THE INHIBITION OF PURIFIED, HUMAN PLASMA CHOLINESTERASE WITH DIISOPROPYL FLUOROPHOSPHATE*

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Previous communications from this Laboratory have shown that the inhibition of α-chymotrypsin by diisopropyl fluorophosphosphate (DFP) is a stoichiometric reaction that comprises the introduction of a single diisopropyl phosphate group into the molecule of α-chymotrypsin, the elimination of fluorine as HF, and the production of an undenatured, crystallizable, inhibited enzyme protein (1–3). Several substances analogous to DFP in composition were found to inhibit α-chymotrypsin in a similar manner. In each case the resulting inhibited protein was obtained in crystalline form and found to contain one phosphate group per mole (4). This reaction, leading to the inhibition of α-chymotrypsin, is therefore not confined to DFP.

There is also evidence that other esterases react with DFP in the same way as α-chymotrypsin. Thus β- and γ-chymotrypsins behave toward DFP in quite the same manner as the α enzyme (5) (which may not be astonishing in view of their general similarity); however, DFP also reacts with trypsin to produce a crystallizable, inhibited trypsin whose equivalent weight with respect to phosphorus is about 20,000 (5). The reaction has also been investigated with non-proteolytic esterases to an extent that at least shows their behavior to be similar to that of the proteolytic esterases. Boursnell and Webb (6) demonstrated that highly purified horse liver esterase bound phosphorus upon inhibition with radioactive DFP, and recently Michel and Krop (7) obtained a similar result with purified cholinesterase from the electric eel. In every case the inhibition of a susceptible enzyme by DFP has resulted in the introduction of the phosphorus of the inhibitor.

The specific activity of cholinesterase is now a matter of considerable interest. Despite many efforts at purification, it seems doubtful whether preparations of outstanding purity have been obtained from any source. The work reported here is an attempt to deduce the specific activity of

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cholinesterase from human plasma, in a greatly purified but still impure preparation, by observing the activity and the amount of DFP required to inhibit it. The inhibition resulted in the attachment of phosphorus to the protein of the preparation. However, the amount of phosphorus found in the completely inhibited enzyme (0.0023 per cent) was considerably smaller than that found in the chymotrypsins and trypsin when inhibited by DFP (2, 5). The low phosphorus content was due to the fact that the plasma enzyme was impure.

Materials and Methods

Purified human plasma cholinesterase was obtained through the courtesy of Dr. F. F. Johnson and Dr. J. H. Hink,1 who used Fraction IV of Cohn et al. (8) as a starting material for the purification of the enzyme. They were able to purify the cholinesterase 750-fold over the activity of the whole plasma protein, as judged by the Michel method of assay (9). The purified enzyme had been lyophilized.

The enzymic activities were determined at 30° by a continuous potentiometric titration procedure (1) at pH 7.5 with an acetylcholine concentration of 1.0 \( \text{M} \). Higher substrate concentrations were slightly inhibitory and lower concentrations failed to give full activity. Under these conditions the reaction was one of zero order. The results are expressed as specific activities; i.e., mM of acetylcholine hydrolyzed per minute per mg. of enzyme preparation or per mg. of total nitrogen therein, 1 mM per minute being 1 unit.

Since it had previously been demonstrated that isopropanol solutions of DFP are very stable (1), the radioactive DFP\(^2\) (DFP\(^*\)) was dissolved in isopropanol to give a concentration of 0.127 \( \text{M} \). The desired dilutions were made from this stock solution prior to their addition to solutions of cholinesterase. The determinations of radioactivity were made with a Geiger-Müller scaler. After treatment, portions of the various solutions were dried under an infra-red lamp and were then compared with known amounts of radioactive phosphorus in the form of alkali-hydrolyzed DFP*, which had been dried similarly under an infra-red lamp.

Results

Protein Nitrogen-Total Nitrogen Relationship of Cholinesterase—The cholinesterase preparation used in this work was found to have 12.4 per cent total nitrogen as determined by the Kjeldahl method. Attempts to determine the protein nitrogen of the enzyme by the method used in this

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1 Cutter Laboratories, Berkeley, California.

2 Purchased from Edward S. Gilfillan, Jr., consulting engineer, Manchester-by-the-Sea, Massachusetts.
Laboratory (10) failed, since no protein precipitated when an apparently ample quantity of the enzyme (0.18 mg. per ml. of 2.5 per cent trichloroacetic acid (TCA)) was heated to 70° for 5 minutes and cooled. Attempts to precipitate the same concentration of cholinesterase by the method of Rothenberg and Nachmansohn for eel cholinesterase (11) likewise failed. These authors used 10 per cent TCA at room temperature for 18 hours for precipitation. However, when the human cholinesterase was heated to 70° for 5 minutes in 10 per cent TCA (0.18 mg. of enzyme per ml.) and cooled, 75 per cent of the nitrogen of the preparation was precipitated. The behavior of this enzyme is reminiscent of that of purified polygalacturonase, which failed to precipitate with 2.5 per cent TCA when heated to 70° for 5 minutes and cooled (12). The cholinesterase prevented the precipitation of relatively small amounts of easily precipitable protein; e.g., a mixture of 1.64 mg. of cholinesterase and 0.7 mg. of crystalline β-lactoglobulin in 10 ml. of 2.5 per cent TCA on heating to 70° and cooling formed only a faint, non-centrifugal cloud. However, in agreement with the results of Michel and Krop (7), relatively large amounts of a carrier protein resulted in the complete precipitation of all of the protein, including that of the cholinesterase preparation. When a mixture of 4.20 mg. of β-lactoglobulin and 1.02 mg. of the cholinesterase preparation in 10 ml. of 2.5 per cent TCA was treated by the usual procedure (10), more than 98 per cent of the total nitrogen of the cholinesterase preparation was found in the precipitate. It is apparent from these results that specific activities of cholinesterase preparations based on TCA precipitation must be considered only with reservations. Since plasma cholinesterase is associated with mucoprotein (13) and may itself be a mucoprotein, and since another mucoprotein, ovomucoid, is resistant to precipitation by TCA (14), this behavior may be a general property of mucoproteins.

Specific Activity of Cholinesterase—Under the conditions of assay described above, the purified plasma cholinesterase had a specific activity of 0.0245 unit per mg. or 0.198 unit per mg. of nitrogen. The two preparations of eel cholinesterase used by Michel and Krop (7) possessed, when calculated in terms of the unit used here, 0.266 and 0.051 units per mg. Hence the preparation used in this work had approximately a 13-fold greater activity than Fraction IV-6 (13), one-third the activity of Fraction IV-6-3 (15), and approximately 0.1 of the activity of the better preparation from eel used by Michel and Krop.

3 Under the conditions of assay used by Surgenor et al. (13), the preparation had an activity of 0.0098 unit per mg. Calculated in terms of their unit, the preparation possessed 588 units per gm. Their fraction of greatest activity, No. IV-6, contained 45 units per gm. However, Goldstein and Doherty (15) have recently worked with preparations of Fraction IV-6-3 obtained from Surgenor which contained 700 to 2000 units per gm.
Inhibition of Cholinesterase by DFP and Phosphorus Content of Inhibited Enzyme—The results obtained on the inhibition of purified cholinesterase are given in Table I. For the concentration of enzyme used, the amount of DFP needed for approximately 50 per cent inhibition would, if all combined with this enzyme, introduce phosphorus into the enzyme to the extent of 0.0124 per cent, provided the inhibition reaction was the same as that with chymotrypsin (1-3). However, it will be seen later that this value is considerably greater than that actually found; hence the reaction was not as rapid as that with chymotrypsin.

Two preparations of essentially completely inhibited cholinesterase were made with DFP*. In the first preparation 100 mg. of enzyme were dissolved in 20 ml. of 0.1 M phosphate buffer of pH 7.75 and treated with 0.8 ml. of 0.0127 M DFP* in isopropanol; in the second, the same amount of enzyme was dissolved in 2 ml. of the same buffer and treated with 0.1 ml. of 0.127 M DFP*. In both cases, less than 2 per cent of the activity remained after 20 minutes incubation at 25°. Subsequently the preparations were dialyzed in the cold against frequent changes of distilled water until the dialysates possessed essentially no radioactivity. The analyses of these preparations are reported in Table II. The amount of phosphorus found in the DFP*-inhibited enzyme was 0.0023 per cent. This was essentially the same amount of phosphorus that Michel and Krop (7) found in a similarly inhibited preparation of the eel enzyme that possessed, however, approximately twice the activity of the plasma preparation used here. This difference may merely reflect a difference in the molecular weights of the enzyme from the two sources. The foregoing results demonstrate, however, that the inhibitor introduced phosphorus into the preparation of plasma cholinesterase. They point again to the inhibition reaction as being a general one.

Table I

<table>
<thead>
<tr>
<th>Molarity of DFP × 10^{-7}</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
</tr>
</tbody>
</table>

*The cholinesterase was dissolved in 0.1 M phosphate buffer at pH 7.75 to give a concentration of 0.05 mg. of enzyme per ml. and was treated with a given concentration of DFP for 20 minutes at 25° prior to assay.
DISCUSSION

From the values quoted by Goldstein and Doherty (15) the activity of the human plasma cholinesterase preparation used in this work corresponds to an enzyme content of 1.9 per cent of the preparation. The phosphorus content of the inhibited enzyme preparation (Table II) would then correspond to an equivalent weight of 26,000 for this cholinesterase on the assumption that the reaction proceeds on a mole for mole basis analogous to the combination of DFP with chymotrypsin (1-3). Furthermore, the

**Table II**

*Phosphorus Content of DFP*-Inhibited Cholinesterase*

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Inhibited enzyme*</th>
<th>Radioactivity† of P*</th>
<th>Phosphorus content‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen per 0.1 ml</td>
<td>Radioactivity per 0.1 ml</td>
<td>mg.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>1§</td>
<td>0.045</td>
<td>131</td>
<td>6400</td>
</tr>
<tr>
<td>2</td>
<td>0.450</td>
<td>177</td>
<td>785</td>
</tr>
</tbody>
</table>

* After DFP* inhibition (see the text), the inhibited enzyme was dialyzed against frequent changes of distilled water in the cold until the dialysate possessed a negligible radioactivity as measured with a dipping Geiger-Müller tube. 6 days were necessary.

† All radioactivities are corrected for the background. The phosphorus was in the form of alkali-decomposed DFP*. The phosphorus content was determined on a stock solution by a slight modification of Allen's method (16).

‡ These values were calculated from the activities and nitrogen content of the inhibited enzyme.

§ Because of the low protein content of this preparation and hence low radioactivity, it was necessary to count it and the corresponding known amount of radio-phosphorus closer to the Geiger-Müller tube than was necessary for Preparation 2.

The specific activities of several highly purified or crystalline hydrolytic enzymes are given in Table III. When several substrates are known for an enzyme, the most rapidly hydrolyzed was chosen for the calculation of the specific activity. Moreover, only the initial rates of hydrolysis were considered. Table III demonstrates that the specific activities of all of the enzymes considered fall within one order of magnitude.

4 Enzymes involved in phosphate transfer have lower specific activities; e.g., crystalline hexokinase (26) has a specific activity of 0.09 and phosphorylase a (27) 0.6 when acting on glucose 1 phosphate in 1 per cent glycogen in the presence of adenylic acid and cysteine. It is suggested, however, that the mechanism of a...
A calculation of the specific activity of the purest preparation of eel cholinesterase made by Rothenberg and Nachmansohn (11) (assuming a nitrogen content of 15 per cent) indicates a specific activity of 51 for that enzyme. On the other hand, if it is assumed that 1.9 per cent of the plasma preparation used in this work was cholinesterase (an assumption not unreasonable in light of the values quoted by Goldstein and Doherty (15)), the specific activity of the plasma enzyme when pure would be 10. The eel enzyme thus appears to be uniquely powerful among hydrolytic enzymes, or else the activities reported are in error, possibly due to the incompleteness of precipitation by TCA. If the latter is true, considerable doubt would be cast upon the value of the equivalent weight of eel cholinesterase calculated from DFP inhibition by Michel and Krop (7).

**SUMMARY**

When a preparation of purified, human plasma cholinesterase was inhibited by radioactive DFP, the phosphorus of the inhibitor was introduced into the inhibited enzyme. Hence the inhibition reaction of this enzyme by DFP was in this respect a similar reaction to the inhibition of the esterolytic proteinases. The amount of phosphorus introduced into the still impure cholinesterase was 0.0023 per cent.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Specific activities, units* per mg. N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline carboxypeptidase</td>
<td>Chloroaeryl-DL-β-phenyllactic acid</td>
<td>1.5 (17)</td>
</tr>
<tr>
<td>&quot; trypsin</td>
<td>α-Toluenesulfonyl-L-arginine methyl ester</td>
<td>1.8 (18)</td>
</tr>
<tr>
<td>&quot; α-chymotrypsin</td>
<td>N-Acetyltirosine ethyl ester</td>
<td>2.2 (19)</td>
</tr>
<tr>
<td>&quot; α-amylase</td>
<td>Starch</td>
<td>2.4 (20)</td>
</tr>
<tr>
<td>&quot; β-amylase</td>
<td>&quot;</td>
<td>9.6 (21, 22)</td>
</tr>
<tr>
<td>&quot; urease</td>
<td>Urea</td>
<td>12 (23)</td>
</tr>
<tr>
<td>Purified invertase</td>
<td>Sucrose</td>
<td>7.4 (24)</td>
</tr>
<tr>
<td>&quot; pectinesterase</td>
<td>Sucrose</td>
<td>1.7 (25)</td>
</tr>
<tr>
<td>&quot; polygalacturonase</td>
<td>Pectin</td>
<td>1.4 (12)</td>
</tr>
</tbody>
</table>

* The units (mM per minute) were calculated from data given in the references cited in this column.

Transfer reaction, catalyzed by a single enzyme, presents a different picture from that of simple hydrolysis. It does not seem logical to expect two types of reaction to proceed necessarily at the same level of activity.
BIBLIOGRAPHY

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