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STUDIES OF THE INHIBITION OF LIPOLYTIC ENZYMES: I. THE INHIBITION OF A CANINE KIDNEY AND LIVER LIPASE *IN VITRO* AND *IN VIVO* BY n-BUTYL CARBAMIC ACID METHYL ESTER (U-14641)

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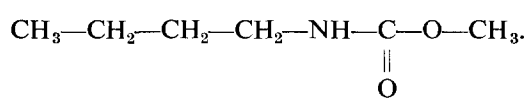
Abstract—n-Butyl carbamic acid methyl ester has been shown to be a potent inhibitor of canine liver and kidney lipases both *in vitro* and *in vivo*. The nature of this inhibition has been investigated and mechanisms consistent with the data are proposed.

When this inhibitor is intravenously administered to dogs in suitable doses, a marked degree of inhibition of liver and kidney lipases has been demonstrated both by direct assay and by the greatly diminished rate of elimination of procaine from the plasma of dogs treated with this compound.

A remarkable species specificity has been demonstrated, since butyl carbamic acid methyl ester does not inhibit liver and kidney lipases obtained from five other species.

FOR many years the esterases (lipases) of mammalian liver have been the subject of intensive biochemical investigation.[1–5] The esterase activity of the kidney, in contrast, has received much less attention. Recent work[6–8] has demonstrated that the liver plays an important role in the uptake of unesterified fatty acids (UFA), as well as triglycerides from the blood. Synthesis and degradation of triglycerides occur in the liver, which is in a state of dynamic equilibrium, not only with the UFA and triglycerides of adipose tissue, but with other organs as well.

While many organic compounds have been shown to inhibit liver esterases from various animal species *in vitro*,[1–4] because of a lack of physiologically acceptable specific inhibitors the role of these enzymes *in vivo* in relation to the metabolism of lipids and other esters has remained obscure. This report is concerned with the discovery, as well as some of the enzymic and physiological properties of a potent inhibitor of canine liver and kidney esterase: U-14641—butyl carbamic acid methyl ester (BCME),



MATERIALS AND METHODS

Preparation of enzymes

A dog was killed by intravenous injection of magnesium sulfate, and the liver and kidneys were immediately removed and buried in ice. All further operations were carried out in a deep freeze at −30°. The organs were blended in at least 10 vols.

* With the technical assistance of Norman J. Crittenden.

acetone for 1 to 2 min. Next the blended material was rapidly filtered on a Buchner funnel and when most of the acetone had been removed, a piece of rubber dam was stretched over the top of the funnel and a vacuum of 10 to 15 μ Hg was applied for a period of 5 to 6 hr. At the end of this time the liver residue was light pink and dry, and the kidney residue was light tan in color. The filter cake was then removed from the freezer, rapidly crumbled manually, and sifted with a flour sifter. Further manual tumbling was continued until the powder felt warm and there was no appreciable odor of acetone. The preparation was stored in a vacuum desiccator over Drierite or activated alumina for a period of 12 to 18 hr at 0 to 3° before use. If kept dry and cold, the lipolytic activity of these acetone powders is stable for months.

To prepare the enzymes for use, 1 g of kidney or liver acetone powder was extracted in a polypropylene centrifuge tube with 10 ml of cold water for 10 min. The mixture was then centrifuged at 34,000 $\times$ g for 5 min, and the clear supernatant fluid was used as such without further fractionation. The usual yield of supernatant fluid was 7·5 to 8 ml, containing 300 to 350 mg of soluble protein.

To assay the lipolytic activity of fresh tissues, the organs were removed from the animal and chilled in ice. They were weighed and blended in a Waring Blendor in 5 volumes of cold water for 60 sec at top speed. The homogenate was then centrifuged in the cold at 34,000 $\times$ g for 10 min, and the slightly turbid supernatant fluid was used for assay.

Initially the kidney enzyme, prepared either from fresh kidney or from acetone powders, was used to test potential inhibitors. The test system was as follows. To 15-ml centrifuge tubes was added 700 μmoles ammonium chloride buffer (pH 8·35), 10 μmoles emulsified tributyrin,* enzyme sufficient to form 2 to 3 μmoles of glycerol during a 10-min incubation, and water to a final volume of 2·0 ml. Potential inhibitors, when soluble, were made up as 0·1 M solutions in water. Otherwise the compounds were dissolved in the minimal amount of 95% ethanol required to keep the compound in solution at 0·1 M concentration. Ten μmoles of each compound added in 0·1 ml volume was used for test. The reaction was followed by glycerol determinations with the method of Lambert and Neish.[9] Under the conditions used, the enzyme reaction was zero order over a 12-min incubation period.

To determine the degree of inhibition induced by BCME *in vivo*, as well as the activity of liver and kidney enzymes from other species, a more rapid nephelometric assay was used which depended on the rate of "clearing" of a tributyrin emulsion. Into a 1-cm cuvette was added 1 mmole of ammonium chloride buffer, pH 8·35, 50 to 75 units of enzyme (an enzyme unit is arbitrarily defined as that amount of enzyme inducing an optical density change of 0·001/min under the conditions of assay), 0·1 ml of inhibitor solution, and water to 2·95 ml. The contents of the cuvette were mixed by inversion and allowed to stand for 5 min at 26°. Then 0·05 ml of 0·1 M tributyrin emulsion previously described was added to the cuvette and the contents were rapidly mixed by inversion. A reading was immediately taken in a Beckman DU spectrophotometer at 420 mμ against a blank containing all components of the reaction mixture except the tributyrin emulsion, and readings were taken at 1-min intervals

* This emulsion was made by weighing 1·5118 g tributyrin into a 100-ml beaker. To this was added 40 ml of a 0·025% solution of Pluronic F-68 (Wyandotte Chemical Co.) and treated for 60 sec with a Branson sonifier set at 8 to 10 amp output. The emulsion was then made up to 50 ml with more Pluronic solution.

thereafter for a period of 5 min. Temperature was maintained at 26° with thermospacers. Under the conditions specified, and without inhibitors, the reaction is zero order for the kidney and liver enzymes of the dog, cat, rat, mouse, rabbit, monkey, and man.

Unfortunately, because the emulsion is not permanently stable (the particles of lipid tend to coalesce and settle with a concomitant decrease in optical density within a matter of hours), it has not been practical to quantitate this assay on a molar basis. It has proved useful, however, for rapid determinations of enzyme activity, and this assay was used for all determinations of the degree of inhibition induced *in vivo* by BCME.

When the ability of the dog liver enzyme to hydrolyze procaine was determined, essentially the same conditions as described by Terp[10] were used. To 15-ml centrifuge tubes was added 1 mmole potassium phosphate buffer, pH 7·4, 60 μg procaine HCl, 33,900 units* of dog liver lipase (acetone powder extract), and water to a final volume of 3 ml. Appropriate controls and blanks were included. The tubes were incubated at 37° for 1 hr in a water bath, after which the tube contents were rapidly transferred to 4-ounce polyethylene bottles containing 60 ml ethylene dichloride extraction mixture in accordance with the method for procaine of Brodie and Axelrod.[11] The tubes were washed with 3 ml 0·8 M borate buffer, pH 9·0, which was added to the extraction mixture. The procedure of Brodie and Axelrod was used to complete the determinations without substantial modification.

The data from kinetic studies were initially plotted by the methods of Lineweaver and Burk.[12] Where linearity was apparent, curves were fitted by least-squares calculations. When the plots were obviously nonlinear, equations were devised and programmed for the IBM 1620 computer, and appropriate constants were selected to obtain calculated curves which most closely fitted the data. The mathematical treatment and the constants derived will be discussed in the following section.

When an inhibitor was discovered by the methods outlined, it was tested as follows. Dogs were anesthetized with intravenous doses of 15 mg thiopental/kg and 200 mg barbital/kg. A tracheal cannula was inserted, the abdomen was opened, and one kidney and a piece of liver were removed. Enzyme extracts were made as described, and the units of lipase activity per milliliter of extract were determined for initial control values. BCME was then administered intravenously, and at the indicated times a piece of liver was removed for assay. At the conclusion of the experimental period the animal was killed by intravenous injection of magnesium sulfate, and the remaining kidney and a piece of liver were removed for the nephelometric lipase assay. The degree of enzyme inhibition was determined by subtracting the lipase units per milliliter of extract found in the sample of tissue removed after BCME administration from the units per milliliter of extract found in the initial control samples. This value, divided by the units per milliliter in the controls, multiplied by 100, gives the percentage inhibition.

RESULTS

Butyl carbamic acid methyl ester was found to inhibit the dog kidney lipase 100 per cent at a concentration of $3{\cdot}3 \times 10^{-4}$ M (in the nephelometric assay) and 93 per

* The units referred to are the same as those defined earlier in the text.

cent at a concentration of $3{\cdot}3 \times 10^{-5}$ M. Under identical conditions of assay the dog liver enzyme was inhibited 100 per cent at a concentration of $3{\cdot}3 \times 10^{-5}$ M and 61 per cent at $3{\cdot}3 \times 10^{-6}$ M. These results were an indication that the active groups of the kidney and liver enzymes were similar, but the liver enzyme appeared more sensitive to inhibition by BCME.

Preliminary experiments to establish the dosage range required to inhibit *in vivo* the enzymes of dog liver and kidney indicated that when liver and kidney samples were assayed 2 hr after administration of BCME, a gradation of response could be expected only with doses below 25 mg/kg. Above this dose practically 100 per cent inhibition of both enzymes was found.

In Fig. 1 are shown the results of an experiment designed to establish a dose–response relationship. One dog was used at each dose indicated. The animal was

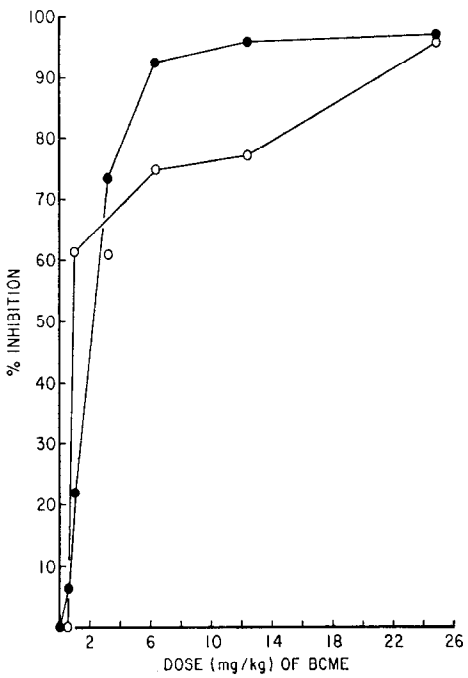


FIG. 1. The relationship of increasing intravenous doses of BCME to inhibitory responses of liver and kidney lipases in the dog. —●—●—●— liver lipase; —○—○—○— kidney lipase.

anesthetized and operated upon as described and was given a dose of BCME; it was then held for 2 hr without further treatment before it was killed with an intravenous injection of magnesium sulfate. Kidney and liver samples were immediately removed for assay, and calculations of the percentage inhibition were made by means of data derived from liver and kidney removed as controls prior to administration of BCME. It was found that neither the anesthetics used nor a lethal dose of magnesium sulfate had demonstrable effect on either the kidney or liver enzyme. It is apparent from Fig. 1 that the enzymes from both organs are remarkably sensitive to BCME *in vivo*, and small increments in dosages below 6 mg/kg result in marked increases in enzyme inhibition.

In unanesthetized animals, doses as high as 50 mg BCME/kg do not produce gross effects. When a dose of 125 mg BCME/kg was administered intravenously to a dog, the animal began to salivate and lacrimate immediately. This was followed by defecation and inability to stand on its feet. These symptoms were transitory, lasting about 10 min, after which they became progressively less acute. The animal was depressed but appeared otherwise normal within an hour.

To ascertain the length of time the inhibition induced by BCME lasts after an intravenous dose of 25 mg/kg, a dog was anesthetized and operated upon as described, and liver samples were excised at 2, 4, and 6 hr for assay of enzymic activity. At 2 hr, 100 per cent inhibition was observed, a value in close agreement with the data of Fig. 1. At 4 and 6 hr, 97 per cent and 94 per cent inhibition, respectively, were seen. The animal was sacrificed at 8 hr. At this time the liver showed 94 per cent inhibition, and the kidney had no demonstrable lipolytic activity. Because of the traumatic surgical procedures employed, it is considered impractical to keep such an animal preparation alive for longer periods and still obtain meaningful data. However, these results indicate that the inhibitory effects of BCME persist at essentially initial levels for at least 8 hr.

To determine whether the prolonged time the animal was held under anesthesia without further treatment had any effect on the activity of the liver and kidney enzymes, another dog was treated in the same fashion, but without any BCME. Liver and kidney samples were removed right after the animal was anesthetized, and again 8 hr after sacrifice. There was no difference in lipolytic activity between the two kidney samples. The liver sample removed 8 hr after induction of anesthesia was 13·5 per cent less active than the initial sample. This compares with a 94 per cent diminution in activity observed after administration of BCME.

While the data from such experiments are strongly suggestive that BCME is a remarkably potent and long-lasting inhibitor of canine liver and kidney lipase, the possibility remained that the liver or kidney could concentrate or retain BCME at sites where the drug was not in contact with the enzymes in question. Disruption of cellular structure by homogenization could then release the compound to the enzyme surface causing an inhibition that would not occur *in vivo*. Terp[10] has demonstrated that the dog, unlike man, has very little "procaine esterase" in the blood and appreciable quantities in the liver, kidney, and lung. To determine whether this enzyme is the same or similar to the enzymes this study is concerned with, an *in-vitro* experiment was set up using essentially the same conditions as those of Terp.[10] The results showed that with the procaine substrate, the dog liver enzyme was inhibited 80 per cent by BCME at a concentration of $3{\cdot}3 \times 10^{-5}$ M, a value very similar to that observed with tributyrin.

To assess the effect of BCME on procaine disappearance *in vivo*, a "crossover" experiment using four dogs was set up in which each dog served as its own control. On a given day, two dogs were used. One was given an intravenous dose of 25 mg BCME/kg. Five minutes later both dogs were given 15 mg procaine/kg intravenously, and blood samples were taken in heparinized syringes, at the times indicated in Table 1, for analysis by the procedure of Brodie and Axelrod.[11] A week later the experiment was conducted in reverse on the same set of two dogs. The dog which had formerly served as the control now received the inhibitor, and the former experimental dog now served as the control. This experiment was repeated on another set of two dogs; the results

obtained with these four dogs are shown in Table 1. For each dog, column a shows the procaine levels observed without inhibitor; column b shows the blood levels observed after administration of BCME. It is apparent from the data of Table 1 that in three of the four dogs used, the half-life of procaine was markedly increased. While dog number 2 did not show so dramatic an effect, still a definite elevation and prolongation of procaine levels in blood is evident.

TABLE 1. THE EFFECT OF BCME ON THE DISAPPEARANCE OF PROCAINE BLOOD LEVELS IN THE DOG

Results are given in microliters procaine/ml plasma.
Column a, blood levels of procaine at various times after administration of 15 mg procaine i.v./kg.
Column b, the same dog given BCME—25 mg/kg i.v. and 5 min later 15 mg procaine i.v./kg.

Dog Number	1		2		3		4		
Treatment	a	b	a	b	a	b	a	b	
Time 0	0		0		0	0	0	0	0 Time
5 min	6·0	5·2	4·8	7·9	6·2	5·6	5·0	4·8	5 min
30 min	3·9	4·6	2·1	4·7	3·0	4·1	2·3	3·7	30 min
1 hr	2·8	3·5	1·0	3·4	1·6	2·8	1·0	2·6	1 hr
2 hr	1·0	2·5	0·4	1·9	0·2	0·8	0·3	1·4	3 hr
4 hr	0·3	1·7	0·2	1·0	0	0·7	0·1	0·7	5 hr
6 hr	0·1	1·0	0·1	0·6		0·5	0·1		7 hr
8 hr	0	0·7	0·1	0·3	0	0·4	0	0·1	9 hr
10 hr	0	0·4	0·1	0·2					
Half-life estimates*	1·2	3·1	2·0	2·4	0·67	5·5	0·96	3·7	

*Half-life estimates (hours) of procaine removal for the period 2 to 10 hr post injection.

TABLE 2. THE DEGREE OF INHIBITION INDUCED BY A CONCENTRATION OF $3{\cdot}3 \times 10^{-3}$ M BCME AGAINST THE LIVER AND KIDNEY ENZYMES OF SIX SPECIES

Species	Inhibition of liver (%)	Inhibition of kidney (%)
Mouse	11	12
Rat	0	13
Cat	4	0
Rabbit	22	32
Monkey	44	35
Man	62	16·5

*Results derived from the tributyrin nephelometric assay.

These results further indicate that BCME is a true inhibitor of these hydrolytic enzymes *in vivo* and is not bound to inactive sites from which it could be released to the catalytic sites of the enzymes after cellular disruption *in vitro*.

In Table 2 are shown the results of experiments in which the kidney and liver lipases from mouse, rat, cat, rabbit, monkey, and man were assayed by the nephelometric method as described to assess their sensitivity to BCME. As can be seen from the data, the enzymes of these species are much less sensitive to BCME then is the canine enzyme and, for this reason, BCME was not evaluated *in vivo* in these species.

Mechanisms of inhibition

The enzymes under consideration in this report are prototypes of a broad class of esterolytic enzymes found in nature for which few potent and specific inhibitors are known. For this reason a detailed study of the kinetics of BCME inhibition of the canine liver and kidney lipases was undertaken.

The inhibition by BCME of tributyrin hydrolysis was studied with both the liver and kidney enzymes under essentially the same conditions described for testing potential inhibitors. The glycerol method of Lambert and Neish[9] was used to follow the reactions, and five substrate concentrations were used for each level of inhibitor added. Incubation periods were such that initial rates of reaction were studied, and it was assumed that steady-state conditions existed for the enzyme complexes. Since, in the hydrolysis of tributyrin, three ester linkages can theoretically be attacked by the enzyme, initial-rate kinetics will correspond to the average rate of all the ester linkages broken during the reaction period. The kinetic constants obtained will be definitive only under the experimental conditions employed since tributyrin has low solubility and, in these experiments unless otherwise indicated, was emulsified in the presence of the surfactant Pluronic F-68.

When the aqueous extracts of liver acetone powder are used as such without resort to further purification, the standard Lineweaver–Burk plot[12] of uninhibited and inhibited tributyrin hydrolysis produces curved lines instead of straight ones, as shown in Fig. 2. When the assumption is made that a fixed amount of tributyrin (7×10^{-4} M)

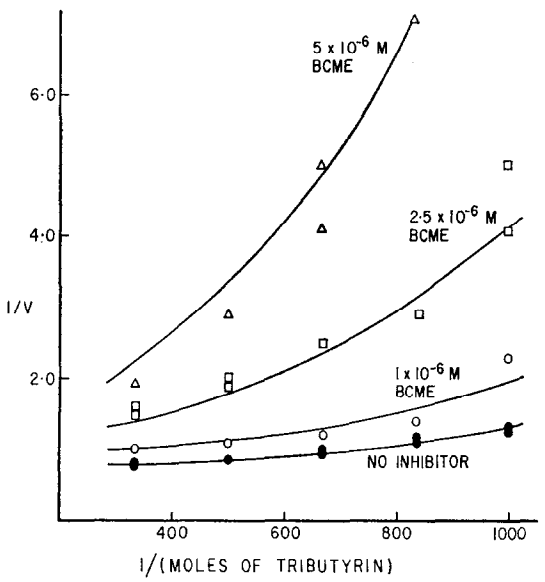


FIG. 2. Inhibition of a crude preparation of dog liver lipase by BCME. ——— Calculated curves (equation 1).

has been tightly bound to some inactive component of the crude enzyme preparation, (K_p, the binding contant of the site $\approx$ 0) then the kinetics of complete exclusive ("competitive") inhibition (nomenclature of Reiner[13]) are obtained from the following calculations.

Terms used throughout are defined as:

$E_0 \equiv$ moles of active center of the enzyme initially present
$S_0 \equiv$ moles of tributyrin added to the reaction mixture
$S \equiv$ moles of tributyrin available to the enzyme
$V \equiv$ velocity of diglyceride or glycerol formation
$Ei \equiv$ enzyme-inhibited complex
$E \equiv$ unassociated enzyme
$I_0 \equiv$ moles of inhibitor added
$I \equiv$ unbound inhibitor in the incubation mixture
$C_1 \equiv$ moles of active enzyme tributyrin complex
$C_2 \equiv$ moles of inactive enzyme–double substrate complex
$C_{1i} \equiv$ moles of inactive complex produced by C_1 binding BCME
$C_{2i} \equiv$ moles of complex produced by C_2 binding BCME

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C_1;\ C_1 \xrightarrow{k_{+2}} E + \text{butyrate} + \text{(diglyceride or glycerol)}$$

$$E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_i;\ S = S_0 - 0{\cdot}0007\ \text{M}$$

with the conservation equations

$$E_0 = E + C_1 + E_i$$
$$I_0 = I + E_i$$

The inhibited rate, V, of hydrolysis is represented by (13)

$$V = k_2\{-(k - mD) + \sqrt{(k - mD)^2 + 4\ kmE_0}\}/2km \tag{1}$$

where $k = 1 + K_1/S$, $m = K_1/K_3S$, $K_1 = (k_{-1} + k_{+2})/k_{+1}$, $K_3 = k_{-3}/k_{+3}$, $D = E_0 - I_0$.

Constants thus derived are: $K_1 = 2{\cdot}7 \times 10^{-4}$ M; $k_2E_0 = 0{\cdot}57$ M/10 min; $K_3 = 1{\cdot}8 \times 10^{-7}$ M; $K_p = 0$.

Two alternative explanations were possible for the failure of all the added tributyrin to participate in the hydrolysis reaction. On one hand the tributyrin could be bound to inactive sites on the enzyme molecule. In this case purification of the enzyme from crude aqueous extracts of dog liver acetone powder would not result in linear kinetics. If, however, the tributyrin is bound to an enzymically inert component of the crude extract, then purification of the enzyme should produce linearity. A purification procedure was developed (which will be published separately), and three preparations were isolated and pooled. The resulting enzyme was 26-fold purified, and when this enzyme was employed in the same inhibition experiments at low substrate concentrations, the partially purified enzyme produced linear Lineweaver–Burk plots. This indicated that the tributyrin which had formerly been unavailable to the catalytic sites of the

enzyme was now participating in the reaction. This was also evidence that the tributyrin was, indeed, bound to an inactive component of the crude extract, and the purification procedure had removed this material. Surprisingly, at high substrate concentrations, the purified enzyme was partially inhibited by tributyrin, as shown in Fig. 3.

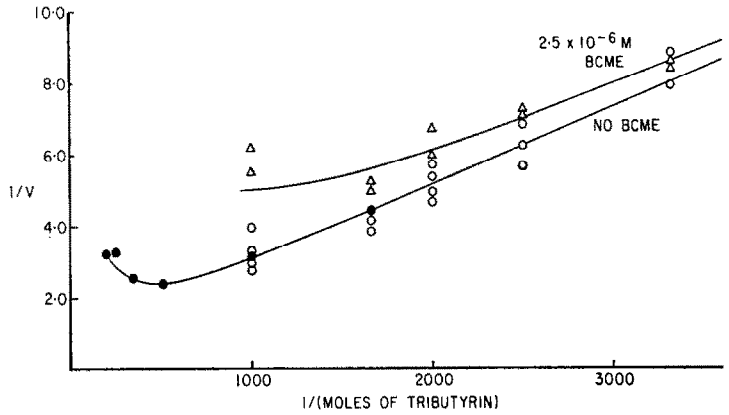


FIG. 3. Inhibition of purified dog liver lipase by BCME. ○, No BCME. ●, No BCME: the average of duplicate readings corrected for enzyme concentration. (A different concentration of enzyme was used for this part of the data so the I/V values were adjusted by multiplication with a suitable constant.) △, $2{\cdot}5 \times 10^{-6}$ M BCME. ——— Calculated curves (equation 2).

The kinetics of tributyrin hydrolysis with the purified canine liver lipase were consistent with the following scheme:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C_1$$

$$C_1 \xrightarrow{k_{+1a}} \text{diglycerides} + \text{butyrate} + E \quad (\text{or glycerol})$$

$$C_1 + S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} C_2$$

$$E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_i \qquad I + C_1 \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} C_{1i} \qquad C_2 + I \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} C_{2i}$$

with the conservation equation $E_0 = E + E_i + C_1 + C_{1i} + C_2 + C_{2i}$. The velocity of tributyrin hydrolysis, by the method of King and Altman[14] is:

$$k_{1a}E_0/V = (1 + I/K_3)K_1/S + (1 + I/K_4) + (1 + I/K_5)\,S/K_2 \qquad (2)$$

where $K_1 = (k_{-1} + k_{1a})/k_{+1}$; $K_2 = k_{-2}/k_{+2}$; $K_3 = k_{-3}/k_{+3}$; $K_4 = k_{-4}/k_{+4}$; $K_5 = k_{-5}/k_{+5}$.

Owing to technical difficulties in obtaining reliable data with low substrate concentrations, or high concentrations of BCME, the mechanism and constants derived must

be regarded as provisional. However, the mechanism most consistent with the data was one of reversible binding of BCME by the ternary enzyme substrate complex, C_2 (*ESS*) to form C_2i (*ESSI*) as per equation 2. Constants* calculated are:

$$Ek_{1a} = 2{\cdot}0 \text{ M}/2{\cdot}5 \text{ min}; K_1 = 4{\cdot}5 \times 10^{-3} \text{ M}; K_2 = 1{\cdot}1 \times 10^{-3} \text{ M};$$
$$K_5 = 6 \times 10^{-7} \text{ M}; K_3 = K_4 = \infty.$$

Since K_3 and K_4 are large, terms involving these may be left out of equation 2. However, in the interest of future experimental work, the more general equation is presented.

The mechanism of inhibition of the enzyme by relatively high concentrations of tributyrin (where the data have much less scatter) was found to be amenable with a model of inhibition of the *ES* complex through addition of another molecule of tributyrin to form *ESS*.

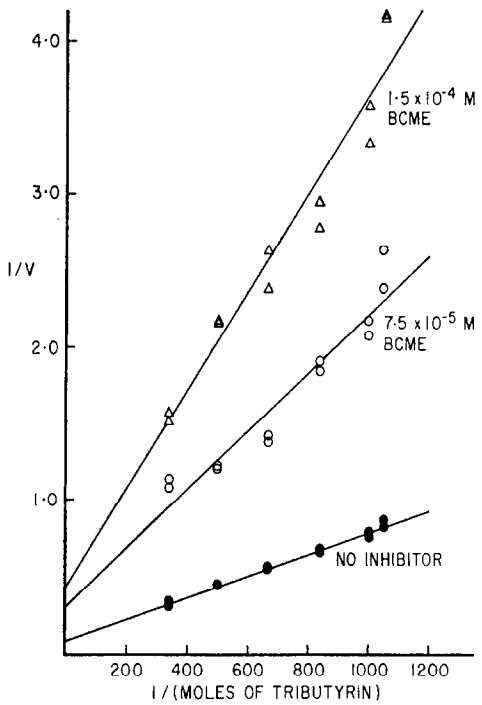


FIG. 4. Inhibition of dog kidney lipase by BCME. ——— Least-square line.

A difference was also observed in rates of hydrolysis in the presence and absence of Pluronic F-68. The addition of surfactant results in a decrease in K_1 which, in the model assumed, corresponds to increased binding of the substrate by the enzyme and/or a decreased breakdown rate of the enzyme substrate complex. Within 95 per cent confidence limits in each case, when Pluronic F-68 is present the $K_1 = (2{\cdot}7 \pm 0{\cdot}5) \times 10^{-3}$ M. In the absence of the surfactant, the $K_1 = (5{\cdot}8 \pm 1{\cdot}3) \times 10^{-3}$ M.

* These constants were calculated from experiments in which the substrate was added immediately after preparation without the presence of the surfactant Pluronic F-68. The emulsion is stable for approximately 30 min in this state before visible coalescence takes place.

Comparison of the mechanisms and constants obtained with the crude and partially purified enzyme preparations shows marked differences which might be due to a preferential selection of isozymes by the purification procedure used. Alternatively, there may have been chemical alteration of the purified lipase resulting in an alteration in the number of binding sites available for tributyrin.

BCME was also an inhibitor of dog kidney lipase, and the mechanism of inhibition of tributyrin hydrolysis catalyzed by the dog kidney lipase was found to be consistent with a model similar to Reiner's[13] "partial inhibition". As seen in Fig. 4, the reciprocal plots of velocity and substrate concentration gave increasing slopes and intercepts. In addition, however, a plot of inhibition concentration (BCME) I, versus $i/(1 - i)$:$(1 - i = r)$ is the (velocity with inhibition)/(velocity without inhibition), produces a plot in which extension of the final slope of the curve forms a negative I intercept and a positive $i/(1 - i)$ intercept (Fig. 5). Such experimental results are in accord with a

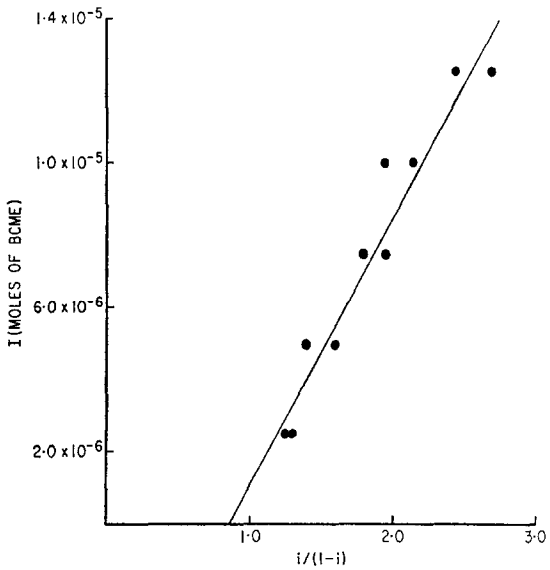


FIG. 5. Inhibition of dog kidney lipase by BCME. Substrate: 0·03 M tributyrin; ——— least-square line.

mechanism of partial inhibition (cf. Reiner[13]) which has been generalized to include inhibition of the enzyme substrate complex C_1, namely

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} C_1 \xrightarrow{k_2} E + \underset{\text{(or glycerol)}}{\text{diglycerides}} + \text{butyrate}; \quad E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_i$$

$$E_i + S \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} C_1 + I \qquad C_1 + I \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} C_{1i}$$

with the conservation equation $E_0 = E + E_i + C_1 + C_{1i}$
where $K_1 = (k_{-1} + k_2)/k_{+1}$; $K_3 = k_{-3}/k_{+3}$; $K_4 = k_{-4}/k_{+4}$; $K_5 = k_{-5}/k_{+5}$;

and where the usual definitions for the symbols are as previously defined. Assuming steady state conditions, the inhibited velocity, V, is such that

$$k_2E_0/V = (1/S)(k_{-1} + k_2 + k_{-4}I)(1 + I/K_3)/(k_1 + k_4I/K_3) + 1 + I/K_5 \qquad (3)$$

With $i = 1 - r$ (where r = inhibited velocity/uninhibited velocity), then

$$i/(1 - i) = \left\{(k_{-1} + k_2 + k_{-4}I)\left(\frac{I/K_3 + 1}{k_1 + k_4I/K_3}\right) + SI/K_5 - K_1\right\}/(K_1 + S) \qquad (4)$$

and for large I

$$i/(1 - i) \cong \{[K_1(k_1 - k_4) + k_{-4}K_3]/k_4 + (K_4 + S/K_5)I\}/(K_1 + S) \qquad (5)$$

Estimates of the various constants (with 95 per cent confidence limits) are shown as follows:

$$(k_{+2} + k_{-1})/k_{+1} = K_1 = (7{\cdot}4 \pm 3{\cdot}3) \times 10^{-3}\ \text{M}$$
$$k_{-3}/k_{+3} = K_3{}^* = (3{\cdot}8 \pm 1{\cdot}0) \times 10^{-5}\ \text{M}$$
$$k_{-4}/k_{+4} = K_4 = 4080 \pm 2410$$
$$k_{-5}/k_{+5} = K_5 = (3{\cdot}1 \pm 2{\cdot}9) \times 10^{-5}\ \text{M}$$

K^* is an estimate obtained by assuming $k_4 \ll k_1$.

From equation 3 and the value of the constants, it is concluded that:

1. Both enzyme and enzyme-tributyrin complex are inhibited.

2. The inhibited BCME-kidney lipase complex may, to a small extent, not only dissociate into the free enzyme again, but also form an active substrate enzyme complex by the exchange of a substrate molecule for an inhibitor molecule.

DISCUSSION

Welch and Bueding[15] and Hunter and Lowry,[16] in their discussion of the effects of drugs on enzyme systems, concur on three important criteria which should be met before an enzyme inhibition observed *in vitro* may be said to occur *in vivo*. These points are: (1) there must be definitive proof that the inhibitor is selectively inhibiting the same enzyme in experimental animals, (2) in animals this inhibition should occur at approximately the same concentration of inhibitor as that observed *in vitro*, and (3) a relationship should exist between dose of inhibitor and degree of enzyme inhibition observed *in vivo*.

With regard to the first point it has been demonstrated, both by assay of the enzymes after administration of BCME in the dog and by the diminished rates of disappearance of procaine from the plasma, that BCME is an effective inhibitor of liver and kidney lipase in the dog.

The second postulate is more difficult to assess because no measurements of the actual amounts of BCME in the liver and kidney of dogs have been made. If one assumes, however, that a dog is 70% water—and that after administration BCME is distributed uniformly throughout this water without extraneous binding, inactivation, or excretion of any kind taking place—a dose of 25 mg/kg (molecular weight = 178)

should result in a concentration of 2×10^{-4} M. Doses of 12·5, 6·25 and 3·12 mg/kg should result in molar concentrations of 1×10^{-4}, 5×10^{-5}, and $2{\cdot}5 \times 10^{-5}$ respectively. Extrapolating from *in-vitro* observations, at all but the lowest dose, practically 100 per cent inhibition of both the liver and kidney enzymes should have been observed within a very short time after drug administration. While the data of Fig. 1 are derived from organs removed 2 hr after drug administration, when it could be expected that a not inconsiderable amount of inactivation or excretion of the drug could have taken place, the very marked degree of inhibition observed at all but the lowest dose is consistent with what could be predicted from the concentrations of BCME required to inhibit the enzymes *in vitro*.

With respect to the third postulate, it would take a prohibitive number of dogs to establish a statistically valid relationship between the dose of BCME and the resulting degree of enzyme inhibition. Nevertheless, the data of Fig. 1 are adequately suggestive that this relationship exists.

With regard to the kinetic analysis of the inhibition mechanism induced by BCME, the question may arise as to how many other theories of equal complexity could fit the data equally well. An analysis of the fitting errors shows that equation 1, together with the condition for tight binding of tributyrin, represents the data in Fig. 2 significantly better (by the F test $P < 0{\cdot}01$) than a simultaneous least-square fit (15) to all the data according to the usual equation $E_0/V = \phi_0 + \phi_1/S + \phi_2 I + \phi_{12} I/S$. Thus, a mechanism involving tributyrin binding together with complete exclusive enzyme inhibition (competitive) fits the data significantly better than a mechanism of complete inclusive enzyme inhibition. Some of the curvature in the data is, therefore, consistent with tight tributyrin binding by nonhydrolytic sites in the enzyme preparation. It is conceivable that some other four-parameter equation may represent the data better, but the analysis of fitting errors shows that the usual four-parameter equation does not give statistically as good a fit as the mechanism proposed.

The fit of equation 2 to the data in Fig. 3 for $I = 0$ is significantly better ($P < 0{\cdot}01$ by the F test) than is a linear least-square fit. One cannot, therefore, choose any mechanism resulting in a linear equation and get a representation of the data equally as good as equation 2.

The diagnostic feature of the mechanism represented by equations 3 and 4 is that a plot I vs. $i/(1 - i)$ will have a negative intercept for large I as is evident also for the data represented by Fig. 5. The usual mechanisms, however, for inclusive complete inhibition lead to a positive intercept for I in a plot of I vs. $i/(1 - i)$ (Reiner[13]). Thus there is an essential feature that is described by equations 3 and 4, which is not described by the usual mechanism of inclusive complete inhibition.

The role of the kidney and liver lipases in lipid metabolism is at present obscure. Exploratory experiments have indicated that inhibition of these enzymes had no effect on the rate of "clearing" of a lipemia induced in dogs by intravenous administration of 1500 mg of the cottonseed oil emulsion Lipomul/kg. Further, BCME does not inhibit "clearing factor lipase" from appearing in the plasma of dogs after administration of suitable doses of heparin.

The unusual species specificity of BCME inhibition of canine liver and kidney lipase is noteworthy. This phenomenon implies that, whereas the enzymes of species other than the dog can catalyze the same hydrolytic reactions, the active groups of the canine enzymes are different in some way and are, therefore, inhibited by BCME.

To carry the analogy farther, since BCME appears to be an almost equally potent inhibitor for canine liver and canine kidney enzymes, this is suggestive evidence that the active groups of these enzymes possess a marked degree of similarity.

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