BBA 66038

SUBSTRATE SPECIFICITY AND INHIBITION CHARACTERISTICS OF A FLUORIDE-SENSITIVE TRIBUTYRINASE

L. RONALD CRUM, RICHARD G. HARBECKE, JOHN J. LECH AND DEANE N. CALVERT
Department of Pharmacology, Marquette School of Medicine, Milwaukee, Wisc. 53233 (U.S.A.)

(Received August 18th, 1969)

SUMMARY

In this paper the results of further investigations on the characteristics of a previously reported F^--sensitive tributyrinase isolated from rat adipose tissue are presented. This tributyrinase showed a specificity for esters of butyric acid (1-monoglycerides, 1,2-diglycerides, triglycerides, and methyl esters). No activity against the cholesteryl esters tested was evident. F^- inhibition was maximum with triglycerides and was also seen with mono- and diglycerides; this inhibition was absent, however, when methyl esters were employed as substrates. In addition, the F^--sensitive tributyrinase was shown to be unaffected by various lipolytic blocking agents, but was almost completely inhibited by the organophosphorous insecticides (DFP, malathion, parathion). It is suggested that this enzyme may be an esterase involved in drug metabolism, a nonspecific esterase, or possibly an enzyme related to a hormone-activated lipase.

INTRODUCTION

Although there is much evidence supporting the involvement of adenyl cyclase in the mechanism of hormonally regulated lipolysis in adipose tissue[1-5], the exact nature of the enzymes involved has not been elucidated. One possible lipolytic enzyme which has been partially purified and investigated, however, is a F^--sensitive tributyrinase isolated from rat adipose tissue[6,7]. This enzyme has been shown to be similar to tributyrinase in rat heart, lung, liver and kidney. It has an alkaline pH optimum, is inhibited by DFP, but is insensitive to eserine, Mg^{2+} and Ca^{2+}. Preliminary work has shown that this tributyrinase shows a specificity for short-chain glycerol esters and has little activity on long-chain glycerol esters. It is the purpose of this paper, therefore, to investigate further the substrate specificity and inhibition characteristics of the F^--sensitive tributyrinase in order to gain more insight into its physiological role in adipose tissue.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

METHODS AND MATERIALS

Preparation of tributyrinase

The purification of a F^--sensitive tributyrinase from rat adipose tissue has been previously reported[6]. The enzyme was thus prepared according to this procedure from adipose tissue of Holtzman rats (female retired breeders), dialyzed against distilled water and stored at 0°. The enzyme is stable for months under these conditions.

The purity of the enzyme preparation was established by disc-gel electrophoresis, as has been previously reported[7]. The protein concentration of the enzyme solution was determined *via* ultraviolet absorption at 280 mμ.

Tributyrinase assay

Tributyrinase activity was assayed by adding 0.1 ml of enzyme solution to a reaction mixture containing 50 μmoles of phosphate buffer (pH 8.5), 10 μmoles of emulsified tributyrin (or other substrate) and sufficient distilled water to make a final volume of 1.0 ml. The mixture was incubated at 37° in a Dubnoff Metabolic Incubator, with slow shaking, for 30 min; and the reaction was stopped by the addition of 5 ml of extraction mixture[8]. The butyric acid (or other fatty acid) produced during the reaction was determined according to the method of DOLE AND MEINERTZ[8] and appropriate corrections were made for acid partition in the extraction mixture.

Preparation of substrates and inhibitors

10 mmoles of the desired substrate were added to 50 ml of distilled water containing 0.25 ml of Triton X-100 and the mixture was sonicated with a Branson Sonifier Cell Disruptor Model S125 at a setting of 8 (microtip) for 2 min. The resulting emulsion was diluted to a final concentration of 0.1 M with distilled water.

Emulsions of the insecticides used were prepared by sonication of the oil in distilled water containing Triton X-100 (0.25 ml/50 ml of emulsion) with a Branson Sonifier Cell Disruptor Model S125 at a setting of 8 (microtip) for 1 min.

Materials

The materials used in the study were obtained as follows: Tributyrin, Aldrich Chemical Co., Milwaukee, Wisc.; 1-monolaurin, C. P. Hall Co. of Illinois, Chicago, Ill.; 1-monoolein, Calbiochem, Los Angeles, Calif.; triolein, Nutritional Biochemical Corp., Cleveland, Ohio; 1-monobutyrin, 1,2-diacetin, 1,2-dibutyrin, 1-monoacetin, methyl butyrate, 1-monostearin and trilaurin, Eastman Organic Chemicals, Rochester, N.Y.; triacetin, methyl stearate, tricaprylin, tricaprin, methyl laurate, methyl palmitate and 1,2-dipalmitin, Sigma Chemical Co., St. Louis, Mo.; cholesteryl acetate and cholesteryl butyrate, K & K Laboratories, Plainview, N.Y.; materials for disc-gel electrophoresis, Canal Industrial Corp., Bethesda, Md.; Sephadex G-200, Pharmacia Fine Chemicals, Inc., New Market, N.J.; Triton X-100, Rohm and Haas, Philadelphia, Pa.; DFP, cholesteryl palmitate, cholesteryl propionate, cholesteryl stearate, General Biochemicals, Chagrin Falls, Ohio; DDT, parathion, dieldrin, malathion, City Chemicals' Corp., New York, N.Y. All other reagents were of the highest purity commercially available.

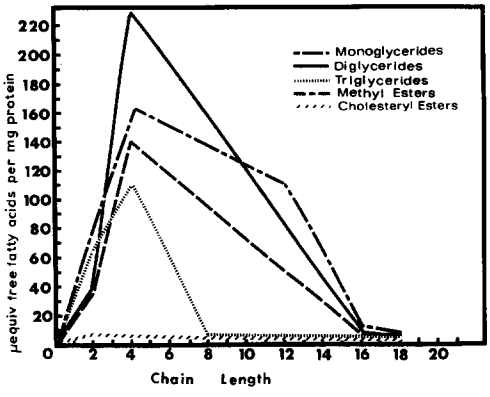

Fig. 1. Hydrolysis of esters of fatty acids of various chain lengths. 1-Monoglycerides, 1,2-diglycerides and cholesteryl-3-esters were used. Chain length refers to the number of carbon atoms in the saturated fatty acid component of the ester. Incubation period was 30 min at 37°.

RESULTS

Substrate specificity

Fig. 1 shows the results of assays of the activity of the F^--sensitive tributyrinase against various substrates. Five classes of compounds were used: monoglycerides, diglycerides, triglycerides, methyl esters and cholesteryl esters. The enzyme showed the greatest activity against 1-monoglycerides, followed by methyl esters, 1,2-diglycerides and finally triglycerides. No activity against cholesteryl esters was demonstrated. The greatest amount of hydrolysis was seen with esters of butyric acid in all four of the classes of compounds which were hydrolyzed.

It can also be seen that with the methyl esters as substrates the widest range of activity was observed, with hydrolysis apparent in esters with chain lengths of 2–14 carbons. Triglycerides, on the other hand, showed the narrowest range, with hydrolytic activity apparent upon compounds having fatty acid chain lengths of 2–6 carbons only. Hydrolytic activity against mono- and diglycerides was intermediate between that against methyl esters and triglycerides.

Table I gives the results of further substrate studies. With oleic acid esters maximum activity was seen against the monoglyceride, followed by the methyl ester and the diglyceride with no detectable activity against the triglyceride. However,

TABLE I

TRIBUTYRINASE HYDROLYSIS OF ADDITIONAL SUBSTRATES

Incubation period was 30 min at 37°.

Substrates	*μequiv free fatty acids/mg protein*	*Inhibition (%) by 1 mM F^-*
1-Monoolein	5.0	20
1,2-Diolein	1.4	0
Triolein	0	0
Trilinolein	0	0
Methyl oleate	7	0

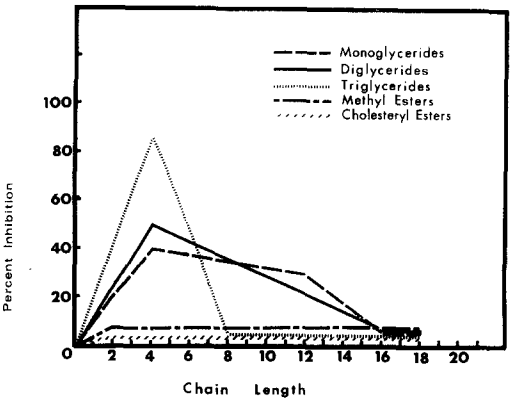

Fig. 2. Percent inhibition of hydrolysis of esters of fatty acids of various chain lengths produced by 1 mM NaF. Incubation period was 30 min at 37°.

with these substrates activity was quite low when compared with the level of activity against butyric acid esters.

Inhibition characteristics

Fig. 2 shows the results of assays of enzyme activity in the presence of 1 mM NaF using the substrates shown in Fig. 1. It can be seen that F^- produced inhibition of the hydrolysis of all the glycerol esters tested. The greatest degree of inhibition was seen with triglycerides (approx. 80% with butyric acid esters). Less inhibition was seen with mono- and diglycerides (approx. 50% with dibutyrin and 40% with monobutyrin). However, no F^- inhibition of the hydrolysis of methyl esters was observed.

In Fig. 3 the results of assays of tributyrin hydrolysis in the presence of various concentrations of five different insecticides are presented. It can be seen that little inhibition of tributyrinase activity was produced by the halogenated hydrocarbon insecticides DDT and dieldrin. Organophosphorous compounds, on the other hand, produced a significant degree of inhibition at the dosages used: 1 mM DFP gave 70%

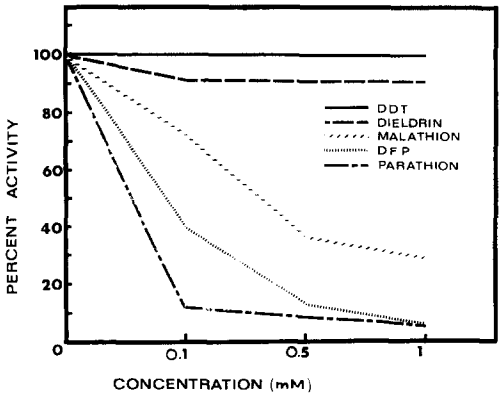

Fig. 3. Inhibition of tributyrin hydrolysis by various insecticides. DFP, malathion and parathion emulsions were prepared by sonication. Incubation period was 30 min at 37°.

TABLE II

EFFECTS OF VARIOUS LIPOLYTIC INHIBITORS ON TRIBUTYRIN HYDROLYSIS

Incubation period was 30 min at 37°.

Inhibitor	*Concn.* $\times 10^4$ *(M)*	*Inhibition (%)*
(1) Phentolamine	1	0
	5	0
	10	0
(2) Kö 592	1	0
	5	0
	10	0
(3) Methysergide	1	20
	5	26
	10	30
(4) Chlorpheniramine	1	0
	5	0
	10	0
(5) Insulin	0.01 munit/ml	0
	0.1 munit/ml	0
	1.0 munit/ml	0
(6) Nicotinic acid	1	17
	5	20
	10	23
(7) Prostaglandin E_1	10 μg/ml	0
(8) *N*-Ethylmaleimide	1	0
	1	0
	10	0
(9) Glycerol	1	0
	1	0
	10	0

inhibition; 1 mM malathion and 1 mM parathion both produced approx. 95% inhibition of tributyrinase activity.

Table II shows the effects of other possible lipase inhibitors on tributyrin hydrolysis. Only methysergide and nicotinic acid produced observable inhibition of tributyrinase activity. Phentolamine, Kö 592, chlorpheniramine, insulin, prostaglandin E_1, *N*-ethylmaleimide and glycerol were without effect at the concentrations used.

DISCUSSION

Since the mechanism of hormonally-induced lipolysis has been a topic of recent interest, several lipolytic enzyme preparations have been reported in attempts to further investigate this mechanism. Many of these preparations have been shown to hydrolyze tributyrin. Thus, the F^--sensitive tributyrinase has been shown to be similar, on the basis of substrate specificity, pH optimum, and inhibition characteristics, to: (1) a nonspecific esterase[9], (2) a tween hydrolase[10], and (3) a monolaurin lipase[11]. It is of value, therefore, to further compare the F^--sensitive tributyrinase with these preparations in the light of the information contained herein.

The F^--sensitive tributyrinase appears to be most similar to a nonspecific esterase obtained from rat adipose tissue[9]. The nonspecific esterase, however, shows a wider range of substrate hydrolysis, with significant activity seen against tripal-

mitin and monoolein, in addition to tri- and monobutyrin. Furthermore, the inhibition characteristics of this preparation are not uniform when different substrates are employed. It is probable, therefore, that the nonspecific esterase activity observed in whole homogenates consists of more than one enzyme: possibly (1) a triglyceride lipase which is sensitive to *p*-chloromercuribenzoate (PCMB) and DFP, (2) a monoglyceride lipase, which is sensitive to PCMB and insensitive to DFP, and (3) the F^--sensitive tributyrinase, which is sensitive to DFP but insensitive to sulfhydryl reagents.

Likewise, a monolaurin lipase from rat skeletal muscle[11] has many characteristics in common with the F^--sensitive tributyrinase. This monolaurin lipase, however, shows marked hydrolytic activity against monolaurin and only slight activity against monobutyrin. The F^--sensitive tributyrinase, on the other hand, shows the reverse situation with maximum hydrolysis of monobutyrin and only slight activity against monolaurin. In view of this difference and the fact that these two preparations are from different tissues, it is probable that they are similar but separate enzymes.

The F^--sensitive tributyrinase, therefore, is probably an enzyme which is involved in the hydrolysis of simple esters. It shows a marked activity against simple butyric acid esters (tri-, di-, and monoglycerides and methyl esters), but little activity against cholesteryl esters. This substrate specificity appears to depend on the overall size of the presenting substrate, for a wider variety of chain lengths is hydrolyzed as the alcohol moiety becomes smaller. Thus the tributyrinase shows a wide range of activity against methyl esters (from 2 to 16 carbons in the fatty acid component) and a narrow range of activity against triglycerides (from 2 to 6 carbons).

In addition, the F^- sensitivity of the tributyrinase may depend on an interaction with a glycerol binding site or with the glycerol component itself, for F^- inhibition is absent with the methyl ester series. An alternative explanation is that the enzyme preparation contains a second enzyme with similar activity against methyl esters but which is insensitive to F^-; however, by the criterion of purity used in this work (disc-gel electrophoresis) only one distinct protein was present in the enzyme preparation[7].

The tributyrinase studied here, at least in the form isolated, does not appear to be involved in the hormonally-induced lipolytic response. The action of this enzyme on tributyrin, for example, is not blocked by a series of pharmacological blocking agents, many of which have been shown to block lipolysis either *in vivo* or *in vitro*. Thus, an alpha adrenergic blocker (phentolamine), a beta adrenergic blocker (Kö 592), and antihistamine (chlorpheniramine), a reaction product (glycerol), insulin and prostaglandin E_1 produced no inhibition of tributyrin hydrolysis. The antiserotonin drug methysergide and the lipolytic inhibitor nicotinic acid produced only slight blockade.

The inhibition of the F^--sensitive tributyrinase produced by DFP, parathion, and malathion appears similar to the inhibition of an aliesterase from rat liver by organophosphorous insecticides shown by DuBois *et al.*[12]. This aliesterase catalyzes the hydrolysis of simple esters such as diethyl succinate. This similarity suggests the possibility that this tributyrinase is such an aliesterase which may be involved in drug metabolism.

A second possibility is that the enzyme investigated herein is an inactive form of the hormone-sensitive lipase. Recent interconversion between a lipase and an

esterase by the addition of a lipid cofactor has been demonstrated by OKUDA AND FUJII[13–15]. The F^--sensitive tributyrinase may thus represent an esterase which, when combined with a cofactor or activator, becomes a true lipase.

ACKNOWLEDGMENTS

This work was supported in part by grant AMO7681 U.S. Public Health Service, Wisconsin Heart Association, and National Institutes of Health Training Grant GM 370 (L.R.C.). We wish to thank Miss Patricia Sandwell and Miss Patricia Baugher for their technical assistance.

REFERENCES

1 M. A. RIZACK, *J. Biol. Chem.*, 236 (1961) 657.
2 M. A. RIZACK, *J. Biol. Chem.*, 239 (1964) 392.
3 M. BLECHER, N. S. MERLINO AND J. T. RO'ANE, *J. Biol. Chem.*, 243 (1968) 3973.
4 M. RODBELL, *J. Biol. Chem.*, 242 (1967) 5744.
5 R. W. BUTCHER, C. E. BAIRD AND E. W. SUTHERLAND, *J. Biol. Chem.*, 243 (1968) 1705.
6 J. J. LECH AND D. N. CALVERT, *Can. J. Biochem.*, 46 (1968) 707.
7 L. R. CRUM AND J. J. LECH, *Biochim. Biophys. Acta*, 178 (1969) 508.
8 V. P. DOLE AND H. J. MEINERTZ, *J. Biol. Chem.*, 235 (1960) 2595.
9 Y. BAILE, E. GORIN AND E. SHAFRIR, *Biochim. Biophys. Acta*, 152 (1968) 28.
10 D. P. WALLACH, H. KÖ AND N. B. MARSHALL, *Biochim. Biophys. Acta*, 59 (1962) 690.
11 D. P. WALLACH, *J. Lipid Res.*, 9 (1968) 200.
12 K. B. DUBOIS, F. K. KINOSHITA AND J. P. FRAWLEY, *Toxicol. Appl. Pharmacol.*, 12 (1968) 273.
13 H. OKUDA AND S. FUJII, *J. Biochem.*, 60 (1966) 154.
14 H. OKUDA AND S. FUJII, *J. Biochem.*, 61 (1967) 313.
15 H. OKUDA AND S. FUJII, *J. Biochem.*, 64 (1968) 377.