SUMMARY

1. The soluble and insoluble fractions of eight species of bacteria were separated by centrifuging the material obtained after disintegrating the cells by shaking with glass beads.

2. The insoluble material, believed to be at least mainly derived from the cell wall, accounted in most cases for about half of the dry weight of the cell.

3. Estimations were performed on both fractions for ash, nitrogen, phosphorus, hexose, pentose, deoxypentose and nucleic acid, the last being determined spectroscopically.

4. Wide variations were found among different organisms but certain regularities were observed. Notably, nucleic acid was confined to the soluble fraction, which was also much richer in phosphorus, pentose and deoxypentose.

5. Concepts of the bacterial cell wall are discussed in the light of these findings.

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The Mechanism of Inhibition of Cholinesterases by Organophosphorus Compounds

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In a previous paper it was concluded that organophosphorus compounds inhibit cholinesterase by phosphorylating the enzyme and a chemical mechanism was suggested for both inhibition and reversal of inhibition (Aldridge, 1953*a*). A general formula of organophosphorus inhibitors may be represented as $(R)_2(X)PO$ where X is the group labile to hydrolysis and R is usually an alkoxy group. According to the phosphorylation theory, provided the R group is maintained constant, even though the X group is varied, the same inhibited enzyme must result. Wilson (1952) has shown that the inhibition of electric-eel cholinesterase after both tetraethyl pyrophosphate and diethyl phosphorofluoridate reverses at the same extremely slow rate and is also increased to the same degree by the presence of high concentrations of choline.

True cholinesterase activity returns quickly after inhibition by dimethyl *p*-nitrophenyl phosphate (Aldridge, 1953*a*) and the inhibition of rat pseudo cholinesterase by diethyl *p*-nitrophenyl phosphate has also been shown to reverse readily (Davison, 1953). Thus after inhibition of these enzymes by a series of inhibitors the identity or otherwise of the

inhibited enzyme may be established by an examination of the rate of return of enzyme activity under standard conditions. In this paper experiments will be described using rabbit erythrocyte true cholinesterase with inhibitors containing two methoxy groups and rat serum pseudo-cholinesterase with inhibitors containing two ethoxy groups.

METHODS

The methods for studying reversal of inhibition are those previously described (Aldridge, 1953*a*). The recovery of activity was measured by first treating the enzyme with inhibitor, then removing excess inhibitor by means of enzymes which will hydrolyse it rapidly and finally testing the activity of the inhibited enzyme after the addition of substrate. Rabbit serum will hydrolyse dimethyl and diethyl *p*-nitrophenyl phosphates (Aldridge, 1953*b*) dimethyl and diethyl phosphorofluoridates (Mazur, 1946), and *OO*-dimethyl and *OO*-diethyl-*S*-*p*-nitrophenyl phosphorothiolates. Tetramethyl and tetraethyl pyrophosphates are hydrolysed by erythrocytes (Aldridge & Davison, 1952*a*), and rat erythrocytes were added for this purpose in studying the reversal of inhibition of rat pseudo-cholinesterase.

The enzyme activity was determined using 0·015 M acetylcholine as substrate for the true cholinesterase of rabbit erythrocytes and 0·03 M *n*-butyrylcholine perchlorate for the pseudo-cholinesterase of rat serum. The buffer used contained 0·0357 M-$NaHCO_3$ and 0·164 M-NaCl. All inhibitors were dissolved freshly in buffer for each experiment. Dimethyl and diethyl phosphorofluoridates were synthesized from the corresponding phosphorochloridates by the method of Saunders & Stacey (1948), and tetramethyl pyrophosphate by the method of Toy (1949).

It should be noted that with inhibitions which are reversible, the inhibitory reaction is stopped upon the addition of substrate and the enzyme activity is returning throughout its determination. Although under these circumstances the output of CO_2 is not linear with respect to time, the best straight line through these points (Aldridge, Berry & Davies, 1949) has been calculated and the slope used as a measure of the activity over the period of determination.

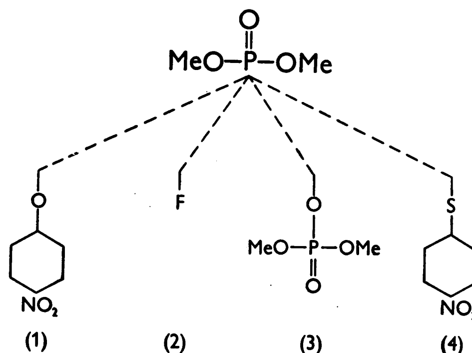


Fig. 1. Inhibitors used containing two methoxy groups. (1) dimethyl *p*-nitrophenyl phosphate. (2) dimethyl phosphorofluoridate. (3) Tetramethyl pyrophosphate. (4) *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate.

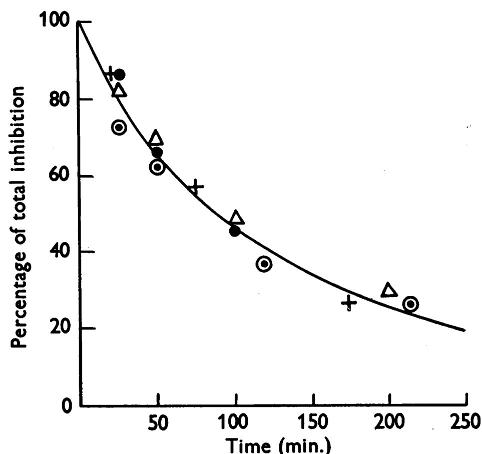


Fig. 2. The rate of return of enzyme activity after the treatment of cholinesterase with inhibitors containing two methoxy groups. After treatment of a suspension of rabbit erythrocytes (15 ml.) with inhibitor for 10 min. at 37°, the preparation was incubated with A-esterase (3 ml.) for 15 min. at 37°. Portions were taken and at various times acetylcholine tipped in to determine enzyme activity. The following concentrations of inhibitors were used: +, dimethyl *p*-nitrophenyl phosphate, $2·6 \times 10^{-7}$ M; ●, tetramethyl pyrophosphate, $1·4 \times 10^{-6}$ M; ⊙, dimethyl phosphorofluoridate, $1·1 \times 10^{-6}$ M; △, *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate, $3·6 \times 10^{-7}$ M.

RESULTS

The return of true cholinesterase activity of rabbit erythrocytes after dimethyl phosphorus compounds. In Fig. 1 are shown the four inhibitors which have been examined. They are all potent inhibitors of cholinesterase. According to the phosphorylation theory the first three compounds should all produce a dimethyl phosphorylated enzyme. The compound containing a *P*-*S*-*p*-nitrophenol grouping will only do so if the bond breaks between the phosphorus and sulphur atoms. The results shown in Fig. 2 show that the enzyme activity after inhibition by all four compounds returns at the same rate. The half-life for the inhibited enzyme at 37° and pH 7·8 is about 85–90 min.

The return of pseudo-cholinesterase activity of rat serum after diethyl phosphorus compounds. The parallel experiments have been carried out using the corresponding diethyl inhibitors and pseudo-cholinesterase of rat serum. As Fig. 3 shows, the rate of return of enzyme activity is the same for all four compounds. The half-life of this inhibited enzyme is approximately 450 min.

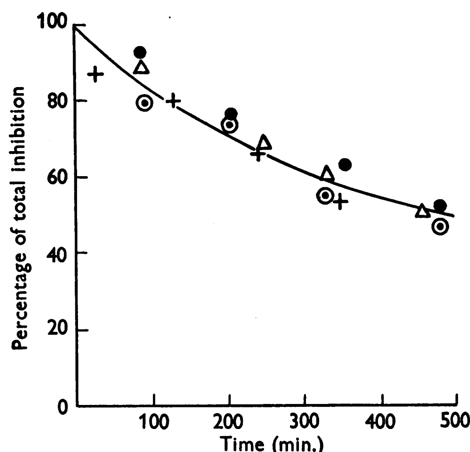
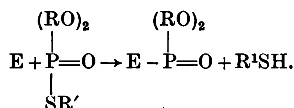


Fig. 3. The rate of return of enzyme activity after treatment of cholinesterase by inhibitors containing two ethoxy groups. After treatment of rat serum (4 ml.) with inhibitor for 30 min. at 37°, the preparation was incubated with A-esterase (1 ml.) for 30 min. at 37°. Portions were taken and at various times butyryl choline tipped in to determine enzyme activity. The following concentrations of inhibitors were used; +, diethyl *p*-nitrophenyl phosphate, 1×10^{-6}M; ●, tetraethyl pyrophosphate, 1×10^{-6}M; △, diethyl phosphorofluoridate, 3×10^{-6}M; ⊙, *OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate, 1×10^{-6}M.

DISCUSSION

Our evidence for the identity of the inhibited enzymes is based on determinations of the rate of return of enzyme activity under standard conditions. On this basis the results described show that, amongst inhibitors of the general formula $(R)_2(X)PO$ (X is the group labile to hydrolysis), if X is varied and R maintained constant the same inhibited enzyme results. This conclusion has been reached with true cholinesterase of rabbit erythrocytes inhibited by dimethyl phosphorus compounds and also for pseudo-cholinesterase of rat serum using diethyl phosphorus compounds. This is a direct confirmation of the theory of the mechanism of inhibition previously put forward (Aldridge, 1953*a*).

Since the inhibitors containing *P*-*S*-*p*-nitrophenyl groups are more unstable to hydrolysis than the corresponding compounds with *P*-*O*-*p*-nitrophenyl groups (Topley, 1950; Aldridge & Davison, 1952*b*) it would be expected that the corresponding enzyme phosphate would have different stabilities if the phosphorus atom were linked to the enzyme through a sulphur or oxygen atom, respectively. For these inhibitors this is not so. A direct attachment of the phosphorus atom to some atom of the enzyme protein would explain this observation, i.e.



When carboxylic esters are hydrolysed by base catalysis, the C—O—R bond is broken between the carbon and oxygen (Remick, 1949). It has also been shown that for a similar hydrolysis of trimethylphosphate, the bond breaks between phosphorus and oxygen atoms (Blumenthal & Herbert, 1945). Since *p*-nitrothiophenol has a distinctly different colour from *p*-nitrophenol it is easy to show that, when compounds containing *P*-*S*-*p*-nitrophenyl groups are hydrolysed at pH above 7, *p*-nitrothiophenol is liberated. In confirmation the colour slowly disappears upon standing (oxidation) owing to the formation of the insoluble disulphide. A-esterase (Aldridge, 1953*b*) also breaks the bond between P and S. It is clear therefore that during base catalysis, enzymic hydrolysis by A-esterase and inhibition of the true and pseudo-cholinesterase the labile ester bond is broken next to the phosphorus atom. The mechanism put forward by Bergmann, Wilson & Nachmansohn (1950) for the hydrolysis of acetylcholine by electric-eel cholinesterase also involves a break next to the electrophilic group. It would appear, therefore, that the ester bonds break in the same place for both enzymic and non-enzymic hydrolysis.

SUMMARY

1. Reaction of organophosphorus inhibitors (of the general formula $(R)_2(X)PO$ where X is the group labile to hydrolysis) with cholinesterase produces the same inhibited enzyme provided the R groups are maintained constant even though X is varied.

2. This is a direct confirmation of the phosphorylation mechanism for inhibition.

We are grateful to Dr J. A. Cohen (Leiden, Holland) for the specimen of dimethyl phosphorochloridate, to Dr H. Coates (Albright and Wilson Ltd.) for the trimethyl phosphate, *OO*-diethyl *O*-*p*-nitrophenyl phosphate, *OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate and tetraethyl pyrophosphate and to Dr R. A. E. Galley for obtaining the *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate. Our thanks are also due to Miss J. E. Cremer and Miss J. Wheatley for valuable technical assistance.

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Physical Studies on a Soluble Protein Obtained by the Degradation of Elastin with Urea

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By the treatment of elastin with oxalic acid at 100°, Adair, Davis & Partridge (1951) obtained a soluble protein which, on gentle warming in pH 4–6 buffer solution, turned milky in appearance; cooling reversed the phenomenon. The effect was due to the separation of a second phase consisting of minute drops of protein solution at a different concentration. Electrophoresis failed to show the existence of more than one component except at an ionic strength of 0·02 and pH 3–5 when there was partial separation of two components. Porous dialysis separated the protein into two components of molecular weight (by osmometry) 6000 and 84000. Only the component of higher molecular weight showed the reversible separation of another phase on warming. Both fractionated and unfractionated material showed only one boundary on sedimentation in the ultracentrifuge, the sedimentation constant being the same in each case.

Hall (1951) showed that elastin could also be brought into solution in boiling 40% (w/v) urea. The protein obtained after removing the urea by dialysis showed great similarity to that obtained by oxalic acid degradation. This protein has been studied in the work described here. Evidence is presented which can be interpreted in terms of a reversible dissociation of the protein. The dissociation is favoured by dilution.

METHODS AND RESULTS

Preparation of the protein

Bovine ligamentum nuchae was stripped of extraneous material as thoroughly as possible by mechanical means, then finely minced. Approx. 500 g. of the minced material was mixed with 1·5 l. of 1% aqueous acetic acid and boiled under reflux for 2–3 hr. to convert collagen into gelatin. The mixture was filtered through cloth, squeezed as dry as possible and then weighed approx. 300 g. In order to prepare the urea breakdown product, about 100 g. of this elastin was refluxed with 5½ l. of 40% (w/v) aqueous urea until a clear solution was obtained. This usually took about 40 hr. The mixture was filtered through cloth and the filtrate dialysed in cellophan for 4 days against running tap water to remove urea, amino acids, etc. The solution was next evaporated under reduced pressure to 2 l. The concentrated solution was brought to pH 10 with NaOH and any residue spun off. HCl was then added to bring the solution to pH 2, any residue being spun off and rejected. The calculated quantity of solid NaCl was added to make the solution 2·5 M, when the protein separated out as a dense opalescent suspension which was centrifuged. The protein was redissolved most easily, giving a pale-yellow solution, in water brought to pH 10 with NaOH. The material obtained resembled that obtained by treating elastin with oxalic acid (Adair *et al.* 1951) in showing the reversible separation of another phase on heating. The temperature at which the milky appearance due to the separation of fine droplets of the second phase appears depends chiefly on the pH. The precise nature of the buffer present is of much less importance than the pH and the concentration of the material. At pH 2–3 an approx. 1% (w/v) solution shows the phenomenon when merely warmed in the hand, but the higher the pH of the solution the higher is the temperature required. The material gave a negative result when tested for carbohydrate by Molisch's test. It gave an intense biuret reaction.

Physical properties

Sedimentation in the ultracentrifuge. The experiments were carried out with the Spinco electrically driven ultracentrifuge using the analytical cell with 12 mm. channel. Observations were made on solutions containing 0·259, 0·106, and 0·042% N (w/v). Estimation of concentration was by