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Studies on Drug Metabolism by Use of Isotopes. XVI.[1] Species Differences in Metabolism of 1-Butyryl-4-cinnamylpiperazine Hydrochloride[2]

Shigeo Baba and Shin-ichi Morishita

Tokyo College of Pharmacy[3]

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Species differences in the metabolism of 1-butyryl-4-cinnamyl[γ-^{14}C]piperazine (I-^{14}C) hydrochloride in mice, rabbits, guinea pigs, and rats were studied by a tracer technique in an attempt to select the most appropriate species of animals for the evaluation of safety use of this drug in man. After the administration of a single dose of 20 mg/kg I-^{14}C, radioactive metabolites excreted in urine were analyzed. The excretion of I-^{14}C and its metabolites in mice and rabbits was mostly in the urine (69.3%, 89.6%), while in rats and guinea pigs approximately a half of the total radioactivity appeared in the feces (57.1%, 41.7%).

In these experimental animals, the main metabolic route of compound I was through *p*-hydroxylation, but the ratio of the *p*-hydroxylated metabolites to the total metabolites identified was differed considerably among these species (mice 95.4%, rabbits 97.7%, guinea pigs 68.7%, rats 74.2%). In guinea pigs and rats, 5—12% (percentage of the ^{14}C-activity in the urine) of unchanged I-^{14}C was detected in the urine, but in mice and rabbits, the amount of the unchanged compound was rather small (0.2—0.9%). The amount of benzoic acid, hippuric acid and their hydroxylated metabolites varied and were found to be 1—17% in guinea pigs and rats. In rabbits, a larger part of metabolites of I-^{14}C were excreted as its conjugated form.

An important aspect in evaluating the safety use of new drugs is to collect information on their metabolic fate in man. In this regard, one of the difficulties encountered is that administration of radioactive isotopes to man is regulated by the recommendations of the International Commission on Radiological Protection. Therefore, the drug metabolism in man is usually predicted from the data obtained from experimental animals. It is, however, not easy to select the most appropriate species of animals for this purpose because of the fact that wide species difference in the metabolism of drugs is usually the case.[4] In recent years, there have been several reviews[5] and articles[6] dealing with studies of metabolism in man by use of a stable isotope tracer technique. However, many problems still remain to be solved in this technique. In future, both the stable and radioactive isotope tracer techniques will be complementally used for the studies on drug metabolism. A comparison of the metabolic fate of a drug between experimental animals and man will serve as a good index on selecting the most suitable species of experimental animals. The animals thus selected could also be a good substitute for man in carrying out tests for toxicity, malformation, and so on.

It has been shown that 1-butyryl-4-cinnamylpiperazine (I) hydrochloride synthesized by Irikura *et al.*[7] possesses an analgesic activity.[8] Furthermore, the metabolism of I in rat

1) Part XV: K. Kawai and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **23**, 920 (1975).
2) This work was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
3) Location: *20–1, Kitashinjuku 3-Chome, Shinjuku-ku, Tokyo, 160, Japan.*
4) H.B. Hucker, *Ann. Rev. Pharmacol.*, **10**, 99 (1970).
5) S. Baba, *Rinsho Yakuri*, **4**, 279 (1973); H. Miyazaki, M. Ishibashi, and H. Takayama, *ibid.*, **4**, 288 (1973); K. Hasegawa, *ibid.*, **4**, 310 (1973); M. Iio, *ibid.*, **4**, 318 (1973).
6) S. Baba, K. Kawai, M. Horie, and Y. Shida, *Yakugaku Zasshi*, **92**, 1569 (1972); P.O. Klein and S.V. Peterson (eds.), "Proceedings of the First International Conference on Stable Isotopes in Chemistry, Biology, and Medicine," Argonne, Illinois., May, 1973, pp. 291—359.
7) T. Irikura, K. Masuzawa, K. Nishino, M. Kitagawa, H. Uchida, N. Ichinoseki, and M. Ito, *J. Med. Chem.*, **11**, 801 (1968).
8) T. Irikura, K. Nishino, N. Ito, M. Ito, and H. Ohkubo, *Japan. J. Pharmacol.*, **20**, 287 (1970).

has been studied by using gas chromatography[9] and by radioactive isotope tracer technique.[10] Before comparing the metabolic fate of I in man with that in experimental animals, a model experiment was designed to clarify the species difference in the metabolism of this drug among various experimental animals. The present study is concerned with the species difference in the metabolism of I among experimental animals such as mice, guinea pigs, and rabbits.

Experimental

Labeled Compound——1-Butyryl-4-cinnamyl[γ-^{14}C]piperazine (I-^{14}C) hydrochloride (3.73 μCi/mg) was prepared according to a method reported from this laboratory.[10]

Nonlabeled Compounds——1-Butyryl-4-(4′-hydroxycinnamyl)piperazine (II) hydrochloride,[9] 1-cinnamylpiperazine (III) dihydrochloride,[7] 1-(4′-hydroxycinnamyl)piperazine (IV) dihydrochloride monohydrate,[9] and *p*-hydroxyhippuric acid (VIII)[11] were synthesized in this laboratory. Benzoic acid (V), *p*-hydroxybenzoic acid (VI), hippuric acid (VIII), and cinnamic acid (IX), which are all guaranteed grade, were purchased from Tokyo Kasei Kogyo Co.

Animal Care——Nine dd male mice (*ca.* 20 g), three Hartley male guinea pigs (*ca.* 300 g), and three white male rabbits (*ca.* 2 kg) were used in one experimental group. The animals were placed individually in a suitable metabolic cage except for mice, which were divided into three groups and a group of three mice was placed in one metabolic cage. All the animals were fed with commercially available diet and tap water (only in the case of guinea pigs, 10 mg of ascorbic acid was added to 50 ml of water). After a 7 day-control period a single dose of 20 mg/kg of I-^{14}C hydrochloride was injected subcutaneously to each animal, and the urine and feces were collected daily for 7 days.

Measurement of Radioactivity——The ^{14}C-activity was measured with a liquid scintillation counter (Aloka LSC-502) in the same manner as described in the previous paper.[10] The ^{14}C-activity in feces was measured as follows: The fecal sample was subjected to an automatic combustion system (Aloka ASC-111) and the $^{14}CO_2$ thus produced was introduced into the scintillation fluid (toluene-ethanolamine-MeOH-PPO-dimethyl POPOP) for the subsequent liquid scintillation counting. The ^{14}C-activity on thin-layer plates were scanned with a thin-layer radiochromatoscanner (Aloka TLC-2B).

Analysis of Metabolites——A portion of the 24-hr urine sample (in case of rabbits, 48-hr urine) was hydrolyzed with β-glucuronidase according to the method described in the previous paper.[10] The hydrolyzed sample and another portion of untreated urine sample were subjected to thin-layer chromatography (TLC). The spotted silica gel plates (Wakogel B-5F, Wako Pure Chemical Ind. Ltd., 0.3 mm thick, 5 cm × 20 cm) were developed with three different solvent systems; (a) BuOH–AcOH–H_2O (4: 1: 2 v/v), (b) benzene–acetone–MeOH–28% NH_4OH (100: 24: 12: 2, v/v), (c) benzene–acetone–AcOH (100: 100: 2, v/v). The possible metabolites I, II, III, IV, V, VI, VII, VIII, and IX in the urine and hydrolyzed urine samples were quantitatively determined by the inverse isotope dilution analysis according to a method described previously.[10] The chemical structures of these compounds in question are given in Fig. 1.

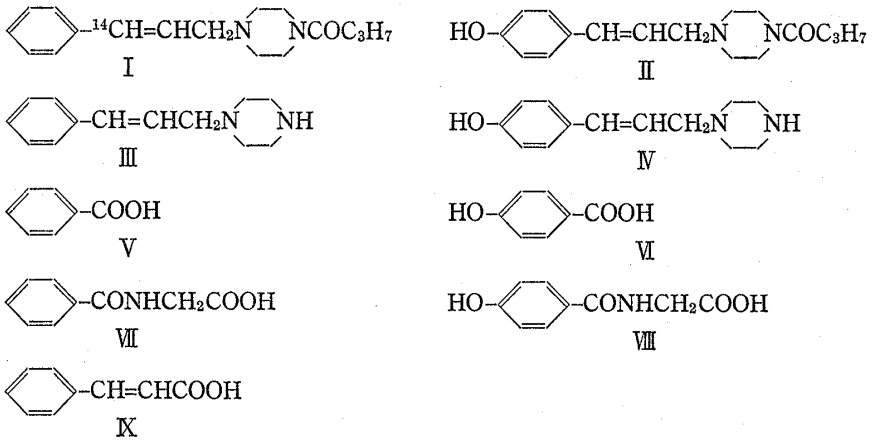

Fig. 1. Chemical Structures of 1-Butyryl-4-cinnamyl[γ-^{14}C]piperazine and Its Possible Metabolites

9) H. Terayama, T. Naruke, S. Kasai, M. Numata, H. Nakayama, T. Saito, and T. Irikura, *Chem. Pharm. Bull.* (Tokyo), **21**, 12 (1973).
10) T. Irikura, Y. Nagatsu, S. Baba, and H. Sone, *Yakugaku Zasshi*, **94**, 802 (1974).
11) E. Fisher, *Chem. Ber.*, **41**, 2875 (1908).

Results and Discussion

In general, species difference in the drug metabolism *in vivo* is caused not only by the difference in "drug-metabolizing" enzyme activity, but also by the difference in the excretion route and in the excretion rate of a drug and its metabolites. It has been demonstrated that the metabolic fate of *l*-ephedrine differed markedly among the species of experimental animals used.[12] Compound I has a phenyl group as in *l*-ephedrine and a nitrogen atom in a form different from *l*-ephedrine. In this regard, it would be interesting to compare the species difference in the metabolism of these two compounds.

Excretion in Urine and Feces

The excretion of radioactivity at every 24 hr for 7 days after subcutaneous injection of I-^{14}C to mice, rabbits, guinea pigs, and rats are shown in Table I. The data obviously demonstrate the species difference with respect to the distribution of radioactivity in urine and feces. Mice and rabbits excreted 70—90% of the total radioactivity in the urine, while in rats and guinea pigs 30—55% of the administered radioactivity appeared in the urine. In the case of ^{14}C-labelled *l*-ephedrine, only a slight difference among these species was observed in the excretion of the radioactivity in feces.[12] The observation that more radioactivity was detected in feces of rats and guinea pigs than in those of mice and rabbits is similar to the excretion patterns of SQ 11 290-^{14}C (4-[3-(7-chloro-5,11-dihydrodibenz[b,e][1,4]oxazepin-5-yl)propyl]-α,β-$^{14}C_2$-1-piperazineethanol dihydrochloride)[13] and Bromohexine[14] in the urine and feces.

Table I. Excretion of ^{14}C-Activity by Various Animals after 1-Butyryl-4-cinnamyl[γ-^{14}C]-piperazine Administration

Day	Recovered radioactivity (%) in each day				Recovered radioactivity (%) in each day			
	Mice		Rabbits		Guinea pigs		Rats	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
1	66.33±6.31	13.51±0.72	67.63±12.33	5.24±1.81	49.97±5.77	25.42±6.42	26.48±0.33	48.76±3.66
2	2.31±1.31	2.58±2.43	21.06±12.39	1.16±0.13	3.59±2.03	10.31±1.90	2.21±1.14	5.95±1.18
3	0.34±0.11	0.50±0.28	0.55± 0.28	0.40±0.11	0.91±0.61	2.84±1.53	0.67±0.49	1.37±0.41
4	0.13±0.07	0.16±0.11	0.17± 0.05	0.25±0.08	0.20±0.08	2.73±2.17	0.47±0.39	0.42±0.06
5	0.09±0.02	0.09±0.04	0.07± 0.01	0.13±0.03	0.07±0.02	0.28±0.15	0.19±0.15	0.24±0.06
6	0.06±0.01	0.07±0.02	0.05± 0.01	0.04±0.02	0.04±0.02	0.05±0.02	0.09±0.06	0.22±0.08
7	0.05±0.02	0.04±0.01	0.05± 0.01	0.03±0.02	0.03±0.00	0.01±0.01	0.06±0.03	0.22±0.08
Total	69.32±4.95	16.95±3.44	89.57± 0.34	7.30±1.74	54.82±3.07	41.65±0.92	30.18±1.23	57.13±3.67

The data are expressed as mean ± SD (n=3).

For the subsequent analyses, the 24 hr urine of mice and guinea pigs, and the 48 hr urine of rabbits were used, because the former and the latter excreted the majority of radioactivity within 24 and 48 hr, respectively.

Metabolites in Urine

In Fig. 2 are shown TLC data of the untreated and enzymatically hydrolyzed urine samples after developing with the solvent system (a).

Each peak was numerically named, according to its *Rf* value, *i.e.*, peak-1, peak-2, peak-3, and peak-4. Peak-1 which had the lowest *Rf* value disappeared after the treatment of the

12) S. Baba, K. Enogaki, T. Matsuda, and Y. Nagase, *Yakugaku Zasshi*, **92**, 1270 (1972).
13) J. Dreyfuss, J.M. Shekosky, J.J. Ross, Jr., and E.C. Schreiber, *Toxicol. Appl. Pharmacol.*, **22**, 105 (1972).
14) Z. Kopitar, R. Jauch, R. Hankwitz, and H. Pelzer, *Eur. J. Pharmacol.*, **21**, 6 (1973).

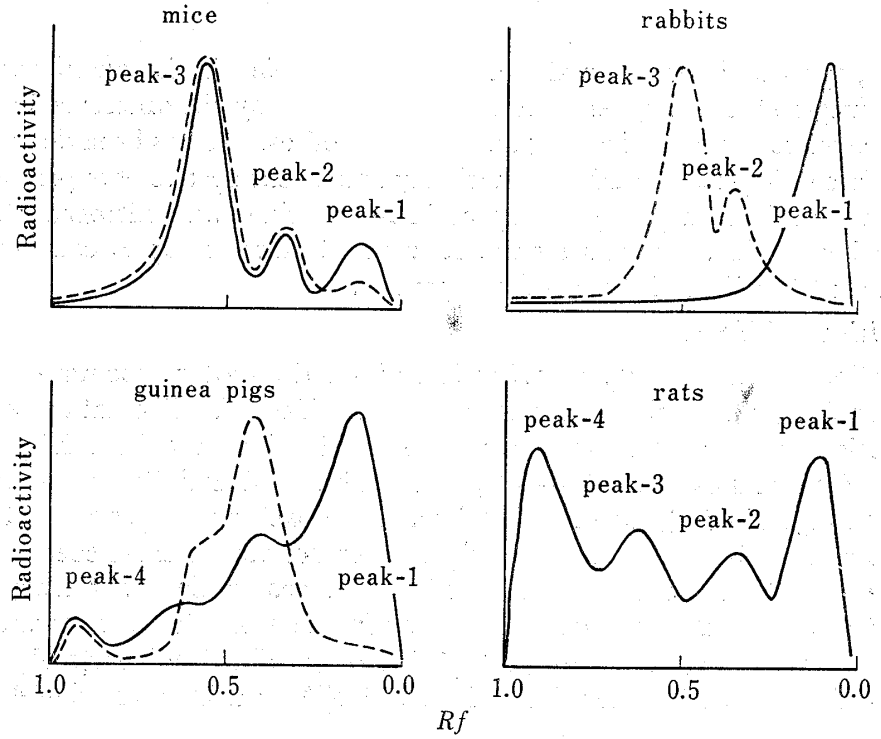

Fig. 2. Radiochromatograms of Urine of 1-Butyryl-4-cinnamyl[γ-^{14}C]-piperazine-Administered Animals

Developing solvent (a) BuOH–AcOH–H_2O (4: 1: 2, v/v)
——: before treatment with β-glucuronidase
----: after treatment with β-glucuronidase

urine sample with β-glucuronidase, and only peak-2 and peak-3 were observed still after that processing, which indicated the presence of glucuronides. The zone corresponding to each peak was scraped off, eluted with methanol, and the eluate was respotted on a thin-layer plate, and the plate was developed with solvent systems (b) and (c). The *Rf* value of each eluate was compared with the reference compound. Chromatographic behaviors of peak-2 and peak-3 were similar to those of references III and IV, and I and II, respectively. Peak-4 corresponded to the references V, VI, VII, VIII, or IX. In guinea pigs, a peak of which *Rf* value was close to that of peak-2 did not correspond to any of these reference compounds.

The results shown in Fig. 1 also indicate that these animal species differed significantly in the metabolic patterns of compound I in the urine. Moreover, it is especially interesting to note that the metabolites in rabbits were mostly excreted as a conjugated form in the urine, because conjugates generally tend to be excreted into feces *via* the bile. In the case of Bromohexine, rabbits also excreted its metabolites in large amount in the conjugated form.[14]

Quantitative determination of the metabolites of compound I-^{14}C in the urine of various species was carried out by the inverse isotope dilution analysis. The results are given in Table II. Although mice and guinea pigs gave several unidentified urinary metabolites, it would still be interesting to compare the metabolic patterns among these animal species. Table III shows, on the basis of radioactivity of metabolites, the percentage of each metabolite to the total amount of identified metabolites in various animal species. In Table IV, the metabolites were classified into the following three groups; group A, metabolites carrying a butyryl group; group B, metabolites lacking a piperazine ring; and group C, hydroxylated metabolites. In rats, the figures given in Table III and IV were calculated on the basis of combining urinary and biliary metabolites by taking account of the difference in excretion ratio between urine and bile, because in rats a greater extent of the total radioactivity was excreted in feces than in urine and fecal and biliary metabolites of rats were quantitatively and qualitatively similar.[10]

As can be seen from Table II and III, the major metabolite in all the animals used here was compound II, but its amount varied widely from species to species. The next major metabolite was compound IV, although in the urine of rats its amount was smaller than metabolite V. Significant amounts of metabolites V, VI, VII, and VIII were detected in guinea pigs and rats urine, while in mice and rabbits urine the amounts of these metabolites were negligible.

From Table III, it became apparent that mice and rabbits excreted more metabolite II than guinea pigs and rats. Mice and rabbits excreted less parent compound I than guinea

Table II. Metabolites of 1-Butyryl-4-cinnamyl[γ-^{14}C]piperazine in the Urine of Various Animals and the Bile of Rats

Metabolite[a]	Mice[b]	Rabbits[c]	Guinea pigs[b]	Rats[d]	
				Urine[b]	Bile[b]
I	0.9±0.2(0.4)	0.2±0.1(0.2)	4.3±0.3(1.9)	12.0	4.6
II	42.1±2.5(12.7)	70.3±4.6(70.3)	18.9±1.2(6.3)	21.7(12.0)	39.6(33.7)
III	0.3±0.1(0.1)	0.4±0.1(0.4)	1.0±0.1(0.6)	1.1	1.1
IV	5.7±1.4(2.5)	13.2±3.7(13.2)	7.4±1.6(3.3)	10.5(6.7)	19.9(13.8)
V	0.6±0.2(0.3)	0.2±0.1(0.2)	5.4±0.8	14.1	0.1
VI	1.8±1.0(1.0)	0.4±0.1(0.4)	1.1±0.2	4.8(4.8)	1.2(1.2)
VII	0.3±0.2(0.1)	0.9±0.4(0.9)	1.8±1.5(1.2)	16.7	—
VIII	0.3±0.0(0.1)	0.7±0.1(0.7)	1.3±0.7(0.5)	2.4(2.4)	1.5(1.5)
IX	0.3±0.1(0.1)	0.4±0.2(0.4)	0.6±0.2(0.2)	—	—
Total	52.3±2.9(17.3)	86.6±1.4(86.6)	41.7±4.9(13.7)	83.3(25.9)	68.5(50.2)
UK-A	11.8±2.1(2.9)				
UK-B	24.3±1.6(6.6)				
^{14}C-Activity in urine or bile	66.3[e]	88.7[e]	50.0[e]	26.5[e]	51.0[e]

The data are expressed as mean ± SD (n=3).
Figures in parentheses indicate the percentage of ^{14}C-activity in the form of glucuronides.
a) I: 1-butyryl-4-cinnamylpiperazine,
II: 1-butyryl-4-(4′-hydroxycinnamyl)piperazine,
III: 1-cinnamylpiperazine,
IV: 1-(4′-hydroxycinnamyl)piperazine,
V: benzoic acid,
VI: *p*-hydroxybenzoic acid,
VII: hippuric acid,
VIII: *p*-hydroxyhippuric acid,
IX: cinnamic acid,
UK-A: unidentified metabolites in peak-2,
UK-B: unidentified metabolites in peak-3
b) percentage of the ^{14}C-activity in the 24 hr urine or bile
c) percentage of the ^{14}C-activity in the 48 hr urine
d) quoted from the previous paper[10]
e) percentage of administered ^{14}C-activity

Table III. Species Differences in the Metabolism of 1-Butyryl-4-cinnamyl[γ-^{14}C]piperazine

Metabolite	Mice[a]	Rabbits[a]	Guinea pigs[a]	Rats[b]
I	1.7	0.2	10.3	9.7
II	80.5	81.2	45.3	45.8
III	0.6	0.5	2.4	1.5
IV	10.9	15.2	17.7	22.4
V	1.1	0.2	12.9	6.7
VI	3.4	0.5	2.6	3.3
VII	0.6	1.0	4.3	7.8
VIII	0.6	0.8	3.1	2.5
IX	0.6	0.5	1.4	—

a) Percentage of the metabolite to the total metabolites identified in the 24 hr or 48 hr urine.
b) Percentage of the metabolite to the total metabolites identified in the 24 hr urine and bile.

Table IV. Percentage of Various Groups of Metabolites

Group of metabolites	Mice[a)]	Rabbits[a)]	Guinea pigs[a)]	Rats[a)]
Group A	82.2	81.4	55.6	55.5
Group B	6.3	3.0	24.3	20.3
Group C	95.4	97.7	68.7	74.2

a) Percentage of the metabolites in the following group to the total metabolites identified.
group A: metabolites carrying a butyryl group
group B: metabolites lacking a piperazine ring
group C: hydroxylated metabolites

pigs and rats. A similar observation was reported in a metabolic study of *l* ephedrine,[4] that is, the parent compound was excreted unchanged in guinea pigs and rats in a greater quantity than in rabbits. On the other hand, mice excreted mainly unchanged *l*-ephedrine. This urinary excretion of a large amount of the unchanged *l*-ephedrine in mice was explained to be due to the higher excretion rate rather than the low metabolic activity in mice.[4]

These animal species were classified into two groups according to the excretion patterns of compound I; the first group of mice and rabbits and the second guinea pigs and rats. To obtain further information on the difference in the metabolic patterns among these animal species, the difference in the amount of other metabolites than compound I in the urine were taken into consideration. As shown in Table IV, the group A metabolites accounted for nearly 80% in mice and rabbits, and approximately 56% in guinea pigs and rats. The group B metabolites were calculated to be 20—24% in guinea pigs and rats, and only 3—6% in mice and rabbits, respectively. That is, in mice and rabbits compound I was hardly metabolized through N-deacylation, while in guinea pigs and rats N-deacylation, the elimination of the piperazine ring and/or the cleavage of the side chain doublebond occurred to a larger extent than in mice and rats. The group C metabolites reached 95—98% in mice and rabbits, and 59—74% in guinea pigs and rats. These facts suggest that mice and rabbits have a higher metabolic activity for compound I than guinea pigs and rats. It is also indicated that the main metabolic pathway was *p*-hydroxylation in mice and rabbits, while guinea pigs and rats gave metabolites of I through *p*-hydroxylation as well as degradation of the side chain.

As discussed earlier compound I and *l*-ephedrine were found to behave similarly both in the excretion of radioactive metabolites in the urine and feces and in the amount of the unchanged parent compound excreted in the urine. However, the metabolic pathways of these two compounds seemed to be different. That is, the main metabolic route of *l*-ephedrine in each species is as follows; deamination in rabbits; demethylation in guinea pigs; and *p*-hydroxylation in rats. On the other hand, the main metabolic pathway of compound I in these animals was through *p*-hydroxylation. This difference seemed to have resulted from the difference in the chemical structure of these two compounds, namely in the chemical nature of their side chain. It is known that *l*-ephedrine, a *β*-phenethylamine derivative, is easily deaminated and demethylated through the microsomal oxidation. However, nitrogen atoms in the piperazine ring of compound I do not seem to be readily oxidized by the microsomal enzyme systems. In this regard, even the experimental animals which can metabolize the side chain of *l*-ephedrine seem to metabolize compound I through *p*-hydroxylation.

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