# CCXVI. THE SPECIFICITY OF CHOLINE-ESTERASE

BY LESLIE HILTON EASSON AND EDGAR STEDMAN

*From the Department of Medical Chemistry, University of Edinburgh*

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THE parallelism between the hydrolytic activities of human blood serum towards choline esters and tributyrin, originally observed by Stedman *et al.* [1933], has recently been confirmed by Vahlquist [1935], who therefore suggests that a name other than lipase should be found for the enzyme responsible for the activities in question. The implication that the name choline-esterase, proposed for the enzyme by Stedman *et al.* [1932], is not appropriate compels us to summarize our results on its specificity and to offer further experimental evidence in support of our previous conclusions.

Our earlier publications show that choline-esterase is specific to the extent that its principal action is to hydrolyse simple esters of choline. Judging from the behaviour of human serum, it probably also exercises a small but readily detectable action on tributyrin. Its action, however, on methyl butyrate, if any, is so small as to lie within the limits of experimental error of the procedure hitherto employed. Vahlquist, as a result of his examination of human serum, does not claim that the hydrolysis of methyl butyrate is brought about by choline-esterase, although he states that the degree of inhibition of the enzymic activity by eserine was "practically the same whether acetylcholine, tributyrin or methyl butyrate were used as substrates". This statement is, perhaps, scarcely borne out by his experimental results, which show that the concentration of eserine required to produce a 50 % inhibition of the hydrolysis of acetylcholine is twice that necessary to cause the same inhibition in the case of methyl butyrate. Nevertheless, it does appear probable from the results presented below that choline-esterase exercises a small hydrolytic action on methyl butyrate and is, in fact, responsible for the whole of the activity of human serum towards this substrate. Such a conclusion is not, however, in contradiction with our previous results. Human serum is peculiar in possessing only a small activity towards methyl butyrate. It was, indeed, classified by Stedman *et al.* [1933] with those sera in which a high choline-esterase activity was associated "with an almost negligible...esterase activity". By modifying the method of estimation it has now been possible to measure its activity towards methyl butyrate more accurately, with the result that this has proved to be only 1/80 of that towards butyrylcholine. An activity of this magnitude lies within the limits of error of the method of estimation hitherto used. For example, Stedman & Stedman [1935] found that the quantity of an extensively purified preparation of choline-esterase from horse serum which liberated in 20 min. from butyrylcholine an amount of acid equivalent to 6·35 ml. of 0·02*N* alkali caused no detectable liberation of acid from methyl butyrate under the same conditions. If the relative activities of horse choline-esterase towards methyl butyrate and butyrylcholine are the same as for human serum, a titration figure of 0·08 ml. should have been obtained when the simple ester was used as substrate, a figure which is quite within the limits of experimental error.

It is thus possible that choline-esterase from the horse, like that in human blood, does attack methyl butyrate to a small extent, although such activity can hardly be regarded as significant.

The fact that human serum apparently contains only one esterase, namely choline-esterase, does not imply that a similar state of affairs holds for sera from other species. We have, indeed, already shown that the contrary is the case, and further evidence in confirmation of our earlier results will be found below.

Some reference must, finally, be made to a paper by Shaw [1935], in which considerable criticism is made of our work on choline-esterase. It will suffice to say, as a reference to the literature will show, that all such criticism is based on misstatements of our results.

*Inhibition of serum esterases*

Shaw states: "It is remarkable that Stedman, having purified the enzyme [choline-esterase], should not have tried the effect of eserine on its action." The reason for the delay in publishing the results of such experiments, which were naturally carried out, is explained elsewhere [Easson & Stedman, 1936]. It might, however, be mentioned in connexion with the present work that, as far back as July 1933, we observed that a concentration of miotine which produced a 75% inhibition in the choline-esterase activity of guinea-pig serum had no effect on its activity towards methyl butyrate, nor was any inhibition of the activity of the enzyme responsible for the hydrolysis of the latter substrate produced when the concentration of the drug was increased tenfold. As this result appeared merely to confirm the conclusion of Stedman *et al.* [1933] that serum from this species contained, in addition to choline-esterase, at least one other esterase, the matter was not pursued further and consequently the single experimental result was not published. In view of the fact that Vahlquist has now applied this method to human serum, using eserine as inhibitor, with the results discussed above, it appeared to us desirable to extend our unpublished experiment. We have therefore examined the inhibitory actions of prostigmine and, in some cases, eserine on the enzymes present in horse, guinea-pig and human blood sera, using butyrylcholine, methyl butyrate and tributyrin as substrates.

The method adopted for the estimation of the various activities was that of continuous titration in the form described by Stedman *et al.* [1933]. Two slight modifications have, however, been made: the normality of the alkali employed was reduced to 0·01, while a slow stream of $CO_2$-free air was passed through the flask, but not through the liquid, during the titration. In some cases the tributyrin activity was determined by the stalagmometric method [Stedman & Stedman, 1931]. The substrates in the titration experiments consisted of 0·25 g. of butyrylcholine bromide, 0·25 ml. methyl butyrate and 0·1 ml. tributyrin respectively. All estimations were made at *p*H 8·0. In measuring the inhibition produced by the inhibitor, the serum was mixed with an equal volume of a solution of the drug of suitable concentration and left overnight. For the control, water was used in place of the solution of the drug. The results obtained with guinea-pig, horse and human sera are collected in Tables I, II and III respectively. In these Tables the following abbreviations have been used: B.C.B. = butyrylcholine bromide; M.B. = methyl butyrate; T.B. = tributyrin; $[I]$ = molar concentration of inhibitor in the serum-inhibitor mixture; $i$ = fractional inhibition. The inhibitor employed was usually prostigmine; an asterisk indicates the use of eserine.

Table I. *Inhibition of esterases present in guinea-pig serum*

| Substrate | Vol. of serum mixture (ml.) | [*I*] | ml. 0·01 *N* alkali | Diminution in drop no. | *i* |
|---|---|---|---|---|---|
| B.C.B. | 2 | 0 | 10·05 | — | |
| | 2 | $10^{-7}$ | 0·75 | — | 0·925 |
| M.B. | 2 | 0 | 8·55 | — | |
| | 2 | $10^{-7}$ | 8·85 | — | Activation |
| T.B. | 0·1 | 0 | — | 15 | |
| | 0·1 | $10^{-7}$ | — | 15 | 0 |
| M.B. | 1 | 0 | 3·35 | — | |
| | 1 | $10^{-5}$ | 4·05 | — | Activation |
| T.B. | 0·5 | 0 | — | 23 | |
| | 0·5 | $5 \times 10^{-4}$ | — | 22 | 0 |
| M.B. | 1 | 0 | 6·10 | — | |
| | 1 | $5 \times 10^{-3}$ | 1·70 | — | 0·72 |
| T.B. | 0·1 | 0 | — | 9 | |
| | 0·1 | $5 \times 10^{-3}$ | — | 5 | 0·44 |

A general confirmation of our previous conclusions can at once be drawn from these results. In the case of the guinea-pig, it can be seen that a concentration of prostigmine ($10^{-7}$ *M*) which produces a fractional inhibition of 0·925 in the choline-esterase activity of the serum exerts no detectable inhibition on its activities towards methyl butyrate and tributyrin. It is not, indeed, until a concentration of the inhibitor of $5 \times 10^{-3}$ *M* is reached that definite inhibition of these activities becomes perceptible. The conclusion appears to be inescapable that guinea-pig serum contains at least two ester-hydrolysing enzymes, namely, choline-esterase, which attacks esters of choline but has no appreciable action on methyl butyrate, and an enzyme, which we designate by the old term esterase, capable of hydrolysing simple esters. Esterase is almost certainly responsible for at least part of the activity of the serum towards tributyrin, but we do not wish to exclude the possibility that a third enzyme is involved in the case of this substrate or that choline-esterase itself does exert a relatively small activity

Table II. *Inhibition of esterases present in horse serum*

| Substrate | Vol. of serum mixture (ml.) | [*I*] | ml. 0·01 *N* alkali | Diminution in drop no. | *i* |
|---|---|---|---|---|---|
| B.C.B. | 1 | 0 | 7·45 | — | |
| | 1 | $10^{-7}$ | 1·10 | — | 0·85 |
| M.B. | 2 | 0 | 2·25 | — | |
| | 2 | $10^{-7}$ | 2·70 | — | Activation |
| B.C.B. | 1 | 0 | 7·65 | — | |
| | 1 | $2{\cdot}5 \times 10^{-4}$* | 0·95 | — | 0·88 |
| M.B. | 2 | 0 | 1·55 | — | |
| | 2 | $2{\cdot}5 \times 10^{-4}$* | 2·35 | — | Activation |
| B.C.B. | 1 | 0 | 4·20 | — | |
| | 1 | $2{\cdot}5 \times 10^{-3}$* | 0·35 | — | 0·92 |
| | 1 | $2{\cdot}5 \times 10^{-6}$* | 0·75 | — | 0·82 |
| M.B. | 2 | 0 | 1·20 | — | |
| | 2 | $2{\cdot}5 \times 10^{-3}$* | 1·10 | — | 0 |
| | 2 | $2{\cdot}5 \times 10^{-6}$* | 1·30 | — | 0 |
| T.B. | 0·5 | 0 | — | 3 | |
| | 0·5 | $2{\cdot}5 \times 10^{-3}$* | — | 3 | 0 |
| M.B. | 2 | 0 | 2·00 | — | |
| | 2 | $1{\cdot}25 \times 10^{-3}$* | 2·55 | — | Activation |
| T.B. | 2 | 0 | — | 19 | |
| | 2 | $1{\cdot}25 \times 10^{-3}$* | — | 15 | 0·21 |

* See text.

towards it. The stalagmometric method used in these experiments is not, perhaps, sufficiently accurate to permit of a definite decision on these points. Attention might also be drawn to the fact that concentrations of prostigmine which produce almost complete inhibition of choline-esterase apparently cause a small activation of esterase. This activation, although small, is, we believe, real.

The results for the horse, whether prostigmine or eserine be used as inhibitor, are entirely similar and need not, therefore, be discussed in detail. It is thus evident that horse serum contains at least two ester-hydrolysing enzymes, namely, choline-esterase and esterase.

Human serum occupies a special position. The results in Table III show that, in confirmation of our previous results, little activity towards methyl butyrate is exhibited. The rate of hydrolysis of this substrate is, indeed, so small that it has been necessary, in order to obtain figures for which a moderate degree of accuracy could be claimed, to use four times as much serum as was employed with butyrylcholine and to prolong the experimental period from 20 to 60 min. An increased volume of serum has also been employed in the case of tributyrin.

Table III. *Inhibition of esterases present in human serum*

| Substrate | Vol. of serum mixture (ml.) | $[I]$ | Titration period (min.) | ml. 0·01 $N$ alkali | $i$ |
|---|---|---|---|---|---|
| B.C.B. | 1 | 0 | 20 | 5·85 | |
| | 1 | $10^{-7}$ | 20 | 1·80 | 0·69 |
| M.B. | 4 | 0 | 60 | 0·90 | |
| | 4 | $10^{-7}$ | 60 | 0·25 | 0·72 |
| T.B. | 2 | 0 | 20 | 1·50 | |
| | 2 | $10^{-7}$ | 20 | 0·50 | 0·67 |

Calculation from these figures of the relative rates of hydrolysis of the three substrates gives the following result: B.C.B. : T.B. : M.B. = 5·85 : 0·75 : 0·075. In other words, the activity of human serum towards butyrylcholine is approximately 8 and 80 times greater respectively than towards tributyrin and methyl butyrate. The inhibitory action of prostigmine is, however, within the limits of experimental error, independent of the substrate. It can therefore be concluded that, in all probability, only one enzyme is involved. It should perhaps be pointed out that it must not be concluded that the choline-esterase from other species possesses the same degree of activity towards methyl butyrate and tributyrin. It is possible that this is the case, although our results do not provide any evidence in favour of such a conclusion.

### *Action of serum esterases on mixed substrates*

The results in the preceding section appear quite definitely to prove that at least two ester-hydrolysing enzymes are present in the sera from the guinea-pig and the horse, while only one is present in human serum. Further confirmation of these results has been obtained by the use of mixed substrates. If one and the same enzyme were responsible for the hydrolysis of, for example, butyrylcholine and methyl butyrate, the total amount of hydrolysis effected in a given time with both substrates present should not exceed that which occurs with one of them alone, assuming, of course, that optimum substrate concentrations are employed. If, on the other hand, two enzymes are involved, it is clear that the two actions should be additive in the experiment with mixed substrates except in so far as one substrate might inhibit the enzyme by which it is not attacked.

The results obtained in such experiments are recorded in Tables IV, V and VI. The method adopted in carrying out these experiments was to measure the amount of hydrolysis with one substrate for a fixed period (10 or 20 min.) and then to add to the reaction mixture the second substrate, the titration being continued for an identical period. In some cases this was followed by the addition of a third substrate. The action of a mixture of serum and inhibitor on mixed substrates has also, in some instances, been examined.

The results obtained with guinea-pig serum appear to be quite conclusive. The figures show that, using butyrylcholine bromide and methyl butyrate as substrates, the enzymic activity in the presence of both substrates is nearly equal to the sum of the activities towards the two substrates separately. This would not be the case if only one enzyme were involved, and it must therefore be concluded, in confirmation of previous results, that guinea-pig serum contains the two enzymes esterase and choline-esterase. That the two activities are not quite additive must be attributed to an inhibition of one or both of the enzymes by the second substrate. Some evidence is, in fact, available which indicates that butyrylcholine exerts a small inhibitory action on esterase. Thus, when the serum is treated with $10^{-5}$ *M* prostigmine, the choline-esterase is almost completely inhibited while the esterase undergoes a slight activation. If the activity of such an inhibited preparation towards the mixed substrates is compared with that towards methyl butyrate alone it is found that the former is smaller than the latter. It must be concluded from this that butyrylcholine exerts a small inhibitory action on esterase. This method is, unfortunately, not available for ascertaining whether methyl butyrate inhibits choline-esterase, for esterase cannot be inhibited by drugs of the type available without causing a greater inhibition of choline-esterase.

Table IV. *Hydrolysis of mixed substrates by guinea-pig serum*

| Material | Vol. (ml.) | Substrate 1st | Substrate 2nd | [*I*] | ml. of 0·01 *N* alkali: 1st substrate | ml. of 0·01 *N* alkali: 1st and 2nd substrate |
|---|---|---|---|---|---|---|
| Diluted serum | 1 | M.B. | B.C.B. | 0 | 2·35 | 4·05 |
| Diluted serum | 1 | B.C.B. | M.B. | 0 | 2·15 | 3·85 |
| Serum-water | 1 | M.B. | B.C.B. | 0 | 3·35 | 7·40 |
| Serum-prostigmine | 1 | M.B. | B.C.B. | $10^{-5}$ | 4·15 | 3·70 |
| Serum-water | 1 | M.B. | B.C.B. | 0 | 5·35 | 9·65 |
| Serum-prostigmine | 1 | M.B. | B.C.B. | $5 \times 10^{-4}$ | 4·25 | 3·75 |
| Serum-water | 1 | B.C.B. | M.B. | 0 | 5·30 | 9·50 |
| Serum-prostigmine | 1 | B.C.B. | M.B. | $5 \times 10^{-4}$ | 0·35 | 3·95 |

Table V. *Hydrolysis of mixed substrates by horse serum*

| Material | Vol. (ml.) | Substrate 1st | Substrate 2nd | Substrate 3rd | ml. of 0·01 *N* alkali: 1st substrate | ml. of 0·01 *N* alkali: 1st and 2nd substrate | ml. of 0·01 *N* alkali: 1st, 2nd and 3rd substrate |
|---|---|---|---|---|---|---|---|
| Serum | 0·5 | B.C.B. | M.B. | T.B. | 3·85 | 3·95 | 4·40 |
| ,, | 0·5 | M.B. | B.C.B. | T.B. | 0·50 | 4·25 | 4·70 |
| ,, | 0·5 | T.B. | B.C.B. | M.B. | 1·00 | 4·50 | 4·65 |
| ,, | 0·5 | B.C.B. | T.B. | — | 3·80 | 4·15 | — |
| ,, | 1·0 | M.B. | T.B. | — | 1·15 | 2·50 | — |
| ,, | 1·0 | T.B. | M.B. | B.C.B. | 1·95 | 2·65 | 8·90 |

*Note.* Experiments by the stalagmometric method showed that neither B.C.B. nor M.B. in concentrations used in the above experiments inhibited the hydrolysis of T.B.

Table VI. *Hydrolysis of mixed substrates by human serum*

| Material | Vol. (ml.) | Substrate 1st | Substrate 2nd | Substrate 3rd | [*I*] | ml. of 0·01 *N* alkali: 1st substrate | ml. of 0·01 *N* alkali: 1st and 2nd substrate | ml. of 0·01 *N* alkali: 1st, 2nd and 3rd substrate |
|---|---|---|---|---|---|---|---|---|
| 50 % serum | 1 | B.C.B. | T.B. | — | 0 | 5·85 | 6·00 | — |
| ,, | 1 | B.C.B. | T.B. | — | $10^{-7}$ | 1·80 | 2·40 | — |
| ,, | 4 | M.B. | B.C.B. | — | 0 | 0·30* | 22·35 | — |
| ,, | 4 | M.B. | B.C.B. | — | $10^{-7}$ | 0·08* | 8·55 | — |
| ,, | 2 | T.B. | M.B. | B.C.B. | 0 | 1·50 | 1·45 | 11·30 |
| ,, | 2 | T.B. | M.B. | B.C.B. | $10^{-7}$ | 0·50 | 0·55 | 4·85 |

* These figures have been corrected for an experimental period of 20 min., the actual time of the experiments being 60 min.

In the case of horse serum the results are not, perhaps, so definite owing to the much smaller activity of this serum towards methyl butyrate. Nevertheless, the total activity in the presence of the two substrates (methyl butyrate and butyrylcholine) is greater than in the presence of butyrylcholine alone. When it is recalled that, according to the experiments with guinea-pig serum, butyrylcholine inhibits esterase, this result can only be interpreted on the assumption that two enzymes, esterase and choline-esterase, are also present in this serum. If a third substrate (tributyrin) be added to the reaction mixture the total enzymic activity is increased. A definite interpretation of this result cannot be given at the present time. It is probable that a third enzyme, which hydrolyses tributyrin, is present.

The results obtained with human serum are consistent with the conclusion that only one esterase, namely choline-esterase, is present. Thus, when the three substrates are examined in pairs, it is found that the total enzymic activity of the serum is, in each case, considerably less than the sum of its activities towards the two substrates separately. Only in one case is the total activity slightly greater than the activity towards the substrate which is the more readily hydrolysed by the enzyme. These results do not, however, hold when inhibited serum is employed, a fact for which we cannot offer any definite explanation.

## Summary

The suitability of the name choline-esterase for the enzyme present in blood which accelerates the hydrolysis of acylcholines is reiterated.

In confirmation of previous work from this laboratory it is shown (*a*) that there exist in blood sera from the guinea-pig and horse at least two ester-hydrolysing enzymes, namely, choline-esterase and an enzyme recognized by its action on methyl butyrate; (*b*) that choline-esterase is specific to the extent that, while attacking esters of choline, its action on methyl butyrate is inappreciable; (*c*) that the choline-esterase present in human serum is almost certainly responsible for the relatively small action of this serum towards tributyrin. It is possible that the choline esterase in sera from the horse and guinea-pig also exercises a small action on tributyrin, but this is less certain.

In the absence of any knowledge regarding the physiological function of the enzyme which hydrolyses methyl butyrate, this enzyme is provisionally designated by the general term esterase.

Guinea-pig serum contains a relatively large amount of esterase; horse serum contains less; while human serum apparently contains none. The small esterase

activity of human serum, which is only 1/80 of its activity towards butyrylcholine is, however, real and is due to the choline-esterase present in this serum.

The activity of serum esterase (guinea-pig) on methyl butyrate is inhibited to an appreciable extent by moderate concentrations of butyrylcholine.

No conclusion has yet been reached as to a possible small action of serum esterase on butyrylcholine.

It is possible that a third ester-hydrolysing enzyme, which attacks tributyrin, is present in blood sera from certain species. The action on this substrate of the sera examined can, however, be explained by assuming that both esterase and choline-esterase attack tributyrin, the former enzyme being much more active than the latter in this respect.

The inhibitory actions of eserine, prostigmine and analogous drugs on esterase are less marked than on choline-esterase.

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