Studies on Ali-esterases and Other Lipid-hydrolysing Enzymes

1. INHIBITION OF THE ESTERASES AND ACETOACETATE PRODUCTION OF LIVER

By D. K. MYERS and B. MENDEL
Pharmaco-therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland

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Mammalian liver contains several types of esterase which might be concerned with the hydrolysis of liver lipid. The best known of these esterases are ali-esterase, lipase (Loevenhart, 1906; Willstätter & Memmen, 1924; Sobotka & Glick, 1934), lecithinase or phospholipase (King, 1931; Belfanti, Contardi & Ercoli, 1936) and cholesterol esterase (Kondo, 1910; Klein, 1938). In the present investigation interest was initially centred on the ali-esterases, that is, those esterases which act preferentially on simple aliphatic esters and triglycerides such as ethyl butyrate and tributyrin (Richter & Croft, 1942).

A fatty acid ester may be hydrolysed by several different esterases, and generally a complex mixture of esterases is present in animal tissues. Consequently it has been difficult to determine the range of specificity of the various ali-esterases. Also, the differences between ali-esterase and lipase have not been clearly defined; frequently it is assumed that the high ali-esterase activity and low lipase activity of liver are due to one and the same enzyme (cf. reviews by Bloor, 1943; Longenecker, 1946; Ammon & Jaarma, 1950).

The physiological function of lipase, lecithinase and cholesterol esterase is probably the hydrolysis and/or synthesis of substrates similar to those upon which these enzymes act most readily *in vitro*. However, the primary functional role of the ali-esterases and their physiological substrates is still largely a matter of conjecture (cf. Baker & King, 1935). Although the action of ali-esterases upon simple fatty acid esters does suggest that these enzymes are important in some stage of fat metabolism, their precise role remains problematical. Insight into their function in cellular metabolism could be obtained by inhibiting these enzymes, but the lack of a potent inhibitor of ali-esterases has previously hampered such investigations. Recently, however, it has been found that certain alkyl phosphate derivatives, commonly used as irreversible cholinesterase inhibitors, can also be employed as *in vitro* inhibitors of ali-esterase activity (Webb, 1948). This has made it possible to study the effects of ali-esterase inhibition on metabolic processes *in vitro*.

Liver tissue was used in the present investigation for the following reasons: (1) the liver is considered to be of primary importance in lipid metabolism; (2) liver lends itself readily to a study of the effects of ali-esterase inhibition on fat metabolism since fatty acid oxidation in liver cells results in an accumulation of free acetoacetate, the amount of which can easily be determined; (3) the liver is characterized by a high content of ali-esterases and virtual absence of lipase; and, if the ali-esterases play some significant role in fat metabolism, this should be especially evident in experiments with liver tissue; (4) rat liver in particular was chosen because its ali-esterase activity can be inhibited *in vivo* by administration of tri-*o*-cresyl phosphate without causing any inhibition of the true cholinesterase as it does in most other animal species (Mendel & Mortimer, 1944; cf. Mendel & Rudney, 1944). Thus in rats it is possible to compare the effects of *in vitro* and *in vivo* ali-esterase inhibition upon liver metabolism.

During the course of their studies on fatty acid oxidation in liver, Jowett & Quastel (1935*b*) investigated the relation of the ali-esterases to the spontaneous acetoacetate production of liver slices. Using atoxyl as inhibitor of the ali-esterase activity towards tributyrin (Rona & Bach, 1920), they found that the ali-esterase activity and the spontaneous acetoacetate production of rat-liver slices were inhibited to the same extent, while the oxidation of exogenous free fatty acids to acetoacetate was not greatly affected. From these data, they concluded that atoxyl inhibits the oxidation of the lipids in liver slices by depressing the 'lipase activity'.

Jowett & Quastel's results seemed to offer a satisfactory explanation of the physiological function of the ali-esterases in liver, and they were readily confirmed in the present investigation, when atoxyl was used as ali-esterase inhibitor. When, however, the more potent alkyl phosphate derivatives were used, no appreciable effect on the spontaneous acetoacetate production of liver slices could be observed in spite of the almost complete inhibition of the ali-esterase activity. This observation was surprising in view of Jowett & Quastel's results, and only after considerable investigation did we feel justified in concluding that the ali-esterases are not essential for lipid catabolism in liver slices. Our experiments

further showed that the phospholipase, lipase and ali-esterase activities of rat-liver homogenate are probably due to separate enzymes. Of these three types of esterase, only the phospholipase seems to be directly essential for lipid catabolism in liver.

METHODS

Animals and diet. In the initial experiments on this problem, Wistar rats fed on a commercial chow diet were used; later this work was continued with a crossed strain of Wistar piebald rats fed on a diet containing 70% wheat flour, 17% milk powder, 6% fat, 2·5% yeast, 2·5% meat, 0·9% cod-liver oil, 0·093% wheat-germ oil and 0·16% salt mixture. No striking differences in liver metabolism or ali-esterase activity were noted between the two groups. Male rats (200–250 g.) of the latter strain were used in most of the experiments reported below.

Measurement of esterase activity. As in previous investigations (cf. Mendel & Rudney, 1943; Hawkins & Mendel, 1949; Myers, 1950), the activity of the ali-esterases was measured manometrically at pH 7·4 by the Warburg method at 37·5° in a medium containing 0·025 M-$NaHCO_3$ saturated with 5% CO_2 and 95% N_2 (v/v). Liver homogenates were made with a glass Potter-Elvehjem homogenizer; 0·05 ml. of a 50-fold diluted homogenate was usually employed for determination of the ali-esterase activity of normal liver. The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the inhibitor was added to the enzyme-bicarbonate mixture in the main compartment. The total fluid volume was usually 5 ml. After equilibration of the vessels at 37·5° for 30–40 min., the substrate was tipped in from the side arm. Readings were started 2 min. later and repeated at appropriate intervals, usually every 3 or 5 min.

Esterase activity was calculated from the initial rate of hydrolysis of substrate; the rate of hydrolysis was usually constant for at least 30 min. when tributyrin or triacetin were used as substrate for the ali-esterase, but tended to diminish markedly after 10 min. when Tween 20 or Tween 80 (Honeywill and Stein, Ltd., 21 St James's Square, S.W. 1) were used. In all cases the final concentration of ester was 1% (v/v); routine determinations of ali-esterase activity were done with a tributyrin emulsion stabilized by the addition of 0·5% gum acacia (final concentration). Other esters used as substrates for the ali-esterases in this investigation were triacetin, methyl butyrate, ethyl acetoacetate, Tween 20 and Tween 80. The two last-mentioned are water-soluble lauric and oleic acid esters, respectively, of a polyoxyalkylene derivative of sorbitan (Archibald, 1946).

The esterase activity of liver slices was measured in a bicarbonate-Ringer medium; the dry weight of these slices was 3–10 mg. (depending on the esterase inhibition) and esterase values were corrected for the small initial rate of CO_2 production due to the breakdown of carbohydrate in the slices. Neither the ali-esterase activity of liver homogenate nor the degree of inhibition was affected when a simple bicarbonate medium was replaced by a bicarbonate-Ringer medium. The esterase activity was measured over a period of 10–30 min., depending on how long the initial rate of hydrolysis remained constant; but in all cases the esterase activity, after correction for non-enzymic hydrolysis of the substrate, was calculated as a Q_{ester} value, i.e. μl. CO_2 produced/mg. dry wt. of enzyme preparation/hr.

Experiments with inhibitors. Ali-esterase inhibitors used were sodium arsanilate (atoxyl), tri-*o*-cresyl phosphate (TOCP), di*iso*propyl fluorophosphonate (DFP), tetraethyl pyrophosphate (TEPP), and diethyl *p*-nitrophenyl phosphate (E 600). The last three of these inhibitors had to be prepared freshly in aqueous solution for each experiment; anhydrous propylene glycol, the usual solvent for preparing stable stock solutions, could not be used since it was found that propylene glycol in concentrations of 0·2–0·5% is itself a strong inhibitor of acetoacetate production.

The TOCP used in this investigation had been purified by distillation at low pressure of a technical product (Eastman-Kodak); the resulting preparation, a clear oil ($n_D^{5.5°} = 1·5538$) had no trace of the slightly yellowish tinge usually found in less pure preparations. Pure TOCP has very little toxicity for rats (Mendel & Rudney, 1944); the ali-esterases of liver are about 70% inhibited, and that of serum almost completely, by the intramuscular injection of 5 μl. TOCP/100 g. body wt. (Mendel & Mortimer, 1944; Myers & Mendel, 1949). In the present investigation, rats of 200–250 g. were usually injected intramuscularly with 0·1 ml. TOCP every second day beginning 5 days before the rats were sacrificed. This treatment resulted in a 95–97% inhibition of the tributyrin-esterase activity of the liver.

For experiments with liver slices, the rat was killed by stunning and decapitation, the liver removed rapidly and dropped into ice-cold Ringer solution. Three slices of 25–30 mg. total dry wt. were placed in a Dickens-type respiration vessel containing 2·5 ml. cold phosphate-Ringer solution (pH 7·4). The vessel with manometer was filled with O_2, equilibrated at 37·5° for 15 min. and the rate of O_2 uptake measured over a subsequent period of 120 min. The total acetoacetate produced in the 135 min. was determined in a 2 ml. sample of the fluid medium by the method of Jowett & Quastel (1935*a*). O_2 uptake was calculated as μl. O_2 taken up/mg. dry wt. of liver slice/hr. ($-Q_{O_2}$) and acetoacetate production as μl. CO_2 formed on decarboxylation of the acetoacetate produced/mg. dry wt. of liver slice/hr. ($Q_{acetoacetate}$).

RESULTS

Ali-esterases and acetoacetate production of liver slices

Typical values obtained in individual experiments which show the effects of various ali-esterase inhibitors upon the oxygen uptake, acetoacetate production and tributyrin-esterase activity of rat-liver slices are presented in Table 1. It is found, in agreement with the results of Jowett & Quastel (1935*b*), that atoxyl causes a relatively selective inhibition of the spontaneous acetoacetate production and of the tributyrin-esterase activity. On the other hand, E 600 has no effect upon acetoacetate production when present in concentrations which inhibit the tributyrin hydrolysis almost completely.

The values for percentage inhibition of ali-esterase activity and of acetoacetate production by atoxyl are very similar to those reported by Jowett & Quastel (1935*b*). The interpretation of these

Table 1. *Effect of ali-esterase inhibitors on the O_2 uptake, acetoacetate production and tributyrin-esterase activity of rat-liver slices*

(Rat-liver slices incubated in phosphate-Ringer medium at 37·5° for 135 min. with and without inhibitor in the medium. Average O_2 uptake during the period 15–135 min. is given as $-Q_{O_2}$; average acetoacetate production during the total period 0–135 min. as Q_{Ac}. Extra acetoacetate production in the presence of sodium butyrate is calculated from the differences in Q_{Ac} values in the presence and absence of butyrate. The tributyrin-esterase activity of slices from the same liver was measured in bicarbonate-Ringer medium as described in the text, with and without inhibitor in the medium; average calculated as Q_{TB}. Typical results in experiments with five different esterase inhibitors are given below. The values for percentage inhibition by $1·2 \times 10^{-2}$M-atoxyl, as calculated from the results obtained by Jowett & Quastel (1935*b*) with a similar method, are given in brackets.)

	In absence of butyrate				In presence of 0·01 M-sodium butyrate							
Inhibitor and concentration	$-Q_{O_2}$ (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{O_2} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Extra Q_{Ac} (μl./mg. dry wt./hr.)	Inhibition (%)	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition (%)
None	10·2	—	0·68	—	14·7	—	1·74	—	1·06	—	141·8	—
10^{-2}M-Atoxyl	8·7	15 (37)	0·13	81 (80)	9·8	33 (35)	1·11	36 (37)	0·98	8 (28)	41·3	71 (74)
None	11·4	—	0·80	—	15·9	—	2·04	—	1·24	—	148·5	—
10^{-4}M-E 600	12·0	−5	0·81	−1	16·1	−1	2·07	−2	1·26	−2	2·4	98
None	12·3	—	1·01	—	15·2	—	3·50	—	2·49	—	162·4	—
10^{-6}M-DFP	11·8	4	0·98	3	15·9	−5	3·50	0	2·52	−1	29·2	82
None	11·4	—	0·72	—	—	—	—	—	—	—	168·5	—
10^{-4}M-TEPP	11·7	−3	0·71	1	—	—	—	—	—	—	10·3	94
None	12·9	—	0·75	—	—	—	—	—	—	—	111·5	—
Approx. 5×10^{-4}M-TOCP (sat.)	12·9	0	0·76	−1	—	—	—	—	—	—	45·1	60

results is complicated by the fact that atoxyl causes a progressive inhibition of the respiration of the liver slices, the Q_{O_2} values given in Table 1 being an average of the results obtained during the 2 hr. period of incubation. The effect that this inhibition of respiration might have upon acetoacetate production is not known; this point will be discussed later.

The tributyrin-esterase activity is strongly inhibited by low concentrations of TEPP, DFP, TOCP and E 600; with none of these inhibitors was there any appreciable effect on the rate of oxygen uptake or the spontaneous acetoacetate production of the liver slices. The other compounds were less suitable than E 600. TEPP is much less stable than E 600 under the experimental conditions. The maximal inhibition of ali-esterase activity by TOCP *in vitro* was usually less than 60 %, whereas a 98–99 % inhibition was readily obtainable with E 600. Both respiration and acetoacetate production were depressed when the ali-esterase was inhibited more than 75 % by DFP.

Thus E 600 seemed to be the best choice as an innocuous *in vitro* inhibitor of the ali-esterase activity of rat-liver slices and most of the subsequent experiments with liver slices were therefore carried out with this compound. Although the results of individual experiments showed small differences in the acetoacetate production and oxygen uptake, whether E 600 was present or not, no definite trend could be observed which would indicate that these variations were due to esterase inhibition (Table 5). The average results of eighteen experiments showed no significant difference between the rate of oxygen uptake, or of acetoacetate production, in the presence ($Q_{O_2} = -11·16$; $Q_{\text{acetoacetate}} = 0·78$) and in the absence ($Q_{O_2} = -11·14$; $Q_{\text{acetoacetate}} = 0·75$) of an initial concentration (10^{-4}M) of E 600 which inhibited the ali-esterase activity of the slices by 98 %. The small variations of less than 5 % in the results of individual experiments with and without E 600 are attributable to experimental errors.

These results would seem to indicate, first, that the ali-esterases are not essential for any step in the lipid catabolism of surviving liver slices, and secondly, that the effect of atoxyl on the spontaneous acetoacetate production must be explained upon some other basis than the inhibition of ali-esterase activity. However, the possibility of methodological errors had to be taken into account before the above conclusions could be drawn. The possibilities considered are given in detail below.

(1) An enzyme catalysing the hydrolysis of DFP is present in rat liver (Mazur, 1946). Since E 600 and the other phosphate inhibitors might also be hydrolysed by the same or a similar enzyme, it was necessary to ascertain whether the ali-esterases of the liver slices remain inhibited throughout the period of incubation in the respiration vessels.

Therefore, after the 135 min. period of incubation with 10^{-4} M-E600, the slices were removed from the respiration vessels, rinsed well, and the tributyrin-esterase activity was determined in bicarbonate-Ringer solution in the absence of inhibitor. The esterase in these slices was found to be inhibited 93% when compared to the esterase activity of control slices. Since an initial concentration of 10^{-4} M-E600 caused a 98% inhibition, the average ali-esterase inhibition in the liver slices during the period of respiration measurements must have been at least 95–96%. In the following section it will be shown that even this estimate is probably too low.

(2) The tributyrin-esterase activity of a liver homogenate is usually 6–7 times greater than the esterase activity of slices from the same liver (Tables 2 and 5). Thus the possibility existed that the tributyrin hydrolysis by liver slices represented only the esterase activity of the broken exterior cells of the slice, and that the ali-esterases within the intact cells were not affected by the alkyl phosphates. To determine whether this was the case, liver slices were allowed to stand in phosphate-Ringer solution with and without 10^{-4} M-E600 for 0·5 hr. at room temperature (18°) and were then washed thoroughly during another 0·5 hr. to remove any free E600 from the slices. This procedure was used to minimize any loss of ali-esterases from the control slices (cf. Rona & Lasnitzki, 1926; Jowett & Quastel, 1935*b*). After the slices were washed five times in 50 ml. of phosphate-Ringer stirred by bubbling O_2 through the solution, some of them were used directly for measurement of esterase activity; others were first ground with water in a close-fitting Potter homogenizer and the resulting homogenate was used for measurement of esterase activity.

The ali-esterase activity of the washed slices was found to have been 88% inhibited by incubation with E600, which would seem to indicate that the irreversible combination of E600 with the ali-esterase was still incomplete after 0·5 hr. at 18°. (The inhibition of the tributyrin activity of liver homogenate by E600 approaches completion within a few min. at 37·5° and is not reversible by dilution.) A homogenate of the same washed slices showed 96% inhibition of the ali-esterase activity in place of the 88% observed with the slices (Table 2). Thus E600 inhibits the esterase in the intact cells throughout the liver slice. It would also appear that the inhibition of the ali-esterases in the liver cells must actually be significantly greater than it would appear to be from measurements on the slice itself. This latter fact can probably be explained by the low solubility of the substrate and its slow diffusion into the intact cells, factors which limit the amount of substrate available to the ali-esterase and thus limit the maximal rate of hydrolysis in normal liver slices. From these results (Table 2) it can be concluded that the inhibition of the ali-esterase in a liver slice by an initial concentration of 10^{-4} M-E600 is probably greater than 99%.

(3) The amount of acetoacetate which accumulates in the fluid medium represents the balance between the rate of production from fatty acid oxidation and the rate of further breakdown of the acetoacetate in the slices. The further oxidation of acetoacetate can be selectively inhibited to a large extent by addition of 0·02 M-malonate to the medium (Jowett & Quastel, 1935*b*). Nevertheless, in the presence of malonate the same value for spontaneous $Q_{acetoacetate}$ was obtained with or without E600 (Table 3). Thus it seems unlikely that E600 itself might possibly be inhibiting

Table 2. *Comparison of the ali-esterase activities of rat-liver slices and liver homogenate*

(Washed E600-treated and control slices were incubated in phosphate-Ringer solution with and without 10^{-4} M-E600, and subsequently washed in phosphate-Ringer solution as described in the text. The tributyrin-esterase activity of the control slices was determined in bicarbonate-Ringer solution, with and without 10^{-4} M-E600; the tributyrin-esterase activity of the washed E600-treated slices similarly, without any added inhibitor. TOCP slices were obtained from the liver of a rat which had been injected with 0·1 ml. of TOCP intramuscularly 1, 3 and 5 days before the rat was killed. A portion of the slices was ground with water in each case and the resulting homogenate was also tested for tributyrin-esterase activity, all esterase values being expressed as Q_{TB}.)

	Slices		Homogenate of similar slices	
Material	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition of esterase activity (%)	Q_{TB} (μl./mg. dry wt./hr.)	Inhibition of esterase activity (%)
Washed control slices	193	—	1282	—
Washed control slices + 10^{-4} M-E600	(4)	(98)	—	—
Washed E600-treated slices	23	88	55	96
TOCP slices	31	84	62	95

Table 3. *Effect of sodium malonate on the acetoacetate production of rat-liver slices in the presence and absence of ali-esterase inhibitor*

(Oxygen uptake (Q_{O_2}) and acetoacetate production (Q_{Ac}) of liver slices measured in phosphate-Ringer medium as in Table 1. TOCP slices obtained from the liver of a rat injected intramuscularly with TOCP as in Table 2.)

	Control values		Average values in the presence of 0·02 M-sodium malonate	
Material	$-Q_{O_2}$	Q_{Ac} (μl./mg. dry wt./hr.)	$-Q_{O_2}$	Q_{Ac}
Normal slices	10·4	0·92	8·8	2·16
Normal slices + 10^{-4} M-E600	10·3	0·89	9·1	2·21
TOCP slices	12·0	0·83	9·2	2·05

simultaneously both acetoacetate formation and acetoacetate oxidation to the same extent.

(4) Previous evidence has indicated that the activity of beef- and pig-liver homogenates towards tributyrin might represent the activity of two different forms of ali-esterase (Fodor, 1946; Falconer & Taylor, 1946). In the present investigation it was found possible to obtain a selective inhibition of the esterase activities of rat-liver homogenate towards different aliphatic esters (Table 4). These results can only be explained by the assumption that rat-liver homogenate contains three different ali-esterases; their relative substrate specificities appear to overlap, but all three seem to have the characteristics of an ali-esterase.

The presence of more than one ali-esterase in the liver made it necessary to determine whether E 600 inhibits the esterase activities towards all of these different aliphatic esters. This was found to be true (Table 5), although slight differences in sensitivity to inhibition by E 600 were observed.

(5) Evans (1951) has shown that the ali-esterases of bone marrow hydrolyse acetoacetate esters. This would suggest that such an esterase might play an important role in the metabolism of acetoacetate. Ethyl acetoacetate was also hydrolysed by liver ali-esterase, but the esterase activity towards this acetoacetate ester was inhibited by E 600 just as well as the activity towards tributyrin (Table 5).

The conclusion that the ali-esterases are not required for any step in the acetoacetate production of liver cells was corroborated by experiments in which the liver esterases were inhibited *in vivo* by the intramuscular injection of TOCP into rats. The degree of esterase inhibition in the liver is again greater when the inhibition is measured in homogenates as compared with that measured in slices (Table 2); however, the inhibition of ali-esterase is never as great as that obtainable with E 600 *in vitro*. The tributyrin-esterase activity of liver homogenate is reduced to about 5 % of the normal level (Tables 2 and 4) and there is no appreciable change in the rate of oxygen uptake or acetoacetate production of liver slices (Table 3) despite the fact that the ali-esterases had been inhibited *in vivo* for 5 days.

Although these experiments show that ali-esterase activity is not essential for the catabolism of the lipids in isolated liver slices, nevertheless

Table 4. *Experiments on the inhibition of the various esterase activities of rat-liver homogenate*

(Rat-liver homogenate containing 1 part by weight of fresh liver plus 9 vol. of water was exposed to high temperature or alkaline pH; the esterase activities towards different substrates were measured in bicarbonate buffer and percentage inactivation calculated by comparison with the esterase activities of the original homogenate. The inhibitory effect of TOCP *in vitro* was measured by incubating the liver homogenate with 0·005 % TOCP in the bicarbonate buffer for 30 min. before determining the esterase activities. The inhibition of esterase activities caused by injection of TOCP *in vivo* was determined by comparison of normal Q values with those obtained on liver homogenates from three rats injected intramuscularly with TOCP as in Table 2.)

	Amount of the esterase activity remaining			
Substrate	After exposure of the liver homogenate to 62° for 10 min. (%)	After exposure of the liver homogenate to pH 10 for 40 min. (%)	After incubation with 0·005 % TOCP *in vitro* (%)	After injection of TOCP into the rat (%)
Triacetin	6	59	68	14
Tributyrin	9	76	46	5
Tween 20	54	91	52	4
Tween 80	74	100	80	4

Table 5. *Effect of various concentrations of E 600 upon the ali-esterase activities and acetoacetate production of rat liver*

(Esterase activities of twelve different liver homogenates were measured in a bicarbonate buffer and expressed as average Q_{ester} values for various esters present in a concentration of 1 % (v/v). The effect of E 600 upon the esterase activity was determined after incubation with E 600 in the bicarbonate buffer for 30 min. before the substrate was added. Oxygen uptake, acetoacetate production and tributyrin-esterase activity of slices from one of the livers were determined as in Table 1.)

	Average Q_{ester} values of liver homogenate with the following esters					Liver slices in phosphate-Ringer		
Concentration of E 600 (M)	Triacetin	Tributyrin	Ethyl acetoacetate	Tween 20	Tween 80	Q_{TB}	$-Q_{O_2}$	Q_{Ac}
			(μl./mg. dry wt./hr.)					
0	551	828	164	68	23	142	10·2	0·60
10^{-8}	485	670	123	56	20	137	10·2	0·62
10^{-7}	—	49	8·9	—	—	105	10·1	0·61
10^{-6}	50	33	6·2	2·0	1·6	55	10·1	0·65
10^{-5}	—	8·1	1·9	—	—	11	10·1	0·59
10^{-4}	7·0	0·8	0·0	0·4	0·2	3	10·3	0·64

synthetic fatty acid esters added to the medium were not catabolized unless they were first hydrolysed by the esterases.

Small amounts (0·01%, i.e. 0·001 M) of the water-soluble ester methyl butyrate were added to the phosphate-Ringer solution in the respiration vessels and incubated with liver slices for 60 min. with and without E 600. (Higher concentrations of methyl butyrate could not be used because the hydrolysis of this ester was rapid enough to produce excessive free acid in the cells.)

Under these conditions, the presence of methyl butyrate causes an increase in acetoacetate production (Table 6); methyl butyrate was even more effective than was sodium butyrate. However, methyl butyrate has little effect on acetoacetate production when the hydrolysis of this ester by ali-esterase is inhibited by addition of E 600 to the medium. Thus it seems justified to conclude that hydrolysis of methyl butyrate is essential before acetoacetate can be formed from the butyrate residue. Experiments with the water-soluble lauric acid ester Tween 20 gave similar results (Table 6).

Table 6. *Effect of E 600 on the oxidation of fatty acid esters by rat-liver slices*

(Rat-liver slices were allowed to stand 30 min. at 20° in Ringer solution with and without 10^{-4} M-E 600. The same slices were then incubated in phosphate-Ringer medium at 37·5° for 60 min. with and without inhibitor in the medium. $-Q_{O_2}$ was calculated from the average O_2 uptake during the period 15–60 min.; Q_{Ac} from the average acetoacetate production during the total period 0–60 min. Sodium butyrate, methyl butyrate or Tween 20 were added to some of the respiration vessels as substrate for the fatty acid oxidative enzymes.)

Added substrate	Control values		Values obtained with 10^{-4} M-E 600 in the medium	
	$-Q_{O_2}$	Q_{Ac}	$-Q_{O_2}$	Q_{Ac}
	(μl./mg. dry wt./hr.)			
None	11·5	0·62	10·9	0·65
0·001 M-Sodium butyrate	13·8	1·56	13·7	1·60
0·001 M-Methyl butyrate	14·5	1·98	11·6	0·71
None	10·6	0·84	10·1	0·80
0·4% Tween 20	11·9	1·67	10·8	1·08

From this last experiment it might also be presumed that the constituent fatty acids of the liver lipids can be oxidized only after the lipids have been hydrolysed. Inhibition of ali-esterase activity, whether by E 600 or by atoxyl, does not affect lipid catabolism in the liver cells; nevertheless, atoxyl does inhibit the catabolism of liver lipids although it does not interfere appreciably with the oxidation of free fatty acids. Thus it seemed logical to suppose that atoxyl inhibits the hydrolysis of the liver lipids, and that the enzyme responsible for the hydrolysis of liver lipids is distinct from the ali-esterases.

Lipid hydrolysis in liver homogenates

Fairbairn (1945*b*) has shown that a fairly rapid liberation of free fatty acids occurs in liver homogenates and indicated that this was probably due to hydrolysis of phospholipins. Other investigators have found that the total amount of phospholipins decreases during incubation of liver slices (Fishler, Taurog, Perlman & Chaikoff, 1941; Sperry, Brand & Copenhaven, 1942). Thus it was of interest to determine what effect the ali-esterase inhibitors would have upon phospholipin hydrolysis and whether the differences in the effects of E 600 and atoxyl upon spontaneous acetoacetate production could be explained upon this basis.

To determine the rate of lipid hydrolysis it was necessary to use a liver homogenate since phospholipin is not only hydrolysed but also re-synthesized in liver slices (Fishler *et al.* 1941), and since the free fatty acids liberated by lipid hydrolysis would be catabolized by intact liver cells. Several experiments were performed using a procedure similar to the example described below. The methods of lipid analysis were based upon those recommended by Bloor (1943) and Fairbairn (1945*a*).

Three adult male rats of the Wistar-piebald strain were killed by stunning and decapitation; the livers were dissected out immediately and dropped into a large volume of ice-cold Ringer solution. The livers (approx. 25 g.) were then weighed and homogenized with 100 ml. of cold phosphate buffer (0·143 M, pH 7·4) for 30 sec. in a Waring Blendor. Two 10 ml. samples of the suspension were each pipetted into 70 ml. of boiling ethanol; other portions of the suspension were added to a small volume of water or inhibitor solution in a conical flask, mixed thoroughly and placed in a water bath at 37·5°. The whole procedure from the time of killing the first rat to this point took less than 5 min. Samples were then removed from the incubated suspensions for determination of pH, ali-esterase activity and lipid content.

The ethanolic suspensions were kept just below boiling for 30 min., filtered, the residue re-extracted with smaller volumes of ethanol, the combined filtrates evaporated to remove the ethanol and re-extracted with light petroleum (Bloor, 1943). Acetone-soluble and acetone-insoluble fractions were isolated and the free fatty acids of the acetone-soluble fraction were determined by the method of Fairbairn (1945*a*). The total fatty acid in the same sample

was subsequently determined after hydrolysis of the esterified lipids with alkali. All lipid values were expressed as m-moles of fatty acid/g. dry wt. of liver.

The results obtained in five such experiments are shown in Table 7. The lipid composition in the livers from rats which had been injected with TOCP for 5 days before they were killed was well within the range of normal values. In other words, 97% inhibition of the ali-esterase activity of rat liver *in vivo* had no marked effect upon the liver lipids in a short-term experiment.

The rate of liberation of free fatty acids by hydrolysis of the lipids in the liver homogenates was fairly well reproducible from one experiment to another (Table 8), and was not affected significantly when the ali-esterase activity was inhibited 80–90% by addition of DFP or E600 to the liver homogenate. (The esterase inhibition by DFP and E600 is somewhat less than previously noted in Tables 1, 2 and 5, due probably to the use of much more concentrated liver suspensions in this case. Undoubtedly more inhibitor would be needed to inhibit the esterase in more concentrated suspensions, but a second factor which appears is the progressive decrease in inhibition during incubation of the concentrated liver homogenates. This phenomenon was not found to occur to an appreciable extent during incubation of liver slices or very dilute liver suspensions, suggesting that concentrated liver suspensions contain a factor which catalyses the destruction of combined inhibitor.) The ali-esterase activity of a homogenate of livers from rats injected with TOCP remained almost completely inhibited throughout the 3 hr. period of incubation and again the rate of lipid hydrolysis was within the normal range.

Atoxyl is the only one of the ali-esterase inhibitors tested which inhibits ali-esterase activity and lipid hydrolysis to a similar extent, just as atoxyl was the only inhibitor which inhibited ali-esterase activity

Table 7. *Lipid composition of rat-liver homogenate*

(Lipid analyses as described in the text. Each analysis was performed on a combined homogenate of three rat livers. Exps. 1–3 were performed with livers from normal rats; exps. 4 and 5 with the livers obtained from rats which had been injected intramuscularly with 0·4 ml. of TOCP spread over a period of 5 days, beginning with an initial dose of 0·2 ml. followed by two injections of 0·1 ml. on the third and fifth days.)

	Lipid content of normal rat liver				Lipid content of livers from rats injected with TOCP		
		Acetone-soluble lipids			Acetone-soluble lipids		
Exp. no.	Acetone-insoluble lipids	Esterified fatty acid	Free fatty acid	Exp. no.	Acetone-insoluble lipids	Esterified fatty acid	Free fatty acid
	(m-moles fatty acid/g. dry wt. of liver)				(m-moles fatty acid/g. dry wt. of liver)		
1	0·156	0·069	0·008	4	0·189	0·089	0·015
2	0·171	0·076	0·012	5	0·165	0·078	0·009
3	0·198	0·113	0·007				

Table 8. *Liberation of free fatty acids during incubation of the liver homogenate in the presence and absence of ali-esterase inhibitors*

(The above liver homogenates were incubated at 37·5° in the presence and absence of ali-esterase inhibitors; pH of the homogenate was 7·4 at 0 min. and 7·2 after 180 min. incubation. The free fatty acids contained in a 10 ml. sample of homogenate were determined after 180 min. by the method of Fairbairn (1945*a*). Ali-esterase activity of the homogenate was measured in a bicarbonate buffer without further addition of inhibitor.)

		Ali-esterase activity of the liver homogenate (Q_{TB})				Change in composition of the lipids of the liver homogenate after 180 min. incubation (as m-moles fatty acid/g. dry wt. of liver)			
Exp. no.	Added inhibitor	15 min. incubation	70 min. incubation	180 min. incubation	Average inhibition (%)	Increase in free fatty acid	Inhibition (%)	Decrease in neutral lipid	Decrease in phospholipin
1	None	766	736	716	—	0·053	—	0·011	0·042
	10^{-2}M-Atoxyl	272	272	278	63	0·029	45	0·012	0·016
	10^{-4}M-DFP	70	117	191	83	0·053	0	0·011	0·041
2	None	821	783	748	—	0·045	—	0·009	0·036
	10^{-4}M-E600	30	61	177	89	0·041	9	0·007	0·035
3	None	707	674	647	—	0·042	—	—	—
	Approx. 5×10^{-4}M-TOCP (sat.)	312	345	373	49	0·043	0	—	—
4	TOCP liver	25	25	22	(97)	0·042	—	—	—

and spontaneous acetoacetate production of liver slices to approximately the same extent. A study of the lipid changes involved (Table 8) shows that most of the free fatty acid liberated arises from the hydrolysis of phospholipin, as suggested by Fairbairn (1945*b*), although the hydrolysis of neutral fat contributes about 20% to the total free fatty acids under these conditions. The total fatty acid of the three fractions (phospholipin plus neutral lipid plus free fatty acid) remained fairly constant in all of the determinations; this confirms the reliability of the methods used and excludes the possibility that free fatty acids might be destroyed in the buffered liver homogenates. It appears from Table 8 that there must be at least two different enzymes involved in the hydrolysis of liver lipids to free fatty acid, a lipase and a phospholipase; both of these lipid-hydrolysing enzymes are distinct from any E600- or DFP-sensitive ali-esterase in the liver suspension. The lipase activity is not significantly affected by atoxyl in the concentrations used, while the phospholipin hydrolysis is inhibited to about the same extent by atoxyl as is the total ali-esterase activity. This latter result may explain the inhibition of the spontaneous acetoacetate production of liver slices by atoxyl.

DISCUSSION

The pertinent data concerning the selectivity of inhibition of various biochemical reactions by atoxyl and the alkyl phosphate derivatives (E600 and DFP) are summarized in Table 9.

DFP and E600 are selective inhibitors of the ali-esterases in rat liver and have little or no effect upon oxygen uptake, spontaneous acetoacetate production, oxidation of butyrate to acetoacetate, phospholipin hydrolysis, or neutral-lipid hydrolysis. Atoxyl also inhibits ali-esterases, but this inhibition could not be responsible for the inhibition of spontaneous acetoacetate production by atoxyl, since no interference with acetoacetate production is seen when the ali-esterases are almost completely inhibited by E600 or DFP. However, in contrast to DFP and E600, atoxyl inhibits not only the ali-esterase but also the phospholipase activity. From these results and from the other data obtained in this investigation, it seems justifiable to conclude that the inhibition of the spontaneous acetoacetate production by atoxyl is due to the inhibition of phospholipin hydrolysis.

As is true for most enzyme inhibitors, neither of these types of inhibitor is absolutely specific for any one enzyme or for any one metabolic process; the above concentrations proved to be about the best for demonstrating the described selective effects. E600 has very little effect on the oxygen uptake of liver slices even when present in high concentrations, but 2×10^{-4}M-DFP does cause an appreciable inhibition of the respiration of liver slices (cf. Brooks, Ransmeier & Gerard, 1949). The range of concentrations of atoxyl in which a definitely selective inhibition of spontaneous acetoacetate production can be demonstrated is fairly small (cf. Jowett & Quastel, 1935*b*). Atoxyl (10^{-2}M) does inhibit the oxygen uptake of liver slices a little but inhibits the spontaneous acetoacetate production to a much greater extent; slightly higher concentrations (e.g. 2×10^{-2}M) cause a very appreciable inhibition of the rate of oxygen uptake and the selective effect upon spontaneous acetoacetate production is no longer clear-cut. It is not certain whether this inhibition of respiration is due in part to an inhibition of lipid oxidation or whether it merely reflects the ability of atoxyl and DFP in high concentrations to combine non-specifically with a great many types of enzymic and non-enzymic proteins. If we assume the latter to be true, then it would be necessary to make a small correction in the acetoacetate production for the inhibition of general respiration.

Another factor which has to be taken into consideration is the possibility that less of the natural substrates in the liver cells would be oxidized in the

Table 9. *Summary of data on selectivity of inhibition of various biochemical reactions by atoxyl and the alkyl phosphate derivatives (E600 and DFP)*

	Inhibition by atoxyl (10^{-2}M) (%)	Inhibition by E600 (10^{-4}M) (%)	Inhibition by DFP (10^{-6}M) (%)
Liver slices (data from Table 1):			
Oxygen uptake	15	0	0
Spontaneous acetoacetate production	81	0	0
Extra acetoacetate production in the presence of 0·01 M-sodium butyrate	8	0	0
Ali-esterase activity	71	98	82
Liver homogenate (data from Table 7):	(10^{-2}M)	(10^{-4}M)	(10^{-4}M)
Ali-esterase activity	63	89	83
Phospholipin hydrolysis	62	0	0
Neutral lipid hydrolysis	0	0	0

presence of the salt of a more readily oxidizable free fatty acid such as sodium butyrate. These problems could probably be answered most effectively by the use of isolated enzyme systems capable of oxidizing various forms of esterified and free fatty acids. The necessary corrections of the data given above would be relatively small in the case of $Q_{acetoacetate}$ values, where the addition of sodium butyrate causes an increase of 150–250%; the overall rate of oxygen uptake of liver slices, however, is increased only by 25–45% on addition of sodium butyrate and thus these values are less reliable criteria.

The experimental data obtained in this investigation seem sufficiently clear-cut to permit several significant conclusions. They fail to give any indication of an essential function of ali-esterases in the normal oxidative processes of rat-liver cells. On the other hand, they have tended to corroborate the generally accepted theories regarding lipid oxidation in liver (Jowett & Quastel, 1935*b*; Stadie, 1945).

It appears that the lipids of rat liver must be hydrolysed before the constituent fatty acids can be oxidized, or, at least, before their oxidation can give rise to free acetoacetate. This is indicated by the results with synthetic esters as presented in Table 6, and by the fact that atoxyl inhibits the spontaneous acetoacetate production of liver slices when present in a concentration which also inhibits the hydrolysis of liver lipids. It also seems probable that the phospholipins must play an important role in the lipid catabolism of normal liver. Most of the free fatty acid liberated by lipid hydrolysis in liver homogenates is derived from the phospholipins, and a concentration of atoxyl which selectively inhibits phospholipin hydrolysis (as opposed to the hydrolysis of neutral fat) also inhibits selectively the spontaneous acetoacetate production of liver cells (as opposed to the extra acetoacetate production in the presence of a free fatty acid).

Thus the main pathway of lipid oxidation in normal rat liver would involve, first, hydrolysis of phospholipin and, secondly, oxidation of the free fatty acids liberated. If this conclusion is justified, it would automatically eliminate ω-oxidation (Verkade & van der Lee, 1934) or oxidation of unsaturated bonds (Hove, 1943) as a significant source of free acetoacetate from esterified fatty acid. Thus it would not be expected that ephemeral lipid esters containing short-chain fatty acids would ever be produced in the normal lipid catabolism of liver, and consequently ali-esterase activity should not be of importance in lipid catabolism.

This conclusion seems justified by the fact that the liver ali-esterases can be almost completely inhibited *in vitro* or *in vivo* without any effect upon either the acetoacetate production or the total oxygen uptake of liver slices. The fact that the ali-esterases also hydrolyse acetoacetate esters would seem to be merely incidental; these esterases have no absolute substrate specificity and will hydrolyse a wide variety of synthetic esters structurally related to those upon which they act preferentially.

Ali-esterase inhibition *in vivo* does not cause any marked change in the composition of the liver lipids in short-term experiments, and does not affect the regeneration of liver adversely (Mendel, Myers & Simons, 1952). Thus our investigations have not disclosed the primary functional role or physiological substrate of the large amount of ali-esterase in liver. The possible identity of certain types of ali-esterase with other hydrolytic enzymes such as cholesterol esterase (cf. Swell, Cassidy & Treadwell, 1951) or some amidases remains to be investigated.

SUMMARY

1. The ali-esterases of rat liver are highly sensitive to inhibition by various alkyl phosphate derivatives such as diethyl *p*-nitrophenyl phosphate (E 600). They can be selectively inhibited *in vivo* by intramuscular injection of tri-*o*-cresyl phosphate.

2. Both the ali-esterases and the phospholipase are inhibited 60–70% by 10^{-2}M-atoxyl. The lipase activity is not significantly affected either by the phosphate derivatives or by atoxyl in concentrations which inhibit ali-esterase activity.

3. The spontaneous acetoacetate production, ali-esterase activity and phospholipid hydrolysis of liver are all inhibited to a similar extent by atoxyl. Selective inhibition of ali-esterases by the phosphate derivatives *in vitro* or *in vivo* has no effect upon the acetoacetate production or oxygen uptake of liver slices.

4. The results fail to give any indication of an essential function of ali-esterases in the normal lipid metabolism of rat liver.

5. The results tend to confirm the generally accepted theories regarding lipid oxidation in liver. The main pathway of lipid oxidation would involve, first, hydrolysis of phospholipin, and secondly, oxidation of the free fatty acids liberated.

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Amino-acid Utilization in Bacterial Growth

1. PEPTIDE UTILIZATION BY A LEUCINE-REQUIRING MUTANT OF *ESCHERICHIA COLI*

By MARIE-LOUISE HIRSCH and G. N. COHEN

Centre National de la Recherche Scientifique, Laboratoire de Chimie Bactérienne, Institut Pasteur, Garches, France

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In the course of studies on the quantitative determination of amino-acids with lactic acid bacteria, numerous authors have studied the availability of peptides in place of the corresponding amino-acids. In all cases so far published, it has been found that the peptides either show no activity, or an activity inferior or equal to that of the constituent amino-acids. The magnitude of the response of the organisms depended on the nature of the amino-acids (other than the one missing in the assay) present in the peptide and on their sequence in the peptide molecule.

In two cases only, a growth response greater with peptides than with the sum of equimolar quantities of the constituent amino-acids has been found. This effect has been observed (*a*) with the strain SF, a member of the genus *Pseudomonas*, studied by Simmonds & Fruton (1949, 1950, 1951); (*b*) with the two proline-requiring mutants of *Escherichia coli* (K 12–679–183 and 58–6317), studied by the same authors (Simmonds & Fruton, 1948). In the case of the strain 'SF', the organism can grow on leucine or glycine as the sole source of carbon and nitrogen, but a better growth is obtained on equimolar amounts of L-leucylglycine. However, the organism can also be grown on a variety of amino-acids as nitrogen source, such as L-phenylalanine, L-alanine, L-glutamate, L-isoleucine and L-valine. Thus, this organism exhibits no specificity as to its amino-acid requirement.

On the contrary, the proline-requiring mutants of *Esch. coli* present an absolute requirement for proline. A number of proline dipeptides and tripeptides show growth-promoting activity 1·5–3