TABLE I

INCORPORATION OF [^{35}S]CYSTINE INTO VASOPRESSIN

Expt.	Time of infusion h	Posterior pituitary		Hypothalamus		Ratio of specific activities H.P
		Total units	Counts/min/unit	Total units	Counts/min/unit	
1*	16	16.0	8.0	3.2	23.0	2.9
2*	24	19.0	14.2	1.6	28.4	2.0
3*	36	13.0	9.7	2.7	31.1	3.2

* Specific activities have been corrected for dilution by carrier and radioactive decay; they represent the mean values for the purification steps outlined (see text) *e.g.* Expt. 2, post. pit. spec. act. after XE-64, 15.4; paper ionophoresis, 15.3; rechromatography on carboxymethylcellulose and dividing the biologically active peak into 3 fractions, 13.5, 12.6, 15.0; Expt. 3, post. pit. gave spec. activities of 11.0, 9.8, 11.4, 7.5, 9.0 respectively; Expt. 1 given as counts/min/2.5 μg (approx. 1 unit vasopressin) of oxidized vasopressin eluted from paper after ionophoresis and measured colorimetrically by the FOLIN procedure[10].

biosynthesis of vasopressin both *in vivo* and *in vitro* as well as to an investigation of the intracellular sites of synthesis and storage of this hormone.

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Chemical nature of the DFP-binding site of pseudocholinesterase

DFP is known to phosphorylate a number of esterases and proteases at their enzymically active sites. Information on the chemical nature of the active sites of these enzymes has been derived from degradation of the DFP-inhibited enzymes and analysis of the P-containing peptides produced.

In the present note we report the analysis of a P-peptide obtained from DFP-inhibited pseudocholinesterase. An enzyme preparation, obtained from about 800 l

horse serum[1], corresponding to $6 \cdot 10^5$ units enzyme activity and purified 1000 times as compared with the crude serum, was completely inhibited by DFP (7 mg [^{32}P]DFP at 20° and pH 7.0 for 1 h; vol., 690 ml). Following dialysis against water to remove the excess of DFP it was found that 6.9 µmoles ^{32}P were bound per 17 g protein. This P-enzyme preparation was fractionated using $Ca_3(PO_4)_2$ gel adsorption followed by ethanol precipitation to obtain 2.3 g protein with 4.6 µmoles bound ^{32}P.

The P-protein (1 % solution) was digested with pepsin (0.005 %) at 37° and pH 2.0 for 6.5 h. At the end of this period the solution was neutralized and residual proteins were removed by precipitation with ethanol (80 %). The peptide mixture was subjected to chromatography on 200–400 mesh Dowex-50 X4 (H^+) (3 × 11 cm column operated with 0.05 *M* acetic acid, pH 3.1). Radioactive eluate fractions were pooled and lyophilized. The resulting material was re-chromatographed on Dowex-50 X4 (1.2 × 60 cm column at pH 3.1). The eluate was collected in 10-ml fractions. The fractions 28–39 contained a ^{32}P-peptide (yield, 2.0 µmoles). This peptide was homogeneous on paper chromatography in several solvents and on paper electrophoresis (pH 3.6 and 6.5) providing evidence for its purity. Moreover NH_2– end-group determinations of the P-peptide with FDNB revealed the presence of one free NH_2–group per phosphoryl group.

The peptide yielded the following amino acids on total hydrolysis (6 *N* HCl at 110° for 16 h): alanine (3), glutamic acid (1), glycine (2), phenylalanine (1) and serine (2) in addition to P (1) (by quantitative amino acid analysis with ninhydrin using paper chromatography). The structure of the P-peptide could be partially established using the EDMAN degradation technique as described by FRAENKEL CONRAT *et al.*[2]. PTH's obtained were identified as such by paper chromatography with the solvent F of EDMAN AND SJÖQUIST[3] and also as the parent amino acids regenerated from the PTH's on hydrolysis (6 *N* HCl at 150° for 16 h). Upon completion of each degradation cycle the shortened P-peptide was purified by electrophoresis (pH 3.6, 60 V/cm for 2 h). The degradation technique was applied on 1.0 µmole of the P-peptide. Successively, phenylalanine, glycine and glutamic acid were found indicating the N-terminal sequence Phe·Gly·Glu for the P-peptide. The shortened P-peptide obtained after three successive degradations was coupled with FDNB for identification of the fourth amino acid and further sequential analysis. A small amount of the DNP-peptide was heated with 2 *N* HCl at 105° for 3 h. N-DNP-serine phosphate could be identified in the hydrolysate by co-chromatography with synthetic N-DNP-serine phosphate in several solvents, as described previously[4]. The bulk of the DNP-peptide was partially hydrolyzed (12 *N* HCl at 35° for 16 h). Essentially only two radioactive DNP-derivatives were obtained. These were extracted from the solution at pH 1.0 with methyl acetate and separated by paper chromatography in BAW (R_F's, 0.50 and 0.60). The DNP-derivative at R_F 0.60 produced alanine on total hydrolysis indicating the sequence DNP–Ser·Ala (with P attached to Ser). The other DNP-derivative (R_F, 0.50) produced glycine and alanine in an approximately 1:1

Abbreviations: DFP, diisopropyl phosphorofluoridate; P–, phosphoryl group which is bound to the enzyme after reaction with DFP; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP–, 2,4-dinitrophenyl; PTH, phenylthiohydantoin derivatives; BAW, butanol–acetic acid–water (4:1:5); DP–, diisopropyl phosphoryl; MP–, monoisopropyl phosphoryl; DIP, diisopropyl phosphate; MIP, monoisopropyl phosphate.

ratio on total hydrolysis; hence it represents the tripeptide DNP-Ser(P)·Ala·Gly. These results establish the structure of the P-peptide as follows: Phe·Gly·Glu·Ser(P)·Ala·Gly·(Ala$_2$, Ser)*. Part of this amino acid sequence viz. Gly·Glu·Ser·Ala·Gly is identical with the sequence in the DFP-binding site of horse-liver aliesterase[1] and strikingly similar to the sequence Gly·Asp·Ser·Gly in the DFP-binding site of the proteolytic enzymes thrombin[5], chymotrypsin[6,7] and trypsin[8]. Most striking in all these structures is the presence of the common sequence dibasic amino acid-serine. Recently[9] we have discussed how a serine-OH and a COO^- group from a dibasic amino acid might be functionally involved in the reaction of these enzymes with their substrates or DFP.

The P-peptide of pseudocholinesterase carried a MP-group instead of the DP-group found in the P-peptides from DFP-inhibited chymotrypsin[6], trypsin[8] and aliesterase[1]. This could be shown by heating a sample of the P-peptide at 100° and pH 12.0 (NH_4OH) for 10 min. All ^{32}P was released from the ^{32}P-peptide as MIP, identified by paper chromatography in phenol-water, BAW and by electrophoresis (pH 3.6, 60 V/cm for 30 min). It is very unlikely that MIP is formed from a DP-substituent on alkaline treatment of the P-peptide since all other P-peptides produce DIP under the experimental conditions employed. The presence of MP in the peptide is compatible with its acidic nature. Also the parent P-pseudocholinesterase produced MIP on alkaline treatment. A freshly prepared DFP-inhibited pseudocholinesterase preparation, however, produced a mixture of DIP and MIP on alkaline treatment. These findings suggest that pseudocholinesterase reacts with DFP in the usual way to form the DP-enzyme which is then spontaneously converted into an MP-enzyme. It has been shown in this laboratory that this conversion of DFP-inhibited pseudocholinesterase forms the chemical basis of its "ageing" viz. conversion of a reactivatable into a non-reactivatable inhibited enzyme[10].

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* It has not yet been determined whether the P-peptide contains tryptophan.