The chemical basis of the "ageing process" of DFP-inhibited pseudocholinesterase

Esterases inhibited with DFP and analogous compounds may be reactivated by means of nucleophilic agents like hydroxamic acids and oximes. In the case of cholinesterases the inhibited enzyme is converted from a reactivatable into a non-reactivatable form upon storage ("ageing")[1-3].

The results reported by JANSZ *et al.*[4] suggested that the DP-group introduced into PsChE by reaction with DFP is converted into the MP-group spontaneously. This reaction might give a satisfactory explanation for the loss of reactivatability of the inhibited enzyme. We now present the results of some investigations made to confirm the occurrence of this conversion and to find a relation with the ageing process.

Horse serum PsChE, purified according to a modified STRELITZ procedure[5] (specific activity, 580 units/mg N) was completely inhibited with [^{32}P]DFP[6] (concn. of enzyme, 180 units/ml; DFP, $2 \cdot 10^{-5}$ *M*; 75 min incubation at 0° in 0.06 *M* veronal, pH 8.6). The inhibited enzyme was dialyzed against distilled water at 0–2° until free from non-protein-bound ^{32}P-compounds (25 h) and subjected to ageing by storage at 24°. At intervals two samples were taken simultaneously; one was heated at pH 12.6 (NaOH) for 10 min at 100° in a sealed Pyrex tube to release the phosphoryl group from the protein, the other was incubated with PAM (0.1 *M*, pH 7.4 at room temperature) to accomplish reactivation.

The alkaline-treated samples were subjected to paper chromatography (BAW). Autoradiography of the chromatograms revealed the presence of two radioactive compounds which were identified as DIP (R_F, 0.81) and MIP (R_F, 0.42) by comparison with reference compounds. The amounts were calculated from radioactivity measurements. In the sample taken immediately after dialysis almost all the ^{32}P was accounted for as [^{32}P]DIP while in the subsequent samples an increasing fraction of the ^{32}P was found as [^{32}P]MIP. The results summarized in the columns A and B of Table I are consistent with a conversion of DP- into MP-enzyme.

In the samples incubated with PAM the reactivation was determined by comparing the restored enzymic activity with the activity of uninhibited but otherwise identically treated samples of the enzyme (titration in $5 \cdot 10^{-4}$ *M* phosphate, pH 7.4, 0.02 *M* acetyl choline with 0.01 *N* NaOH at 25°). After 4–6 days of incubation optimal reactivation was achieved; at this stage part of each sample was chromatographed in BAW, the remainder was treated as indicated below. Autoradiograms showed two radioactive spots on the chromatograms, protein-bound ^{32}P on the start and [^{32}P]DIP. The percentage of the ^{32}P present in the form of [^{32}P]DIP corresponded with the percentage of reactivation obtained in the same sample (Table I, columns C, D and E). This result shows that upon reactivation a DP– group is released from the inhibited enzyme with simultaneous restoration of the enzymic activity.

The remainder of the PAM-containing samples (see above) were treated with NaOH (10 min, pH 12.6, 100°) to establish the nature of the protein-bound ^{32}P

Abbreviations: PsChE, pseudocholinesterase; DFP, diisopropyl phosphorofluoridate; DP–, diisopropyl phosphoryl–; MP–, monoisopropyl phosphoryl–; DIP, diisopropyl phosphate; MIP, monoisopropyl phosphate; PAM, pyridine-2-aldoxime methiodide; BAW, butanol–acetic acid–water (4:1:5).

TABLE I

PROPERTIES OF [^{32}P]DFP-INHIBITED PSEUDOCHOLINESTERASE IN RELATION TO AGEING

[^{32}P]DFP-inhibited PsChE was dialyzed at 0° and stored at 24°. At the times indicated two samples were taken; one was subjected to alkaline hydrolysis, the other was incubated with PAM for 4–6 days. After this period part of the latter sample was hydrolyzed with alkali. See text for further details.

Time of ageing at 24° (h)	Alkaline treatment. All ^{32}P released		PAM incubation			PAM incubation followed by alkaline treatment. All ^{32}P released	
	as [^{32}P]DIP	as [^{32}P]MIP	Restored enz. act.	^{32}P released as [^{32}P]DIP	^{32}P remaining on the enzyme	as [^{32}P]DIP	as [^{32}P]MIP
	(A) %	(B) %	(C) %	(D) %	(E) %	(F) %	(G) %
0	95	5	87	94	6	93	7
1	88	12	77	79	21	82	18
2	76	24	63	72	28	71	29
3	67	33	—	—	—	—	—
4	59	41	47	53	47	51	49
6	44	56	36	40	60	38	62
8	33	67	26	29	71	26	74

substituent. The solutions were acidified (pH 3) and percolated through a column containing 2.5 ml of IRC 50 (H^+) on top of 2.5 ml of Dowex 50 X4 (H^+) ion-exchange resin to remove proteins and cations. The radioactive eluate fractions were concentrated and chromatographed (BAW). Two ^{32}P compounds were found which were identified as [^{32}P]DIP and [^{32}P]MIP. For each sample the percentages of [^{32}P]DIP and [^{32}P]MIP were equal to the percentages of [^{32}P]DIP and protein-bound ^{32}P found before the alkaline treatment (Table I, columns D, E, F and G) indicating that the phosphoryl group refractory to the oxime is a MP– group.

It will be seen from the figures in the columns A, C and D of Table I that all DP-enzyme present in the mixture of DP- and MP-enzyme at the start of the PAM incubation is reactivated. This means that the conversion of DP- into MP-enzyme is terminated completely by the presence of PAM. In similar experiments carried out with isonitrosoacetone as a reactivator (0.1 *M*, pH 7.4) only a slight retardation of the ageing process was found. These results suggest that the protection against ageing observed with PAM is due to the quaternary ammonium structure of the oxime. The effectiveness in this respect of quaternary ammonium compounds in general is under investigation.

The fate of the isopropyl group which is removed from the phosphoryl group during the conversion of DP- into MP-enzyme was studied in experiments with PsChE inhibited with [^{14}C]- and [^{32}P]DFP. It was found that upon ageing the ^{14}C content of the protein (measured in washed trichloroacetic acid precipitates) gradually decreased while the ^{32}P content remained constant. Free [^{14}C]isopropanol could be demonstrated in the supernatant of the precipitates.

From our experiments it may be concluded that the reaction of PsChE with DFP results in a DP-substituted enzyme, which may be reactivated by oximes; upon storage this DP-enzyme is converted into the non-reactivatable MP-enzyme by the release of isopropanol.

Our results are not in favour of the conception of JANDORF *et al.*[2] and of DAVIES AND GREEN[7], who suggested that ageing is due to a migration of the phosphoryl group

in the inhibited enzyme from the imidazole-N of a histidine residue to the OH of a serine residue.

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Received May 8th, 1959

An extreme example of the coding problem, Avian PPLO 5969*

A problem in the relation of protein to DNA is whether or not a given nucleotide sequence uniquely corresponds to a given protein molecule. This equivalence relation may be termed one to one coding. Among the alternative possibilities would be overlap coding where a segment of DNA may encode portions of the peptide sequence of several different proteins. An approach to this problem is to find an organism where the DNA code is short enough to begin to distinguish between some of the possible hypotheses.

In an attempt to find an organism where the amount of DNA presents a coding limitation to the number of possible proteins, we have directed our attention to small cells capable of reproduction on a non-living medium. While a number of small bacteria have been looked at, the most extreme case we have encountered is the pleuropneumonia-like organism Avian 5969[1].

The individual cells are spherical and in the electron microscope show a diameter of 0.25 μ. This extremely small size has the following consequences. The cell has a volume of $8.2 \cdot 10^{-5}$ ml, and assuming a 75% water content has a dry weight of $2.0 \cdot 10^{-15}$ g, which corresponds to a molecular weight of $1.2 \cdot 10^{9}$. Or from a somewhat different point of view we see that we have a cell which contains in the non-aqueous portion the order of 200,000,000 atoms.

We have recently carried out DNA measurements on this type of cell in the following manner. A large culture grown on a medium of Difco Tryptose Broth plus 1% Difco PPLO Serum Fraction is centrifuged and washed. Portions are taken for dry-weight determination, while other portions are first extracted in 75% ethanol and then in 5% trichloroacetic acid (100° for 45 min). The precipitate is centrifuged out and DNA determinations are carried out on the trichloroacetic supernatant by

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

* PPLO indicates pleuropneumonia-like organism. This strain is also known as *Mycoplasma gallisepticum*.