Biochemical Pharmacology, Vol. 34, No. 15, pp. 2779–2785, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

A RADIOCHEMICAL ASSAY METHOD FOR CARBOXYLESTERASE, AND COMPARISON OF ENZYME ACTIVITY TOWARDS THE SUBSTRATES METHYL [1-^{14}C] BUTYRATE AND 4-NITROPHENYL BUTYRATE

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(*Received* 31 *December* 1984; *accepted* 8 *March* 1985)

Abstract—A radiochemical assay for carboxylesterase based on the substrate methyl[1-^{14}C]butyrate is described. The blank value corresponds to 1.04 μg (liver)–1.44 mg (plasma) of tissues with the highest and lowest activity respectively, which constitute the sensitivity of the method. The hydrolysis of methyl butyrate and 4-nitrophenyl butyrate by plasma, liver, lung, heart, diaphragm, cerebrum, kidney and duodenum of rat have been compared. The results showed that the two substrates were hydrolysed preferentially by two different groups of the enzyme. The effect of selective esterase inhibitors showed that both groups can be characterized as carboxylesterase, because bis-4-nitrophenyl phosphate inhibits the hydrolysis of both substrates, physostigmine has only a slight effect and EDTA is no inhibitor. The exception is the enzyme in the duodenum which is inhibited by all three inhibitors. The effect of phenobarbital induction and soman treatment on enzyme activity towards the two substrates were similar. Sex difference in the plasma activity towards methyl butyrate, but not 4-nitrophenyl butyrate, indicates that the group of carboxylesterase preferentially hydrolyzing 4-nitrophenyl butyrate may be the most important for the detoxification of soman.

Previous work in our laboratory has shown that carboxylesterase (EC 3.1.1.1) may be strongly involved in the detoxification of small doses (0.5 LD_{50}) of the organophosphorus anticholinesterases, soman or sarin, administered repetitively in rodents [1–6]. The detoxification is due to the ability of this enzyme to bind organophosphorus compounds covalently at the active site [7, 8], just as does cholinesterase. A variety of isoenzymes of carboxylesterase have been shown to exist with widely different affinities towards carboxylesterase substrates and inhibitors (see refs. 9–11 for review). Several different carboxylesters may be used as substrate in carboxylesterase measurement. Several assays, involving titrimetric or manometric methods, which are both laborious and time-consuming have been described. The present paper describes a rapid radiochemical method for measuring the hydrolysis of methyl butyrate, a commonly used substrate for carboxylesterase. The hydrolysis of methyl butyrate has been compared with that of 4-nitrophenyl butyrate, another carboxylesterase substrate that may be measured spectrophotometrically. The two substrates are hydrolysed differently by different rat tissues and may be hydrolysed by different groups of isoenzyme.

MATERIALS AND METHODS

Chemicals

Methyl butyrate (purity: reference substance for gas chromatography min. 99.5%), butyric acid sodium salt, hydrochloric acid, diethyl ether, chloroform, EDTA (all from E. Merck AG), bis-4-nitrophenyl phosphate sodium salt (BPNP), physostigmine salicylate, 4-nitrophenyl butyrate (all from Sigma Chemical Co.), phenobarbital (Norsk Medisinaldepot) and *n*-[1-^{14}C]butyric acid sodium salt (56 Ci/mole) (Amersham International plc) were purchased. Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) was synthesized in our laboratory and the purity found to be better than 97% by nuclear magnetic resonance spectroscopy.

Synthesis of methyl[1-^{14}C]butyrate

About 0.2 g of butyric acid sodium salt and 4 × 250 μCi of *n*-[1-^{14}C]butyric acid sodium salt was dissolved in 2 ml of 2 N HCl. The solution was dehydrated with diethyl ether by repetitive extraction (20 × 25 ml) and evaporation (Rotavapour). The final residue, containing ^{14}C-labelled butyric acid, was dissolved in 2 ml of diethyl ether and cooled on ice. The solution was mixed with 8 ml of diethyl ether containing 0.5 M diazomethane, synthesized as previously described [12], and stirred in ice/water for 60 min. The mixture was then concentrated by distillation on a Vigroux column at 39° (water bath) to about 300 μl volume. At this volume the mixture contained about 30% of methyl[1-^{14}C]butyrate. Methyl[1-^{14}C]butyrate was isolated from this volume by preparative gas chromatography in a Varian aerograph Series 200. The conditions were: 60° oven, 210° injector, 210° detector, 2 min 53 sec retention time, 10% Apiecon column 2 m length 0.4 mm thickness. The synthesis of methyl[1-^{14}C]butyrate led to a yield of 31%, purity better than 99% and specific radioactivity 0.41 Ci/mole. Due to its high volatility the compound was kept stored at −20° in a screw-cap

vial with Mininert Teflon valve (Dynatech Precision Sampling Corp.).

Animals

Wistar rats (220–270 g weight) were purchased from National Institute of Public Health, Oslo. Male rats were used if not otherwise indicated.

Administration of soman and phenobarbital

Soman was diluted in 0.9% NaCl solution and administered subcutaneously (50 μg/kg). Phenobarbital was injected intraperitoneally (100 mg/kg) daily for 3 days. Injected volumes were 0.5 per thousand of the rat body weight.

Preparation of tissues

Rats were anaesthetized with ether, and the heart was exposed and punctured with a heparinized syringe for withdrawal of 2 ml blood. Then 40 ml of 0.9% NaCl solution was injected through the heart ventricles to remove contamination of blood in the tissues. The organs were dissected and 10% (w/v) homogenates of tissue in 20 mM sodium phosphate buffer pH 7.4 were prepared with a Potter Elvehjem homogenizer (liver and cerebrum) or an Ultra-Turrax homogenizer (lung, diaphragm, kidney, duodenum and heart). Rats treated with soman were lightly ether anaesthetized 24 hr in advance to remove 2 ml blood by heart puncture. Blood was centrifugated (1000 *g* 10 min) for isolation of plasma. The tissue preparations were used undiluted or were adequately diluted in 20 mM sodium phosphate buffer pH 7.4 before enzyme assays. In case of extensive dilution (0.1% w/v liver and 0.25% w/v lung) the buffer contained 1% bovine serum albumin.

Measurement of carboxylesterase activity

Carboxylesterase activities were measured spectrophotometrically with 4-nitrophenyl butyrate or radiochemically with methyl[1-^{14}C]butyrate as the substrates.

(A) *Spectrophotometric assay.* The assay of Ljungquist and Augustinsson [13] was used with modifications. The assay mixture at 30° consisted of 0.1 M sodium phosphate buffer pH 7.8, 2 mM 4-nitrophenyl butyrate and tissue in a total volume of 3.02 ml. The stock solution of 4-nitrophenyl butyrate was 0.6 M in aceton. The absorbance of 4-nitrophenol at 400 nm was followed in a Varian Cary 118 spectrophotometer, with assay mixture except tissue as reference. A molar absorption coefficient of 17,000 M^{-1} cm^{-1} was used [13].

(B) *Radiochemical assay.* The routinely used substrate solution, containing 1.9 mM methyl[1-^{14}C] butyrate (0.037 Ci/mole) in 0.1 M sodium phosphate buffer pH 7.8, was prepared by rapid transfer of 1 μl methyl[1-^{14}C]butyrate (0.41 Ci/mole) and 10 μl unlabelled methyl butyrate into 50 ml of the buffer. The solution was stable for one week when kept refrigerated in a glass stoppered Erlenmeyer flask. Tissues were diluted to hydrolyse less than one third of the substrate. The assay method (see Results) was performed as follows: 50 μl of tissue and 0.5 ml of substrate solution was incubated in glass stoppered test tubes (Quickfit MF 24/0 10/19) at 30° for 30 min. Then 0.5 ml of 0.1 M sodium phosphate buffer pH 7.4 and 1 ml of chloroform was added, and the mixture was shaken 20–30 sec to extract unhydrolysed methyl butyrate into the chloroform phase. After centrifugation (1000 *g* 5 min) 0.5 ml of the aqueous phase containing [1-^{14}C]butyric acid anion was transferred to a scintillation vial with 0.5 ml H_2O and 10 ml of Insta-gel scintillation mixture (Packard Instrument Co.). The radioactivity was measured in a Packard Tri-Carb model 3380 scintillation counter, with counting efficiency 79%. Blanks were run without tissue, and were 186 ± 11 cpm (mean ± S.E.M.) for 30 measurements.

Measurement of cholinesterase activity

Cholinesterase activities were measured by the radiochemical method of Sterri and Fonnum [14].

RESULTS

Stability of methyl[1-^{14}C]butyrate solutions

Methyl butyrate in small incubation volumes escaped during incubation as shown in Table 1. We were unable to improve the incubation conditions by the presence of 1% Triton X-100 or 5% methanol, ethanol, isopropylalcohol or *N,N*-dimethyl formamid in the buffer. Nor could a layer of cyclohexan on top of the solution during incubation prevent the escape. Methyl butyrate in all concentrations tested,

Table 1. Effect of volume and glass stoppering on stability of methyl[1-^{14}C]butyrate solution

	Radioactivity after incubation (% of control)	
Volume (μl)	without glass stopper	with glass stopper
10	5	55
100	28	84
200	49	92
300	56	94
400	67	99
500	73	98

Indicated volumes of 1.9 mM methyl butyrate in 0.1 M sodium phosphate buffer pH 7.8 (spec. act. 0.037 Ci/mole) were incubated 30 min at 30° in Quickfit test tubes.
Control radioactivity (=100%) is without incubation.
The numbers are mean values of two experiments.

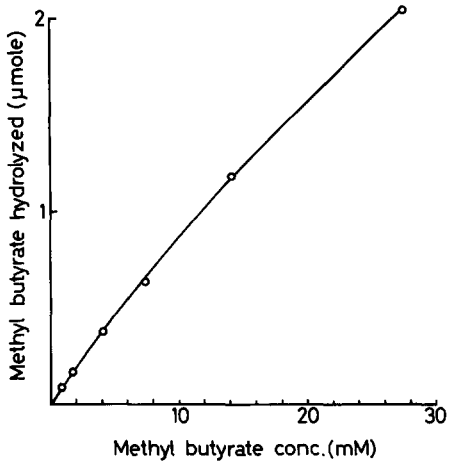

Fig. 1. Methyl butyrate hydrolysis by plasma as a function of methyl butyrate concentration in assay. Fifty μl rat plasma and 0.5 ml of 0.1 M sodium phosphate buffer pH 7.8 containing indicated concentrations of methyl[1-^{14}C]butyrate were incubated 30 min at 30°.

1.9–27.5 mM, was stable in 0.5 ml buffer volume during 30 min incubation at 30° in glass stoppered test tubes (Table 1).

Radiochemical assay method

Chloroform extracted methyl butyrate almost completely (>99%) from 0.1 M sodium phosphate buffer pH 7.8. The two phases were separated by brief centrifugation. Radiochemical assay of carboxylesterase was investigated by incubation of rat plasma with 0.5 ml vol. of buffer (0.1 M sodium phosphate pH 7.8) containing methyl[1-^{14}C]butyrate, and subsequent scintillation counting of radioactivity in aqueous phase after chloroform extraction.

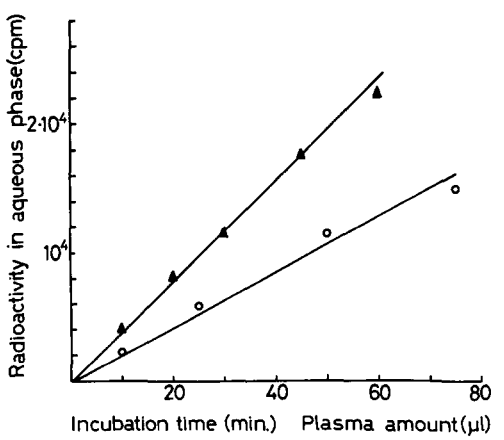

Fig. 2. Methyl butyrate hydrolysis by plasma as a function of incubation time and plasma amount. Indicated volumes (○) of rat plasma and 0.5 ml substrate (0.1 M sodium phosphate buffer pH 7.8 containing 1.9 mM methyl[1-^{14}C] butyrate, spec. act. 0.037 Ci/mole) were incubated 30 min at 30°, or 50 μl plasma and 0.5 ml substrate was incubated at 30° in the times indicated (▲).

Table 2. Relative carboxylesterase activities

Substrate	Plasma	Liver	Lung	Heart	Diaphragm	Cerebrum	Kidney	Duodenum
I. Methyl butyrate	1	1390 ± 90	421 ± 51	32 ± 3	36 ± 3	1.3 ± 0.3	25 ± 1	132 ± 15
II. 4-nitrophenylbutyrate	1	80 ± 4	25 ± 1	2.3 ± 0.1	2.7 ± 0.8	2.4 ± 0.1	16 ± 1	80 ± 9
I/II	1	17.4	16.8	13.9	13.3	0.54	1.56	1.65

The numbers are mean values ±S.E.M. of 4–12 rats.
Plasma activity ±S.E.M. with methyl butyrate = 9 ± 1 nmole/hr/mg tissue.
Plasma activity ±S.E.M. with 4-nitrophenyl butyrate = 139 ± 9 nmole/hr/mg tissue.

Effect of substrate concentration

The amount of methyl butyrate hydrolysed by 50 μl rat plasma during 30 min incubation at 30° increased with increasing substrate concentration (0.88–27.5 mM, spec. act 0.0037–0.001 Ci/mole) without reaching any saturation (Fig. 1).

Effect of incubation time and plasma amount

With 1.76 mM methyl butyrate in the assay mixture the radioactivity in aqueous phase increased proportionally with incubation time (0–60 min) and with the amount of rat plasma used (0–75 μl) (Fig. 2).

Carboxylesterase activities in different tissues

Several different tissues were examined for the ability to hydrolyse methyl butyrate and 4-nitrophenyl butyrate. The results showed that cerebrum, kidney and duodenum showed similar relative activities towards the two substrates as plasma, whereas liver, lung, heart and diaphragm were much more active towards methyl butyrate (Table 2). The arylesterase inhibitor EDTA [15, 16] did not influence the activities of the tissues, except for duodenum with methyl butyrate as the substrate (Table 3). The cholinesterase inhibitor physostigmine [17] strongly inhibited the duodenum activity with both substrates, and also inhibited the hydrolysis of methyl butyrate by kidney (Table 3). Bis-4-nitrophenyl phosphate, a carboxylesterase inhibitor [18], arrested the hydrolysis of methyl butyrate to a greater extent than the hydrolysis of 4-nitrophenyl butyrate by several tissues (Table 3).

Comparison of esterase activities in female and male rats

The hydrolysis of methyl butyrate, 4-nitrophenyl butyrate and acetylcholine by male and female rat tissues were compared. The results showed that both methyl butyrate and acetylcholine were hydrolysed to a higher extent by female plasma than male plasma, whereas no difference was observed in hydrolysis of 4-nitrophenyl butyrate (Table 4). Lung and liver activities were similar in male and female with both carboxylesterase substrates (Table 4).

Effect of phenobarbital treatment

The hydrolysis of methyl butyrate and 4-nitrophenyl butyrate by liver tissue was increased to a similar extent after phenobarbital treatment of rats (Table 5).

Effect of soman

The ability of plasma to hydrolyse methyl butyrate and 4-nitrophenyl butyrate was inhibited about 40% and 60% respectively 1 hr after soman injection (0.5 LD_{50} s.c.), and within 24 hr only the former activity was completely recovered (Table 6). The ability of lung to hydrolyse the two substrates was not inhibited, and no sex difference was observed with respect to the effect of soman treatment (Table 6). Incubation of plasma, liver and lung tissue with soman *in vitro* led to similar extent of inhibition of methyl butyrate and 4-nitrophenyl butyrate hydrolysis (Table 7).

Table 3. Effect of selective esterase inhibitors on carboxylesterase activities in different tissues

Substrate	Inhibitor (conc.)	Carboxylesterase activity (% of control) ± S.E.M. (N = 3–7)							
		Liver	Plasma	Lung	Heart	Diaphragm	Cerebrum	Kidney	Duodenum
Methyl butyrate	BPNP (10^{-4} M)	3 ± 1	30 ± 2	1 ± 0.1	2 ± 0.1	5 ± 1	46 ± 2	12 ± 1	8 ± 1
	Physostigmine (10^{-5} M)	90 ± 4	90 ± 3	97 ± 3	102 ± 3	105 ± 4	92 ± 5	73 ± 2	40 ± 6
	EDTA (10^{-4} M)	95 ± 3	102 ± 3	97 ± 2	100 ± 2	103 ± 5	102 ± 3	114 ± 1	66 ± 12
4-Nitrophenylbutyrate	BPNP (10^{-4} M)	18 ± 2	38 ± 4	4 ± 0.4	32 ± 3	30 ± 5	81 ± 7	51 ± 3	9 ± 2
	Physostigmine (10^{-5} M)	83 ± 6	95 ± 5	91 ± 4	90 ± 7	88 ± 9	89 ± 4	96 ± 9	29 ± 4
	EDTA (10^{-4} M)	100 ± 7	99 ± 5	98 ± 1	96 ± 7	109 ± 4	111 ± 3	117 ± 10	96 ± 8

Inhibitors were preincubated with tissue 30 min 30°, and were also present in the assays.
Control activity (=100%) in tissue without inhibitor.

Table 4. Esterase activities in female rat

Substrate	Activity (% of male) ±S.E.M. (N = 4–5)		
	Liver	Plasma	Lung
Methyl butyrate	86 ± 18	253 ± 25	113 ± 15
4-nitrophenyl butyrate	91 ± 7	91 ± 5	94 ± 7
Acetylcholine	180 ± 22	264 ± 21	94 ± 15

Male rat activity = 100%.

Table 5. Effect of phenobarbital treatment on esterase activities in rat

Substrate	Activity (% of control) ±S.E.M. (N = 6)		
	Plasma	Lung	Liver
Methyl butyrate	114 ± 7	115 ± 22	177 ± 6
4-nitrophenylbutyrate	122 ± 5	93 ± 10	156 ± 9
Acetylcholine	104 ± 8	79 ± 5	100 ± 9

Phenobarbital (100 mg/kg i.p.) daily in 3 days, animals taken for analysis 48 hr after the last injection.
Control (100% activity) is untreated animals.

Table 6. Effect of soman treatment on esterase activities in rat

Substrate	Sex	Activity (% of control) ±S.E.M. (N = 3)			
		Plasma		Lung	
		1 hr	24 hr	1 hr	2 hr
Methyl butyrate	Male	59 ± 3	94 ± 6	109 ± 18	115 ± 8
	Female	64 ± 11	97 ± 6	123 ± 13	101 ± 9
4-nitrophenylbutyrate	Male	39 ± 5	80 ± 8	94 ± 11	97 ± 5
	Female	35 ± 5	68 ± 8	119 ± 10	106 ± 8
Acetylcholine	Male	25 ± 4	63 ± 2	19 ± 6	43 ± 4
	Female	12 ± 1	66 ± 3	11 ± 1	34 ± 7

Animals taken for analysis at the time indicated after soman injection (50 μg/kg s.c.).
Plasma control activity (=100%) by heart puncture before soman.
Lung control activity (=100%) in untreated rats.

Table 7. Effect of soman on carboxylesterase activities in different tissues

Substrate	Soman conc.	Carboxylesterase activity (% of control) ± S.E.M. (N = 3–5)		
		Plasma	Liver	Lung
Methyl butyrate	$5 \cdot 10^{-7}$ M	71 ± 4	87 ± 8	24 ± 1
	10^{-6} M	55 ± 5	61 ± 4	10 ± 2
	10^{-5} M	1	4 ± 1	1
4-nitrophenylbutyrate	$5 \cdot 10^{-7}$ M	89 ± 7	72 ± 8	21 ± 5
	10^{-6} M	74 ± 5	59 ± 10	12 ± 3
	10^{-5} M	7 ± 2	20 ± 3	4 ± 1

Soman was preincubated with tissue (plasma, or 10% (w/v) homogenates of liver and lung) 15 min 30°.
Control activity (=100%) in tissue without soman.

DISCUSSION

One objective of the work was to develop a rapid and sensitive method for carboxylesterase measurement with methyl butyrate as the substrate. Several carboxylic esters hydrolysed by carboxylesterase are also substrates for arylesterase and cholinesterase [16, 19–22]. However, butyric acid esters may be generally better substrates for carboxylesterase than acetic acid esters, whereas vice versa holds for arylesterase [16, 19, 22]. We have previously assayed carboxylesterase with Warburg technique and tributyrin as substrate [2], in accordance with other investigators [23, 24]. With several samples this technique is highly impractical, in addition tributyrin has to be used as an emulsion due to its low solubility. The most simple aliphatic butyric acid ester, methyl butyrate, is commonly used with titrimetric assay [25–28], which we also find highly time-consuming [29].

We were able to synthesize highly pure methyl-[1-^{14}C]butyrate. Based on previous experience with radiochemical enzyme methods [14, 30], we have now described a radiochemical method for carboxylesterase measurement which is rapid and reproducible (Figs 1 and 2). The sensitivity is restricted due to the volatility of methyl butyrate (Table 1), but it is far higher than that of titrimetric method which requires incubation volumes at least 10-fold higher than for the present method.

The other objective of the work was to investigate substrate specificity of the carboxylesterase in different tissues.

Isoenzymes of carboxylesterase have been shown to have different specificity towards methyl butyrate and 4-nitrophenyl acetate [31]. This might be ascribed to different alkyl groups or to different acyl groups of the two esters, although the authors only stress aromatic versus aliphatic ester [31]. We decided to compare the hydrolysis of the two butyric acid esters, the aliphatic methyl butyrate and the aromatic 4-nitrophenyl butyrate. For all tissues tested, except duodenum, there seemed to be only minor contribution by cholinesterase and aryl esterase in the hydrolysis of the two substrates (Table 3). In duodenum, a tissue with high carboxylic ester hydrolysing capacity [32] (Table 2), the enzyme may be a special type of carboxylic ester hydrolase. Thus, the activity with both substrates were highly sensitive to the cholinesterase inhibitor physostigmine, and with methyl butyrate also sensitive to the arylesterase inhibitor EDTA (Table 3). In addition, both activities were almost completely inhibited by BPNP (Table 3), which is regarded as a very specific inhibitor of carboxylesterase [18, 33].

The results indicate different carboxylesterases to be important for methyl butyrate and 4-nitrophenyl butyrate hydrolysis. Thus, the tissues seemed to separate into two main groups with respect to the relative activities towards the two substrates. Cerebrum, kidney and duodenum showed similar relative activities as plasma (Table 2). In contrast, liver, lung, heart and diaphragm showed great differences between the relative activities, 13–17× in favour of methyl butyrate (Table 2). In addition, BPNP inhibited the hydrolysis of 4-nitrophenyl butyrate to a lesser extent than the hydrolysis of methyl butyrate in several cases (Table 3). This is consistent with different isoenzymes of carboxylesterase having different affinity to BPNP [34].

That methyl butyrate and 4-nitrophenyl butyrate may involve different groups of carboxylesterase was also demonstrated from the comparison between male and female rats. It is well established that cholinesterase activity in female rat plasma (and liver) is higher than in male [35–37], and this was also found for the hydrolysis of napthyl ester by carboxylesterase [38]. We found high activity for the hydrolysis of methyl butyrate, but not 4-nitrophenyl butyrate, in plasma of females compared to males (Table 4). This is most interesting with respect to the toxicity of soman, which does not differ significantly between male and female rats (Sterri *et al.*, unpublished). Previous work in our laboratory has shown that carboxylesterase in plasma might be a major factor in modifying soman toxicity in rodents [1–6]. The present results (Table 4) therefore indicate that the carboxylesterases with highest specificity for 4-nitrophenyl butyrate may be most important in this respect.

The results also demonstrate that the hydrolysis of 4-nitrophenyl butyrate and methyl butyrate are inhibited to a similar extent by incubation of the tissues with soman *in vitro* (Table 7). In addition, the inhibition and recovery *in vivo* of the hydrolysis of both substrates by plasma were similar, but not identical, after soman treatment (Table 6). Thus, there may obviously be over-lapping specificity for the two substrates by different carboxylesterases. This was also found for methyl butyrate and 4-nitrophenyl acetate with purified isoenzymes from liver [31].

Unexpectedly, the lung activity with both substrates were not affected after the injection of 0.5 LD_{50} of soman, although soman does reach the lung based on the cholinesterase activities (Table 6). One could speculate that this may be a matter of soman affinity and/or enzyme concentration, since the lung carboxylesterase was inhibited to a lesser extent than cholinesterase also by injection of 1 LD_{50} of soman [39].

The hydrolysis of methyl butyrate and 4-nitrophenyl butyrate by liver was induced to a similar extent after phenobarbital treatment (Table 5). The increase in activity (60–70%) was similar to that for hydrolysis of naphthyl acetate after corresponding treatment [40]. In agreement, tributyrin, procaine and phenacetin hydrolysis in rat and rabbit liver also increased in parallel after phenobarbital treatment [32]. It may reflect a general increase in microsomal carboxylesterase content. The results (Table 5) differ from that found for phenobarbital induction of carboxylesterase in mice, which showed similar enhanced activities in both liver and plasma [41].

In conclusion, the results stress the importance of using more than one substrate in carboxylesterase measurement, due to both inter- and intra-tissual diversity of the enzyme.

The results show that methyl butyrate and 4-nitrophenyl butyrate may be relevant substrates to use in parallel. Also, an attractive radiochemical

method for measuring methyl butyrate hydrolysis is presented.

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