by the short influence of alkali, which was used for the purification of this protein. Also the higher rates of release of amino acids from the Hb-B and Hb-C may be explained by a denaturation more or less of these proteins caused by their being kept longer before the experiment was carried out, since they were sent to us from the Netherlands Antilles (Curaçao).

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The chemical structure of the reactive group of esterases

It is well known that a number of esterases are able to combine with $DF^{32}P^*$ to yield $D^{32}P$-proteins. At a recent symposium of the Faraday Society[1] it was reported that on mild enzymic hydrolysis by means of a polyvalent proteolytic pancreas preparation (cotazym) essentially one single radioactive $D^{32}P$-peptide can be obtained from each of these $D^{32}P$-proteins. It was assumed that these peptides would contain all or part of the original enzyme active site bound to the DP-radical and that therefore their analysis would provide valuable information on the chemical structure of this biologically very important site. Preliminary results were reported on DP-peptides prepared from the following esteratic proteins: true red cell cholinesterase, red cell ali-esterase, serum pseudo-cholinesterase, liver ali-esterase, α-chymotrypsin and trypsin. All peptides behaved in a very similar way during various electrophoretic and chromatographic procedures. The peptides obtained from DP-α-chymotrypsin, DP-trypsin and DP-liver-ali-esterase were examined in more detail; on acid hydrolysis they produced the amino acids reported in Table I (nos. 1, 2 and 3) in addition to DP. The sequence of the amino acids in the DP-peptide from chymotrypsin, reported earlier[1,2], was as follows: Gly. Asp. Ser. Gly. [Pro, Leu, (Gly)]. It will be noted that the composition of the trypsin peptide is in good agreement with the larger peptide recently isolated by DIXON *et al.*[3] by α-chymotrypsin hydrolysis of DP-trypsin.

In the present paper experiments are described which confirm and extend the preliminary results reported above. This was made possible by the development of new isolation techniques which will be described.

First optimal conditions for the cotazym hydrolysis at 37° C were established by running samples, taken at intervals from the incubation mixture, on a paper chromatogram using b.a.w. (butanol–acetic acid–water 4:1:5) as a solvent. Thus the time and pH necessary to split off the $D^{32}P$-peptides in optimal yield from the corresponding $D^{32}P$-proteins was found. The bulk of the incubation mixture was then hydrolysed accordingly. In the case of the $D^{32}P$-chymotrypsin the peptide was then isolated in the pure state as follows. The incubation mixture was first deproteinised by precipitation with alcohol 70% (v/v). The supernatant was concentrated *in vacuo* and chromatographed on a Dowex-50 column (8% cross-linkages 200–400 mesh) at pH 5.0 (Na-acetate buffer 0.1 *M*). The radioactive substance passed rapidly together with the acid amino

The following abbreviations will be used: DFP = di*iso*propylphosphorofluoridate; DP-, DP = di*iso*propylphosphoryl- and di*iso*propylphosphate respectively; DNFB = dinitrofluorobenzene; DNP- = dinitrophenyl-.

TABLE I

ACID HYDROLYSIS OF DP-PEPTIDES

	α-Chymotrypsin	*Trypsin*	*Ali-esterase*	
	1	*2*	*3*	*4*
DP	1	1	1	1
Glycine	2–3	2–3	2	1–2
Alanine	—	—	1	1
Aspartic acid – asparagine	1	1	—	—
Glutamic acid – glutamine	—	—	1	1–2
Serine	1	1	1	1
Proline	1	1	1	—
Leucine-*iso*leucine	1	—	1	—
Valine	—	1	—	—

acids through the column. The radioactive fraction of the eluate was run through again at pH 3.75 (Na-acetate buffer 0.1 *M*) followed by elution at pH 5.0 as before. From the eluate a fraction could be obtained which on column zone electrophoresis (Na-acetate 0.05 *M*; pH 4.60) yielded the $D^{32}P$-peptide described in Table I in the pure form. A single DNP-derivative resulted from treatment of the peptide by DNFB by Sanger's method, thus proving its purity. As before, glycine was found to be the N-terminal residue.

The $D^{32}P$-peptide from purified[4] liver ali-esterase was isolated from the cotazym incubation mixture by chromatography on the cation exchanger Amberlite IRC50 (120–200 mesh). Acetic acid at pH 4.9 was used as the elution fluid. This procedure resulted in a single radioactive peptide contaminated by the following amino acids: tryptophane, leucine and phenylalanine. These could be removed by paper chromatography in b.a.w. followed by a run in b.w. (butanol-water). Paper electrophoresis (Na-acetate 0.1 *M*; pH 4.6) resulted in the final pure peptide the amino acid content of which is shown in Table I, No. 3. From $D^{32}P$-liver-ali-esterase a second peptide could be obtained by pepsin hydrolysis (pepsin concentration 0.01 %; pH 1.5–2.0; temp. 37° C, incubation period 4 hours). Purification was now effected by chromatography on IRC50 (pH 3.0) and paper electrophoresis as described above, followed by paper chromatography in b.a.w. and phenol NH_3. This procedure yielded a peptide which could be transformed by Sangers DNFB method into a single DNP-derivative, thus proving its purity. The peptide consisted of a $D^{32}P$ radical in 1:1 relationship to the dinitrophenyl group and in addition a number of amino acids as described in the table (No. 4).

It is clear (Table I) that all esterases so far investigated produced chemically very similar DP-peptides. This result gives strong support to the hypothesis that the peptides isolated represent at least part of the enzyme-active site of the enzyme concerned. It seems reasonable to assume that the similarity observed is related to a similar chemical site on each protein, capable of esterolysis and combination with DFP. MASSEY *et al.*[5] have suggested that esterases might have a group concerned with binding the substrate to the active centre and another one (the DFP-reacting site), which is necessary for the activation of the enzyme substrate complex. It is suggested that our peptides are related to the latter group.

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