mechanism is not yet certain, the phenomenon is interesting since many butyric acid bacteria have been described as nitrogen fixers, and hydroxylamine or ammonia have been proposed for other organisms as primary products of nitrogen fixation. The present experiments indicate that the hydroxylamine theory of Virtanen & Laine (1939, see also Virtanen, 1947), and the ammonia theory of Burris & Wilson (1945, 1946; see also Wilson & Burris, 1947), are not necessarily incompatible, since both substances give rise to aspartic acid with either fumarate or oxaloacetate. Woods (1938) has shown that hydroxylamine is reduced by molecular hydrogen to ammonia in the presence of suspensions of *Cl. welchii*. It is now shown that such reduction can also occur with other hydrogen donors; this point will be further studied. Another type of reaction between hydroxylamine and fumarate leading to hydroxyaspartic acid, which is not further utilized, has been reported by Jacobsohn & Soares (1936-7) for suspensions of *Esch. coli*.

SUMMARY

1. A study has been made of the comparative rates of fermentation of fumarate, malate, oxaloacetate and pyruvate by cell suspensions of *Clostridium saccharobutyricum* GR 4. The relative proportions of butyric and acetic acids formed were also measured.

2. The results indicate the following sequence of reactions

Malate is not an intermediate in the transformation of fumarate to oxaloacetate. This is supported by experiments with various inhibitors.

3. The organism reduces hydroxylamine to ammonia in the presence of fumarate or oxaloacetate.

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The Fate of Certain Organic Acids and Amides in the Rabbit

8. TOLUIC ACIDS AND AMIDES

By H. G. BRAY, W. V. THORPE AND P. B. WOOD

Department of Physiology, Medical School, University of Birmingham

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The metabolism of the toluic acids and their amides was investigated primarily in order to determine the effect of a nuclear substituted methyl group upon the hydrolysis of the carbamyl group *in vivo*. It was also of interest to compare the excretion products of the acids with those of the xylenes (Bray, Humphris & Thorpe, unpublished results) from which they are

derived *in vivo*. Further, since the toluamides provide examples of compounds with two different potential 'centres for conjugation' (cf. Bray, Ryman & Thorpe, 1948), the metabolic fates of these compounds could be compared with those of the xylenes, in which the two centres are identical in character.

Kraut (1856) and Gleditsch & Moeller (1889) isolated the toluric (methylhippuric) acids, $\text{Me.C}_6\text{H}_4.\text{CONH.CH}_2.\text{COOH}$, as metabolites of the toluic acids in man. Quick (1932) found that all three toluic acids gave rise to increased excretion of glucuronic acid and glycine by the dog. *o*-Toluic acid was conjugated only slightly with glycine, but to a considerable extent with glucuronic acid; a small amount was excreted unconjugated. *p*-Toluic acid was conjugated with glycine to a much greater extent than was *m*-toluic acid, but both these isomers were conjugated with glucuronic acid to a smaller extent than was *o*-toluic acid. Bernhard (1937), on the other hand, found that the dog excreted all three toluic acids unconjugated.

In this investigation the effect of the acids and amides on the excretion of ether-soluble acid, reducing material and ethereal sulphate by rabbits was studied and the nature of the main metabolites determined.

MATERIALS AND METHODS

Materials. The acids used were obtained either from British Drug Houses Ltd. or from Hopkin and Williams Ltd. The amides were prepared from the corresponding nitriles (from the same sources) by the method of Noller (1933). Phthalide was prepared from phthalimide (British Drug Houses Ltd.) by the method of Reissert (1913).

Diet and dosage. The rabbits used were does of 2–3 kg. and received the diet previously described (Bray, Ryman & Thorpe, 1947) throughout the investigation. The compounds were administered by stomach tube, the acids as solutions of their sodium salts and the amides as suspensions in water. The only compound which possessed marked toxic properties at dose levels up to 0.3 g./kg. was *o*-toluamide. At a dose level of 0.25 g./kg. this compound caused a deep narcosis lasting several hours, although the animals recovered completely. Doses of *o*-toluic acid corresponding to 0.75 g./kg. were in some cases fatal and *m*-toluamide occasionally caused slight narcosis. The other compounds were without toxic effect at the dose levels used.

Quantitative methods. The methods used for the estimation of ether-soluble acid, reducing material and ethereal sulphate have already been described (Bray, Thorpe & Wood, 1949). Toluene fractionation of the ether-soluble acid was not carried out, since the differences in the solubilities of the toluic and toluric acids were not sufficiently well defined. The presence of labile ether-soluble glucuronides which might decompose during toluene extraction would also complicate the interpretation of the results obtained.

RESULTS

Quantitative studies

Normal excretion of metabolites. The average daily output of ether-soluble acid, reducing material and ethereal sulphate for individual rabbits ranged as follows: 629–852 mg. (calc. as hippuric acid), 130–244 mg. (as glucuronic acid) and 33–45 mg. SO_3 . In sixty-one experiments the average percentages, by which the weekly average figures used as base-

line values for the calculation of results differed from the individual overall averages, were, for ether-soluble acid, $\pm 7\%$, and, for reducing values, $\pm 8\%$. The corresponding difference for ethereal sulphate in sixteen experiments was $\pm 8\%$ (± 3 mg. SO_3).

Metabolism of *o*-toluic acid and amide. The results obtained are given in Table 1. It can be seen that the percentages of the doses of *o*-toluic acid excreted as ether-soluble acid and as ester glucuronide are approximately the same. The experiments recorded in Table 2 show that the reducing power of the ether-soluble acid was, within experimental error, identical with that of the urine as determined directly. Thus ether-soluble ester glucuronide appears to be the principal metabolite of *o*-toluic acid in the rabbit.

Table 1. *Metabolism of o-toluic acid and amide in the rabbit*

Rabbit no.	Percentage of dose excreted as	
	Ether-soluble acid	Ester glucuronide
<i>o</i> -Toluic acid (dose level 0.3 g./kg.)		
134	77	70
134	67	83
216	72	64
216	71	61
216	84	61
216	78	58
221	70	71
228	84	86
228	83	86
228	78	86
228	—	80
228	—	66
229	79	81
Average	77	73
<i>o</i> -Toluamide (dose level 0.25 g./kg.)		
134	46	13
134	18	—
134	10	—
168	22	9
168	17	11
168	0	—
168	29	15
190	23	—
216	37	12
216	50	—
217	0	77
227	0	—
227	42	13
227	38	15
228	31	31
228	45	16
228	0	—
229	0	6

The analytical results obtained from *o*-toluamide urine were very variable. Further, the 'extra' reducing material could not, as in the case of *o*-toluic acid urines, always be completely extracted with ether, and presumably, therefore, did not consist only of the ester glucuronide metabolite of *o*-toluic acid. In view of the toxicity of *o*-toluamide it seems possible that the extra reducing material represents a response similar to that observed with *o*- and *m*-acetotoluides (Bray & Thorpe, 1948). A similar effect has also been

Table 2. Ether-soluble nature of reducing material excreted by rabbits after administration of o-toluic acid

Rabbit no.	Dose* (g.)	24 hr. period	Reducing value (expressed as mg. glucuronic acid)			
			Urine	Urine after ether extraction (a)	Ether-soluble material (b)	Total (a) + (b)
228	1	1st	253	205	12	217
		2nd	1408	298	1038	1336
		3rd	285	239	16	255
228	1	1st	199	192	15	207
		2nd	1276	281	950	1231
		3rd	251	270	24	294
228	1	1st	250	267	14	281
		2nd	1105	266	907	1173
		3rd	212	211	34	245

* Dose administered at beginning of second 24 hr. period.

observed in some cases after dosage with o-xylene, when the urine contains extra reducing material which is insoluble in ether, in addition to the usual amount of ether-soluble reducing material (presumably o-toluyglucuronic acid) which is excreted alone in the majority of the experiments (Bray *et al.* unpublished results). o-Toluamide is the only compound of the six studied which had any effect on the ethereal sulphate excretion, but this effect was very small and almost within experimental error.

Metabolism of m-toluic acid and amide. Table 3 summarizes the results obtained. The percentages of the doses of acid and amide excreted as ether-soluble acid are comparable, but the

extent of glucuronide conjugation is much less in the case of the amide. The quantitative results considered without reference to the nature of the metabolites of the amide suggest that the carbamyl group is completely hydrolysed, whereas, as will be described later, the acid arises only in part from the hydrolysis of the amide group, the remainder being the result of oxidation of the methyl group. The ester glucuronide is completely extracted by ether and is, therefore, included in the ether-soluble acid total. Neither acid nor amide caused any increase in ethereal sulphate excretion.

Metabolism of p-toluic acid and amide. The results obtained with these compounds are summarized in Table 4. The ether-soluble acid from the amide represents the extent of amide hydrolysis more nearly than in the case of m-toluamide,

Table 3. Metabolism of m-toluic acid and amide in the rabbit

Rabbit no.	Percentage of dose excreted as	
	Ether-soluble acid	Ester glucuronide
<i>m</i> -Toluic acid (dose level 0.3 g./kg.)		
134	104	33
134	96	40
182	90	23
185	—	25
191	—	28
215	100	28
229	75	27
229	81	29
231	100	27
233	87	9
233	103	18
234	—	26
244	—	30
Average	93	26
<i>m</i> -Toluamide (dose level 0.3 g./kg.)		
134	89	15
134	108	26
134	84	13
182	102	6
215	98	5
231	96	2
247	84	3
248	84	5
249	97	8
250	94	3
Average	94	9

Table 4. Metabolism of p-toluic acid and amide in the rabbit

Rabbit no.	Percentage of dose excreted as	
	Ether-soluble acid	Ester glucuronide
<i>p</i> -Toluic acid (dose level 0.3 g./kg.)		
134	88	31
213	—	14
219	—	24
228	—	21
229	—	19
234	—	13
244	—	4
244	102	7
247	93	9
247	110	11
247	—	13
248	99	10
249	105	14
249	89	7
Average	98	14
<i>p</i> -Toluamide (dose level 0.3 g./kg.)		
182	70	5
215	67	3
220	—	2
221	88	5
221	57	4
231	71	2
234	92	4
Average	74	4

though here also a small proportion of the dose (at least 0.5%) is excreted with the carbamyl group intact and the methyl group oxidized to carboxyl (see p. 49). The ester glucuronide formed is completely extracted by ether. Neither acid nor amide caused any increase in ethereal sulphate conjugation.

Hydrolysis of toluamides by rabbit-liver extracts. Solutions of the three amides (0.01204M) in phosphate buffer (pH 7.4) were incubated with rabbit-liver extracts prepared as previously described (Bray, James, Ryman & Thorpe, 1948) and the degree of hydrolysis measured by estimation of the NH_3 liberated (Bray, James, Raffan, Ryman & Thorpe, 1949). Table 5 summarizes the results obtained from three

Table 5. *Hydrolysis of toluamides and p-nitrobenzamide by rabbit-liver extracts at pH 7.4*

Amide	Average percentage hydrolysis by liver extract in	
	3 hr.	20 hr.
<i>o</i> -Toluamide	2.5	3.0
<i>m</i> -Toluamide	7.0	13.0
<i>p</i> -Toluamide	19.0	48.5
<i>p</i> -Nitrobenzamide	13.0	42.0

experiments with each isomer. In one of each of these, formol titrations gave results agreeing with the NH_3 values within the limits of experimental error. Values for *p*-nitrobenzamide are also included in order to enable comparison to be made with the behaviour of an amide which has been shown to be completely hydrolysed in the intact animal (Bray, Thorpe & Wood, 1949).

Qualitative studies

Metabolites of *o*-toluic acid. No metabolite of *o*-toluic acid could be isolated from the ether extract of the urine as collected from rabbits dosed with this acid. Continuous extraction of the acidified urine, however, yielded a brown syrup which gave strongly positive naphthoresorcinol and reducing (Benedict's) tests. The quantitative results recorded in Tables 1 and 2 suggested that this consisted primarily of ester glucuronide, but attempts to obtain this in a crystalline form were unsuccessful, probably owing to its instability. When a concentrated solution of the syrup in aqueous ethanol was allowed to evaporate at ordinary temperature in air, crystals of *o*-toluic acid were deposited, but whether from the decomposition of the glucuronide or because the acid was present as an individual excretion product could not be determined. It is possible that a small amount of *o*-toluic acid may be excreted unconjugated since in some experiments the ether-soluble acid excretion was greater than the excretion of ester glucuronide (Table 1). Glycine conjugation, if it occurred at all, took place to only a slight extent since examination of the ether-soluble material by toluene fractionation as described for the other isomers yielded no *o*-toluic acid.

We failed to isolate the glucuronide by the usual lead precipitation method which yielded only syrups from which crystalline products could not be obtained, even by means of ether extraction. Since the only product identified after hydrolysis of the ether extracts was *o*-toluic acid it is probable that the glucuronide is *o*-toluylglucuronide.

Metabolites of *o*-toluamide. The 24 hr. urine of ten rabbits which had each received 0.5 g. *o*-toluamide was continuously

extracted with ether for 24 hr., first as collected (extract *A*) and then after acidification with 2N- H_2SO_4 (extract *B*). The residue from the second extraction was hydrolysed by boiling and then continuously extracted with ether (extract *C*). Extract *A* was a clear yellow syrup which crystallized. Recrystallization from water gave white plates (m.p. 70°). During recrystallization they melted to form a pale yellow oil having an odour resembling that of the tolunitriles. The compound, which was nitrogen free, was identified as phthalide and gave no depression of melting point on mixing with an authentic specimen (m.p. 70°). (Found: C, 71.3; H, 4.4. Calc. for $\text{C}_8\text{H}_5\text{O}_2$, C, 71.7; H, 4.5%.) The total yield of phthalide was approx. 1 g.

Extract *B* was a dark brown syrup which slowly deposited crystals. The first crop (200 mg.) was separated and after recrystallization from water had m.p. 116–118°. It appeared to be crude benzoic acid, giving no depression of melting point when mixed with the pure acid (m.p. 120°). The residual syrup finally crystallized almost completely. The solid material was separated on a porous tile and recrystallized from water (charcoal), and yielded 700 mg. pure benzoic acid (m.p. 120°). (Found: C, 69.2; H, 5.0. Calc. for $\text{C}_7\text{H}_5\text{O}_2$, C, 68.9; H, 4.9%.) Its identity was confirmed by conversion to benzanilide (m.p. 163°), which did not depress the m.p. 163° of an authentic specimen. No metabolite of *o*-toluamide could be isolated from extract *C*.

Metabolites of *m*-toluic acid. The ether-soluble material from acidified *m*-toluic acid urine was fractionated by refluxing with toluene. The dark brown syrup was almost completely soluble in the hot solvent, but crystals and syrup were deposited on cooling. Recrystallization of this material from water (charcoal) gave needles (m.p. 140°), which were strongly acidic to congo red and were identified as *m*-toluric acid. (Found: N, 7.0; equivalent (by titration) 190. Calc. for $\text{C}_{10}\text{H}_{11}\text{O}_3\text{N}$, N, 7.3%; equivalent 193. Gleditsch & Moeller (1889) gave m.p. 139°.) The toluene filtrate obtained after separation of the crude toluric acid was evaporated to dryness under reduced pressure and yielded a brown syrup. This, on treatment with dilute aqueous ethanol, gave needles which after recrystallization had m.p. 110° and were identical with an authentic specimen of *m*-toluic acid (mixed m.p. 110°). It was difficult to establish the yields of the two metabolites described, since it is certain that a proportion remained in the intractable syrupy residues. The amounts of toluric and toluic acids isolated were 1.0 and 0.5 g. respectively from doses of *m*-toluic acid totalling 3 g. The only product isolated after hydrolysis of the syrupy residues was *m*-toluic acid.

Metabolites of *m*-toluamide. Extraction of *m*-toluamide urine as collected gave no recognizable crystalline product, suggesting that little or none is excreted unmodified.

During continuous ether extraction of acidified *m*-toluamide urine solid material separated from the ethereal solution. After 24 hr. extraction this solid was separated, and, after exhaustive extraction with boiling toluene, recrystallized from aqueous ethanol. The product had m.p. 278° and on acid hydrolysis gave isophthalic acid (m.p. 334°). Analysis confirmed the identity of the metabolite with isophthalic acid (*m*-carbamylbenzoic acid). (Found: N, 8.2; equivalent (by titration) 160. Calc. for $\text{C}_8\text{H}_7\text{O}_3\text{N}$, N, 8.5%; equivalent 165.) Yield of isophthalic acid 1.7 g. from a total dose of 5 g. *m*-toluamide. The toluene-soluble material appeared to be crude hippuric acid contaminated mainly with isophthalic acid.

After removal of the crude isophthalic acid from the original extract the ethereal filtrate was evaporated to

dryness and the residual syrup fractionated in the same way as the ether-soluble material from *m*-toluic acid urine. *m*-Toluic and *m*-toluric acids were thus isolated in yields of 0.3 and 1.8 g. respectively from an original total dose of 5 g. *m*-toluamide. The final fraction from the *m*-toluric acid mother liquors appeared to be crude acid similar to that extracted by toluene from the crude *isophthalamic* acid.

Metabolites of *p*-toluic acid. The ether-soluble material obtained by continuous extraction of acidified rabbit urine, corresponding to a dose of 3 g. *p*-toluic acid, was a light-brown syrup which crystallized almost completely on standing at room temperature. By trituration with water in the cold most of the reducing material was removed, leaving a residue which, after recrystallization from water (charcoal), gave white plates (m.p. 146–147°). These were extracted with boiling toluene and resolved into two fractions: (a) crystals (100 mg.), readily soluble in toluene (m.p. 178–180°), which did not depress the m.p., 179°, of an authentic specimen of *p*-toluic acid; and (b) crystals (2 g.) only slightly soluble in toluene (m.p. 163°). These were strongly acidic to congo red and proved to be *p*-toluric acid. (Found: N, 7.3; equivalent (by titration) 189. Calc. for $C_{10}H_{11}O_3N$, N, 7.3%; equivalent 193. Gleditsch & Moeller (1889) found m.p. 161–162°.)

The aqueous tritrate (above) was extracted by shaking with ether. Evaporation of the ether from the extract yielded only a small amount of *p*-toluic acid. The residue after extraction reduced Benedict reagent, but we were not able to isolate *p*-toluyglucuronic acid. Attempts to obtain it by the normal lead precipitation procedure were also unsuccessful.

Metabolites of *p*-toluamide. The chief metabolite of *p*-toluamide is *p*-toluric acid which was isolated (2.5 g. corresponding to the administration of 4 g. amide), together with small amounts of *p*-toluic acid (120 mg.), by the method described above. The amounts obtained were similar to those isolated from *p*-toluic acid urine. It was also observed that a small amount of material was much less soluble in water than were toluric or toluic acids. This was separated and recrystallized from aqueous ethanol. It was acid to congo red and did not melt, but sublimed at about 300°. These properties were consistent with those of terephthalamic acid (*p*-carbamylbenzoic acid). (Found: N, 8.1%. Calc. for $C_8H_7O_3N$, N, 8.5%.) Yield 20 mg.

Extraction of *p*-toluamide urine as collected gave needles melting at 170° which were not *p*-toluamide or *p*-toluic acid. The amount isolated (5 mg.) was not sufficient for identification.

DISCUSSION

In an earlier paper of this series (Bray, Ryman & Thorpe, 1948) we discussed the metabolism of various aromatic compounds from the point of view of the presence in their molecules of groupings which can be directly conjugated and of groupings which can be modified so that conjugation can then take place. The metabolism of the toluamides is of particular interest in this connexion. These compounds contain no directly conjugable groups, but both the methyl and carbamyl groups are potential centres for conjugation inasmuch as they may be converted to carboxyl groups, by oxidation and hydrolysis respectively. The three isomers show a gradation in metabolic behaviour. In the *para*

isomer the carbamyl group is largely hydrolysed and the excretion products are very similar to those of the corresponding acid. Hydrolysis is not quite complete, however, since a small amount of terephthalamic acid is formed, indicating that oxidation of the other potential centre for conjugation does occur. The terephthalamic acid isolated appears to have been excreted unconjugated. In *m*-toluamide the extent of the hydrolytic and oxidative reactions is markedly different from that in the *para* isomer. Nearly half the dose is excreted as *isophthalamic* acid, and the remainder as derivatives of *m*-toluic acid. Since both terephthalamic and *isophthalamic* acid are excreted unconjugated, it appears that potential centres for conjugation may be converted into actual centres even if conjugation cannot or does not then take place.

The excretion product of *o*-toluamide isolated merits individual consideration. Phthalide may arise from *o*-toluamide by means of the following reactions:

The fact that the studies *in vitro* suggested that the carbamyl group in *o*-toluamide is biologically stable may be interpreted as evidence against a metabolic pathway involving reactions (3), (4) and (5), as may also the fact that phthalide could not be isolated from *o*-toluic acid urine. Failure to isolate *o*-hydroxymethylbenzoic acid should not be regarded as final evidence against reaction (5), owing to the ease with which this compound is converted to phthalide. It is unfortunate that *o*-hydroxymethylbenzamide has not been prepared, since its properties might determine whether phthalide could be formed directly by reaction (2), or only through *o*-hydroxymethylbenzoic acid (reactions (6) and (5)). It thus appears that the first metabolic reaction which *o*-toluamide undergoes in the rabbit is oxidation of the methyl group. The resulting primary alcohol is however labile, undergoing, directly or indirectly, ring closure to form phthalide. So far as we are aware there is only one cited example of the isolation of this intermediate stage in the oxidation *in vivo* of a methyl group attached to the aromatic nucleus. This is the isolation of *o*-nitrobenzyl alcohol as a metabolite of *o*-nitrotoluene (Jaffé, 1878–9). It is often difficult to isolate intermediate products in biological reactions of this type and lactonization such as occurs in the formation of phthalide may provide a means of ‘fixing’ and identifying the intermediate products in other oxidations. We are

examining some other alkyl derivatives of benzamide with this end in view.

It is clear from the *in vivo* experiments that *m*- and *p*-toluamides can act as substrates for two different enzyme systems, one oxidizing the methyl group and another hydrolysing the carbamyl group. The results of *in vitro* experiments with liver extract recorded in Table 5 were obtained by estimation of ammonia and thus represent the hydrolysis of the carbamyl group only. Since corresponding results were obtained by formol titration, it seems probable that the principal, if not the only, reaction occurring in digests with liver extracts is hydrolysis of the carbamyl group. The use of extracts appears, in effect, to isolate one of the enzyme systems.

In view of the fact that only phthalide was isolated as a metabolite of *o*-toluamide it is difficult to comment upon the analytical results obtained (Table 1). The significance of the reducing values has already been discussed. The benzoic acid isolated from acidified but unhydrolysed *o*-toluamide urine is presumably a normal metabolite of the rabbit, though it is not usually isolated in such comparatively large amounts. If *o*-toluamide inhibits glycine conjugation it would not be expected that the amount of ether-soluble acid would differ greatly from the normal amount, since *in vitro* experiments suggest that the carbamyl group of *o*-toluamide is stable. The analytical results, however, suggest that extra ether-soluble acid is usually excreted, although the amount is variable. We have not yet been able to determine the nature of this extra acid.

SUMMARY

1. The metabolism of the toluic acids and their amides in the rabbit has been studied both quantitatively and qualitatively.

2. The acids are excreted as conjugates of glycine or glucuronic acid, the latter being ether-soluble. Small amounts are excreted unchanged.

With the amides, the methyl group may be oxidized or the carbamyl group hydrolysed (or both), and the resulting carboxylic acids excreted free or conjugated as above. With the possible exception of *o*-toluamide, there is no evidence of ethereal sulphate formation.

3. The percentages of the dose excreted as ether-soluble acid were: acids—*ortho*, 77 %; *meta*, 93 % and *para*, 98 %; amides—*meta*, 94 % and *para*, 74 %. These values include the ether-soluble ester glucuronides.

4. *m*- and *p*-Toluic acids are excreted unchanged (17 and 3 % respectively isolated), and conjugated with glucuronic acid (26 and 14 %), and with glycine (23 and 46 % isolated). *o*-Toluic acid is conjugated with glucuronic acid, but glycine conjugation was not observed.

5. After dosage with *m*- and *p*-toluamides, toluic (6 and 3 % respectively), toluric (25 and 43 %), and *isophthalamic* (28 %) or *terephthalamic* (0.4 %) acids were isolated. Glucuronide excretion accounted for 9 and 4 % respectively.

6. The only metabolite of *o*-toluamide isolated was phthalide (20 % of the dose), which probably arose from *o*-hydroxymethylbenzoic acid or amide. Consistent analytical results could not be obtained.

7. Rabbit-liver extracts hydrolyse *o*-, *m*- and *p*-toluamides to the extent of 3, 13 and 49 % respectively.

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