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Serum Esterases

1. TWO TYPES OF ESTERASE (A AND B) HYDROLYSING *p*-NITROPHENYL ACETATE, PROPIONATE AND BUTYRATE, AND A METHOD FOR THEIR DETERMINATION

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Huggins & Lapides (1947) have developed a sensitive method for the determination of esterases using as substrates *p*-nitrophenyl esters of the lower fatty acids. Although these esters are esters of phenols it was assumed that they were hydrolysed by what is usually termed 'ali-esterase'; no evidence was given on this point. It has been shown that other enzymes will hydrolyse *p*-nitrophenyl esters: Zeller, Fleischer, McNaughton & Schweppe (1949) have demonstrated that a purified erythrocyte cholinesterase will hydrolyse *p*-nitrophenyl acetate, and Whittaker (1951) has produced some evidence that both the true and pseudocholinesterases of human blood will hydrolyse a variety of substituted phenyl acetates. It is, therefore, not at all clear what enzymes were being determined in the methods developed by Huggins & Lapides (1947).

During the course of work on the enzyme which will hydrolyse diethyl *p*-nitrophenyl phosphate (E 600) it was found that there are in most sera two quite distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One type is inhibited, like cholinesterase, by 10^{-8} M-E 600, while the other is not inhibited at all. This paper describes the work leading to the development of quantitative methods for the estimation of these two types of esterase and which is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

METHODS

p-Nitrophenyl acetate, propionate and butyrate were prepared by a procedure essentially the same as described by Huggins & Lapides (1947). *p*-Nitrophenol was esterified by the corresponding acyl chloride in benzene in the presence of magnesium turnings and it was found that far better yields of ester were obtained in benzene saturated with water than in dry benzene. The acetate and propionate were crystallized from methanol. The solubilities of these esters in water at 25° are approximately 10 times less than those given by Huggins & Lapides (1947).

Estimation of esterase activity. Since these esters are unstable, a colorimetric method based on the determination of *p*-nitrophenol is not suitable. A manometric method has been developed, based on the CO_2 liberated from bicarbonate buffer by the 2 moles of acid produced during the hydrolysis. The substrate suspension was prepared by dissolving 100 mg. of the esters in 0·5 ml. of methanol with warming and then blowing the solution rapidly from a Pasteur pipette into 25 ml. of bicarbonate buffer ($NaHCO_3$, 0·031 M; NaCl, 0·162 M; gelatin, 0·1% w/v) and shaking vigorously to obtain a fine suspension of the ester. 1·0 ml. of the enzyme preparation (suitably diluted with the bicarbonate buffer) in the side arm of a Warburg flask was tipped into 3·0 ml. of substrate suspension after the flask had been gassed with 5% (v/v) CO_2 + 95% N_2 and equilibrated. The output of CO_2/min. is calculated from readings taken every 5 min.

from 10 to 40 min. using the method of Aldridge, Berry & Davies (1949). The non-enzymic hydrolysis has been obtained by extrapolating to zero enzyme concentration the straight line obtained when the output of CO_2/min. is plotted against the enzyme concentration.

Throughout this paper the enzyme(s) hydrolysing *p*-nitrophenyl acetate, propionate and butyrate have been termed simply the esterase(s). The cholinesterases have been excluded by their inhibition by eserine.

RESULTS

Enzymic hydrolysis of p-*nitrophenyl esters and its sensitivity to* E 600

It was found that rabbit, rat and horse sera hydrolyse *p*-nitrophenyl acetate, propionate and butyrate actively and under the conditions of determination this activity is such that a 50- to 100-fold dilution of the serum must be used. Rabbit serum hydrolyses the acetate at a higher rate than the butyrate, and for rat serum the reverse is true.

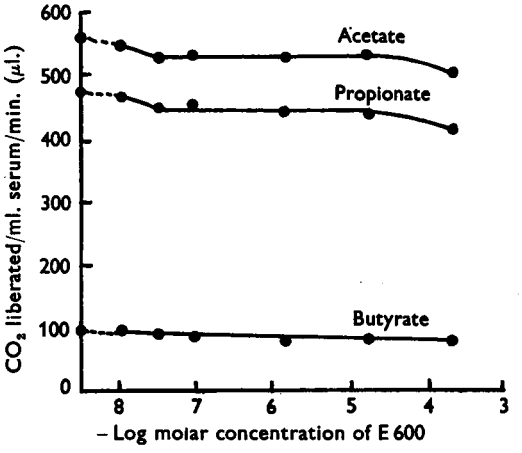

Fig. 1. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by rabbit serum after incubation for 30 min. at 37° with various concentrations of E600. Original activity determined without inhibitor.

When the sensitivity of these esterases to incubation with E 600 was examined it was found that the hydrolysis of *p*-nitrophenyl acetate by rabbit serum was hardly prevented at all by previous incubation with 10^{-4}M-E 600 while the hydrolysis of *p*-nitrophenyl butyrate by rat serum was nearly stopped by 10^{-8}M-E 600. The hydrolysis of all these *p*-nitrophenyl esters by rabbit, rat and horse sera has therefore been determined after incubation with various concentrations of E 600 at 37° for 30 min. The results are given in Figs. 1–3 which show differences in the sensitivity of the esterase in the rabbit, rat and horse sera towards E 600. In rabbit serum the enzymes are hardly inhibited by concentrations of E 600 as high as 10^{-3}M. In rat serum hydrolysis of all three esters is inhibited by 10^{-7} to 10^{-8}M-E 600. In horse serum hydrolysis of *p*-nitrophenyl propionate and butyrate is strongly inhibited by

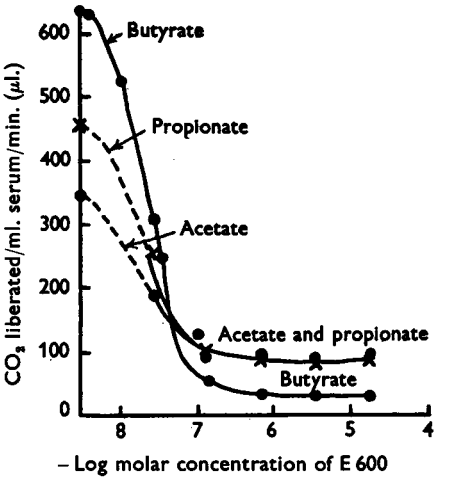

Fig. 2. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by rat serum after incubation for 30 min. at 37° with various concentrations of E600. Original activity determined without inhibitor.

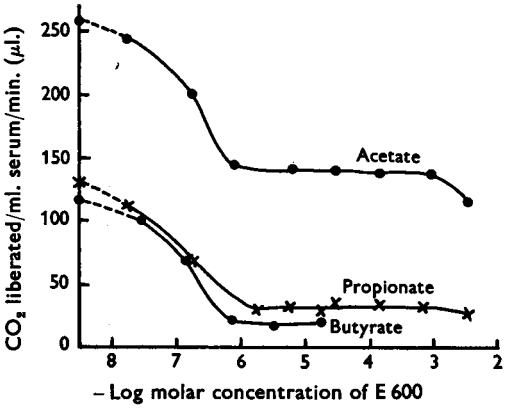

Fig. 3. The hydrolysis of *p*-nitrophenyl acetate, propionate and butyrate by horse serum after incubation for 30 min. at 37° with various concentrations of E600. Original activity determined without inhibitor.

10^{-6} to 10^{-8}M-E 600, while hydrolysis of *p*-nitrophenyl acetate is only partly inhibited by 10^{-3}M-E 600. These results could be accounted for by the existence of two separate esterases, one inhibited by 10^{-7}M-E 600 and another which is not inhibited by 10^{-3}M-E 600. The data plotted in the graphs (Figs. 1–3) may be used to calculate the rates of hydrolysis of the three substrates for the resistant and the sensitive types of enzyme.

The results of these calculations are given in

Table 1. Inspection of Table 1 and Figs. 1–3 show that in all three sera the resistant type of esterase hydrolyses the acetate more rapidly than the butyrate: in rat serum the sensitive type hydrolyses the butyrate more rapidly than the acetate, while in horse serum the rate of hydrolysis of the three substrates is similar. Individual sera vary to some degree in their content of the two types of esterase.

The predominant esterases in rabbit, rat and horse sera which hydrolyse *p*-nitrophenyl esters may be clearly differentiated by their widely differing sensitivity to E 600. There has been no previous indication in the literature that these esterases may be separated by the effects of selective inhibitors. However, serum lipase and human milk lipase have been shown to be inhibited by tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate (Bloch, 1943; Hottinger & Bloch, 1943) and Webb (1948) has also demonstrated that liver esterase and human milk lipase are inhibited by di*iso*propyl fluorophosphonate.

Sensitivity of the esterases to other cholinesterase inhibitors

In view of this differing sensitivity of the esterases to E 600, other members of the broad group of organophosphorus inhibitors were tried. Tetraethyl pyrophosphate and di*iso*propyl fluorophosphonate were chosen as representative types of this class of inhibitors. Figs. 4–6 show the inhibition of the same enzyme preparations by tetraethyl pyrophosphate, di*iso*propyl fluorophosphonate and E 600. The enzyme in rat serum was inhibited by low concentrations of each inhibitor. In Table 2 the concentrations of inhibitor giving 50% inhibition (after 30 min. incubation at 37°) are listed together with the values for the inhibition of true and pseudocholinesterase. The values for inhibition of the cholinesterases agree well with those in the literature (Jones, Meyer & Karel, 1948; Mackworth & Webb, 1948; Grob & Harvey, 1949). It is interesting that the sensitivity to inhibition by these inhibitors is of

Table 1. *The presence in rabbit, rat and horse sera of esterases resistant and sensitive to treatment with* E 600

(Enzyme activities are expressed as the output of CO_2/ml. serum/min. calculated from the output of CO_2 obtained on the addition of a suitable dilution of serum to a suspension of the *p*-nitrophenyl ester in the bicarbonate buffer after gassing with 5% (v/v) CO_2 in N_2. Total esterase is the enzyme activity determined without E 600, resistant esterase is the activity after incubation for 30 min. with 10^{-5} M-E 600 and sensitive esterase is the difference between the total and resistant esterase.)

Material	*p*-Nitrophenyl ester as substrate	Enzyme activity (μl. CO_2/ml./min.) Total esterase	Resistant esterase	Sensitive esterase
Rabbit serum	Acetate	565	525	—
	Propionate	475	445	—
	Butyrate	100	80	—
Rat serum	Acetate	340	80	260
	Propionate	460	80	380
	Butyrate	635	30	605
Horse serum	Acetate	255	140	115
	Propionate	130	30	100
	Butyrate	120	20	100

Table 2. *The sensitivity of esterase in rat serum, true cholinesterase in sheep erythrocytes and pseudocholinesterase in horse serum to organophosphorus inhibitors*

(Enzyme preparations were incubated for 30 min. at 37° with the inhibitor prior to the addition of substrate. The concentrations of inhibitor are those during the incubation period. The esterase preparation used was 0·5 ml. rat serum diluted 50-fold; true cholinesterase preparation was 0·5 ml. sheep erythrocytes washed with saline twice and resuspended and diluted to the volume of the original whole blood; pseudocholinesterase preparation was 0·5 ml. horse serum diluted 5-fold. All dilutions were made in the bicarbonate buffer. The results for rat serum are corrected for the small amount of butyrate hydrolysis which is not inhibited by 10^{-5} M inhibitor. True and pseudocholinesterases are completely inhibited by 10^{-5} M inhibitor.)

Inhibitor	Molar concentrations for 50% inhibition: Butyrate hydrolysis (rat serum)	True cholinesterase (sheep erythrocytes)	Pseudocholinesterase (horse serum)
Tetraethyl pyrophosphate	$4·8 \times 10^{-8}$	$1·1 \times 10^{-8}$	—
Di*iso*propyl fluorophosphonate	$4·5 \times 10^{-8}$	$4·6 \times 10^{-7}$	$2·3 \times 10^{-9}$
Diethyl *p*-nitrophenyl phosphate	$1·8 \times 10^{-8}$	$2·1 \times 10^{-8}$	$5·4 \times 10^{-8}$

the same order for cholinesterase and the esterase in rat serum hydrolysing *p*-nitrophenyl butyrate. Fig. 4 shows that with this particular sample of rabbit serum about 15 % inhibition was produced by 10^{-7} M-E 600. This is in agreement with the results obtained with tetraethyl pyrophosphate and di*iso*-propyl fluorophosphonate (Figs. 5 and 6) and is due to the presence of a small amount of the sensitive type of the esterase in this sample of serum.

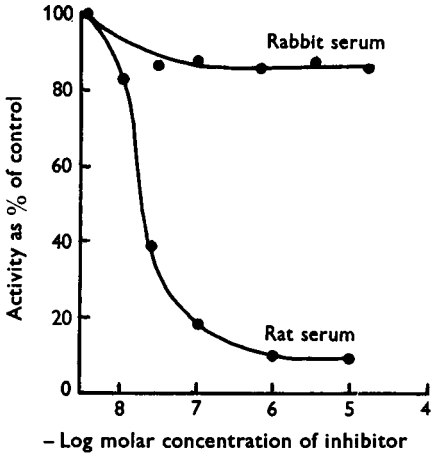

Fig. 4. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with E 600.

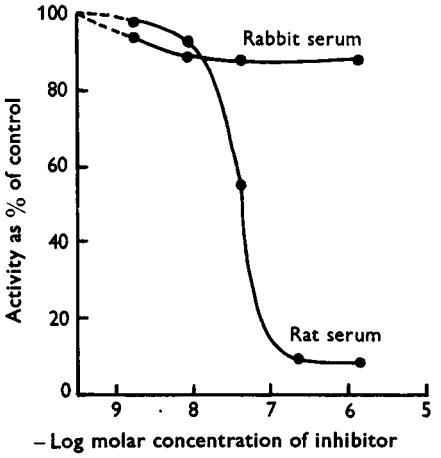

Fig. 5. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with tetraethyl pyrophosphate.

Zeller *et al.* (1949) and Whittaker (1951) have shown that *p*-nitrophenyl acetate is hydrolysed by true and pseudocholinesterase. Eserine is the most satisfactory agent for the differentiation of cholinesterases from the aliphatic esterases (Richter & Croft, 1942; Myers & Mendel, 1949). The results in Table 3 show that neither the rabbit nor rat enzymes are inhibited until a concentration of 10^{-3} M-eserine is reached and then only slightly. With horse serum 14 % inhibition is produced by 10^{-6} M-eserine and this remains constant up to 10^{-4} M. Horse serum contains a high pseudocholinesterase activity (Mendel, Mundel & Rudney, 1943), and Sturge & Whittaker (1950) have shown that a whole range of esters is hydrolysed by a purified cholinesterase from horse serum.

The results in Table 4 are in agreement with the view that the activity against *p*-nitrophenyl esters, which is inhibited by treatment with 10^{-5} M-eserine, is due to the presence of cholinesterases. Any method for the determination of these esterases must take this into account.

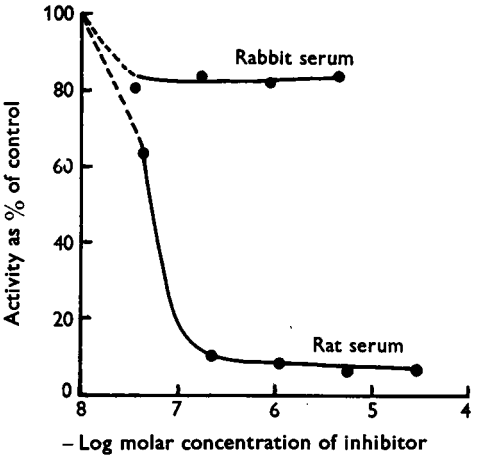

Fig. 6. The hydrolysis of *p*-nitrophenyl acetate by rabbit serum and of *p*-nitrophenyl butyrate by rat serum after incubation for 30 min. at 37° with di*iso*propyl fluorophosphonate.

Sensitivity of the esterases to other inhibitors

In Table 5 the results are given of an examination of the sensitivity of rabbit and rat serum esterase to copper, nickel and *p*-chloromercuribenzoic acid. The concentrations of rabbit and rat serum used with *p*-nitrophenyl acetate and butyrate as substrates, respectively, are not sufficiently different to cause much error due to non-specific absorption of the inhibitors. The esterase in rabbit serum appears to be more sensitive to metallic inhibitors than the esterase in rat serum. The ratio of the concentration for 50 % inhibition of the rat serum enzyme to that for the rabbit serum enzyme is 10 for copper sulphate and 30 for *p*-chloromercuribenzoic acid.

Inhibition of the rat serum esterase by nickel sulphate could not be determined since a precipitate, presumably of a basic carbonate, was produced when above 4×10^{-4}M-nickel sulphate was used. Utilizing the fact that a preparation of the esterase resistant to E 600 may be made by incubating serum with E 600 (5 μg./ml.) for 30 min. at 37° to destroy all the sensitive enzyme, it has been shown that the hydrolysis of *p*-nitrophenyl acetate by such a preparation from horse serum is also sensitive to *p*-chloromercuribenzoic acid; the concentration for 50 % inhibition is 1×10^{-5}M. The rat serum esterase appears to be slightly more sensitive to sodium fluoride.

Table 3. *Effect of eserine on the hydrolysis of* p-*nitrophenyl esters by rabbit, rat and horse sera*

(Enzyme preparations were incubated with eserine for 15 min. at 37° prior to the addition of substrate. The concentrations of eserine given are the final concentrations after the addition of substrate. Enzyme preparations were 0·5 ml. rabbit serum diluted 100-fold, 0·5 ml. rat serum diluted 50-fold and 0·5 ml. horse serum diluted 20-fold.)

Concentration of eserine (M)	Rabbit serum with *p*-nitrophenyl acetate as substrate (% activity)	Concentration of eserine (M)	Rat serum with *p*-nitrophenyl butyrate as substrate (% activity)	Concentration of eserine (M)	Horse serum with *p*-nitrophenyl butyrate as substrate (% activity)
$3·9 \times 10^{-3}$	86	$1·1 \times 10^{-3}$	42	$2·6 \times 10^{-3}$	74
$3·9 \times 10^{-4}$	94	$1·1 \times 10^{-4}$	95	$5·2 \times 10^{-4}$	89
$3·9 \times 10^{-5}$	101	$1·1 \times 10^{-5}$	98	$1·04 \times 10^{-4}$	88
$3·9 \times 10^{-6}$	101	$1·1 \times 10^{-6}$	97	$2·1 \times 10^{-5}$	88
$3·9 \times 10^{-7}$	100	$1·1 \times 10^{-7}$	98	$4·2 \times 10^{-6}$	86

Table 4. *Effect of eserine on the esterase in horse serum*

(Enzyme was incubated with eserine for 15 min. at 37° prior to the addition of the substrates. The final concentration of eserine after the addition of substrate was $3·8 \times 10^{-5}$M. Enzyme preparations were 0·5 ml. serum diluted 20-fold with the *p*-nitrophenyl esters as substrates, 0·5 ml. serum diluted 13-fold with the choline esters.)

Substrate	Activity (μl. CO_2/ml./min.) Without eserine	With eserine	Difference
p-Nitrophenyl acetate	229	193	36
p-Nitrophenyl propionate	164	132	32
p-Nitrophenyl butyrate	152	107	45
Acetylcholine	84	Nil	84
Benzoylcholine	29	Nil	29

Method for the determination of the two types of esterase

On the basis of the differing sensitivities of esterase to inhibition by E 600, quantitative methods for the determination of these two distinct types of esterase have been developed.

Reagents. These are identical with those for total esterase estimation given earlier with the addition of: (1) E 600 solution, 10 μg./ml. ($3·6 \times 10^{-5}$M). Stock solution (0·1 ml.) of E 600 in absolute ethanol (10 mg./ml.) is diluted to 100 ml. with the bicarbonate buffer. The dilution is prepared freshly every day. (2) Eserine solution, 8×10^{-5}M. This is prepared from a stock solution of eserine sulphate in water (2·6 mg./ml.) by diluting 100 times with the bicarbonate buffer. The dilution is freshly prepared every day.

Procedure. Into two test tubes are placed 5 ml. of a suitable dilution of serum. To one, 5 ml. of the eserine solution are added and to the other, 5 ml. of the E 600 solution. They are incubated at 37° for 30 min. Meanwhile, the buffer and substrates are prepared as previously described and 3 ml. are pipetted into the centre compartment of each Warburg flask. After the 30 min. incubation with eserine or E 600, 1 ml. of the serum solution is pipetted into the side arm of a Warburg flask, after making any necessary dilutions with eserine solution or with buffer in the case of the one incubated with E 600. The procedure, thereafter, is identical with that given earlier for the determination of esterase. The results are calculated as μl. CO_2/ml. serum/min. The determination using the serum incubated with eserine gives the total esterase (1). The sample incubated with E 600 gives the resistant enzyme (2) (A-esterase). The sensitive esterase (B-esterase) is given by the difference between (1) and (2). (See below for explanation of nomenclature.)

The determination of total esterase must be carried out in

Table 5. *Sensitivity of esterases of rabbit and rat sera to other inhibitors*

(Inhibitor and diluted serum incubated for 15 min. at 37° prior to tipping into the substrate. The substrate contains the same concentration of inhibitor as in the serum during incubation. The concentrations of serum given are those during the 15 min. incubation.)

Inhibitor	Molar concentrations for 50 % inhibition: 1·33 % rabbit serum with *p*-nitrophenyl acetate as substrate	2·0 % rat serum with *p*-nitrophenyl butyrate as substrate
Nickel sulphate	$1·3 \times 10^{-5}$	—
Copper sulphate	$1·4 \times 10^{-5}$	$1·6 \times 10^{-4}$
p-Chloromercuribenzoic acid	$1·4 \times 10^{-5}$	4×10^{-4}
Sodium fluoride	8×10^{-3}	$2·3 \times 10^{-3}$

the presence of eserine in order to prevent the hydrolysis of *p*-nitrophenyl esters by cholinesterase. Since eserine is a reversible inhibitor any dilutions of the original incubation mixture must be made with solutions containing eserine and the final concentration in the flask should be approximately 10^{-5} M. After addition of E 600, which produces an irreversible inhibition *in vitro*, the solutions may be diluted with buffer. The sensitive esterase is almost completely inhibited by 10^{-7} M solutions of E 600. It is necessary, therefore, to be very careful to avoid the accidental contamination of solutions of the enzymes by the use of pipettes that have been used for dilutions of E 600.

For convenience and clarity we have used a simple method of nomenclature for these enzymes. The substances which both of these enzymes are hydrolysing are esters and both are, therefore, called esterases. The observation that the enzyme not inhibited by E 600 hydrolysed acetate at a higher rate than butyrate suggested that this enzyme be called A-esterase and the sensitive enzyme, therefore, B-esterase. This avoids placing any emphasis on their specificity to a particular substrate.

A-esterase is, therefore, the esterase which is not inhibited by E 600, and B-esterase is the esterase which is inhibited by E 600.

A- and B-esterases in various sera

Sera from various species have been examined using the methods for the determination of A- and B-esterase. The list is rather an arbitrary collection and in many cases only one sample of serum has been examined (Fig. 7). However, it is shown that the methods have a general application, but the finer differentiation of substrate-specificity patterns is not possible with such a small number of samples from each species. With one exception A-esterase is present in the sera from thirteen species. Duck serum has no detectable A-esterase activity and fowl serum has very little. In some sera, e.g. of the sheep, cow and goat, only A-esterase appears to be present. Sera from the rabbit sometimes contain only A-esterase but usually there is some B-esterase present. B-esterase is present in the sera of the cat, guinea pig, rat, horse and rabbit.

All the A-esterases demonstrated in the sera examined hydrolyse *p*-nitrophenyl acetate at a higher rate than the butyrate under the conditions of determination. The substrate patterns of the A-esterase from different species vary considerably, for instance A-esterase from rabbit serum hydrolyses the propionate at roughly 75 % of the rate of the acetate, while the A-esterase of the horse, sheep and cow hydrolyses the propionate at only 30 % of the rate of the acetate. In two species, the ferret and the cat, the propionate is hydrolysed at a higher rate than the acetate. Even with these differing

Table 6. *Summation experiments on A-esterase of rabbit serum using* p-*nitrophenyl butyrate, and ethyl acetate and butyrate as substrates*

(A-esterase prepared by incubation of a suitable dilution of rabbit serum with 5 μg. E 600/ml. for 30 min. at 37° prior to summation experiments. Substrate concentrations for ethyl acetate and butyrate were 0·4 ml./25 ml. bicarbonate buffer and for *p*-nitrophenyl butyrate suspension, 4 mg./ml.)

Enzyme activities (μl. CO_2/ml./min.)				
p-Nitrophenyl butyrate (*a*)	Ethyl acetate (*b*)	*p*-Nitrophenyl butyrate + ethyl acetate (*c*)	(*a*) + (*b*)	(*c*) − (*a*)
65·6	8·4	51·2	74·0	−14·4
64·1	7·6	49·9	71·7	−14·2
p-Nitrophenyl butyrate (*a*)	Ethyl butyrate (*b*)	*p*-Nitrophenyl butyrate + ethyl butyrate (*c*)	(*a*) + (*b*)	(*c*) − (*a*)
58·4	0	47·5	58·4	−10·9
55·4	0	37·7	55·4	−17·7

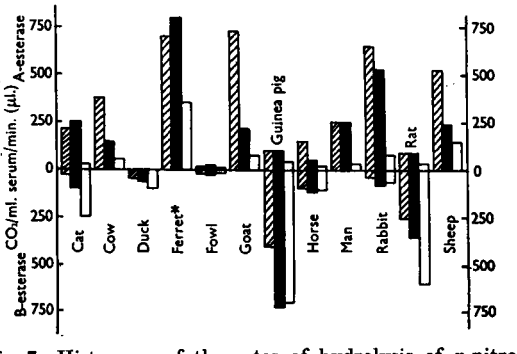

Fig. 7. Histogram of the rates of hydrolysis of *p*-nitrophenyl esters by the sera of various species. Substrates: ▨, *p*-nitrophenyl acetate; ■, propionate; □, butyrate. * For convenience the rates of hydrolysis plotted for ferret serum are one-half the rates actually determined.

specificity patterns no exceptions have so far been found to the general rule that A-esterase hydrolyses the acetate at a higher rate than the butyrate.

Hydrolysis of aliphatic esters by A-esterase

Huggins & Lapides (1947) assumed that the enzyme which hydrolyses *p*-nitrophenyl acetate, propionate and butyrate, is the same as that which hydrolyses aliphatic esters such as ethyl acetate. No evidence is given in their paper for this point of view. Summation experiments have been carried out on the A-esterase of the rabbit using *p*-nitrophenyl butyrate, and ethyl acetate and butyrate as substrates. The results are given in Table 6 and indicate that there is competition between *p*-nitrophenyl butyrate and ethyl acetate for A-esterase. If ethyl butyrate was hydrolysed the amount was too small to measure, but some lowering of the rate of hydrolysis of *p*-nitrophenyl butyrate was caused by the presence of this compound. This probably indicates competition for the same active centre by both esters.

DISCUSSION

With the aid of E 600 it has been demonstrated that the esterases present in many sera may be divided into two distinct types. One type (A-esterase) is not inhibited by E 600 up to concentrations of 10^{-3} M, whereas the other type (B-esterase) is extremely sensitive to E 600 and is inhibited by concentrations as low as 10^{-8} M. E 600 is an excellent inhibitor for this purpose. It is easy to prepare pure, is relatively stable to hydrolysis and is extremely active. It is clear that the organophosphorus inhibitors are by no means specific inhibitors for cholinesterase even though this action may govern their toxicity. Chymotrypsin is inhibited by di*iso*propyl fluorophosphonate (Jansen, Nutting, Jang & Balls, 1949) and by E 600 (Hartley & Kilby, 1952). Acetylesterase from oranges is inhibited by tetraethyl pyrophosphate and di*iso*propyl fluorophosphonate (Jansen, Nutting & Balls, 1948). In this paper it has been shown that B-esterase from rat serum is inhibited by E 600, tetraethyl pyrophosphate and di*iso*propyl fluorophosphonate.

The A-esterase in the sera examined hydrolyses *p*-nitrophenyl acetate at a higher rate than the butyrate. B-esterases appear to hydrolyse the butyrate at the same or a higher rate than the acetate, though caution must be used since we have as yet no evidence whether the B-type of esterase includes other esterases known as lipase. It should be noted that the relative rates of hydrolysis of substrates refer only to the conditions of determination used in this paper. The relative amounts of A- and B-esterase vary from one animal to another. For example, the A-esterase activity against *p*-nitrophenyl acetate in sera from four rabbits varied from 660 to 521 μl. CO_2/ml./min., while the B-esterase activity varied from 0 to 300 μl. CO_2/ml./min. With such variation in the amounts of two esterases in each serum, a study of their total activity in serum with substrates alone must, therefore, give misleading results. The apparent specificity pattern will vary from sample to sample. A combination of the use of inhibitors of the E 600 type and substrates will probably help to solve the problem of the specificity of mixtures of esterases.

If the activity of A-esterase of rabbit serum acting on *p*-nitrophenyl acetate is taken as 100 then its activity against the butyrate is 16. Since, in Table 6, this A-esterase has been shown to hydrolyse *p*-nitrophenyl butyrate approximately 8 times faster than ethyl acetate, *p*-nitrophenyl acetate will therefore be hydrolysed by this enzyme approximately 40–50 times faster than ethyl acetate. Before it can be decided whether the activity called ali-esterase is identical with that hydrolysing *p*-nitrophenyl esters, much more work requires to be carried out on purified preparations of A- and B-esterases from different species. It can be said, however, that other enzymes hydrolyse aromatic esters at a higher rate than aliphatic esters, e.g. the phosphatases (King & Delory, 1939).

It is very difficult to decide the function of an enzyme and especially of those with a low specificity. Among the sera we have examined those from the ruminants (cow, sheep and goat) all hydrolyse the acetate at a much higher rate than the propionate or the butyrate. It is known that large amounts of acetic acid are produced in the rumen by the digestion of cellulose (Stephenson, 1949; Gray, Pilgrim, Rodda & Weller, 1951). It is tempting to speculate that there is some connexion between these two facts.

SUMMARY

1. Serum esterases of many species may be separated into two distinct types, A- and B-esterase.

2. The A-type is not inhibited by E 600 and hydrolyses *p*-nitrophenyl acetate at a higher rate than *p*-nitrophenyl butyrate.

3. The B-type is inhibited by 10^{-7} to 10^{-8} M-E 600 and hydrolyses *p*-nitrophenyl butyrate at the same or a higher rate than *p*-nitrophenyl acetate.

4. It is possible that both the A-type and B-type esterases contribute to the ali-esterase activity of serum.

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Serum Esterases

2. AN ENZYME HYDROLYSING DIETHYL *p*-NITROPHENYL PHOSPHATE (E 600) AND ITS IDENTITY WITH THE A-ESTERASE OF MAMMALIAN SERA

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It has been stated (Roche, 1950): 'The orthophosphoric tri-esters which are easily hydrolysed by acids, bases or even boiling water, are not hydrolysed enzymatically.' If a solution of diethyl *p*-nitrophenyl phosphate (E 600) is added to rabbit serum the serum rapidly turns yellow. This is due to the liberation of *p*-nitrophenol by the hydrolysis of E 600.

E 600 is an orthophosphoric tri-ester and has been studied primarily for its anticholinesterase properties. It is a very active inhibitor (Aldridge, 1950; Aldridge & Davison, 1952) and will inhibit the cholinesterase activity of sheep erythrocytes by 50% after incubation at 37° for 30 min. at a concentration of 2×10^{-8} M. It is also very toxic, the LD_{50} for rats being about 0·5 mg./kg. It is therefore of interest that this substance can be hydrolysed in mammals. In this paper, methods for the determination of this enzyme (E 600-esterase) are given followed by a study of its properties and distribution in some species.

It has been shown (Aldridge, 1953) that there are two distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One esterase (A-esterase) is not inhibited by E 600 while the other (B-esterase) is inhibited by concentrations as low as 10^{-8} M. In this paper evidence will be presented which shows that the enzyme hydrolysing E 600 and A-esterase are identical enzymes. A preliminary report of these observations has been published (Aldridge, 1951).

The work described in this paper is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

EXPERIMENTAL

Materials

Diethyl *p*-nitrophenyl phosphate was first prepared by Schrader (1947) who called it E 600. It is a pale yellow liquid, sp.gr. 1·27, practically non-volatile at room temperature and atmospheric pressure, b.p. 160° at 0·05 mm. Hg pressure. It is soluble in water at 25–37° to 2·37 mg./ml. and is slowly hydrolysed in buffers at pH 7·8 to give diethyl phosphoric acid and *p*-nitrophenol, no orthophosphate being produced. The first-order constant for its hydrolysis at 37° in Sørensen's M/15 phosphate buffer, pH 7·6, is $3·9 \times 10^{-5}$ (min.$^{-1}$), the half life under these conditions being 11·2 days. I am grateful to Mr B. Topley (Albright and Wilson Ltd.) for a generous supply of E 600.