Peptides combined with ^{14}C-di*iso*propyl phosphoryl following degradation of ^{14}C-DIP-trypsin with α-chymotrypsin

The first report by JANSEN[1,2] of the specific inhibitory action of DFP* and related organophosphorus compounds upon esterolytic and proteolytic enzymes, with the formation of stable, inactive enzyme derivatives, offered an attractive avenue for the elucidation of the chemical nature of the active sites in these enzymes. SCHAFFER *et al.*[3] were able to show that degradation of DF^{32}P labelled chymotrypsin led to O-serine phosphate on acid hydrolysis, and later isolated labelled di- and tri-peptides with the sequence, aspartic acid (or asparagine), O-serine phosphate and glycine[4]. However, the possibility existed that under the conditions of acid hydrolysis a migration of the DIP* group from its original site of reaction to the hydroxyl of serine occurred. In order to avoid degradation involving acid hydrolysis, OOSTERBAAN *et al.*[5] have used a pancreas preparation (Cotazym) containing a mixture of proteolytic enzymes to degrade DI^{32}P-chymotrypsin and have isolated a DIP-peptide containing one residue each of proline, leucine, aspartic acid (or asparagine) and serine and two or three residues of glycine. The properties of this enzyme system are unusual in that the protein molecule is entirely degraded to amino acids with the exception of the peptide containing the above amino acids and which is combined with DIP.

In our study of the chemical nature of the active site of trypsin, we have used a single proteolytic enzyme, α-chymotrypsin, to degrade DIP-trypsin labelled with ^{14}C in the *iso*propyl groups. The advantages of using a single enzyme for degradation are that its specificity is well established and longer peptide sequences might be expected to result. A disadvantage not seen with cotazym is that a complex mixture of peptides results from the chymotryptic degradation and the fractionation problem becomes correspondingly greater.

DFP was synthesized from 1,3 ^{14}C *iso*propanol by condensation of *iso*propyl iodide with silver monofluorophosphate[6] and possessed a specific activity of 10,390 c.p.m./μmole. Trypsin (2 × crystallized, Worthington) was dialyzed against 0.001 *M* HCl to remove salts, then reacted with ^{14}C DFP, dialyzed and twice recrystallized[7].

The DIP-trypsin was subjected to degradation by α-chymotrypsin (substrate: enzyme ratio 5:1) at pH 7.5 in 0.1 *M* Tris* buffer at 25° C for 24 hours. The progress of degradation was followed by the release of both total phosphorus (analyzed by the FISKE AND SUBBAROW method[8]) and ^{14}C into the supernatant solution, following precipitation of an aliquot of the digest with an equal volume of 30% trichloroacetic acid; the results are given in Table I. The chymotryptic digest was lyophilized at 24 hours and stored as a dry powder at 4° C.

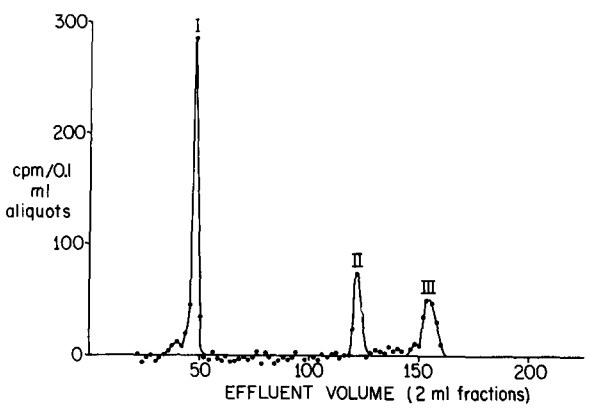

Fig. 1. Elution diagram of the chymotryptic digest of ^{14}C-DIP-trypsin. 300.4 mg of the lyophilized digest was applied to a column (18 × 32 mm) of Dowex-50 × 2 resin in the ammonium form and elution was by an increasing gradient of ionic strength (0.1 *M* ammonium acetate pH 3.2 to 1.0 *M* ammonium acetate pH 5.5). The flow rate was 22 ml/h, and 2 ml fractions were collected.

TABLE I

Digestion time (minutes)	*% Release of total phosphorus into the TCA supernatant*	*% Release of ^{14}C into the TCA supernatant*
0	1.6	0
65	22.7	21.0
180	37.6	35.5
1320	71.4	71.5

Conditions. The digest was carried out at pH 7.5 in 0.1 *M* Tris buffer, 25° C using a 5:1 ratio of ^{14}C-DIP-trypsin to α-chymotrypsin. Precipitation by TCA and phosphorus estimation was as described in the text and the ^{14}C was counted in a gas flow counter after removal of TCA by ether extraction.

* The following abbreviations will be used herein:
DFP = di*iso*propyl phosphofluoridate; DIP = di*iso*propyl phosphoryl-; Tris = tris(hydroxymethyl)aminomethane; DNFB = dinitrofluorobenzene.

The complex hydrolyzate was applied to a column of Dowex-50 × 2 ion-exchange resin in the ammonium form and a gradient elution of the peptide components was carried out (0.1 *M* ammonium acetate pH 3.0 to 1.0 *M* ammonium acetate pH 5.5); the effluent fractions were assayed for radioactivity by plating 0.2 ml aliquots (using lens paper discs to ensure uniform area). The elution diagram is shown in Fig. 1; three radioactive peaks were obtained and are labelled I, II and III, respectively. The fractions comprising each radioactive peak were pooled and taken to dryness *in vacuo*, the ammonium acetate buffer being removed by vacuum sublimation at 40° C[9].

Peak I was suspected to be free di*iso*propyl phosphate (DIP) and this was confirmed by paper chromatography with an authentic sample of DIP in ethanol: 0.1 *M* ammonium acetate, pH 7.5, solvent. The ^{14}C in peak I was quantitatively accounted for as DIP by elution of the DIP spot from the chromatogram.

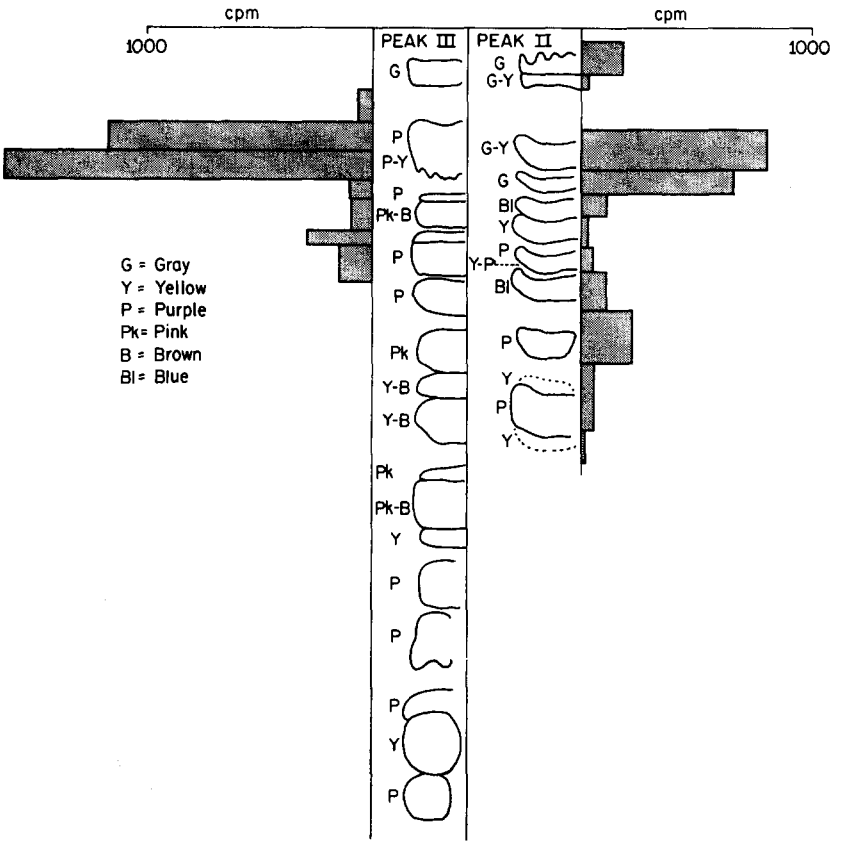

Fig. 2. Tracing of a ninhydrin-stained guide strip of a chromatogram (Whatman #3 paper developed in butanol:acetic:water) of peaks II and III. The radioactivity of material corresponding to each spot and eluted from horizontal sections of the unstained chromatogram is also plotted.

Aliquots of peaks II and III were chromatographed on Whatman #3 paper in butanol: acetic acid:water (4:1:5) for 12–13 hours. On spraying a guide strip with ninhydrin (0.4% in 95 ml of 90% *iso*propanol/water + 5.0 ml collidine) to visualize peptides[10], 10–15 ninhydrin positive spots were seen in each case. The remainder of the chromatogram corresponding to each spot was eluted chromatographically with water and its radioactivity determined; the results are seen in Fig. 2. The major radioactive peptide fractions were rechromatographed on Whatman #3 and developed for 36 hours in butanol:acetic acid:water. Peak II now showed a single ninhydrin reactive spot (grey) and peak III showed two spots, one purple and a faster running

yellow spot. Strips of the chromatogram corresponding to these spots were eluted as above and the grey spot from peak II and the purple spot from peak III were found to be radioactive.

Each peptide was reacted with DNFB* in triethylamine[11] and hydrolyzed in 5.7 *N* HCl for 16 hours at 105° C. The ether soluble DNP amino acid fractions were examined by two-dimensional chromatography[12] but no N-terminal group could be determined in either case. The water soluble fractions of each hydrolysate were examined by two-dimensional paper chromatography (butanol:acetic acid:water followed by phenol:cresol:borate buffer[12]) and the following amino acids were found:

Peak II – cystine, aspartic, glutamic, glycine, serine, alanine, proline and valine.

Peak III – cystine, aspartic, glutamic, glycine, serine, alanine, proline, valine and ε-DNP-lysine. The water soluble ε-DNP lysine spot from peak III was eluted and its concentration determined by its absorption at 350 mμ. It was found to be stoichiometric with the radioactivity indicating a 1:1 ratio between DIP group and ε-DNP lysine. The concentration of amino acids was too small for quantitative estimation but a visual examination of the chromatograms indicated that only glycine was present in greater than molar ratio (probably 2 molecules).

If it is assumed, as seems probable, that the same peptide sequence occurs in both peptides II and III, then the position of the lysine in peptide III must be either N- or C-terminal, but since reaction with DNFB gives rise to ε-DNP lysine rather than α,ε-bis-DNP lysine, the latter position is indicated. This focuses attention upon the presence of a peptide with C-terminal lysine in a chymotryptic digest. Since the DIP-trypsin subjected to degradation was totally inactive by the esterase determination[13] and since the peptide bond formed by a lysine carboxyl group is not susceptible to chymotryptic attack, the possibility exists that a C-terminal lysine is present in the intact trypsin molecule. This has been suggested by other studies in this laboratory using carboxypeptidase[14].

The lack of a demonstrable N-terminal residue by the DNP method is not inconsistent in view of the known lability to acid hydrolysis of the DNP derivatives of glycine, serine and half-cystine.

Further work is in progress to elucidate the sequence of amino acids in these peptides, and to explore other controlled enzymic degradations of DIP-trypsin. In view of recent evidence which suggests that DFP inhibition may be a two-phase process, the primary reaction being the phosphorylation of the imidazole side chain of a histidine residue followed by a secondary shift of the DIP group to serine hydroxyl[15,16], particular attention will be paid to the rapid degradation of freshly inhibited trypsin.

Our thanks are due to Miss DOROTHY KAUFFMAN for technical assistance. This work has been carried out in part, under Contract No. Nonr-477-04 between the University of Washington and the Office of Naval Research, Department of the Navy.

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[1] E. F. JANSEN, M. D. FELLOWS NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 189.
[2] E. F. JANSEN, M. D. FELLOWS NUTTING AND A. K. BALLS, *J. Biol. Chem.*, 179 (1949) 201.
[3] N. K. SCHAFFER, S. C. MAY AND W. H. SUMMERSON, *J. Biol. Chem.*, 206 (1954) 201.
[4] N. K. SCHAFFER, S. HARSHMAN, R. R. ENGLE AND R. W. DRISKO, *Federation Proc.*, 14 (1955) 275.
[5] R. A. OOSTERBAAN, P. KUNST AND J. A. COHEN, *Biochim. Biophys. Acta*, 16 (1955) 299.
[6] B. C. SAUNDERS AND G. J. STACEY, *J. Chem. Soc.* (1948) 695.
[7] L. W. CUNNINGHAM, *J. Biol. Chem.*, 211 (1955) 13.
[8] C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
[9] C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
[10] P. N. CAMPBELL AND T. S. WORK, *Brit. Med. Bull.*, 10 (1954) 196.
[11] F. SANGER AND E. O. P. THOMPSON, *Biochem. J.*, 53 (1953) 353.
[12] H. FRAENKEL-CONRAT, J. IEUAN HARRIS AND A. L. LEVY, in *Methods of Biochemical Analysis*, edited by D. GLICK, Interscience Pubs., New York, Vol. II, 1955, p. 359.
[13] G. W. SCHWERT, H. NEURATH, S. KAUFMANN AND J. E. SNOKE, *J. Biol. Chem.*, 172 (1948) 221.
[14] E. DAVIE AND H. NEURATH, *J. Am. Chem. Soc.*, 74 (1952) 6305.
[15] B. J. JANDORF, H. O. MICHEL, N. K. SCHAFFER, R. EGAR AND W. H. SUMMERSON, *Discussions Faraday Soc.* (1955), in press.
[16] I. B. WILSON, *Discussions Faraday Soc.* (1955), in press.

Received November 2nd, 1955