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# Determination of Carboxylesterase in Rat Tissues and Blood using Riboflavin-5′-monobutyrate

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Riboflavin-5′-monobutyrate (R-MB, mp 244—245°) was synthesized from riboflavin and butyric anhydride (yield: 10%) as well as from riboflavin-2′,3′,4′,5′-tetrabutyrate and hydrazine hydrate (yield: 44%). Riboflavin-5′-monopalmitate (R-MP, mp 228—230°) was also prepared from riboflavin and palmitic anhydride (yield: 9.8%). The absorption, fluorescence, and infrared spectra, and *Rf* values of R-MB and R-MP are given.

R-MP was hardly hydrolyzed by pure hog hepatic carboxylesterase. However, R-MB was readily hydrolyzed to riboflavin by pure hog hepatic carboxylesterase, though not by pure pancreatic lipase (triacylglycerol lipase), acetylcholinesterase, or cholinesterase. The activity of carboxylesterase could therefore be estimated by fluorometric determination of riboflavin hydrolyzed from R-MB. The optimum incubation conditions for the assay of carboxylesterase with R-MB were pH 7, 37°, 15 min. Studies on the rat organs and liver subcellular distributions of carboxylesterase showed that the enzyme is localized in the small intestine, kidneys, lungs, heart, and blood, and high carboxylesterase activity was demonstrated in the microsomal fraction in rat liver. R-MB was a rather specific substrate for carboxylesterase, permitting selective assay of this enzyme activity.

**Keywords**——riboflavin; riboflavin-5′-monobutyrate; riboflavin-5′-monopalmitate; periodate oxidation; carboxylesterase in rat tissues; carboxylesterase in rat blood; determination of carboxylesterase

In 1961, Yagi and Okuda (one of the authors) reported chemical syntheses of riboflavin-2′,3′,4′,5′-tetrapalmitate, -tetracaprate, -tetrabutyrate (R–TB), -tetrapropionate, and -tetraacetate.[2] Among these esters, R–TB was found to be most easily hydrolyzed to free riboflavin (FR) through various intermediates (riboflavin-tri, -di, and -monobutyrates) by a crude pancreatic lipase preparation.[3]

As it was found recently that pure hog liver carboxylesterase [EC 3.1.1.1]hydrolyzes R–TB, we hoped to obtain a fatty acid monoester of FR suitable for use as a substrate for a simple fluorescence microdetermination procedure for carboxylesterase activity in animal tissues and blood, because a fatty acid monoester of FR should be hydrolyzed solely to FR by the enzyme, and the fluorescence of FR can be estimated by fluorometry after separating it from its fatty acid monoester. For this purpose, we synthesized riboflavin-5′-monobutyrate (R–MB) and -monopalmitate (R–MP). As expected, R–MB (but not R–MP) was readily hydrolyzed to FR by a homogenate of rat tissues, blood, and pure hog liver carboxylesterase, but not by other esterases, *e.g.*, hog pancreatic lipase [EC 3.1.1.3], electric eel acetylcholinesterase [EC 3.1.1.7] or horse serum cholinesterase [EC 3.1.1.8].

Specific substrates for carboxylesterase have not previously been reported, so it is useful to obtain a specific and sensitive fluorescent substrate for carboxylesterase. In this paper, chemical syntheses and some physico-chemical properties of R–MB and R–MP are described, together with a new and specific micromethod for the determination of carboxylesterase in rat tissues and blood using R–MB.

1) Location: *Tempaku-cho, Tempaku-ku, Nagoya, 468, Japan.*
2) K. Yagi, J. Okuda, A.A. Dmitrovskii, R. Honda, and T. Matsubara, *J. Vitaminol.*, 7, 276 (1961).
3) K. Yagi, J. Okuda, S. Niwa, and Y. Yamamoto, *J. Vitaminol.*, 7, 281 (1961).

## Experimental

**Rat Tissues and Blood**——Heart, lungs, liver, pancreas, spleen, kidneys, small intestine, and blood were obtained from adult Wistar male rats weighing *ca.* 200 g (body weight).

**Materials**——FR, butyric anhydride, palmitic anhydride, hydrazine hydrate, phenyl acetate and eserine were of reagent grade. R-TB was supplied by Tokyo Tanabe Co., Ltd., and PMSF (phenylmethylsulfonyl fluoride, a potent inhibitor of carboxylesterase) was purchased from Sigma Chemical Co.

Pure hog hepatic carboxylesterase and hog pancreatic lipase (type VI) [triacylglycerol lipase] were purchased from Boehringer Mannheim GmbH and Sigma Chemical Co., respectively. Electric eel acetylcholinesterase and horse serum cholinesterase were purchased from Worthington Biochem. Corp.

**Apparatus**——Visible and ultraviolet absorption spectra of R-MB and R-MP were measured with a Hitachi 124 double-beam recording spectrophotometer. Fluorescence and infrared (IR) spectra of R-MB and R-MP were recorded with a Hitachi MPF 2A recording spectrometer and a Jasco infrared spectrophotometer (model IRA-1), respectively. A Kotaki UM-2S fluorometer was used for the determination of FR formed by hydrolysis from R-MB in this experiment.

**Methods**——For the determination of esterified fatty acids in R-MB and R-MP, the method of Entenman[4] was used. This method is based on the conversion of fatty acid esters to colored ferric salts of hydroxamic acid. To determine the position of esterified butyric and palmitic acids in the ribityl residue of R-MB and R-MP, periodate oxidations of R-MB and R-MP were carried out. The amount of periodate was determined by the method of Honda *et al.*[5]

**Esterase Determination using Phenyl Acetate**——Esterase activity in the samples was measured according to the procedure of Hashinotsume[6] using phenyl acetate as a substrate.

**Subcellular Fractionation of Rat Liver**——Subcellular fractions of livers obtained from adult Wistar male rats were prepared in 0.25 M sucrose by differential centrifugation of the homogenates according to the method of Hogeboom.[7]

**Protein Determination**——The protein concentrations in tissue extracts and various subcellular fractions were determined spectrophotometrically by the biuret method using bovine serum albumin as a standard.[8]

## Results

### A. Syntheses and Properties of R-MB and R-MP

### 1. Syntheses of R-MB and R-MP

1) **R-MB from FR**——FR (100 mg) was dissolved in 50 ml of dried pyridine, and butyric anhydride (150 μl) was added. The mixture was shaken at 37° for 14 hr. Three major and three minor yellow fluorescent substances were detected by thin layer chromatography (TLC) [on silica gel H developed with chloroform–methanol mixture (CM mixture, 82/18, v/v)] under ultraviolet irradiation. The *Rf* values of the three major substances (FR, R–MB, and R–TB) were 0.11, 0.52, and 0.91, respectively, and those of the three minor unknown substances were 0.34, 0.41, and 0.44. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in the CM mixture and washed with saturated sodium bicarbonate aqueous solution to neutralize butyric acid formed. After washing, the chloroform layer was dried with anhydrous sodium sulfate, then concentrated under reduced pressure. The residue obtained was subjected to TLC using the CM mixture as a developing solvent. The pure R–MB fraction (*Rf* 0.52) was extracted with the CM mixture. Pure R–MB (mp 244—245°) was obtained by recrystallization from methanol (yield: 10%).

**From R-TB**——R–MB was also obtained from R–TB by hydrolysis. R–TB (100 mg) was dissolved in 1 ml of 10% hydrazine hydrate ethanol solution. The solution was shaken at 20° for 15 hr in a dark room, then the reaction mixture was diluted with 4 ml of the CM

4) C. Entenman, "Methods in Enzymology," Vol. 3, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, 1957, p. 323.
5) S. Honda, K. Adachi, K. Kakehi, H. Yuki, and K. Takiura, *Anal. Chim. Acta*, 78, 493 (1975).
6) M. Hashinotsume, K. Higashino, T. Hada, and Y. Yamamura, *J. Biochem.*, 84, 1325 (1978).
7) G.H. Hogeboom, "Methods in Enzymology," Vol. 1, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, 1955, p. 16.
8) B. Hagiwara, "Kosokenkyuho," Vol. 1, ed. by S. Akabori, Asakura Shoten, Tokyo, 1955, p. 168.

mixture, and this solution was subjected to silica gel column chromatography (2.7×25 cm). The CM mixture was used as the mobile phase. The R–MB fraction was eluted with the same CM mixture and concentrated. R–MB was purified by recrystallization from methanol (yield: 44%).

2) **R-MP from FR**——FR (100 mg) was dissolved in 50 ml of dried pyridine, 50 ml of chloroform containing 171 mg of palmitic anhydride was added, and the mixture was refluxed in an oil bath at 120—140° for 3 hr and then concentrated under reduced pressure. R–MP in the residue was purified by TLC as described for R–MB. R–MP (*Rf* 0.65) was extracted with the CM mixture. Pure R–MP (mp 228—230°) was obtained by recrystallization from methanol (yield: 9.8%).

## 2. Structures of R-MB and R-MP

1) **R-MB**——a) Hydrolysis: When an ethanol solution (119 μg/ml) of the sample (R–MB) was mixed with an equal volume of 1 N NaOH at room temperature in the dark for 30 min, R–MB was completely hydrolyzed to FR.

b) Butyric Acid in R–MB: The amount of butyric acid in the sample was determined by the method of Entenman;[4] 81 μg of butyric acid was found in 476.1 μg of the sample. The molar ratio of FR to butyric acid was calculated to be 1.00: 1.02.

c) Periodate Consumption of R–MB: To determine the position of the esterified butyric acid in the ribityl moiety of R–MB, R–MB (29.2 μg) was incubated at 25° in the presence of an excess of periodate for 24 hr. The amount of periodate consumed was 27.8 μg, and the molar ratio of R–MB to the consumed periodate was calculated to be 1.00: 2.00. In the experiment with FR as a standard, 3 mol of periodate per 1.0 mol of FR was consumed. The oxidized product from R–MB was identical on TLC with 6,7-dimethyl-isoalloxazine-9-acetaldehyde obtained by oxidation of FR with periodate.[9]

d) Elemental Analysis: *Anal.* Calcd for $C_{21}H_{26}N_4O_7$: C, 56.48; H, 5.88; N, 12.55. Found: C, 56.99; H, 5.95; N, 12.44.

2) **R-MP**——a) Hydrolysis: R–MP was converted to FR by hydrolysis with 1 N NaOH, as in the case of R–MB.

b) Palmitic Acid in R–MP: As in the case of R–MB, the molar ratio of FR to palmitic acid was found to be 1.00: 0.98.

c) Periodate Consumption of R–MP: Periodate oxidation of R–MP was carried out as in the case of R–MB. The molar ratio of R–MP to periodate consumed was found to be 1.00: 1.90.

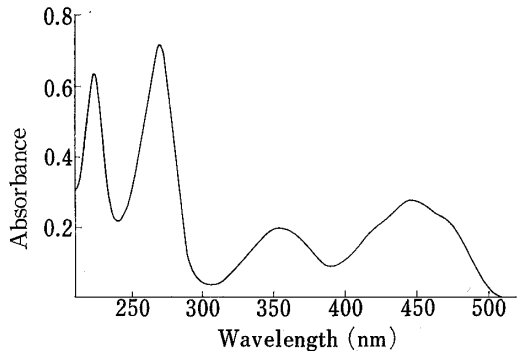


Fig. 1. Absorption Spectra of Riboflavin-5′-monobutyrate and Monopalmitate in Ethanol

Table I. Molar Absorption Coefficients ($\varepsilon$) of R–MB and R–MP

| Wavelength (nm) | R–MB | R–MP |
|---|---|---|
| 223 | 28300 | 28400 |
| 270 | 31800 | 31700 |
| 352 | 8700 | 8900 |
| 445 | 12200 | 12200 |

9) K. Yagi and Y. Matsuoka, *J. Biochem.*, **48**, 93 (1960).

d) Elemental Analysis: *Anal.* Calcd for $C_{33}H_{50}N_4O_7$: C, 64.46; H, 8.21; N, 9.11. Found: C, 64.09; H, 8.21; N, 8.87.

### 3. Physicochemical Properties of R-MB and R-MP

Both esters have an orange-yellow color, and they are soluble in methanol, ethanol, and pyridine. R–MP is insoluble in water, but R–MB is slightly soluble in water (237 μg/ml). The absorption spectrum of R–MB in ethanol is shown in Fig. 1. The spectra of R–MB and R–MP cannot easily be distinguished from each other. The maxima of R–MB are at 223, 270, 352, and 445 nm, almost the same as those of R–MP. The molar absorption coefficients of R–MB and R–MP at the four maxima are listed in Table I.

The fluorescence and excitation spectra of R–MB in ethanol are shown in Fig. 2. The maximum of the fluorescence spectrum was at 512 nm. The fluorescence and excitation spectra of R–MP were almost the same as those of R–MB.

IR spectra of R–MB and R–MP in KBr are shown in Fig. 3 (A) and (B), respectively. The absorptions of R–MB in the 1725 $cm^{-1}$ and 1170 $cm^{-1}$ regions, and the absorptions of R–MP in the 1715 $cm^{-1}$ and 1170 $cm^{-1}$ regions are attributable to the ester bonds.

The *Rf* values of R–MB and R–MP on TLC with three solvents are listed in Table II.

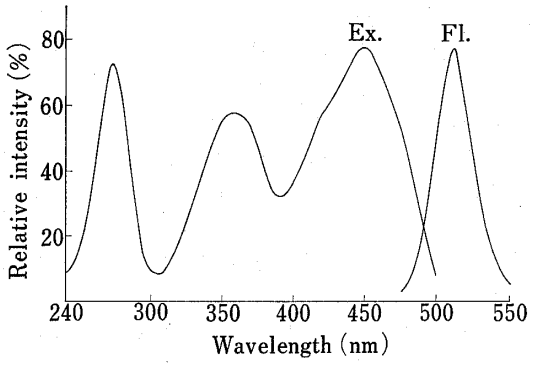


Fig. 2. Excitation and Fluorescence Spectra of Riboflavin-5′-monobutyrate and Monopalmitate in Ethanol

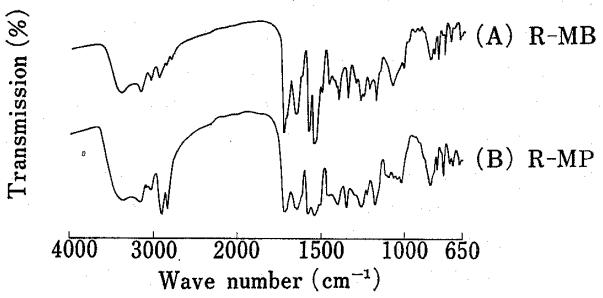


Fig. 3. IR Spectra of Riboflavin-5′-monobutyrate and Monopalmitate in KBr

Table II. The *Rf* Values of Riboflavin and Its Esters

| | Solvents | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Riboflavin | 0.11 | 0.10 | 0.45 |
| Riboflavin-5′-monobutyrate (R–MB) | 0.52 | 0.44 | 0.68 |
| Riboflavin-5′-monopalmitate (R–MP) | 0.65 | 0.54 | 0.80 |
| Riboflavin tetra-butyrate (R–TB) | 0.91 | 0.87 | 0.96 |

1: $CHCl_3$–$CH_3OH$ (82/18, v/v).
2: $CHCl_3$–$CH_3OH$–$CH_3COOH$–$H_2O$ (79/11/8/2, v/v).
3: $CHCl_3$–$CH_3OH$–$H_2O$ (100/40/6, v/v).

## B. Determination of Carboxylesterase using R-MB (R-MP)

### 1. Standard Procedure for the Determination of Carboxylesterase using R-MB (R-MP)

**Principle of the Determination**——Since carboxylesterase in animal tissues was found to hydrolyze R–MB (but not R–MP) to FR, R–MB was used as a substrate. R–MB remaining in the reaction mixture after incubation was extracted with chloroform–*n*-butanol mixture (1/1, v/v, CB mixture) and FR remained in the water layer. The fluorescence of FR thus formed was measured with a fluorometer.

**Standard Procedure**——R–MB solution (0.5 ml; 95 μg/ml in 0.1 M phosphate buffer, pH 7.4) is added to glass centrifuge tubes (14 ml) A and B and the enzyme solution (50 μl of rat serum) is added to tube A. Tubes A and B are then stoppered and incubated at 37° for 15 min. After the incubation, 2.0 ml each of water and CB mixture are added to tubes A and B, and the same volume of the enzyme solution is pipetted into tube B. Tubes A and B are centrifuged at 3500 rpm for 5 min. One ml of the upper layer is transferred into 5 ml of *n*-butanol in centrifuge tubes A′ and B′. After mixing well, tubes A′ and B′ are centrifuged again, and the fluorescence intensities of FR in the supernatants (5.0 ml) are measured.

The reproducibility of this method was 100±5.1% (average±SD) in 10 successive assays of the same sample.

**Definition of the Unit of Carboxylesterase Activity**——One unit of carboxylesterase is defined as the amount of enzyme that catalyzes the hydrolysis of R–MB to give 10 ng of FR per min under the conditions described in "Standard Procedure."

## 2. Examination of Optimum Conditions for the Determination of Carboxylesterase Activity using R-MB

To examine the optimum conditions for determination of carboxylesterase activity in animal tissues, the authors used rat serum as an enzyme solution.

1) **Optimum pH**——Activity of rat serum carboxylesterase was measured in 0.1 M phosphate buffers of various pHs. Fig. 4 shows a maximum at pH 7.6, so a pH of 7.6 was selected for the determination.

2) **Optimum Temperature**——Carboxylesterase activity of rat serum was measured at various temperatures. Fig. 5 shows that high activity was obtained over a wide range of temperature from 35—50°. In the standard determination, incubation was carried out at 37°.

3) **Determination of $K_m$ Value**——The velocities at various concentrations of R–MB were plotted according to Lineweaver and Burk. Fig. 6 shows that the apparent $K_m$ was 0.22 mM.

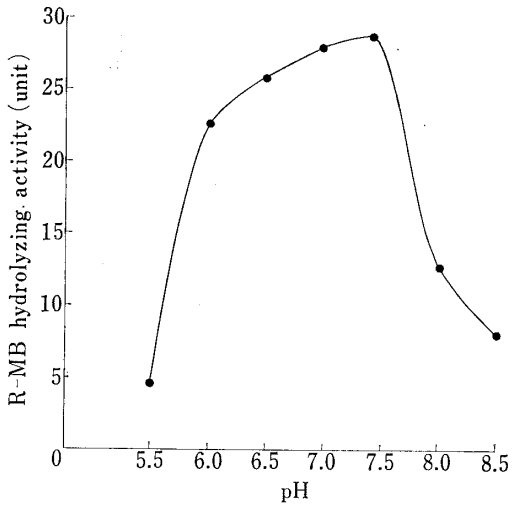


Fig. 4. Optimum pH of Carboxylesterase Activity in Rat Serum (Substrate: Riboflavin-5′-monobutyrate)

Activity was measured as described in the text.

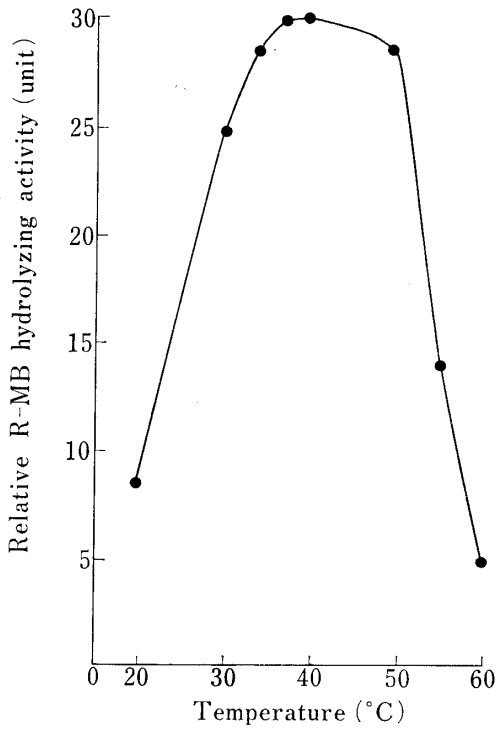


Fig. 5. Optimum Temperature of Carboxylesterase Activity in Rat Serum (Substrate: Riboflavin-5′-monobutyrate)

Activity was measured as described in the text.

4) **Volume of Rat Serum**——As shown in Fig. 7, carboxylesterase activity was proportional to the amount of rat serum in the range of 20—100 μl.

5) **Reaction Time**——Figure 8 shows that the hydrolysis of R-MB by rat serum carboxylesterase was linear with respect to the reaction time for at least 30 min.

### 3. Hydrolyses of R-MB and R-MP by Various Other Esterases

Pure hog liver carboxylesterase, electric eel acetylcholinesterase, horse serum cholinesterase, and pure hog pancreatic lipase were each dissolved in 0.02 M potassium phosphate buffer

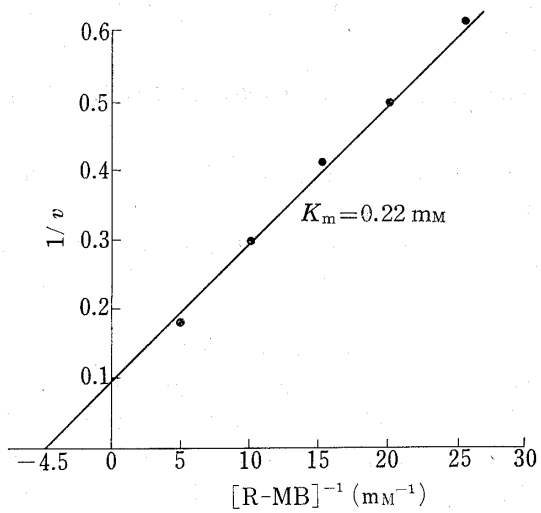


Fig. 6. $K_m$ Value of Carboxylesterase in Rat Serum (Substrate: Riboflavin-5′-monobutyrate)

Activity was measured as described in the text.

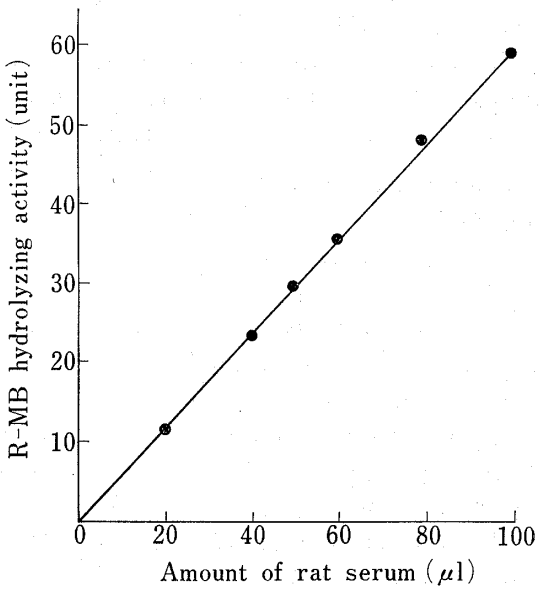


Fig. 7. Relation between the Amount of Rat Serum and the Carboxylesterase Activity (Substrate: Riboflavin-5′-monobutyrate)

Activity was measured as described in the text.

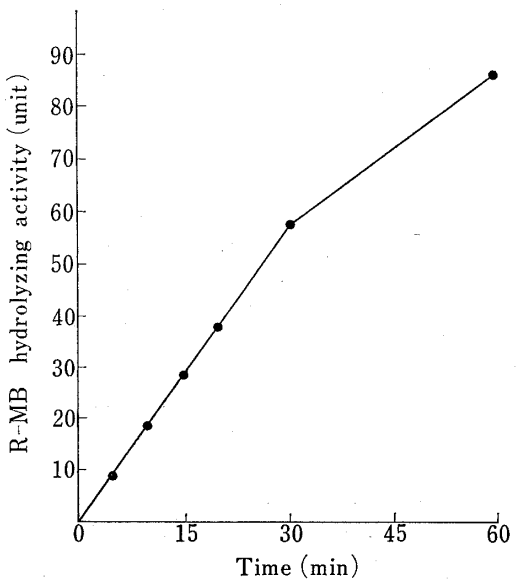


Fig. 8. Effect of Incubation Time for Carboxylesterase Activity in Rat Serum (Substrate: Riboflavin-5′-monobutyrate)

Activity was measured as described in the text.

Table III. Distribution of Carboxylesterase in Rat Organs

| Organs | Specific activity[a)] |
|---|---|
| Heart | 36.4 |
| Lungs | 52.9 |
| Pancreas | 26.9 |
| Spleen | 35.1 |
| Kidneys | 63.7 |
| Small intestine | 193.6 |
| Liver | 17.8 |
| Serum (50 μl) | 29.5 |

a) ng of FR hydrolyzed from R-MB × min$^{-1}$ × mg protein$^{-1}$.

(pH 7.0) containing 1% albumin. R–MB solution was added to each enzyme solution, and the mixtures were incubated as described above. The results showed that R–MB was readily hydrolyzed to FR by even 0.1 unit of pure hog liver carboxylesterase at 37° within 15 min, but was not hydrolyzed by either 3.0 units of acetylcholinesterase or 8.8 units of cholinesterase at 37° for 60 min. R–MB was not hydrolyzed by 99 units of pure hog pancreatic lipase at 37° for 60 min.

R–MP was hardly hydrolyzed to FR by rat serum or by any of the esterases described above.

### 4. Effects of PMSF and Eserine on the Enzyme Activity

Based on the above data, the R–MB hydrolyzing enzyme in rat blood appeared to be a carboxylesterase. The effects of PMSF and eserine (cholinesterase inhibitor) were next examined. PMSF at a final concentration of 0.1 mM completely inhibited the enzymatic activity of rat serum toward R–MB, while eserine had almost no effect at a final concentration of 0.1 mM. These findings were identical to those for pure hog liver carboxylesterase.

### 5. Distribution of Carboxylesterase Activity in Several Organs of Rats

Table III shows the distribution of the carboxylesterase activity in several organs of rats; high carboxylesterase activity was demonstrated in the small intestine, kidneys, lungs, heart, etc.

Table IV. Subcellular Distribution of Carboxylesterase in Rat Liver

| Subcellular fraction | Specific activity[a)] |
|---|---|
| Whole homogenate | 13.8 |
| Nuclei | 10.2 |
| Mitochondria | 7.0 |
| Microsomes | 35.2 |
| Sup | 13.8 |

*a*) ng of FR hydrolyzed from R–MB × $min^{-1}$ × mg $protein^{-1}$.

### 6. Subcellular Distribution of Carboxylesterase Activity in Rat Liver

Although the enzyme activity was relatively low in rat liver, the subcellular distribution of the enzyme was studied, because the liver consists mainly of parencymal cells with only small amounts of connective tissues and other cells. Fresh rat liver was homogenized and separated into nuclear, mitochondrial, microsomal, and soluble fractions by the method of Schneider and Hogeboom.[7] The enzyme activity in each fraction was determined, and the results are shown in Table IV. The highest activity was found in the microsomal fraction, and the specific activity in the microsomal fraction was 4 times that of the whole liver homogenate.

## Discussion

In 1968, Tanaka *et al.*[10] synthesized the O-isopropylidenedibutyl ester of FR, and hydrolyzed the compound to obtain R–MB. They reported elemental analysis data, melting point, and periodate consumption of R–MB, but the yield of R–MB from FR appeared to be less than 5%.

Our method for the synthesis of R–MB from R–TB with hydrazine hydrate, described above, is simpler than Tanaka's method, and our yield was 44%. As R–TB is now com-

10) T. Tanaka, H. Tanaka, and T. Tsubaki, *Bitamin*, **38**, 229 (1968).

mercially available, our synthetic method for R–MB from R–TB is convenient. The yield in a direct synthesis of R–MB from FR with butyric anhydride was low (10%), and the purification procedure by TLC is rather tedious.

R–MP was newly synthesized from FR with palmitic anhydride in a yield of 9.8%.

We studied the ratios of esterified fatty acids, the periodate consumptions, and the absorption, fluorescence, and IR spectra of R–MB and R–MP. The spectrophotometric properties of R–MB are almost the same as those of R–MP. From the above results, the structures of the synthesized R–MB and R–MP were determined to be riboflavin-5′-monobutyrate and riboflavin-5′-monopalmitate.

We also investigated whether R–MB and R–MP could serve as substrates for the determination of esterases in tissues and blood of higher animals.

In 1959, Augustinsson[11] demonstrated the presence of as many as three different types of esterases in mammalian plasma and designated them as arylesterase (aromatic esterase), aliesterase (aliphatic esterase) and cholinesterase. These results were confirmed by Holmes *et al.*[12] For the determination of these esterases, various substrates, *e.g.*, ethyl butyrate,[13,14] α-naphthyl acetate,[12,15] *p*- or *o*-nitrophenyl acetate,[16] *m*-hydroxybenzoate,[17] phenyl acetate,[6,18] and 2,3-dimercaptopropan-1-ol-tributyroate,[19] have been reported. However, these substrates are nonspecific, and phenyl acetate, for instance, is hydrolyzed by arylesterase, aliesterase, and even by cholinesterase in rat serum.[20]

As mentioned above, R–MB was hydrolyzed to FR by pure hog liver carboxylesterase, and this hydrolysis was inhibited by PMSF. However, R–MB was not hydrolyzed by pure hog pancreatic lipase, electric eel acetylcholinesterase or cholinesterase. The rat organ distribution of R–MB hydrolyzing enzyme activity (see Table III) was similar to that of carboxylesterase reported by Holmes *et al.*[12] Furthermore, the fact that the highest R–MB hydrolyzing activity was found in microsomes in rat liver (see Table IV) is consistent with the finding that carboxylesterase is also localized in microsomal fractions in rat organs.[21–24]

Recently, we partially purified carboxylesterase from rat blood using Sephadex G 200[19] and found that R–MB is specifically hydrolyzed by carboxylesterase, and not by arylesterase or lipase.[25] It may be concluded that R–MB can be used as a rather specific substrate for carboxylesterase in tissues and blood of higher animals. We are further purifying the R–MB hydrolyzing carboxylesterase activity from animal tissues and blood, and the results will be reported in detail shortly.

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