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Some Esterases of the Rat

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There is much confusion in the literature on the identity of esterases and lipases. Much of this has arisen from the different conditions under which they have been examined and in particular to the variety of substrates which have been used. In most cases the preparations probably contained more than one esterase whose activities against different substrates have not been separated. For instance, although it was pointed out over 20 years ago (Cherry & Crandall, 1932) that tributyrin is not a specific substrate for pancreatic lipase, this fact has not always appeared to be appreciated. It is shown in this paper that tributyrin is an excellent substrate for both lipase and esterase.

The esterases which are discussed in this paper are not cholinesterases and do not hydrolyse choline esters. It is first necessary to try to separate the activities of the esterases (including lipases) so that the effects of alterations in the chemical nature and physical state of the substrates may be examined under strictly comparable conditions. Previous work (Aldridge, 1953*a*, *b*) has shown that the esterases of serum may be separated into two distinct types (A and B) by their sensitivity to organophosphorus inhibitors. This method has since been used for the esterases of erythrocytes (Mounter & Whittaker, 1953) and for tissue esterases (Mendel, Myers, Uyldert, Ruys & Bruyn, 1953). Using this method an initial examination of the esterases of the intestinal mucosa and pancreas of the rat soon showed that they differed markedly from those present in rat serum. A detailed examination has been made of the A-type esterases of rat serum and pancreas and the B-type esterases of intestinal mucosa. After the activities of esterases and pancreatic lipase had been clearly separated, an examination of the effect of the physical state of the substrate on the activity of both enzymes was made. This has provided a clue to the essential difference between these two enzymes. The characterization and a knowledge of the distribution of esterases should provide clues to the physiological function of this group of enzymes. The techniques described in this paper should help in approaching this problem.

MATERIALS AND METHODS

In the methods used for determining the activity of the esterases examined in this paper no claim is made that optimum conditions have been produced. No attempt has been made to determine the activity of the enzymes at their optimum pH because where several substrates are being used the pH optima may vary considerably from substrate to substrate. This has been well shown for alkaline phosphatase by Delory & King (1943) and for pancreatic lipase by Schønheyder & Volqvartz (1945*a*).

Substrates. The esters used as substrates were phenyl acetate, propionate and *n*-butyrate and triacetin and tri-*n*-butyrin from British Drug Houses Ltd.; tripropionin, trivalerin, tri-*iso*valerin, tricaproin were from Eastman Kodak Ltd. Triolein (Olive Oil B.P.) showed only a trace of free fatty acid when examined by running a 50 g. sample through an alumina column as described by Sylvester, Ainsworth & Hughes (1945). Phenyl *iso*butyrate and *iso*valerate were synthesized by the method given by Huggins & Lapides (1947). Tween 20 and Tween 80 (polyoxyethylene derivatives of sorbitan laurate and oleate respectively) were from Honeywill and Stein Ltd., London. All the phenyl esters as purchased or prepared contain free phenol which cannot be separated by distillation. The free phenol may be readily removed by passing 100 ml. of 20% (w/v) solution in dry light petroleum (B.P. below 40°) down an alumina column (type H; Peter Spence, Widnes). A further 100 ml. of light petroleum is run through and the combined solutions evaporated *in vacuo* at 50°. This procedure removes all phenol and the controls without enzyme contain only the traces of phenol liberated by the non-enzymic hydrolysis of the substrate.

Enzyme preparations. Tissue suspensions were prepared in water using a Folley & Watson type homogenizer (Folley & Watson, 1948). For many purposes acetone-dried tissue powders were used (Willstätter & Waldschmidt-Leitz, 1923). The tissue was removed and homogenized in acetone at room temperature. For rat intestinal mucosa, the small intestine of a rat was removed and washed through with running tap water. After the intestine had been cut open the mucosa was scraped off with a blunt spatula and dropped into the acetone at room temperature and then homogenized. The precipitates were centrifuged down and washed three times with acetone. The powders so obtained were dried *in vacuo* until free from acetone, ground up and finally sieved through muslin to give a fine flour-like powder which was stored at $-5°$. Aqueous extracts were found to be more reproducible than those prepared in buffer and to prepare extracts the powders were treated with water for 10 min. at room temperature, centrifuged and the supernatant filtered through a Whatman no. 54 filter paper. It is important when using the manometric method to add buffer to the aqueous extract before gassing with 5% CO_2 in N_2. A considerable loss of activity was observed when unbuffered extracts were used.

Pancreatic juice was obtained by Dr K. K. Cheng from rats under Nembutal anaesthesia supplemented by ether. The juice was collected through a fine polythene cannula inserted into an isolated segment of bile duct into which the pancreatic duct has been shown to drain. Secretion was stimulated by the administration of arachis oil (2 ml.) orally and by pilocarpine nitrate (160 mg./kg. body wt.) subcutaneously.

Manometric determination of enzyme activity. All determinations were carried out in the presence of 0·44% (w/v) gelatin (Bacteriological, G. T. Gurr Ltd., London) which was added to prevent inactivation of the enzymes. Buffer containing $NaHCO_3$, 0·0357 M and gelatin, 0·5% (w/v) was used except for some determinations such as the hydrolysis of the higher glycerides when buffer containing $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·040 M, and gelatin, 0·5% (w/v) was used. During the determination each flask contained 0·5 ml. of enzyme extract in water and 0·5 ml. of buffer in the side arm and 3 ml. of emulsion of substrate in buffer in the main compartment. The gaseous phase was 5% CO_2 in N_2. The final buffer concentrations during the determination of enzyme activity were therefore $NaHCO_3$, 0·0312 M; NaCl, 0·143 M; $MgCl_2$, 0·035 M; gelatin, 0·44% (w/v). The emulsions of all substrates except Tweens 20 and 80 were prepared at a concentration of approx. 20 mg./ml. in the correct buffer by homogenizing in a Folley & Watson (1948) type homogenizer at 10000 rev./min. for 2 min. The Tweens were used in 10% (v/v) concentration in buffer. During the manometric determination, readings were taken at intervals of 5 min. for the first 30 min. The output of CO_2 was in most cases linear with respect to time and the results have been obtained by calculation using the method of Aldridge, Berry & Davies (1949). At all times the rate of CO_2 evolution so obtained was proportional to enzyme concentration up to at least an output of 12 μl. of CO_2/min.

Colorimetric determination of enzyme activity using phenyl esters as substrates. A method for the estimation of enzyme activity using phenyl esters as substrates has been developed, the phenol liberated being estimated colorimetrically by the method of Gottlieb & Marsh (1946) using 4-aminoantipyrine.

Reagents: (1) Sørensen's 0·0667 M phosphate buffer, pH 7·4. (2) 0·5% (w/v) 4-Aminoantipyrine in phosphate buffer, pH 7·4; the solution is filtered and stored in the dark. The reagent is diluted 10 times with phosphate buffer before use. (3) 4% (w/v) Potassium ferricyanide in phosphate buffer, pH 7·4. The reagent is stored in the dark and diluted 10 times with phosphate buffer before use. (4) Buffered substrate. Approximately 40 mg. ester is shaken vigorously with 50 ml. of phosphate buffer, pH 7·4.

Procedure: buffered substrate mixture (3 ml.), previously warmed to 37° was added to 1·0 ml. of the enzyme preparation and the solution incubated at 37° for 15 min. The reaction was stopped by the addition of the colorimetric reagents in the following order: 4-aminoantipyrine solution (3 ml.) was added and, after mixing, 2 ml. of the potassium ferricyanide solution; the mixture was left for 5 min. and then 10 ml. of water was added to dissolve the excess substrate. The extinction at 510 mμ. was measured using the Unicam D.G. spectrophotometer. Under these conditions the rate of liberation of phenol is linear with respect to time and there is a linear relation between enzyme concentration and the phenol liberated. Occasionally with some enzyme preparations the final solution was slightly turbid; such solutions could usually be clarified by the addition of 1 ml. of dialysed iron (British Drug Houses's preparation, diluted 3 times) which removed the insoluble material but not the pigment.

Experiments with inhibitors. Solutions of inhibitors were always prepared freshly in the buffer solutions used for the determination of enzyme activity. To 0·5 ml. of tissue extract in water was added 0·5 ml. of inhibitor in buffer. After a definite incubation period the substrate was added and the enzyme activity determined. An A-type esterase is defined as one which is not inhibited (in the irreversible or phosphorylating sense, Aldridge, 1953*c*) by organophosphorus inhibitors while B-type esterases are those which are so inhibited. The inhibitors used in this paper were: di-*iso*propylphosphorofluoridate (DFP) from Allan and Hanbury Ltd., diethyl (E600) and di-*iso*propyl *p*-nitrophenylphosphates, tetraethylpyrophosphate (TEPP) from Dr H. Coates, Albright and Wilson, Ltd., *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) and di-*iso*propylphosphorodiamidic anhydride (DPDA) from Dr G. S. Hartley, Pest Control Ltd.

RESULTS

Esterases of serum

As previously shown (Aldridge, 1953*a*) the esterases of rat serum may be clearly differentiated into A- and B-types by their sensitivity to organophosphorus inhibitors (Table 1). After treatment with either E 600, TEPP, DFP or mipafox there is a residual activity which is due to an A-type esterase. It was previously demonstrated (Aldridge, 1953*b*) that this enzyme can hydrolyse E 600. It is therefore possible for E 600 to act as a competitive substrate in the hydrolysis of phenyl acetate. However, the concentration of substrate is much higher than that of the inhibitor and no interference with the general method for the differentiation of serum esterases into A- and B-types by means of this inhibitor is encountered.

The A-esterase of serum is sensitive to acetone and the activity of this enzyme is much reduced in acetone-dried powders of serum. This may indicate a general sensitivity to denaturation by solvents. Since in some respects the substrates may act as solvents it may explain why a linear relation between serum concentration and activity is not obtained. It was found that this could be corrected by the addition of gelatin to the buffer. A possible explanation is that the gelatin is protecting the enzyme from the denaturing action of the substrate.

In Fig. 1 is shown the substrate pattern for this enzyme against a series of phenyl esters. It has a high activity against the acetate and does not hydrolyse at a measurable rate such esters of glycerol as triacetin and tributyrin. So far as the results in this paper show, this enzyme may be termed an aromatic esterase (Mounter & Whittaker, 1953). However, it is important to remember that the aromatic esters used are more liable to non-enzymic aqueous hydrolysis than the aliphatic esters and also that aromatic rings do increase the adsorptive capacity of some compounds for protein

Table 1. *Activity of various rat-tissue preparations against esters with and without* NN′-*di*-iso*propyl-phosphorodiamidic fluoride* (*mipafox*)

Enzyme extracts or tissue suspensions were prepared in water and enzyme activities were determined by the manometric method. The buffer composition was $NaHCO_3$, 0·0357 M; gelatin, 0·5% (w/v). Enzyme activities are all expressed as μl. CO_2/min. obtained from 1 ml. tissue extract prepared from 2 mg. acetone-dried powder of fresh tissue. PA = Phenyl acetate, PB = phenyl butyrate, TB = tributyrin. Resistant esterase (A-type) is the esterase which remains after incubation of equal volumes of enzyme extract (in water) and 2×10^{-4} M mipafox (in buffer) for 15 min. at 37°. Total esterase was determined after a similar incubation of equal volumes of enzyme extract and buffer. For the determination of enzyme activity each flask contained 1 ml. of these mixtures or their dilution with buffer of the same concentration in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) in buffer and was gassed with 5% CO_2 in N_2. The sensitive esterase (B-type) is the difference between total and resistant esterases. The same sample of rat serum was used for the experiments labelled serum (native) and serum (acetone dried); 1 g. of native serum will produce approximately 100 mg. acetone-dried powder.

Enzyme preparation	Substrate	Enzyme activity (μl. CO_2/ml./min.) Total esterase	Resistant esterase (A-type)	Sensitive esterase (B-type)
Intestinal mucosa (acetone dried)	PA	32	3	29
	PB	130	1	129
	TB	147	28	119
Pancreas (acetone dried)	PA	7	6	Nil
	PB	8	6	Nil
	TB	755	765	Nil
Pancreas (fresh tissue dispersion)	PA	—	Nil	—
	PB	—	Nil	—
	TB	—	360	—
Pancreatic juice	PA	0·39	0·26	0·13
	PB	0·65	0·55	0·10
	TB	468	455	Nil
Liver (acetone dried)	PA	17·9	2·3	15·6
	PB	17·1	Nil	17·1
	TB	3·7	Nil	3·7
Serum (native)	PA	2·56	2·18	0·38
	PB	0·79	0·09	0·70
	TB	0·098	Nil	0·098
Serum (acetone dried)	PA	5·90	3·20	2·70
	PB	5·47	0·12	5·35
	TB	0·79	Nil	0·79

(Klotz, 1946). King & Delory (1939) have also shown that phenyl phosphates are hydrolysed by alkaline phosphatase at higher rates than are simple aliphatic phosphates.

The B-type esterase of serum, like that of intestinal mucosa, is extremely sensitive to organophosphorus compounds. However, it differs from the B-type esterase of intestinal mucosa since the ratio of the rates of hydrolysis of tributyrin and phenyl *n*-butyrate is approximately 0·14 instead of 0·89.

Esterases of pancreas

The hydrolysis of tributyrin by rat serum and intestinal mucosa is prevented by treatment with 10^{-7}M mipafox. However, it was soon found that an acetone-dried powder of rat pancreas hydrolysed tributyrin rapidly but this activity was unaffected by up to 10^{-3}M mipafox (Table 1). This enzyme can therefore be classified as an A-type esterase but is clearly quite different from the A-type esterase of serum for it has little activity against phenyl esters. This preparation of pancreas does not contain any B-type esterase and this A-type enzyme appears to be the same as that present in pancreatic juice (Table 1). This A-type esterase hydrolyses higher glycerides such as triolein and is clearly pancreatic lipase.

Initially much trouble was encountered in developing a reliable method for the determination of this enzyme. Two factors are important. A solution of more consistent activity was obtained when water instead of buffer solution was used to extract the acetone-dried powder. The most important factor is however the emulsification of the substrate. The results in Table 2 indicate that for this enzyme preparation both the hydrolysis of tributyrin and triolein is increased when the substrate is emulsified. The hydrolysis of triolein is increased by magnesium while the hydrolysis of tributyrin is practically unaffected by the presence of magnesium. This is in agreement with the results and suggestion of Schønheyder & Volqvartz (1945*a*) that magnesium and calcium activate lipase by removing the higher fatty acids as they are formed as insoluble soaps. Such an activation would occur with triolein as substrate but since magnesium and calcium butyrates are soluble it should not occur with tributyrin.

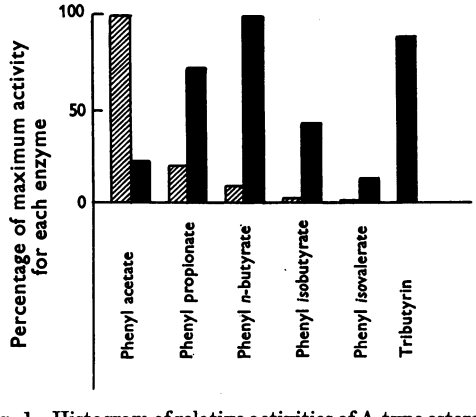

Fig. 1. Histogram of relative activities of A-type esterase of rat serum and B-type esterase of rat intestinal mucosa against phenyl esters. The preparation of A-type esterase was serum after incubation with an equal volume of 2×10^{-4}M *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) in buffer for 15 min. at 37°. The preparation of B-type esterase of intestinal mucosa was an extract of 20 mg. acetone-dried powder in 10 ml. of water with the addition of an equal volume of buffer after removal of the insoluble material. The buffer contained $NaHCO_3$, 0·0357M, and gelatin, 0·5% (w/v). For the determination of enzyme activities by the manometric method each flask contained 1 ml. of these preparations or their dilutions in the side arm and 3 ml. emulsion of substrates (approx. 20 mg./ml.), and was gassed with 5% CO_2 in N_2. The activity of the B-type esterase against tributyrin is added so that Figs. 1 and 2 may be compared. ▨, A-Type esterase; ■, B-type esterase.

Table 2. *Hydrolysis of tributyrin and triolein under various conditions by extract of acetone-dried rat intestinal mucosa and pancreas*

Enzyme activities were determined by the manometric method. Buffer contained $NaHCO_3$, 0·0357M; NaCl, 0·164M; $MgCl_2$, 0·04M; gelatin, 0·5% (w/v). For the determination of enzyme activity each flask contained 0·5 ml. enzyme extract in water and 0·5 ml. buffer in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. Enzyme activities are expressed as μl. CO_2/min. for 1 ml. of an extract of 2 mg. acetone-dried powder. For the experiments with unemulsified substrates, the ester was still in the form of large droplets at the end of the experiment.

Constituents of buffer				Enzyme activity (μl./CO_2/ml./min.)
$NaHCO_3$	NaCl	$MgCl_2$		
Pancreas extract (substrate, tributyrin)				
+	−	−	Substrate not emulsified	319
+	+	+		284
+	−	−	Substrate emulsified	986
+	+	+		1070
Pancreas extract (substrate, triolein)				
+	−	−	Substrate not emulsified	Nil
+	+	+		Nil
+	−	−	Substrate emulsified	6·7
+	+	+		61·2
Intestinal mucosa extract (substrate, tributyrin)				
+	−	−	Substrate not emulsified	178
+	+	+		166
+	−	−	Substrate emulsified	195
+	+	+		174

The hydrolysis of tributyrin and triolein by an extract of acetone-dried pancreas is not inhibited by TEPP, DFP, mipafox and DPDA. However E 600 does inhibit both activities, although the inhibition curves are not the same. Previous experience with inhibitors of this type suggested that it was unlikely that only one out of a series of such compounds would inhibit the enzyme in the phosphorylating sense. Dilution experiments (Table 3) have shown that in this case E 600 is acting as a reversible inhibitor. The differing physical conditions when tributyrin and triolein are used as substrates will probably alter the effective concentration of E 600 in solution. This would account for the different inhibition curves obtained for the two substrates.

The A-type esterase of rat serum also hydrolyses E 600 as well as aromatic carboxylic esters (Aldridge, 1953*b*). The A-type esterase from rat pancreas was therefore tested for its ability to hydrolyse organophosphorus compounds. The most sensitive method to detect hydrolysis of these compounds is to incubate a high enzyme concentration with a low concentration of organophosphorus compound. Any drop in the concentration of organophosphorus compound may be detected by a drop in the inhibitory power of the solution on a test enzyme such as pseudo-cholinesterase. Table 4 gives the results of some experiments which show that no measurable hydrolysis of DFP, TEPP or mipafox occurred. Any hydrolysis of E 600 may be detected by the formation of the yellow colour of *p*-nitrophenol. No hydrolysis was observed.

Table 3. *Reversible inhibition by diethyl* p-*nitrophenylphosphate* (E 600) *of A-type esterase from rat pancreas*

Enzyme extract was prepared by extracting 20 mg. acetone-dried pancreas with 10 ml. of water. Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). *A* = a mixture of equal volumes of extract in water and $3·9 \times 10^{-4}$ M E 600 in buffer; *B* = a mixture of equal volumes of extract and buffer. *A* and *B* were incubated for 30 min. at 37°. After dilution the enzyme activities were determined by the manometric method. For the determination each flask contained 1·0 ml. of diluted enzyme in the side arm and 3·0 ml. emulsion of tributyrin (approx. 20 mg./ml.) in buffer and was gassed with 5% CO_2 in N_2.

Dilution procedure	Enzyme activity (μl. CO_2/ml./min.)
100-Fold dilution of *A* with buffer (half-strength) containing $1·95 \times 10^{-4}$ M E 600	Nil
100-Fold dilution of *A* with buffer (half-strength)	5·85, 5·90
100-Fold dilution of *B* with buffer (half-strength)	6·75, 6·80

Table 4. *Destruction of organophosphorus compounds by A-type esterase from rat pancreas*

Enzyme extract was prepared by extracting 20 mg. acetone-dried pancreas with 10 ml. of water. Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). Equal volumes of enzyme extract and inhibitor in buffer were mixed and incubated for 30 min. at 37°. The inhibitory power of this solution was compared against a control containing only inhibitor. Pseudo-cholinesterase was the test enzyme (0·5 ml. of four-fold diluted horse serum). For the determination of cholinesterase activity by the manometric method each flask contained 0·5 ml. inhibitory solution and 0·5 ml. diluted horse serum in the side arm and 3 ml. substrate (acetylcholine; 0·015 M final concentration) in buffer and was gassed with 5% CO_2 in N_2. The cholinesterase and inhibitory solutions were incubated in the side arm for 30 min. at 37° prior to tipping into the substrate.

Inhibitor concentration	Inhibition of cholinesterase (%)	
	Control	With pancreas extract
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	64	66
Tetraethylpyrophosphate (TEPP)	86	80
Di-*iso*propylphosphorofluoridate (DFP)	83	85

Table 5. *Effects of different treatments on the ratio of the rates of tributyrin and triolein by various preparations of rat pancreas*

Buffer contained $NaHCO_3$, 0·0357 M; NaCl, 0·164 M; $MgCl_2$, 0·04 M; gelatin, 0·5% (w/v). Enzyme determinations were made using the manometric method. Each flask contained 0·5 ml. enzyme preparation in water and 0·5 ml. buffer in the side arm and 3 ml. emulsion of substrate (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. Nitrogen was determined by a colorimetric Nessler procedure (King, 1951). Enzyme activity (μl. CO_2/μg. N/min.) was determined using tributyrin as substrate. The substrate ratio is the ratio of the rates of hydrolysis of tributyrin and triolein.

Treatment	Enzyme activity (μl. CO_2/μg. N/min.)	Substrate ratio (tributyrin/triolein)
Fresh pancreas disrupted in water, centrifuged and filtered. Use filtrate	5·75	10·5
Water extract of freshly prepared acetone-dried pancreas	14·9	10·3
Water extract of acetone-dried pancreas (after storing for 6 months at −5°)	11·5	9·9
Precipitate water extract of acetone-dried pancreas with 19% (v/v) ethanol containing acetate buffer, pH 6·0 (cf. Cohn *et al.* 1950)	32·0	11·1
Heat water extract of acetone-dried pancreas for 10 min. at 53°	1·4	10·8

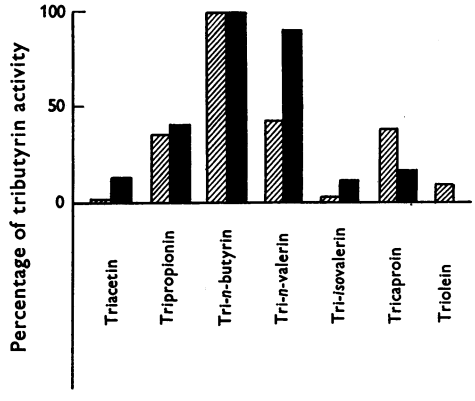

Fig. 2. Histogram of relative activities of A-type esterase of rat pancreas and B-type esterase of rat intestinal mucosa against triglycerides. The preparation of A-type esterase of pancreas was an extract of 20 mg. acetone-dried powder with 10 ml. of water with an equal volume of buffer added after removal of insoluble material. The preparation of B-type esterase of intestinal mucosa was an extract of 20 mg. acetone-dried powder in 10 ml. of water after heating for 20 min. at 56° to remove any A-type esterase present. After cooling to room temperature an equal volume of buffer was added. For the experiments with intestine the buffer contained $NaHCO_3$, 0·357 M, and gelatin, 0·5% (w/v). For the experiments with pancreas the buffer contained in addition NaCl, 0·164 M; $MgCl_2$, 0·04 M. For the determination of enzyme activities with the manometric method, each flask contained 1 ml. of these preparations or their dilutions in the side arm and 3 ml. emulsion of substrates (approx. 20 mg./ml.) and was gassed with 5% CO_2 in N_2. ▨, A-Type esterase; ■, B-type esterase.

Before the substrate pattern of the A-type enzyme of pancreas was determined it was essential to know that all the activities are mediated by the same enzyme. The results in Table 5 show that the ratio of the rates of tributyrin/triolein hydrolyses remains unaltered by a variety of treatments. It is concluded that the hydrolytic activity of this preparation against tributyrin and triolein are mediated by the same enzyme and it is assumed that the other glycerides examined are also hydrolysed by this enzyme. The relative rates of hydrolysis of these glycerides are shown in Fig. 2.

Esterases of intestinal mucosa

An acetone-dried powder of the intestinal mucosa of the rat is a very potent preparation of a B-type esterase (cf. Table 1). The equivalent of an extract of 10 μg. of such a powder will liberate 100 μg. phenol from phenyl butyrate in 15 min. under the conditions of the colorimetric technique. An advantage of the use of acetone-dried powder for this work is that although rat intestinal mucosa contains pseudo-cholinesterase (Ord & Thompson, 1950; Davison, 1953) the acetone-dried powders are almost inactive against choline esters. Its activity against butyrylcholine is only 0·5% of its activity against phenyl butyrate.

Upon treatment of this preparation with E 600 and other organophosphorus compounds its activity against phenyl butyrate is completely abolished. In Table 6 is shown its sensitivity to a variety of inhibitors of this type. It has long been known that there exist in animal tissues esterases which will hydrolyse esters such as methyl butyrate and tributyrin. These esterases are commonly called ali-esterase (Richter & Croft, 1942). Huggins & Lapides (1947) used aromatic esters and assumed that the hydrolysis obtained was due to the ali-esterase of Richter & Croft. The tributyrin hydrolysing power of rat liver is inhibited by low concentrations of E 600 (Myers & Mendel, 1953) so that it seemed probable that the B-type esterase which hydrolyses aromatic esters also will hydrolyse tributyrin. This point has been examined using initially as a test of identity the sensitivity of the activities against phenyl butyrate and tributyrin to various organophosphorus compounds. It was soon found that although its activity against phenyl butyrate was completely inhibited by organophosphorus compounds only approximately 80% of its activity against tributyrin was removed and the remaining 20% was quite resistant. Fig. 3 shows the results obtained when mipafox was used as an inhibitor. Similar results are obtained when TEPP and DFP are used but with E 600 the last 20% is inhibited by higher concentrations of inhibitor (10^{-5} M). There are present therefore in intestinal mucosa two enzymes which hydrolyse tributyrin, one of which (B-type) is inhibited by low concentrations of organophosphorus inhibitors and another which is extremely resistant (A-type).

The tributyrin hydrolysing activity of the B-type component appears to be identical with the enzyme hydrolysing phenyl butyrate. In Table 6 is shown the very close agreement of the sensitivity of both activities to treatment with five organophosphorus inhibitors. As shown in the preceding section the A-type esterase of pancreas is easily heat-denatured in unbuffered aqueous solution. While the B-type esterase of intestinal mucosa is more resistant the A-type present is also heat sensitive and by heating for 10 min. at 56° it is possible to lower the A-type esterase activity by more than 90% and only reduce the activity of the B-type esterase by 20–30%. The ratio of the rates of hydrolysis of tributyrin and phenyl butyrate (0·89) for the B-type esterase obtained after correction for the activity resistant to mipafox is the same as that obtained from a heated sample containing virtually no A-type esterase. Further heating to lower the activity of the B-type esterase itself by 70% does not alter the tributyrin to phenyl butyrate ratio. It is concluded

Table 6. *Sensitivity of B-type esterase of rat intestinal mucosa to organophosphorus compounds*

Enzyme extract (0·5 ml.; in water) was incubated with 0·5 ml. inhibitor solution in buffer for 30 min. at 37°. The residual enzyme activities were determined by the manometric method except for those marked (*), when the colorimetric method was used. Details of these methods and the buffer used are as given in the text. $pI_{50} = \log_{10}$ M concentration of inhibitor to produce 50% inhibition.

Inhibitor	Substrate: pI_{50} Phenyl butyrate	Tributyrin	Tween 80
Diethyl *p*-nitrophenylphosphate (E 600)	8·5*	8·5	—
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	7·2	7·2	7·0
Di-*iso*propylphosphorodiamidic anhydride (DPDA)	3·8*	4·1	3·9
Di-*iso*propylphosphorofluoridate (DFP)	9·0*	9·0	—
Tetraethylpyrophosphate TEPP	8·2	8·2	—
Di-*iso*propyl *p*-nitrophenylphosphate	8·6*	—	—

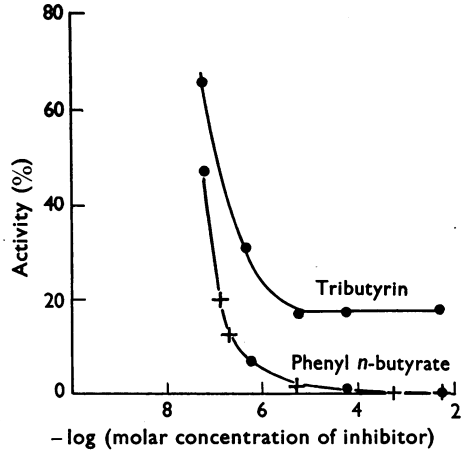

Fig. 3. Inhibition by mipafox of the hydrolysis of tributyrin and phenyl butyrate by an extract of rat intestinal mucosa. Equal volumes of an aqueous extract of the acetone-dried tissue powder and inhibitor in buffer were incubated for 30 min. at 37° prior to the addition to substrate. Enzyme activities were determined by the manometric method (●) and by the colorimetric method (+). Details of these methods are as given in the text.

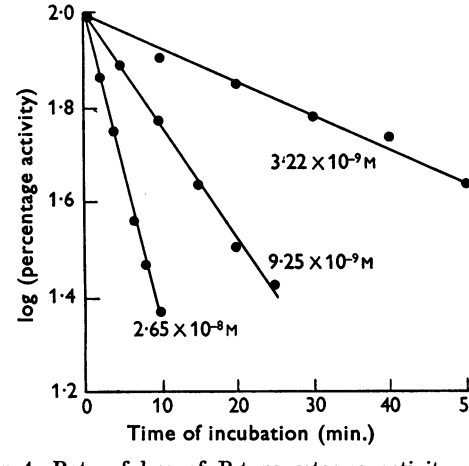

Fig. 4. Rate of loss of B-type esterase activity of rat intestinal mucosa after incubation with diethyl *p*-nitrophenylphosphate (E 600). Scrapings of rat intestinal mucosa were homogenized in 0·0667 M phosphate buffer, pH 7·4, for 2 min. at room temp. with a Folley & Watson (1948) type homogenizer. Dilutions of this preparation were incubated with E 600 in phosphate buffer. After various times of incubation the residual enzyme activity was determined by the colorimetric method. The addition of substrate stops the inhibitory reaction. Concentration of inhibitor during incubation shown against each curve.

therefore that these two hydrolytic activities are mediated by the same enzyme. The substrate patterns for the hydrolysis of triglycerides and phenyl esters are given in Figs. 1 and 2. The activity of this enzyme is not increased by emulsification of the substrate (Table 2). The reaction of erythrocyte cholinesterase and E 600 follows the kinetics of a first-order reaction and is bimolecular (Aldridge, 1950). Similarly, the inhibition of B-type esterase of intestinal mucosa by E 600 also shows the characteristics of a first-order reaction and is bimolecular (Fig. 4). The A-type esterase present in intestinal mucosa seems to be identical with the A-type esterase or lipase in pancreas. Neither enzyme is inhibited by TEPP, DFP, mipafox or DPDA. The A-type esterase of pancreas is inhibited by 10^{-5} M E 600 but this is a reversible type of inhibition. The A-type esterase of intestinal mucosa is also inhibited by 10^{-5} M E 600. The activity of the enzymes from both tissues against tributyrin and triolein is increased by emulsification of the substrate and both are equally sensitive to heat treatment. The ratio of the rates of hydrolysis of tributyrin and triolein for the intestinal enzyme approximates to that of the A-type esterase of pancreas. It is therefore concluded that these two enzymes are identical.

Esterases of other tissues

The B-type esterase present in the acetone-dried powder of liver, although very sensitive to organophosphorus inhibitors appears to have a substrate pattern (TB/PB = 0·22) which is different from both

Table 7. *Hydrolysis of various esters by rat brain dispersion after treatment with organophosphorus compounds*

Brain dispersions were prepared by homogenizing a rat brain in 15 ml. buffer for 2 min. at room temp. in a Folley & Watson (1948) type homogenizer. The buffer contained $NaHCO_3$, 0·0357 M; gelatin, 0·5% (w/v). Tissue dispersions (3 ml.) were incubated for 30 min. at 37° with 10^{-3} M inhibitor (0·3 ml.) in buffer. For the determination of the residual enzyme activity by the manometric method each flask contained 1 ml. of these solutions or their dilutions in buffer in the side arm and 3 ml. of substrate in buffer and was gassed with 5% CO_2 in N_2. Emulsions of the non-choline substrates (approx. 20 mg./ml.) were used. PA = Phenyl acetate, PB = phenyl butyrate, TB = tributyrin, TC = tricaproin. The choline esters were used at a final concentration of 0·015 M. AcCh = Acetylcholine, BuCh = butyrylcholine. Enzyme activities are given as μl. CO_2/min. for 1 ml. of original tissue dispersion.

Inhibitor	Substrate					
	AcCh	BuCh	PA	PB	TB	TC
	Enzyme activity (μl. CO_2/ml./min.)					
None	10·0	2·6	56·0	20·8	7·8	5·4
NN'-Di-*iso*propylphosphorodiamidic fluoride (mipafox)	0·9	Nil	21·9	10·0	3·7	3·7
Tetraethylpyrophosphate (TEPP)	Nil	Nil	9·8	2·3	1·5	2·9
Diethyl *p*-nitrophenylphosphate (E 600)	0·2	Nil	8·7	2·0	1·0	2·7

the B-type esterase of intestinal mucosa and of serum.

Recently Mendel *et al.* (1953) have stated that there is in rat brain an esterase which is less sensitive to E 600 than that in rat serum. In Table 7 is shown the results of some experiments with preparations of rat brain. This work is not yet complete but illustrates the complexity of the problem. In the first place there is a considerable activity remaining against phenyl acetate, phenyl butyrate, tributyrin and tricaproin after treatment with mipafox. The activity against phenyl butyrate cannot all be due to A-type esterase of serum and is an unusual finding. TEPP and E 600 reduce the activity against all four substrates further. These results indicate that the esterases in brain are different from those present in the other tissues previously discussed.

A comparison of the A-type esterase from pancreas with B-type esterase from intestinal mucosa

An examination of the experimental results illustrated in Fig. 2 shows that A-type esterase from pancreas and the B-type esterase from intestinal mucosa both hydrolyse the lower triglycerides but that triolein is only hydrolysed by the A-type esterase. It is clear from the evidence previously discussed that this A-type enzyme is pancreatic lipase whereas the B-type esterase is intestinal esterase. Since these two enzymes have been definitely separated, it is possible to compare their properties under identical conditions. Using tributyrin as substrate it has been shown that the activity of the B-type esterase is unaffected by emulsifying the substrate. The replacement of hydrolysed substrate is therefore not a rate-determining step. However emulsification of tributyrin increases the activity of A-type esterase three- to four-fold. These observations could be explained if it were assumed that the B-type esterase only hydrolyses the substrate in solution in the aqueous phase while the A-type esterase can adsorb on the surface of the substrate and is dependent on the surface area of the substrate available. On this basis the reason the B-type esterase does not hydrolyse triolein is not because it is incapable of hydrolysing esters of higher fatty acids but because triolein is so insoluble in water; conversely a water-soluble oleate should be hydrolysed by the B-type esterase. That this is so is shown by the fact that the B-type esterase will hydrolyse Tween 80, a water-soluble oleate. That this activity is due to B-type esterase is shown by the fact that it is inhibited by mipafox and DFP to the same extent as the hydrolysis of tributyrin and phenyl butyrate (Table 6). Tween 20 (a laurate) is also hydrolysed by B-type esterase. The ratio of the tributyrin and Tween 80 hydrolyses for B-type esterase is approximately 150 and for A-type esterase 450. The B-type esterase is therefore a little more efficient (relative to the hydrolysis of tributyrin) at the hydrolysis of the water-soluble oleate than is the A-type esterase.

The hydrolysis of choline esters by A- and B-type esterases

It has been shown by many workers that both true and pseudo-cholinesterases hydrolyse a variety of esters other than choline esters (Whittaker, 1951). It is of interest therefore to examine the esterases discussed in this paper to see if they will hydrolyse choline esters at all. The results in Table 8 show that choline esters are hardly if at all hydrolysed by these esterases. This indicates that substrates which contain a strong positive charge are probably unable to reach the enzyme active centre. This is in agreement with the view put forward by Wilson, Levine & Freiberger (1952) based on results obtained using the inhibitor prostigmine and horse-liver esterase.

Table 8. *Hydrolysis of choline esters by rat esterases*

A-Type esterase (serum) was rat serum after incubation with an equal volume 2×10^{-4}M *NN'*-di-*iso*propylphosphorodiamidic fluoride (mipafox) in buffer for 15 min. at 37° to remove B-type esterase and cholinesterases. A-Type esterase (pancreas) was an extract prepared from 80 mg. acetone-dried pancreas in 10 ml. of water after incubation with an equal volume of 2×10^{-4}M mipafox in buffer. B-Type esterase (intestine) was an extract prepared from 80 mg. acetone-dried intestinal mucosa after incubation with an equal volume of $2{\cdot}2 \times 10^{-5}$M di-*iso*propylphosphorodiamidic anhydride (DPDA) for 30 min. at 37° to remove pseudo-cholinesterase. Buffer for the experiments with serum and intestine contained $NaHCO_3$, 0·0357M, and gelatin, 0·5% (w/v). For the experiments with pancreas the buffer contained in addition NaCl, 0·164M, and $MgCl_2$, 0·04M. For the determinations of enzyme activity by the manometric method the flasks contained 1 ml. of these solutions or their dilutions and 3 ml. substrate in buffer and was gassed with 5% CO_2 in N_2. Emulsions of phenyl acetate and tributyrin (approx. 20 mg./ml.) were used and acetyl- and butyryl-choline to give a final concentration of 0·015M. Enzyme activities are given as μl. CO_2/min. for 1 ml. serum or 1 ml. of an extract of 8 mg. acetone-dried powder.

Substrate	Enzyme activity (μl. CO_2/ml./min.)	Ratio
A-Type esterase (serum)		
Phenyl acetate	401	$>10^3$
Acetylcholine	<0·4	
B-Type esterase (intestinal mucosa)		
Tributyrin	638	
Butyrylcholine	3·8	$1{\cdot}7 \times 10^2$
A-Type esterase (pancreas)		
Tributyrin	2800	
Butyrylcholine	<0·2	$>1{\cdot}4 \times 10^4$

DISCUSSION

Persistent attempts have been made to characterize the group of enzymes able to hydrolyse esters. The results given in this paper show why attempts using only a variety of substrates have not been generally successful. A-Type esterase in rat pancreas hydrolyses the same triglycerides as the B-type esterase of intestinal mucosa. A method of separating the esterases in sera, previously described by Aldridge (1953*a*), has been shown to be generally applicable to the esterases of tissues. In the work on the esterases of serum, E600 was the inhibitor chiefly used though TEPP and DFP gave the same results. It is important to consider only the 'irreversible' or 'phosphorylating' type of inhibition (Aldridge, 1953*c*). The results in this paper show that it is unwise to rely on only one inhibitor. For instance, although the A-type esterase of pancreas is not inhibited by TEPP, DFP or mipafox, it is inhibited by 10^{-5}M E600. However, this inhibition is reversible. Most of the work described has been concerned with characterizing three enzymes which, when separated, are quite dissimilar. The A-type esterase of pancreas readily hydrolyses triglycerides but aromatic esters hardly at all, while for the serum A-type esterase the reverse is true. The B-type esterase of intestinal mucosa hydrolyses both triglycerides and phenyl esters with equal facility. Although these enzymes when separated have very different characteristics much difficulty has been experienced by earlier workers in devising methods for their determination. The Tween compounds (fatty acid esters of polyoxyethylene derivatives of sorbitan) have been used as specific substrates for lipase (Archibald & Ortiz, 1946; Boissonas, 1948; Gomori, 1945, 1949). Recently, however, the use of Tween 20 (laurate) has been recommended for the determination of esterase (Copenhaver, Stafford & McShan, 1950). Nachlas & Seligman (1949) have pointed out that the distribution of esterase in the rat, shown histochemically using β-naphthyl acetate, is very similar to that obtained by Gomori (1945) using Tween 20. Using organophosphorus inhibitors it has been shown conclusively that both Tween 80 (oleate) and Tween 20 are hydrolysed both by the intestinal esterase (B-type) and by pancreatic lipase (A-type). These esterases are extremely difficult to separate by the conventional protein purification methods, and the results above illustrate the difficulty of characterizing them by the use of different substrates. Although tributyrin is often used as a substrate for lipase it is clear that this substrate is quite unsuitable for this purpose. This was pointed out by Cherry & Crandall (1932). The use of organophosphorus compounds will undoubtedly help to characterize the enzymes which have been named after the substrates they hydrolyse, e.g. vitamin A esterase (Ganguly & Deuel, 1953; Krause & Alberghini, 1950; McGugan & Laughland, 1952), cholesterol esterase (Fodor, 1950) aspirin esterase (Vincent & Lagreu, 1949) and esterases hydrolysing indoxyl esters (Barrnet, 1952). That for histochemical methods the specificity conditions must be extremely stringent has been illustrated by Denz (1953) who showed that at the myoneural junction the high concentrations of cholinesterase produced staining when using β-naphthyl acetate as substrate.

Recently Mendel *et al.* (1953) have reported an enzyme in rat brain which is less sensitive to inhibition by E600 than that of serum (Aldridge, 1953*a*). They have called this enzyme C-esterase in contrast to the B-esterase of serum. It has now been agreed in discussion with Professor Mendel that such differences in sensitivity do not warrant a separate classification. Accordingly, the esterase of Mendel *et al.* (1953) will be classified as a B-type esterase. The esterases examined in the paper have all been classified into the two groups, A- or B-types.

The substrate patterns of the A-type esterase of pancreas (lipase) and the B-type esterase of intestine (esterase) against triglycerides are similar. The differences between lipase and esterase have never been clearly defined. Now that their activities have been separated using organophosphorus compounds, certain differences may be discussed. Lipase hydrolyses triolein much more readily than esterase and esterase hydrolyses aromatic esters more readily than lipase. The effects of emulsification of the substrates on the activity of both enzymes provides a clue to a more fundamental difference. Desnuelle (1951), in a discussion of this question, has asked 'Et ne faudrait-il pas simplement baser la distinction entre substrat esterasique et substrat lipasique sur la solubilite dans l'eau?'. A comparison of the activity of lipase and esterase against tributyrin under identical conditions shows that only the activity of lipase is increased by emulsification of the substrate. The activity of esterase is the same whether the substrate is emulsified or added as drops from a Pasteur pipette. The replacement of hydrolysed substrate to the aqueous phase cannot be a rate-determining factor. Schønheyder & Volqvartz (1945*b*) have also shown that the affinity of pig pancreas for tricaproin is very low in homogeneous solution. With increasing amounts, as soon as a heterogeneous system is produced, there is a large increase in reaction velocity which rapidly reaches a maximum with increasing surface area of the substrate. It is suggested that esterase acts on the substrate in solution in the aqueous phase while lipase has the ability to catalyse the hydrolysis of the substrate at the oil-water interface. On this basis esterase will not be able to hydrolyse triolein because it is so insoluble, but it should be able to hydrolyse an ester of a higher fatty acid if it were in solution. Tween 80 (oleate) is such an ester and it has been shown that relative to tributyrin, esterase hydrolyses it more readily than lipase. The locations where these enzymes exert their activity in a physiological sense does not rule out such an idea.

Using the information gained by studies such as those described on a particular tissue, organophosphorus compounds might well provide useful tools to examine physiological problems. For instance there are the rival theories of Frazer (1948) and Verzár (1948) for the mechanism of fat absorption. Recent work, using the techniques of partition chromatography and radioactive isotopes, has shown that after ingestion of free fatty acids triglycerides are found in the lymph (Borgstrom, 1952*a*). If triglycerides containing labelled glycerol are ingested then the triglycerides recovered from the lymph contain much unlabelled glycerol (Reiser, Bryson, Carr & Kuiken, 1952). Further, if monoglycerides are ingested containing labelled fat and labelled glycerol, triglycerides are recovered from the lymph and the relative activities of their fatty acid and glycerol contents indicates that there must have been considerable hydrolysis of the monoglycerides before resynthesis (Reiser & Williams, 1953). Monoglycerides are resistant to hydrolysis by lipase (Borgstrom, 1952*b*, 1953), and it is therefore clear that considerable chemical activity is taking place between the gut and the lymph. This suggests the need for an examination of the necessity of esterase and for lipase in these processes. It would be relatively easy to maintain animals with their intestinal esterase at a low level by feeding with low concentrations of an organophosphorus compound. Di-*iso*propyl *p*-nitrophenylphosphate would appear to be a suitable compound. It is stable, has an extremely high activity against B-type esterase, and is at least 1000 times less active against true cholinesterase (Aldridge, 1953*d*).

SUMMARY

1. With the method of using organophosphorus compounds for the differentiation of esterases into A- and B-types, another A-type esterase in pancreas of the rat has been demonstrated.

2. The properties of the A-type esterases from serum and pancreas and the B-type esterase of intestinal mucosa have been examined.

3. From a comparison of the A-type esterase of pancreas (lipase) and the B-type of intestinal mucosa, suggestions have been discussed on the difference between esterase and lipase.

4. The uses of organophosphorus compounds for the characterization of esterases have been discussed.

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A Study of Some Simple Peptide Complexes with Zinc and Cadmium Ions in Aqueous Solution

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Previous studies (Perkins, 1952, 1953*a*) of metal complexes in aqueous solution were restricted to amino acids. It has already been shown (Monk, 1951*a–c*; Perkins, 1952) that for the simplest peptide, glycylglycine, complexes are formed which have smaller stability constants than those of glycine. Dobbie, Kermack & Lees (1951) studied the copper complexes of glycylglycine and diglycylglycine and found that the effect of the second peptide bond was to further reduce the stability constant. In this paper a range of simple peptide complexes with zinc and cadmium ions are examined. Some of the results have been the subject of a preliminary communication (Perkins, 1953*b*).

EXPERIMENTAL

Materials. The following AnalaR (British Drug Houses Ltd., Poole) salts were used in 0·01 M aqueous solution: $ZnSO_4$, $7H_2O$ and $3CdSO_4$, $8H_2O$. All the peptides were vacuum-dried over P_2O_5 at 100° for 48 hr., weighed and made up in 0·02 M solution. The peptides and complexes of glycyl-L-tryptophan and glycyl-L-tyrosine were relatively insoluble, and were therefore used in 0·004 M and 0·002 M solutions respectively. All the peptides were obtained from Roche Products Ltd. unless otherwise stated. The sample of triglycylglycine was kindly given by Dr Hanby, Courtaulds Ltd., Maidenhead. The peptides, glycyl-DL-serine (Fischer & Roesner, 1910), glycyl-DL-phenylalanine (Fischer & Schoeller, 1907), DL-alanyl-DL-alanine (Fischer & Raske, 1906) and glycyl-DL-isoleucine (Abderhalden, Hirsch & Schuler, 1909) were synthesized by the methods cited.

The purity of the peptides was checked by two-dimensional filter paper chromatography and nitrogen content using the micro-Kjeldahl procedure.

Potentiometric titration. A Cambridge Instrument Co. Ltd. bench type pH meter was used with a glass electrode and saturated calomel half-cell. The titrations were carried out as outlined by Albert (1950) with minor modifications. The dissociation constant for the α-amino group was determined at 25° under the conditions subsequently used for the titrations in the presence of the metal. These titrations were made on solutions in which the ratio of acid to metal was 2:1. For most titrations equal volumes of stock metal and amino acid solutions were mixed and then stirred with O_2-free N_2 for 10 min. At the mid-point of the titration the volume was 50 ml. All measurements were made at 25·0±0·1° in a thermostatically controlled water bath.

Calculations. The system to be considered in calculating the stability of each complex is

$$M^{s+} + \text{Peptide} \rightleftharpoons \text{Complex } I^{+} \text{ (association constant} = K_1).$$