THE TURNOVER NUMBER OF ALI-ESTERASE, PSEUDO- AND TRUE CHOLINESTERASE AND THE COMBINATION OF THESE ENZYMES WITH DI*ISO*PROPYLFLUOROPHOSPHONATE

by

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In the course of a study of the mechanism of action of anticholinesterases a hypothesis was developed concerning the chemical structure of cholinergic neuroreceptors[1,2]. It was proposed that in addition to anionic sites the receptor possessed a so-called B group, which was identical to a group occurring in or near the catalytic group of DFP sensitive esterases (including cholinesterases). Therefore it was decided to study the chemical structure of the enzyme-active group of esterases because this might not only provide valuable information on the mechanism of action of esterases and their inhibition by anticholinesterases but also on the structure of neuroreceptors.

The study was started on mammalian cholinesterase and later extended to chymotrypsin, pseudo-cholinesterase and ali-esterase. It included the development and application of methods to determine turnover numbers and analyses of products of the action of $DF^{32}P$* on various enzymes [3,4,5,6]. The latter method of investigation was greatly stimulated by recent discoveries by SCHAFFER *et al.*[7,8]. They found that partial hydrolysis of the reaction product of $DF^{32}P$ and chymotrypsin yielded serine phosphate. This compound contained 30% of the radioactive ^{32}P initially combining with chymotrypsin. The remaining ^{32}P appeared mainly as inorganic phosphate. Similar results were obtained by the same authors with electric eel cholinesterase.

In the present paper detailed results are described on mammalian enzymes which on the strength of their sensitivity to DFP could be assumed to possess B groups. Highly purified bovine red-cell cholinesterase has recently become available[9] and was used. Other preparations studied were the crude cholinesterase and ali-esterase of bovine red-cell stroma and purified horse serum cholinesterase. It will be shown that for these enzymes the methods previously described for the determination of turnover numbers proved adequate and that in all substances ^{32}P containing serine phosphate could be isolated from the hydrolysate of the reaction products of the enzymes with $DF^{32}P$.

* The abbreviations used in this paper are as follows: DFP for di*iso*propylfluorophosphonate; $DF^{32}P$ for the same compound labelled with ^{32}P; $D^{32}P$-cholinesterase and $D^{32}P$-esterase for the combination products of the enzymes involved with a di*iso*propylphosphatyl radical resulting from interaction of these enzymes with $DF^{32}P$; T.O.N. for turnover number; B.A.W. for butanol-acetic acid-water and *iso*b-NH_3 for *iso*butyric acid-NH_3 mixtures used as solvents for paper chromatography.

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EXPERIMENTAL METHODS

Incubation of enzymes with $DF^{32}P$

The combination product of $DF^{32}P$ with the true cholinesterase of crude stroma was prepared by method B described in a previous communication[6]. Stroma was made as already reported[10]. True cholinesterase was purified by the method of WARRINGA AND COHEN[9]. It was treated by both methods, A and B, recommended by COHEN *et al.*[6]. Pseudo-cholinesterase was purified through stage 6 of the STRELITZ method[11] by Dr. C. v. d. MEER of this laboratory. The combination product of $DF^{32}P$ with the ali-esterase of stroma was prepared by a method based on the principle of method B quoted above[7]. In this case protection of the enzyme-active groups was afforded not by butyrylcholine but by tributyrine, a substrate for ali-esterase. A complication is introduced by the fact that this substance not only protects a large proportion of the ali-esterase groups against attack by DFP but also a small proportion of the true cholinesterase present in the stroma. To prevent attachment of $DF^{32}P$ to these groups during the second incubation of procedure B butyrylcholine is added. This compound prevents any reaction of $DF^{32}P$ with true cholinesterase.

The methods for the estimation of the enzyme activity of true and pseudo-cholinesterase and of the radioactivity of the various preparations were the same as described before[6]. The same applies to the synthesis of $DF^{32}P$ which however was used at a considerably higher specific activity, *viz.* 200 μc/mg. Ali-esterase activity was measured by a continuous titration method at 24° C using tributyrine (1%) as a substrate in an unbuffered solution of 10 ml. The activity is expressed in units representing the number of μl 0.01 molar NaOH which must be added per hour to maintain the pH of the reaction mixture at 7.4 under the experimental conditions. Partial hydrolysis of the combination products of enzyme preparations and $DF^{32}P$ was effected by the action of 2 *N* HCl at 100° C during 18 hours according to SCHAFFER *et al.*[7,8]. After filtration the hydrolysates were evaporated at 30° C and dried *in vacuo* over KOH.

The fractions were separated on a Dowex-50 ion exchange column of 17 mm diameter and 750 mm length. Elution was effected by 0.05 *N* HCl at 30 ml/h.

Paper chromatography was performed on Whatman No. 1 using mixtures of butanol–acetic acid–water 4:1:5 and *iso*butyric acid-ammonia (0.5 *N*) 10:6 at pH 3.8 as solvents.

The solvents were allowed to ascend at 25° C for a period of 18 hours.

EXPERIMENTAL RESULTS

I. *The reaction of $DF^{32}P$ with red-cell stroma components*

Three grams of freeze-dried stroma (COHEN *et al.*[10]) were taken up in 150 ml 0.01 molar phosphate buffer (pH 7.5). The cholinesterase activity of this solution was 4700 units/ml; its N content 1.97 mg/ml. The solution was divided in 3 equal portions of 50 ml (1 g) which were processed as follows:

Prep. 1. 50 ml stroma (1 g) were incubated with 1 ml $DF^{32}P$ $5 \cdot 10^{-6}$ w/v during 40 minutes at 24° C. The mixture was consecutively dialysed overnight against 1% NaCl solution, during 5 days against tap water and overnight against distilled water. Analysis in a liquid Geiger Müller counter revealed that 4.60 μg of $DF^{32}P$ had reacted with the stroma. This quantity represents all DFP reactive groups of the stroma including those of cholinesterase and ali-esterase.

Prep. 2. In this preparation the stroma was first treated with non-radioactive DFP under protection of the true cholinesterase by butyrylcholine according to method B described by COHEN *et al.*[6]. Next the $DF^{32}P$ treatment and dialysis followed as before resulting in a radioactivity corresponding to 0.10 μg of originally reacting $DF^{32}P$.

Prep. 3. This preparation was made on the same principle as the previous one. Only this time we aimed at protecting the ali-esterase instead of the cholinesterase during the incubation with non-radioactive DFP. For this purpose 1% tributyrin was used instead of butyrylcholine. Enzyme activity tests revealed that only approx. 50% of the available ali-esterase could have reacted with $DF^{32}P$ under the prevailing conditions. The remaining 50% of the potentially reactive esterase groups kept their enzyme activity as a result of the protective action of a proportion of the tributyrin which could not

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be dialysed out. With thus treated stroma 2.50 μg of DF^{32}P were found to react. Consequently, 5.00 μg would have reacted if all esterase groups had been available. Preparation 3 owed its capacity to combine with DF^{32}P exclusively to the presence of ali-esterase, provided that tributyrin affords a specific protection of this enzyme. It has only half the ^{32}P activity of prep. 1 but *also* only half of the ali-esterase present in prep. 3 has reacted. This would mean that practically all of the DFP combining power of the red-cell stroma is due to its ali-esterase, and only a very small proportion to its cholinesterase content. It should be stressed that the correctness of this statement depends on the assumption that under the experimental conditions employed tributyrin protects the ali-esterase in a specific way. A similar assumption has been proved for the DFP-butyrylcholine-true cholinesterase, but so far not for the DFP-tributyrin-ali-esterase system. The fact that the degree of protection by tributyrin of stroma ali-esterase corresponds to the amount of ^{32}P found in the treated stroma might still depend on an aspecific fencing-off by tributyrin of various reactive proteins, including ali-esterase, to the same extent. This however is made unlikely by the observation that the protection of ali-esterase by tributyrin is vastly superior to that of the cholinesterase of the same stroma preparation. It seems therefore reasonable to assume that the presence of a considerable number of groups other than ali-esterase but capable of binding ^{32}P from DF^{32}P would have interfered with the correspondence found between protection by tributyrin of the enzymic activity of ali-esterase and the amount of ^{32}P bound by the whole stroma.

II. *The turnover of true cholinesterase, pseudo-cholinesterase and ali-esterase*

A recently developed purification procedure[9] allowed us to re-estimate the T.O.N. of true cholinesterase using this time both of the previously recommended methods (method A and B)[6]. The T.O.N. is defined as the number of molecules of substrate broken down per active centre per minute under the normal conditions of the activity test. Freeze-dried material was dissolved in 45 ml water to give a solution containing 3880 units/ml and 0.0117 mg N/ml (330,000 units/mg N). Dialysis was performed as has been described above for stroma and the T.O.N. was estimated. By method A a value of 372,000, by method B one of 278,000 was found. The latter corresponds very well with previous estimations. The former is somewhat higher. No explanation can be offered for this discrepancy but it should be remembered that theoretically method B might be expected to yield the more accurate results.

10 ml of a purified pseudo-cholinesterase preparation containing 244,000 units and 2 mg N were incubated with 1 ml DF^{32}P $2 \cdot 10^{-6}$ (w/v) during 40 minutes at 24° C resulting in complete inhibition of the enzyme activity. The solution was thoroughly dialysed as described before. A comparison of the radioactivity of the dialysed solution and the enzyme activity of the original protein permitted a calculation of the T.O.N. of pseudo-cholinesterase. This was found to be approx. 50,000.

An attempt was made to interpret the results with preparation 3 to arrive at an estimation of the T.O.N. of ali-esterase. A procedure based on the principles of method B and using tributyrin as a protective agent was used. The figure thus obtained was 780. It should be considered as highly tentative due to the serious limitations mentioned earlier inherent in the use of tributyrin and the uncertainty regarding its specificity as a protective agent. It cannot be directly compared with the values reported for cholinesterases on account of the differences in the respective testing procedures.

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III. *The nature of the bond resulting from the action of DFP on esterases*

The three stroma preparations described in part I were partially hydrolysed by HCl. A small insoluble fraction was filtered off; this contained 5–10% of the total radioactivity. The filtrates were separated on a Dowex-50 column. The data corresponding

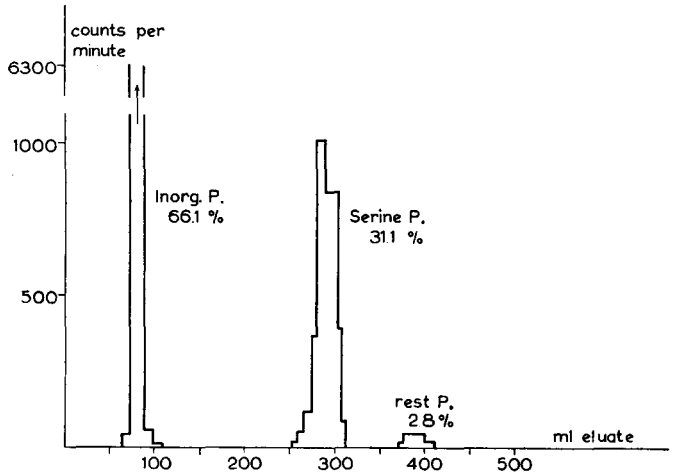

Fig. 1. Dowex-50 [H+] chromatogram of prep. 1. The eluate was collected in portions of 6.5 ml. The radioactivity is given in c/m as indicated by a liquid G.M. counter. 100 c/m represents a radioactivity corresponding to $22.1 \cdot 10^{-4}$ μg DFP per ml.

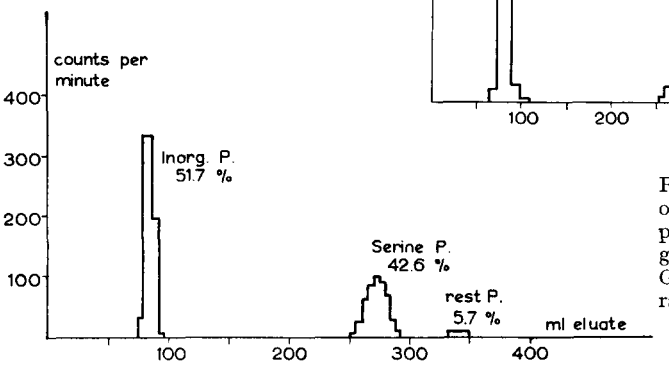

Fig. 2. Dowex-50 [H+] chromatogram of prep. 2. The eluate was collected in portions of 5 ml. The radioactivity is given in c/m as indicated by a liquid G.M. counter. 100 c/m represents a radioactivity corresponding to $14.0 \cdot 10^{-4}$ μg DFP per ml.

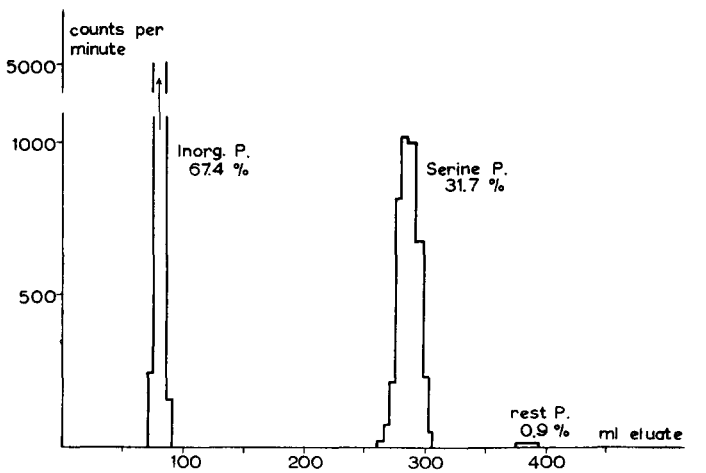

Fig. 3. Dowex-50 [H+] chromatogram of prep. 3. The eluate was collected in portions of 5 ml. The radioactivity is given in c/m as indicated by a liquid G.M. counter. 100 c/m represents a radioactivity corresponding to $14.9 \cdot 10^{-4}$ μg DFP per ml.

to the eluates obtained from preparations 1, 2 and 3 are represented in Fig. 1, 2 and 3 respectively. Table I provides additional information on the fractionation. Column 1 gives the amount of ^{32}P in the filtrate calculated in μg of the original DFP. Only a limited percentage of this, tabulated in column 2 of Table I, could be eluted by 0.05 *N*

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HCl. Figs. 1, 2 and 3 and the percentages given in columns 3, 4 and 5 of Table I obviously only refer to this elutable fraction of material brought on the column. Of the two main fractions obtained from the column the fast moving one could be recognized as inorganic phosphate; it represented 66, 52 and 67% of the eluted material in preparations 1, 2 and 3. The other fraction representing 31, 43 and 32% was identified as serine phosphate. The identification was carried out by comparing the behaviour of both unknown fractions with that of synthetic serine-o-phosphate and inorganic phosphate as markers on the Dowex column and on paper chromatograms using various solvents. Complete agreement could be established between the positions of the unknowns and the markers on the Dowex column and on paper chromatograms using *iso*b-NH_3 or B.A.W. as solvents. The position of the unknowns on the paper could be revealed by the usual staining methods and by autoradiography of the papers on X-ray films.

TABLE I

	1 μg DFP	2 %	3 % IP	4 % SP	5 % rest	6 T.O.N.
Prep. 1 stroma	4.60	58.0	66.1	31.1	2.8	
Prep. 2 true cholinesterase (stroma)	0.10	90.0	51.7	42.6	5.7	
Prep. 3 ali-esterase (stroma)	2.50	63.7	67.4	31.7	0.9	780*
Purified true cholinesterase			52.0	48.0		278,000
Purified pseudo-cholinesterase			± 66	± 33		50,000

1 = ^{32}P in filtered hydrolysate — expressed in μg DFP.
2 = recovery in 0.05 *N* HCl eluate from Dowex-50 of ^{32}P present in 1.
3 = per cent of 2 present as inorganic phosphate.
4 = per cent of 2 present as serine phosphate.
5 = ^{32}P not accounted for in fractions 3 and 4.
6 = T.O.N.

* This figure represents only a highly tentative estimate.

A small unidentified additional fraction was found (column 5 of Table I). Preparations 1 and 3 yield essentially similar results as might be expected from the assumption made earlier, that in both preparations it is chiefly ali-esterase that is responsible for the reaction of DFP with stroma. Preparation 2 might possibly differ in having relatively more radioactivity in the serine phosphate and less in the inorganic phosphate fraction. It is essentially a true cholinesterase preparation.

The purified preparation of true cholinesterase described in part II and dealt with in Table I yields a distribution pattern of the radioactivity, which is essentially similar to that of preparation 2 as might be expected. It is characterized by a relatively high serine phosphate content. The hydrolysate prepared as described before was brought on a

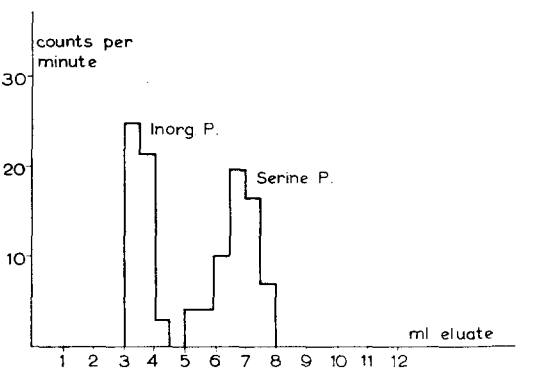

Fig. 4. Dowex-50 [H^+] chromatogram of purified true cholinesterase. The eluate was collected in portions of 0.5 ml. 100 c/m represents a radioactivity corresponding to $4.1 \cdot 10^{-3}$ μg DFP.

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Dowex-50 column of 30 mm length and 0.9 cm diameter. The fractions were concentrated by evaporation and quantitatively transferred to paper. The results are summarized in Fig. 4 and Table I. The position of the fractions on the paper after developing in B.A.W. in the presence of markers was assessed by X-ray film exposure during 6 weeks. The positions of the blackening of the photographic emulsion due to the radioactivity of the fractions coincided with those of the coloured spots on the paper due to staining of the added markers. 48% of the radioactivity collected from the Dowex column was present in the inorganic phosphate and 52% in the serine phosphate fraction.

The reaction product of DFP and pseudo-cholinesterase, as used for the determination of the corresponding T.O.N. in part II, was hydrolysed as described and dried. The dried product was taken up in 100 μl water. 5 μl were chromatographed on paper using B.A.W. and *iso*b-NH_3 as solvents. Inorganic phosphate and serine phosphate were added as markers. Again the 3 week-autoradiogram revealed two spots of blackening corresponding exactly in position and shape to the coloured spots on the paper due to the staining of the markers. The ratio of the distribution of radioactivity between the inorganic and the serine phosphates could in this case only be guessed approximately by visual comparison of the corresponding areas of blackening. It was estimated as being approximately 2:1.

DISCUSSION

In the introduction attention was drawn to the work of SCHAFFER *et al.*[7,8] who demonstrated, that serine phosphate could be recovered from the partial hydrolysates of the D.I.P. derivatives of chymotrypsine and electric eel cholinesterase. In the present work we were able to extend these observations to the reaction products of DFP with purified mammalian red-cell cholinesterase, plasma pseudo-cholinesterase and red-cell ali-esterase. On the strength of their reactivity towards DFP all these proteins should possess a common B group in the terms of the hypothesis quoted in the introduction. The present results suggest that the serine group might be a component of this B group since for all enzymes investigated a large part of the ^{32}P radioactivity from the DFP is recovered combined with serine in the hydrolysates. Although all enzymes investigated are esterases this conclusion does not necessarily imply that serine itself is important for their esteratic action. Serine might be situated so near to the actual hydrolysing group that its combination with D.I.P. might produce steric hindrance of the latter. Experiments reported recently[4] on $D^{32}P$-chymotrypsin have shown that this compound can be broken down to form a radioactive peptide. This peptide possesses an intact di*iso*propylphosphoryl group; serine phosphate is formed on acid hydrolysis. The peptide is formed from $D^{32}P$-chymotrypsin under extremely mild conditions of enzymic hydrolysis. In addition to one serine the following amino acid residues are present: 2–3 glycine, 1 proline, 1 aspartic acid and 1 leucine. It is not known where the D.I.P. group is situated on the peptide, but in view of the mild treatment applied it seems reasonable to assume that this localisation will not be different from the original point of attack of DFP on the protein.

Several authors[12,13,14] have stressed the lability of peptides containing serine residues, in particular the transfer of N-linked acyl radicals to form esters with the hydroxyl group of serine. Correspondingly acid hydrolysis of the peptide described might involve migration of the ^{32}P from a labile P–N linkage to a stable P–O-serine

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linkage with loss of the *iso*propyl groups. The serine phosphate thus produced would at least partly resist the acid hydrolysis. The possibility of such a mechanism has been suggested by WAGNER-JAUREGG[15]. The inorganic radioactive phosphate found would presumably rise from two sources: from the ^{32}P escaping the migration process and from the hydrolysis of serine phosphate.

The work of SCHAFFER *et al.*[7,8] and WAGNER-JAUREGG[15] would suggest that imidazol N might be the primary point of attack of DFP. The absence of histidine in our peptide renders this assumption less attractive.

The distribution of radioactivity over the two main fractions is identical for the ali-esterase preparations 1 and 3 and somewhat different for the cholinesterase preparation 2. This suggestion that the relation inorganic phosphate: serine phosphate might be different for ali-esterase and cholinesterase is supported by the results obtained with the purified cholinesterase. Fig. 4 shows that relatively large amounts of serine phosphate are present in the hydrolysate. For pseudo-cholinesterase no valid conclusions can be drawn in this respect since the relationship involved could only be estimated by crude visual evaluation of the degree of blackening of the photographic emulsion.

The value for the turnover number of true cholinesterase reported earlier (295,000) could be confirmed on a preparation of far higher purity than was formerly available. The value of 50,000 for pseudo-cholinesterase was the result of a direct determination; that is to say, no attempt was made to correct for possible reactions of groups other than those connected with the active sites of pseudo-cholinesterase with DFP; the actual value might therefore be higher than the reported one.

SUMMARY

1. Bovine red cell stroma was capable of combining with $DF^{32}P$. The combination was found to be mainly due to the ali-esterase present in the stroma. The true cholinesterase of the stroma accounts for only a minor percentage of the ^{32}P bound.

2. The reaction products of $DF^{32}P$ with highly purified preparations of true and pseudo-cholinesterase were prepared by incubation of the enzymes concerned with $DF^{32}P$.

3. For these enzyme preparations turnover numbers could be established. The figure found for true cholinesterase confirmed the value previously reported (295,000). A turnover number of 50,000 was found for pseudo-cholinesterase.

4. The reaction products of DFP with ali-esterase, true and pseudo-cholinesterase were hydrolysed and subjected to chromatography on Dowex-50.

5. In all three cases the bulk of the radioactivity proved to be associated with the inorganic phosphate and serine phosphate fractions of the chromatogram.

6. The results suggest that the OH groups of serine might be of importance in the combination of DFP with the active centre of the esterases concerned.

RÉSUMÉ

1. Le stroma des globules rouges du boeuf est capable de se combiner avec $DF^{32}P$. La liaison a lieu principalement par l'intermédiaire de l'ali-estérase présente dans le stroma. La cholinestérase vraie du stroma n'est responsable que d'un faible pourcentage du ^{32}P lié.

2. Les produits de la réaction du $DF^{32}P$ avec des préparations fortement purifiées de cholinestérase vraie et de pseudo-cholinestérase ont été préparées par incubation des enzymes respectifs avec $DF^{32}P$.

3. Des nombres de turnover ont pu être établis pour ces préparations d'enzymes. La valeur trouvée pour la cholinestérase vraie confirme la valeur publiée précédemment (295,000). Un nombre de turnover de 50,000 a été trouvé dans le cas de la pseudo-cholinestérase.

4. Les produits de la réaction du DFP avec l'ali-estérase, la cholinestérase vraie et la pseudo-cholinestérase ont été hydrolysés et soumis à la chromatographie sur Dowex-50.

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5. Dans les trois cas l'essentiel de la radioactivité a été retrouvé dans les fractions du chromatogramme correspondant au phosphate minéral et à l'ester phosphorique de la sérine.

6. Les résultats suggèrent que les groupes OH de la sérine pourraient jouer un rôle important dans la combinaison entre le DFP et le centre actif de l'estérase mise en jeu.

ZUSAMMENFASSUNG

1. Stroma aus roten Blutkörperchen des Rindes tritt mit $DF^{32}P$ in Verbindung. Es wurde bewiesen, dass die Verbindung hauptsächlich der im Stroma gegenwärtigen Ali-Esterase zuzuschreiben ist. Die wahre Cholinesterase des Stromas ist nur für einen kleineren Prozentsatz des gebundenen ^{32}P verantwortlich.

2. Die Reaktionsprodukte von $DF^{32}P$ mit weitgehend gereinigten Präparaten wahrer und Pseudo-Cholinesterase wurden durch Inkubation der betreffenden Enzyme mit $DF^{32}P$ erhalten.

3. Die Umsetzungszahlen für diese Enzympräparate konnten festgestellt werden. Der für die wahre Cholinesterase gefundene Wert bekräftigte die vorher erhaltene Umsetzungszahl (295,000). Eine Umsetzungszahl von 50,000 wurde für Pseudo-Cholinesterase festgestellt.

4. Die Reaktionsprodukte von DFP mit Ali-Esterase, wahrer und Pseudo-Cholinesterase wurden hydrolysiert und einer chromatographischen Untersuchung mit Dowex-50 unterworfen.

5. In allen 3 Fällen wurde der Hauptteil der Radioaktivität in Verbindung mit den inorganischen Phosphat- und Serinphosphatfraktionen des Chromatogrammes wiedergefunden.

6. Die Ergebnisse führen zur Annahme, dass die Serin-OH-Gruppen in der Verbindung zwischen DFP und dem aktiven Zentrum der betreffenden Esterasen eine wichtige Rolle spielen könnten.

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