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RETINOID ACID ESTERASE ACTIVITIES: TISSUE AND SUBCELLULAR DISTRIBUTION IN MICE*

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Abstract—Methyl retinoate, ethyl retinoate and a trimethylmethoxyphenyl (TMMP) analog of ethyl retinoate are cleaved by enzymes widely distributed in tissues of mice; brain, liver and ovary contain high activity and plasma contains low activity for all three substrates. In the lung, enzymatic activity is primarily localized in the microsomal fraction, with some activity also in the soluble cytoplasmic fraction. From studies on subcellular distribution, the effect of pH, sensitivity to inhibitor and thermal stability, it appears that there are several esterases that act upon retinoid acid esters.

Retinoids (vitamin A compounds and their synthetic analogs) are potent agents for controlling differentiation and growth and for preventing the process of chemical carcinogenesis in several different epithelial tissues including lung [1–3]. However, the usefulness of natural retinoids as anticarcinogenic agents is limited by excessive toxicity [3]. Since the undesirable toxic effects may be associated with the presence of a free terminal carboxyl group, carboxyl esters of retinoic acid and an ester of a aromatic retinoic acid analog have been synthesized in an attempt to reduce toxicity of these compounds while retaining their biological activity [4]. We present in this report information on retinoid acid esterase activity in lung and other tissues of mice.

MATERIALS AND METHODS

Methyl retinoate, ethyl retinoate, and the trimethylmethoxyphenyl (TMMP)† analog of ethyl retinoate [ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate] were prepared by Hoffmann-La Roche, Nutley, New Jersey, and Basel, Switzerland, and were supplied to us by the Lung Cancer Segment of the National Cancer Institute. Diethyl *p*-nitrophenyl phosphate, glucose-6-phosphate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, and *p*-nitrophenyl-β-D-glucuronide were purchased from Sigma Chemical Company, St. Louis, Missouri.

For retinoid acid esterase, the standard assay mixture contained 0.1 ml of buffer of desired pH, 0.025 ml of 20% ethanol containing 0.025 μmole of substrate, and enzyme preparation in a total of 0.5 ml. All buffers, potassium phosphate, pH 6.6-pH 7.4; sodium barbital, pH 8.4; sodium glycinate, pH 9.2-pH 10.4, had an ionic strength of 0.2. Appropriate blanks lacking substrate or enzyme were included in the assays, all of which were performed in duplicate or triplicate. No detectable nonenzymatic hydrolysis occurred under the assay conditions. Preparations were incubated for 2 hr at 37°, and unhydrolyzed substrate was extracted by adding 2 ml of ethanol-petroleum ether (1:2, v/v). After centrifugation, the upper layer was removed and the extraction repeated with 1 ml of the same solvent. The extracts were combined and the unhydrolyzed ester measured spectrophotometrically at 370 nm. At this wavelength, the absorption due to interfering substances was minimal. In control experiments, the recovery was always greater than 98 per cent. Under these conditions, the solvent did not extract retinoid acids. *N*-Acetyl-β-D-hexosaminidase (EC 3.3.1.30), β-glucuronidase (EC 3.2.1.31) and glucose-6-phosphate (EC 3.1.3.9) activities were determined as previously described [5]; protein was measured by the Lowry method [6].

RESULTS AND DISCUSSIONS

Table 1 shows the distribution of esterase activity for methyl retinoate, ethyl retinoate and the TMMP analog of ethyl retinoate in mouse tissues. The data show that (a) for all three substances, brain, ovary and liver have high activity; (b) the TMMP analog of ethyl retinoate is hydrolyzed at faster rates than methyl retinoate and ethyl retinoate by brain, lung, spleen, and ovary enzymes; and (c) there is no large difference in the hydrolysis of methyl retinoate and ethyl retinoate by all tissues examined.

The results of esterase assays following differential centrifugation of homogenized lung tissue are shown in Fig. 1. The subcellular distribution pattern of methyl retinoate esterase, ethyl retinoate esterase and TMMP analog of ethyl retinoate esterase are similar to that of glucose-6-phosphatase, a microsomal marker enzyme, and distinct from β-glucuronidase and *N*-acetyl-β-D-hexosaminidase, which are lysosomal markers. Both the retinoid acid ester hydrolyase(s) and glucose-6-phosphatase have highest activity in the microsomal fraction; the lysosomal enzymes, β-glucuronidase and *N*-acetyl-β-D-hexosaminidase, have the highest activity in the mitochondria + lysosome fraction. The relative specific activity of the hydrolases in the soluble cytoplasmic fraction

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† Abbreviations used are: TMMP, trimethyl methoxyphenyl analog.

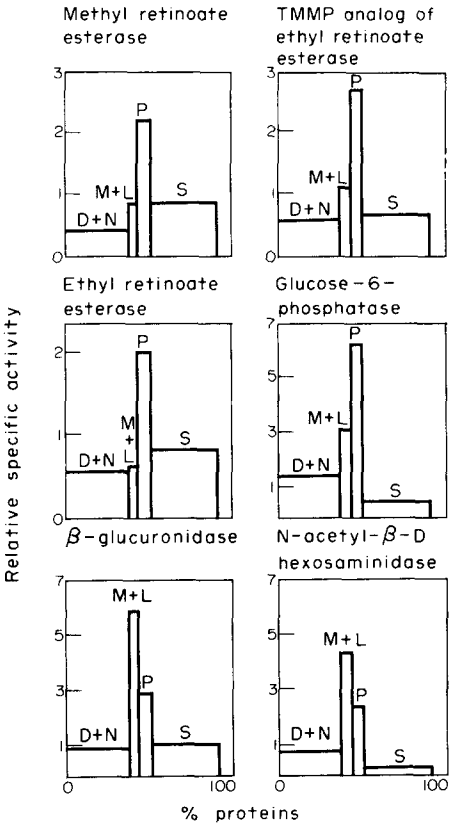

Fig. 1. Intracellular distribution of retinoid acid esterase and 3 marker enzymes in mouse lung. The tissues were homogenized in 9 vol. of 0.25 M sucrose and centrifuged at 2000 *g* for 10 min to give a nuclear fraction containing cellular debris (D + N). The resulting supernatant was centrifuged at 13000 *g* for 10 min to give a crude mitochondria-lysosome (M + L) fraction. To prepare the microsomal fraction (P), the post M + L supernatant was centrifuged at 105000 *g* for 45 min. The supernatant constituted the soluble cytoplasmic fraction. Each pellet isolated was appropriately diluted in 0.25 M sucrose so that 0.15 ml contained 0.5 mg of protein, the amount used for assay. Similar subcellular distribution patterns were observed when lower protein concentrations (i.e., 0.3 and 0.15 mg) were used. Other conditions were as described in the text. The buffer used was Na glycinate, pH 9.5, except for assay of TMMP analog of ethyl retinoate esterase, which was sodium barbital, pH 8.4. Other enzymes were assayed as described in the text. The results are presented by the method of DeDuve *et al.* [13]. Relative specific activity = percentage of total activity/percentage of total protein. On the abscissa, the fractions are represented by their relative protein content. The recovery for the enzyme activities are: methyl retinoate esterase, 87.7%; ethyl retinoate esterase, 75.2%; TMMP analog of ethyl retinoate esterase, 80.6%; glucose-6-phosphate, 123%; *N*-acetyl-β-D-hexosaminidase, 91%; β-glucuronidase, 152%; protein, 106%.

is higher than that of glucose-6-phosphate, a result suggesting the presence of an extra-microsomal esterase in this fraction.

The dependence of hydrolysis of retinoid esters on pH is shown in Fig. 2. Although the activities of methyl retinoate esterase in the microsomal and supernatant fractions show similar pH optima at pH 9.5, the soluble esterase activity for the TMMP analog of ethyl retinoate and the microsomal esterase

Table 1. Distribution of esterases for methyl retinoate, ethyl retinoate and the TMMP analog of ethyl retinoate in mouse tissues

Tissue	Percent of substrate hydrolyzed*		
	Methyl retinoate	Ethyl retinoate	TMMP analog of ethyl retinoate
Brain	15.6 ± 1.4	14.6 ± 0.8	18.0 ± 0.3
Ovary	13.9 ± 0.4	11.2 ± 1.0	17.5 ± 0.2
Liver	12.0 ± 1.6	14.4 ± 3.0	11.9 ± 1.0
Kidney	7.0 ± 2.8	8.4 ± 1.9	10.0 ± 1.5
Spleen	6.0 ± 1.2	4.3 ± 1.5	10.3 ± 2.7
Lung	5.2 ± 1.1	6.8 ± 2.7	12.1 ± 0.3
Heart	3.7 ± 1.6	4.7 ± 0.8	5.7 ± 1.1
Plasma	2.5 ± 1.9	1.7 ± 0.3	1.4 ± 1.0

* DBA/2♀ mice were killed by CO_2 suffocation and tissues were homogenized in 10 vol. of 0.25 M sucrose. Appropriate dilutions were made so that 20 μl contained 60 μg of protein, the amount used for assay. With larger amounts the enzymatic activity did not increase proportionally for all tissues. Incubations were performed as described in the text. The buffer used was phosphate, pH 7.4. Actual absorbance values for controls (incubation of substrates without enzyme) were 0.47 ± 0.02 (mean ± standard error) for methyl retinoate, 0.51 ± 0.02 for ethyl retinoate and 0.51 ± 0.02 for TMMP analog of ethyl retinoate.

activity for this substrate show distinct pH-activity profiles. The supernatant enzyme has an activity optimum at pH 8.4; but the microsomal enzyme displays a broad activity optimum at pH 9.5–10.0.

Microsomal esterase activity for the TMMP analog of ethyl retinoate was completely inhibited by 1 μM diethyl *p*-nitrophenyl phosphate (Table 2). Similar

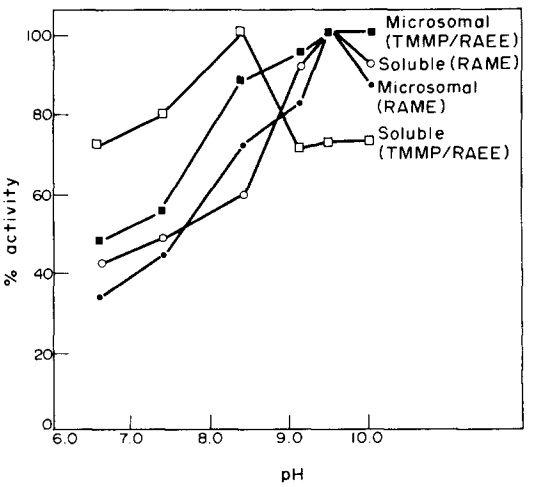

Fig. 2. Effect of pH on the hydrolysis of methyl retinoate by lung microsomal (●) and supernatant (○) esterases, and of TMMP analog of ethyl retinoate by lung microsomal (■) and supernatant (□) esterases. Tissues were homogenized in 3 vol. of 0.25 M sucrose, and microsomal and supernatant fractions were prepared by centrifugation as described in the legend to Fig. 1. Portions of 150 μl were used for assay. Other conditions were the same as those described in the text. Abbreviations used are: RAME, methyl retinoate; TMMP/RAEE, TMMP analog of ethyl retinoate.

Table 2. Effect of Diethyl *p*-nitrophenyl phosphate on microsomal and supernatant esterase activities*

Substrate	Subcellular fraction	Concentration of inhibitor (M)	% Inhibition of enzyme activity
TMMP analog of ethyl retinoate	Microsomal	1×10^{-7}	62
		1×10^{-6}	100
		1×10^{-5}	99
	Soluble cytoplasmic	1×10^{-7}	0
		1×10^{-6}	0
		1×10^{-5}	0
Methyl retinoate	Microsomal	1×10^{-7}	59
		1×10^{-6}	100
		1×10^{-5}	100
	Soluble cytoplasmic	1×10^{-7}	57
		1×10^{-6}	100
		1×10^{-5}	95

* Enzyme preparation and assay conditions were the same as for Fig. 3, except the enzymes were preincubated with buffer and inhibitor for 30 min at 0° before the addition of substrates.

results were obtained with both microsomal and supernatant methyl retinoate esterases. In contrast, the soluble esterase activity for the TMMP analog of ethyl retinoate was insensitive to the inhibitor even at a concentration of 10 μM. The microsomal and supernatant esterases hydrolyzing the TMMP analog of ethyl retinoate can also be differentiated by their thermal stability, as revealed by heating the enzymes at 52° (Fig. 3A). The microsomal enzyme loses about 25 per cent of its activity after 10 min of incubation but retains the remaining activity for up to 2 hr. In contrast, activity of the supernatant esterase initially increases about 10 per cent, but this is followed by a decrease to about 50 per cent of its original activity.

The difference in pH optima, sensitivity to inhibitor, and thermal stability demonstrate the presence in mouse lungs of different microsomal and supernatant forms of enzymes hydrolyzing the TMMP analog of ethyl retinoate. As a further indication of a possible multiplicity of esterases acting upon retinoid esters, there is also a difference in the thermal stability of microsomal and supernatant methyl retinoate esterases (Fig. 3B). The microsomal enzyme activity has a stability similar to that of microsomal enzyme hydrolyzing the TMMP analog of ethyl retinoate, losing about 30 per cent of its activity after 10 min of incubation but retaining the remaining activity for up to 2 hr. In contrast, activity of the supernatant methyl retinoate esterase initially increases about 10 per cent, stays at this level for 20 min, then gradually decreases to about 70 per cent of original activity after 2 hr of incubation.

These data indicate the presence in mouse lung of at least one microsomal and two soluble esterases utilizing retinoid esters as substrates. The main microsomal localization of retinoid acid esterase is similar to those of retinyl acetate hydrolase [7] and non-specific esterase [8] but is distinct from the nucleic and mitochondrial-lysosomal localization of retinyl palmitate hydrolase [7].

By the use of inhibitors, enzyme fractionation and a comparison of enzyme properties, Ganguly [9] concluded that liver retinyl acetate esterase, non-specific esterase, and cholesterol esterase are independent

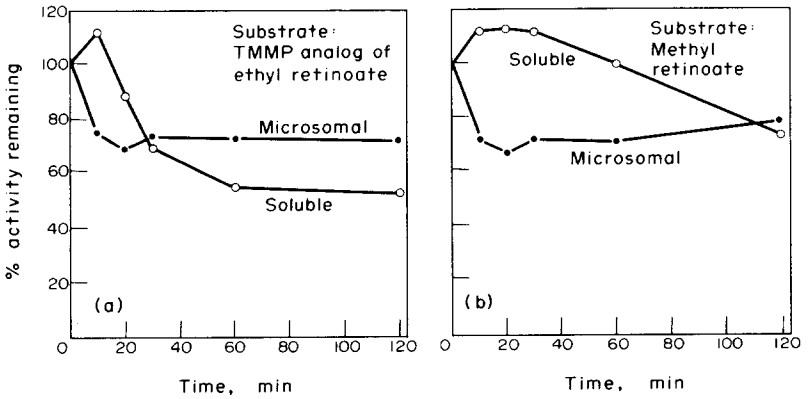

Fig. 3. (A). Heat inactivation of lung microsomal (●) and soluble (○) esterase for TMMP analog of ethyl retinoate at 52°. The enzyme fractions were prepared as described in the legend to Fig. 2. Following the heat treatment, the mixtures were cooled in ice and analyzed for esterase activity. The assay conditions were as described in the legend to Fig. 2, except that sodium barbital, pH 8.4, was used as the buffer. (B). Heat inactivation of lung microsomal (●) and soluble (○) esterase for methyl retinoate at 52°. The enzymes were assayed at pH 9.5. Other details were as for Fig. 3A.

entities. On the other hand, it was recently shown that retinyl acetate is also a substrate for a highly purified non-specific esterase from liver and kidney [10]. It is questionable, therefore, whether the distinction between retinyl acetate esterase and non-specific esterase is still justified. We do not know from the present work whether retinoid acid ester hydrolase and non-specific esterase are similar or independent enzymes. However, since the non-specific esterase is known to exist in multiple forms, it is likely that one or more of its forms participates in the hydrolysis of retinoid acid esters.

The occurrence of an unidentified, "physiological" ester of retinoic acid in all tissues of rats [11, 12] and the present finding that retinoid acid esterase activity is present in all tissues suggest that the enzymes have a role in the action of retinoic acid.

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