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POTENT COMPETITIVE URICASE INHIBITORS—2,8-DIAZAHYPOXANTHINE AND RELATED COMPOUNDS

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Abstract—The inhibitory activities of 2,8-diazahypoxanthine and related compounds on uricase of bovine kidney and rat liver homogenates were compared *in vitro*. 2,8-Diazahypoxanthine was found to be even more inhibitory than 2-azahypoxanthine, 8-azaxanthine, 8-azahypoxanthine or oxonate. From Lineweaver–Burk plots, 2,8-diazahypoxanthine and 8-azaxanthine were shown to be competitive inhibitors of uricase.

2,8-Diazahypoxanthine had no influence on the activity of milk xanthine oxidase or rat erythrocyte hypoxanthine-guanine phosphoribosyltransferase at the concentrations tested.

The inhibitory activities of 2,8-diazahypoxanthine and related compounds on rat liver uricase *in vivo* were compared by measuring their effects on serum urate and allantoin. 2,8-Diazahypoxanthine, administered intraperitoneally, caused the greatest increase in the urate level with a concomitant decrease in the allantoin level.

8-Azaxanthine and 8-azahypoxanthine were both relatively strong inhibitors of uricase *in vivo* although the latter was not a strong inhibitor *in vitro*.

IN MOST mammalian species uric acid is converted to allantoin by uricase (urate: oxygen oxidoreductase, EC 1.7.3.3), which is found predominantly in the liver.[1,2] Uricase is lacking in man and higher apes, and in these species uric acid rather than the more water-soluble allantoin is the end-product of purine metabolism found in urine.[3] This absence of uricase in man is one reason for gout.

To obtain an animal model for studies on hyperuricemia and on the physiological and psychological functions of uric acid, hepatic uricase must be blocked with a selective inhibitor.

Previously, we reported that the analog of hypoxanthine, 2-azahypoxanthine, was a potent inhibitor of uricase *in vitro*, although it also caused slight inhibition of xanthine oxidase and hypoxanthine-guanine phosphoribosyltransferase.[4] Therefore, other analogs of hypoxanthine and xanthine were tested to find a more specific inhibitor of uricase.

We found that 2,8-diazahypoxanthine was a stronger and more specific inhibitor of uricase than 2-azahypoxanthine. We tested the effect of this compound in rats in an attempt to inhibit uricase and induce hyperuricemia. The results of this investigation are described here.

MATERIALS

Enzymes. Bovine kidney uricase (soluble powder Type II) was purchased from Sigma Chemical Company. Milk xanthine oxidase was prepared by the method of Klenow

and Emberland.[5] Hypoxanthine-guanine phosphoribosyltransferase from a hemolysate of rat erythrocytes was prepared by the method of Kelley *et al.*:[6] the erythrocytes were washed twice with five volumes of isotonic saline and hemolyzed by rapid freezing and thawing three times in a dry-ice acetone bath.

Compounds. 2-Azahypoxanthine monohydrate was prepared as described by Shealy *et al.*[7] Analysis: calculated for $C_4H_3ON_5.H_2O$: C, 30·95; H, 3·25; N, 45·12 per cent. Found: C, 30·63; H, 3·34; N, 45·36 per cent. 2,8-Diazahypoxanthine monohydrate was prepared as the sodium salt as described by Shealy *et al.*[7] Analysis: calculated for $C_3HN_6ONa.H_2O$: C, 20·22; H, 1·68; N, 47·19 per cent. Found: C, 20·23; H, 1·54; N, 46·50 per cent. Oxonic acid was prepared as the potassium salt as described by Brandenberger.[8] Analysis: calculated for $C_4H_2O_4N_3K$: C, 24·61; H, 1·04; N, 21·53 per cent. Found: C, 24·26; H, 1·00; N, 21·78 per cent. 8-Azahypoxanthine and 8-azaxanthine were prepared by the method of Roblin *et al.*[9] Oxipurinol was prepared by the method of Elion *et al.*[10] 4-Hydroxypyrazolo[3,4-*d*]-*v*-triazine monohydrate (4-HPT) was prepared by the method of Cheng *et al.*[11] Allopurinol was a gift from Mr. M. Takahashi, Kohjin Co., Ltd. Other chemicals were obtained from commercial sources.

METHODS

Inhibition of uricase was assayed with bovine kidney uricase *in vitro* by a modification of the method of Fridovich[12] in 67 mM borate buffer, pH 8·5. Disappearance of urate in 1 hr at 30° was estimated from the absorption at 292 nm. The assay mixture contained 0·5 ml of 3×10^{-4} M uric acid, 0·5 ml of enzyme solution (2 mg of soluble powdered bovine kidney uricase Type II/5 ml of water), 0·5 ml of various concentrations of drugs or water and 3·0 ml of 100 mM borate buffer, pH 8·5.

Inhibition of xanthine oxidase *in vitro* was assayed as described previously.[4]

Inhibition of hypoxanthine-guanine phosphoribosyltransferase activity of rat erythrocyte hemolysates was assayed isotopically by a modification of the method of Krenitsky.[13] Incubation mixture contained 5 mM magnesium chloride, 50 mM Tris–HCl buffer, pH 7·4, 0·0214 mM 8-^{14}C-hypoxanthine (20·7 mCi/mM), 1 mM 5-phosphoribosyl 1-pyrophosphate (dimagnesium salt) and drug solution in a final volume of 100 μl. Reactions, started by addition of enzyme (1·0 mg protein), were incubated for 10 min at 37°, and were stopped by addition of 20 μl of 4 M formic acid. Twenty μl of the reaction mixture were placed on filter paper with the carriers for separation of product (IMP) and substrate (hypoxanthine), and chromatographed in 5% Na_2HPO_4–isoamyl alcohol (2:1 by vol.) for 20 hr.

Inhibition of uricase in rat liver homogenates was assayed using the system of Leeling and Lata.[14] Male rats, weighing 250–300 g, were fasted overnight and then killed by decapitation. The liver was perfused *in situ* with cold physiological saline to remove blood and then cut out. It was stripped of gross connective tissue, finely cut up with scissors and homogenized in cold Krebs–Ringer phosphate buffer, pH 7·1, in a Potter–Elvehjem homogenizer with a Teflon pestle for no more than 45 sec. A 5 per cent liver homogenate was used as the uricase preparation. The reaction mixture contained 1·5 ml of Krebs–Ringer phosphate buffer, pH 7·1, 1 ml of uric acid solution (330 μg/ml), 0·5 ml of liver homogenate and 0·3 ml of drug solution or water. The reaction mixture was incubated for 15 min at 28° and the reaction was stopped by adding a mixture of 10% sodium tungstate and 2/3 N sulphuric acid. Uric acid

metabolized by the liver homogenate was determined by subtracting the amount remaining in solution after incubation from that initially added. Uric acid was measured by the method of Henry *et al.*[15]

Assay of uric acid and allantoin in rat serum: male Sprague–Dawley rats, weighing 120–150 g, were killed by decapitation and their blood was stood for 15 min at room temperature. Then serum was obtained by centrifugation at 5000 rev/min for 10 min at 4°. Serum uric acid was determined spectrophotometrically by the method of Henry *et al.*[15] Protein was precipitated by mixing 0·2 ml of serum and 0·3 ml of water with 0·5 ml of deproteinizing solution (5% sodium tungstate in 1/3 N sulphuric acid). This mixture was left to stand for 10 min and then 4·0 ml of water was added. The mixture was then centrifuged at 5000 rev/min at 4° and 3 ml of the resulting supernatant was used for the assay. Serum allantoin was determined spectrophotometrically by the method of Young and Conway.[16] Protein was precipitated by mixing 0·3 ml of serum with 1·0 ml of 5% TCA solution and 1·2 ml of water. This mixture was left to stand for 10 min and then centrifuged at 5000 rev/min for 10 min at 4°. TCA in the supernatant was removed by washing the solution twice with 3 ml of ether saturated with water and the TCA-free supernatant was used for the assay.

RESULTS

In vitro *inhibition of bovine kidney uricase and other enzymes by* 2,8-*diazahypoxanthine and related compounds.* Previously we showed that 2-azahypoxanthine strongly inhibited uricase *in vitro.*[4] The inhibitory effects of the various hypoxanthine and xanthine analogs shown in Table 1, including 2-azahypoxanthine, on bovine kidney uricase were tested *in vitro.*

TABLE 1. INHIBITION OF URICASE AND OTHER ENZYMES BY PURINE ANALOGS

Compound	Inhibition (%)			
	Uricase		X.O.*	HG-PRTase†
	1×10^{-6} M	1×10^{-5} M	1×10^{-5} M	1×10^{-3} M
2,8-Diazahypoxanthine	64	90	0	0
2-Azahypoxanthine	35	80	5	16
8-Azaxanthine	21	74	5	0
8-Azahypoxanthine	0	5	27	2
Hypoxanthine	0	0	—	—
Xanthine	0	35	—	4
Uric acid	—	—	0	0
8-Azaguanine	0	9	4	0
2, 6-Diaminopurine	0	0	4	10
6-Chloropurine	0	0	0	37
6-Mercaptopurine	0	9	5	37
Caffeine	6	9	0	0
Allopurinol	0	3	72	14
Oxipurinol	0	4	73	0
4-HPT‡	0	11	48	6
Oxonate	30	77	0	0

* Xanthine oxidase.
† Hypoxanthine-guanine phosphoribosyltransferase.
‡ 4-Hydroxypyrazolo (3,4-*d*)-*v*-triazine.

The most inhibitory was the purine analog, 2,8-diazahypoxanthine. This compound caused 90 and 64 per cent inhibition at concentrations of 1×10^{-5} M and 1×10^{-6} M, respectively. 2-Azahypoxanthine and 8-azaxanthine were both strong inhibitors, while 8-azahypoxanthine was slightly inhibitory, as observed by Norris and Roush[17] and Bergmann *et al.*[18]

Hypoxanthine and xanthine, which are both substrates for xanthine oxidase, had different effects on uricase; hypoxanthine had no effect on enzyme activity, while xanthine was a relatively strong inhibitor. The observed inhibition by xanthine confirms the report of Bergmann *et al.*[18]

Among pyrazoles, allopurinol, oxipurinol and 4-HPT are known to inhibit xanthine oxidase strongly. However, they all showed little or no inhibitory effect on uricase, as previously reported.[4]

8-Azaguanine, 2,6-diaminopurine, 6-chloropurine, 6-mercaptopurine and caffeine all had no effect to uricase at the concentrations tested.

Oxonate, which is a *s*-triazine compound reported to inhibit uricase,[12,19,20] caused 30 and 77 per cent inhibition at concentrations of 1×10^{-6} M and 1×10^{-5} M, respectively.

We examined the specificities of the inhibitory effects of these uricase inhibitors by testing their effects on xanthine oxidase and hypoxanthine-guanine phosphoribosyltransferase activities. 2,8-Diazahypoxanthine caused no inhibition of these enzymes at the concentrations tested with uricase, while 2-azahypoxanthine inhibited both enzymes slightly, as we reported previously.[4,21]

6-Chloropurine and 6-mercaptopurine were shown to inhibit hypoxanthine-guanine phosphoribosyltransferase. 8-Azahypoxanthine, 8-azaxanthine, 8-azaguanine, 2,6-diaminopurine and caffeine caused little or no inhibition of either this enzyme or xanthine oxidase.

Dose-response curve and I_{50} of uricase inhibition by 2,8-diazahypoxanthine, 2-azahypoxanthine and oxonate. Figure 1 shows a plot of the concentration of 2,8-diazahypoxanthine against the observed inhibition, which gave a sigmoidal curve. For comparison, similar plots for 2-azahypoxanthine and oxonate are also shown.

From these plots the concentrations giving 50 per cent inhibition (I_{50}) were deduced

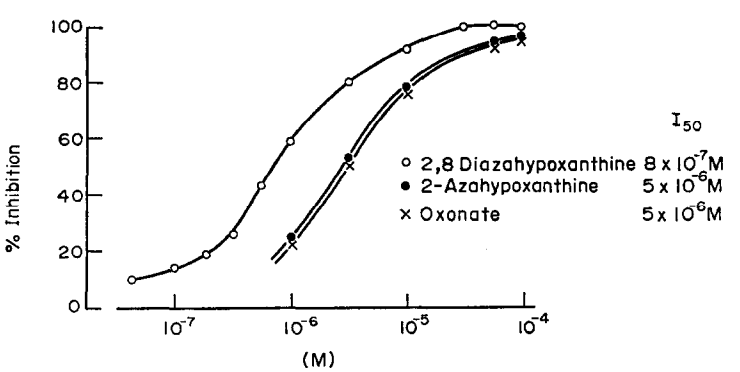

FIG. 1. Dose-response curves of uricase inhibition by 2,8-diazahypoxanthine, 2-azahypoxanthine and oxonate.

for 2,8-diazahypoxanthine, 2-azahypoxanthine and oxonate to be 8×10^{-7} M, 5×10^{-6} M and 5×10^{-6} M, respectively.

Inhibitory effect of 2,8-diazahypoxanthine and related compounds on uricase in rat liver homogenates. The abilities of 2,8-diazahypoxanthine and related compounds to inhibit uricase in rat liver homogenates are shown in Table 2.

2,8-Diazahypoxanthine was the strongest inhibitor. 2-Azahypoxanthine was also strongly inhibitory, causing 9 and 35 per cent inhibition at concentrations of 1×10^{-6} M and 1×10^{-5} M, respectively. Allopurinol caused no inhibition at the concentrations tested.

TABLE 2. COMPARISON OF INHIBITORY EFFECT OF 2,8-DIAZAHYPOXANTHINE AND RELATED COMPOUNDS ON URICASE ACTIVITY OF RAT LIVER HOMOGENATE

Compound	Inhibition (%)		
	1×10^{-6} M	1×10^{-5} M	1×10^{-4} M
2,8-Diazahypoxanthine	14	60	76
2-Azahypoxanthine	9	35	43
Xanthine	0	12	—
Allopurinol	0	0	—
Oxonate	10	47	57

Kinetics of inhibition of bovine kidney uricase by 2,8-diazahypoxanthine and 8-azaxanthine. 2,8-Diazahypoxanthine and 8-azaxanthine were both effective uricase inhibitors. The mechanism of this inhibition was tested by measuring uricase activity in the presence and absence of the inhibitors over a wide range of substrate concentrations at pH 8·5.

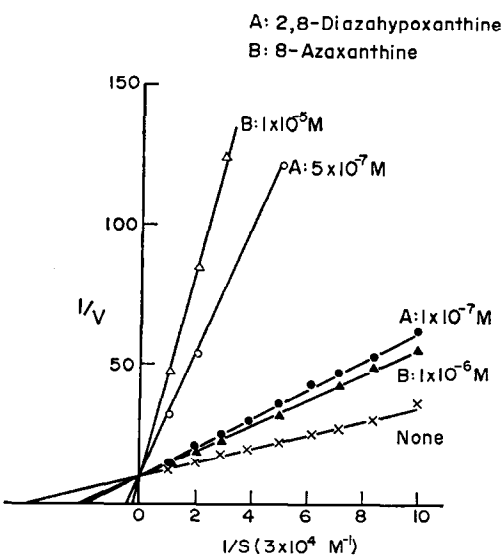

FIG. 2. Competitive inhibition of uricase by 2,8-diazahypoxanthine and 8-azaxanthine.

Double reciprocal (Lineweaver–Burk) plots of the velocity against substrate concentration (Fig. 2) showed that both 2,8-diazahypoxanthine and 8-azaxanthine are competitive inhibitors using uric acid as substrate.

The apparent K_m for urate observed in these studies was $8{\cdot}3 \times 10^{-6}$ M. The apparent K_is for 2,8-diazahypoxanthine and for 8-azaxanthine were calculated to be $8{\cdot}0 \times 10^{-8}$ M and $9{\cdot}0 \times 10^{-7}$ M, respectively.

Effects of 2,8-*diazahypoxanthine and related compounds on the serum levels of uric acid and allantoin in rats.* 2,8-Diazahypoxanthine, 2-azahypoxanthine, 8-azahypoxanthine and 8-azaxanthine (125 mg/kg) were administered intraperitoneally to rats. The animals were sacrificed 1 hr later and uric acid or allantoin in their serum was measured. In control animals, the concentrations of uric acid and allantoin were $3{\cdot}1 \pm 0{\cdot}4$ mg/100 ml and $3{\cdot}2 \pm 0{\cdot}4$ mg/100 ml of serum, respectively.

As shown in Table 3, 2,8-diazahypoxanthine caused a marked decrease of allantoin and increase of uric acid.

On the other hand, 2-azahypoxanthine caused neither a reduction in the allantoin level nor an increase in the uric acid level, although it strongly inhibited uricase *in vitro*.

TABLE 3. EFFECT OF TREATMENT WITH PURINE ANALOGS ON URATE AND ALLANTOIN LEVELS OF RAT SERUM

Treatment (No. of rats)	Uric acid (mg/100 ml)	Allantoin (mg/100 ml)
None (5)	$3{\cdot}1 \pm 0{\cdot}4$	$3{\cdot}2 \pm 0{\cdot}4$
2,8-Diazahypoxanthine (5)	$5{\cdot}4 \pm 0{\cdot}8$	$1{\cdot}4 \pm 0{\cdot}2$
2-Azahypoxanthine (5)	$3{\cdot}4 \pm 0{\cdot}3$	$3{\cdot}0 \pm 0{\cdot}2$
8-Azahypoxanthine (5)	$5{\cdot}0 \pm 0{\cdot}5$	$1{\cdot}6 \pm 0{\cdot}1$
8-Azaxanthine (5)	$4{\cdot}6 \pm 0{\cdot}4$	$1{\cdot}6 \pm 0{\cdot}2$

The purine analogs (125 mg/kg) were given intraperitoneally 1 hr before sacrifice.

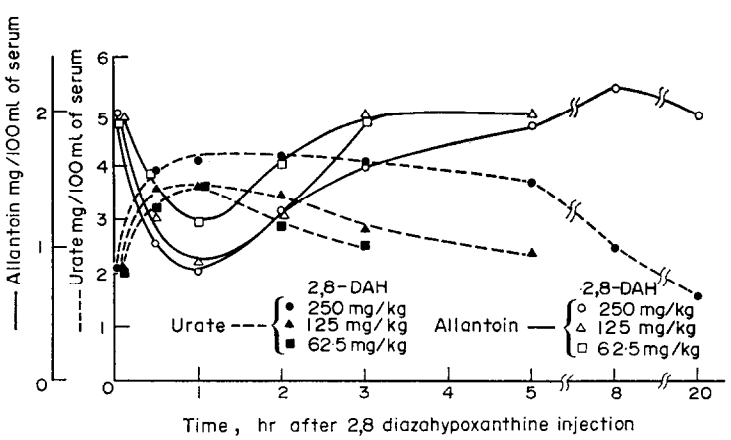

FIG. 3. Effects of single injections of 2,8-diazahypoxanthine (2,8-DAH) on urate and allantoin levels in rat serum.

Treatment with 8-azaxanthine also increased the level of uric acid with concomitant decrease in the level of allantoin. 8-Azahypoxanthine had a similar effect, although *in vitro* it only slightly inhibited uricase at a concentration of 1×10^{-5} M. These results suggest that 8-azahypoxanthine is converted to 8-azaxanthine *in vivo* and that the latter compound inhibits uricase in rat liver, resulting in an increase of uric acid.

Time course of changes in serum urate and allantoin levels in rats induced by 2,8-*diazahypoxanthine.* Figure 3 shows the time courses of changes in the serum urate and allantoin levels in rats after various doses of 2,8-diazahypoxanthine (62·5, 125 and 250 mg/kg, i.p.). The maximum decrease of allantoin, and maximum increase of urate occurred 1 hr after treatment with 250 mg/kg and the changes lasted for at least 5 hr.

DISCUSSION

2-Azapurines, 2-azahypoxanthine and 2,8-diazahypoxanthine, were obtained by diazotization of the appropriate aminohetero-cycles.[7,22] 2-Azahypoxanthine and 2,8-diazahypoxanthine were found to cause moderate inhibition of tumour growth in mice and rats in this laboratory[23] and by Shealy *et al.*[7,24] and 2-azahypoxanthine was found to have slight antimicrobial activities by Yamamoto[25] and by Pershin and Shcherbakova.[26]

Previously we demonstrated that 2-azahypoxanthine was a potent inhibitor of uricase *in vitro*, although it also caused slight inhibition of xanthine oxidase and hypoxanthine-guanine phosphoribosyltransferase.[4] In this work, we found that 2,8-diazahypoxanthine inhibited uricase even more strongly than 2-azahypoxanthine and also that, unlike 2-azahypoxanthine, it had no effect on xanthine oxidase or hypoxanthine-guanine phosphoribosyltransferase at the concentrations tested.

There is a considerable difference in the inhibitory activities of 2,8-diazahypoxanthine and related compounds on uricase shown in Tables 1 and 2. The compounds showed more potent inhibitions in Table 1 than in Table 2. The following possibilities may explain the differences: first, there is a difference in substrate concentration. Uric acid concentrations are $3{\cdot}33 \times 10^{-5}$ M in Table 1 and $5{\cdot}95 \times 10^{-4}$ M in Table 2. As these compounds showed competitive inhibition (Fig. 2), in which inhibitory activity decreases as substrate concentration increases, it is expected that inhibitions are more potent in Table 1 than in Table 2. Second, there is the possibility that these compounds are metabolized by liver homogenate in experiments shown in Table 2. 2-Azahypoxanthine is known to be attacked by xanthine oxidase,[27] but we have no information about the metabolism of 2,8-diazahypoxanthine or oxonate.

8-Azapurines, 8-azaxanthine, 8-azahypoxanthine and 8-aza-2-hydroxypurine, and many thiouric acids had been reported to be effective inhibitors of mammalian uricase *in vitro*.[18] Accordingly, we compared the inhibitory effects on uricase of 2,8-diazahypoxanthine, 8-azaxanthine and 8-azahypoxanthine. 2,8-Diazahypoxanthine was found to inhibit uricase more strongly than the 8-azapurines. The present studies also showed that 2,8-diazahypoxanthine, when administered intraperitoneally, caused about a two-fold increase in the serum urate level with a concomitant decrease in serum allantoin and that it strongly inhibited uricase in rat liver homogenates. Hence it appears that 2,8-diazahypoxanthine should also inhibit uricase in rat liver *in vivo*. However, even at a higher dose it failed to induce a rapid and marked increase in the uric acid level in rat serum. This suggests that uric acid formation in rats may be relatively slow.

2-Azahypoxanthine itself is attacked by xanthine oxidase with consequent changes

in the u.v. spectrum, as described by Shaw and Woolley.[27] In solution in 0·1 M phosphate buffer (pH 7·5) 2,8-diazahypoxanthine was not attacked by xanthine oxidase or uricase since even in large excess, these enzymes did not affect the u.v. spectrum (unpublished data). Hence it is not destroyed by these enzymes *in vivo*. However, the effect of a single dose of 2,8-diazahypoxanthine (125 mg/kg, i.p.) was of relatively short duration and frequent injections were required to obtain sustained inhibition of uricase, so this compound seems to be metabolized or excreted quite rapidly. Kinetic analysis demonstrated that the inhibitions of uricase by 2,8-diazahypoxanthine and 8-azaxanthine were competitive. The ideal uricase inhibitor for induction of hyperuricemia would be one which is irreversible and non-competitive, so that its activity would be independent of the level of the substrate, uric acid, and effective inhibition could be attained at low dosage levels. It is hoped that this ideal inhibitor may be found by appropriate substitution on 2,8-diazahypoxanthine.

8-Azaxanthine was a relatively strong inhibitor, while 8-azahypoxanthine was only moderately inhibitory *in vitro*. These results agree with those of Norris and Roush,[17] and Bergmann *et al.*[18] We found that 8-azahypoxanthine and 8-azaxanthine were both strong inhibitors of uricase *in vivo*. We are aware of no previous reports on these inhibitory effects *in vivo*. This finding suggests that 8-azahypoxanthine is converted to 8-azaxanthine *in vivo* by xanthine oxidase or by other enzymes involved in the hydroxylation of purine or pyrazolopyrimidine ring, such as hypoxanthine and allopurinol.[28–30]

To our knowledge, no drugs except oxonate have been used to induce hyperuricemia in animals.[20] In this work, 2,8-diazahypoxanthine was found to be the strongest inhibitor of uricase among the triazines and various purine analogs.

In man, hyperuricemia and hyperuricosuria lead to deposition of urate in the kidney tubules and interstitial tissues. This condition can be stimulated in rats by treatment with 2,8-diazahypoxanthine and urate, as described in the following paper.[31] We hope that our animal model will be valuable in studies on the physiological and psychological significance of uric acid.

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