Summary

The seven compounds of N-alkanoyl-8-ethoxy-5-quinolinesulfonamide were synthesized and their *in vitro* and *in vivo* effects were tested on the Nakayama strain of Japanese B encephalitis virus. Among them, N-decanoyl-8-ethoxy-5-quinolinesufonamide was selected as the most effective. The optimum effective dose, effective time, acute toxicity, and minimum hemolytic action of this compound were examined and it was found to be more promising than PANS-610 in view of *in vitro* activity, *in vivo* effect, and effective time.

(Received December 25, 1959)

UDC 577.164.12 : 543.544

130. Satoru Kuwada, Toru Masuda, and Mitsuko Asai: Application of Chromatography. XL.*1 Riboflavin-activity of 6,7-Dimethylribolumazine in Animal Body.

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In 1955, the authors[1] discovered a green fluorescent substance in the mycelium of *Eremothecium ashbyii*, named it 6,7-dimethylribolumazine, and established its structure. Thereafter, from the assumption that the biosynthesis of riboflavin in the mycelium of the microörganism might be participated by 6,7-dimethylribolumazine, an investigation was made on the mechanism of the biosynthesis starting from 6,7-dimethylribolumazine.[2]

Studies so far made were confined to the mechanism of the biosynthesis of riboflavin in the mycelium of *Er. ashbyii*, but later, the interest was directed to the inquiry of whether 6,7-dimethylribolumazine can be converted to riboflavin in the animal body as well. The direct motive for this was the report of Katagiri, *et al.*,[3] that an enzyme present in the bovine liver, despite its inability to produce riboflavin in the normal state, showed a remarkable activity in changing 6,7-dimethylribolumazine into riboflavin.

On the other hand, Suzuoki, *et al.**3 of these Laboratories investigated the possibility of 6,7-dimethylribolumazine being converted to riboflavin in the animal body. According to their curative tests on riboflavin-deficient rats, riboflavin activity after oral administration of 815 γ (2.5 μM) per day per rat of 6,7-dimethylribolumazine (*per os*) was very faint and far less than the administration of 3.75 γ (0.01 μM) per day per rat of riboflavin. Intraperitoneal injection of 6,7-dimethylribolumazine also gave the same result. These facts led to the conclusion that 6,7-dimethylribolumazine is not transformed into riboflavin *in vivo*.

Although the animals in the above two cases are different in kind, the result obtained by Suzuoki is too different from that of Katagiri, *et al.*, and this made it necessary

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*3 J. Suzuoki, *et al.*: J. Vitaminology, **6**, 145(1960).

1) S. Kuwada, T. Masuda, *et al.*: Vitamins (Japan), **9**, 368(1955); **10**, 530(1956); This Bulletin, **4**, 71, 375(1956); **5**, 28(1957); **6**, 447(1958); **7**, 361(1959).

2) T. Masuda: This Bulletin, **5**, 136(1957); S. Kuwada, *et al.*: *Ibid.*, **6**, 618(1958); **7**, 515(1959); J. Vitaminology, **4**, 217(1958).

3) H. Katagiri, J. Takeda, K. Imai: J. Vitaminology, **4**, 285(1958).

to ascertain whether the enzyme which converted 6,7-dimethylribolumazine into riboflavin in the bovine liver is also present in the liver of rats.

A fresh bovine liver was made into a homogenate with a phosphate buffer (pH 7.0) and the product was left standing at 37° for a definite period, with or without 6,7-dimethylribolumazine. The reaction mixture, after being deproteinized with trichloroacetic acid, was subjected to paper partition chromatography, and the spot of riboflavin detected from its yellow fluorescence and its location was extracted with an aqueous solution of saccharin. Riboflavin in the extract was determined by the lumiflavin method and the increase of riboflavin due to the addition of 6,7-dimethylribolumazine was expressed by percentage. As shown in the experimental part, the increase of riboflavin was 50~60%, and this value was about the same as in the authors' duplication of the method of Katagiri, *et al.* Therefore attempt was made to apply this technique to experiments with rats.

Remarkably differing from the case of the bovine liver, however, no increase of riboflavin was observed in the liver homogenate of rats. As this result was suspected to be due to the absence of a carbon-donor necessary for the biosynthesis of riboflavin from 6,7-dimethylribolumazine, the same reaction was conducted in the presence of diacetyl or sodium acetate, but the result was the same as before. This fact indicates that in the liver of rats there is no enzyme which converts 6,7-dimethylribolumazine into riboflavin or some factor is present which prevents biosynthesis. At any rate, the final conclusion should be drawn after conducting many more experiments.

It may not be proper to regard the liver as the sole organ where riboflavin is produced from 6,7-dimethylribolumazine, but at least the result obtained by Suzuoki, *et al.* in their curative tests on riboflavin-deficient rats is in good agreement with that of the present experiment with the liver homogenate of the animal. From these results it must be concluded that the function of the liver differs according to animal species and this is clearly reflected on the biosynthesis of riboflavin in this organ.

Similar experiments were carried out on the liver of rabbits. A homogenate was prepared from a fresh liver and it was incubated with 6,7-dimethylribolumazine as usual, but though the experiment was conducted only three times, the result was almost the same as in the case of the liver homogenate of rats. In parallel with this experiment, examination was made to see whether 6,7-dimethylribolumazine administered to a rabbit is excreted in the urine in the form of riboflavin. A rabbit was fasted for 48 hours and the urine excreted during the latter 24 hours was collected as "fore-urine." The rabbit was then administered with 6,7-dimethylribolumazine by intraperitoneal injection and the urine excreted during the subsequent 24 hours was collected as "after-urine." Both kinds of urine were subjected to paper partition chromatography and the chromatograms obtained are shown in the experimental part. As seen in the chromatograms, a comparatively strong spot of riboflavin was observed in the "fore-urine" but the spot became faint in the "after-urine," and instead a fairly strong green fluorescent spot of 6,7-dimethylribolumazine which was not detected in the "fore-urine" was observed. From this result, it was assumed that the 6,7-dimethylribolumazine given in the abdominal cavity of a rabbit was excreted into the urine through the liver without undergoing any change. If this assumption is right, it will also serve to explain the fact that 6,7-dimethylribolumazine was not converted into riboflavin in the liver homogenate.

From the experimental data mentioned above, such a biosynthesis of riboflavin from 6,7-dimethylribolumazine as in the mycelium of *Er. ashbyii* seems not to occur in the liver of rats and rabbits. Although the present authors also believe that the bovine liver contains an enzyme which is able to transform 6,7-dimethylribolumazine into riboflavin, it still remains hypothetical because there has so far been no chance to conduct experiments on such a large kind of animals.

Experimental

Procedure—A portion of 13~70 g. of the fresh liver of a cattle, rat, or rabbit was homogenized for 2 min. in a homogenizer with 1.5 volumes of a phosphate buffer (pH 7.0) under cooling with ice. The resulting homogenate was divided evenly and placed in brown flasks. A definite amount of 6,7-dimethylribolumazine was added as a substrate to the homogenate in some of the flasks and left to stand at 37° for 3~24 hr. The homogenate not containing 6,7-dimethylribolumazine was also treated in the same manner to be used as a control. The reaction mixture was centrifuged and the supernatant, after being deproteinized with 0.5% its weight of trichloroacetic acid, was again centrifuged. 0.1~0.2 cc. of the supernatant thus obtained was applied in a line on a strip of filter paper of 4 cm. width and developed with EtOH-BuOH-H_2O (15:50:35). The yellow fluorescent spot of riboflavin was cut out, extracted with 5 cc. of 2% aqueous solution of saccharin at 80° for 30 min., and riboflavin in the extract was determined by the lumiflavin method as follows: A mixture of 2 cc. of 2*N* NaOH added to 2 cc. of the extract in a test tube was photodecomposed by irradiating with a 20-w fluorescent lamp from a distance of 10 cm. for 1 hr. The decomposition product was mixed with 0.5 cc. of AcOH, shaken with 8 cc. of $CHCl_3$, and $CHCl_3$ layer was dried over anhyd. Na_2SO_4. The intensity of the fluorescence of the $CHCl_3$ solution was measured by Kotaki fluorometer and the riboflavin content in the sample was calculated from the value.

Result

A) Change of 6,7-Dimethylribolumazine by Bovine Liver Homogenate—i) 13 g. of the liver was made into 25 cc. of a homogenate with 25 cc. of phosphate buffer. The product was divided in two and placed in brown flasks, 2 mg. of 6,7-dimethylribolumazine was added to one of the flasks, both flasks were incubated at 37° for 3 hr., and riboflavin in the flasks was determined as above.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 4 expts.)
Homogenate + 6,7-dimethylribolumazine	3.18 (R=0.27)
Control (homogenate alone)	1.99 (R=0.38)
Increase of riboflavin	+59.8%

R: Maximum value minus minimum value.

ii) 30 g. of the same liver was processed with 50 cc. of the phosphate buffer and 80 cc. of the homogenate obtained was then divided into four equal portions. Two mg. each of 6,7-dimethylribolumazine was added to two of them, and one pair, consisting of the homogenate containing 6,7-dimethylribolumazine and that not containing it was incubated at 37° for 6 hr., and the other pair, for 24 hr. under the same conditions. Riboflavin in them was determined as above.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 2 expts.)
a) Homogenate + 6,7-dimethylribolumazine (after 6 hr. of incubation)	5.05 (R=0.15)
Control	3.38 (R=0.13)
Increase of riboflavin	+49.4%
b) Homogenate + 6,7-dimethylribolumazine (after 24 hr. of incubation)	6.30 (R=0.30)
Control	3.99 (R=0.14)
Increase of riboflavin	+57.9%

iii) 30 g. of the liver from a different cattle was worked up with 50 cc. of the phosphate buffer and 80 cc. of the homogenate so obtained was divided into four equal portions. 2 mg. each of 6,7-dimethylribolumazine was added to two of them and all the four samples were incubated for 5 hr. Riboflavin in them was determined twice to obtain mean values.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 4 expts. on 2 samples)
Homogenate + 6,7-dimethylribolumazine	3.62 (R=0.08)
Control	2.37 (R=0.035)
Increase of riboflavin	+52.7%

iv) 50 g. of the liver of another cattle was processed together with 75 cc. of the phosphate buffer and the resulting homogenate (120 cc.) was divided into six equal portions. Two mg. each of 6,7-dimethylribolumazine was added to three of them, all the samples were incubated at 37° for 20 hr., and riboflavin in them was determined as before.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 samples)
Homogenate + 6,7-dimethylribolumazine	4.73 (R=0.43)
Control	3.72 (R=0.52)
Increase of riboflavin	+27.1%

B) Change of 6,7-Dimethylribolumazine by Rat Liver Homogenate—The homogenate was prepared from 3~5 pieces of liver.

i) 34 g. of the liver was worked up with 51 cc. of the phosphate buffer into 80 cc. of a homogenate, which was then divided into four equal portions of 20 cc. each. Two mg. each of 6,7-dimethylribolumazine was added to two of them, all the samples were incubated at 37° for 5 hr., and riboflavin in the sample was determined as usual.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 4 expts.)
Homogenate + 6,7-dimethylribolumazine	3.42 (R=0.02)
Control	3.81 (R=0.24)
Increase of riboflavin	−10.3%

ii) 38 g. of the liver was made into 80 cc. of a homogenate together with 57 cc. of the phosphate buffer and the product was divided into four equal portions. Riboflavin in the samples which have been treated as above was determined.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 4 expts.)
Homogenate + 6,7-dimethylribolumazine	3.86 (R=0.15)
Control	3.48 (R=0.26)
Increase of riboflavin	+10.8%

iii) 43.5 g. of the liver and 70 cc. of the phosphate buffer were processed into 120 cc. of a homogenate and divided into six equal portions. Two mg. each of 6,7-dimethylribolumazine was added to three of them and all the samples were incubated at 37° for 5 hr.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + 6,7-dimethylribolumazine	4.87 (R=0.27)
Control	5.06 (R=0.26)
Increase of riboflavin	−3.75%

iv) 120 cc. of a homogenate was prepared from 48 g. of the liver and 72 cc. of the phosphate buffer, and divided into six equal portions. Two mg. of 6,7-dimethylribolumazine and 4 mg. of diacetyl ($10^{-1}M$, 0.5 cc.) were added to three of them, 0.5 cc. each of the phosphate buffer was added to the remaining three samples, and all the samples were left to stand at 37° for 2 hr.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + 6,7-dimethylribolumazine + diacetyl	5.94 (R=1.05)
Control	6.12 (R=0.32)
Increase of riboflavin	−2.9%

v) 57 g. of the liver and 85 cc. of the phosphate buffer were processed into 80 cc. of a homogenate, which was divided into four parts of 20 cc. each. One cc. of AcONa solution (100 mg./50 cc. $=2.44\times10^{-2}M$) was added to each of them, 2 mg. each of 6,7-dimethylribolumazine was added to three of them, and all the samples were incubated at 37° for 5 hr.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + AcONa + 6,7-dimethylribolumazine	4.16 (R=0.26)
Control (homogenate + AcONa)	4.15 (R=0.20)
Increase of riboflavin	+0.24%

C) Change of 6,7-Dimethylribolumazine by Rabbit Liver Homogenate—i) 70 g. of the liver was processed with 105 cc. of the phosphate buffer as in the case of the liver of a cattle or rat, and 120 cc. of the resulting homogenate was divided into six equal portions. Two mg. each of 6,7-dimethylribolumazine was added to three of them and all the samples were incubated at 37° for 4 hr. Riboflavin in the samples was determined as before.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + 6,7-dimethylribolumazine	1.19 (R=0.16)
Control	1.35 (R=0.10)
Increase of riboflavin	−11.9%

ii) A homogenate was prepared from 50 g. of the liver and 75 cc. of the phosphate buffer, and 120 cc. of the product was divided into six equal portions. Two mg. each of 6,7-dimethylribolumazine was added to three of them, and all the samples were incubated at 37° for 3 hr.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + 6,7-dimethylribolumazine	0.610 (R=0.1)
Control	0.623 (R=0.125)
Increase of riboflavin	−2.1%

iii) The same experiment as in ii) was repeated.

Quantity of Riboflavin detected (γ/cc.)

	(Mean value of 3 expts.)
Homogenate + 6,7-dimethylribolumazine	0.632 (R=0.125)
Control	0.620 (R=0.100)
Increase of riboflavin	+1.93%

D) Observation on the Urine of Rabbits treated with 6,7-Dimethylribolumazine—A rabbit was fasted for 48 hr. and urine excreted during the latter 24 hr. was collected as "fore-urine." The rabbit was then administered with 2 cc. of 2.5 mg./cc. solution of 6,7-dimethylribolumazine in physiological saline by intraperitoneal injection and the urine excreted during the subsequent 24 hr. was collected as "after-urine." 0.1 cc. of both urines was applied on a strip of filter paper, 4×35 cm., and developed with a solvent of EtOH-BuOH system mentioned before, to give the chromatograms shown below.

Rf Values, Color of Fluorescence, and Fluorescence Intensity

Fore-urine	0.04	0.15	0.16		0.25	0.58
	B+	B+	Y+		Y##	V±
After-urine	0.05	0.14		0.20	0.28	0.56
	B±	Y±		G##	Y+	V±

Color of fluorescence: V—violet, Y—yellow, G—green, B—blue.

As seen in the above chromatograms, the yellow fluorescent spot of riboflavin at Rf 0.25 was fairly strong and no green fluorescent spot was observed in the "fore-urine," whereas, a yellow fluorescent spot of riboflavin at Rf 0.28 became faint in the "after-urine" and a fairly strong green fluorescent spot of 6,7-dimethylribolumazine which was not observed in the "fore-urine" appeared at Rf 0.20.

The authors are grateful to Dr. Jiro Suzuoki, Mr. Hajime Yokotani, and Mr. Koji Furuno for their kind advice and help in the treatment of animals.

Summary

There is a remarkable contradiction between the report of Katagiri, *et al.*, that the liver of the cattle contains an enzyme which converts 6,7-dimethylribolumazine into riboflavin, and that of Suzuoki, *et al.* that the former was nearly ineffective in their curative tests on the riboflavin-deficient rats.

The present authors duplicated the method of Katagiri, *et al.*, and confirmed that a phosphate buffer extract of the liver of the cattle transformed 6,7-dimethylribolumazine into riboflavin *in vitro*. The same technique was applied to the liver of rats, but no increase of riboflavin was observed. Addition of diacetyl or sodium acetate as a carbon donor also gave the same result. These data are in good agreement with the result of Suzuoki, *et al.* in their tests on the whole body of the animal.

Further the same experiments were attempted with the liver of rabbits and the result was the same as in the case of the liver of rats. On the other hand, a rabbit was fasted for a definite time and administered with 6,7-dimethylribolumazine. The urine excreted before and after the administration was collected separately and subjected to paper partition chromatography. As a result, a distinct spot of riboflavin was observed in the "fore-urine" but it became faint in the "after-urine," and a conspicuous spot of 6,7-dimethylribolumazine appeared in its stead. Although this result is merely qualitative, it is consistent with the result of the experiment on the liver homogenate of animals.

(Received December 25, 1959)