

## BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—IV.

### UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY RESORCINOLS, TROPOLONES AND DIONES

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**Abstract**—Resorcinol derivatives, tropolones and diones, having  $pK_a$ 's in the range 3-7 uncouple oxidative phosphorylation in liver mitochondria. Many substituted resorcinols and tropolones are more potent uncouplers than the related phenols and it is possible to relate this enhanced potency to their lower  $pK_a$ 's than those of the corresponding phenols.

Optimal uncoupling activity was observed with compounds having  $pK_a$  close to 4.0 (e.g. 3, 5, 7-tribromotropolone, 2-phenyl-indan-1, 3-dione), and considerable lipophilic character.

The anti-inflammatory activity of some lipophilic resorcinols and a cycloheptimidazolone drug (RCH-314) is discussed in relation to the theory that ability to uncouple oxidative phosphorylation may determine anti-inflammatory activity.

Tetrabromocatechol inhibits yeast hexokinase.

LIGHTOWLER and Rylance<sup>1</sup> have recently shown that some dihydroxybenzenes, in particular resorcinol and 5-methylresorcinol (orcinol) derivatives, have anti-inflammatory (anti-oedema) activity in rats. Adams and Cobb<sup>2</sup> first suggested that anti-inflammatory drugs might uncouple oxidative phosphorylation: it has now been shown that no less than ten different types of anti-inflammatory drugs actually do so.<sup>3</sup> In view of the fact that certain resorcinols (derivatives of  $\beta$  and  $\gamma$  resorcylic acids) appeared to be rather more potent as uncouplers of oxidative phosphorylation than the corresponding phenols (salicylates),<sup>4, 6</sup> we compared the uncoupling activity of some simple resorcinol derivatives with that of the related phenols.

It is well known that some resorcinol derivatives, for example n-hexylresorcinol, have bacteriocidal properties which are rather superior to those of the corresponding phenols. In this respect they are paralleled by the tropolones.<sup>7-9</sup> We have now obtained evidence that several tropolone and resorcinol compounds may be more potent drugs than the related phenols at the subcellular level.

#### EXPERIMENTAL

##### *Materials*

Dibromo-orcinol was a gift from Dr. H. J. Rylance (Edinburgh Pharmaceutical Industries Ltd.),  $\beta$ -Tropolone was a gift from Dr. O. L. Chapman (Iowa State University, Ames, U.S.A.),  $\alpha$ -Tropolone was obtained from Koch Laboratories Ltd., Haverhill, Suffolk and  $\beta$ -thujaplicin from L. Light & Co. Ltd., Colnbrook. Other

compounds were obtained from Aldrich Chemical Co., Milwaukee, U.S.A.; Kodak Ltd., Liverpool; British Drug Houses Ltd., Poole; and Fluka A.G., Buchs, Switzerland; or synthesised by standard procedures. 4, 5-Benzotropolone was synthesised by condensing 1-methoxyacetone with *o*-phthaldialdehyde and hydrolysing the resulting methyl ether.<sup>10</sup> N-Picryl-2-aminophenol and N-picryl-2-aminoresorcinol were prepared by reacting the amine with 2, 4, 6-trinitrobenzene sulphonic acid in the presence of aqueous sodium bicarbonate according to the procedure described by Okuyama and Satake.<sup>11</sup> All compounds tested were first purified by recrystallisation to the correct melting point.

### *Biochemical procedures*

Oxidative phosphorylation by the mitochondrial fraction of rat livers was measured at 30° with succinate as substrate with and without added drugs.

Livers from white female rats (Oxford Wistar strain) were homogenised with 4 volumes of 0.44 M sucrose (containing 1 mM EDTA) at pH 6.8. The homogenate was first centrifuged at 600 *g* for 10 min to remove cell debris, nuclei, etc. and the supernatant fraction then centrifuged at 3000 *g* for 15 min to give the mitochondrial fraction used in all incubations. All operations were carried out below 4°. The mitochondrial fraction isolated from approximately 1 g wet weight of liver and resuspended in 2 ml of a medium containing 0.21 M mannitol and 0.07 M sucrose was added to each Warburg flask which contained 50  $\mu$ moles inorganic phosphate as 0.1 M sodium phosphate pH 6.8, 30  $\mu$ moles sodium succinate at pH 6.8, 25  $\mu$ moles potassium chloride, 2.5  $\mu$ moles adenosine triphosphate (ATP),\* 30  $\mu$ moles, glucose, 15  $\mu$ moles potassium fluoride and 15  $\mu$ moles magnesium chloride in a total volume of 1 ml in the main compartment; 20  $\mu$ g (approximately 2.0 international units) of crystalline yeast hexokinase (Boehringer & Soehne, Mannheim) contained in 0.2 ml 1% (w/v) aqueous glucose was added to the side arm of each flask and 0.2 ml 1% (w/v) aqueous sodium hydroxide to the centre wall.

Drugs were added to the incubation medium in 25  $\mu$ l of N, N-dimethylformamide (DMF); 25  $\mu$ l of DMF being added to drug-free controls. Following a 7 min pre-incubation period, the side arm and main compartment contents were mixed and oxygen uptake was measured for a period not exceeding 25 min. The reaction was terminated by adding 5 ml 5% (w/v) aqueous trichloroacetic acid. The contents of two flasks were acidified after the 7 min preincubation period. Phosphate uptake was calculated as the difference between the phosphate content of these flasks and those flasks incubated after mixing side arm and main compartment contents. Although it was found that the absolute phosphorylation quotient (P/O ratio) of the drug-free controls (at least two for each mitochondrial preparation) varied from preparation to preparation within the limits 1.2 to 1.7, the degree of uncoupling by a given concentration of a drug was fairly constant with different preparations (see Ref. 6).

The effect of drugs on the hexokinase was determined by measuring glucokinase activity at 30° at pH 8–9 by the method of Darrow and Colowick.<sup>12</sup> Mitochondrial adenosinetriphosphatase (ATPase) activity was measured at room temperature.<sup>6</sup>

\* Abbreviations used throughout the text are:

ATPase = Adenosinetriphosphatase.

ATP = Adenosine triphosphate.

DMF = N, N-Dimethylformamide.

### *Partition coefficients*

These were determined approximately at  $18 \pm 2^\circ$  as follows; 25  $\mu$ l of a solution of the compound in DMF was added to 5 ml 0.1 M phosphate buffer pH 6.8 and the buffer pH 6.8 and the light absorption in 1 cm cells, measured with a spectrophotometer at a suitable absorption maximum. The solution was extracted by shaking with 5 ml of the organic solvent (chloroform or *n*. octanol). After separation of the layers by centrifugation the light absorption of the aqueous layer was measured again. The partition coefficient was calculated as the difference in these two values divided by the residual absorption of the aqueous phase: the values given are the mean of at least three determinations.

### *Acid dissociation constants*

pK<sub>a</sub>'s were measured spectrophotometrically at  $18 \pm 2^\circ$ ,<sup>13</sup> if not available.<sup>14-16</sup>

## RESULTS

The P/O ratio for the mitochondrial fraction incubated without drugs never attained the "theoretical" value of 2.0 with succinate as a hydrogen donor, and was sometimes as low as 1.2. This ratio was not significantly raised by: (a) washing the mitochondria (i.e. resuspending the mitochondria in sucrose and recentrifuging them) or (b) by including serum albumen in the medium for isolating the mitochondria. However, when serum albumen (free of citrate, 3 mg/ml) was included in the incubation medium, P/O values were obtained which were up to 0.4 greater than P/O values for parallel incubations without albumen. This would indicate that fatty acids were being continually liberated during incubation at  $30^\circ$ ; these acids are known to uncouple oxidative phosphorylation. Unfortunately it was not possible to include serum albumen routinely in the incubation medium when testing for (uncoupling) drug activity, as so many of the compounds examined in this study were to some degree bound to serum albumen.

### *Uncoupling by tropolones (Table 1)*

Hagihara *et al.*<sup>5</sup> have shown that derivatives of 5-nitrosotropolone uncouple oxidative phosphorylation.

Tropolone itself, which is a stronger acid than either phenol or the dihydroxybenzenes, at submillimolar concentrations uncoupled oxidative phosphorylation (in contradistinction to phenol and the dihydroxybenzenes). In this respect it is more potent than salicylic acid<sup>6</sup> and approximately 1/10th as active as the classic uncoupling agent, 2, 4-dinitrophenol (Table 1). The following tropolone congeners did not significantly affect oxidative phosphorylation when tested at 1 mM;  $\alpha$ -tropolone methyl-ether, 2 chlorotropolone, 2-aminotropolone, the isomeric  $\beta$ -tropolone and 4, 5-benzotropolone (which is a much weaker acid than  $\alpha$ -tropolone). Colchicine, a naturally occurring tropolone methyl ether, was likewise inactive but its desmethyl derivative, colchicine (a true tropolone) did uncouple oxidative phosphorylation. Neither colchicine nor  $\beta$ -thujaplicin (hinokitiol, 4-isopropyltropolone) were more potent than tropolone itself even though both were more lipophilic than  $\alpha$ -tropolone, as suggested by their higher coefficients for partitioning into two organic solvents.

With tropolone,  $\beta$ -thujaplicin and colchicine it was impossible to obtain complete uncoupling of oxidative phosphorylation even at millimolar concentrations. Table 2

illustrates this phenomenon, which was not seen in the more potent tropolone derivatives. The "levelling off" response is not due to any readily discernible saturation of the incubation medium with these tropolones. Tropolone added to the incubation medium in 25  $\mu$ l DMF to give a final concentration of 10 mM remained in solution while colchicine was soluble at 5 mM.

Tropolone behaved like known uncoupling agents (salicylate, 2, 4-dinitrophenol) and consistently stimulated the hydrolysis of ATP by (latent) mitochondrial ATPases; for example, 1 mM tropolone caused a 350 per cent stimulation of ATPase activity.

TABLE 1. CORRELATION OF THE UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY CERTAIN PHENOLS, RESORCINOLS AND TROPOLONES WITH THEIR ACID DISSOCIATION CONSTANTS AND LIPOPHILIC CHARACTER

Drug	Concentration $\times 10^{-4}$ M	P/O (% of control)	pK <sub>a</sub>	Partition coefficient <i>n</i> , octanol    chloroform	
Phenol	50	86	9.9	0.6	2.6
Resorcinol	25	95	9.4		
$\alpha$ -Tropolone	2	50	6.9	2.0	
$\beta$ -Tropolone	20	100	5.4	0.25	
Colchicine	5	50	6.7	8.4	4
Colchicine	10	100			
4, 5-Benzotropolone	20	50	10.0	> 25	> 25
$\beta$ -Thujaplicin	2	47	7.3	7.0	
2-nitrophenol	5	95	7.1	12	> 25
2-nitroresorcinol	2	65	6.4	6.5	> 10
4-nitrophenol	2	24	7.1	3.8	5.7
4-nitroresorcinol	1	25	6.3	2.7	0.5
5-nitrotropolone	1	30	3.3	0.4	< 0.05
2, 4-dinitrophenol	0.2	50	4.0	0.3	0.3
2, 4-dinitroresorcinol	1	66	3.1	0.2	< 0.05
4-nitrosophenol	10	65	6.5		
4-nitrosoresorcinol	7	48	5.2		
5-nitrosotropolone	2	20	5.7	0.5	
4, 6-dibromo- <i>o</i> -cresol	2.0	59			
2, 4-dibromorcinol	2.5	96			
2, 4, 6-tribromophenol	0.5	26	5.9		
2, 4, 6-tribromoresorcinol	2.0	70	5.3		
2, 4, 6-tribromorcinol	2.0	32			
2, 4, 6-tribromo-phloroglucinol	7.5	65	5.7		
3, 5, 7-tribromotropolone	0.5	4	4.3	0.9	

TABLE 2. RELATION BETWEEN PERCENTAGE COUPLING AND CONCENTRATION OF UNCOUPLING AGENT FOR SOME TROPOLONES

Concentration $\times 10^{-4}$ M	Tropolone	Colchicine	$\beta$ -Thujaplicin
	% coupling (as % of control P/O ratio)		
0.5			82
1.0	61		64
2.0	50		46
2.5	43	67	
5.0	41	50	44
10.0	41	48	32

The mononitroso, mononitro and tribromo derivatives of tropolone were rather more potent in uncoupling oxidative phosphorylation than tropolone itself, so resembling the corresponding phenol derivatives in their relationship to phenol. With the exception of 5-nitrotropolone, these substituted tropolones were more active than the corresponding substituted phenols.

#### *Uncoupling by resorcinols*

Table 1 shows that both the 2- and 4-mononitroresorcinols are each more active drugs (and stronger acids) than the corresponding 2- and 4-mononitrophenols. By contrast, 2, 4-dinitroresorcinol and tribromoresorcinol are each less potent uncouplers of oxidative phosphorylation than the related phenols. Likewise a dibromomethylresorcinol (orcinol) is less active than a dibromo-methylphenol (*o*. cresol). Table 3 indicates that a number of other resorcinol derivatives uncouple oxidative phosphorylation and that this property is reasonably specific to 1, 3-dihydroxy compounds. 4-*n*-Hexanoylresorcinol was consistently more potent than 4-*n*-hexylresorcinol in repeated assays suggesting that the  $\alpha$ -ketonic group confers some drug activity, being *ortho* to a phenolic group (cf. *o*. hydroxyacetophenone<sup>6</sup>), which is additive to that of the resorcinol. Tribromophloroglucinol considerably inhibited respiration (by more than 50 per cent) at concentrations at which it also uncoupled oxidative phosphorylation.

#### *Uncoupling by catechols*

Only at relatively high concentrations (5 mM) did catechol itself have any effect on oxidative phosphorylation. 4-Nitrocatechol was a less potent drug than either 4-nitroresorcinol or 5-nitrotropolone. Tetrabromocatechol appeared to be a powerful uncoupler of oxidative phosphorylation comparable with dinitrophenol but it also inhibited hexokinase at concentrations above 10  $\mu$ M. At 25  $\mu$ M it virtually abolished oxidative phosphorylation but inhibited hexokinase by approximately 70 per cent. However, at 50  $\mu$ M tetrabromo-catechol stimulated mitochondrial ATP-ase fourfold and in this respect behaved like an uncoupling agent.

All the other active compounds in Table 1 and 3 had no effect on hexokinase and should therefore be considered as true uncouplers.

#### *Uncoupling by 1, 3-diketones*

Dihydroresorcinol and dihydrocatechol (cyclohexane-1, 3 and 1, 2-diones and dimedone (5, 5'-dimethyl-cyclohexane-1, 3-dione) did not uncouple oxidative phosphorylation at 5 mM, but partially inhibited respiration at this concentration. In this respect they resembled some of the barbiturates (e.g. amytal) which are also 1, 3-dioxo compounds and inhibit respiration.

Certain more lipophilic  $\beta$ -diketones were found to be fairly potent uncoupling agents (Table 3). Dibenzoylmethane was too insoluble for adequate testing but failed to uncouple oxidative phosphorylation at 0.25 mM. Indane-trione (ninhydrin) was more potent than indane-1, 3-dione in uncoupling.

A few dimedone derivatives of aliphatic aldehydes were tested and found to uncouple oxidative phosphorylation: their activity increased with increasing chain length of the aldehyde. Formaldehyde dimedone was inactive in saturated solutions at pH 6.8 (*ca.* 1 mM).

TABLE 3. UNCOUPLING ACTIVITIES OF SOME MONO- AND DIHYDROXY-PHENOLS, TROPOLONES AND DIONES

Drug	Concentration $\times 10^{-4}$ M	P/O (% of control)	Drug	Concentration $\times 10^{-4}$ M	P/O (% of control)
2-aminoresorcinol	20	100	Catechol	50	70
N-picryl-2-aminoresorcinol	0.5	10	4-nitrocatechol	1	58
4-( <i>p</i> -nitrophenyl)-azoresorcinol, "Magneson"	0.2	25	3, 4, 5, 6-tetrabromocatechol	0.25	4
4-phenylazoresorcinol	0.2	80	Benzoylacetone	25	40
4-pyridylazoresorcinol	0.5	100	Indan-1-one	10	100
2, 4-dinitrosorcinol	2	90	Indan-1, 3-dione	5	32
4- <i>n</i> . hexylresorcinol	10	53	Indan-1, 2, 3-trione	1	25
4- <i>n</i> . hexanoylresorcinol	4	51	2-Phenylindan-1, 3-dione	0.5	35
1, 3-dihydroxynaphthalene	4	54	Dimedone	50	90
2, 7-dihydroxynaphthalene	10	100	Dimedone-derivatives of acetaldehyde	10	100
N-picryl-2-aminophenol	10	100	isobutyraldehyde	10	55
4-phenylazophenol	0.2	100	2-methylvaleraldehyde	5	20
5-phenylazotropolone	0.2	5			

## DISCUSSION

This investigation has shown that many resorcinol and tropolone derivatives are able to uncouple oxidative phosphorylation and often do so to a greater degree than the corresponding phenols at the same concentration. It is possible to collate this superior uncoupling activity of the resorcinol or tropolone derivatives with at least three different, but not mutually exclusive properties of these compounds. They are (i) chemical structure, particularly as it governs the ability to complex metal ions or form internal hydrogen bonds; (ii) relative strengths as acids; (iii) lipophilic character, i.e. ability to pass from a neutral aqueous medium across lipid membranes and to concentrate within the lipid-rich mitochondria, the site of oxidative phosphorylation. We have tried to obtain some estimate of this last parameter by measuring the relative partitioning of a series of related compounds between either octanol or chloroform and a quasi-physiological salt solution at pH 6.8.

*Tropolones*

$\alpha$ -tropolone itself is considerably more acidic than phenol or catechol ( $pK_a$  9.45) and forms complexes with many metal ions (e.g. cupric, ferric) so resembling catechol and salicylic acid. Tropolone is evidently more lipophilic than these latter phenols (metal chelators) probably because of hydrogen bonding between the two oxygen atoms which diminishes the hydrophilic character of the hydroxyl group. (This effect is also seen in the higher partition coefficients and volatility of 2-nitrophenol compared with 4-nitrophenol). That lipophilic character alone does not determine uncoupling activity is shown by the inactivity of 4, 5-benzotropolone, a weak acid because the benzotropolone anion is not stabilised by  $\pi$  electron delocalisation to the same extent as is the tropolone anion. In this instance it is improbable that the fused benzene nucleus abolishes uncoupling activity by some bulk effect since analogous molecules, e.g. naphthoresorcinol (Table 3) and *o*. hydroxynaphthoates<sup>6</sup> are more active in uncoupling oxidative phosphorylation than resorcinol and salicylic acid respectively. Therefore the inactivity of 4, 5-benzotropolone must be due rather to its high  $pK_a$ : that of  $\beta$ -tropolone to its considerable hydrophilic character indicated by a low partition coefficient.

*Resorcinols*

Like tropolones, resorcinols form complexes with many metal ions more readily than do the corresponding phenols. Thus naphthoresorcinol (1, 3-dihydroxynaphthalene) gives a red brown coloration with ethanolic ferric chloride whereas  $\beta$ -naphthol and 2, 7-dihydroxynaphthalene give a yellow-green coloration.

Resorcinol exhibits some of the character of its tautomeric  $\beta$ -diketone (cyclohexene-1, 3-dione)<sup>18</sup> and as shown here, several such diketones will uncouple oxidative phosphorylation. The mono-enol tautomer of such  $\beta$ -diketones possess a structure also present in those congeners of salicylic acid which uncouple oxidative phosphorylation,<sup>6</sup> and in phenylbutazone, which carry anti-inflammatory activity.

Naphthoresorcinol ( $pK_a$  7.3) and *n*. hexanoylresorcinol ( $pK_a$  7.3) were more potent uncoupling agents than 2, 7-dihydroxynaphthalene ( $pK_a$  9.6) and *n*. hexylresorcinol ( $pK_a > 9.5$ ) indicating that the acidic ionisation constant is one determinant of uncoupling activity amongst resorcinols. The finding that 2, 4-dinitroresorcinol is a poorer uncoupling agent than 2, 4-dinitrophenol suggests that it is too acidic for

optimum activity but it is actually a more potent drug in this context than salicylic acid ( $pK_a$  3.0).<sup>6</sup> Since both 2- and 4-nitroresorcinols are respectively more acidic and more potent drugs *in vitro* than the 2- and 4-nitrophenols it would seem that optimum drug activity is approached with a  $pK_a$  of approximately 4 (i.e. that of 2, 4-dinitrophenol or 3, 5, 7-tribromotropolone). A number of lipophilic arylacetic acids with  $pK_a$ 's of this order, but no other simple property in common with salicylic acid or phenylbutazone are known to uncouple oxidative phosphorylation and to manifest anti-inflammatory (anti-rheumatic) activity.<sup>19</sup>

### 1, 3 Diones

The uncoupling activity of these compounds can apparently be correlated with their acid ionisation constants. The  $pK_a$  of indan-1, 3-dione was found to be approximately 7.2, that of its 2-phenyl derivative is reported as 4.2.<sup>20</sup> The latter is therefore close to that of 2, 4-dinitrophenol and it is a fact that 2-phenyl-indan-1, 3-dione closely resemble this dinitrophenol in its uncoupling potency.

Benzoylacetone and dibenzoylmethane have  $pK_a$ 's greater than 8.4<sup>21</sup> and despite their considerable lipophilic character, this evidently precludes them from being very active inhibitors of mitochondrial phosphorylation. Dimedone and dihydroresorcinol have  $pK_a$ 's of 5.2 and 5.3 respectively and might be expected to exhibit uncoupling activity (cf. tribromoresorcinol,  $pK$  5.3) but are evidently too hydrophilic to do so. However, some far less water soluble derivatives, the aldehyde dimedones, were moderately effective in uncoupling oxidative phosphorylation. That this was due to increased lipid character, rather than increased acidity, was shown when the  $pK_a$  of the dimedone derivative of isobutyraldehyde was found to be 5.15.

These findings support the general conclusion that the mono-enolone structure is an uncoupling pharmacophore whether it be present in saturated 1, 3-diketones, with at least one hydrogen atom at the (intermediate) methylene group, or in resorcinols, or in other drugs (salicylate, etc.).

### Relationships to *in vivo* pharmacological activity

Lightowler and Rylance<sup>1</sup> found that only lipophilic resorcinols among the isomeric dihydroxybenzenes examined exhibited anti-inflammatory activity in rats, and that while 2, 4, 6-tribromo-5-methylphenol (at 150 mg/Kg) and 2, 4, 6-tribromoresorcinol (at 300 mg/Kg) were inactive, 2, 4, 6-tribromo-oreinol and 2, 4, 6-tribromophloroglucinol were effective drugs *in vivo* (at 150 mg/Kg and 38 mg/Kg respectively). The *in vivo* inactivity of the phenol, which would be expected to be more potent than the tribromoresorcinol in uncoupling oxidative phosphorylation *in vivo*, might be explained by its hyperlipophilic character; i.e. by its being too lipophilic to be able to dissociate from plasma proteins, resist excretion and/or detoxification to be able to concentrate within the connective tissues. (Some evidence for this has been presented;<sup>22</sup> *o*, hydroxynaphthoates were found to be less potent than salicylic acid *in vivo* even though they are much more potent uncouplers of oxidative phosphorylation *in vitro* than salicylates.) The *in vivo* activities of tribromoresorcinol and tribromo-oreinol parallels their relative *in vitro* activities in uncoupling oxidative phosphorylation. Together with other evidence<sup>3</sup> this suggests that ability of a drug to uncouple oxidative phosphorylation within the inflamed (and other?) tissues may largely determine its anti-inflammatory activity.



The high *in vivo* activity of tribromophloroglucinol<sup>1</sup>, a feeble uncoupling agent, may be due in part to its property of inhibiting cellular respiration. Other respiratory inhibitors are known to exhibit anti-inflammatory activity.<sup>23</sup>

1-Benzylcycloheptimidazol-2-(1H)-one (RCH-314) has been recently disclosed as a novel type of anti-inflammatory (anti-oedema) agent.<sup>24</sup> We have found it to be a very feeble uncoupler of oxidative phosphorylation (50 per cent at 3 mM) but it does activate mitochondrial ATPase(s) at these high levels. It is conceivable that it might be degraded *in vivo* to yield tropolone, which is approximately ten times as potent *in vitro* as RCH-314.

The  $pK_a$ 's for 2- and 4-mononitroresorcinols given in Table 1 differ from those in the literature.<sup>14, 15</sup> Indantrione hydrate (ninhydrin) is with an acid  $pK$  of approximately 9.0, as determined spectrophotometrically and by direct titration of aqueous solutions.

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