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CHARACTERISTICS AND DISTRIBUTION OF FLUORIDE-SENSITIVE TRIBUTYRINASE IN RAT TISSUES

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SUMMARY

The characteristics of a fluoride-sensitive soluble tributyrinase isolated from rat adipose tissue were investigated. The purity of the preparation was demonstrated by disc gel electrophoresis. This enzyme was shown to be similar to tributyrinase in rat heart, lung, liver and kidney. It has a pH optimum of 8.5 in Tris–phosphate buffer. Furthermore, the enzyme is inhibited by DFP, but is insensitive to eserine, Mg^{2+} and Ca^{2+}. The fluoride-sensitive tributyrinase shows a specificity for short chain glycerol esters (mono-, di-, and tributyrin) and little activity on long chain glycerol esters (monolaurin, monoolein, triolein and tripalmitin).

INTRODUCTION

Tributyrinase activity in adipose tissue has been reported by several investigators. Similar activity has also been demonstrated in rat kidney, spleen, heart, lung[1], liver[1,2] and skeletal muscle[1,3], and in human liver, lung, heart, kidney, adrenal, thyroid, ovary, testis, spleen, brain and muscle[4]. Due to differences in the methodology employed, tributyrinase activity has been described under a variety of terms. Thus such activity has been associated with: (1) a tributyrinase from pig adipose tissue[5], (2) a Tween hydrolase from rat adipose tissue[6], (3) a monolaurin lipase from rat skeletal muscle[3], (4) a nonspecific esterase from rat tissues[1,2], (5) an alkaline lipase from human tissues[4], and (6) a monoglyceride lipase from rat adipose tissue[7]. Recently adipose tissue has been shown to contain two types of tributyrinase activity, fluoride sensitive and fluoride insensitive[8]. It is the purpose of this paper, therefore, to further investigate this fluoride sensitive tributyrinase and to discuss its characteristics in relation to the above preparations.

MATERIALS AND METHODS

Preparation of tissue extracts

Perirenal, parametrial and mesenteric adipose tissue was excised from ex-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

sanguinated female retired breeder Holtzman rats, rinsed in cold distilled water, blotted dry, and homogenized in cold distilled water in a Waring blender for 2 min at maximum speed. The homogenate was then centrifuged at 12 000 × *g* for 15 min in a refrigerated (0°) centrifuge, and fluid below the fat cake was removed and kept on ice until assay. The same procedure was followed for other tissues (lung, heart, liver, kidney), except that the clear supernatant was used as the extract. For all assays, the homogenate contained 3 ml of cold distilled water for each gram of tissue.

Preparation of ammonium sulfate fractions

Enzyme grade ammonium sulfate was added with stirring at 0° to the above extracts to a concentration of 0.45 saturation. The solution was then centrifuged at 12 000 × *g* and the clear supernatant was collected. Ammonium sulfate was then added to this supernatant to a concentration of 0.65 saturation; the solution was centrifuged as above and the precipitate was dissolved in cold distilled water (up to 1/20 the original volume). This stage of purification is hereafter termed the ammonium sulfate fraction.

Tributyrinase purification

The purification of a fluoride sensitive tributyrinase from rat adipose tissue as a modification of the procedure for the isolation of a Tween hydrolyzing enzyme[6] has been previously reported[8]. The enzyme was thus prepared according to this procedure from tissue obtained from Holtzman female retired breeders by gel filtration on Sephadex G-200. The gel bed in this procedure was 1.5 cm × 75 cm and the eluant consisted of 0.05 M phosphate buffer (pH 8.6). The flow rate was 4 ml/h. As an alternative procedure, the enzyme was prepared according to the method of WALLACH[6], dialyzed against distilled water for 24 h at 0° (see ref. 8), and was further purified by chromatography on DEAE Sephadex A-50. In this procedure, the column bed was 1.5 cm × 75 cm and the eluant consisted of 0.05 M phosphate buffer over a pH gradient of 7.5 to 6.5. The flow rate of the eluant was 10 ml/h. The contents of the tubes containing tributyrinase activity were pooled, dialyzed against distilled water and stored at 0°. The enzyme was stable for months under these conditions. Hereafter, it is termed the Sephadex or DEAE fraction.

Disc gel electrophoresis

Disc gel electrophoresis was carried out at 0° on polyacrylamide gel, with a 7.5% separating gel at pH 8.6, a 2.5% spacer gel at pH 6.6 and a reservoir buffer consisting of Tris 3.0 g and glycine 14.4 g per l at pH 8.6. Sucrose (200 mg/ml) was added to tissue extracts to aid in layering the preparations on the spacer gel under the reservoir buffer. The columns were 5 mm × 50 mm and were run at 3–4 mA each until the tracking dye was 3 mm from the bottom of the column. One of three procedures was then carried out on the gels: (1) they were stained for protein with Amido Schwarz dye in 7% acetic acid (1 g/100 ml); (2) they were rapidly cut into 2.2-mm slices which were assayed for tributyrinase activity as previously described[8]; or (3) they were assayed for tributyrinase activity, after dialysis in distilled water at 0° overnight, by development in a 0.02% cresol red stain containing 5 ml of tributyrin emulsion per 100 ml of solution. In the third procedure the gel is stained

red, while the zone of acid produced by the action of the enzyme on the tributyrin added turns yellow.

Tributyrinase assay

Extracts and purified preparations were assayed by adding 0.1–0.2 ml of enzyme to a reaction mixture containing 50 μmoles of phosphate buffer (pH 8.0), 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 the required period of time, usually 30 min, and the reaction was stopped by addition of 5 ml of extraction mixture[9]. The butyric acid (or other fatty acid) produced during the reaction was determined according to the method of DOLE AND MEINERTZ[9] and appropriate corrections were made for acid partition in the extraction mixture. For the assays of the enzyme on polyacrylamide gels following electrophoresis, the slices were crushed in the buffer with a glass rod before addition of tributyrin to start the reaction.

Preparation of substrates

10 mmoles of tributyrin, 1,2-dibutyrin, 1-monobutyrin, 1-monolaurin, tripalmitin, 1-monoolein, or triolein (99% as verified by thin-layer chromatography) were added to 50 ml of distilled water containing 0.25 ml of Triton X-100 and the mixture was sonicated with a Bronson Sonifier Cell Disruptor Model S125 at a setting of 8 (microtip) for 2 min. The resulting emulsion was diluted to a final concentration of 100 μmoles/ml with distilled water. For those studies on substrates in solution, the emulsion was centrifuged at 12 000 $\times$ g for 15 min and the resulting infranatant fluid was used as the substrate preparation.

Materials

The materials used in this study were obtained as follows: tributyrin, Aldrich Chemical Co., Milwaukee, Wisc.; monolaurin, C. P. Hall Co. of Illinois, Chicago, Ill.; monoolein, Calbiochem, Los Angeles, Calif.; triolein, Nutritional Biochemicals Corp., Cleveland, Ohio (purified by column chromatography on silicic acid according to the method of BORGSTRÖM[10]), materials for disc gel electrophoresis, Canal Industrial Corp., Bethesda, Md.; Sephadex G-200 and DEAE Sephadex A-50, Pharmacia Fine Chemicals, Inc., New Market, N.J.; Triton X-100, Rohm and Haas, Philadelphia, Pa.; DFP, General Biochemicals, Chagrin Falls, Ohio. All other reagents were of the highest purity commercially available.

RESULTS

Tributyrinase activity of various tissue extracts

The results of the direct assay of tissue extracts are presented in Fig. 1. It can be seen that fluoride sensitive tributyrinase activity is detectable in all five tissues tested. The greatest concentration of the enzyme is found in adipose tissue with moderate amounts also present in liver, kidney and lung. Heart extracts show only minimal activity. It is also apparent that adipose tissue contains a greater proportion (about 80%) of fluoride sensitive tributyrinase than do the other tissues (approx. 50% in each case).

Disc gel electrophoresis of various tissues

The results of disc gel electrophoresis of various tissue extracts are shown in Fig. 2. Electrophoresis of adipose extracts produces two peaks of activity, a major peak at slice 4 and a minor peak around slice 9. The major peak has been demonstrated to correspond to fluoride sensitive activity, while the minor peak contains fluoride insensitive activity[8]. Similar assays of liver extracts show a major peak (slices 4 and 5) corresponding to the fluoride sensitive tributyrinase; no minor peak is present with this tissue. Kidney extracts also show a major fluoride sensitive peak at slice 4 and

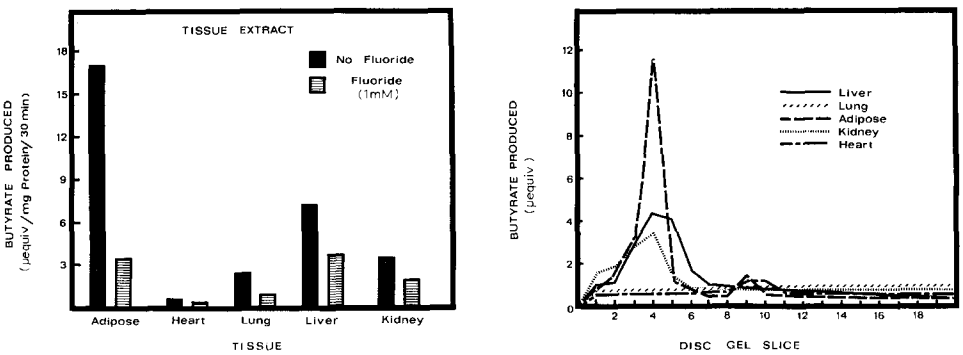


Fig. 1. Tributyrinase activity of crude extracts of various tissues. Assay procedure is described in METHODS. The solid bars represent assays of crude (12 000 × *g* supernatant) aqueous extracts of various tissues; the broken bars represent assays of the same extracts in the presence of 1 mM sodium fluoride. Incubation time was 30 min at 37°.

Fig. 2. Disc gel electrophoresis and assay of tributyrinase in crude extracts of various tissues Experimental procedure outlined under METHODS. Approx. 0.1 ml of crude extracts of various tissues corresponding to 30 mg of whole tissue were used for electrophoresis on polyacrylamide gel. After development, gel slices were assayed for tributyrinase activity. Incubation time was 2.5 h at 37°.

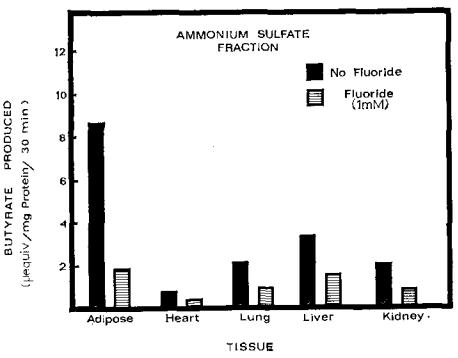
no minor peak. Heart extracts, on the other hand, show only the minor peak at slice 9, presumably indicating a predominance of fluoride insensitive enzyme. No significant tributyrinase activity was demonstrable in lung by this method.

Tributyrinase activity of various ammonium sulfate fractions

Fig. 3 shows the results of the assay of the ammonium sulfate fraction (0.45-0.65 saturation) from the above five tissues. The results are similar to those found in tissue extracts, again demonstrating a greater proportion of enzyme and of fluoride sensitivity in adipose tissue. These results also point out that in each of the tissues tested (adipose, heart, liver, lung and kidney) the fluoride sensitive tributyrinase is precipitated from extracts in the 0.45–0.65 ammonium sulfate fraction.

Disc gel electrophoresis of the DEAE Sephadex fraction

Fig. 4 shows the results of disc gel electrophoresis of the DEAE Sephadex fraction. One major protein band can be seen on the gel stained for protein (A). In addition, one or two slower moving, minor bands were detected. The main protein band corresponds to the zone of acid production (light area) on the gel stained with



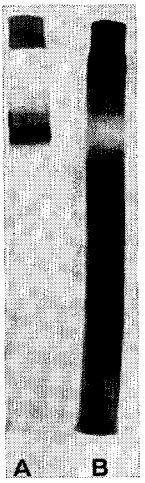


Fig. 3. Tributyrinase activity of ammonium sulfate fractions of various tissues. The solid bars represent assays of the ammonium sulfate fractions (0.45–0.65 saturation) of various tissues; the broken bars represent assays of the same ammonium sulfate fractions in the presence of 1 mM NaF.

Fig. 4. Disc gel electrophoresis of the purified tributyrinase (DEAE-Sephadex fraction). Experimental procedure is outlined under METHODS. DEAE-Sephadex fraction (0.05 mg protein per 0.1 ml) was added. The gels were run at the same time. Gel A was fixed and stained for 30 min in Amido Schwarz and destained electrophoretically. Gel B was stained in cresol red–tributyrin for 30 min.

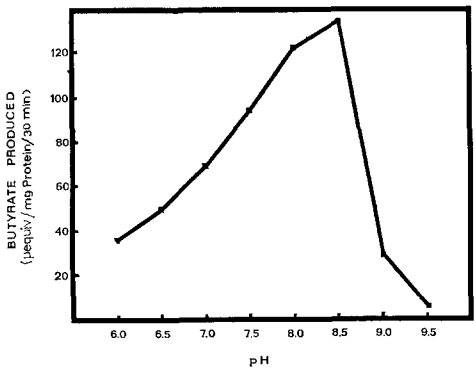
tributyrin–cresol red (B). The blurred nature of the edges of the acid is probably due to rapid diffusion in the gel matrix of the acid liberated. The minor protein bands may represent isozymes or subunits of the fluoride sensitive tributyrinase or simply protein contaminants similar in nature to this enzyme. These results indicate that the purified enzyme does exhibit tributyrinase activity.

Characteristics of the enzyme

pH optimum. The assays for pH optimum were carried out as described above with one exception: Tris–phosphate buffer was substituted for the phosphate buffer used in other assays. The results (Fig. 5) show that this enzyme has maximum activity in the alkaline range with a peak at pH 8.5.

Halide effects. Fig. 6 shows that the purified tributyrinase from adipose tissue is inhibited by fluoride. The enzyme is about 50% inhibited at 4 mM fluoride and almost 90% inhibited at 3 mM fluoride. Chloride, bromide and iodide show no significant inhibitory action toward the enzyme. These results suggest that the fluoride sensitivity of the tributyrinase is specific and cannot be considered a generalized halide effect, a sodium effect, or an inhibition due to increasing ionic strength alone.

Inhibitors. Table I shows the effects of four reported inhibitors of various enzymes exhibiting tributyrinase activity on the fluoride sensitive tributyrinase. Three concentrations of each compound were used: 0.1 mM, 0.5 mM and 1.0 mM, Physostigmine produced only slight inhibition at all concentrations tested. DFP on the other hand, produced 61% inhibition at 0.1 mM, 87% inhibition at 0.5 mM and 93%



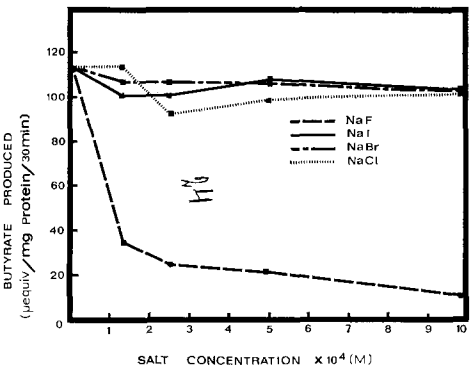


Fig. 5. pH optimum of the purified tributyrinase (DEAE-Sephadex fraction). The assays were run in 0.05 M (final concentration) Tris–phosphate buffer. Incubation time was 30 min at 37°.

Fig. 6. Halide effects on the purified tributyrinase (Sephadex G-200 fraction). Assay procedure described under METHODS. Incubation time was 30 min at 37°.

inhibition at 1 mM. $MgCl_2$ and $CaCl_2$, however, did not produce significant inhibition at any of the concentrations tested.

Substrate specificity. Two types of substrate preparations were used (see METHODS). Among the emulsified substrates tested, the fluoride sensitive tributyrinase shows a specificity for glycerol esters of short chain fatty acids. The results (Table II) show a greater specificity for 1,2-dibutyrin (150% greater than tributyrin hydrolysis) and 1-monobutyrin (50% greater than the tributyrin effect) than for tributyrin itself. In addition, a slight action on monoglycerides (monolaurin and monoolein) was detected. Considering the substrates in solution, the enzyme shows the same specificity toward butyric acid esters as demonstrated with emulsified substrates and the

TABLE I

INHIBITOR EFFECTS ON THE PURIFIED TRIBUTYRINASE (DEAE-SEPHADEX FRACTION)

Assay procedure described under METHODS. Incubation was for 30 min at 37°.

Inhibitor	*Concn. × 10⁻⁴ (M)*	*Inhibition (%)*
Physostigmine	1	4
	5	7
	10	4
DFP	1	61
	5	87
	10	93
$MgCl_2$	1	0
	5	0
	10	0
$CaCl_2$	1	0
	5	0
	10	0

TABLE II

SUBSTRATE SPECIFICITY OF THE PURIFIED TRIBUTYRINASE (DEAE-SEPHADEX FRACTION)

Assay procedure and preparation of substrates are described under METHODS. Incubation was for 30 min at 37°.

Substrate	*μequiv per mg protein per 30 min*	
	Emulsified	*In solution*
Tributyrin	109.1	25.5
Dibutyrin	245.5	—
Monobutyrin	149.0	—
Triolein	0	0
Monoolein	4.5	0
Monolaurin	16.9	9.1
Tripalmitin	0	—

same slight action on monolaurin as seen in emulsified form. The addition of bovine serum albumin did not alter the substrate specificity to any significant degree.

DISCUSSION

The mechanism of lipolysis in adipose tissue and its hormonal regulation has been a subject of much interest in recent years. Ultimately such investigation must involve the isolation and characterization of the specific enzymes involved in the hormonally regulated lipolytic system. Many studies have employed tributyrin as a substrate for the lipases being investigated[11–14]. The existence of a soluble tributyrinase bears a direct relation to these studies, since, unless the lipase preparation is extensively purified, observed hydrolysis of tributyrin may reflect the activity of the tributyrinase rather than that of a specific lipolytic enzyme. Furthermore, this tributyrinase may not be a true lipase but merely a nonspecific esterase, such as that reported by BIALE *et al.*[1]. True lipases are believed to be of two types: (1) a triglyceride lipase, which hydrolyzes triglycerides of long chain fatty acids and (2) a monoglyceride lipase which hydrolyzes monoglycerides of long chain fatty acids. The fluoride-sensitive tributyrinase reported here, however, does not act on such natural tri- and monoglyceride substrates, but rather hydrolyzes short chain glycerol esters and other simple compounds containing esteratic linkages, such as α-naphthol acetate. The substrate specificity of this enzyme, therefore, more closely resembles that of an esterase than that of a true lipase.

The importance of this fluoride-sensitive tributyrinase is further increased by the observation that it occurs in a wide variety of tissues (adipose, heart, lung, liver, kidney). It is possible that the activity reflected in these various tissues is due to the same tributyrinase. Three lines of evidence support this conclusion: (1) assays of tissue extracts have demonstrated fluoride-sensitive tributyrinase in all of the tissues tested. In all cases the proportion of the enzyme inhibited by fluoride has represented at least 50% of the total tributyrinase activity; (2) assays of ammonium sulfate fractions from various tissues have indicated that the tributyrinase is precipitated between 0.45 and 0.65 saturation with ammonium sulfate, the proportion of fluoride

TABLE III

SOLUBLE TRIBUTYRINASES, PARTIALLY PURIFIED

Substrates: TB, tributyrin; DB, 1,2-dibutyrin; MB, 1-monobutyrin; TC, tricaproin; TO, triolein; MO, 1-monoolein; ML, 1-monolaurin; TP, tripalmitin. Substrates: the quantification represents relative hydrolysis rates. Inhibitors: ++++, approx. 100% inhibition; +++, 75%; ++, 50%; +, 25% or less.

Ref.	*Designation*	*Species*	*Tissue*	*pH optimum*	*Substrates*		*Inhibitors*	
6	Tween hydrolase	Rat	Adipose	6.8–7.2	TB +++ TO o	MO o TP o	NaF ++++ Eserine + PCMB ++	$MgCl_2$ + $CaCl_2$ +
7	Monoglyceride lipase	Rat	Adipose	7.4–7.6	TB + MB ++++ TO o	MO +++ ML ++	NaF ++ NaCl ++ Eserine o	PCMB ++++ DFP ++
3	Monolaurin lipase	Rat	Skeletal muscle	8.45	TB + MB + TO o	MO o ML ++++ TP o	DFP ++++ Eserine + PCMB +	
3	Tricaproin lipase	Rat	Skeletal muscle	6.4–6.6	TB ++++ MB ++ (TC ++++) TO o	MO o ML +++ TP o	DFP ++++ Eserine + PCMB ++	
5	Adipose lipase	Pig	Adipose	4.5–7.0	TB +++ (DB ++++++) MB ++++	TO o TP +	DFP ++++ PCMB ++++ $CaCl_2$ o	

TABLE IV

SOLUBLE TRIBUTYRINASES, NOT PURIFIED
For details see Table III

Ref. No.	*Designation*	*Species*	*Tissue*	*pH optimum*	*Substrates*	*Inhibitors*
1	Nonspecific esterase	Rat	Adipose and others	8.5	TB +++ MB ++++ TO + MO ++	DFP ++++ PCMB o $MgCl_2$ +++ in liver and kidney $CaCl_2$ +++
4	Alkaline lipolytic activity	Human	Adipose and others	8.0	Only TB used	NaF ++++ Eserine +

sensitivity being retained in all cases; and (3) the tissues with the greatest tributyrinase activity (kidney, adipose and liver) show similar mobility on disc gel electrophoresis. The somewhat wider band of activity shown by liver extracts may reflect the presence of more than one enzyme with tributyrinase activity in this tissue.

Since several reported lipase preparations have shown tributyrinase activity, it is of value to compare the characteristics of the fluoride-sensitive tributyrinase described herein with those of the above preparations (Tables III and IV). The first basis for comparison is the purity of the preparations. The most extensively purified enzyme presented in Tables III and IV is that of LYNN AND PERRYMAN[5]. These workers claim a 900-fold purification of their porcine adipose lipase. WALLACH's Tween hydrolase[6] is also more extensively purified (75 fold) than the other enzymes presented. This fluoride-sensitive tributyrinase involves a further purification of Wallach's Tween hydrolase and thus constitutes one of the most highly purified tributyrinases reported. This purity has also been demonstrated by disc gel electrophoresis (Fig. 4) where only one or two minor protein bands remain and the major protein band corresponds to the zone exhibiting tributyrin hydrolysis.

On the basis of pH optimum, the fluoride-sensitive tributyrinase preparation corresponds most closely to the nonspecific esterase of BIALE *et al.*[1]. Monolaurin lipase[3] and the alkaline lipolytic activity observed in human tissues by CORTNER AND SCHNATZ[4] also show an alkaline pH optimum. pH optima, however, may vary with the buffer used and are not, therefore, the best criteria for comparison of different preparations. It has also been observed that unless a final buffer concentration of 0.05 M is used, the desired pH is not maintained throughout the incubation period.

Substrate specificity is also a difficult criterion with which to compare lipases, since substrate purity and preparation methods often vary. Using this criterion, however, the fluoride-sensitive tributyrinase shows marked similarity to the porcine adipose lipase of LYNN AND PERRYMAN[5], with maximum activity against 1,2-dibutyrin, 1-monobutyrin and tributyrin. There is also a close correspondence between the above tributyrinase and the nonspecific esterase of BIALE *et al.*[1].

Inhibition characteristics, however, still constitute the single best criterion for comparing different enzyme preparations. On this basis, the fluoride-sensitive tributyrinase appears most similar to (1) WALLACH's Tween hydrolase[6], due to NaF sensitivity, and the very slight inhibitory effects of eserine, PCMB, Ca^{2+} and Mg^{2+};

(2) WALLACH's monolaurin lipase[3], due to DFP sensitivity and the slight inhibition produced by eserine and PCMB; (3) the nonspecific esterase of BIALE *et al.*[1], due to PCMB insensitivity and DFP sensitivity; and (4) the alkaline lipolytic activity of CORTNER AND SCHNATZ[4], due to fluoride sensitivity and eserine insensitivity. It may be that all or part of the activity of some of these preparations is due to the presence of the fluoride-sensitive tributyrinase described herein.

The fluoride-sensitive tributyrinase does not appear similar, however, to the monoglyceride lipases of KUPIECKI[7] where the fluoride inhibition appears to be due to ionic strength (0.2 M fluoride, compared with 1 mM fluoride in our preparation). Likewise, WALLACH's tricaprion lipase[3] and LYNN AND PERRYMAN's adipose lipase[5] appear to be distinct from the tributyrinase. All of these enzymes show PCMB sensitivity, while sulfhydryl reagents show little inhibitory effect on the fluoride sensitive tributyrinase.

Overall, the fluoride-sensitive tributyrinase most closely resembles the nonspecific esterase activity of BAILE *et al.*[1], which has been demonstrated in crude extracts of rat adipose, liver, kidney, skeletal muscle, lung and spleen. The two preparations are similar in several ways: (1) both show a pH optimum of 8.5, (2) both show a marked specificity for tri- and monobutyrin, and (3) both preparations are inhibited by DFP and are unaffected by PCMB. It is possible that the fluoride-sensitive tributyrinase is responsible for part of the esteratic activity reported[1].

The physiological role of this tributyrinase is uncertain at this time. It is almost certain that tributyrin is not its natural substrate. The observation that the enzyme is more active against emulsified substrate than against substrate in solution suggests that this enzyme may be a true lipase which acts at an oil–water interface. If such is the case, the enzyme shows a marked affinity for short chain glycerol esters. Another possibility is that the enzyme is an esterase which acts on substrate in solution. In this case, the apparent specificity for emulsified substrate may merely reflect a larger substrate pool, represented by the emulsion particles which may gradually release substrate into solution. The lack of inhibition by eserine indicates that the enzyme is not acetylcholine esterase or pseudocholinesterase. It is difficult, however, to say with certainty whether this enzyme is a true lipase or an esterase showing tributyrinase activity.

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