[1] K. LINDERSTRØM-LANG, *Proceedings of the 9th Solvay Congress* (Brussels 1953) 247.
[2] A. V. GÜNTELBERG, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1954) 27.
[3] A. V. GÜNTELBERG AND M. OTTESEN, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 29 (1954) 36.
[4] C. F. JACOBSEN AND JOSÉ LÉONIS, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, 27 (1951) 333.
[5] M. KUNITZ, *J. Gen. Physiol.*, 24 (1940) 15.
[6] C. B. ANFINSEN, *J. Biol. Chem.*, 196 (1952) 201.
[7] A. TISELIUS AND I. B. ERIKSSON-QUENSEL, *Biochem. J.*, 33 (1939) 1752.
[8] C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
[9] D. M. BROWN, C. A. DEKKER AND A. R. TODD, *J. Chem. Soc.*, (1952) 512.
[10] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
[11] L. VANDENDRIESSCHE, *Acta Chem. Scand.*, 7 (1953) 699.
[12] G. PERLMANN, *Nature*, 173 (1954) 406.
[13] R. R. PORTER, *Biochem. J.*, 53 (1953) 320.

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The nature of the reaction between diisopropylfluorophosphate and chymotrypsin

JANSEN *et al.*[1,2] were the first to study the inhibition of chymotrypsin using ^{32}P labeled diisopropylfluorophosphate ($DF^{32}P$). They showed that one mole $DF^{32}P$ was bound per mole of active enzyme, that a fluorine ion was released during the reaction and that the reaction product consisted of the original protein with a diisopropylphosphate (DIP) rest[3]. Moreover it was demonstrated by SCHAFFER *et al.*[4,5] that this reaction product yielded O-serinephosphate on acid hydrolysis.

In the course of our study of the pharmacological action of organophosphates we became interested in the character of the bond resulting from the reaction of DFP on mammalian cholinesterase[6]. In view of the limited supply of purified cholinesterase available at the time, we decided first to obtain more basic information about the inhibition of crystalline chymotrypsin by $DF^{32}P$. It was assumed that this inhibition could serve as a satisfactory model for the corresponding reaction on cholinesterase. This hypothesis was supported by the fact that other esterases could be demonstrated to produce serinephosphate on acid hydrolysis. This was shown by SCHAFFER *et al.*[7] for electric eel cholinesterase, and by us for purified bovine cholinesterase, serum pseudo-cholinesterase and stroma ali-esterase[8]. SCHAFFER *et al.*[5] have repeatedly stressed that these findings do not necessarily mean that the primary point of attack of DFP on the chymotrypsin molecule would involve the hydroxyl group of a serine molecule. The isolated serinephosphate might be produced during and as a result of the acid hydrolysis. It appeared to us that breakdown of chymotrypsin-DFP by enzymic methods, avoiding the acid hydrolysis, might yield a product which on analysis would provide information about the primary side of reaction of DFP.

For the breakdown of the chymotrypsin-DIP a polyvalent commercial pancreatic enzyme preparation was used*. This preparation was able to split a number of proteins, including chymotrypsin, into amino acids. It seems reasonable to assume on the basis of the specificity of proteolytic enzymes, that the presence of an unusual group in the protein molecule (like the DIP group in the chymotrypsin-DIP) might interfere with the normal breakdown of such a protein. Thus a peptide might result, containing this unusual group (in the present case the ^{32}P labeled DIP) in addition to a number of amino acids. Hydrolysis of chymotrypsin-DIP by cotazym indeed produced a single peptide which contained 50 to 60% of the original DIP group. The isolation of this peptide from the hydrolysate could be effected by distribution between water and phenol, followed by paper chromatography in butanol-acetic acid-water and butanol-water, or better still by zone electrophoresis on a starch column[9] at pH 4.6, followed by paper chromatography in butanol-acetic acid-water.

The isolated peptide had the following properties:

1. It was stable against hydrolysis by cotazym.

2. It moved rapidly towards the anode on the starch column, suggesting a high acidity (oddly it hardly moved on paper electrophoresis).

3. On alkaline treatment (pH 11.0) and heating at 100° C for 5 minutes all labeled phosphorus was split off as DIP, indicating that the labeling group was still present as DIP.

4. On acid treatment (pH 2.0) at 100° C the peptide did not produce DIP and yielded serinephosphate on continued acid hydrolysis with 2 *N* HCl (15 h 100° C).

5. Per DIP group the original peptide was shown to consist of the following amino acids: proline (1), leucine (1), aspartic acid (1), serine (1), and glycine (2 or 3).

The sequence phosphoseryl-glycine, demonstrated by SCHAFFER *et al.*[10], could obviously occur in the present peptide. We do not know yet where the DIP group is situated, but in view of the mild treatment we applied, it seems reasonable to assume that this localisation will not be different

from the original point of attack of DFP on the protein. Further work is under way to elucidate the sequence of the amino acids in the isolated peptide, the localisation of the DIP group and the chemical composition and properties of the peptide resulting from the alkaline treatment.

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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] E. F. JANSEN, M. D. FELLOWS NUTTING, R. JANG AND A. K. BALLS, *J. Biol. Chem.*, 185 (1950) 209.
[4] N. K. SCHAFFER, S. C. MAY AND W. H. SUMMERSON, *Federation Proc.*, 11 (1952) 282.
[5] N. K. SCHAFFER, S. C. MAY AND W. H. SUMMERSON, *J. Biol. Chem.*, 202 (1953) 67.
[6] J. A. COHEN, R. A. OOSTERBAAN AND M. G. P. J. WARRINGA, *Résumés des comm. II Congrès Intern. Biochimie*, Paris 1952, 231.
[7] N. K. SCHAFFER, S. C. MAY AND W. H. SUMMERSON, *J. Biol. Chem.*, 206 (1954) 201.
[8] J. A. COHEN, R. A. OOSTERBAAN AND M. G. P. J. WARRINGA, *Arch. Int. de Physiol.*, 62 (1954) 574.
[9] P. FLODIN AND J. PORATH, *Biochim. Biophys. Acta*, 13 (1954) 175.
[10] N. K. SCHAFFER, S. HARSHMAN AND R. R. ENGLE, *Federation Proc.*, 13 (1954) 289.

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* Cotazym Organon Oss (Holland).

Purification of cholinesterase from ox red cells

In a previous communication[1] a method was reported for the preparation of soluble cholinesterase from ox red cells presenting a 250–400 fold purification. These preparations still suffered from various disadvantages; they were still of limited purity and had a tendency to lose solubility particularly after freeze drying.

A new method will be described which yields preparations approx. 10 times as pure as that of the previous ones which can be satisfactorily freeze dried without loss of solubility.

Analytical methods. As described previously[1].

Procedure. Stroma was prepared as before[1]. Instead of being freeze dried it was now added to one l of phosphate buffer 0.02 *M* pH 8.0 containing 20 g NaCl and 2 ml cotazym (a pancreatic extract purchased from N.V. Organon, Oss; 3 tablets are dissolved in 22 ml of distilled water and centrifuged to remove insoluble material). This mixture is adjusted to pH 8.0 with NH_4OH 25% and after vigorous shaking incubated for 16 hours at 37° C. After incubation the pH is readjusted to 8.0 and the mixture centrifuged in the Servall vacuum centrifuge SS2 (approx. 14,000 rpm). The supernatant is saturated to 0.7 with ammonium sulphate and allowed to stand at 5° C for 1 hour. After centrifugation in the Servall centrifuge the precipitate is dissolved in distilled water. This solution is dialysed overnight against three different lots of distilled water at pH 7.0. The dialysate is centrifuged and the supernatant adjusted to pH 6.2. Alcohol is added to a concentration of 9% *v/v* at —2° C. The precipitate is discarded by centrifugation at 2,000 rpm at —2° C. The alcohol concentration of the supernatant is increased to 32.8% *v/v* at —5° C. The precipitate is dissolved in 100 ml phosphate buffer pH 7 (0.01 *M*).

To samples of one ml of the solution calcium phosphate gel[2] is added until the supernatant on centrifugation is optimal as regards enzyme concentration and purity. Usually two volumes of the gel have to be added in two stages. The pH should be kept at 7.0. The bulk is then treated with calcium phosphate gel according to the results obtained on the sample. The gel is separated by centrifugation and the supernatant saturated to 0.8 with ammonium sulphate at pH 6.5. After standing overnight at 5° C the precipitate is spun down and dissolved in 0.01 *M* phosphate buffer (pH 7.0), dialysed and freeze dried.

Preparations with an average activity of 100,000–350,000 units per mg N were obtained.

To our knowledge the mammalian cholinesterase prepared in this way is considerably purer than any reported in the literature or commercially available.

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[1] J. A. COHEN AND M. G. P. J. WARRINGA, *Biochim. Biophys. Acta*, 10 (1953) 195.
[2] D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc.*, 124 (1938) 399.

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