Inhibition of Cholinesterases by Fluoride *in vitro*

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(*Received* 6 *October* 1965)

1. Series of colorimetric dynamic assays allowed the study of the inhibition of cholinesterases by F^- ions *in vitro*, by using, as sources of enzyme, whole human blood, human serum, homogenized rat brain and two preparations of red blood cells (human and bovine) whose enzymic purity was ascertained. 2. The first evidence of inhibition of human serum pseudocholinesterase by fluoride was noticed at 15–25 μM-fluoride. Ten times as much fluoride was needed to start inhibition of acetylcholinesterase of the red blood cells. 3. The action of fluoride on the enzymic reaction was immediate. The reversibility of the inhibition was shown by dialysis and dilution. 4. Kinetic measurements showed that the inhibition under study was not dependent on the substrate concentration and was of the uncompetitive type, similar to that observed in the presence of a heavy metal (cadmium). 5. The activity of serum cholinesterase did not change in the absence of Mg^{2+} and Ca^{2+} ions. Fluoride was shown to inhibit the enzyme in the absence of these ions as well as of phosphate. 6. Fluoride could inhibit cholinesterases in the presence of three different substrates and had no action on the non-enzymic hydrolysis. 7. It is thought that the halide is bound reversibly to the enzyme molecule, with the probable exclusion of the active site, but no firm conclusion could be reached on this point.

Although the action of fluoride as prophylactic agent against dental caries can be partially explained by its incorporation within the mineral constituent of enamel (apatite), increasing interest is being focused upon the effects of fluoride on the metabolism of the oral flora (Goose & Hartles, 1964). Further, the pathogeny of fluoride intoxication, as pointed out by Davis (1961), still remains obscure.

These effects may quite possibly be mediated by inhibitory actions on individual enzymes or enzyme systems. Two categories of inhibition might be considered: first, where the enzyme has a metal-ion cofactor that will form a complex with fluoride; secondly, where there is no such cation cofactor requirement and fluoride would act by direct reaction with the enzyme. There are many examples of the first type, of which enzymes with magnesium requirement such as enolase (Lohman & Meyerhof, 1934; Warburg & Christian, 1942), myokinase (Siekevitz & Potter, 1953) and phosphoglucomutase (Najjar, 1948) have been studied in detail. No such studies are available for enzymes of the second category.

Cholinesterases do not require metal ions for optimum activity (Nachmahnsohn & Wilson, 1955; Augustinsson, 1960); they represent a class of enzymes whose distribution and function have been comprehensively investigated in the past few years (Wilson, 1960; Augustinsson, 1960; Kitz, 1964). The sequence of events thought to take place at their active site during hydrolysis of choline esters has been postulated as the possible mechanism applicable to other enzymic hydrolysis (Dixon & Webb, 1958*b*).

It was therefore thought that an extensive investigation of the inactivation of cholinesterases by fluoride would be useful, especially as previous studies in this field (Gomori & Chessik, 1953; Dybing & Loe, 1956; Harris & Whittaker, 1963) were concerned almost exclusively with the problem of doses of fluoride.

MATERIALS AND METHODS

Measurement of enzyme activity

The activity of the cholinesterases was determined by the colorimetric method of Ellman, Courtney, Andres & Featherstone (1961). Unless otherwise indicated, 20 μl. of the enzyme solution and 100 μl. of dithiobis(nitrobenzoic acid) (0·01 M) were added to 2·8 ml. of 0·1 M-sodium phosphate buffer, pH 8. A 20 μl. portion of the substrate (acetylthiocholine iodide or butyrylthiocholine iodide), dissolved in water, was pipetted into the cuvette at zero time of the recording (usual final substrate concentration 0·5 mM). The change in extinction was recorded at 412 mμ in a Beckman DU spectrophotometer for 8–10 min. All the

measurements were made at room temperature. Occasionally, 0·1 M-sodium barbital buffer, pH 8, and two 0·1 M-glycine buffers, pH 9 and 12, were used. Magnesium was determined spectrofluorimetrically (Schachter, 1961) and calcium by a titrimetric method (Klass, 1962). Inhibition by F^- ions was studied over the concentration range 5·2 μM-fluoride (0·1 mg./l.) to 23 mM-fluoride (450 mg./l.) by dissolving sodium fluoride in the buffer solutions.

To study the kinetics, in a first series of assays, eight concentrations of acetylthiocholine iodide, from 0·051 to 0·51 mM, were used. The suitability of the range of substrate concentrations used was tested in the presence of inhibitors having known kinetic properties: the competitive inhibitor 62C47 [1,5-bis-(4-trimethylammoniumphenyl)pentan-3-one di-iodide, antiacetylcholinesteratic; Wellcome and Co., Beckenham, Kent] (Copp, 1953), and Cd^{2+} ions, as heavy metals are known to inhibit cholinesterases in an uncompetitive fashion (Kitz, 1964). In a second series of assays, six different concentrations of fluoride were used, from 3 to 90 mg. of fluoride/l., in the presence of two given concentrations of substrate (0·1 mM and 0·5 mM-acetylthiocholine iodide).

Sources of enzyme

Whole human blood. This was diluted (1:50, v/v) with 0·1 M-sodium phosphate buffer, pH 8·0, and 0·3 ml. of this solution was used for each assay. The use of acetylthiocholine iodide as substrate gave satisfactory readings with this source of both 'true' and 'pseudo' cholinesterases (Mendel & Rudney, 1943).

Human serum. This was diluted (1:3, v/v) in the phosphate buffer and 20 μl. of this solution was used in each assay. Both acetylthiocholine and butyrylthiocholine iodides could be used successfully with this source of pseudocholinesterase.

Human haemolysed red blood cells. A portion (10 ml.) of human blood was centrifuged, the serum and top layer of white cells were discarded and the red blood cells washed three times with physiological saline (0·9%, w/v). They were then frozen to −20° and brought back to room temperature, this operation being repeated three times. Finally, the clear supernatant obtained by centrifugation (3000 rev./min.) was removed and stored at −20°; it showed a considerable cholinesterase activity ('true' cholinesterase). The purity of the enzyme from this source was checked by testing its hydrolytic properties with the two substrates, acetylthiocholine and butyrylthiocholine iodides, and by the use of the two specific inhibitors: tetraisopropylpyrophosphoramide (antibutyrylcholinesteratic; L. Light and Co. Ltd., Colnbrook, Bucks.) and 62C47. The enzyme prepared from haemolysed red blood cells was indeed active on acetylthiocholine iodide, but not on butyrylthiocholine iodide; the former was completely inhibited by 62C47 (10 μM) but not at all by tetraisopropylpyrophosphoramide (0·1 mM). A suitable portion of haemolysed red-blood-cells was diluted 1:50 in buffer when needed and 20 μl. of this solution was used in each assay.

Bovine red blood cells. A commercial preparation of purified bovine red-blood-cell cholinesterase (Sigma) was used. It was dissolved in 0·1 M-phosphate buffer to obtain an activity of about 5 i.u./ml.; 20 μl. of this solution was used in each assay. Its purity was assessed by the same procedure described for the human red-blood-cell preparation.

Homogenized rat brain. Tissue (10 mg.) was homogenized in 10 ml. of 0·1 M-phosphate buffer, pH 8·0, in a Potter–Elvehjem homogenizer. In each assay 100 μl. of this solution was used. Satisfactory readings could be obtained at this concentration of the enzyme with acetylthiocholine iodide as the substrate.

RESULTS AND DISCUSSION

Enzyme activity and fluoride concentration. The activities of cholinesterases from the various sources were determined in the presence of increasing concentrations of fluoride. Generally, four assays were run at one time, two in the absence and two in the presence of fluoride. The effects of ionic strength were checked by including equivalent concentrations of Cl^- ions in some control assays. Preliminary work had shown that the inhibition of cholinesterases by fluoride is immediate, and that its degree remains constant for long periods of time, up to 48 hr. at room temperature, or for 30 min. at 37°. When percentage activity was plotted against the logarithm of the concentrations of the halogen ion, straight lines were obtained for most of the enzyme preparations. For human serum in the presence of acetylthiocholine iodide and for rat-brain homogenate in the presence of the same substrate, the points on the semilogarithmic graph fell on two straight lines rather than one. The pI_{50} values extrapolated from such graphs are reported in Table 1. The pI_{50} values for human red-blood-cell acetylcholinesterase, bovine red-blood-cell acetylcholinesterase and esterases from human blood were approximately the same. Human serum pseudocholinesterase, in the presence of butyrylthiocholine iodide as substrate, was about seven times as sensitive to fluoride as red-blood-cell acetylcholinesterase. This difference was smaller when the substrate acetylthiocholine iodide was used for the serum pseudocholinesterase. However, if one compares the concentrations of the halide needed for liminal inhibition, serum pseudocholinesterase, with either of these substrates, was ten

Table 1. pI_{50} *values (molar concentration of fluoride) for the various enzyme preparations*

Assays were performed in 0·1 M-phosphate buffer, pH 8. The substrates were either acetylthiocholine iodide (A) or butyrylthiocholine iodide (B) at 0·5 mM concentration.

Source of enzyme	Substrate	pI_{50} (mM-fluoride)
Human red blood cells	A	2·9
Bovine red blood cells	A	2·6
Human blood	A	2·9
Human serum	B	0·4
Human serum	A	1·3
Rat brain	A	1·6

times as sensitive to fluoride as red-blood-cell acetylcholinesterase. Inhibition of the serum enzyme was first noticed in the presence of 0·3–0·5mg. of fluoride/l.(15–25 μM-F^- ions).

Reversibility. The reversibility of the inactivation of cholinesterases by fluoride was studied both by dialysis and dilution, with human haemolysed red blood cells as source of enzyme.

In the first instance (dialysis), three solutions of the enzyme in phosphate buffer were used, identical in volume and enzyme concentration. The first was F^- ion-free; the second, also F^- ion-free, was dialysed against phosphate buffer; the third contained a F^- ion concentration of 90mg./l. (4·7mM) and was similarly dialysed. The enzyme activity of the two F^- ion-free control solutions did not significantly change during the experimental time. After dialysis for about 5hr. the three solutions showed approximately the same enzyme activity. That the reversibility was due to the loss of F^- ions from the dialysis bag was shown in a similar experiment, where a solution of the enzyme, F^- ion-inhibited, was dialysed against buffer containing active enzyme: three identical human red-blood-cell acetylcholinesterase solutions in phosphate buffer were used, one inhibited by F^- ions and dialysed against the second, which was F^- ion-free. The third solution was also inhibited and was dialysed against buffer. An increase in activity was found in the two dialysed F^- ion-inhibited solutions, although limited as would be expected by this short time of dialysis (45min.). The acetylcholinesterase in the dialysing fluid of the first dialysis showed a rapid exponential drop in activity, indicating the passage of fluoride into the surrounding solution of the enzyme.

For the experiments involving dilution two portions of a human red-blood-cell acetylcholinesterase solution inhibited with fluoride were used. One was diluted in a photometer cell containing pure buffer, the other in a cell containing buffer plus fluoride. The first assay showed a much greater enzyme activity (ΔE/min.) than the second one. Thus the F^- ion inhibition of acetylcholinesterase is reversible by dilution, in the conditions of the present experiment.

Kinetics. The kinetics of the inhibition of acetylcholinesterase by fluoride were studied in various series of assays, either by varying the substrate (acetylthiocholine iodide) concentration, in the presence of a given amount of fluoride, or by changing the concentration of the halogen ion in the presence of two given concentrations of acetylthiocholine iodide.

The results of the kinetic studies, with human haemolysed red blood cells as source of acetylcholinesterase, are reported in Fig. 1. The type of inhibition that has just been described, characterized by parallel lines on the Lineweaver–Burk type of plot, is called 'uncompetitive' or 'coupling' inhibition (Webb, 1963). The K_m value obtained from the intercept of the control line on the abscissa ($-1/K_m$) is 0·086mM (concentration of acetylthiocholine iodide).

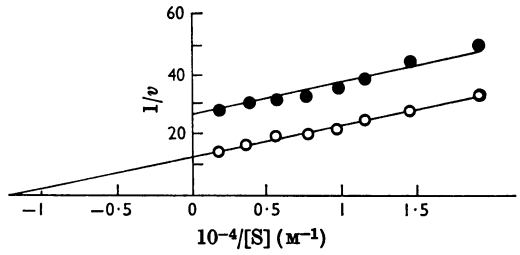

Fig. 1. Lineweaver–Burk plots of human red-blood-cell acetylcholinesterase activity: ○, in pure phosphate buffer (0·1M, pH8); ●, in the presence of 2·3mM-fluoride. Eight substrate concentrations (acetylthiocholine iodide) were used, as described in the Materials and Methods section. $10^3 \times \Delta E$/min. was used as a measure of v.

When using the bovine red-blood-cell source of enzyme, the experiment was not limited to a single concentration of fluoride, but included a wide range of halide concentrations (1·5, 4·7 and 14mM) which are plotted on the $1/v$–$1/[S]$ type of plot (Fig. 2). The results are similar to those obtained with the human source of enzyme. The highest concentration of fluoride (14mM-F^- ions) gave, however, a line that was not strictly parallel to the control plot. It will be noticed that, with increasing concentrations of fluoride, the maximum velocity (V, ordinate intercept) is decreased and so is K_m (determined from the intercept on the abscissa, not shown on Fig. 2), in such a way as to yield parallel lines. At various concentrations fluoride is an uncompetitive inhibitor of acetylcholinesterase. The value for K_m, obtained by extrapolation, is 0·085mM; it is lower than that indicated by Ellman *et al.* (1961).

When two known inhibitors, Cd^{2+} ions (0·25mM, in a veronal buffer, pH8) and 62C47 (0·1 μM), were tested, parallel lines were obtained for the former on the Lineweaver–Burk type of plot, and converging lines for the latter, as expected.

As for the assays performed while changing the concentration of fluoride in the presence of two given concentrations of substrate, they gave parallel lines on a $1/v$–[I] type of plot (Dixon & Webb, 1958*a*), thus confirming the results presented above, for both the human and bovine source of acetylcholinesterase.

Studies on activators. Attention was particularly focused upon Mg^{2+} ions, since it has been repeatedly said that the formation of a fluoride–magnesium

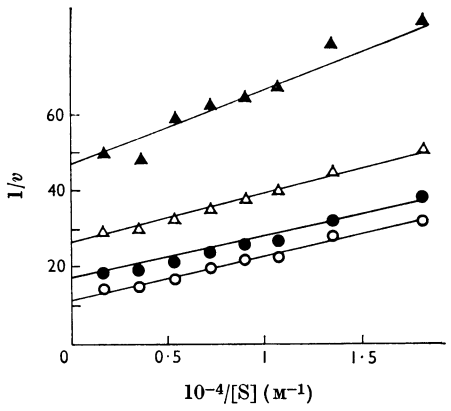

Fig. 2. Lineweaver–Burk plots of bovine red-blood-cell acetylcholinesterase activity: ○, in pure phosphate buffer; ●, in the presence of 30mg. of fluoride/l. (1·5mM); △, in the presence of 90mg. of fluoride/l. (4·7mM); ▲, in the presence of 270mg. of fluoride/l. (14mM). Eight substrate concentrations were used, as described in the Materials and Methods section. $10^3 \times \Delta E$/min. was used as a measure of v.

complex is a likely mechanism by which enzyme activity is inhibited by fluoride. The hypothetical role of Ca^{2+} and PO_4^{3-} ions was also investigated.

First, the solution of bovine red-blood-cell acetylcholinesterase used for the kinetic study reported in Fig. 1 was analysed for Mg^{2+} and Ca^{2+} ions and was found to be free of these. Secondly, with human serum diluted 1:4 in the glycine buffers of either pH 9 or pH 12, and the two substrates acetylthiocholine iodide and butyrylthiocholine iodide, a series of assays was run in the presence or in the absence of 2mM-disodium ethylenediaminetetra-acetate (Complexon III; Siegfried, Zofingue, Switzerland). The solutions containing the complex-forming agent were free from Mg^{2+} and Ca^{2+} ions. Enzyme activities were found to be identical in the control and in the chelator-containing solutions. At pH 12, as could be expected, the enzyme activity was greatly impaired. Hence, in the conditions of the present experiment, the absence of Mg^{2+} and Ca^{2+} ions (and of any other ion forming chelate rings with Complexon III) had no effect upon the activity of cholinesterases.

The role which phosphate could play in the inhibition of cholinesterases by fluoride was checked by studying the action of the halide upon the enzyme in veronal buffer. In the presence of increasing concentrations of fluoride, the enzyme activities in veronal buffer decreased in a manner comparable with that observed when phosphate buffer was used.

Furthermore, the effect of Ca^{2+} ions on the activity of acetylcholinesterase was studied in the presence of acetylthiocholine iodide as substrate. With haemolysed human red blood cells in veronal buffer, pH 8, assays were run in the presence of calcium chloride or sodium chloride at the concentrations of 0·01 and 0·1M. The activity of acetylcholinesterase was not impaired in the sodium chloride-containing solutions, whereas in the presence of 0·01M- and 0·1M-calcium chloride there was 10% and 30% inhibition respectively. If, as pointed out by Hofstee (1960), Ca^{2+} ions can greatly enhance cholinesterase activity in the presence of non-polar substrates such as fatty acid esters, this does not seem to be the case when polar substrates, such as acetylthiocholine, are used. It is thought (Hofstee, 1960) that the action of Ca^{2+} or other bivalent cations would be to neutralize and inactivate the anionic part of the cholinesterase active site (Wilson, 1962), which normally facilitates the approach of polar substrates, but hinders the approach and proper orientation of a non-polar substrate.

Studies on the substrate. The possible participation of the substrate in the inhibitory action of fluoride upon cholinesterases was studied either by using a substrate different from the two utilized in the previous assays or by recording the action of the halide upon the non-enzymic hydrolysis of the substrate acetylthiocholine iodide.

In the first instance, 40μl. of human serum was dissolved in 2ml. of a solution containing the non-specific substrate α-naphthyl acetate, and Fast Garnet GBC salt, prepared according to Pearse (1961); a black azo-dye is formed in the test tube upon hydrolysis of α-naphthyl acetate. In the presence of 90mg. of fluoride/l. (4·7mM) it was repeatedly observed that no black colour could be formed.

In the second instance, non-enzymic hydrolysis of the substrate acetylthiocholine iodide could be recorded by allowing the assay to last 30 or 60min. at room temperature, or 10min. at 60°, in the absence of any enzyme. In these conditions, extinction values were identical in the absence or in the presence of fluoride (90mg./l.).

In conclusion, the inhibition of cholinesterases by fluoride does not take place through the formation of complexes of fluoride with magnesium, phosphate or calcium; nor does the substrate play a role in the mechanism of inhibition. It can therefore be postulated that the enzyme molecules themselves are directly affected by the halide ions.

The author is deeply grateful to Dr D. Young, Department of Clinical Biochemistry, University of Geneva, for his interest and encouragement. The help of Dr R. Scherrer, Dr M. C. Sanz and Dr C. Rosenbush is also gratefully acknowledged. The inhibitor 62C47 was kindly supplied

by Dr H. T. Openshaw of Wellcome Laboratories. This research was aided by grants of the Swiss National Fund for Scientific Research (no. 2478) and of the Swiss Odontological Society.

REFERENCES

Augustinsson, K. B. (1960). In *The Enzymes*, vol. 4, p. 521. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Copp, F. C. (1953). *J. chem. Soc.* p. 3116.

Davis, R. K. (1961). *J. occup. Med.* **3**, 52.

Dixon, M. & Webb, E. (1958*a*). *Enzymes*, p. 25. New York: Academic Press Inc.

Dixon, M. & Webb, E. (1958*b*). *Enzymes*, p. 310. New York: Academic Press Inc.

Dybing, O. & Loe, L. V. (1956). *Acta pharmacol. toxicol., Copenhagen*, **12**, 364.

Ellman, G. L., Courtney, K. D., Andres, V., jun. & Featherstone, R. M. (1961). *Biochem. Pharmacol.* **7**, 88.

Gomori, G. & Chessik, R. D. (1953). *J. cell. comp. Physiol.* **41**, 51.

Goose, D. H. & Hartles, R. L. (1964). *Principles of Preventive Dentistry*, p. 104. Oxford: Pergamon Press Ltd.

Harris, H. & Whittaker, M. (1963). *Ann. hum. Genet., Lond.*, **27**, 53.

Hofstee, B. H. (1960). *J. Pharmacol.* **128**, 299.

Kitz, R. (1964). *Acta anaesth. scand.* **8**, 197.

Klass, C. S. (1962). *Amer. J. clin. Path.* **37**, 655.

Lohman, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.

Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.

Nachmahnsohn, D. & Wilson, I. B. (1955). In *Methods in Enzymology*, vol. 1, p. 642. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Najjar, V. A. (1948). *J. biol. Chem.* **175**, 281.

Pearse, A. G. E. (1961). *Histochemistry: Theoretical and Applied*, p. 886. London: J. and A. Churchill Ltd.

Schachter, D. (1961). *J. Lab. clin. Med.* **58**, 495.

Siekevitz, P. & Potter, V. R. (1953). *J. biol. Chem.* **200**, 187.

Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.

Webb, L. J. (1963). *Enzyme and Metabolic Inhibitors*, vol. 1, p. 160. New York: Academic Press Inc.

Wilson, I. B. (1960). In *The Enzymes*, vol. 4, p. 501. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Wilson, I. B. (1962). In *Ciba Found. Symp.: Enzymes and Drug Action*, p. 4. Ed. by Mongar, J. L. & de Reuck, A. V. S. London: J. and A. Churchill Ltd.