3. *S. cerevisiae* can synthesize diphosphopyridine nucleotide from nicotinamide as well as from nicotinic acid and can rapidly deamidate nicotinamide under both growing and resting conditions. Cells of *L. mesenteroides* form diphosphopyridine nucleotide from nicotinic acid but not from nicotinamide.

4. Experiments with [^{14}C]nicotinic acid and [^{14}C]nicotinamide confirm that the biosynthesis of diphosphopyridine nucleotide is effected by suspensions of *S. cerevisiae* almost as efficiently from nicotinamide as from nicotinic acid and that there is a rapid deamidation of nicotinamide.

5. It is concluded that the nicotinic acid pathway of biosynthesis of diphosphopyridine nucleotide is the predominant mechanism under normal conditions in *L. mesenteroides* and *S. cerevisiae*.

We wish to thank Dr B. C. Johnson and Dr H. P. Sarett for various gifts mentioned in this paper. Grateful acknowledgement is also due to the Indian Council of Medical Research for financial assistance.

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The Amino Acid Sequence around the Reactive Serine Residue of some Proteolytic Enzymes

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The three endopeptidases of the pancreas, chymotrypsin, trypsin and elastase, are stoicheiometrically inhibited by di*iso*propyl phosphorofluoridate, which combines with an unusually reactive serine residue in the enzymes. In chymotrypsin and trypsin this residue has been shown to be present in the sequence Gly.Asp.Ser.Gly (Turba & Gundlach, 1955; Schaffer, Simet, Harshman, Engle & Drisko, 1957; Oosterbaan, Kunst, Van Rotterdam & Cohen, 1958; Dixon, Kauffman & Neurath, 1958). [For definitions of the abbreviations used for amino acids in this paper see *Biochem. J.* (1957), **66**, 6.] That elastase contains the same sequence was concluded from experiments in which a partial acid hydrolysate of the di*iso*propoxy[^{32}P]phosphinyl derivative of elastase was subjected to paper ionophoresis and shown to contain the same radioactive peptides as did similar hydrolysates of the di*iso*propoxy[^{32}P]-phosphinyl derivatives of chymotrypsin and trypsin (Hartley, Naughton & Sanger, 1959). The present paper describes some further experiments with two-dimensional ionophoresis which confirm the above-mentioned result.

Considerable difficulties were encountered when we attempted to identify the various radioactive peptide spots present in these hydrolysates. The

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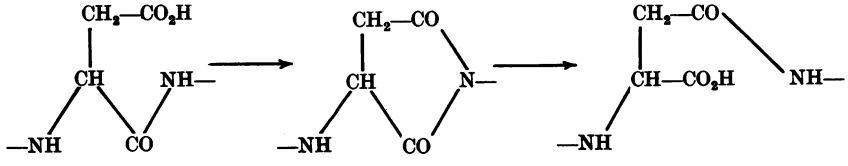
mixture was much more complex than would have been expected and the results could not be explained on the simple assumption that the only reaction taking place during the partial hydrolysis of a protein by acid was the splitting of peptide and amide bonds. Thus, for instance, when hydrolysis was carried out for 3 days in 12N-hydrochloric acid, at least three peptides were present which appeared to have the structure aspartyl–serine phosphate (Asp.SerP) and were interconvertible on further hydrolysis. Another three appeared to have the structure Asp.SerP.Gly. Since the serine phosphate residue in the radioactive proteins was present as the di*iso*propyl derivative heterogeneity was to be expected if the removal of the *iso*propyl groups by acid was not complete (Schaffer *et al.* 1957), and in fact small amounts of mono*iso*propyl derivatives were detected. The main difficulties, however, appeared to be due to some interconversions of the aspartyl residues and the results could best be explained by assuming that the reactions shown in Scheme 1 were taking place.



α-Aspartyl αβ′-Aspartyl β-Aspartyl

Scheme 1

That the reaction in Scheme 1 can occur during acid hydrolysis was shown by Swallow & Abraham (1958), who isolated ε-(αβ-aspartyl)lysine [referred to by them as ε-(aminosuccinyl)lysine] from a partial hydrolysate of bacitracin A. In model experiments they also showed that ε-(α-aspartyl)-lysine and ε-(β-aspartyl)lysine were interconvertible but were both largely converted into the αβ-aspartyl derivative. They found that an αβ-aspartyl derivative was formed from α-(α-aspartyl)-lysine but was not as stable as the ε-(αβ-aspartyl)-lysine. The present results agree with those of Swallow & Abraham, and suggest that the above-mentioned reactions will be of general occurrence in experiments where partial acid hydrolysis is used on proteins or peptides containing aspartic acid.

One of the objects of this investigation was to try to develop methods for identifying the amino acid sequence in the vicinity of a known isotopically labelled residue when only small amounts of material are available. Such methods would involve studying the electrophoretic and chromatographic rates of the radioactive peptides produced on partial hydrolysis and the effect of degradations such as acid hydrolysis and the Edman procedure on these peptides.

Thus, for instance, it should be possible to determine the number of residues in a labelled peptide from the products it gives on partial acid hydrolysis. A dipeptide should give only two labelled products, unchanged dipeptide and the free amino acid. A tripeptide in which the labelled residue is terminal would give itself, the amino acid and one new dipeptide. A tripeptide with the labelled residue in the middle should give two dipeptides and so forth. Similarly, some of the interrelationships of the different peptides may be deduced by studying the products produced after removal of the *N*-terminal residues by the Edman procedure. It was considered that the present system, in which a single residue in a known sequence is labelled, would be a good one on which to work out the methods. We have thus studied the effect of partial acid hydrolysis on the various bands in some detail and these results have contributed considerably to their identification.

Where enough material was available we have identified the amino acids present in the peptides after complete hydrolysis and in some cases have determined the *N*-terminal residues by the 2:4-dinitrophenyl technique.

EXPERIMENTAL

The following abbreviations for amino acid residues will be used in this paper: SerP, *O*-phosphoserine; SerIP, mono*iso*propyl phosphoserine; SerDIP, di*iso*propyl-phosphoserine.

Materials

Trypsin and chymotrypsin were crystalline salt-free preparations from Worthington Biochemical Corp. Elastase was prepared by a method to be described (Naughton & Sanger, 1960).

Di*iso*propyl [^{32}P]phosphorofluoridate (DF^{32}P) was a gift from Dr R. Davies and usually contained between 1 and 30 μc of ^{32}P/mg.

*Preparation of di*iso*propoxy*[^{32}P]*phosphinyl enzymes*

Various preparations of the di*iso*propoxy[^{32}P]phosphinyl (formerly 'di*iso*propylphosphoryl') derivatives were made. A satisfactory procedure was as follows.

The enzyme (25 mg.) was dissolved in 5 ml. of 0·1 M-phosphate buffer, pH 8·0, and treated with 0·63 ml. of 0·02 M-DF^{32}P in propan-2-ol. After incubation at room temperature for 3hr. the mixture was dialysed against two changes of 5 l. of 0·01 M-acetic acid at 2° and freeze-dried.

Hydrolysis of diisopropoxy[^{32}P]phosphinyl enzymes and peptides

Three methods of hydrolysis were used: (*a*) hydrolysis with 12 N-HCl at 37°; (*b*) hydrolysis with 5·7 N-HCl at 100° in a boiling-water bath; (*c*) hydrolysis with 5·7 N-HCl at 37°.

In general, where only small amounts of protein or peptides were to be hydrolysed the reaction was carried out in small test tubes with about 0·2 ml. of HCl. The HCl was then removed *in vacuo* over NaOH, water was added and the residue dried off about three times. In some experiments redistilled 5·7 N-HCl was used, but this made no apparent difference to the course of hydrolysis. As a general approximation it would appear that treatment for 10 min. by method (*b*) is equivalent to 24 hr. by method (*a*).

Ionophoresis of peptides

Paper ionophoresis was normally carried out by the method of Michl (1951) (Ryle, Sanger, Smith & Kitai, 1955). Except where otherwise stated, the separations were done on Whatman no. 52 filter paper with a voltage gradient of 40 v/cm. The most useful buffer for the fractionation of the phosphoserine peptides was pyridine–acetate, pH 3·5 (pyridine–acetic acid–water; 1:10:189, by vol.). The relative rates of the different peptides are very sensitive to slight changes in pH and it will be noted that the fractionations obtained in this work are rather different from those reported previously (Hartley *et al.* 1959). Although the buffer used was the same it seems that the actual pH on the paper will depend on the amounts of pyridine and acetic acid dissolved in the toluene which will be in equilibrium with the buffer on the paper. In the present experiments the toluene contained 0·3% (v/v) of acetic acid. The buffer at pH 6·5 contained pyridine–acetic acid–water (10:0·4:90, by vol.) and the toluene used in the tank with it contained 8% (v/v) of pyridine.

Each peptide fraction to be analysed, which should not contain more than about 0·1 mg. of material, was applied as a thin line over 2 cm. to the dry paper. Samples to be compared, as for instance in comparing the hydrolysates of the di*iso*propoxyphosphinyl derivatives (e.g. Fig. 11), or where peptides were run with a control mixture for identification, were applied with no gap between them but with slight overlapping (about 0·2 cm.). In this way a rigorous test for the identity of any two bands is obtained, since identical substances should give a continuous band between the two samples. After application of the samples the rest of the paper was wetted with buffer in such a way that it flowed evenly in towards the origin from each side, thus sharpening the bands of the applied samples.

In order to obtain satisfactory radioautographs each sample applied should contain at least 1 μmc of ^{32}P. To prepare the pH:mobility curves (Fig. 10) ionophoresis was carried out between cooled plates (Gross, 1955), since there was less flow of buffer into the paper from the electrode vessels than in the toluene-cooled system.

Two-dimensional ionophoresis. In order to prepare comparative two-dimensional ionophoresis patterns as shown in Fig. 12, the following technique was found useful. The samples to be compared were applied side by side and run in one dimension; the paper was then dried and a radioautograph was prepared (Fig. 11). A strip 0·5 cm. wide was then cut from the part of the paper that was to be run in a second dimension and the peptides were transferred to another sheet of paper by blotting. To do this a pad of clean filter paper slightly larger than the strip was wetted with water and blotted so that there was no excess of moisture. The strip was then wetted by pressing on to the pad with a thin glass plate. It was then transferred and pressed firmly on to the origin line of a clean sheet of paper. The process was repeated once or twice. This method transferred about 50% of the material to the new paper, which was then subjected to ionophoresis in a different buffer. An alternative method of doing two-dimensional ionophoresis which gives rather compact spots is to use cellulose acetate membrane (Kohn, 1957) as support during the first run. The cellulose acetate strip was wetted with buffer (pH 3·5) before application of the sample. Otherwise it was run under toluene in the same manner as the paper strips and a radioautograph prepared. The bands were sharp but usually rather uneven (see Fig. 5*a*). There was considerably more electroendosmosis than on paper and the relative rates of some of the peptides were slightly different although the buffer was the same. A strip about 0·5 cm. wide was cut from the cellulose acetate at a place where the bands were running evenly. A sheet of Whatman

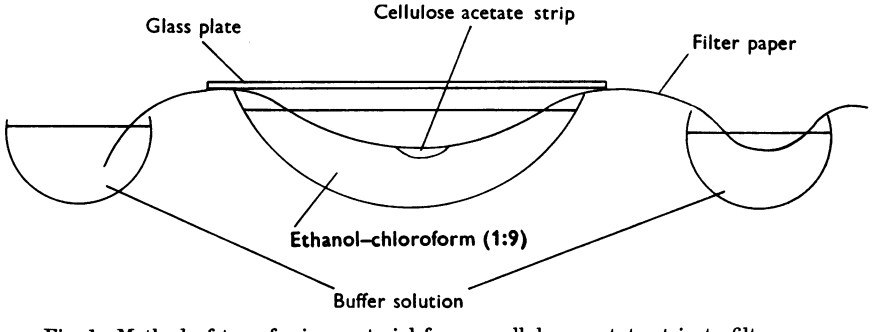


Fig. 1. Method of transferring material from a cellulose acetate strip to filter paper for two-dimensional ionophoresis.

no. 52 paper was wetted with buffer at pH 6·5 and the cellulose acetate strip pressed on to the origin line so that it was wetted with the buffer. While in this position it was wetted with a small amount of ethanol–chloroform (1:9) from a pipette. This was allowed to evaporate, thus sticking the cellulose acetate to the paper. The whole sheet was then inverted and the part around the origin line was soaked in ethanol–chloroform (1:9) as shown in Fig. 1. The paper was kept wet by immersion of its ends in troughs containing the buffer solution. The cellulose acetate dissolved in the ethanol–chloroform, leaving the radioactive spots on the paper. After soaking for about 2 hr. the paper was transferred to the ionophoresis tank for running at pH 6·5. Fig. 5*b* is a radioautograph of a paper on which a two-dimensional ionophoresis had been carried out in this way.

Isolation of peptides

In most experiments in which the peptides were studied by radioautography only small amounts of the hydrolysates were used and no preliminary separation of the phosphoserine peptides was necessary. However, in order to obtain sufficient amounts of peptides to determine their amino acid content and end groups, two experiments were done on a somewhat larger scale. Di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin (100–200 mg.) was hydrolysed with acid (method *a* for 2 days or method *b* for 20 min.). The HCl was removed *in vacuo* with a rotary evaporator and the residue was dissolved in 2 ml. of water and applied to a column (2 cm. diam.) of Dowex-50 (10 g., in the acid form). The column was developed with water. The material coming through with the solvent front was collected and contained over 90% of the added radioactivity. This treatment, which removes the majority of the peptides not containing serine phosphate (Flavin, 1954), was necessary to avoid overloading during ionophoresis. After repeated evaporation *in vacuo* the peptide mixture was applied as a 35 cm. band 10 cm. from the cathode end of a sheet of Whatman no. 52 paper. Ionophoresis was carried out at pH 3·5 at 40 v/cm. for 2·5 hr. A radioautograph was prepared, a part of which is shown in Fig. 4. The bands were cut out and eluted with water. Each peptide was purified by ionophoresis at pH 6·5. Samples were taken for hydrolysis and for end-group determination by the 2:4-dinitrophenyl technique. Where possible 0·1 μmole of peptide was used for hydrolysis. The amounts of the various peptides were estimated from the radioactivity.

The amino acids in the hydrolysates were identified by ionophoresis in 2·5% (v/v) formic acid for 25 min. at 80 v/cm. This system gives very sharp bands and clearly separates all the amino acids expected in these peptides (Ser, Asp, Gly, Glu, Ala). *N*-Terminal residues were determined by the 2:4-dinitrophenyl technique, dinitrophenyl-amino acids being identified by paper chromatography on *tert.*-amyl alcohol–phthalate buffer, pH 5·0 (Blackburn & Lowther, 1951).

Edman degradation

In the Edman degradation it is usual to identify the *N*-terminal residue as the phenylthiohydantoin or as the amino acid missing from a hydrolysate of the phenylthiocarbamyl peptide. We have used a somewhat different approach and have identified the radioactive product produced. This gives considerable information about the relationships between the different peptides. Various modifications of the original Edman method have been employed. Most of the results reported here were obtained by the procedure described below, which is a combination of various published methods (Edman, 1950; Fraenkel-Conrat & Harris, 1954).

The radioactive peptide (containing at least 1 μmc of ^{32}P) was taken to dryness in a small test tube and dissolved in 0·1 ml. of water. Phenyl*iso*thiocyanate–pyridine (0·1 ml.; 1:9, v/v) was added and the mixture shaken at room temperature for 3–5 hr. Benzene (2 ml.) and a few drops of water were added, and the mixture was shaken and the benzene layer removed. The extraction was repeated and the aqueous residue taken to dryness over H_2SO_4 in a desiccator. After addition of water and evaporation two or three times, 0·2 ml. of 3 N-HCl was added and the mixture allowed to stand for 5 hr. at room temperature. The HCl was removed *in vacuo* over NaOH and the residue subjected to ionophoresis at pH 3·5. A partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin was run in parallel as a control in order to be able to identify the product.

We have also used the method of Sjöquist (1957) on some of the peptides. Little difference was noticed between the methods, but it was observed that some peptides gave much clearer results than others. Those containing *iso*propyl groups appeared to react better than those with unesterified phosphate. When peptides containing *N*-terminal serine phosphate (e.g. peptide 10 and some peptides from ovalbumin; F. Sanger, unpublished work) were subjected to this procedure, the only product present in significant amounts migrated at the same rate as inorganic phosphate. It is unlikely that the phenylthiohydantoin of serine phosphate, which is the expected product, would move so fast and it appears that the compound is unstable and breaks down to give phosphate. Similarly, peptide 11 (SerIP.Gly) breaks down to give *iso*propyl phosphate (band 2).

Radioautographs

The filter paper to be radioautographed was stuck with cellulose self-adhesive tape into a cardboard filing folder, one side of which was covered with lead sheeting (0·25 mm. thick). One or two sheets of Ilford Industrial G X-ray film were then stuck in the folder, which was kept in a light-proof box until ready for developing. Where individual bands from a sample put on over 2 cm. paper contained 1 μmc of ^{32}P the film could be developed after about 24 hr. If the bands contained 0·1 μmc about 10–15 days were required.

In order to mark the radioautographs and to be able to align them with the filter papers, the papers were marked with a radioactive pencil: commercial propelling pencil leads were immersed overnight in radioactive residues (^{14}C or ^{35}S) of high specific activity and then dried in an oven at 105°. The leads were then used in a propelling pencil.

*Time course of hydrolysis of di*iso*propoxy[^{32}P]phosphinyl enzymes*

The di*iso*propoxy[^{32}P]phosphinyl enzyme was dissolved in HCl and samples were measured into small test tubes, the size of the sample being adjusted so that the amounts put on the ionophoresis paper would be constant (about 1 μmc of ^{32}P/cm.). In most experiments each sample contained about 2 μmc and was put on the ionophoresis paper

over a 2 cm. width; however, in the experiment illustrated in Fig. 2 the size of the samples varied. Each sample was incubated at 37° or 100° for the appropriate period and after removal of the HCl *in vacuo* they were subjected to ionophoresis at pH 3·5, being put on side by side in the appropriate order (see Figs. 2 and 3).

RESULTS

Most of the results reported in this section were obtained with chymotrypsin or trypsin. However, they apply equally well to elastase since no major differences could be detected in the radioactive peptides obtained from the di*iso*propoxy[^{32}P]-phosphinyl derivatives of the three enzymes (see below).

*Course of hydrolysis of di*iso*propoxy[^{32}P]phosphinyl enzymes*

Fig. 2 shows the course of hydrolysis of di*iso*-propoxy[^{32}P]phosphinyl-trypsin by method (*b*). It can be seen that the main stable derivatives are in bands 1, 4 and 10. Bands, 3, 6, 7 and 9B are also relatively stable but are present in smaller amounts. It seemed that a period of hydrolysis of 20–30 min. gave a suitable mixture of peptides, and most of the work was carried out under these conditions. In some experiments method (*a*) was used and it was found that a suitable time of hydrolysis was 2–3 days. There were no apparent qualitative differences between the two methods of hydrolysis, so that to save time method (*b*) was normally used.

In an attempt to identify larger peptides, hydrolysates prepared by method (*c*) were also studied. The course of this reaction is shown in Fig. 3 and appears to be somewhat different from that with methods (*a*) and (*b*). A 3-day period of hydrolysis gave relatively high concentrations of a number of bands, such as 7B, 9A, 11, 14, 15 and 18, which were absent or present only in small amounts in the other more complete hydrolysates, and this time of hydrolysis was then used to study these slower-moving peptides.

Identification of radioactive bands

Fig. 4 shows a radioautograph of a typical ionophoresis experiment of a partial acid hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. On running again at pH 6·5, most of the bands were essentially pure though there were usually smaller amounts of other contaminants. Fig. 5 shows a two-dimensional ionophoresis run first on cellulose acetate at pH 3·5 and then on paper at pH 6·5, as described in the Experimental section, and the numbering of the various peptide spots is

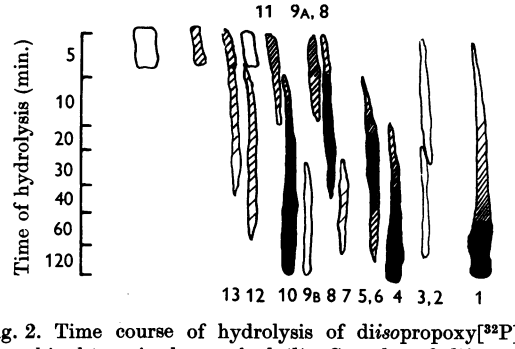


Fig. 2. Time course of hydrolysis of di*iso*propoxy[^{32}P]-phosphinyl-trypsin by method (*b*). Samples of di*iso*-propoxy[^{32}P]phosphinyl-trypsin were hydrolysed for different periods and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr.

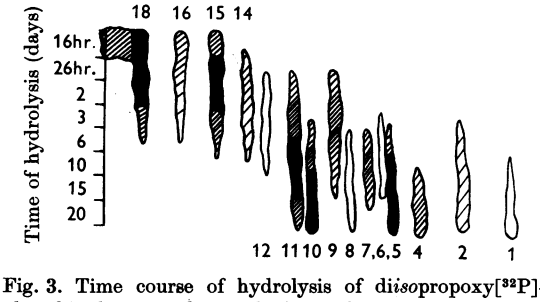


Fig. 3. Time course of hydrolysis of di*iso*propoxy[^{32}P]-phosphinyl-trypsin by method (*c*). Samples were hydrolysed for different periods and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr.

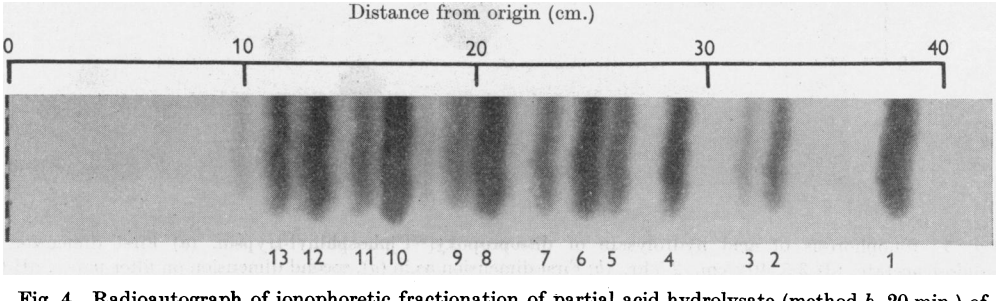


Fig. 4. Radioautograph of ionophoretic fractionation of partial acid hydrolysate (method *b*, 20 min.) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. Ionophoresis at pH 3·5, 40 v/cm., 2·5 hr.

shown in Fig. 6. This is a diagram of a two-dimensional ionophoresis pattern carried out entirely on filter paper and is thus slightly different from Fig. 5. It incorporates all the main peptides studied in this work, including those in the hydrolysate obtained by method (*c*) (3 days).

The yields of peptides from a hydrolysate (method *a*, 2 days) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin are given in Table 1. The total recovery of ^{32}P is low (62 %) so that there have been considerable losses, probably mainly during elution of the peptides from the filter paper and running again at pH 6·5.

The results of the large-scale experiments, in which some of the peptides were hydrolysed to identify the amino acids present, and in which the end groups of some peptides were determined, are shown in Table 2. This method of analysis clearly requires that the peptides should be free of any contaminating non-radioactive peptides. Most of these should have been removed on the Dowex-50 column and should not move as acids at pH 3·5.

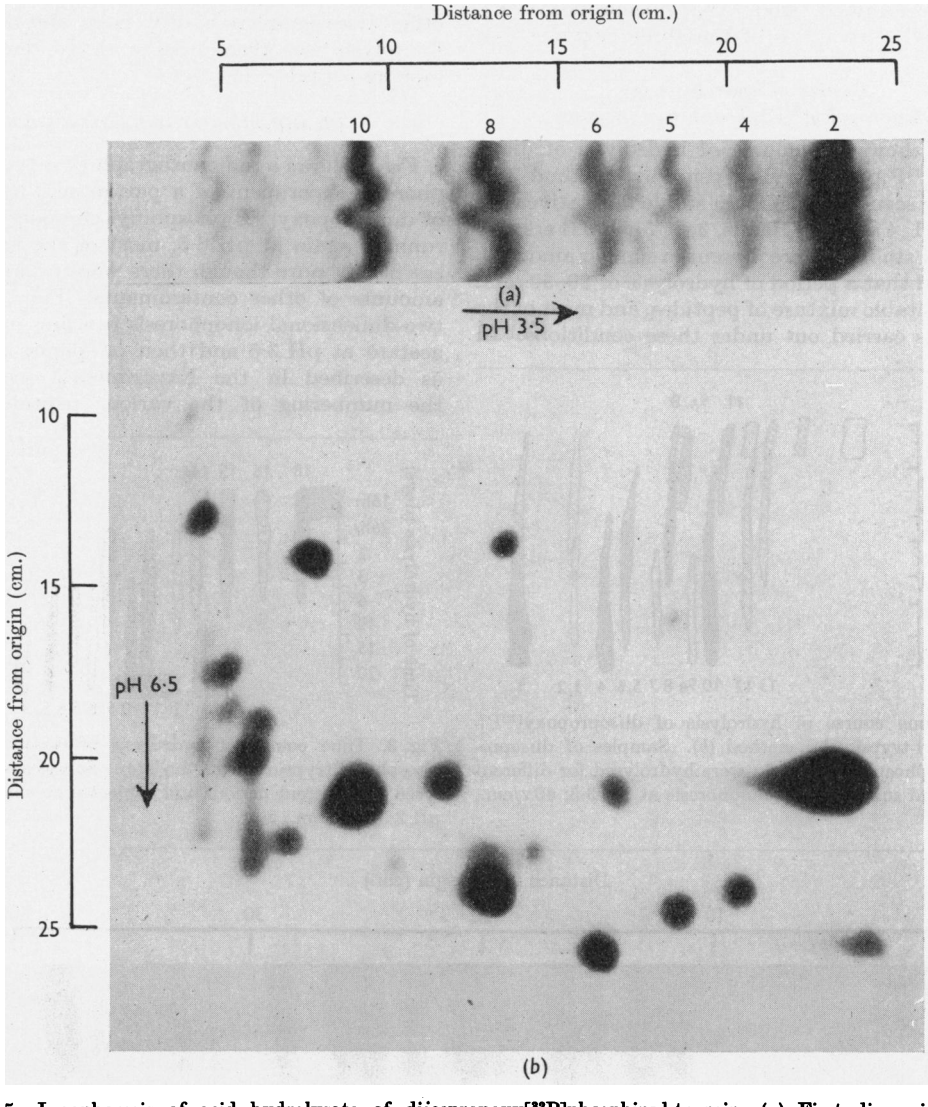


Fig. 5. Ionophoresis of acid hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-trypsin. (*a*) First dimension on cellulose acetate, pH 3·5, 40 v/cm., 2·5 hr. (*b*) First dimension as in (*a*), second dimension on filter paper, pH 6·5, 40 v/cm., 1·75 hr. The strong spot 2 is due to contamination of the di*iso*propoxy[^{32}P]-phosphinyl-trypsin with DF^{32}P, caused by inadequate dialysis of the preparation.

However, the presence of glutamic acid in the hydrolysate of band 5A before purification at pH 2·1 indicates that some contamination may take place. It was at first considered that peptide 5A was [Asp, SerP, Gly, Glu]. However, this could not be reconciled with the partial-hydrolysis results, so that further purification was attempted.

Each band was partially hydrolysed (usually by method *b*, 30 min.) and subjected to ionophoresis at pH 3·5. A sample of a hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin was run adjacent to each peptide hydrolysate as a control in order to be able to identify the various degradation products. The results are given in Table 3. The yields given were estimated by eye from the darkening of the radioautographs and indicate the relative amount of each product obtained from a given quantity of each peptide band. The letters 'tr' indicate the presence of a small but significant amount of the product. A small amount of free phosphate (band 1) was obtained from each band but this is not included in the Table 3.

Since the course of hydrolysis by method (*c*) is somewhat different, the main products obtained by this method were also subjected to a further hydrolysis for 3 days to show their inter-relationships. These results are given in Table 4.

Samples of some of the peptides were subjected to the Edman degradation procedure and the product was identified by ionophoresis at pH 3·5. The results were not always entirely clear-cut. Unchanged material was frequently present and varying amounts of free phosphate. The results of experiments where the main product of the degradation was clear are given in Table 3.

Two of the bands could be identified directly by comparison of their rates with marker compounds of known structure. These were band 1, which was identified as free phosphate, and band 4, which was serine phosphate. Band 2 is present together with band 1 in a mild acid hydrolysate (e.g. method *c*, 1–3 days) of DF^{32}P. On hydrolysis it gives rise to band 1 only and appears to be mono*iso*propyl phosphate.

The only peptide band that was readily identifiable was band 10, which behaved essentially as a dipeptide on partial acid hydrolysis, giving only serine phosphate in significant amounts. It was

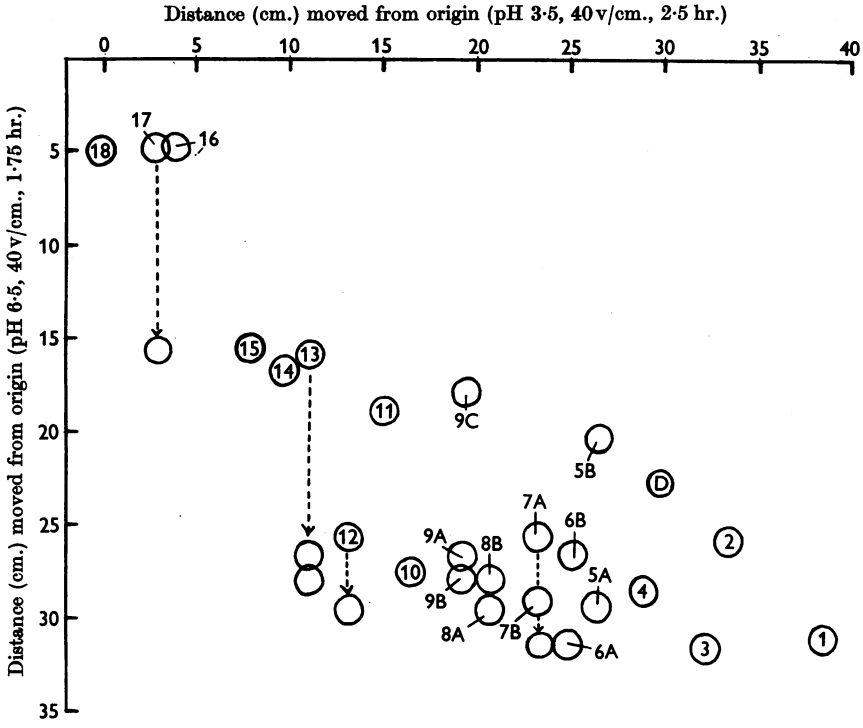


Fig. 6. Diagram showing the migration rates at pH 3·5 and pH 6·5 of radioactive compounds obtained from partial acid hydrolysates of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. Ionophoresis on Whatman no. 52 filter paper. The materials were applied near to the cathode end of the paper. There was considerable flow of buffer into the paper from both ends during the ionophoresis. Spot D shows the position occupied by di*iso*propyl phosphate. The broken arrows indicate the changes that take place during running at pH 6·5.

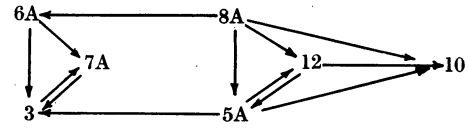
the most stable peptide (Fig. 2) and gave serine and glycine on complete hydrolysis. It was clearly SerP.Gly.

Although the main product of partial hydrolysis of SerP.Gly was serine phosphate, two other bands were given in small amounts. They moved at the rates of bands 9B and 10*x* (Fig. 7). Peptide 9B appears during the later stages of hydrolysis by method (*b*) and is very stable. On partial hydrolysis it gives band 4 and a trace of band 10. It is formed in larger amounts if peptide 10 is hydrolysed in more dilute acid (e.g. 3N-HCl, 100°; Fig. 7, no. 4). On Edman degradation it gave serine phosphate. It seems probable that it is Gly.SerP formed by inversion of SerP.Gly via the intermediate anhydride. This reaction has been shown to occur during hydrolysis in 2N-HCl (Schaffer, Harshman & Engle, 1955) and also appears to occur in 5·7N-HCl at 100°, though it was probably not significant when method (*a*) was used.

The nature of band 10*x* is unknown. Its yield varies considerably and is greatly increased if the partial hydrolysis is carried out in the presence of a small amount of glucose (Fig. 7, no. 5) or of filter paper. It would thus appear to be an artifact formed from SerP.Gly due to the presence of impurities (probably carbohydrate) extracted from the filter paper. Its slow rate of migration at pH 3·5 suggests decarboxylation or else that one of the acidic groups is substituted.

The results with the other main peptide bands could not readily be explained in any simple way. A number of bands were interconvertible. For instance, peptide 5A on partial hydrolysis gave 12 and 12 gave 5A. When certain bands were run again as controls at pH 3·5 they were found to be converted into other bands and these conversions could be brought about by incubation overnight at pH 6·5 (Fig. 8). Thus 12 was completely converted into 5A, 13 which appears to be a mixture was converted into two peptides moving at the rates of 6B and 8B respectively. Band 7 was partly converted into band 3 and was partly unchanged. It seemed to contain two compounds: 7A, which is converted into 3 at pH 6·5, and 7B, which is stable at pH 6·5. The decomposition of bands 12 and 13 can be seen in Fig. 5 by the streaks originating from spots 12 and 13. Evidently in this experiment the conversion was taking place during the ionophoresis at pH 6·5.

The various interconversions of peptides 3, 6A, 7A, 5A, 8A, 12 and 10 are summarized in Scheme 2.



Scheme 2

The fact that 3, 6A and 7A are related and on partial hydrolysis give only serine phosphate and one another suggested that they were all different forms of the same dipeptide, presumably Asp.SerP. Peptides 5A, 8A and 12 were also similarly related and broke down to give 10 (SerP.Gly) and different forms of Asp.SerP. They thus appeared to be the corresponding three forms of the tripeptide Asp.SerP.Gly.

Table 1. *Yield of peptides from hydrolysate (method* a, *2 days) of di*iso*propoxy[*^{32}*P]phosphinyl-chymotrypsin*

Yield is expressed as ^{32}P recovered in peptide band as percentage of ^{32}P of original di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin.

Band	Yield
1	5·0
2	1·5
3	2·4
4	3·0
5A	11·0
5B	0·2
6A	1·5
6B	1·0
7 (A + B)	5·0
8A	9·0
8B	1·4
9 (A + B + C)	3·0
10	10·7
11	1·6
12	2·3
13 (A + B)	3·2
Total yield	61·8

Table 2. *Amino acid composition and* N*-terminal residues of peptides from di*iso*propoxy[*^{32}*P]phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Amino acids present	Approx. strength	*N*-Terminal residue
1	None	—	—
2	None	—	—
4	Ser	× × ×	—
	SerP	×	—
	Glu	tr	—
5A*	Ser	× ×	Asp
	Gly	× ×	—
	Asp	× ×	—
6A	Ser	× ×	Asp
	Asp	× ×	—
8A	Ser	× ×	Asp
	Gly	× ×	—
	Asp	× ×	—
	Glu	tr	—
10A	Ser	× ×	—
	Gly	× ×	—

* Band 5A, after purification by ionophoresis at pH 3·5 and 6·5, contained glutamic acid (× × ×). This was not present after further ionophoresis in 2·5% (v/v) formic acid (pH 2·1).

Table 3. *Partial hydrolysis and Edman degradations of serine phosphate peptides from diisopropoxy[^{32}P]phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Peptide bands given after hydrolysis (method *b*, 30 min.)									Main product of Edman degradation	Probable structure
	3	4	5	6	7	8	10	12	Other bands		
3	× ×	× ×	—	—	×	—	—	—	—	—	β-Asp.SerP
4	—	×	—	—	—	—	—	—	—	1	SerP
5A	× ×	× ×	× × ×	—	×	—	× × ×	×	—	—	β-Asp.SerP.Gly
5B	—	×	—	—	—	—	—	—	—	—	SerIP
6A	tr	× ×	—	× ×	tr	—	—	—	—	4	α-Asp.SerP
6B	×	× ×	×	—	tr	—	× ×	tr	—	—	β-Asp.SerIP.Gly
7A*	× ×	× × ×	—	—	×	—	—	—	—	—	αβ-Asp.SerP
7B*	tr	× × ×	—	× ×	tr	—	—	—	—	5B	α-Asp.SerIP
8A	tr	× ×	tr	× ×	tr	× × ×	× × ×	tr	—	10	α-Asp.SerP.Gly
8B	×	× ×	× ×	—	tr	×	× × ×	×	13 (×)	5†	Gly.β-Asp.SerP.Gly?
9A	tr	× × ×	tr	× ×	tr	× ×	× × ×	tr	—	11	α-Asp.SerIP.Gly
9B	—	× ×	—	—	—	—	tr	—	9 (× ×)	4	Gly.SerP
9C	×	× × ×	—	—	× ×	—	—	—	9 (× ×)	—	?
10	—	×	—	—	—	—	× × ×	—	9B (tr), 10*x* (tr)	1	SerP.Gly
11	—	×	—	—	—	—	× × ×	—	9B (tr), 10*x* (tr)	2	SerIP.Gly
12	× ×	× ×	× × ×	—	×	tr	× × ×	×	—	—	αβ-Asp.SerP.Gly
13A‡	×	× ×	×	—	×	—	× ×	tr	13 (tr)	—	αβ-Asp.SerIP.Gly
13B‡	×	× ×	×	—	×	tr	× ×	×	13 (×)	—	Gly.αβ-Asp.SerP.Gly?
14	tr	× × ×	—	× ×	tr	—	—	—	—	16	α-Asp.SerDIP
15	tr	× ×	tr	×	tr	×	× ×	tr	9A (tr)	18	α-Asp.SerDIP.Gly
16	—	×	—	—	—	—	—	—	—	—	SerDIP
17	tr	× ×	tr	×	×	tr	× ×	tr	—	—	αβ-Asp.SerDIP.Gly?
18	—	×	—	—	—	—	× × ×	—	2 (× ×)	—	SerDIP.Gly

* Band 7 was incubated at pH 6·5 overnight and refractionated at pH 3·5. 7A was the fast-moving band (3), 7B the slower-moving one. 7B was obtained in higher yield directly from the 3-day hydrolysate by method (*c*).

† Result of a single experiment. There was a relatively large amount of unchanged peptide present.

‡ Band 13 was incubated at pH 6·5 overnight and refractionated at pH 3·5. 13A moved at the rate of band 6, 13B at the rate of band 8.

Table 4. *Partial hydrolysis (method c) of peptides from diisopropoxy[^{32}P]phosphinyl-chymotrypsin*

Band no. (Fig. 6)	Peptide bands given after partial hydrolysis (method *c*, 3 days)									
	5*	7B	9A	10	11	14	15	17	18	Other bands
7B	tr	× ×	—	—	—	—	—	—	—	—
9A	tr	×	× ×	—	×	—	—	—	—	8 (tr), 13 (tr)
11	tr	—	—	tr	× ×	—	—	—	—	2 (tr)
14	×	×	—	—	—	× ×	—	—	—	—
15	tr	×	×	tr	×	×	× ×	tr	×	2 (tr)
18	—	—	—	tr	× ×	—	—	—	×	2 (× ×)

* Bands 5 and 6 were not well resolved. It is probable that the band in this position is 5B though it could in some cases have been 5A, 6A or 6B.

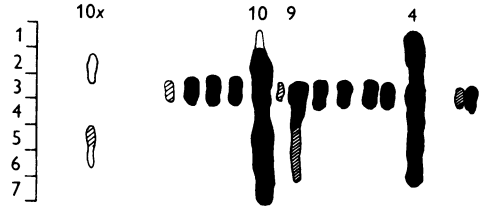


Fig. 7. Breakdown of peptide 10 under various conditions of hydrolysis. Samples of peptide 10 (containing ^{32}P, 5 μmc) hydrolysed as below and subjected to ionophoresis at pH 3·5, 40 v/cm., 2·5 hr. 1, Method (*b*), 3 hr.; 2, method (*a*), 3 days; 3, control hydrolysate (method *b*, 30 min.) of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin; 4, 3 N-HCl, 100°, 6 hr.; 5, as for 6 with a trace of glucose added; 6, method (*b*), 35 min.; 7, untreated.

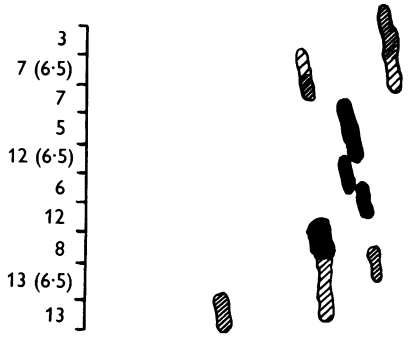


Fig. 8. Decomposition of peptides 7, 12, 13 after incubation at pH 6·5, for 16 hr. Other markers were eluted from papers on which they had been fractionated by ionophoresis, dried down and run again (1 μmc per sample). Ionophoresis: pH 3·5, 40 v/cm., 2 hr.

These results are best explained by assuming that 6A and 8A are normal α-aspartyl peptides, 7A and 12 the relatively unstable ring forms (αβ-aspartyl derivatives) and 3 and 5A the β-aspartyl peptides. The assignment of the β form to 3 and 5A was at first based largely on their greater ionophoretic rate at pH 3·5. Since the aspartic acid is *N*-terminal in both peptides, the aspartic acid carboxyl group in the β form will be adjacent to an α-amino group and will have a lower p*K* than

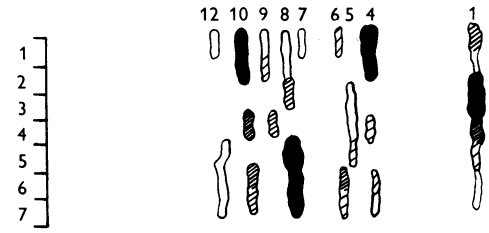


Fig. 9. Breakdown of peptide 8 under various conditions. Samples of peptide 8 (containing ^{32}P, 1 μmc) were treated as below and subjected to ionophoresis at pH 3·5, 40 v/cm., 2 hr. 1, Method (*b*), 55 min.; 2, 2 N-HCl, 100°, 2 hr.; 3, water, 100°, 6 hr.; 4, 20% (v/v) formic acid, 100, 4 hr.; 5, thionyl chloride, 18°, 18 hr.; 6, 6 N-HCl, 120°, 3 min.; 7, method (*b*), 25 min.

the normal β-carboxyl group and hence move faster at pH 3·5. Peptide 3 is formed during the later stages of hydrolysis and seems to be relatively stable. John & Young (1954) showed that α-Asp.Val can be converted into β-Asp.Val by heating in water at 100° for 6 hr. Under these conditions considerable conversion of 8A into 5A and of 6A into 3 occurred (see Fig. 9). The various results given in Tables 2–4 are in agreement with the above interpretation and can thus be regarded as confirmation of it.

Probably the best method of characterizing the various aspartyl derivatives would be by their titration curves (see Swallow & Abraham, 1958). Since we had insufficient material for this we attempted to determine the p*K* values of the various groups by measuring the ionophoretic mobility of the peptides at different pH values. If the mobility is plotted against pH, the resulting curve should be analogous to a titration curve. In order to compensate for electroendosmosis, for the flow of buffer into the paper from the electrode vessels and for variations in conditions of running, the results were expressed relative to a marker of known titration curve. For this purpose serine phosphate was used and the radioactive material (from band 4) was run together with the peptides at each pH value. A glucose marker was also used

to determine the displacement of the origin. A titration curve of serine phosphate was then prepared in the normal way, and the net charge was calculated for the different pH values. From this the theoretical mobility of serine phosphate was determined, assuming a value at pH 5·1 of 13 cm. This was the experimental value found after ionophoresis for 1·5 hr. at 30v/cm. The relative mobilities for the peptides were calculated as

$$\frac{\text{Distance of peptide from glucose marker}}{\text{Distance of serine phosphate from glucose marker}} \times \text{theoretical mobility for serine phosphate.}$$

The results are, then, the distance travelled by the peptide in 1·5 hr. at 30v/cm. under the experimental conditions (heating etc.) that prevailed during the experiment at pH 5·1. Since the absolute mobilities could not be determined it was considered preferable to express the results in these arbitrary units.

Some of the curves so obtained are shown in Fig. 10. Although they cannot be regarded as very accurate owing to the limitations of the method, they do give an indication of the p*K* values of the various groups involved and clearly show that one of the carboxyl groups and the amino groups in 3 and 5A are considerably stronger than the corresponding groups in 6A and 8A. Unfortunately 7A and 12 were too unstable at most pH values to determine their mobilities. One of the p*K* values of the phosphate group is about 5·5–6·0 (Fölsch & Österberg, 1959), and this is absent from peptide 11, thus confirming that it is a mono*iso*propyl derivative (see below). The p*K* values of the amino groups decrease with increased peptide size (4 > 6A > 8A), as would be expected.

Fig. 9 shows the results of various treatments on peptide 8 (α-Asp.SerP.Gly). The main products of hydrolysis are normally 4, 6, 10 and 12 (e.g. in sample 7), the relative amounts of each depending on the time of hydrolysis. Treatment with 20% (v/v) formic acid (sample 4) leads almost exclusively to peptide 10 (SerP.Gly) and its inversion product 9B. This is in agreement with the results of Partridge & Davis (1950), who showed a preferential splitting of bonds involving aspartyl residues in weak acid.

The action of thionyl chloride was studied to see if it converted an α-aspartyl peptide (e.g. peptide 8) into the αβ form (e.g. peptide 12). This does take place to a small extent, though most of 8 was unchanged under the conditions used. A more appreciable conversion of peptide 6 into 7 was brought about by treatment with thionyl chloride at 100° for 15 min. This can be regarded as further support for the conclusion that 7A and 12 are αβ forms.

A number of peptides, especially in the hydrolysate prepared by method (*c*) for 3 days, contain the phosphate as the mono*iso*propyl or di*iso*propyl derivatives. These can be recognized by their relative rates of migration at pH 3·5 and 6·5, since the second ionization constant (p*K* 5·5–6·0) of the phosphate is blocked in them. Also, on treatment with alkali (4% triethylamine, 30 min., 105°) the mono*iso*propyl derivatives gave mono*iso*propyl phosphate (band 2), which could be identified. The following bands were shown in this way to be mono*iso*propyl derivatives: 5B, 7B, 9A, 11. Their probable structure was deduced from the products of their hydrolysis and is shown in Table 3. Peptides 14, 15, 16, 17 and 18 appeared from their behaviour on degradation to be di*iso*propyl derivatives. When they were treated with triethylamine and the product was subjected to iono-

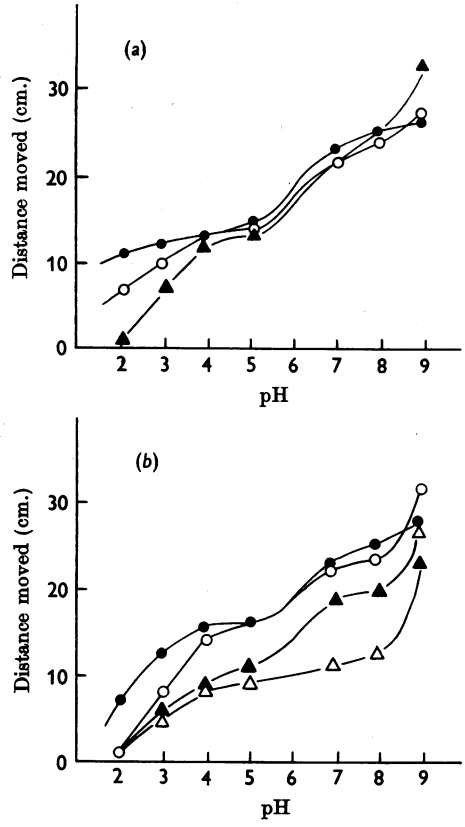


Fig. 10. Mobility–pH curves for ^{32}P-containing bands from partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin. The curves are plotted relative to the theoretical distance of migration of serine phosphate at 30v/cm. for 1·5 hr. (see text). (*a*) ●, Serine phosphate (theoretical curve); ○, band 5A; ▲, band 8A. (*b*) ●, Band 3; ○, band 6A; ▲, band 10A; △, band 11.

phoresis at pH 3·5 under toluene the radioactivity was almost completely lost. This also occurred when DF^{32}P was treated in the same way. If, however, the ionophoresis was done between cooled plates a band was obtained from the alkaline degradation of DF^{32}P and of peptide 15 which moved slightly faster than serine phosphate. It is presumably di*iso*propyl phosphate. It appears that it is extracted into toluene, possibly in the form of its triethylamine salt.

Schaffer *et al.* (1957) identified the peptide Gly.Asp.SerP.Gly in hydrolysates of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin (method *a*, 3 days). It seems most likely that band 8B has this structure, although it was present in rather small amounts and was not completely characterized. It is clearly in the β form and 13B is the corresponding $\alpha\beta$ form. Schaffer & Lang (1959) have reported the identification of a labelled peptide having the amino acids [Ser.Gly.Glu.Ala] (see also Turba & Gundlach, 1955). We have been unable to detect any peptide that could have this structure. There are a number of weak bands that have not been thoroughly characterized. The most likely structure for 9C is $\alpha\beta$-Asp.SerIP. On running again at pH 3·5 and on incubation with alkali it gives rise to a peptide moving between bands 3 and 4, which could be β-Asp.SerIP.

When band 10 (SerP.Gly) was repurified by ionophoresis at pH 6·5 a small amount of a rapidly moving band was always present. This proved to be free phosphate. Similarly, when bands 11 (SerIP.Gly) and 18 (SerDIP.Gly) were purified at pH 6·5, faster-moving bands were present which could have been *iso*propyl phosphate and di*iso*propyl phosphate respectively. It would thus appear that these peptides are somewhat unstable to the conditions employed. There were several other bands present in small amounts whose structures could not be determined. The results with these are not recorded here.

Comparison of ^{32}P*-containing peptides from the di-isopropoxy*[^{32}P]*phosphinyl derivatives of chymotrypsin, trypsin and elastase*

Samples of the di*iso*propoxy[^{32}P]phosphinyl derivatives of trypsin, chymotrypsin and elastase were hydrolysed for 20 min. by method (*b*) and subjected to ionophoresis in parallel together at pH 3·5. Fig. 11 is a radioautograph of the paper which shows no difference between the three enzymes. By the use of the blotting method two-dimensional ionophoresis patterns were prepared from the above-mentioned parallel one-dimensional ones and these are shown in Fig. 12. A shorter-time hydrolysis (10 min.) of di*iso*propoxy[^{32}P]phosphinyl-elastase is also shown. These results reveal no differences in the ^{32}P-containing peptides from the three enzymes.

DISCUSSION

The above-described results show that the complexity of the hydrolysates of the di*iso*propoxy-[^{32}P]phosphinyl-enzymes is largely due to two factors: the incomplete hydrolysis of the *iso*propyl

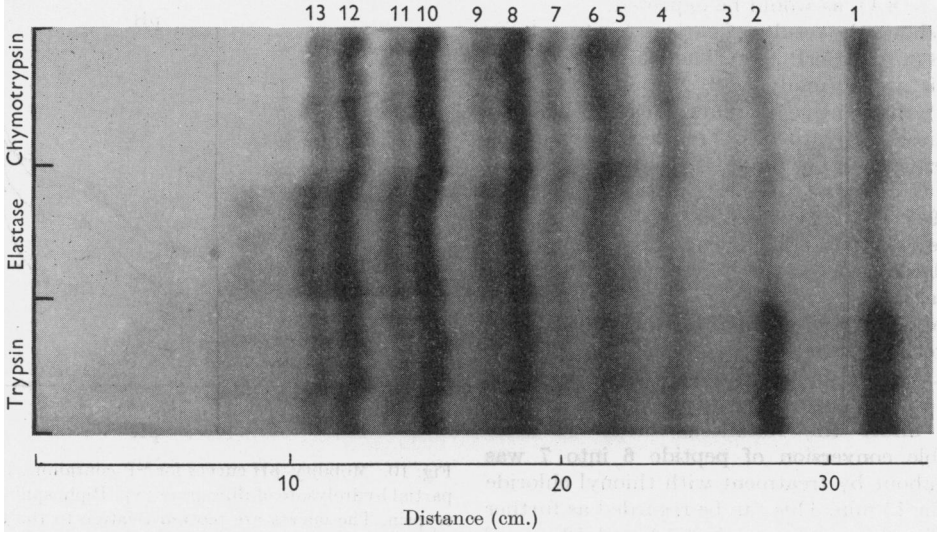


Fig. 11. Ionophoresis of partial hydrolysates (method *b*, 20 min.) of the di*iso*propoxy[^{32}P]phosphinyl derivatives of chymotrypsin, elastase and trypsin, pH 3·5, 40 v/cm., 2 hr. The strong bands 1 and 2 from the trypsin derivative are derived from DF^{32}P, which contaminated the preparation.

ester groups and the aspartyl-interconversion reaction. It seems likely that this interconversion is a general reaction and is to be expected in experiments where proteins or peptides containing aspartic acid are subjected to hydrolysis with acid. It probably occurred during the hydrolysis of fraction A of oxidized insulin with acid (Sanger & Thompson, 1953), since three peptides (Ala9, Ala10, Ala14) with the apparent structure Asp.Tyr were detected. They were explained as being due to the presence of an amide group and to TyrX (chlorotyrosine; Thompson, 1954). It is unlikely that amide groups would in fact be stable under the conditions of hydrolysis and a more likely explanation is that peptide Ala14, which gave a yellow colour with ninhydrin, was the β-Asp.Tyr. John & Young (1954) report that β-aspartyl peptides give a yellow ninhydrin colour.

Although Schaffer *et al.* (1957) did not detect the interconversion in their experiments, which were done under the same conditions as we have used, it is probable that it occurred and that their peptide 2 was the same as our peptide 5A (β-Asp.SerP.Gly), whereas peptide 3 was our 8A. They found that although both reacted with phenyl *iso*thiocyanate in the Edman procedure, the

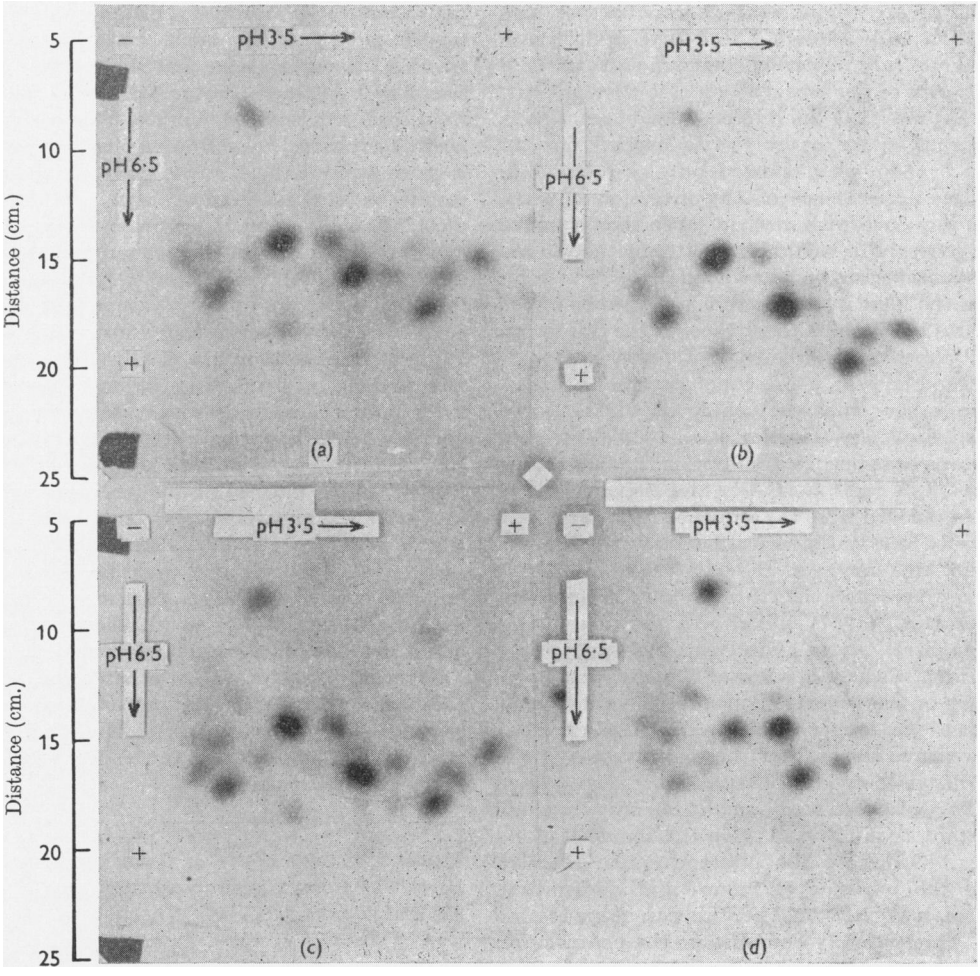


Fig. 12. Comparative two-dimensional ionophoresis patterns of partial hydrolysates (method *b*) of the di*iso*propoxy[^{32}P]phosphinyl derivatives of trypsin, chymotrypsin and elastase. First dimension, pH 3·5, as in Fig. 11; second dimension, pH 6·5, 40 v/cm., 1 hr. Transfer to second dimension was by the blotting technique (see text). (*a*) Di*iso*propoxyphosphinyl-trypsin, 20 min. hydrolysate. (*b*) Di*iso*propoxyphosphinyl-chymotrypsin, 20 min. hydrolysate. (*c*) Di*iso*propoxyphosphinyl-elastase, 20 min. hydrolysate. (*d*) Di*iso*propoxyphosphinyl-elastase, 10 min. hydrolysate.

phenylthiohydantoin of aspartic acid was liberated only from peptide 3 and not from peptide 2, as would be expected if the latter was a β-aspartyl peptide.

Various methods of hydrolysis were studied (e.g. Fig. 9) in an attempt to find conditions in which the interconversions of aspartic acid peptides could be avoided. No such conditions could be found but the reaction seems to be less marked in 5·7N-hydrochloric acid than in 12N-hydrochloric acid. The main difference between hydrolyses in 12N-hydrochloric acid (method *a*) and in 5·7N-hydrochloric acid (method *c*) at 37° is in the stability of the *iso*propyl groups, which are much more resistant under the latter conditions. There was no evidence of any significant amounts of peptides larger than tripeptides in the 3-days hydrolysate (method *c*). Only relatively small amounts of the β and αβ forms of the aspartyl peptides were present. It also appears that the interconversion reaction is less marked when hydrolysis is carried out by method *b* than when carried out by method *a*. There was no evidence of any inversion of a dipeptide sequence with method (*c*) so that it seems that in general this would be a better method to use if side reactions are to be avoided.

From the identity of the radioautographs of the ionograms of partial hydrolysates of the di*iso*propoxy[^{32}P]phosphinyl derivatives of elastase, chymotrypsin and trypsin, it was concluded that elastase, like the other enzymes, contained the sequence Gly.Asp.SerP.Gly (Hartley *et al.* 1959). This conclusion was based on the assumption that only three peptides (Asp.SerP, SerP.Gly and Asp.SerP.Gly) would be derived from the sequence Asp.SerP.Gly. In fact six to seven identical bands were obtained from the three enzymes. Hence it was assumed that they must have more than three residues in common; Schaffer *et al.* (1957) had shown the presence of Gly.Asp.SerP.Gly in hydrolysates of the type we were using. Clearly in view of the rearrangement reactions of the aspartyl peptides, the above conclusions are no longer valid. In fact in the present work we have shown that the hydrolysates of the three di*iso*propoxy[^{32}P]phosphinyl enzymes have about 20 peptides in common but can only conclude for certain that they all have the sequence Asp.Ser.Gly. Owing to the interconversion reaction and to the presence of mono- and di-*iso*propyl esters, each aspartic acid peptide can occur in nine forms, which greatly complicates the composition of the hydrolysates. It is probable that peptide 8B is Gly.Asp.SerP.Gly, and it seems to be present in the hydrolysates of di*iso*propoxy[^{32}P]-phosphinyl-chymotrypsin, -trypsin and -elastase (Fig. 12), suggesting they have the tetrapeptide sequence in common. However, the amount of this peptide is very small and its structure uncertain so that the suggestion cannot be regarded as conclusive.

The possibility has to be considered whether the aspartyl residue in the intact proteins could be the β or αβ form rather than the normal α form. This seems unlikely since the proportion of the β forms increases with time of hydrolysis and the conversion of a β form into an α form (e.g. 5A → 8A) has not been observed on partial acid hydrolysis of the isolated peptides, whereas the reverse reaction does take place (e.g. 8A → 5A). On the other hand the yields of the β and αβ forms do seem to be unexpectedly high. Table 5 shows the relative yields of bands 8A (α-Asp SerP.Gly), 5A (β-Asp.SerP.Gly) and 12 (αβ-Asp SerP Gly) from a partial hydrolysate of di*iso*propoxy[^{32}P]phosphinyl-chymotrypsin and from a partial hydrolysate of peptide 8A. It can be seen that the relative yields of the β and αβ forms are greater from the protein than from the isolated peptide. This can probably best be explained by assuming that the conversion is more rapid if the α-amino group of the aspartyl residue is blocked as in a larger peptide, so that most of the reaction takes place during the early stages of hydrolysis of the protein and the β forms (e.g. 3 and 5A) are probably produced largely from the β and αβ forms of larger peptides rather than from the corresponding α forms (6A and 8A). In this connexion if 8B is Gly.β-Asp SerP.Gly and 13B its αβ form, there is no evidence of the α form which suggests that the longer period of survival of the larger peptide in the acid has led to its complete conversion. Whereas it seems most likely that the natural form of the aspartyl residue is the α form, the possibility cannot be excluded that it may be partly in the β or αβ form. It seems unlikely that the reaction could be involved in the enzymic activity of the proteins, since it appears to be brought about by acid treatment; however, again the possibility cannot be excluded.

It must, of course, be more than coincidence that the three proteolytic enzymes studied have the sequence Asp Ser.Gly around the reactive serine residue. It seems that there are two possible explanations for this.

Table 5. *Yields of the α, β and αβ forms of Asp.SerP.Gly from di*isopropoxyphosphinyl-chymotrypsin *and from α-Asp.SerP.Gly*

Band no. (Fig. 6)	Relative yield of band in hydrolysate (method *b*, 30 min.): Di*iso*propoxy-[^{32}P]phosphinyl-chymotrypsin	Band 8A
5A	36	12
8A	100	100
12	38	12

One is that the sequence is essential for enzymic activity, in which case some function must be ascribed to the aspartyl and glycyl residues for the catalytic activity, presumably in activating the serine residue. Rydon (1958) has suggested a mechanism involving the aspartyl residue, though it is more generally assumed that the activity of the serine residue is due to its spatial proximity to a specific histidine residue. No function has yet been suggested for the glycyl residue. It could perhaps be steric; for instance, it might be that a larger side chain would sterically hinder the catalytic activity. It seems possible, however, that the similarities in the structures of trypsin and chymotrypsin may extend over a larger part of the molecule (see Šorm *et al.* 1957) and this could hardly be explained on the basis of the catalytic activity. An alternative explanation is in the biological origin of the three proteins and it seems likely that they are in fact 'descended' from a common protein and have developed their specific sequences by independent mutations. Thus it may be that some more primitive organism contained a single protease and that, during the evolutionary process, three enzymes with different specificities (trypsin, chymotrypsin and elastase) have developed from it. Thus one can envisage evolution of proteins as taking place within a species, as well as between species. The nature and course of these changes could probably be revealed by a detailed study of the sequence of these and related proteins. A similar situation is found with the pituitary hormones: vasopressin and oxytocin on the one hand (du Vigneaud, Lawler & Popenoe, 1953) and adrenocorticotrophic hormone and melanophore-stimulating hormone on the other (Harris & Roos, 1956). Here again proteins or peptides from the same organ which have different biological activities have similarities in amino acid sequence and this may be expected to be a general phenomenon as more proteins are studied.

SUMMARY

1. The di*iso*propoxy[^{32}P]phosphinyl derivatives of chymotrypsin, trypsin, and elastase were subjected to partial acid hydrolysis and the products investigated after ionophoretic fractionation.

2. During acid hydrolysis an interconversion of the aspartyl peptides occurs in which the normal α form is converted into the $\alpha\beta$ and β forms.

3. By comparison of the radioactive peptides in the hydrolysates of the di*iso*propoxy[^{32}P]phosphinyl derivatives of the three enzymes it is concluded that elastase, like trypsin and chymotrypsin, contains the sequence Asp.Ser.Gly around its reactive serine residue.

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