Biochemical Pharmacology, 1962, Vol. 11, pp. 113–124. Pergamon Press Ltd., Printed in Great Britain.

CHOLINESTERASES IN THE DOMESTIC FOWL AND THE SPECIFICITY OF SOME REVERSIBLE INHIBITORS

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(*Received* 24 *July* 1961; *accepted* 18 *September* 1961)

Abstract—The properties of cholinesterases from several tissues of the domestic fowl have been studied *in vitro*. Substrate-inhibitor relationships showed that the enzyme corresponding to mammalian acetylcholinesterase resembled the mammalian enzyme in most respects but was considerably less active in its ability to combine with bisquaternary substrates or inhibitors. This suggests some difference between the active sites in the mammalian and the fowl enzymes. The enzyme corresponding to mammalian butyrocholinesterase was found to have properties intermediate between those of mammalian butyrocholinesterase and those of the fowl acetylcholinesterase. "Specific" inhibitors of mammalian butyrocholinesterase were less potent against the corresponding enzyme in the fowl while "specific" inhibitors of mammalian acetylcholinesterase possessed considerable inhibitory potency against the fowl plasma enzyme. Thus no specific inhibition could be obtained. The enzyme corresponding to butyrocholinesterase, like the fowl acetylcholinesterases, was unable to combine with bis-quaternary compounds.

INTRODUCTION

PREVIOUS workers[1–3] have shown that, although the properties of plasma cholinesterase in the fowl are essentially those of a butyrocholinesterase, it is capable, unlike its mammalian counterpart, of hydrolysing acetyl-β-methylcholine. Brain cholinesterase in the fowl has been investigated by Myers[3] who reported that the enzyme was a true cholinesterase which showed a higher activity towards proprionylcholine than to acetylcholine. Ferrari[4] found that the serum and brain cholinesterases of hens, ducks, and pigeons were insensitive to the anticholinesterases CT3318 and eucapine, whereas most mammalian cholinesterases were readily inhibited. Reports from this laboratory have shown that some anticholinesterases have markedly different potencies in the fowl and the mammal. The enzyme present in hen skeletal muscle was only slightly inhibited by Ambenonium, a bisquaternary compound usually considered to be a specific inhibitor of mammalian acetylcholinesterase, whilst the enzyme present in the plasma appeared unable to hydrolyse the bisquaternary substrate succinylcholine, in contrast to the plasma enzyme of the cat.[5] Results obtained by Cuthbert[6] using the smooth muscle tissue of the chick amnion, also showed that the properties of the enzyme present differed from those of both acetyl- and butyrocholinesterase of the mammal.

In view of these reports it was felt of interest to make a more thorough investigation of the properties of the cholinesterases present in a number of different tissues of the

* One of us (A.W.C.) was in receipt of a Pfizer Ltd., Research Award during the tenure of this work.

fowl. The results obtained emphasize the possible fallacies that may arise when results obtained with the cholinesterase of one species are used to interpret the effects of anti-cholinesterases in another species.

METHODS

The cholinesterase activity of various tissues from white leghorn hens (*Gallus domesticus* L.) (killed by a blow on the head), weighing between 1–1½ kg, and of chick embryo amniotic membranes were estimated manometrically at 37 °C in an atmosphere of 95% nitrogen and 5% carbon dioxide. The enzyme preparation and the reversible inhibitors were placed in the main compartment of the flasks and 0·5 ml of a solution of the substrate in distilled water (with the exception of tributyrin) was placed in the side arm, the total volume of fluid in each flask was 3 ml. Because of its insolubility, tributyrin was pipetted directly into the main compartment and the enzyme preparation placed in the side arm. Inhibition with di*iso*propylphosphorofluoridate was obtained by incubation of the tissue for 1 hr at 37 °C before it was placed in the main compartment of the flasks. All estimations were carried out in bicarbonate buffer previously adjusted to pH 7·6, the final concentration of $NaHCO_3$ being 0·033 M.

The activity is expressed as Q_{co_2} values (μl CO_2/g weight of tissue or ml of plasma/hr) allowance being made for non-enzymic hydrolysis. Ten minutes was allowed for equilibration after tipping the substrate, and readings taken at 10 min intervals for 30 min. The enzyme preparations were prepared from:

(a) Erythrocytes. Heparinized blood was centrifuged at 3000 rev/min for 10 min, the plasma separated and the cells washed three times in normal saline and finally packed by centrifuging at 3000 rev/min for 10 min. A volume of 0·2 ml of packed erythrocytes was used for each estimation.

(b) Plasma. This was diluted five times in distilled water; 0·5 ml of this dilution was used in each flask.

(c) Brain. This was homogenized in distilled water in an all glass homogenizer, to give a final concentration of 50 mg wet weight/ml of tissue; 0·5 ml of this suspension was used for each estimation.

(d) Small Intestine. This was scraped free of mucus, chopped and homogenized as for brain to give a concentration of 50 mg wet weight/ml of tissue; 0·5 ml was used in each flask.

(e) Gastrocnemius muscle. This was freed from tendon and fibrous capsule, finely chopped, and homogenized in bicarbonate buffer such that 2 ml contained 300 mg of fresh tissue; 2 ml of this suspension were placed in each flask, the final concentration of $NaHCO_3$ being 0·033 M.

(f) Chick embryo amniotic membranes. These were dissected from eggs previously incubated for 8–12 days at 37 °C. They were homogenized as for brain to give a final concentration not exceeding 50 mg wet weight/ml of tissue; 0·5 ml of this suspension being used in each flask.

The substrates used were: acetylcholine chloride, Roche (Ach); butyrylcholine iodide, Roche (Buch); butyrylthiocholine iodide, Roche (BuThch); ± acetyl-β-methylcholine chloride, Light & Co. (Mech); acetylthiocholine chloride, Light & Co. (AThch); propionylcholine chloride, Light & Co. (Prch); benzoylcholine, B.D.H. (Bzch); tributyrin, B.D.H. (Tb); Succinylcholine chloride, Allen & Hanbury (Such).

The inhibitors used were ethopropazine hydrochloride (May & Baker), N-*p*-chlorophenyl-N-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Ro2-1250) (Roche), 1:5-bis-(4-trimethylammonium phenyl) pentan-3-one diiodide (62C47) (Wellcome), D*iiso*propyl phosphoro fluoridate (D.F.P.) (Boots), physostigmine salicylate (B.D.H.), and neostigmine methylsulphate (Roche).

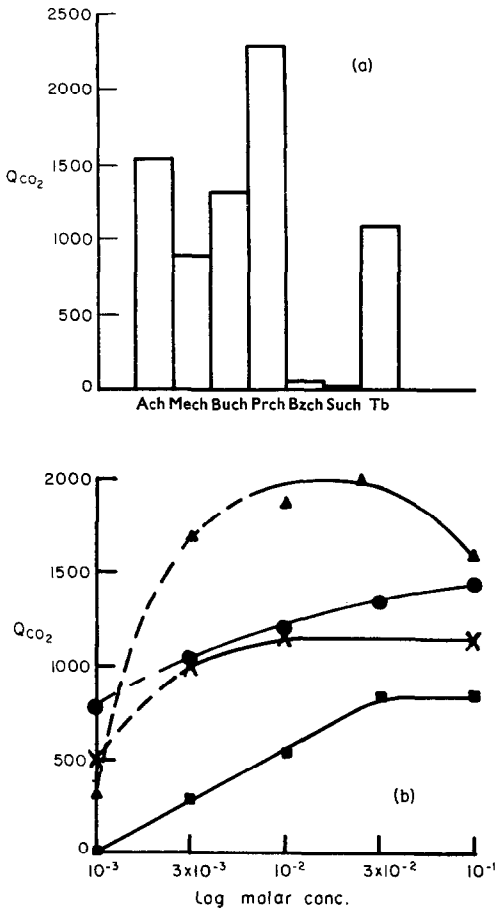

FIG. 1. Plasma cholinesterase. (a) Histogram. All substrates used in a final concentration of 3×10^{-2} M. (b) Substrate-activity curves. Where the output of CO_2 was not linear the curve is shown dotted. ● Ach, ■ Mech, × Buch ▲ Prch.

RESULTS AND DISCUSSION

Pseudocholinesterase

Plasma. Fig. 1 (a) is a histogram showing the activity of the plasma enzyme against seven substrates each at a final concentration of 3×10^{-2} M, and Fig. 1 (b) shows substrate activity curves against four substrates.

The results show that the enzyme is a proprionylcholinesterase and has a high activity against acetyl-β-methyl choline thus confirming the reports of previous

workers.[2, 3, 7] In addition to this anomaly there was very low activity against benzoylcholine and succinylcholine. The activity towards tributyrin was completely inhibited by 10^{-5} M physostigmine and was therefore due to cholinesterase and not to other esterases.[8] The enzyme had approximately twice the activity towards the thio analogues of Ach and Buch as towards the oxygen analogues, which agrees with the findings for mammalian cholinesterase.[9] The shape of the substrate activity curves (Fig. 1 (b)) for Ach, Mech and Buch are typical of a butyrocholinesterase,[10] whereas Prch shows an optimal activity typical of an acetylcholinesterase. This is at variance with the results of Myers[3] who did not obtain an optimum on the Prch substrate-activity curve.

These results indicate that the properties of fowl plasma cholinesterase are intermediate between those of mammalian acetylcholinesterase and butyrocholinesterase.

Ethopropazine HCl has been described by Bayliss and Todrick[11] as a specific inhibitor of butyrocholinesterase in the rat. However, at a concentration of 5×10^{-5} M, the concentration reported by Bayliss and Todrick to inhibit completely butyrocholinesterase in the rat, the enzyme present in fowl plasma was only 80 per cent inhibited. Fig. 2 (a) shows that the hydrolysis of Ach, Mech, and Buch were affected to a similar extent by this inhibitor. The fact that the hydrolysis of all three substrates was affected to the same extent is further confirmation that only one enzyme is present in the plasma.

62C47, a specific inhibitor of acetylcholinesterase of rat brain[12] was found not to inhibit fowl plasma cholinesterase in concentrations as high as 10^{-3} M when Ach or Buch were used as the substrates, although a small amount of inhibition was produced at 10^{-3} M with Mech as the substrate.

Ro2-1250, another specific inhibitor of acetylcholinesterase, in rat erythrocytes and dog brain[13] was found to be more potent than 62C47 against the fowl plasma cholinesterase. Fig. 2 (b) shows that hydrolysis of both Ach and Mech were inhibited to a similar extent; this inhibitor is 10^4 times more potent than the "specific" butyrocholinesterase inhibitor ethopropazine HCl. The curve for Buch approached those of Ach and Mech only if the substrate concentration was reduced from 3×10^{-2} M to 3×10^{-3} M (Fig. 2 b).

D.F.P. inhibition is not reversed by high substrate concentrations, and a concentration of 10^{-9} M was found to inhibit hydrolysis of Ach by 24·9 per cent, of Mech by 23·3 per cent and Buch by 25·4 per cent. Earl and Thompson[2] found 78 per cent inhibition of Mech hydrolysis and 79 per cent inhibition of Buch hydrolysis using 3×10^{-9} M D.F.P.

With Ach as substrate, inhibition by physostigmine and neostigmine was found to be similar (Fig. 2 c) 50 per cent inhibition being produced by $2{\cdot}6 \times 10^{-8}$ M physostigmine and $2{\cdot}2 \times 10^{-8}$ M neostigmine.

It is therefore clear that the cholinesterase present in the fowl plasma consists of only one enzyme which has properties intermediate between those of mammalian acetyl- and butyrocholinesterase, and will henceforth be termed the intermediate cholinesterase.

The differences in inhibition of hydrolysis of various substrates by the reversible inhibitors is probably a consequence of the differing affinities of the various substrates for the enzyme. This was clearly illustrated in the case of Ro2-1250 with Buch as the substrate, the inhibition was reversed by 3×10^{-2} M Buch, but a similar inhibition

curve to that obtained using Ach and Mech as substrates could be produced using a lower concentration of Buch. This indicates that Buch has a greater affinity for the enzyme than either Ach or Mech. The results obtained with 62C47 indicated that Mech had less affinity for the enzyme than either Ach or Buch since only inhibition of Mech hydrolysis was observed. This places the order of affinity of the substrates as Buch > Ach > Mech the order expected for a plasma cholinesterase.

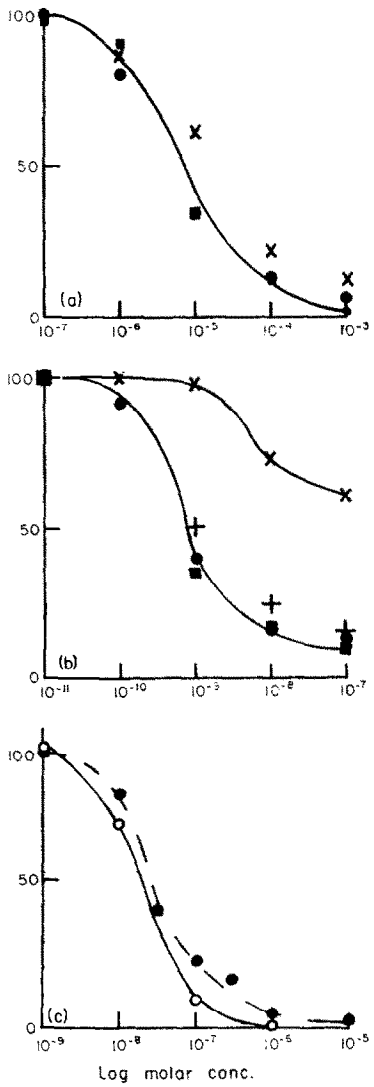

FIG. 2. Inhibition of plasma cholinesterase. (a) ethopropazine HCl. (b) Ro2-1250. Ach and Mech used in a final concentration of 3×10^{-2} M. Buch used at 3×10^{-2} M and 3×10^{-3} M ● Ach, ■ Mech, × Buch, (3×10^{-2} M). + Buch (3×10^{-3} M). (c) Neostigmine methylsulphate and physostigmine salicylate, ——— Neostigmine, - - - - Physostigmine. Final concentration of Ach was $1{\cdot}38 \times 10^{-3}$ M. In all three histograms vertical axes represent percent control activity.

Distribution of the intermediate cholinesterase

Fig. 3 shows a comparative histogram for plasma, intestinal muscle, and chick amnion cholinesterases against various substrates. The correlation is such that the same enzyme can be said to be present in all three tissues, but the possibility of a small contribution by other esterases cannot be excluded.

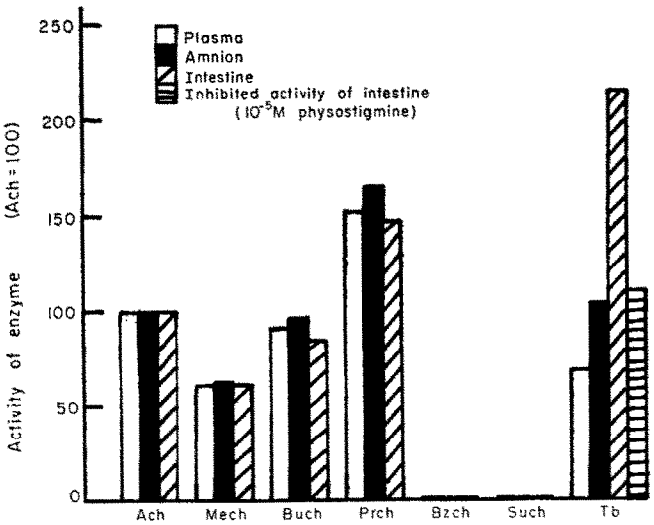

FIG. 3. Comparative histogram. All substrates used in a concentration of 3×10^{-2} M.

The high value for Tb for the intestinal enzyme was shown to be due to the presence of esterase other than cholinesterase. The hydrolysis of Tb attributable to cholinesterase was assessed by inhibition with 10^{-5} M physostigmine[8] and was found to be equivalent to each of the activities of the other two tissues. The substrate activity curve for amnion using Ach as the substrate was found to be of the butyrocholinesterase type.

In the mammal, the intestine contains mainly butyrocholinesterase, whereas in the fowl the intermediate cholinesterase is present. Similarly the amnion would be expected to contain butyrocholinesterase, being an aneuronal smooth muscle structure, but again the intermediate enzyme is present. This report is contrary to that of Kuschinsky *et al.*[14] who concluded that chick amnion cholinesterase was acetylcholinesterase, but this opinion was based only on the ability of the enzyme to hydrolyse Mech and not Bzch.

It appears likely, therefore, that in the fowl the intermediate enzyme is the equivalent of mammalian butyrocholinesterase.

True cholinesterase

Erythrocytes. No enzyme activity could be detected in the erythrocytes against any of the substrates investigated, this confirms the previous findings of Augustinsson[15] and Davies *et al.*[7]

Brain. Fig. 4 (a) shows the substrate-activity curves of fowl brain cholinesterase against four different substrates, the shape of the curves is typical of acetylcholinesterase.[10] The activity of the brain enzyme was found to be very high confirming the findings of Nachmansohn[16] and Davison[17].

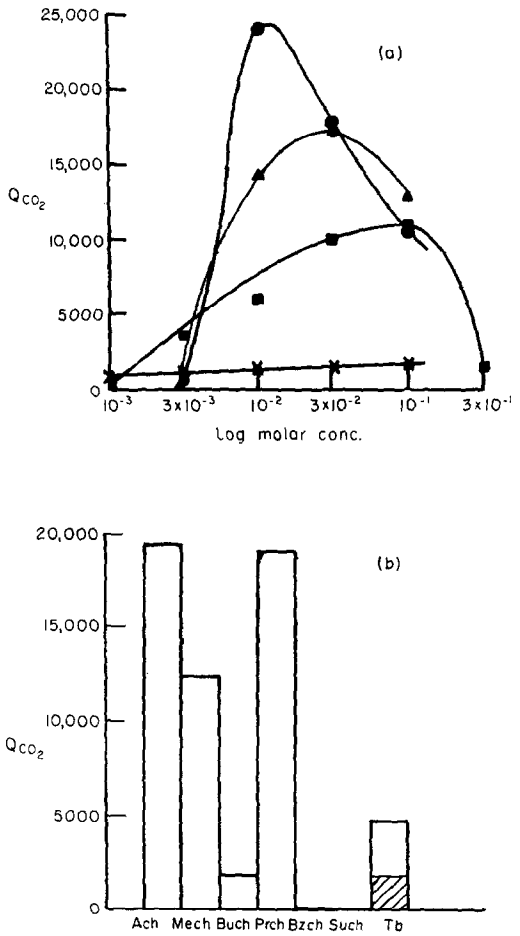

FIG. 4. Brain cholinesterase. (a) Substrate-activity curves. ● Ach, ■ Mech, × Buch ▲ Prch. (b) Histogram. All substrates used in a final concentration of 3×10^{-2} M. The hatched portion represents Tb activity due to cholinesterase.

In contrast to the findings of Myers[3] we did not find the brain cholinesterase to be a proprionylcholinesterase, but the curves indicate a typical acetylcholinesterase. No obvious explanation of this difference is apparent, but Myers carried out his experiments in the presence of 4×10^{-9} M D.F.P. This would undoubtedly inhibit any intermediate enzyme present but may have altered the affinity of the remaining enzyme for the various substrates. The hydrolysis of Buch may represent a low activity of the acetylcholinesterase against the substrate or may indicate a proportion of

another cholinesterase, this proportion must be very low since the Q_{CO_2} value was only 1500 against that of Ach of 25,000.

The histogram (Fig. 4b) shows the activity of the brain enzyme against seven substrates each at a concentration of 3×10^{-2} M, and is again typical of acetylcholinesterase.

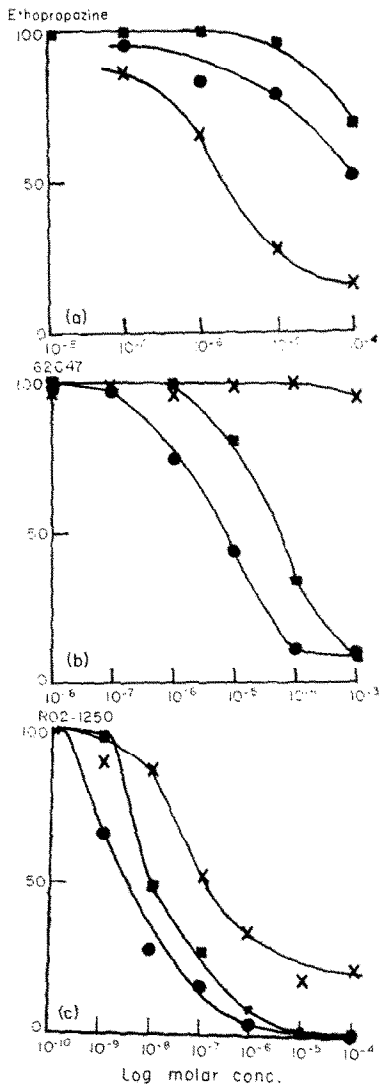

FIG. 5. Inhibition of brain cholinesterase. All substrates used in a final concentration of 3×10^{-2} M, ● Ach, ■ Mech, × Buch. (a) ethopropazine HCl. (b) 62C47. (c) Ro2-1250. Vertical axes represent percent control activity.

Estimation of the percentage inhibition with 3×10^{-9} M D.F.P. gave 9 per cent using Ach, 2·8 per cent using Mech, and 52·8 per cent using Buch, as substrates. The hydrolysis of Buch was, however, too low to permit an accurate estimation of the degree of inhibition. The figures do indicate, however, that activity of the brain

is possibly due to a mixture of acetylcholinesterase with a very low proportion of a second cholinesterase.

Inhibition of fowl brain cholinesterase with ethopropazine HCl, 62C47, and Ro2-1250 is given in Fig. 5, (a), (b) and (c), respectively, and comparison with Fig. 2 shows that no selective inhibition of either the acetylcholinesterase nor the intermediate cholinesterase could be obtained at any concentration of the inhibitors used.

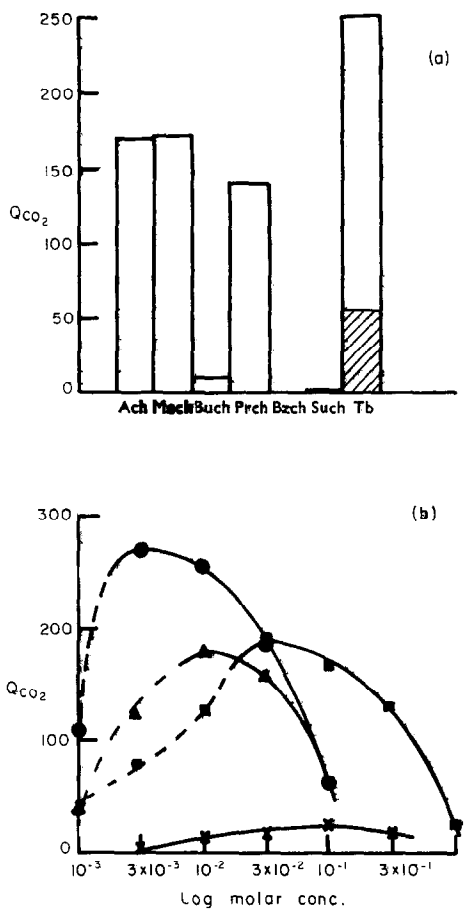

FIG. 6. Skeletal muscle cholinesterase. (a) Histogram. All substrates used in a final concentration of 3×10^{-2} M. The hatched portion represents Tb activity due to cholinesterase. (b) Substrate-activity curves. Where the output of CO_2 was not linear the curve is shown dotted. ● Ach, ■ Mech, × Buch, ▲ Prch.

62C47 is less effective against fowl brain than against rat brain. Fulton and Mogey[12] give the pI_{50} against rat brain as 6·7 whereas for fowl brain the figure was approximately 5 using Ach and Mech as substrates.

The pI_{50} of Ro2-1250 was approximately 8·0 which agrees with the figures of Hawkins and Mendel[13] for human erythrocytes, however, fowl plasma is more sensitive to this inhibitor but not sufficiently so to be in any way selective.

The bisquaternary inhibitor ambenonium is not effective in inhibiting fowl acetylcholinesterase[5] having a pI_{50} of 4·6 compared to 7·6 for cat brain[18] and 8·2 for cat tibialis.[19]

Skeletal muscle. Fig. 6 shows the histogram and substrate activity curves for the cholinesterase of skeletal muscle. The high Tb activity was again mainly due to an esterase other than cholinesterase, that due to cholinesterase being shown by the hatched portion. 3×10^{-9} M D.F.P. gave greater inhibition than with brain, and again the extent of the inhibition varied with the three substrates, Ach hydrolysis was inhibited by 19·6 per cent, Mech hydrolysis by 15·9 per cent and Buch hydrolysis by 25·7 per cent. The Q_{CO_2} for Buch was, again, too low for accurate determination of the percentage inhibition.

The results indicate that the enzymes in fowl skeletal muscle consist principally of an acetylcholinesterase, with a small proportion of a second cholinesterase.

CONCLUSIONS

The domestic fowl does not possess a typical butyrocholinesterase, the plasma and intestinal muscle contain a cholinesterase intermediate in properties between those of fowl acetylcholinesterase and mammalian butyrocholinesterase. Brain and skeletal muscle contain acetylcholinesterase and a small proportion of a second cholinesterase which may be identical with the intermediate enzyme. Further, we suggest that the distribution of the intermediate enzyme is similar to that of butyrocholinesterase in the mammal.

Bisquaternary inhibitors are less effective against fowl acetylcholinesterase than the corresponding enzyme in the mammal and bisquaternary substrates are not hydrolysed. This may represent a difference between the anionic sites in fowl and mammalian cholinesterase.

The monoquaternary selective inhibitor Ro2-1250 has a similar potency against fowl acetylcholinesterase as against mammalian acetylcholinesterase, but the plasma cholinesterase due to its intermediate nature is also inhibited. For the same reason the monoquaternary inhibitor of butyrocholinesterase ethopropazine HCl, is less effective against the plasma enzyme thus preventing selective inhibition.

These results emphasize the possible fallacies that may arise when results obtained with inhibitors in one species are used to interpret results in another.

Acknowledgements—We are indebted to the following firms for their generous gifts of materials: to May & Baker Ltd. for ethopropazine hydrochloride, to Burroughs Wellcome Ltd. for 62C47 and to Roche Ltd. for Ro2-1250. Our thanks are also due to Dr. F. Hobbiger for valuable advice concerning the manuscript.

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