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^{31}P NMR of Apicomplexans and the Effects of Risedronate on *Cryptosporidium parvum* Growth[1]

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High-resolution 303.6 MHz ^{31}P NMR spectra have been obtained of perchloric acid extracts of *Plasmodium berghei* trophozoites, *Toxoplasma gondii* tachyzoites, and *Cryptosporidium parvum* oocysts. Essentially complete resonance assignments have been made based on chemical shifts and by coaddition of authentic reference compounds. Signals corresponding to inorganic pyrophosphate were detected in all three species. In *T. gondii* and *C. parvum,* additional resonances were observed corresponding to linear triphosphate as well as longer chain polyphosphates. Spectra of *P. berghei* and *T. gondii* also indicated the presence of phosphomonoesters and nucleotide phosphates. We also report that the pyrophosphate analog drug, risedronate (used in bone resorption therapy), inhibits the growth of *C. parvum* in a mouse xenograft model. When taken together, our results indicate that all the major disease-causing apicomplexan parasites contain extensive stores of condensed phosphates and that as with *Plasmodium falciparum* and *T. gondii,* the pyrophosphate analog drug risedronate is an inhibitor of *C. parvum* cell growth. © 2001 Academic Press

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The phylum Apicomplexa is composed of a large number of organisms which pose serious health threats to both humans and animals. *Plasmodium* spp. are the etiologic agents of malaria, and are thus the cause of mortality and morbidity in areas native to their *Anopheles* vector, particularly in less developed nations (1). *Toxoplasma gondii* and *Cryptosporidium parvum* are opportunistic parasites which are transmitted to humans from intermediate animal hosts primarily by the encysted forms of these parasites (2, 3). The widespread distribution of these cysts, combined with the severity of the resulting infections (potentially lethal in the immunocompromised) and the lack of effective treatments, make novel aspects of parasite structure and biology attractive as chemotherapeutic leads.

In previous work, we found evidence for remarkably large levels of condensed inorganic phosphates: pyrophosphate, tripolyphosphate and tetrapolyphosphate, in the apicomplexan *T. gondii* (4), as well as in the trypanosomatids *Trypanosoma cruzi* (5, 6), *Trypanosoma brucei* (6) and *Leishmania major* (6). In the trypanosomatids, these condensed phosphates were present as solid aggregates in the acidocalcisomes, calcium, magnesium and phosphate containing organelles ubiquitous to both the apicomplexan and trypanosomatid parasites (7). In the trypanosomatids, the condensed phosphate levels were far higher than those of ADP and ATP (5, 6). We also found that nonhydrolyzable pyrophosphate analogs, bisphosphonates, currently used in bone resorption therapy and known to be potent inhibitors of the enzyme farnesyl pyrophosphate synthase (8–12), were inhibitors of cell growth in *P. falciparum, T. gondii, T. cruzi, T. brucei rhodesiense,* and *L. donovani* (13), the causative agents of malaria, toxoplasmosis, Chagas' disease, East African sleeping sickness, and visceral leishmaniasis.

Possession of some form of condensed phosphate is a trait common to many life forms. For example, the free-living protozoa *Tetrahymena pyriformis* (14), *Saccharomyces cerevisiae* (15), and *Acinetobacter johnsonii* (16) have all been shown to accumulate large amounts of polyphosphates and metals, and to utilize these com-

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pounds for the regulation of intracellular pH, osmotic balance and energy status by the action of polyphosphatases and phosphate transporters. Given that parasitic and nonparasitic organisms use polyphosphate to compensate for drastic environmental changes, it is probable that these compounds likewise play a critical role in the life cycle of the apicomplexan parasites. It is therefore somewhat surprising that there have been so few reports on the phosphate metabolism of these organisms.

The use of ^{31}P NMR spectroscopy for the study of *Plasmodium* spp. has been reported previously (17, 18), but these studies were primarily concerned with identifying the effects of parasitemia on the spectra of whole blood. In these early experiments, no novel metabolites were detected, most probably due to low spectral resolution and sensitivity. In addition, while an earlier NMR spectroscopic study of *T. gondii* tachyzoite extracts detected some condensed phosphates (4), complete resonance assignments were not reported. We therefore report here the ^{31}P NMR spectra of *P. berghei* and *T. gondii* perchloric acid extracts, under high-resolution conditions and using a high sensitivity (750 MHz ^{1}H) NMR spectrometer, together with results for *C. parvum*, a major cause of diarrheal disease worldwide, especially in the immunosuppressed. The NMR results indicate the presence of pyrophosphate in *P. berghei* and very high levels of this and other condensed phosphate species in *T. gondii* and in *C. parvum*. In the trypanosomatids, we previously showed that these condensed phosphates are primarily present in the solid state. We also argued that the sensitivity of these organisms to pyrophosphate analog (bisphosphonate) drugs might be attributable, at least in part, to their selective uptake by the parasites, due to the chemical similarities between bone and the solid-state Ca, Mg-P deposits in the parasites located in their acidocalcisomes. Since *C. parvum* was also found to exhibit major pyrophosphate and polyphosphate resonances, we reasoned that bisphosphonates might also have significant activity against this organism and we report herein preliminary findings using a mouse xenograft model which support this proposal.

MATERIALS AND METHODS

Culture methods. *P. berghei berghei* (strain NK64) were maintained *in vivo* in male Balb/c mice by weekly transfer infection, and trophozoites were isolated as described by Marchesini *et al.* (19). *T. gondii* tachyzoites (RH strain) were cultivated in bovine turbinate cells according to Moreno and Zhong (20) and were purified as described previously (21). *C. parvum* oocysts (AuCp1 isolate) were maintained by passage in newborn male calves, and purified from feces using sugar flotation and CsCl gradient centrifugation, as previously described (22). For NMR, all parasites were extracted with perchloric acid (6) and EDTA added to a final concentration of 20 mM prior to adjusting to pH 8. All extracts contained 10% D_2O (v/v) to provide a field-frequency lock.

Risedronate inhibition of C. parvum growth. To test the effects of the bisphosphonate risedronate (made as described previously, Ref. 13), on the growth of *C. parvum*, we used the mouse xenograft model also described previously (23, 24) in which fetal rabbit intestine is grafted onto an athymic nude mouse. The xenografts were inoculated with 10^7 *C. parvum* oocysts (60% excysted), in the absence (control) or presence of risedronate (100 μM). Xenografts inoculated in the presence of risedronate received additional drug (100 μl of 100 μM risedronate in sterile saline) once a day for 3 additional days. 72 hours following inoculation, mice were sacrificed and the xenografts subjected to tissue sectioning and histochemical staining to detect and quantify the presence of parasites, as previously described (24).

NMR spectroscopy. Phosphorus NMR spectra were acquired at 303.6 MHz using a Varian INOVA 750 MHz NMR spectrometer equipped with a 17.6 Tesla Oxford Instruments magnet. For perchloric acid extracts of *P. berghei* and *T. gondii*, 2048 or 4096 transients were typically collected at room temperature using 23 μs (90°) pulse excitation, a 20 kHz spectral width, 32k data points, and a 10 s recycle time. In the case of the *C. parvum* oocyst extract, 32,768 transients were acquired using a 2-s recycle delay. In most cases, proton decoupling was applied only during data acquisition, to remove NOE's and J-couplings. In some cases, proton-coupled spectra were also recorded for use in the identification of phosphomonoester resonances. The specific assignments of individual resonances were initially based on published chemical shifts (25, 26) and ^{1}H-^{31}P and ^{31}P-^{31}P scalar couplings, then were confirmed by coaddition of authentic reference compounds. The chemical shifts of all ^{31}P NMR spectra were referenced with respect to an 85% phosphoric acid external reference at 0 ppm (26), using the convention that high-frequency, low-field, paramagnetic or deshielded values are positive (IUPAC δ-scale). Processing of NMR spectra and the calculation of the relative intensities of the various ^{31}P NMR resonances were carried out by using the VNMR software package (Varian., Palo Alto, CA), and typically included baseline correction and a 10 Hz exponential line broadening, prior to Fourier transformation.

RESULTS AND DISCUSSION

We show in Fig. 1 the 303.6 MHz (^{1}H decoupled) ^{31}P NMR spectra of perchloric acid extracts of *P. berghei* trophozoites (A), *T. gondii* tachyzoites (B), and *C. parvum* oocysts (C). Resonance assignments obtained by using the methods described above are shown in Table 1. In the phosphomonoester region (3 to 5 ppm) of the *P. berghei* trophozoite and *T. gondii* tachyzoite spectra, phosphoethanolamine (peak A), AMP (B) and phosphocholine (C) were detected, while no significant resonances were detected in this region in the *C. parvum* oocysts. In all three species, inorganic phosphate (D) appeared at ~2.6 ppm and was the most prominent resonance in the spectrum. The observation that the phosphocholine peak is quite intense in the *P. berghei* spectrum may be of significance in the context of the importance of phosphatidylcholine biosynthesis in *Plasmodium spp.* and the growth inhibition generated by the bis-quaternary alkylammonium salts (27).

The upfield regions (−2 to −25 ppm) of all three spectra contain nucleotide phosphate and/or condensed inorganic phosphate resonances, but with markedly different distributions of these molecules amongst the three species. In the diphosphodiester regions (−10 to −12 ppm) of the *P. berghei* and *T. gondii* spectra,

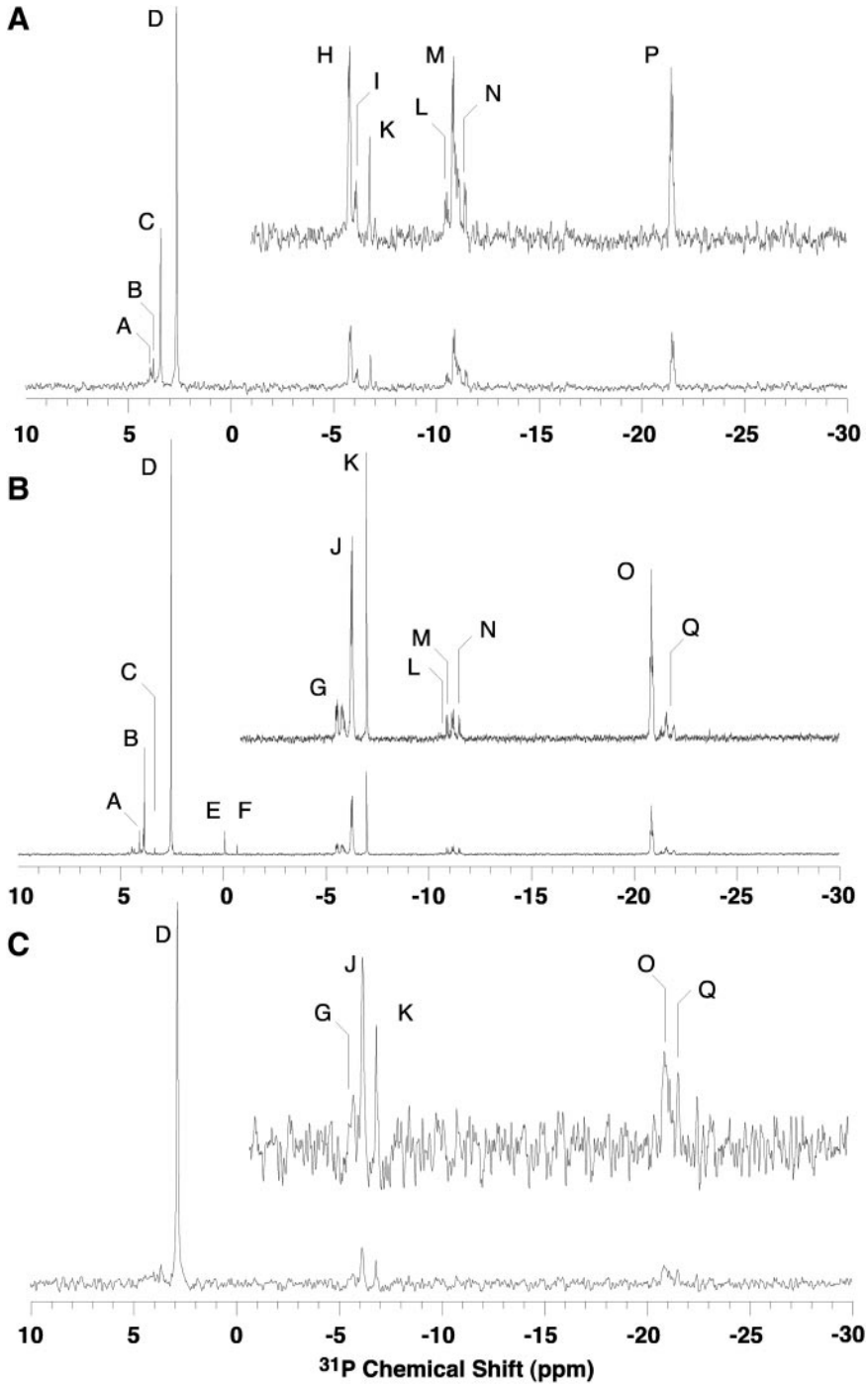

FIG. 1. 303.6 MHz ^{31}P NMR spectra of perchloric acid extracts of (A) *P. berghei* trophozoites, (B) *T. gondii* tachyzoites, and (C) *C. parvum* oocysts. All samples were pH 8, 20 mM EDTA and 10% D_2O. The spectra were the result of 4096 (A), 2048 (B), or 32,768 (C) free induction decays acquired at room temperature with 23 μs (90°) pulse excitation, a 10-s (A and B) or 2-s (C) recycle delay, 20 kHz spectral width and 32k data points. The FID's were zero-filled once and apodized with 10 Hz (A), 3 Hz (B) or 20 Hz (C) line broadening due to exponential multiplication, prior to Fourier transformation. Chemical shifts were referenced to 85% H_3PO_4 at 0 ppm (external reference). The insets show vertical expansions of the upfield (−2 to −23 ppm) region of each spectrum. Essentially complete resonance assignments are given in Table 1.

resonances corresponding to α-ADP (L), α-ATP (M) and the α- and β-phosphates of NADH (N) were detected. In the case of *P. berghei*, the α-phosphate resonance of ATP was accompanied by resonances corresponding to the β- and γ-phosphates (P and H, respectively), and likewise the α-phosphate of ADP was accompanied by the β-ADP resonance, at −6 ppm. In addition to these nucleotide phosphates, inorganic pyrophosphate

TABLE 1

^{31}P NMR Assignments for Perchloric Acid Extracts of *P. berghei* Trophozoites, *T. gondii* Tachyzoites, and *C. parvum* Oocysts

		Chemical shift (δ, ppm) and relative intensity (% total integral)					
		P. berghei trophozoites		*T. gondii* tachyzoites		*C. parvum* oocysts	
Peak	Assignment	δ	% total	δ	% total	δ	% total
A	Phosphorylethanolamine	—	—	3.90	1.4	—	—
B	AMP	—	—	3.85	5.7	—	—
C	Phosphorylcholine	3.34	12.8	3.35	0.5	—	—
D	Inorganic phosphate	2.56	24.9	2.55	46.6	2.59	69.8
E	*sn*-Glycerophosphorylcholine	—	—	−0.06	0.8	—	—
F	*sn*-Glycerophosphoethanolamine	—	—	−0.66	0.1	—	—
G	α-P's of polyphosphate	—	—	−5.57	5.3	−5.98	2.1
H	γ-P of ATP	−5.91	12.4	*	*	—	—
I	β-P of ADP	−6.20	3.2	*	*	—	—
J	α-P of tripolyphosphate	—	—	−6.24	16.5	−6.39	9.3
K	Pyrophosphate	−6.88	6.6	−6.97	7.8	−7.07	4.8
L	α-P of ADP		3.1	−10.50	0.1	—	—
M	α-P of ATP	−10.95	12.5	−10.90	0.3	—	—
N	NADH	−11.40	10.1	−11.33	1.3	—	—
O	β-P of tripolyphosphate	—	—	−20.84	12.7	−21.09	11.0
P	β-P of ATP	−21.56	12.2	*	*	—	—
Q	β-P of polyphosphate	—	—	−21.58	1.1	−21.77	2.9

Note. —, not detected; *, overlapped with other resonances.

($P_2O_7^{4-}$, resonance K) was detected in all three species, and in *P. berghei,* it was present at approximately 25% the level of ATP (in terms of molarity). This assignment was confirmed in some samples by coaddition of authentic pyrophosphate, and in others by the elimination of this signal by yeast inorganic pyrophosphatase. Previous ^{31}P NMR experiments in the literature did not detect any pyrophosphate (or longer chain polyphosphates) in the ^{31}P NMR spectra of whole mouse blood having *P. berghei* parasitemia levels of up to 83% (17). Our detection of pyrophosphate under high-resolution, high sensitivity conditions is not altogether unexpected, however, given the recent discovery of a plant-like proton translocating vacuolar pyrophosphatase (V/H$^+$-PPase) in the plasma and acidocalcisomal membranes of *P. berghei* and *P. falciparum* (the causative agent of human malaria) (19, 28), and the previous observation that all species possessing such a V/H$^+$-PPase also contain large levels of pyrophosphate. Given that little else is known about pyrophosphate-dependent metabolism in *Plasmodium spp.,* the search for other PPi-utilizing enzymes (without mammalian analogs) may reveal other important metabolic pathways which can be targeted for chemotherapy.

The *T. gondii* extract spectrum (Fig. 1B), taken under high-resolution conditions, exhibited a large pyrophosphate resonance (approximately 13 times the molarity of ATP), in addition to other resonances corresponding to the α- and β-phosphates of tripolyphosphate (J and O) and longer chain polyphosphates (G and Q). Interestingly, these extracts show no significant (cyclic) trimetaphosphate (−21.4 ppm), the primary breakdown product of longer chain polyphosphates by chemical degradation, indicating that these short chain inorganic phosphates are unlikely to be the result of very long chain polyphosphate hydrolysis during extract preparation (29, 30). The relative levels of these polyphosphates, together with their average chain length (~3.3 phosphates per molecule) are in remarkable accord with results obtained previously with perchloric acid extracts of trypanosomatids (6). In *T. brucei, T. cruzi* and *L. major,* we also showed that these molecules are localized to the parasite's acidocalcisomes, and that they are present as solid-like complexes with calcium and magnesium (31) and also have an average chain length of 3.3 phosphates per molecule. *T. gondii* tachyzoites also contain acidocalcisomes and a V-H$^+$-PPase (4, 7) and *T. gondii* is known to utilize pyrophosphate in place of ATP as an energy donor by the action of a pyrophosphate dependent phosphofructokinase (PPi-PFK) (32, 33). These results suggest that condensed phosphates in *T. gondii* may play a role as both counter-ions for high levels of divalent cations within the parasite's acidocalcisomes, in addition to being active substrates in cellular metabolism.

Due to the difficulties associated with obtaining large samples of *C. parvum,* the signal-to-noise ratio of the *C. parvum* oocyst spectrum shown in Fig. 1C is noticeably worse than that found with the other organ-

isms investigated, but it is still clear that the spectrum displays only condensed inorganic phosphate resonances. Moreover, the distribution of these compounds is generally similar to that found in the *T. gondii* extract. *C. parvum* oocysts have also been shown to have a pyrophosphate dependent metabolism (33, 34), possessing a PPi-PFK similar to that described for *T. gondii* (33) and *Eimeria tenella* (33), but other aspects of polyphosphate metabolism or storage have not yet been described.

Nevertheless, the fact that the *C. parvum* spectrum showed clear evidence of condensed phosphate species naturally suggested the possibility that that this organism might also have these phosphates present in a solid-state form and might therefore be particularly susceptible to growth inhibition by bisphosphonate inhibitors of the enzyme farnesyl pyrophosphate synthase, which are used extensively in bone resorption therapy. These compounds are known to bind to the calcium hydroxyapatite ($Ca_5(OH)(PO_4)_3$) in bone, increasing their local concentration, and we have argued elsewhere (31) that related uptake into the acidocalcisomes may provide a degree of selectivity against both trypanosomatid and apicomplexan parasites, all of which investigated so far possess these organelles. We therefore investigated whether the bisphosphonate, risedronate, had any effect on *C. parvum* proliferation. We used the mouse xenograft model of *C. parvum* infection and obtained the results shown in Fig. 2. Figure 2A shows a photomicrograph of a *C. parvum* infected mouse xenograft in which large numbers of parasites (small dots, arrowed) can be seen. Figure 2B shows an intestinal section of an infected mouse treated with risedronate (coinjected at the time of infection). Seventy-two hours after infection, there were no parasites detectable in the treated mouse xenograft, in sharp contrast to the result shown in Fig. 2A. In a separate experiment, the addition of 100 μM risedronate to a partially (60%) excysted preparation of *C. parvum* oocysts did not result in observable loss of sporozoite mobility or viability (data not shown).

When taken together, the results we have presented above give clear evidence for the presence of large levels of condensed inorganic phosphates in the apicomplexan parasites *P. berghei*, *T. gondii* and *C. parvum*. Thus, both trypanosomatid as well as apicomplexan parasites have unusually high levels of condensed inorganic phosphates. In *T. cruzi*, *T. brucei* and *L. major*, the polyphosphate stores are located as solid aggregates in the acidocalcisome organelles, and it is likely that the polyphosphate stores in *P. berghei* and *T. gondii* are likewise located in their acidocalcisomes. The presence of a vacuolar proton-pumping pyrophosphatase as well as a pyrophosphate-dependent phosphofructokinase in both *P. berghei* and *T. gondii* supports the idea that PPi-metabolism is of importance to both organisms. Moreover, the presence of major levels of PPi and the presence of a PPi-PFK in *C. parvum*

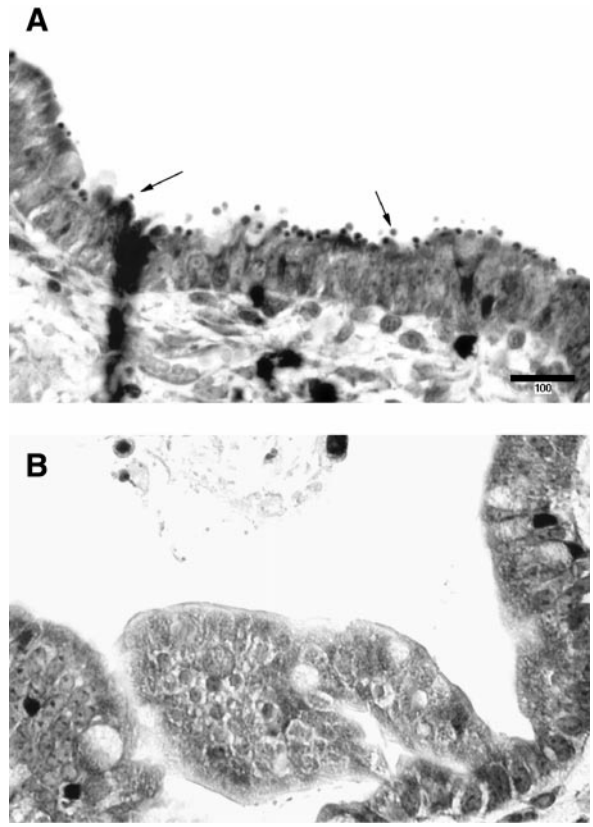

FIG. 2. Photomicrographs of sections of mouse xenografts (fetal rabbit intestine grafted onto athymic nude mice). (A) 72 h after inoculation with 10^7 *C. parvum* oocysts (control); (B) as A but treated with 100 μM risedronate. The parasites are indicated with an arrow in A. No parasites are observable in B.

suggests that PPi-metabolism is important in all of the apicomplexan parasites. At present, it is not known whether *C. parvum* contains acidocalcisomes. However, the fact that all other trypanosomatid and apicomplexan parasites having intense condensed phosphate ^{31}P NMR resonances do have these organelles, and the observation that condensed phosphates in all three systems investigated so far with solid-state NMR (*T. cruzi*, *T. brucei* and *L. major*) were present in the solid-state, suggested (as with *P. falciparum* and *T. gondii*) that pyrophosphate-analog drugs might be effective in inhibiting the growth of *C. parvum*. This was shown to be the case with the bisphosphonate drug risedronate, using a mouse xenograft model for *C. parvum* infection. In summary then: all three major human disease causing apicomplexan parasites contain large levels of condensed inorganic phosphates, an unusual pyrophosphate-based metabolism, and the growth of all three is inhibited by pyrophosphate-analog drugs currently used in bone resorption therapy.

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