dicarboxylic amino acids have free ω-COOH groups and the other four are present as amides.

Ionophoretic rates of peptides. Table 5 shows the distance moved by the various peptides on ionophoresis in 0·05 M ammonium acetate. In this table the results listed in each column were obtained in the same experiment and so are directly comparable. Results from different experiments are not strictly comparable, although in general a peptide moving slightly towards the cathode is neutral and peptides moving towards the anode are acidic.

Table 6 shows the results of the amide determinations on the peptides produced by enzymic hydrolysis of fractions *A* and *B*.

Table 4. *Action of papain on peptide Ap5 (Ap5pa)*

Band no. (Fig. 4)	Strength	Composition	Probable structure
1	× × ×	[Glu, Gly, Val, Leu or Ileu]	Gly.Ileu.Val.Glu
2	×	[Tyr]	Free tyrosine
3	× × ×	[$CySO_3H$, Ser, Val, Leu or Ileu]	Val.$CySO_3H$.Ser.Leu
4	×	[$CySO_3H$, Glu, Ser, Gly, Ala, Val, Leu or Ileu]	Unchanged *Ap5*
5	× × × ×	[$CySO_3H$, Glu, Ser, Ala]	Pyr.$CySO_3H$.$CySO_3H$.Ala.Ser

Band 3. Also contained traces of glutamic acid.

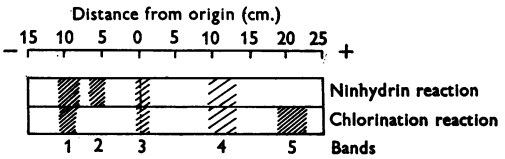


Fig. 4. Ionophoresis of papain hydrolysate of peptide *Ap5* in 0·2M acetic acid, 220v, 16 hr. (Expt. *Ap5pa*) (see Table 4).

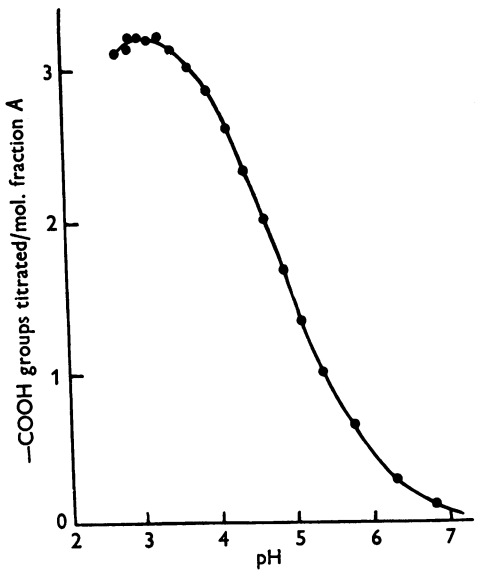


Fig. 5. Titration curve for fraction *A*.

DISCUSSION

Amide groups in the phenylalanyl chain

The amide contents of the peptides from fraction *B* (Table 6) were obtained using the fractions obtained by ionophoresis (Fig. 1), which may not have been completely pure. There may therefore be some error in the results but they are sufficiently accurate for the present purposes.

Sanger (1949*b*) showed that the aspartic and glutamic acid residues in positions 3 and 4 respectively were both in the form of amides, since several different *N*-terminal DNP-peptides containing the same amino acids could be obtained. This is supported by the amide content of peptide *Bp3*, which gives a value approximating to the expected two residues.

Sanger & Tuppy (1951*b*) concluded that the glutamic acid residue in position 13 (see Table 7) contained a free γ-COOH group, since peptides *Bp1* (Val.Glu.Ala+Val.Glu) and *Bp5* (Leu.Val.Glu) from the peptic hydrolysates had the same R_F values as the corresponding peptides from the acid hydrolysates. This is confirmed by the fact that the peptides *Bp1* and *Bp2* (Val.Glu.Ala.Leu) are acidic (Fig. 1) and contain no amide groups (Table 6).

The remaining glutamic acid residue (position 21) is present in peptide *Bp4* (Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe). This is slightly acidic (Fig. 1) and contains no amide groups, so that the glutamic acid residue must have a free γ-COOH group.

Amide groups in the glycyl chains

It has already been shown that the *C*-terminal residue is asparagine (Harris, 1952; Sanger & Thompson, 1953*b*) and this is confirmed by the presence of one amide group in peptide *Ac1* ($CySO_3H$.Asp).

Several of the peptides obtained from the peptic hydrolysate have duplicate spots which contain the same amino acids, but are more acidic. Thus peptides *Ap10* (Tyr.Glu), *Ap4* (Asp.Tyr.$CySO_3H$.Asp), *Ap2* (Glu.Asp.Tyr.$CySO_3H$.Asp) and *Ap7* (Leu.Glu.Asp.Tyr.$CySO_3H$.Asp) are the corresponding forms of peptides *Ap11*, *Ap3*, *Ap1* and *Ap6* respectively. It was at first thought that this was due to deamidation occurring under the action of pepsin, but no loss of amide-N could be detected from fraction *A* when it was incubated with pepsin. More probably the insulin used was already partially deamidated, since Harfenist (1953) has shown that certain preparations contain a component having one less amide group than the normal insulin.

Peptides *Ap11* and *Am17* both contain the sequence Tyr.Glu and are neutral. They are therefore Tyr.$GluNH_2$ and the glutamic acid residue in position 15 is present as an amide. There is considerable confirmation for this. Peptides *Ap15*, *Apa14* (Tyr.Glu.Leu) are also neutral and *Ap15* contains one amide group. Peptide *Am15* (Glu.Leu.Glu) moves more slowly on ionophoresis than Leu.Glu (*Am16*) and must therefore be $GluNH_2$.Leu.Glu.

Table 5. *Ionophoretic rates of peptides in* 0·05 M *ammonium acetate*

The figures listed in each column represent the results obtained in a single experiment.

Peptide	Spot no.	Distance moved towards anode (cm.) (15)*	(10)*	(15)*	(15)*	(14)*	(8·5)*	(15)*	(18)*	(18)*
Tyr.Glu	*Ap*11	—	−1	−2	—	—	—	—	—	—
	*Ap*10	—	—	5·5	—	—	—	—	—	—
	*Am*17	—	—	—	—	—	−1	—	—	—
Tyr.Glu.Leu	*Ap*15	—	−1	−2	—	—	—	—	—	—
	*Apa*14	—	—	—	—	—	—	−1	—	—
Tyr.Glu.Leu.Glu	*Ap*14	—	4·4	—	—	—	—	—	—	—
	*Apa*12	—	—	—	—	—	—	3·6	—	8
Leu.Glu	*Apa*16	—	—	—	—	—	—	6	—	—
	*Am*16	—	—	—	—	8·5	—	—	—	—
Glu.Leu.Glu	*Am*15	—	—	—	—	7	—	—	—	—
Asp.Tyr.$CySO_3H$.Asp	*Ap*3	8·7	7	—	8·2	—	—	—	—	—
	*Ap*4	15	—	14	—	—	—	—	—	—
	*Apa*3	—	—	—	8	—	—	—	—	—
Glu.Asp.Tyr.$CySO_3H$.Asp	*Ap*1	12·3	9	—	11·6	—	—	—	—	—
	*Ap*2	18·7	—	—	—	—	—	—	—	—
	*Apa*2	—	—	—	11·7	—	—	—	—	—
Leu.Glu.Asp.Tyr.-$CySO_3H$.Asp	*Ap*6	—	—	9	9·5	—	—	—	—	—
	*Ap*7	15	—	—	—	—	—	—	—	—
	*Apa*5	—	—	—	9·7	—	—	—	8	—
Gly.Ileu.Val.Glu	*Ap*16	—	—	4·3	—	—	—	—	—	—
	*Apa*15	—	—	—	—	—	—	4·3	—	—
	*Am*20	—	—	—	—	—	5	—	—	—
Ileu.Val.Glu	*Apa*17	—	—	—	6	—	—	—	—	—
Val.Glu	*Am*14	—	—	—	9	9·5	—	—	—	—
Ser.Leu.Tyr.Glu	*Apa*13	—	—	—	—	—	—	−1	—	—
Ser.Leu.Tyr	*Am*22	—	—	—	—	—	−1	—	—	—
Glu.Leu.Glu.Asp.Tyr.-$CySO_3H$.Asp	*Ac*3	—	—	—	—	—	—	—	6·7	—
Glu.Leu.Glu.Asp.Tyr	*Ac*5	—	—	—	—	—	—	—	—	6·7
Glutamine	*Am*10	—	—	—	—	−1	—	—	—	—
Asparagine	*Am*9	—	—	—	—	−1	—	—	—	—
Glutamic acid	—	14·5	—	16	—	—	—	—	—	—

* Time of ionophoresis (hr.).

Table 6. *Amide determinations of peptides*

Peptide	Sequence	No. of estimations averaged	Amide groups (residues/mol.)
*Bp*1	Val.Glu.Ala	2	0·15
*Bp*2	Val.Glu.Ala.Leu	2	0·15
*Bp*4	Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe	3	0·13
*Bp*3	Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.His.Leu	2	1·7
*Ac*1	$CySO_3H$.Asp	1	0·7
*Ap*15	Tyr.Glu.Leu	2	0·98
*Ap*14	Tyr.Glu.Leu.Glu	2	1·03
*Ap*3	Asp.Tyr.$CySO_3H$.Asp	4	2·10
*Ap*1	Glu.Asp.Tyr.$CySO_3H$.Asp	7	1·94
*Ap*5*pa*1	Gly.Ileu.Val.Glu	2	0·15
*Ap*5	Gly.Ileu.Val.Glu.Glu.$CySO_3H$.$CySO_3H$.Ala.Ser.Val.-$CySO_3H$.Ser.Leu	17	1·16

Similarly, Glu . Leu . Glu . Asp . Tyr . $CySO_3H$. Asp (*Ac*3) moves slower than Leu . Glu . Asp . Tyr . $CySO_3H$. Asp (*Apa*5).

In the chymotryptic digests two peptides (*Ac*5, *Ac*7) were isolated having the same amino acid composition but one (*Ac*7) gave no colour with ninhydrin. It was pointed out that this was presumably due to the lability of an *N*-terminal glutaminyl residue which tends to cyclize to a pyrrolidone carboxylic acid residue. Thus *Ac*5 is probably $GluNH_2$. Leu . Glu . Asp . Tyr and *Ac*7 Pyr . Leu . Glu . Asp . Tyr. Also Pyr . Leu (*Apa*19) was found in the papain hydrolysate.

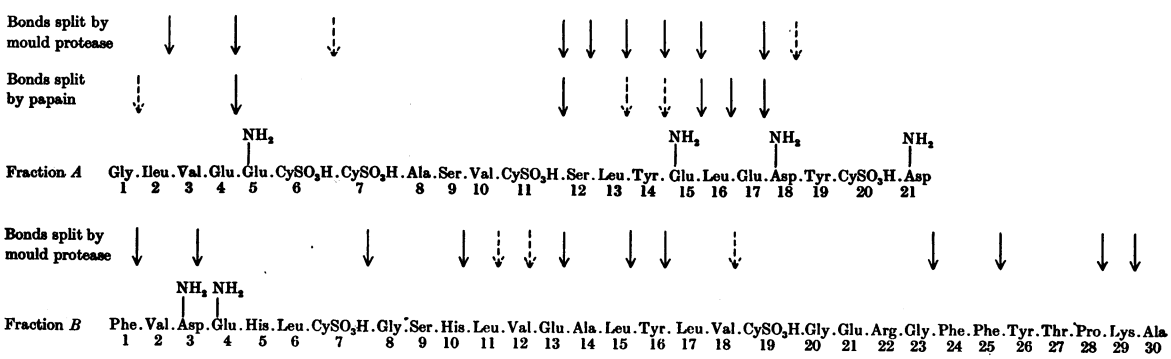
Whereas peptide *Ap*15 (Tyr . $GluNH_2$. Leu) was found to be neutral, peptide *Ap*14 (Tyr . Glu . Leu . Glu) is acidic, which suggests the second glutamic acid residue has a free carboxyl group. This is supported by the fact that Leu . Glu (*Apa*16, *Am*16) and Glu . Leu . Glu (*Am*15) are acidic. The glutamic acid residue in position 17 therefore contains a free γ-COOH group. Amide determination also showed that peptide *Ap*14 (Tyr . $GluNH_2$. Leu . Glu) had only one amide group, as did *Ap*15 (Tyr . $GluNH_2$. Leu). Peptides *Ap*1 and *Apa*2 (Glu . Asp . Tyr . $CySO_3H$. Asp) differ from peptides *Ap*3 and *Apa*3 (Asp . Tyr . $CySO_3H$. Asp) only by one glutamic acid residue but are more acidic on ionophoresis, which confirms that this glutamic acid residue (position 17) is not amidized. Similarly peptide *Ap*6 (Leu . Glu . Asp . Tyr . $CySO_3H$. Asp) is less acidic than *Ap*1 and more acidic than *Ap*3.

Peptide *Ap*3 (Asp . Tyr . $CySO_3H$. Asp) contains two amide groups (Table 6). These can only be located on the aspartic acid residues so that both (positions 18 and 21) must be present as asparagine. Similarly *Ap*1 (Glu . Asp . Tyr . $CySO_3H$. Asp) contains two amide residues, and the glutamic acid residue has a free γ-COOH group. Further evidence for an amide in position 18 is that Glu . Leu . Glu . Asp . Tyr (*Ac*5) moves slower on ionophoresis than Tyr . Glu . Leu . Glu (*Ap*14). These two peptides differ only by the presence of the aspartic acid residue in question, although they contain a different tyrosine residue.

Three enzymes have split the bond between the two glutamic acid residues in positions 4 and 5. Thus, papain gave a high yield of peptide *Apa*15 (Gly . Ileu . Val . Glu). On ionophoresis it moved towards the anode, showing that the glutamic acid was not present as an amide. Similarly, *Ap*16 and *Am*20, which have the same sequence as *Apa*15 are acidic and NH_3 determination showed there was no amide in *Ap*5*pa*1. Peptides *Apa*17 (Ileu . Val . Glu) and *Am*14 (Val . Glu) are also acidic. Peptide *Ap*5*pa*5 was identified as a pyrrolidonoyl peptide (Pyr . $CySO_3H$. $CySO_3H$. Ala . Ser) indicating that the residue in position 5 is glutamine.

From these results it was expected that peptide *Ap*5 (Gly . Ileu . Val . Glu . Glu . $CySO_3H$. $CySO_3H$. Ala . Ser . Val . $CySO_3H$. Ser . Leu) would contain one amide group (on position 5). However, innumerable determinations gave values which varied somewhat but were considerably closer to two than to one. Also a few determinations on peptides *Ap*7, *Ac*2 and *Ac*4 gave similar high results. The *Ap*5 was prepared either from chromatograms or more often by a single ionophoresis in 0·2M acetic acid. Although the apparent absence of aspartic acid and tyrosine from hydrolysates suggested that the *Ap*5 was reasonably pure, it now seems that the high amide value was due to the presence of an amide-rich impurity, since when *Ap*5 was further purified by high-voltage ionophoresis at pH 3·7, values approximating to one were obtained. The figures averaged in Table 6 are all for such purified material. This impurity appears to travel slightly slower than *Ap*5 in the 0·2M acetic acid ionophoresis since a cut taken behind the *Ap*5 band gave a higher amide value than the main band. It seems probable that it is largely unchanged fraction *A* and possibly

Table 7. *Amide distribution in insulin*



other similar large peptides. Fraction *A* has a comparatively high amide content and a small amount present in the *A*p5 would cause considerable error. In our experience it forms very long spread-out streaks both on ionophoresis and chromatography and, if present, would be expected to contaminate *A*p5 and other peptides (*A*p9, *A*c2, *A*c4) which move at similar rates.

On the basis of these combined results it is concluded that the distribution of amide groups in insulin is as shown in Table 7. This agrees well with the results of Chibnall & Rees (1952), who showed that all three aspartic acid and three of the glutamic acid residues are amidized.

The possibility of iso*glutamine residues*

Kandel, Kandel, Kovacs & Bruckner (1954) have recently suggested that insulin contains an *iso*-glutamine residue. This is based on the finding that when insulin is subjected to the Hofmann degradation and hydrolysed, a substance is produced which gives a 2:4-dinitrophenylhydrazone that moves at the same rate on a butanol–3 % NH_3 paper chromatogram as the dinitrophenylhydrazone of 4-oxobutyric acid. This product would be obtained if an *iso*glutaminyl residue were present in the protein. We have repeated the above experiment and have confirmed the presence of a spot that moves on a butanol–3 % NH_3 chromatogram at the same rate as the synthetic dinitrophenylhydrazone of 4-oxobutyric acid. There were, however, rather more spots on the chromatogram than the two recorded by Kandel *et al.* Samples of the dinitrophenylhydrazone spot were eluted from the paper and reinvestigated by chromatography on *tert.*-amyl alcohol–phthalate (pH 6) buffer (Blackburn & Lowther, 1951) and in the 'toluene' solvent of Biserte & Osteux (1951). With the former solvent two strong spots were produced which had moved 6 and 11 cm. down the paper respectively and a fainter spot that had moved 19 cm. A control spot of the synthetic hydrazone had moved 19 cm. Similar results were obtained with the 'toluene' solvent. It thus seems that the spot that was identified by Kandel *et al.* as the above hydrazone, was in fact a mixture of several substances, one of which may have been the hydrazone. It is present in only small quantities and would probably account for about 1–2% of one residue in insulin (mol.wt. 5700). In view of these results and the known complexity of the reactions of hypochlorite with amino acids, peptides and proteins (see e.g. Goldschmidt & Strauss, 1930 and previous papers) it seems that there is no clear evidence for the existence of *iso*glutamine residues in insulin.

The fact that the residues in positions *A*5 and *A*15 are converted to a pyrrolidonoyl group when they are the *N*-terminal residues of a peptide (e.g. in peptides *A*pa1 and *A*c7, respectively) indicates that they are glutaminyl residues. An *iso*glutaminyl residue would not decompose to a pyrrolidonoyl peptide but would give the amide of pyrrolidone carboxylic acid. No such evidence exists for the residue in position *B*4, but a study of the Hofmann degradation of fractions *A* and *B* of oxidized insulin showed that the spot moving at the same rate as the dinitrophenylhydrazone of 4-oxobutyric acid was produced from both fractions.

Action of the proteolytic enzymes

The main sites at which splitting occurred under the action of papain and mould protease are indicated in Table 7. The sites of action of mould protease on fraction *B* are based on results of a single experiment and should not be regarded as conclusive and are not comprehensive. Both enzymes show a wide specificity which is somewhat similar to that of pepsin, though the relative intensities of splitting of the various bonds was markedly different for the three enzymes. Mould protease carried the digestion of fraction *A* further than the other enzymes. For structural studies in the future, this enzyme may prove useful, for example, in degrading longer chains obtained by the use of other enzymes.

It is doubtful if the preparations of the two proteinases represent pure enzymes. In the case of papain we have also studied the action of an activated sample of crystalline mercuripapain prepared by Kimmel & Smith (1954). The specificity appeared to be essentially the same as that found with the crude commercial sample.

It is interesting that papain breaks peptide *A*p5 in a different way from fraction *A*. In fraction *A* strong splitting occurs at the $CySO_3H$.Ser bond (position 11–12), whereas this bond was stable in *A*p5 and splitting occurred largely at the Ser.Val bond (position 9–10). This result could be explained if papain was inhibited by a free carboxyl group on the second residue from the bond. Other explanations are possible, but it does illustrate that the specificity of proteolytic enzymes is not conditioned solely by the nature of the residues involved in the bond in question.

SUMMARY

1. Fraction *A* of oxidized insulin was hydrolysed with mould protease and cyanide-activated papain and the resulting peptides studied. Both enzymes showed a rather wide specificity.

2. The positions of the amide groups of insulin were determined by estimating the relative ionophoretic mobilities and the amide contents of peptides from enzymic hydrolysates of fractions *A* and *B* of oxidized insulin. The results are summarized in Table 7.

We wish to thank Dr R. R. Porter for the mould protease and Dr Emil L. Smith for the crystalline mercuripapain.

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REFERENCES

Bailey, K. (1937). *Biochem. J.* **31**, 1406.
Biserte, G. & Osteux, R. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 50.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Chibnall, A. C. & Rees, M. W. (1952). *Biochem. J.* **52**, iii.
Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*. London: Lockwood.
Crewther, W. G. & Lennox, F. G. (1950). *Nature, Lond.*, **165**, 680.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Goldschmidt, S. & Strauss, K. (1930). *Ber. dtsch. chem. Ges.* **63**, 1218.
Harfenist, E. J. (1953). *J. Amer. chem. Soc.* **75**, 5528.
Harris, J. I. (1952). *J. Amer. chem. Soc.* **74**, 2944.
Kandel, I., Kandel, M., Kovacs, J. & Bruckner, V. (1954). *Naturwissenschaften*, **41**, 281.
Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
Michl, H. (1951). *Monatshefte*, **82**, 489.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
Sanger, F. (1949*a*). *Biochem. J.* **44**, 126.
Sanger, F. (1949*b*). *Biochem. J.* **45**, 563.
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.

Metabolism of Polycyclic Compounds

8.* ACID-LABILE PRECURSORS OF NAPHTHALENE PRODUCED AS METABOLITES OF NAPHTHALENE

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Baumann & Herter (1877) observed that crystals of naphthalene appeared in the distillate when the acidified urine of rabbits dosed with naphthalene was heated. Bourne & Young (1933) noticed that crystals of naphthalene separated from such acidified urine in the cold. They suggested that this might be due to the dehydration of 1:2-dihydro-2-naphthol, which is known to give naphthalene with cold mineral acid (Bamberger & Lodter, 1895), but dihydronaphthol does not appear to have been isolated from urines of animals dosed with naphthalene. Chang & Young (1943) and Young (1947) estimated that the acid-liberated naphthalene accounted for from 4 to 14 % of the dose fed to rats or rabbits. The liberation of anthracene on acidification of the urine of rats and rabbits dosed with anthracene was noticed by Boyland & Levi (1936). Chang & Young (1943) also found that small amounts of hydrocarbons were liberated on acidification of urines of rats dosed with phenanthrene and anthracene. Acid-labile precursors could not be detected in the urines of rats dosed with higher polycyclic hydrocarbons.

* No. 7 of this series: Boyland, E. & Wiltshire, G. M. (1953).

Studies on 1:2:3:4-tetrahydronaphthalene (tetralin) metabolism by Pohl & Rawicz (1919) and by Rockemann (1922) indicated that tetralin was converted into 1- or 2-tetralyl glucosiduronic acids by dogs and rabbits. When these tetralyl glucosiduronic acids were steam-distilled from acid solution, 1:2-dihydronaphthalene and naphthalene were produced. Pohl & Rawicz (1919) also dosed rabbits with 1:2-dihydronaphthalene and found that naphthalene was produced on acidification of the urine; they suggested that a 1:2-dihydronaphthyl glucosiduronic acid had been formed. Corner, Billett & Young (1954) have recently shown that 1:2-dihydronaphthalene-1:2-diol is excreted as a glucosiduronic acid derivative by rabbits dosed with naphthalene.

Evidence that naphthalene is metabolized to 1:2-dihydro-1-naphthyl glucosiduronic acids, which readily yield naphthalene in acid solution, is presented in this paper.

EXPERIMENTAL

Fifty rats (average body weight 250 g.) maintained on rat cake and water, were each injected intraperitoneally with naphthalene (100 mg.) dissolved in arachis oil (1 ml.) on