Studies on Fatty Acid Esters of Flavins

VI. Incorporation of Riboflavin Part of Riboflavin Tetrabutyrates- $2\text{-}^{14}\text{C}$ into Flavin Nucleotides in the Organs of Rat¹

KUNIO YAGI, MASAKO YAMADA AND JUN OKUDA^{2,3}

*Institute of Biochemistry, Faculty of Medicine,
University of Nagoya, Nagoya (Post No. 466)*

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Riboflavin- $2',3',4',5'$ -tetrabutyrates- $2\text{-}^{14}\text{C}$, synthesized from riboflavin- $2\text{-}^{14}\text{C}$, was administered to normal rats *per os* or by injection. At 72 hours after the administration, the radioactivities of FAD, FMN and riboflavin fractions extracted from the liver, kidney, heart, and small intestine were measured. The results show that the same level of specific radioactivities was found in all flavins of these organs. The specific radioactivities in the case of *per os* administration were at the same level as those in the case of injection. From these results, it was concluded that the riboflavin moiety of the butyrates administered *per os* or by injection can be incorporated into FMN and FAD of these organs.

As described in the previous papers, R-BUT (1, 2), a fat soluble riboflavin derivative, was found to have the vitamin B₂ action (3). This fact indicates the utilization of riboflavin part of R-BUT for the synthesis of flavin coenzymes, so far as the participation in the enzymatic reaction is considered to be the major part of the vitamin action. To ascertain this presumption, an incorporation of riboflavin moiety of administered R-BUT- ^{14}C into flavin nucleotides in main organs of rat was investigated, which was reported herein.

EXPERIMENTAL

Materials

1. R-BUT

Riboflavin-2- ^{14}C — It was prepared by our method (4) using *Eremothecium ashbyii* cultivated in the culture medium containing formate- ^{14}C . In 1 liter of the culture medium, 200 μCi of formate was added. Riboflavin- $2\text{-}^{14}\text{C}$ extracted was diluted with authentic riboflavin; the specific radioactivity was 5.47×10^5 cpm/mg in terms of riboflavin.

¹ Following abbreviations are used: R-BUT, riboflavin- $2',3',4',5'$ -tetrabutyrates; R-BUT- ^{14}C , riboflavin- $2',3',4',5'$ -tetrabutyrates- $2\text{-}^{14}\text{C}$.

² Present address; Department of Pharmaceutical Science, Meijo University, Showa-ku, Nagoya.

³ 八木国夫, 山田雅子, 奥田 潤.

Chemical Synthesis of R-BUT-¹⁴C from Riboflavin-2-¹⁴C—Two ml of butyric anhydride was added to 5 mg of riboflavin-2-¹⁴C, then the mixture was heated in an oil bath at 140° for 4 hours. The resultant solution was concentrated *in vacuo*. R-BUT-¹⁴C in the residue was extracted with ethyl alcohol, adsorbed on alumina column, eluted with acidic ethyl alcohol containing acetic acid (final concentration, 1%), and the eluate was evaporated under a reduced pressure. For a further purification of R-BUT-¹⁴C, the sample was dissolved in ethyl alcohol and precipitated by addition of ethyl ether (2). The specific radioactivity of R-BUT-¹⁴C was calculated to be 5.47×10^5 cpm/mg in terms of riboflavin.

2. Animals

Male rats (about 150 g) having received barley and vegetables for a week were used.

Methods

1. Administration of R-BUT-¹⁴C

Each rat was placed in an individual cage for 72 hours and only vegetables were given during the experiment.

R-BUT-¹⁴C (700 μ g, corresponding to 400 μ g of riboflavin; total radioactivity 2.19×10^5 cpm) was suspended in 0.2 ml of soybean oil and given *per os*. In the case of injection, same amount of R-BUT-¹⁴C was dissolved in 1 ml of soybean oil and injected subcutaneously into the back of rat.

2. Analysis of Flavins

The estimation of flavins of the organs of rat was carried out 72 hours after the administration of R-BUT-¹⁴C. Total amount of flavins of each organ was estimated by lumiflavin fluorescence method (5). To determine each amount of FAD, FMN and riboflavin, a method of fractional determination of flavins by paper chromatography employing *n*-butanol: acetic acid: water (4:1:5, v/v) as a solvent was used (6).

3. Estimation of Radioactivities of Flavins

For the measurement of the radioactivity of total flavins, the aqueous extract of flavins (7) was photolyzed to lumiflavin by addition of alkali (5), which was then extracted with CHCl_3 for estimation of flavin. A portion of the lumiflavin- CHCl_3 solution was concentrated and dried up on a disc and the radioactivity was measured by a Q gas flow counter. To measure the radioactivity of each flavin, the separated flavin on filter paper was extracted with water, and was treated in the same way as above.

RESULTS

1. Flavin Quantities in the Organs of Normal Rat

Prior to the administration experiment of R-BUT-¹⁴C, flavin quantities in the organs of normal rat were estimated. The results are shown in Table 1.

2. Incorporation of R-BUT-¹⁴C Administered *per os* into Flavin Nucleotides in the Organs of Rat

As described above, 700 μ g of R-BUT-¹⁴C suspended in 0.2 ml of soybean oil was administered *per os* to rats and the amounts of flavins and their radioactivities

TABLE 1
Quantities of Flavin Compounds in Organs of Normal Rat

Organs	Total flavin	FAD		FMN		Riboflavin	
	$\mu\text{g/g}$	$\mu\text{g/g}$	per cent	$\mu\text{g/g}$	per cent	$\mu\text{g/g}$	per cent
Liver	29.5	22.1	74.9	6.7	22.7	0.7	2.4
Kidney	34.9	24.1	69.0	9.9	28.4	0.9	2.6
Heart	15.9	14.3	90.0	1.5	9.4	0.1	0.6
Small intestine	4.3	3.2	74.4	1.0	23.3	0.1	2.3

TABLE 2
Quantities and Specific Radioactivities of Flavin Compounds in Organs of Rat after Administration of Riboflavin Tetrabutyrate-2- ^{14}C per os

Organs	Total flavin	Counts		Specific radioactivities
	$\mu\text{g/g}$	cpm ^a	cpm/g ^b	cpm/ μg ^c
Liver	30.3	11,920	2,397	79.1
Kidney	34.8	2,640	2,203	63.3
Heart	19.2	480	960	50.0
Small intestine	6.3	1,560	374	59.3

^a Total counts of organ.^b cpm/g of organ.^c cpm/ μg of flavin.

TABLE 3
Quantities and Specific Radioactivities of Each Flavin Compound in Organs of Rat after Administration of Riboflavin Tetrabutyrate-2- ^{14}C per os

Organs		Liver	Kidney	Heart	Small intestine
Total flavin	$\mu\text{g/g}$	30.3	34.8	19.2	6.3
FAD	$\mu\text{g/g}$	23.0	19.6	16.5	4.0
	% ^a	75.9	56.3	85.9	63.5
	cpm/ μg ^b	69.6	59.2	21.3	11.5
FMN	$\mu\text{g/g}$	7.2	14.5	2.7	2.2
	% ^a	23.8	41.7	14.1	34.9
	cpm/ μg ^b	32.9	57.2	64.7	27.9
Riboflavin	$\mu\text{g/g}$	0.1	0.7	^c	0.1
	% ^a	0.3	2.0	^c	1.6
	cpm/ μg ^b	20.0	110.0	^c	75.0

^a Per cent to the total amount of flavins.^b cpm/ μg of flavin.^c Not detectable.

in the main organs were estimated 72 hours after the administration. As shown in Table 2, the amounts of total flavin in these organs were found to be similar to those of normal rat. The total radioactivities appeared in the main organs are also summarized in Table 2. As can be seen from the table, the specific radioactivities of these organs are not largely different from each other, even though the highest radioactivity was found in the liver. The distributions of radioactivities in each FAD, FMN and riboflavin in these organs are shown in Table 3. Although the values of the specific radioactivities are different from each other, it is noted that these values are found in the same order of magnitude.

TABLE 4
Quantities and Specific Radioactivities of Flavin Compounds in Organs of Rat after Injection of Riboflavin Tetrabutyrate-2-¹⁴C

Organs	Total flavin	Counts		Specific radioactivities
	$\mu\text{g/g}$	cpm^a	cpm/g^b	$\text{cpm}/\mu\text{g}^c$
Liver	35.4	9,920	2,991	84.5
Kidney	32.5	919	1,316	40.5
Heart	19.7	339	1,133	57.5
Small intestine	5.2	1,020	363	69.8

^a Total counts of organ. ^b cpm/g of organ. ^c cpm/ μg of flavin.

TABLE 5
Quantities and Specific Radioactivities of Each Flavin Compound in Organs of Rat after Injection of Riboflavin Tetrabutyrate-2-¹⁴C

Organs		Liver	Kidney	Heart	Small intestine
Total flavin	$\mu\text{g/g}$	35.4	32.5	19.7	5.2
FAD	$\mu\text{g/g}$	26.6	23.7	16.1	3.3
	% ^a	75.1	72.9	81.7	63.5
	$\text{cpm}/\mu\text{g}^b$	82.1	40.4	42.8	61.8
FMN	$\mu\text{g/g}$	8.5	8.3	2.8	1.8
	% ^a	24.0	25.5	14.2	34.6
	$\text{cpm}/\mu\text{g}^b$	86.6	28.2	50.0	70.6
Riboflavin	$\mu\text{g/g}$	0.3	0.5	0.8	0.1
	% ^a	0.9	1.6	4.1	1.9
	$\text{cpm}/\mu\text{g}^b$	39.6	100.0	^c	100.0

^a Per cent to the total amount of flavins. ^b cpm/ μg of flavin. ^c Not detectable.

3. Incorporation of Injected R-BUT-¹⁴C into Flavin Nucleotides in the Organs of Rat

The same amount of R-BUT-¹⁴C (700 μg) dissolved in 1 ml of soybean oil was injected subcutaneously into the back of rats. Flavins and their radioactivities in the organs of rats were also determined 72 hours after the injection. As shown in Table 4, the total amounts of flavins in the main organs were at the same level as those in the normal rat. As can be seen from Tables 4 and 5, the radioactivity was found in each flavin of these organs, and the values were at the same level as in the case of the *per os* administration, though the specific radioactivity of riboflavin in the liver was considerably higher than that in the latter case.

DISCUSSION

Flavin levels in the main organs of rat 72 hours after the administration of R-BUT-¹⁴C *per os* or by injection were similar to those of normal rat. Although flavin level could be elevated for rather long time upon administration of 700 μg of R-BUT to a rat, it gradually decreases to normal level at the time such as 72 hours after the administration, as already reported (8).

The foremost result obtained in the present experiment is that all flavin

derivatives, FAD, FMN and riboflavin, in all organs tested became radioactive after the administration of R-BUT- ^{14}C . Considering the fact that riboflavin is not synthesized in animal body, the occurrence of radioactive FMN or FAD observed in the present experiment explains the hydrolysis of R-BUT- ^{14}C resulting the formation of radioactive riboflavin which could be synthesized to FMN or FAD *in vivo*. The previously reported fact that riboflavin is phosphorylated to FMN which is then synthesized to FAD in organs of rat (9) supports this view. To understand, without any contradiction, the fact that the riboflavin part of R-BUT- ^{14}C was incorporated into FMN or FAD even in the case of injection, the hydrolysis of R-BUT in tissues or organs should be presumed. The hydrolysis demonstrated by using pancreatic lipase (10) indicates intuitively the possibility of hydrolysis of the butyrate in the intestinal canal, however, at the same time, it strongly indicates the occurrence of hydrolysis of the butyrate after the absorption⁴.

Radioactive FAD or FMN thus synthesized from radioactive riboflavin part of R-BUT- ^{14}C is exchanged for FAD or FMN present in these organs (11). From the datum of specific radioactivity of FAD in the liver of rat which received R-BUT- ^{14}C by injection, the exchange rate was calculated to be 15.0 %. It means that 15 % of flavins existed in the liver before the injection was exchanged for flavins derived from riboflavin moiety of injected R-BUT. This exchange in the body may also be explained from the data of mixed excretion of ^{14}C -labeled and non-labeled riboflavin into urine and feces after the injection of R-BUT- ^{14}C which will be described in the forthcoming paper (12).

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⁴ Recent results revealed that the butyrate administered *per so* could be mostly absorbed in the form of ester and partly hydrolyzed in the intestinal canal by digestive juice as suggested by the previous paper (10). Details will appear in a forthcoming paper.