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Studies on Ali-esterases and other Lipid-hydrolysing Enzymes

3. INHIBITION OF THE ESTERASES OF PANCREAS*

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Extensive studies of the substrate specificity of crude preparations of pancreatic lipase have been carried out by many investigators (cf. Ammon & Jaarma, 1950), though they usually failed to consider the fact that the pancreas contains more than one enzyme with esterase activity. The results obtained by Loevenhart & Pierce (1907) with sodium fluoride and by Platt & Dawson (1925) with albumin and edestin as inhibitors suggest that different enzymes are responsible for the hydrolysis of ethyl esters and olive oil by pancreas. Although these authors did not draw this conclusion, later experiments by Wolvekamp & Griffioen (1934) and Fodor (1946, 1950*a, b*) showed that the suggestion is justified.

It is generally agreed that pancreatic lipase can hydrolyse tributyrin as well as triolein (cf. Willstätter & Memmen, 1923), and recent evidence presented by Fodor (1950*a*) and Aldridge (1954) supports the assumption that the activity of pancreas preparations towards tributyrin is due largely, if not entirely, to the lipase. Fodor (1950*b*) suggested further that the hydrolysis of esters such as methyl butyrate is due largely to cholesterol esterase, an enzyme which was shown by Kelsey (1939) to be distinct from the lipase of pancreas. The methods used by Fodor (1946, 1950*a, b*) to distinguish these two enzymes (effect of gum acacia, bile salts, trypsin digestion and exposure to high temperatures and to alkali) did not permit of a

definition of their relative substrate specificities. However, Webb (1948) has suggested that organo-phosphorus inhibitors would be useful tools in a study of the substrate specificity of esterases and lipases, since the lipases are relatively resistant to inhibition by these compounds. The results obtained by Aldridge (1953*a*, 1954), Mounter & Whittaker (1953) and Myers *et al.* (1955) with serum and other tissues have confirmed the value of this technique. In the present investigation we have therefore reinvestigated the specificity of the pancreas esterases, using various organo-phosphorus derivatives as esterase inhibitors.

We found that the pancreas enzymes responsible for the hydrolysis of tributyrin and methyl butyrate could indeed be readily distinguished. Further attention was paid to the hydrolysis of salicyl butyrate since Hofstee (1952*a, b*) reported that pancreas preparations contain two esterases capable of hydrolysing salicyl esters, neither of which is identical with lipase. Our experiments confirm the conclusions of Hofstee (1952*a, b*), as well as those of Fodor (1950*a, b*), and show that it is possible to distinguish at least three types of enzymes with esterase activity in pancreas by the use of organo-phosphorus inhibitors.

MATERIALS AND METHODS

Substrates. Many of the substrates were prepared in this laboratory by Miss J. W. Tol according to the methods described in the literature; these esters include phenyl

* Part 2: Myers, Schotte & Mendel (1955).

acetate, propionate and butyrate (Perkin, 1889), *p*-nitrophenyl acetate and butyrate (Huggins & Lapidès, 1947), salicyl butyrate (Hofstee, 1952*b*), the phenyl ester of phenylacetic acid and the ethyl esters of L-tyrosine and L-leucine (Fischer, 1901). The remaining esters tested were commercial preparations from British Drug Houses Ltd. and Eastman Kodak Ltd.

Inhibitors. Diethyl *p*-nitrophenyl phosphate (E 600) and diisopropyl *p*-nitrophenyl phosphate (DINP) were obtained through the courtesy of Albright and Wilson, Ltd., diisopropyl phosphorofluoridate (DFP) was supplied by Dr B. C. Saunders, Cambridge, *NN'*-diisopropyl phosphorodiamidofluoridate (mipafox, Isopestox) by Dr G. S. Hartley, Pest Control Ltd., and *NN*-dimethylcarbamoyl fluoride by Dr G. Schrader, Elberfeld. Tri-*o*-cresyl phosphate (TOCP) was obtained commercially from Eastman Kodak Ltd. and methanesulphonyl fluoride was synthesized in this laboratory by Mr A. Kemp, jun., according to the method described by Davies & Dick (1932).

Manometric determination of esterase activity. Esterase activity was determined by the Warburg method at 37.5° and pH 7.4 in a medium containing 0.025 M-NaHCO₃ and saturated with CO₂+N₂ (5:95, v/v). Rat-pancreas suspensions were prepared with a glass Potter-Elvehjem homogenizer and diluted in the Warburg vessels so that the vessels contained 0.05–2 mg. of fresh pancreas in a fluid volume of 5 ml. for most experiments. The reaction mixture was incubated for 30 min. at 37.5°, with or without inhibitor, before adding a small volume of substrate to determine the residual esterase activity. All esters were prepared in the form of an aqueous emulsion, suspension or solution containing gum acacia such that the final concentrations of ester and gum acacia in the reaction mixture were 0.2 and 0.1% respectively. The values given for the esterase activities are corrected for the spontaneous hydrolysis of the substrate concerned.

Titrimetric determination of lipase activity. The manometric method proved unsuitable for determining the rate of hydrolysis of olive oil (triolein), since the values obtained were always very low. The titrimetric methods described for serum lipase by Cherry & Crandall (1932) and for pancreatic juice by Lips (1951) gave much more satisfactory results. The latter technique was used in most of the experiments in the present investigation. A portion (2 ml.) of a pancreas suspension containing 50–100 mg. of fresh pancreas/ml. were mixed with 2 ml. of 5% (w/v) sodium taurocholate solution and shaken with 10 ml. of olive oil to obtain an emulsion. Samples were withdrawn after incubation at 37.5° for various periods up to 4 hr., pipetted into ethanol and titrated with alkali.

The pH of the reaction mixture usually fell from 6.7 to about 5.3 within a 4 hr. period of incubation. When olive oil was replaced by tributyrin as substrate, the pH dropped much more rapidly; under these conditions, better results were obtained when the reaction mixture was buffered with phosphate. Since the rate of hydrolysis of the oil decreased appreciably with longer periods of incubation, the lipase activity was calculated on the basis of the hydrolysis occurring within the first hour. All values were corrected for a control containing pancreas suspension which had been inactivated by heating at 100°.

Martin & Peers (1953) have reported that the lipase of oat seeds exhibits maximum activity, with olive oil as substrate, in a reaction mixture which contains relatively small amounts of water. Larger amounts of oil did not

improve the results obtained by the method described above with the lipase of pancreas; however, when proportionately less oil was used, a less satisfactory emulsion was obtained and the lipase activity appeared to be lower. The taurocholate was necessary to produce a finely dispersed emulsion but could be replaced by gum acacia when the method of Cherry & Crandall (1932) was applied to pancreas suspensions. Satisfactory results could also be obtained with serum as emulsifying agent when olive oil was used as substrate for the pancreas lipase; the normal sera have no appreciable activity towards olive oil under these conditions.

In several experiments on the reversibility of the inhibition by E 600, the pancreas suspension was first incubated with E 600 alone for 30 min., subsequently an equal volume of rabbit serum was added and the mixture incubated for another 30 min. before adding the taurocholate and oil. In this way any excess of E 600 in the solution was completely destroyed by the A-esterase in the rabbit serum (Aldridge, 1953*b*) before the residual lipase activity of the pancreas was determined. Control suspensions without E 600 but treated in the same way with rabbit serum exhibited a normal lipase activity towards olive oil.

Colorimetric determination of cholesterol-esterase activity

Cholesterol-esterase activity was measured colorimetrically (Sperry & Webb, 1950) with the techniques described by Yamamoto, Goldstein & Treadwell (1949). The pH of the reaction mixture, which contained 1% of sodium taurocholate and 0.4% of cholesterol ester, was maintained at 6.3–6.4. The esterase activity was usually calculated from the hydrolysis occurring within the first 3–4 hr. at 37°. Cholesteryl acetate was used as substrate in most experiments since the cholesterol esterase of pancreas acts most efficiently on the esters of the lower aliphatic acids (Swell, Cassidy & Treadwell, 1951; Swell & Treadwell, 1955). A few experiments were carried out with the same technique to determine the rate of hydrolysis of cholesteryl stearate and the rate of esterification of free cholesterol in the presence of stearic acid (Swell & Treadwell, 1950).

RESULTS

Fresh suspensions of rat pancreas exhibit a very high activity towards tributyrin and a high activity towards phenyl butyrate. The esterase activity towards phenyl butyrate, propionate and acetate is almost completely inhibited by low concentrations of all the organo-phosphorus derivatives investigated, whereas the activity of the lipase towards tributyrin is not significantly affected by low concentrations of most of these compounds (Table 1). A similar result was obtained with the methanesulphonyl and dimethylcarbamoyl fluoride derivatives (Table 1), which have been shown to act similarly to the organo-phosphorus derivatives (Myers & Kemp, 1954). The classical inhibitors for distinguishing esterase activity from lipase activity (Rona & Pavlovic, 1922) proved to be much less satisfactory; high concentrations of atoxyl do indeed inhibit the esterase activity towards phenyl

butyrate without affecting the lipase activity towards tributyrin appreciably, but quinine inhibited both enzymes equally (Table 1). As could be surmised from the results obtained by Loevenhart & Pierce (1907), the esterase activity was more sensitive than the lipase activity to inhibition by sodium fluoride (Table 1).

In confirmation of the results obtained by Aldridge (1954), E 600 exhibits a considerable inhibitory activity against the lipase of rat-pancreas suspensions (Table 1). Its isopropyl analogue,

Table 1. *Sensitivity of the lipase and esterase activities of rat pancreas to various inhibitors*

Rat-pancreas suspensions were incubated for 30 min. at 37° and pH 7.4 with various concentrations of the inhibitors indicated below. Subsequently the esterase activity was measured by a manometric technique, and the negative logarithm of the molar concentration of inhibitor required to produce 50% inhibition of the activity (pI_{50}) was determined graphically. In most experiments with tributyrin, the reaction mixture contained 0.02 mg. of fresh pancreas/ml.; a concentration of 0.1 mg. of fresh pancreas/ml. was used in the experiments with phenyl butyrate as substrate.

Inhibitor	pI_{50} values	
	Phenyl butyrate	Tributyrin
Diethyl <i>p</i> -nitrophenyl phosphate (E 600)	7.6	5.2
Diisopropyl <i>p</i> -nitrophenyl phosphate (DINP)	8.4	2.6
Diisopropyl phosphorofluoridate (DFP)	6.7	(<3)
<i>NN'</i> -Diisopropyl phosphorodiamidofluoridate	5.5	(<3)
<i>NN</i> -Dimethylcarbamoyl fluoride	5.9	2.8
Methanesulphonyl fluoride	4.1	(<3)
Sodium fluoride	2.3	1.4
Quinine hydrochloride	3.2	3.2
Sodium arsenilate (atoxyl)	2.1	(<1.5)

DINP, also exhibits some inhibitory activity. The degree of inhibition of the hydrolysis of olive oil is in all cases almost identical with that observed when tributyrin is used as substrate (see Table 2). These data confirm the conclusion of previous investigators that both substrates are hydrolysed by the same enzyme, a lipase.

As found previously (Myers, 1954), this inhibition by E 600 is progressive (Table 2) and irreversible. The data given in Tables 2 and 3 show that the inhibition of the lipase activity is not affected by a thousand-fold dilution of the incubation mixture containing E 600. Moreover, the inhibition of the pancreas lipase was found to remain unaltered for at least 4 hr. after the excess of E 600 in the reaction mixture had been removed by treatment with rabbit serum in the manner described above.

Table 2. *Progressive inhibition of pancreas lipase on incubation with E 600 or DINP for various periods*

Rat-pancreas suspensions (50 mg. of fresh pancreas/ml.) were incubated at 37° with 3×10^{-4} M E 600 or 3×10^{-4} M DINP for various periods. Subsequently 2 ml. of the suspension were mixed with 2 ml. of 5% sodium taurocholate solution plus 10 ml. of olive oil and the lipase activity was determined titrimetrically as described in the text (cf. Lips, 1951). The activity of the same pancreas suspensions towards tributyrin was determined manometrically after one thousand-fold dilution in the Warburg vessel.

Inhibitor	Time of incubation at 37° (min.)	% inhibition	
		Olive oil	Tributyrin
E 600	5	36	—
	30	70	67
	60	86	84
	120	93	95
	240	94	98
DINP	30	6	12
	240	27	38

Table 3. *Inhibition of pancreas lipase by E 600 under various experimental conditions*

Rat-pancreas suspensions in 0.67 M phosphate buffer, pH 7.3, were incubated with various concentrations of E 600 for 30 min. before determining the lipase activity towards olive oil and tributyrin by a titrimetric or manometric method as described in the text. The results were plotted graphically in order to estimate the pI_{50} value (negative logarithm of the molar concentration of E 600 required to produce 50% inhibition).

Concn. of pancreas suspension (mg./ml.)

Concn. of pancreas suspension (mg./ml.)		Technique used in measuring lipase activity	Substrate	Lipase activity (m-moles of acid/g. pancreas/hr.)	pI_{50} value with E 600
During incubation with E 600	During measurement of activity				
180	25.7	Titrimetric	Olive oil	21	3.5
50	7.1	Titrimetric	Olive oil	23	3.8
50	7.1	Titrimetric	Tributyrin	27	3.8
50	0.05	Manometric	Tributyrin	120	3.8
0.05	0.05	Manometric	Tributyrin	145	5.0
0.005	0.005	Manometric	Tributyrin	260	5.6

The conclusion by Aldridge (1954) that the inhibition by E 600 is reversible may be based on the fact that a given concentration of E 600 produces a much smaller degree of inhibition in a concentrated suspension than in a highly diluted suspension of the same pancreas (Table 3). This phenomenon has been observed previously with other tissues and is probably due to interaction of the organo-phosphorus derivative with the tissue constituents (Hobbiger, 1951). The same type of result is observed not only with the lipase but also with the esterase in rat pancreas which is responsible for the hydrolysis of phenyl acetate and phenyl butyrate. The amounts of E 600, DINP and

phosphorus derivatives (Fig. 1). In this respect, therefore, the cholesterol esterase resembles the enzyme responsible for the hydrolysis of phenyl acetate, propionate and butyrate. The inhibition curves obtained with cholesteryl acetate and with phenyl acetate as substrates were similar but not identical (Fig. 1); the discrepancies observed may be due to the different techniques employed with these two substrates. No marked differences were apparent in the inhibitory effects of E 600, DINP and DFP. Further, the hydrolysis of cholesteryl acetate, the hydrolysis of cholesteryl stearate and the esterification of free cholesterol in the presence of stearic acid are all inhibited to about the same extent by DFP (Table 4); apparently these enzymic activities are all due to the same esterase (cf. Swell & Treadwell, 1955).

Fig. 1. Inhibition of hydrolysis of phenyl acetate (A) and cholesteryl acetate (B) by the cholesterol esterase of rat-pancreas suspensions after incubation with E 600, DINP and DFP. Pancreas suspensions (40 mg. of fresh pancreas/ml.) were incubated for 2 hr. at 37.5° with these inhibitors in various concentrations. The activity towards cholesteryl acetate was determined colorimetrically at pH 6.3 by the method of Yamamoto *et al.* (1949). The activity towards phenyl acetate was determined manometrically at pH 7.4, as described in the text, after diluting the pancreas suspension 200-fold.

DFP required to produce 50% inhibition of this esterase are, on the average, forty times greater with a concentrated suspension (40 mg. fresh pancreas/ml.) than with a dilute suspension (0.1 mg. fresh pancreas/ml.) (see Table 1 and Fig. 1). The differences in the pI_{50} values observed with the lipase under similar conditions (Table 3) are of the same order of magnitude.

The cholesterol esterase activity of pancreas suspensions is readily inhibited by the organo-

Table 4. Effect of diisopropyl phosphorofluoridate on the activity of the cholesterol esterase towards various substrates

The cholesterol esterase activity of rat-pancreas suspensions (40 mg. of fresh pancreas/ml.) was determined colorimetrically at pH 6.3, according to the technique described by Yamamoto *et al.* (1949) and Swell & Treadwell (1950).

Expt.	Substrate	Cholesterol esterase activity (μ moles/g. pancreas/hr.)	Inhibition in the presence of 10^{-4} M DFP (%)
1	Cholesteryl acetate	9.3	82
	Cholesteryl stearate	2.9	74
2	Cholesteryl acetate	10.3	79
	Cholesterol + stearic acid	1.3	80

Both the cholesterol esterase and the lipase of pancreas proved to be relatively resistant to inhibition by TOCP *in vivo*. Whereas the ali-esterases (B-type esterases) of rat serum and liver are almost completely inhibited 1 day after the administration of 50 μ l. of TOCP/kg. (Mendel & Myers, 1953; Myers & Mendel, 1953), a dose of 1 ml. of TOCP/kg. intraperitoneally was required in order to produce an appreciable depression of the cholesterol-esterase activity of the pancreas (Table 5). The same dose of TOCP, which is sufficient to produce a slight diarrhoea, has less effect on the lipase activity of rat pancreas. The hydrolyses of olive oil and of tributyrin by pancreas are affected in the same way by TOCP *in vivo*, but the hydrolysis of phenyl acetate again tends to be somewhat more strongly inhibited than the hydrolysis of cholesteryl acetate (Table 5).

In these experiments (cf. Table 5), the injection of TOCP did not affect significantly the amount of free cholesterol or cholesterol ester in the serum. The total amount of cholesterol in the liver also

Table 5. *Effect of tri-o-cresyl phosphate (TOCP) injection on the lipase and cholesterol esterase of rat pancreas*

Male rats weighing approximately 250 g. were injected with 1 ml. of TOCP/kg. intraperitoneally. At various times after the injection, the rats were killed and the pancreas removed. The activity of the pancreas suspensions towards olive oil was determined titrimetrically, the activity towards cholesteryl acetate colorimetrically and the activities towards tributyrin and phenyl acetate manometrically.

Time after the injection of TOCP (days)	No. of rats	Lipase and esterase activities (m-moles acid/g. pancreas/hr.)			
		Olive oil	Tributyrin	Phenyl acetate	Cholesteryl acetate
0	15	24	290	28	0.0119
1	6	17	205	25	0.0113
3	6	20	240	10	0.0049
5	6	16	195	14	0.0090
7	6	21	255	17	0.0096
9	6	22	270	26	0.0130

Table 6. *Effect of heating on the lipase and esterase activities of rat-pancreas suspension*

A suspension containing 100 mg. of fresh pancreas/ml. was heated in a water bath at 60° for various periods. The activities towards tributyrin and phenyl butyrate were determined in the usual way with 0.01 and 0.1 mg. of pancreas/ml. respectively in the Warburg vessels. The activity towards salicyl butyrate was measured with 10 mg. of pancreas/ml. in the Warburg vessel, in the presence and in the absence of 10^{-3} M E 600; that portion of the activity which was sensitive to inhibition by E 600 is indicated under I, and the remaining portion, which was insensitive to E 600, is indicated under II.

Time of heating at 60° (min.)	Lipase and esterase activities (μl. of CO ₂ /mg. pancreas/hr.)			
	Tributyrin	Phenyl butyrate	Salicyl butyrate I	Salicyl butyrate II
0	7120	1380	3.8	8.0
5	4200	118	0.8	8.2
10	2980	10	0	8.8
20	1980	0	0	8.4
60	260	0	0	8.8

remained unaltered, although the relative proportion of cholesterol ester increased from 10 to 30% of the total cholesterol after injection of 1 ml. of TOCP/kg.

A third type of enzyme in rat-pancreas suspensions could be distinguished when salicyl butyrate was employed as substrate. According to Hofstee (1952*a, b*), the hydrolysis of salicyl esters by pancreatin preparations is due to two enzymes which he designated as esterase I and esterase II. Esterase I was readily inactivated by exposure to high temperatures, but esterase II was more resistant to heating; both enzymes could be distinguished from the lipase and from the cholinesterases. In the present investigation it was found that the activity of rat-pancreas suspensions

towards salicyl butyrate was relatively low (Table 6). Approximately 30–40% of the total activity is readily inhibited by E 600, DINP or DFP in low concentrations and is quickly destroyed at 60°. This enzyme, esterase I, resembles the esterase responsible for the hydrolysis of phenyl butyrate (Table 6). The remaining portion of the activity towards salicyl butyrate was resistant to heating (Table 6) and proved to be completely insensitive to inhibition by E 600 and other organophosphorus derivatives. In both respects this second enzyme, esterase II, differs from the lipase of pancreas and resembles the A-esterase of serum.

Table 7 shows the results obtained in a preliminary investigation of the relative substrate specificities of the lipase and DINP-sensitive esterase in rat pancreas. In most cases it was not possible to demonstrate any significant activity of the esterase II towards the esters tested with the exception of salicyl butyrate. Salicyl acetate and the other acetates tested are not hydrolysed by this enzyme. A small portion of the total activity towards methyl butyrate, amounting to less than 1 μl. of CO₂/mg. pancreas/hr., was insensitive to both E 600 and DINP. However, the small portion of the total hydrolysis of phenyl butyrate which is not due to the DINP-sensitive esterase could be inhibited by E 600 in high concentrations and was therefore ascribed to the lipase.

In confirmation of the results obtained by Fodor (1950*a, b*), it was found that the hydrolysis of methyl butyrate and other non-glyceride esters was due mainly to the DINP-sensitive esterase of the pancreas, although the lipase does exhibit a small activity towards this ester. The lipase appears to hydrolyse triolein and tributyrin with equal facility (Table 3), although tripropionin is hydrolysed more slowly (Table 7). These results correspond with those obtained by Balls, Matlack & Tucker (1937), who found maximal activity

towards tricaproin and triheptylin. The lipase exhibits a much smaller activity towards triacetin under the experimental conditions. A large portion of the total activity towards triacetin is due to the DINP-sensitive esterase of pancreas.

Table 7. *Rate of hydrolysis of various esters by the lipase and esterase of rat pancreas*

The activities were measured manometrically at pH 7.4 and 37.5° with 0.2% (w/v) ester in the presence of 0.1% (w/v) gum acacia. The amounts of pancreas were varied between 0.01 and 10 mg. of fresh pancreas/ml., so that the rate of hydrolysis of the substrate in question was usually in the range 1–6 μ l. of CO₂/min. The portion of the activity which was completely inhibited by 10⁻⁴M, 10⁻⁵M and 10⁻⁶M DINP is given under the heading 'Esterase I'. The portion of the activity which was not inhibited by these concentrations of DINP and which was completely inhibited by 10⁻³M E600 was ascribed to the lipase. No value is given when the activities of other enzymes towards the same substrate were so high that a reliable measurement of the activity in question was not possible.

Substrate	Enzyme activity (μ l. of CO ₂ /mg. of pancreas/hr.)	
	Lipase	Esterase I
Tributyrin	6580	—
Tripropionin	2180	—
Triacetin	20	13
Diacetin	5	5
Monoacetin	2	3
Amyl acetate	2	16
Ethyl acetate	0.5	1.4
Methyl acetate	0.9	2
Methyl butyrate	6	50
Methyl benzoate	1	27
Benzyl acetate	105	160
Benzyl benzoate	1	70
o-Cresyl acetate	—	470
Salicyl acetate	—	1
Salicyl butyrate	—	3
Phenyl acetate	5	660
Phenyl propionate	10	1250
Phenyl butyrate	14	1220
Phenyl phenylacetate	5	90
Phenyl benzoate	1	70
p-Nitrophenyl acetate	8	1090
p-Nitrophenyl butyrate	25	1170

As could be surmised from the results obtained by Fodor (1946) and Aldridge (1954), the activity of the pancreas lipase is strongly influenced by the presence of gum acacia. This is not due simply to protection of the pancreatic lipase by gum acacia against destruction at 37.5° (cf. Child, 1945); the results were not appreciably altered when the pancreas suspension itself was incubated with gum acacia instead of adding the gum acacia to the substrate in the usual manner. The gum acacia is important for the emulsification of insoluble substrates (Aldridge, 1954), but it also influences the

activity towards triacetin which is completely soluble in water at the concentration used in these experiments. The rate of hydrolysis of triacetin by the lipase in the presence of 0.2% (w/v) gum acacia was almost twice as great as in the absence of gum acacia, though the esterase activity was not appreciably altered.

DISCUSSION

The esterases in rat pancreas differ from the various types of ali-esterases found in other tissues. The ali-esterases (B-type esterases) are capable of hydrolysing both triglycerides and phenyl esters at an appreciable rate and are inhibited by low concentrations of E600, DINP and related compounds (Aldridge, 1953a; Mounter & Whittaker, 1953). The substrate specificity of the DINP-sensitive esterase of the pancreas (esterase I) resembles that of the A-esterase (arom-esterase) in serum and liver, i.e. it exhibits a high activity towards phenyl esters, a low activity towards ethyl acetate and triacetin and no appreciable activity towards tributyrin (Aldridge, 1953a, 1954; Mounter & Whittaker, 1953). However, the pancreas esterase is very sensitive to inhibition by the organo-phosphorus derivatives, whereas the A-esterase is not irreversibly inhibited by these compounds, and indeed hydrolyses certain of them, e.g. E600 fairly rapidly (Aldridge, 1953b).

It is still uncertain whether the same esterase is responsible for the hydrolysis of phenyl and cholesteryl esters by pancreas suspensions. The fact that both activities are readily inhibited by the organo-phosphorus derivatives suggests that this may be the case. Another type of cholesterol esterase may be responsible for the esterification of free cholesterol that occurs when serum or plasma is allowed to stand (Sperry, 1935). We have found that this esterification can be prevented by the presence of DFP, E600 or DINP in concentrations of 10⁻⁵M and 10⁻⁴M with rat serum and human serum respectively. However, we have not been able to identify this enzyme by the methods used in the present investigation; possibly the common esterase activity of this enzyme is very small in comparison with that of the other esterases present in serum. In this respect it might be noted that Byron, Wood & Treadwell (1953) have shown differences in the characteristics of the cholesterol esterases present in liver and in pancreas. Although the acetyl esterase of wheat seeds has not been tested for cholesterol-esterase activity, this enzyme does not hydrolyse tributyrin rapidly and in other respects appears to resemble the DINP-sensitive esterase of pancreas (cf. Jansen, Nutting & Balls, 1948; Singer & Hofstee, 1948; Mendel, Myers, Uydert, Ruys & de Bruyn, 1953).

The differentiation of the esterase and lipase of pancreas makes possible a better interpretation of the results of previous investigators. For example, the data on the relative rate of hydrolysis of *p*-nitrophenyl esters by pancreas preparations (Huggins & Moulton, 1948) are concerned with the DINP-sensitive esterase and not with the lipase. It is to be expected that the hydrolysis of naphthyl esters is also due to the same esterase, in which case the results of Seligmann, Nachlas & Mollomo (1949) would indicate that the injection of mecholyl and eserine liberates the DINP-sensitive esterase as well as the lipase and other enzymes from the pancreas into the blood stream. Similarly, it is of interest that a large portion of the total activity towards triacetin was due to the pancreatic esterase in these experiments; this substrate appears therefore to be unsuitable for assay of lipase activity.

The data given in Table 7 for the substrate specificity of pancreatic lipase must be regarded with some caution. In the first place, a single concentration of ester was used with all the substrates. Secondly, the activity towards tributyrin is not always directly proportional to enzyme concentration (Table 3). However, we were not able to find evidence for the presence of a lipase inhibitor in the pancreas suspensions to explain this result. A suspension heated at 70° to inactivate the lipase had no significant effect on the hydrolysis of tributyrin by fresh pancreas suspensions; moreover, the hydrolysis of tripropionin may still be directly proportional to the amount of lipase at a concentration of pancreas where this proportionality no longer exists when tributyrin is used as substrate. This problem appears therefore to be due to the experimental techniques. Despite these complications, we have been able to confirm the previous observations that pancreatic lipase acts rapidly not only on triglycerides but also on benzyl esters, and that it does not hydrolyse benzoates rapidly (Table 7), observations which have been employed as the basis for a theory concerning the mechanism of hydrolysis by pancreatic lipase (Scott, 1953).

It might be noted that the lipase of pancreas resembles that of oat seeds in many respects, i.e. both enzymes hydrolyse olive oil and tributyrin equally well (cf. Martin & Peers, 1953), are moderately sensitive to inhibition by E600 and relatively resistant to inhibition by DFP (cf. Mendel *et al.* 1953). Since the inhibition by E600 is progressive and irreversible, the designation of the lipase as an A-type esterase seems inadvisable, the more so in view of the fact that the substrate specificity of the lipase is completely different from that of the A-esterase of serum.

The third enzyme described, the E600-resistant esterase in the pancreas (esterase II), also appears

to differ from the A-type esterases (arom-esterases) and the B-type esterases (ali-esterases) of other tissues. It does not hydrolyse either tributyrin or phenyl butyrate rapidly and, unlike the A-esterase of serum, does not appear to hydrolyse E600 (cf. Aldridge, 1954). The significance of this enzyme is not known. However, preliminary studies have shown that part of the hydrolysis of certain amino-acid esters, e.g. ethyltyrosine and ethyl-leucine, by rat-pancreas suspensions is also resistant to inhibition by E600. It is possible therefore that this esterase activity may be due to one of the proteolytic enzymes found in pancreas. Although ethyltyrosine is hydrolysed by chymotrypsin under the experimental conditions (Parks & Plaut, 1953), the enzyme in question could not be either chymotrypsin or trypsin, since both of these proteolytic enzymes are inhibited by E600 (Hartley & Kilby, 1952; Kilby & Youatt, 1954) and other organo-phosphorus derivatives.

SUMMARY

1. Fresh rat-pancreas suspensions contain at least three enzymes with esterase activity which can readily be distinguished by their sensitivity to organo-phosphorus inhibitors. All three enzymes differ markedly from the A-esterases and ali-esterases of serum, liver and other tissues.

2. One of these enzymes, the lipase, hydrolyses olive oil and tributyrin rapidly, has very little activity towards triacetin and phenyl esters and is relatively insensitive to inhibition by most of the organo-phosphorus derivatives. It is, however, progressively and irreversibly inhibited by diethyl *p*-nitrophenyl phosphate (cf. Aldridge, 1954).

3. One of the esterases in pancreas hydrolyses phenyl acetate, phenyl propionate and phenyl butyrate, but exhibits little or no activity towards the triglycerides. This enzyme is readily inhibited by the organo-phosphorus derivatives and other esterase inhibitors. Since the cholesterol esterase of pancreas is also inhibited by the organo-phosphorus derivatives in low concentrations, it is possible that the esterase in question may be a cholesterol esterase (cf. Fodor, 1950*b*).

4. Unlike the ali-esterases of serum and liver, neither of these enzymes can be inhibited *in vivo* by subtoxic doses of tri-*o*-cresyl phosphate.

5. A second esterase in pancreas hydrolyses salicyl esters (cf. Hofstee, 1952*a, b*), exhibits little or no activity towards the triglycerides and phenyl esters and is insensitive to all of the organo-phosphorus inhibitors tested. This esterase may be a proteolytic enzyme.

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