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RESISTANCE OF THE RABBIT TO METHOTREXATE: ISOLATION OF A DRUG METABOLITE WITH DECREASED CYTOTOXICITY*

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Abstract—Hematological and toxicological studies revealed a strong resistance of the rabbit to the folic acid antagonist methotrexate. Investigation of its metabolism led to the isolation and purification of a drug metabolite from urine and liver incubation samples, based on solubility characteristics of the compound. The metabolite was characterized by u.v. absorption spectrum, pattern of crystallization, and paper chromatography in phosphate buffers. Elementary analysis was compatible with the 4,7-dihydroxy derivative of methotrexate. Toxicity studies in mice indicated that the metabolite is a product of detoxification with reduced but apparently not fully obliterated antifolic acid activity.

RABBITS, compared with dogs, rats, mice, monkeys, and man, are relatively resistant to the toxic effects of aminopterin.[1, 2] The decreased susceptibility of the rabbit extends also to other chemically related members of the group of folic acid antagonists, since the authors were unable to produce significant hematological and toxicological changes in response to injections of methotrexate.[3] The presence of an enzyme system catalyzing the oxidation of various halogenated analogues of methotrexate and aminopterin was demonstrated with liver tissue of rabbits, rats, mice, rhesus monkeys, and guinea pigs by Loo and Adamson.[4] Johns *et al.*,[5, 6] using a partially purified enzyme fraction from rabbit liver, showed that the nonhalogenated parent compounds methotrexate and aminopterin are likewise oxidized by the same enzyme system. It can therefore be assumed that differences in susceptibility to these agents are most likely related to species-specific variations in the rate of their metabolic degradation; however, nutritional characteristics of the animals as well as variations in distribution or excretion of the antimetabolites would have to be considered.

The presented publication provides data of a toxicological study attesting to the high degree of resistance of the rabbit to methotrexate. It describes the isolation, purification, and characterization of a metabolite of methotrexate obtained from rabbit urine and incubation samples of the drug with liver tissue. The reduced susceptibility to the drug is explained as a manifestation of its rapid metabolic degradation and excretion.

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METHODS

Experimental animals were male white New Zealand rabbits (1·2–2·8 kg) and male CF_1 mice (19–23 g); all received commercial laboratory diets.

*Hematological and toxicological effects of methotrexate**

Eighteen rabbits were used for the hematological study. Three groups of four animals each received i.m. injections of methotrexate: 0·5 mg/kg, 1·0 mg/kg, and 4·0 mg/kg respectively; two rabbits received 25 mg/kg; four served as controls with saline injections. The injection of the first three groups and the control group were given daily during the first week, then reduced to three injections per week and continued for a period of seven weeks. The highest dose (25 mg/kg) was administered daily on six consecutive days of each week for a period of two weeks in one animal and three weeks in the other. Venous blood was obtained from the dorsal ear vein at weekly intervals for peripheral blood counts, measurement of hematocrits, and smears. After completion of the treatment period these studies were combined with an analysis of the bone marrow which was aspirated from the tibia.

Metabolism of methotrexate in vivo

The rabbits were placed in metabolism cages. Food and water were allowed *ad libitum*. Methotrexate was administered by intramuscular or subcutaneous injection, and urine was collected over a period not exceeding 26 hr. The individual portions were frozen immediately after voiding. The first portion served usually for the isolation of the metabolite; it was voided generally 8–18 hr after the injection.

Metabolism of methotrexate in vitro

The rabbits were exsanguinated by heart puncture and killed by injection of air into an ear vein. The liver was rapidly removed, cooled in ice-cold saline, and homogenized in a Potter homogenizer with Teflon pestle in three volumes of precooled phosphate buffer (pH 7·8, 0·1 M). Methotrexate was added to give a final concentration of 0·5 mg/ml, and the samples were incubated for a period of 2–3 hr at 37° in the Dubnoff shaker, with air as the gas phase.

For the establishment of the acute LD_{50} of the metabolite, mice were injected intraperitoneally with a solution of the agent in 1·3% sodium bicarbonate.

Ultraviolet absorption spectra were obtained on a recording Beckman DB spectrophotometer.

Chromatography of the metabolite and methotrexate was performed by the ascending technique on Whatman 3 MM paper. Phosphate buffers (0·1 M) with a pH range from 5·3 to 9·0 served as solvents.

RESULTS

Hematological and toxicological studies with methotrexate

Hematological data of rabbits receiving repeated injections of methotrexate over periods from 2–7 weeks are summarized in Table 1. With exception of the red cells, which were at the upper range of normal, pretreatment levels of the other peripheral blood elements corresponded well with those reported in the literature. Normal ranges may be characterized as follows: erythrocytes 4·0–6·9 million, leukocytes 4,000–12,000, reticulocytes 0·5–7 per cent, hematocrit 29–50 per cent.[7, 8]

A moderate but progressive rise in white cell counts occurred in groups A, B, and

* Supplied by Lederle Laboratories, Pearl River, N.Y. (Dr. J. M. Ruegsegger).

TABLE 1. HEMATOLOGICAL DATA OF RABBITS RECEIVING INJECTIONS OF METHOTREXATE

		No. animals			Pretreatment levels	Treatment period (weeks) 1	2	3	4	5	6	7	8
A	Controls (saline i.m.)	4	erythrocytes (mil. per mm³)	mean	6·72	6·17	7·30	5·80	5·59	5·67	6·33	6·48	5·93
				range	60·7–7·69	5·29–8·34	5·23–10·15	5·21–6·12	4·69–6·21	5·28–6·77	5·58–7·15	5·65–7·48	5·04–6·55
			hematocrit	mean	34%	35·6%	36·5%	34%	34·3%	35%	35·9%	39%	34·8%
			leukocytes (per mm³)	mean	7250	8375	9975	8175	9500	9450	9925	9950	10,550
				range	6350–10,250	6600–9900	7500–11,300	7800–8500	8100–11,700	5800–12,400	8,700–11,200	7700–11,600	9500–11,800
B	Methotrexate (0·5 mg/kg i.m.)	4	erythrocytes (mil. per mm³)	mean	5·50	5·72	5·58	5·64	5·60	6·16	5·47	6·11	6·03
				range	4·95–5·94	5·16–6·17	4·86–6·15	4·85–6·31	5·37–5·83	5·24–7·21	5·12–5·70	5·74–6·68	4·36–6·68
			hematocrit	mean	36·1%	35·6%	35·5%	35·4%	35·9%	37%	36·1%	38 1%	35·3%
			leukocytes (per mm³)	mean	7344	9000	11,725	9150	12,000	11,125	11,600	13,400	14,175
				range	6425–9650	7600–10,700	8900–14,900	7600–11,300	9000–15,700	8600–14,100	10,100–13,600	10,600–17,200	10,900–20,300
			total accum. dose (mg/kg)		—	3	4·5	6·0	7·5	9	10·5	12	—
C	Methotrexate (1·0 mg/kg i.m.)	4	erythrocytes (mil. per mm³)	mean	6·50	7·45	6·18	5·39	5·11	5·23	5·74	5·50	5·26
				range	5·42–7·51	5·05–12·47	5·26–8·24	4·95–6·10	4·47–6·16	4·53–5·84	5·34–6·12	4·84–5·91	4·43–5·69
			hematocrit	mean	38·9%	34·4%	34·8%	32·6%	31%	32·5%	35%	33·1%	33·9%
			leukocytes (per mm³)	mean	9313	10,250	10,975	9375	9050	9000	7350	9875	8350
				range	6350–12,200	8100–14,900	7500–14,100	6800–11,700	5400–11,800	5800–12,600	5200–10,800	5700–14,300	7200–9500
			total accum. dose (mg/kg)		—	6	9	12	15	18	21	24	—
D	Methotrexate (4·0 mg/kg i.m.)	4	erythrocytes (mil. per mm³)	mean	6·79	6·54	6·22	6·55	5·34	5·91	5·83	5·64	5·28
				range	6·08–7·35	6·39–6·73	5·78–7·14	6·20–6·90	5·08–5·68	5·70–6·15	3·99–6·85	4·02–6·66	4·54–6·02
			hematocrit	mean	37·9%	39·5%	35·8%	36·5%	36%	35·1%	33·5%	33·5%	34%
			leukocytes (per mm³)	mean	6813	10,350	10,200	9700	12,125	11,925	10,105	12,550	12,725
				range	6400–7400	8700–13,500	9000–13,200	7800–11,900	8600–17,900	9400–18,200	6600–13,900	9700–16,300	9000–15,400
			total accum. dose (mg/kg)		—	24	36	48	60	72	84	96	—
E	Methotrexate (25 mg/kg i.m.)	2	erythrocytes (mil. per mm³)	mean	7·20	6·52	6·30	7·37					
				range	7·11–7·29	5·67–7·37	6·18–6·42	—					
			hematocrit	mean	46·5%	44·8%	42·0%	41·0%					
			leukocytes (per mm³)	mean	7100	5100	6750	4500					
				range	6600–7600	3400–6800	5300–8200	—					
			total accum. dose (mg/kg)		—	150	300 ↑ one rabbit sacrificed	450					

D over the eight-week treatment period. It is apparently unspecific and can be attributed to the repeated intramuscular injections. Hematocrit, erythrocyte, and reticulocyte counts remained within normal range, and platelets were adequate on peripheral smears. Bone marrow examination at termination of the experiment showed normal cellularity and a normal number of megakaryocytes without morphological changes.

The rabbits in group E showed minor changes that could be interpreted as responses to the drug. The reticulocyte count in one animal dropped to 0·1 per cent after two weeks while total white cells, hematocrit, and red blood cells remained within normal range. Bone marrow examination revealed a decrease in red cell precursors but no change in red cell morphology. All other marrow elements remained normal. The rabbit injected for a period of three weeks responded with a drop in white cells from 6600 to 3400 after one week, but values were back to normal at the end of the second week. Red cell counts, reticulocytes, and hematocrit remained normal. The bone marrow was hypercellular, with an increase in plasma cells. The red cell precursors showed some increased fineness of the nuclear chromatin that might represent slowing of nuclear maturation but no definite megaloblastosis.

The treatment had no effect on the rate of growth of the animals as indicated by their increase in weight (Fig. 1). None of the animals developed diarrhea or showed macroscopic signs of bleeding from the gastrointestinal tract. The oral mucosa was

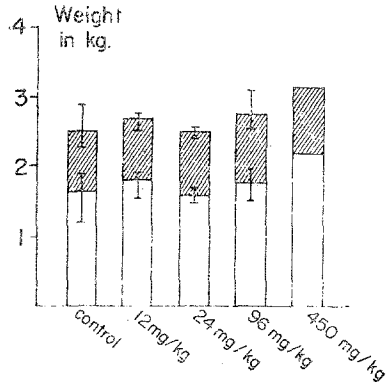

FIG. 1. Average increase in weight of rabbits during eight weeks of observation. Upper and lower bars indicate range of weight at start and end of experiment. Groups and accumulated dose levels are represented according to Table 1.

periodically examined and found to be normal in appearance. A temporary moderate alopecia was observed in both rabbits receiving 25 mg methotrexate/kg daily. It occurred after 8–10 days of treatment and affected mainly regions at the neck, nose, and eyes. Improvement of the condition was noted within a week despite continuation of the injections.

The acute LD_{50} of methotrexate in rabbits could not be established, because excessive amounts of the drug would have been required. Subcutaneous injections up to 490 mg/kg were tolerated without apparent signs of toxicity.

Isolation of a metabolite of methotrexate from urine

After parenteral injection of 100–200 mg methotrexate/kg, 20–45 per cent of the administered dose is excreted within 26 hr in the urine as the metabolite. Attempts to

isolate the metabolite from the liquid portion of urine by column chromatography on cellulose powder gave inconsistent results with low yields and products of insufficient purity. The procedure was much simplified when it was realized that the major fraction of the metabolite was present in the discarded insoluble urine sediment.

The following procedure was used to isolate the metabolite. The collected urine portion (100–200 ml) was centrifuged immediately after thawing, the sediment washed once with cold acetone, resuspended in 10–20 ml of 0·05 N NaOH, and the supernatant separated by centrifugation. This step served to bring the metabolite into solution and to separate it from the bulk of insoluble inorganic matter. Glacial acetic acid was added dropwise to the clear, slightly yellowish supernatant, resulting first in precipitation of a bright yellow material which redissolved on further addition of glacial acetic acid. Crystallization of the metabolite occurred within a few minutes when the samples were adjusted to pH of about 2·0 but continued to give increasing yields when kept overnight at 5°. Optimal conditions for uniform crystallization were obtained with the addition of 0·8 ml glacial acetic acid/ml supernatant. The crystals were separated by centrifugation, washed twice in cold acetone, and dried in a desiccator. Recrystallization involved solubilization in a few ml of 0·05 N NaOH followed by addition of glacial acetic acid as described.

With the described procedure about 80–150 mg of the crystallized metabolite can be obtained from the urine of a rabbit receiving 400–600 mg methotrexate.

Isolation of the metabolite from incubation samples of methotrexate with liver tissue

Incubation was conducted as described, and the sample was cooled in an ice-water bath at the end of the incubation period. The pH was adjusted to approximately 8·2 by dropwise addition of 1 N NaOH, and the sample was centrifuged for 15 min at 0° and 24,000 *g*; the sediment was discarded. The supernatant was heated for 4 min in boiling water, cooled, and the precipitated protein fraction removed by centrifugation. The pH of the supernatant was readjusted to 5·0 by dropwise addition of glacial acetic acid, and the resulting reddish-brown precipitate was collected by centrifugation. The precipitate was redissolved in a few ml of 0·05 N NaOH, again precipitated with glacial acetic acid as described, washed with cold acetone, and dried in a desiccator. The brown residue was suspended in a few ml of 3·5% (v/v) perchloric acid, resulting in the precipitation of an associated protein fraction with the metabolite remaining in solution. Sodium hydroxide (1·0 N) was added slowly with stirring until precipitation of a yellow amorphous substance was observed. The precipitate was separated by centrifugation, dissolved in a few ml of 0·5 N NaOH, and brought to crystallization by adding 0·8 volume of glacial acetic acid.

With the described procedure, about 4–5 mg of the metabolite can be obtained from an incubation sample containing 11 g liver and 22 mg methotrexate.

Characterization of the metabolite

Both fractions of the methotrexate metabolite were obtained as bright yellow crystalline substances. The spheric discoid shape of the crystals is shown in Fig. 2. The metabolite is insoluble in water (pH 7·0) but is readily soluble in alkaline solutions where a marked change in color from bright yellow to faint yellow occurs when the pH is increased above 8·9. The metabolite is almost insoluble in the pH range 3–6 but shows a tendency (especially in higher concentration) to form a gel at pH 4·2.

The substance is stable to heating in 0·05 N NaOH solution (5 min, 100°) but decomposes slowly when kept for a few months in a desiccator at room temperature exposed to light. The melting point of the purified metabolite could not be established; gradual decomposition occurred with continuous heating. Elementary analysis of a recrystallized sample (dried in high vacuum) was conducted by Dr. Alfred Bernhardt, Max Planck Institute, Mülheim, Germany. *Analysis:*

Calc. for $C_{20}H_{22}N_8O_6$ (7-hydroxy derivative of methotrexate): C, 51·06; H, 4·71; N, 23·82; O, 20·41;

for $C_{20}H_{21}N_7O_7$ (4,7-dihydroxy derivative of methotrexate): C, 50·95; H, 4·49; N, 20·80; O, 23·75.

Found: C, 49·71; H, 5·22; N, 20·95; O, 23·98.

It was assumed that water of crystallization, possibly associated with the metabolite, has been removed by drying in high vacuum, since concomitant heating to 145° did not yield further reduction of weight.[9]

The u.v. absorption spectrum of the metabolite is shown in Fig. 3. The material

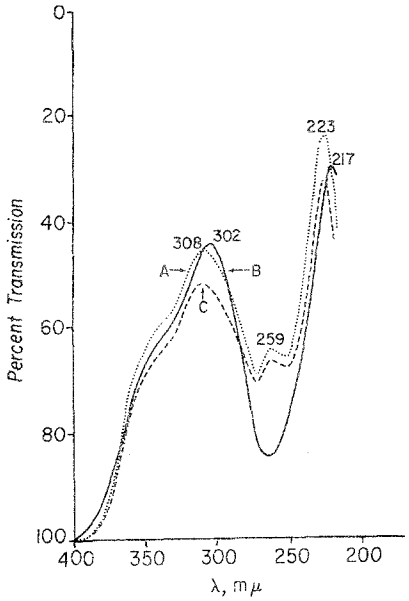

FIG. 3. Absorption spectrum of methotrexate metabolite. A: Metabolite formed *in vivo* (isolated from urine), concentration 6·25 μg/ml in 0·05 N NaOH. B: Same as A but in 0·05 N HCl C: Metabolite formed *in vitro* (isolated from liver homogenate), concentration 5·20 μg/ml in 0·05N NaOH. Numbers indicate position of absorption peaks.

from urine (A) was identical with that isolated from liver incubation samples (C), exhibiting a shoulder at 340 mμ and absorption peaks at 308, 259, and 223 mμ in 0·05 N NaOH. In 0·05 N HCl (B) the two main peaks shifted to 302 and 217 mμ respectively; the peak at 259 mμ disappeared.

Identity of both fractions was also verified by paper chromatography in phosphate buffers. The following R_f values of the metabolite were obtained (those for methotrexate are given in parentheses): pH 9·00, 0·51 (0·56); pH 7·38, 0·51 (0·56); pH 6·88,

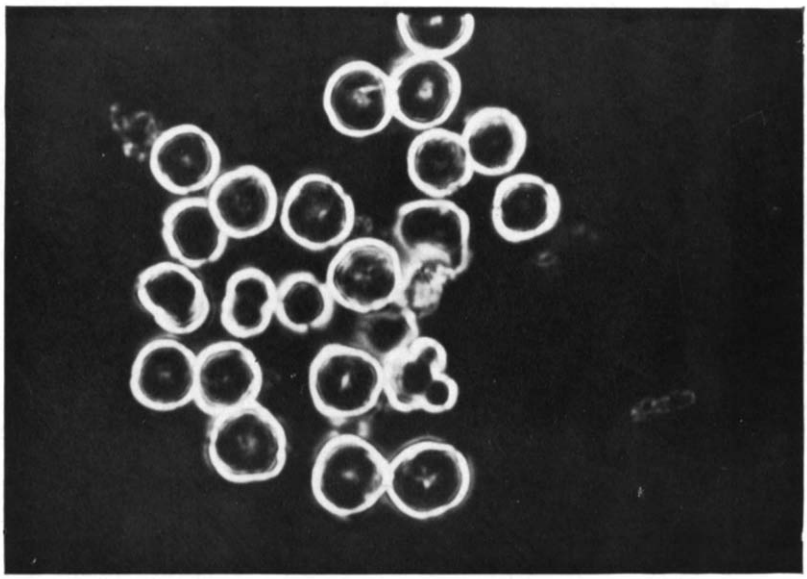

FIG. 2. Crystalline form of the methotrexate metabolite, photographed in dark-field. Crystal size 50–100 μ.

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0·50 (0·60). Trailing due to decreased solubility of the metabolite occurred at pH 6·4 and 5·3. The compound was visible under Woods light as a dark bluish-black spot on the partially dried paper.

Preliminary toxicity studies were performed with mice. The acute LD_{50} of the metabolite was 900 mg/kg $\pm$ 147 (18 animals, single i.p. injection), that of methotrexate 172 mg/kg $\pm$ 20 (46 animals). Death in both groups occurred with similar symptomatology on the third or fourth day after the injection. Loss of weight, inflammation of and secretion from the conjunctivae, diarrhea, drop in circulating white blood cells, and depression of the bone marrow were observed as the most prominent signs in the lethal dose range.

DISCUSSION

The finding of a reduced susceptibility of the rabbit to toxic effects of folic acid antagonists must be considered as a quantitative rather than a qualitative phenomenon. Minnich *et al.*[1] injected five rabbits for periods up to 33 days with daily doses of 2·5–6·2 mg aminopterin/kg and observed the death of two animals with development of diarrhea and loss of weight. Bone marrow changes consisted of hypolasia of all marrow elements without presence of megaloblastic cells. While the study with methotrexate showed only minor indications of toxicity, it must be realized that this compound is less potent than aminopterin.

The fact that rabbits are coprophagic and have been classified as "pseudo-ruminants" indicates that folic and folinic acid are available to them from bacterial synthesis. It is, however, unlikely from consideration of quantitative relationships that this mechanism can account for the lack of toxic response. The amount of folic acid contained in the food was estimated by the producer to be 2·93 ppm,* and the relative ineffectiveness of even large amounts of folic acid to prevent or reverse the effects of the antagonists is well known.

The presented data support the view that the resistance is related to the rapid rate of degradation of the drug. Work of Loo and Adamson[4, 10] and Johns *et al.*[11] showed that this degradation involves only a minor chemical alteration of the compounds. Evidence obtained so far indicated that the oxidation takes place at the pteridine moiety, resulting probably in formation of the 7-hydroxy derivatives of the parent compounds.

In comparing the absorption spectrum of methotrexate with that of the metabolite, the disappearance of the peak at 374 mμ of the former compound in dilute alkali and the occurrence of the shoulder at 340 mμ in the latter are strongly reminiscent of the hysochromic shift from 364 mμ for 2,4-diamino-6-methylpteridine to 338 mμ for 2,4-diamino-7-hydroxy-6-methylpteridine.[10, 12, 13] The ultraviolet absorption characteristics of the described metabolite are in close agreement with the compound isolated by Johns *et al.*[11] from incubation samples of methotrexate with a purified rabbit liver enzyme fraction by means of column chromatography on DEAE-cellulose. The spectrum deviates, however, from that of a synthetically prepared sample of the 7-hydroxy derivative of methotrexate.† Elementary analysis of the purified metabolite also does not support the 7-hydroxy structure but is in good agreement with the

* Ralston Purina Co., St. Louis, Mo.
† Dr. Ti Li Loo, M. D. Anderson Hospital, Houston, Tex., personal communication.

4,7-dihydroxy derivative. This configuration was previously proposed for the oxidation product of dichloromethotrexate, isolated by Loo and Adamson[4] from liver homogenates. These authors have recently reported the isolation of a metabolite of dichloromethotrexate from rabbit urine, identifying it as the 7-hydroxy derivative of the parent drug.[10] While the 4,7-dihydroxy structure could not be fully excluded, it was assumed that deamination at the 4-position of the first described compound was probably due to the drastic procedure used in their earlier degradation study. The 4-amino group of the 2,4-diaminopteridines is comparatively labile and easily undergoes hydrolysis.[14] The finding that a practically identical ultraviolet absorption spectrum of the metabolite is obtainable after simple extraction of the insoluble urine fraction with mild alkali renders it unlikely that 4-deamination of the methotrexate metabolite is likewise unspecific and due to the procedure of isolation.

The fact that the main portion of the metabolite is found in the insoluble urine fraction is of interest, especially since rabbit urine usually has an alkaline pH. The possibility that this insoluble material represents a chelation product with a heavy metal was considered after it was observed that the metabolite is easily precipitated by zinc ions. Methotrexate, which is found mainly in the soluble urine portion, forms also heavy metal complexes and can be isolated in high purity from urine by fractional precipitation with zinc sulfate.

No efforts have been made to isolate the enzyme system responsible for the oxidation of methotrexate. Such studies have been carried out by Johns *et al.*[5, 11] with rabbit liver. These authors obtained a partially purified preparation which in specific activity and susceptibility to different inhibitors appeared to be identical with the previously described enzyme aldehyde oxidase.[15, 16] While the elementary analysis cannot be considered as final identification, recognition of the 4,7-dihydroxy structure for the described metabolite would require a more complicated enzymatic reaction involving multiple steps. It is also not possible to exclude nonenzymatic 4-deamination which might take place during incubation or in the process used to isolate the metabolite.

The pattern of crystallization, ultraviolet absorption spectrum, and R_f values leave little doubt that the metabolite formed *in vivo* and the one formed *in vitro* are identical. It is therefore unlikely that its formation is merely related to degradation by intestinal bacteria, taking place during enterohepatic circulation of the drug.[17]

The toxicity studies indicate that the metabolite is a product of detoxification with reduced toxicity for mice. It is, however, not totally devoid of antifolic activity. These findings correspond well with the observations of Johns *et al.*,[6] who described that the metabolites of methotrexate and aminopterin formed during incubation with a purified rabbit liver enzyme fraction had a reduced ability to inhibit dihydrofolate reductase, as compared with the respective parent drugs.

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