SERINE PHOSPHORIC ACID FROM DIISOPROPYLPHOSPHORYL DERIVATIVE OF EEL CHOLINESTERASE*

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(Received for publication, May 22, 1953)

Diisopropyl fluorophosphate (DFP) is a highly specific inactivator of esterases, such as cholinesterase and chymotrypsin (2), and combines irreversibly with these enzymes (3, 4). In a previous report from this laboratory (5) we have shown that, when the reaction product of DFP and chymotrypsin, namely, diisopropylphosphoryl chymotrypsin, is partially hydrolyzed, serine phosphoric acid may be obtained in a 30 per cent yield (based on phosphorus) from the hydrolysate. It was of interest to see whether or not the reaction between DFP and cholinesterase yielded the same product after partial hydrolysis.

Eel cholinesterase preparations, even though highly active enzymatically, ordinarily contain considerable inert protein. Since the reaction between DFP and such preparations can be prevented by the addition of an excess of acetylcholine (3), it is reasonable to assume that the DFP-binding property is associated with the cholinesterase present and not with concomitant impurities. This reasoning justified the use of these preparations in an effort to determine the nature of the combination between cholinesterase and DFP. The name “diisopropylphosphoryl cholinesterase” (DPChE) has been given to the DFP derivative of cholinesterase.

We have prepared DPChE containing P32, partially hydrolyzed the product, fractionated the hydrolysate on a cation exchange resin, and obtained serine phosphoric acid in a yield of approximately 40 per cent based on phosphorus.

EXPERIMENTAL

P32-Labeled DPChE—A solution of 1 mg. of P32-labeled DFP1 in 2 ml. of water was added to 20 ml. of a 0.3 per cent solution of an eel cholinesterase

* Presented in part at the Forty-fourth annual meeting of the American Society of Biological Chemists at Chicago (1).

1 This was obtained commercially with an initial specific activity of approximately 10 mc. per mg and a purity of 80 per cent or better. The determination of purity was based on the rate and extent of reaction with chymotrypsin and with standardized cholinesterase preparations and was kindly carried out by Dr. H. O. Michel of this laboratory.
preparation\(^\text{3}\) in 0.1 m phosphate buffer at pH 7.3 at room temperature. The reaction was allowed to proceed for 5 to 10 minutes, at which time all enzymatic activity was lost. The solution was dialyzed against running distilled water at 4\(^\circ\) for 2 days. The inactivated enzyme precipitated during the dialysis and was recovered with the supernatant fluid. The cholinesterase solution was found to bind an amount of P\(^{32}\) equivalent to a reaction with 18.4 \(\gamma\) of DFP.\(^3\)

**Partial Hydrolysis of DPChE**—Two methods of partial hydrolysis of DPChE were used with similar results. In Method 1 the hydrolysis of DPChE from 10 ml. of the cholinesterase solution was carried out by the successive steps of peptic digestion, tryptic digestion, and hydrolysis at 100\(^\circ\) in 2 N HCl for 10 hours, similar to the procedure described for diisopropylphosphoryl chymotrypsin (5). The enzymatic digestions were carried out in a volume of about 30 ml. The HCl hydrolysis was carried out in a 5 ml. volume after evaporation in vacuo. A precipitate which formed during the hydrolysis was centrifuged and washed free of radioactive material with dilute HCl. Method 2 was similar to Method 1, except that the preliminary enzymatic digestions were omitted.

**Dowex 50 Chromatography**—After removal of the HCl from the hydrolysate by repeated evaporation to dryness in vacuo, the residue was chromatographed on a column of Dowex 50 (hydrogen form) 0.83 cm. in diameter and 100 cm. in length with use of 0.05 N HCl as the eluent at a flow rate of 6 ml. per hour (5).

Phosphorus was determined by the method of Fiske and Subbarow (7).

**Results**

**Dowex 50 Chromatography of Partially Hydrolyzed DPChE**—The Dowex 50 chromatogram of the partial hydrolysate from a preparation by Method 1 is shown in Fig. 1. The hydrolysate yielded two main phosphorus-containing fractions. The faster moving fraction, amounting to 43 per cent of the added phosphorus, appeared at the front and presumably was orthophosphoric acid (5). The second phosphorus fraction, amounting to 46 per cent of the phosphorus, was in the position previously found for serine phosphoric acid (5).

\(^1\) The enzyme was prepared for us by Dr. D. Nachmansohn of Columbia University (6) and had an activity of 10 units per mg. of dry weight (1 unit = 1 gm. of acetylcholine hydrolyzed per hour).

\(^2\) This is equivalent to \(1.7 \times 10^{-10}\) mole of DFP per unit of enzyme activity, a figure sufficiently close to the \(2.1 \times 10^{-10}\) mole per unit found by Michel and Krop (3) for two preparations which had 26 and 5 per cent of the enzymatic activity, respectively, of the present preparation. This fact further substantiates their conclusion that the reaction between DFP and these cholinesterase preparations involves only cholinesterase.
Detection of Serine Phosphoric Acid in Second Phosphorus Fraction—Owing to the scarcity of eel cholinesterase, not enough material was present in the second phosphorus fraction for paper chromatograms and, hence, isotope dilution experiments were resorted to. The first experiment was a fractional alcohol precipitation. The second phosphorus fraction from a preparation obtained by Method 2 containing 15,500 c.p.m. was mixed with 124 mg. of authentic serine phosphoric acid\(^4\) in a volume of 3.9 ml. and was precipitated with 1.46 volumes of absolute ethanol. The specific activity of the supernatant fluid was about twice as great as that of the precipitate (Table I). The precipitate was redissolved in water and was again partially precipitated with alcohol. This procedure was repeated twice more. After three or four precipitations it appeared that all of the phosphorus-containing material of the fraction that was not serine phosphoric acid had been separated in the previous supernatant fluids. Similar results were obtained with a preparation by Method 1.

In the second isotope dilution experiment Dowex 50 chromatography was utilized. The material used was that represented by the second precipitate of the fractional alcohol precipitation of Table I, weighing 63.3 mg. and containing 850 c.p.m. at the time of this experiment. When chromatographed on a Dowex 50 column, as described above, the radioactive phosphorus was equally distributed in the first two fractions. The results are shown in Fig. 1.

\(^4\) We are grateful to Dr. T. L. McMeekin of the Eastern Regional Research Laboratory, United States Department of Agriculture, for the synthetic serine phosphoric acid.

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**Fig. 1.** Dowex 50 chromatogram of partial hydrolysate of diisopropylphosphorylcholinesterase. Material hydrolyzed by pepsin, trypsin, and 2 N HCl. 89.6 per cent of phosphorus recovered. 250 to 500 mesh resin in hydrogen form. Column 0.83 × 100 cm. Eluent 0.05 N HCl. Filtration under 2.5 cm. of Hg pressure with flow rate of 6 ml. per hour. Fraction size 2 ml.
phorus from the fraction coincided exactly with the phosphorus of the added serine phosphoric acid as determined colorimetrically (Fig. 2).

When the material of the first supernatant fluid of Table I, containing 4500 c.p.m. and weighing 9.9 mg., was chromatographed, the radioactive

<table>
<thead>
<tr>
<th>Original mixture</th>
<th>Precipitation per cent</th>
<th>Specific activity</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ppt. c.p.m. per mg.</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>92</td>
</tr>
<tr>
<td>1st ppt. redissolved</td>
<td>82</td>
<td>89</td>
</tr>
<tr>
<td>2nd &quot; &quot;</td>
<td>80</td>
<td>89</td>
</tr>
<tr>
<td>3rd &quot; &quot;</td>
<td>80</td>
<td>87</td>
</tr>
</tbody>
</table>

Fig. 2. Dowex 50 chromatogram of mixture of radioactive second phosphorus fraction plus serine phosphoric acid. Conditions same as those in Fig. 1.

The counts per minute are corrected to a single day.

phosphorus coincided with the colorimetric phosphorus in the first half of the curve, but in the second half considerably more P32 was recovered than expected from the recovery of the added serine phosphoric acid (Fig. 3). The area of the colorimetric phosphorus curve, representing serine phosphoric acid, was 63 per cent of that of the P32 curve. Assuming that about 90 per cent of the radioactivity in the first precipitate of Table I was attributable to serine phosphoric acid, the yield of serine phosphoric acid in
the second phosphorus fraction was 38.5 per cent. When the material from Tubes 41 to 47 of Fig. 3 was further hydrolyzed in 2.5 N HCl at 100° for another 10 hours and rechromatographed, the radioactive and colorimetric phosphorus curves coincided completely and accounted for 50 per cent of the radioactive phosphorus chromatographed. This indicated that the non-serine phosphoric acid material of the second phosphorus fraction was a peptide of serine phosphoric acid. It could not be a diisopropyl derivative because diisopropylphosphoric acid5 is completely hydrolyzed under the conditions of the acid hydrolysis.

![Graph](image)

**Fig. 3.** Dowex 50 chromatogram of alcohol-fractionated mixture of radioactive second phosphorus fraction plus serine phosphoric acid. The material chromatographed was that of the first alcoholic supernatant fluid of Table I. Conditions same as those in Fig. 1.

The second phosphorus fraction from a preparation by Method 1 mixed with 22.1 mg. of serine phosphoric acid gave a Dowex 50 chromatogram essentially like that shown in Fig. 2, except that the last three P32 values at the bottom of the curve were somewhat above the colorimetric phosphorus figures.

The possibility remained that threonine phosphoric acid, which is known to be acid-stable (8), might behave identically with serine phosphoric acid in the isotope dilution experiments. Therefore 16 mg. of an authentic sample of threonine phosphoric acid6 were chromatographed with radioactive serine phosphoric acid isolated from radioactive diisopropylphosphoryl chymotrypsin (5). The P32 was found to be distributed between Tubes 36 and 42, whereas the colorimetrically determined phosphorus, from the threonine phosphoric acid, was between Tubes 51 and 59. The presence

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5 Obtained by prolonged hydrolysis of P32-labeled DFP at room temperature.

6 We are grateful to Mr. J. J. O'Neill and to Dr. R. Plapinger of these laboratories for samples of synthetic threonine phosphoric acid.
of threonine phosphoric acid in the HCl hydrolysate of DPChE was pre-
cluded because no P31 was found beyond Tube 47 in the hydrolysates.

DISCUSSION

The presence of serine phosphoric acid in partial hydrolysates of the
diisopropylphosphoryl derivatives of both chymotrypsin and eel cholin-
esterase suggests that reactive serine residues occur in both enzymes. In
view of the suggestion (2-4, 9, 10) that DFP may react with the active
centers of these enzymes, the involvement of serine in the active centers is
a possibility. However, DFP might react with a group near the active
center of the esterases and thus by steric hindrance prevent a reaction of
the substrate with the enzyme. As considered previously (5), there re-
mains a possibility that DFP reacts initially with some other residue and
the serine residue becomes phosphorylated in a secondary reaction.

In connection with serine reactivity it is of interest to note that peptide
bonds involving the amino groups of serine and threonine in proteins possess
an unusual lability (11, 12), possibly related to the migration of acyl resi-
dues from the amino group to the hydroxyl group (13).

Threonine appears to be eliminated as a reactive residue in these ester-
ases, since no threonine phosphoric acid was detectable in the acid hydroly-
sates of their diisopropylphosphoryl derivatives. It is of interest that
threonine phosphoric acid has been isolated recently from casein (14).

SUMMARY

The reaction product of diisopropyl fluorophosphate and eel cholin-
esterase, diisopropylphosphoryl cholinesterase, was partially hydrolyzed by
pepsin, trypsin, and 2 N HCl or directly with 2 N HCl. Serine phosphoric
acid was separated from the hydrolysate in approximately a 40 per cent
yield (based on phosphorus) by fractionation with Dowex 50 chromatog-
raphy. Identity was established by comparison with synthetic serine phos-
phoric acid by fractional alcohol precipitation and Dowex 50 chromatog-
raphy.

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