

Pathological characterization of testicular tumours and lymphomas-leukaemias, and of their precursors observed in Sprague-Dawley rats exposed to methyl-tertiary-butyl-ether (MTBE)

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Summary

In a previous report the authors published the results of an experimental carcinogenicity study on methyl-tertiary-butyl-ether (MTBE). The compound was tested on groups of 120 Sprague-Dawley rats from the colony of the Cancer Research Centre (CRC) of Bentivoglio (BT), of the European Ramazzini Foundation (FR), exposing both sexes (60 males and 60 females) by ingestion (gavage), at the doses of 1000, 250, 0 mg/kg b.w., in olive oil, once daily, 4 days weekly, for 104 weeks. The animals were kept under observation until spontaneous death. The results showed that MTBE causes an increase in interstitial cell adenomas (Leydig cell tumours) of the testis, as well as lymphomas and leukaemias and haemolymphoreticular dysplasias in female animals. The present report gives the results of a further systematic collegial review of testicular and haemolymphoreticular neoplasias and correlated pathologies. The results of this review confirm and strengthen the results of the previous report. In the groups of animals exposed at doses of 1000, 250 and 0 mg/kg b.w. of MTBE, the following percent incidences have been found, respectively: 15.0, 13.3 and 6.7 of interstitial cell hyperplasia of the testis; 18.3, 8.3 and 5.0 of interstitial cell adenomas of the testis; 20.0, 26.7, and 5.0 of haemolymphoreticular dysplasias (all lymphoimmunoblastic), and 20.0, 11.7, and 3.3 of haemolymphoreticular neoplasias of lymphatic origin (lymphoblastic lymphomas, lymphoblastic leukaemias, lymphoimmunoblastic lymphomas, these last being the large majority). The lung is the most frequent site of haemolymphoreticular neoplasias. Interstitial cell adenomas of the testis do not seem to be related to regressive changes. The only 4 multifocal tumours of this type were observed in the group exposed to the highest dose.

Key words: leukaemia, testicular tumour, MTBE, rat

Riassunto

In un precedente resoconto gli Autori hanno pubblicato i risultati di uno studio sperimentale di cancerogenicità del metil-ter-butil etere (MTBE). Il composto è stato saggiato su gruppi di 120 ratti Sprague-Dawley della colonia del Centro di Ricerca sul Cancro (CRC) di Bentivoglio, della Fondazione Europea Ramazzini (FR), di entrambi i sessi (60 maschi e 60 femmine) mediante ingestione (gavaggio), alle dosi di 1000, 250, 0 mg/kg p.c., in olio d'oliva, una volta al giorno, 4 giorni alla settimana, per 104 settimane. Gli animali sono stati tenuti sotto osservazione fino a morte spontanea. Da quei risultati scaturiva che l'MTBE produce un aumento di adenomi interstiziali del testicolo (tumori delle cellule di Leydig), e di linfomi e leucemie e di loro precursori, negli animali femmine. Il presente resoconto riferisce i risultati di una ulteriore, sistematica e collegiale revisione della patologia testicolare ed emolinforeticolare. I risultati di questa revisione confermano e rafforzano le risultanze del precedente resoconto. In gruppi di animali esposti alle dosi di 1000, 250, e 0 mg/kg p.c. di MTBE sono state riscontrate rispettivamente le seguenti incidenze percentuali: 15.0, 13.3 e 6.7 di iperplasie interstiziali del testicolo; 18.3, 8.3 e 5.0 di adenomi interstiziali del testicolo; 20.0, 26.7, e 5.0 di displasie emolinforeticolari (tutte linfoimmunoblastiche); e 20.0, 11.7 e 3.3 di neoplasie emolinforeticolari di origine linfatica (linfomi linfoblastici, leucemie linfoblastiche e linfomi linfoimmunoblastici, questi ultimi in larga maggioranza). La localizzazione più frequente delle neoplasie emolinforeticolari è il polmone. Gli adenomi a cellule interstiziali del testicolo non sembrano correlati a lesioni degenerative. Gli unici tumori di questo tipo multifocali sono stati osservati nel gruppo trattato alla dose più alta.

Parole chiave: leucemia, tumori testicolari, MTBE, ratto

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Introduction

In recent years the use of oxygenated additives has been introduced and expanded for the production of formulated gasolines. The proposed oxygenated additives include methyl alcohol, ethyl alcohol, methyl-tertiary-butyl ether (MTBE), ethyl-tertiary-butyl ether (ETBE), tert-amyl-methyl ether (TAME), and di-isopropyl ether (DIPE). The use of oxygenated gasolines is intended to improve the combustion process and more specifically, to significantly reduce motor vehicle CO emission, especially during low temperatures in winter months.

The most commercialized oxygenated additive is at present MTBE, which proves to be nowadays one of the most widely produced synthetic compounds in the world. Presently MTBE is produced worldwide at the rate of about 20 million tons per year. It is mainly consumed in the United States, particularly in the cooler states.

Incredibly, when the use of MTBE was started, sufficient toxicological information, with particular regard to the carcinogenic effect, was not available and, even at present, the existing information on the carcinogenicity of the compound must be considered preliminary and not sufficient to disclose all the carcinogenic potentialities of MTBE. Yet the production of MTBE is predicted to expand on a worldwide scale.

Ten years ago, in the framework of a series of experiments conducted to evaluate the carcinogenic effects of oxygenated additives, we undertook a first pilot long-term experimental research study (long-term bioassay) to assess if, and to what extent, MTBE could pose carcinogenic risks. The study was performed by exposing Sprague-Dawley rats by oral administration. Preliminary results were given at the Collegium Ramazzini Conference on "Present knowledge on the hazards of conventional and new gasolines" held in Carpi, Italy, on October 29, 1993 in the framework of the "Annual Ramazzini Days, 1993". The results were then extensively published in 1995 (Belpoggi, Soffritti and Maltoni, 1995).

Our study showed that MTBE, in the experimental conditions tested, was carcinogenic since it caused a statistically significant increase in testicular tumours, specifically interstitial cell adenomas (Leydig cell tumours), as well as lymphomas and leukaemias, which was dose-related. The compound also caused an increase in dysplastic proliferation of lymphoreticular tissue at various sites.

In another long-term carcinogenicity study (known as the ARCO study), supported in the United States by a group of producers and users of MTBE, the compound was tested on both male and female Fischer 344 rats and CD-1 mice. In this study, MTBE was administered by inhalation at concentrations of 8000, 3000, 400, and 0 ppm. The results of this study (released but not published in 1992) showed MTBE to be carcinogenic (Burleigh-Flayer, Chun and Kintish, 1992; Chun, Burleigh-Flayer and Kintish, 1992): in the studied experimental conditions the compound increases the incidence of a rare type of kidney tumour (renal tubular adenomas and carcinomas) in male rats exposed to high-dose and particularly to mid-dose. This was statistically significant when compared to concurrent controls. Further, a mid-dose-related increase (still statistically significant) in "interstitial cell adenoma of the testes" was noted in male rats. This increase was difficult to evaluate because of the high background of such tumours occurring spontaneously in their historical controls (Haseman *et al.*, 1985).

The results of the ARCO study also showed that MTBE causes an increase in the incidence of hepatocellular adenomas in female mice. In the ARCO study on rats there was a high and apparently dose-related incidence of non-cancer renal toxicity in both sexes, an effect which was not observed in our experiment.

Our results and the results of the ARCO study, although different, converge in exposing the carcinogenic potential of MTBE. Moreover, the combined results of the two studies appear to indicate that MTBE is a trans-species, multistrain, multisite carcinogen.

Some "industry-oriented experts" in USA challenged the fact that in our 1995 reports we aggregated the incidence of lymphomas and leukaemias. To those criticisms we reply that: 1) lymphomas and leukaemias (such terms including all types of haemolymphosarcomas and of leukaemias) are neoplasias arising from the haemolymphoreticular tissues; 2) the aggregation of "lymphomas and leukaemias" in experimental carcinogenesis is a long-standing practice; and 3) in the case of lymphocytic-lymphoblastic sarcomas and lymphatic leukaemias, there are borderline cases in which it is difficult, and some time even impossible, to clearly identify what picture is prevalent. Maliciously we believe that the aim of this criticism was not to know more about pathology but rather, by pathological subdividing, to lower the statistical strength of our data (a well known over-abused practice).

Given the industrial and economic impact of MTBE, we have been urged by many parties involved, and particularly USA Institutes and Agencies, to provide more information on the pathological characteristics of the testicular and haemolymphoreticular oncological lesions observed in our study.

In response to this request, this report provides data on the histocytotypes, sites and incidence of the pathological changes observed in our experiment dealing with the testes and the haematopoietic system.

Materials, methods and previously reported results

The experimental material and methods of the original study can be summarized as follow.

MTBE was supplied by a US gasoline company; its purity was higher than 99%. During the experiment, the compound was stored at a temperature of 4°C.

Male (M) and female (F) Sprague-Dawley rats from the colony of the Bentivoglio (BT) Cancer Research Centre (CRC) of the European Ramazzini Foundation (FR) were used. This colony of rats has been employed for various experiments in the BT Laboratory for nearly 25 years. Historical data are available on more than 10,000 historical controls, kept under observation for life-span and submitted to systematic necropsies and standardized histopathological examination. Therefore, data on the expected incidence of the different types of tumours in control animals and on its fluctuations are available.

After weaning, at 4-5 weeks of age, the experimental animals were identified by ear punch, randomized in order to have not more than one male and one female of each litter in the same group, and housed 5 per cage. The animals were 8 weeks old at the start of the experiment.

The plan of the experiment is shown in Table 1.

The experiment on MTBE was started in September 1988.

Every single dose of MTBE was administered by gavage as 1 ml of extravirgin olive oil solution, once daily, 4 days weekly (Mon-

Table 1 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Plan of the experiment

Group	Daily dose (mg/kg b.w. in olive oil)	Age at start (weeks)	Animals	
			Sex	No.
I	1000	8	M	60
			F	60
			M+F	120
II	250	8	M	60
			F	60
			M+F	120
III	0 (a) (control)	8	M	60
			F	60
			M+F	120

(a) Olive oil alone

day and Tuesday, Thursday and Friday), for 104 weeks. Administration of the compound at the highest dose (5-6 days weekly) would not have been tolerated by the rats. The solutions were prepared weekly and maintained at 4°C. Control animals were given 1 ml of extra virgin olive oil alone.

The animals were kept under observation until natural death, under highly standardized housing, diet and experimental conduct conditions, identical to those used in the BT Laboratory over the last 25 years. In the experiments performed at the BT Cancer Research Centre, the animals are usually allowed to live until natural death. By doing so, it is possible to allow the development of all the neoplastic potentialities. Mean daily feed and drinking water consumption were determined once weekly for the first 13 weeks from the start of the experiment, then every 2 weeks, until 112 weeks of age. Individual animal weight was measured once weekly from 8 weeks of age for the first 13 weeks, and then every 2 weeks until 112 weeks of age, and every 8 weeks until the end of the experiment. In order to detect and register all gross lesions, the animals were examined every week for the first 13 weeks, and then every 2 weeks until the end of the experiment.

The biophase of the experiment terminated after 166 weeks, with the death of the last animal at the age of 174 weeks.

Upon death, the animals underwent systematic necropsy. Histopathology was routinely performed on skin and subcutaneous tissue, the brain, pituitary gland, Zymbal glands, salivary glands, Harderian glands, cranium (with oral and nasal cavities and external and internal ear ducts) (5 levels), tongue, thyroid and parathyroid, pharynx, larynx, thymus and mediastinal lymph nodes, trachea, lung and mainstem bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys and adrenal glands, oesophagus, stomach (fore and glandular), intestine (4 levels), bladder, prostate, uterus, gonads, interscapular fat pad, subcutaneous and mesenteric lymph nodes, and any other organ or tissue with pathological lesions.

All organs and tissues were preserved in 70% ethyl alcohol, except the bones which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution. The normal specimens were trimmed, following the standard procedures of the BT Laboratory: i.e. parenchymal organs were dissected through the hilus to expose the widest surface, and hollow organs were sectioned across the greatest diameter(s). The pathological tissue was trimmed through the largest surface, including normal adjacent tissues. The trimmed specimens were processed as paraffin blocks, and 3-5 micron sections of every specimen were obtained. Sections were routinely stained with haematoxylin-eosin. Specific stainings were performed when needed. All slides were examined microscopically by the same group of pathologists; a senior pathologist reviewed all the tumours and any other lesion of oncological interest. All pathologists followed the same criteria of histopathological evaluation and classification.

Two statistical methods were used for the analysis of tumour incidence. The first method is a prevalence analysis for nonlethal tumours and is described in Hoel and Walburg (1972). The interstitial cell adenomas of the testes were considered nonlethal. The second method assumes lethality of the tumour and the statistical method is a log-rank test and is described in Mantel (1966) and Cox (1972). This was used for the lymphomas/leukaemias.

The incidence of interstitial cell adenomas of the testis, of lymphomas and leukaemias and of dysplastic proliferation of haemolymphoreticular tissues at various sites in females, as given in the report published in 1995, are presented in Tables 2-4.

For the purpose of the present study, all pathological material was

Table 2 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: incidence of interstitial cell adenomas of the testis, in males (M) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Corrected number (a)	Animals bearing Leydig cell tumours		
					No.	% (b)	% (c)
I	1000	M	60	32	11	18.3	34.4 (d)
II	250	M	60	25	2	3.3	8.0
III	0 (e) (control)	M	60	26	2	3.3	7.7

(a) Alive male rats at 96 weeks of age, when the first Leydig cell tumour was observed

(b) Percentages refer to the number at start

(c) Percentages refer to the corrected number

(d) p<0.05

(e) Olive oil alone

Table 3 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: incidence of lymphomas and leukaemias, in female (F) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Corrected number (a)	Animals bearing lymphomas and leukaemias		
					No.	% (b)	% (c)
I	1000	F	60	47	12	20.0	25.5 (d)
II	250	F	60	51	6	10.0	11.8 (e)
III	0 (f) (control)	F	60	58	2	3.3	3.4

(a) Alive female rats at 56 weeks of age, when the first leukaemia was observed

(b) Percentages refer to the number at start

(c) Percentages refer to the corrected number

(d) $p < 0.01$ (e) $p < 0.1$

(f) Olive oil alone

Table 4 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: incidence of dysplastic proliferation of haemolymphoreticular tissues (DPHLT) at various sites, in female (F) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Corrected number (a)	Animals bearing DPHLT		
					No.	% (b)	% (c)
I	1000	F	60	59	9	15.0	15.3
II	250	F	60	59	15	25.0	25.4
III	0 (d) (control)	F	60	60	1	1.7	1.7

(a) Alive female rats at 26 weeks of age, when the first DPHLT was observed

(b) Percentages refer to the number at start

(c) Percentages refer to the corrected number

(d) Olive oil alone

reviewed by several pathologists of our Cancer Research Centre, collegially discussed, and in part reviewed with an outdoor pathologist. Moreover, for what concerns the interstitial cell adenomas of the testis, our diagnostic parameter included the one used by the pathologists of the USA National Toxicology Program (NTP) (Boorman, Chapin and Mitsumori, 1990).

This pathological review has led to some minimal changes with respect to the results reported in our 1995 report.

Results

As anticipated, the results deal with pathological changes to the testis and haematopoietic system.

1. Pathology of the testis

The incidence of regressive, proliferative and neoplastic pathologies of the testis are reported in Tables 5 and 6.

Regressive pathologies (Table 5) include degenerative changes, atrophy and mineralization. No relation has been detected between treatment and degenerative changes and atrophy. On the other hand, there is a direct dose-related parallelism between the

exposure to MTBE and mineralization (both of the vessel walls and of the parenchyma).

Interstitial cell hyperplasia may be diffuse or focal. The incidence of these proliferative changes increases in animals treated with MTBE at both tested doses (Table 6).

A dose related increase in interstitial cell adenomas has been observed in exposed animals (Table 6). These tumours may be of different histocytotypes: they may be composed of monomorphic basophilic cells or of monomorphic and polymorphic eosinophilic cells, or of both histocytotypes. Interstitial cell adenomas may be multifocal: multifocality was observed only in the animals treated with the highest dose of MTBE.

2. Pathology of the haematopoietic system in female rats

Dysplasia of haemolymphoreticular cells increased in the treated groups: this increase did not appear dose-related (Table 7). All the observed dysplasias were of lymphoimmunoblastic type. This dysplastic proliferation was found in the lung, nodes and spleen. A dose-related increased incidence of haemolymphoreticular neoplasias was found in treated animals (Table 8). These neoplasias were of lymphatic origin and included lymphoblastic lymphomas, lymphoblastic leukaemias and lymphoimmunoblastic lym-

Table 5 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: testicular regressive pathologies, in male (M) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Animals bearing testicular regressive pathologies (a)							
				Degeneration				Atrophy		Mineralization	
				Total		MNGC (b)		No.	%	No.	%
No.	%	No.	%								
I	1000	M	60	9 (3)	15.0	5 (1)	8.3	13 (6)	21.7	21 (9)	35.0
II	250	M	60	9 (2)	15.0	5 (1)	8.3	12 (2)	20.0	12 (3)	20.0
III	0 (c) (control)	M	60	12 (2)	20.0	9 (1)	15.0	12 (1)	20.0	8	13.3

(a) Between brackets the number of animals also bearing interstitial cell adenoma
 (b) MNGC = multinucleated giant cells
 (c) Olive oil alone

Table 6 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: testicular interstitial cell proliferative and neoplastic pathologies, in male (M) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Animals bearing interstitial cell proliferative and neoplastic pathologies											
				Interstitial cell hyperplasias (a)				Interstitial cell adenomas							
				Total		Focal		Total		1 single histocytotype		Mixed histocytotype		Multifocal	
				(diffuse and focal)		No.	% (b)	No.	% (c)	No.	% (b)	No.	% (d)	No.	% (d)
I	1000	M	60	9 (4)	15.0	2 (2)	2.2	11	18.3	5	45.5	6	54.5	4	36.4
II	250	M	60	8 (3)	13.3	3 (1)	37.5	5	8.3	5	100.0	0	–	0	–
III	0 (e) (control)	M	60	4	6.7	1	25.0	3	5.0	1	33.3	2	66.7	0	–

(a) Between brackets the number of animals also bearing interstitial cell adenomas
 (b) Percentages refer to the total number of animals
 (c) Percentages refer to the total number of animals bearing interstitial cell hyperplasias
 (d) Percentages refer to the total number of animals bearing interstitial cell adenomas
 (e) Olive oil alone

Table 7 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: haemolymphoreticular dysplasias among female (F) rats

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Animals bearing haemolymphoreticular dysplasias (a) (lymphoimmunoblastic dysplasia)	
				No.	%
I	1000	F	60	12	20.0
II	250	F	60	16	26.7
III	0 (b) (control)	F	60	3	5.0

(a) The dysplasias observed in animals bearing lymphomas and leukaemias are not included
 (b) Olive oil alone

phomas. The anatomical sites involved by these neoplasias and their distribution are presented in Table 9. In the treated groups the lung appears to be the most frequently involved site. In this organ the neoplasias appeared to arise usually from the peribronchial and perivascular lymphoid tissue.

Conclusions

The results of systematic re-examination by different experts of the pathological material of our first MTBE study substantially confirm the data already published (Belpoggi, *et al.*, 1995). The revision and the adoption of the NTP criteria (particularly for what concerns testicular pathologies) led to some increase in the number of testicular interstitial cell hyperplasias and adenomas and of haemolymphoreticular neoplasias and dysplasias. The results presented in this report, on the whole, not only confirm but also reinforce the previous conclusions. MTBE does not seem to increase the incidence of degenerative changes and atrophy of the testis (Table 6), so the increase in interstitial cell adenomas cannot be interpreted as a mere reparative effect. The increased incidence of interstitial cell adenomas in the MTBE treated groups is dose-related, and it is paralleled by a dose-related increase in the incidence of interstitial cell hyperplasia. The increased incidence of the total number of haemolymphoreticular neoplasias in female rats is dose-related. All the observed neoplasias arise from lymphatic tissue and the lymphoimmunoblastic lymphoma is the most frequent histocytotype.

Table 8 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: haemolymphoreticular neoplasias among female (F) rats: incidence and distribution by histocytotype

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	Animals bearing haemolymphoreticular neoplasias							
				Total (a)		Lymphoblastic lymphoma (b)		Lymphoblastic leukaemia (b)		Lympho-immunoblastic lymphoma (b)	
				No.	%	No.	%	No.	%	No.	%
I	1000	F	60	12	20.0	3	25.0	1	8.3	8	66.7
II	250	F	60	7	11.7	0	–	1	14.3	6	85.7
III	0 (c) (control)	F	60	2	3.3	1	50.0	0	–	1	50.0

(a) Percentages refer to the number of animals at start

(b) Percentages refer to the total number of animals bearing haemolymphoreticular neoplasias

(c) Olive oil alone

Table 9 - Long-term carcinogenicity bioassay on methyl-tertiary-butyl-ether (MTBE), administered by stomach tube to Sprague-Dawley rats (Experiment BT 958). Results: distribution of haemolymphoreticular neoplasias (HLRN) in female (F) rats by site

Group No.	Daily dose (mg/kg b.w. in olive oil)	Sex	No. of animals at start	No. of animals with HLRN	Distribution of haemolymphoreticular neoplasias by site (a)				
					Sites involved	Total	Lymphoblastic lymphoma	Lymphoblastic leukaemia	Lympho-immunoblastic lymphoma
						No.	No.	No.	No.
I	1000	F	60	12	Nodes	9	4	1	4
					Spleen	4	2	1	1
					Liver	5	2	2	1
					Lung	11	3	1	7
					Other	3	2	1	0
II	250	F	60	7	Nodes	2	0	0	1
					Spleen	2	0	1	1
					Liver	1	0	1	0
					Lung	5	0	0	5
					Other	0	0	0	0
III	0 (b) (control)	F	60	2	Nodes	2	1	0	1
					Spleen	2	1	0	1
					Liver	1	1	0	0
					Lung	0	0	0	0
					Other	1	0	0	1

(a) Haemolymphoreticular neoplasias may affect many different anatomical sites; in this table all localizations have been taken into account

(b) Olive oil alone

MTBE also causes an increase in haemolymphoreticular dysplasias. The incidence of this last type of change is slightly higher among animals treated at the lower dose: this apparently paradoxical effect is probably due to the fact that more of these dysplasia proliferations might have developed into lymphomas and leukaemias among female rats treated at the higher dose.

In conclusion, on the basis of our results already published and of the present revision, MTBE must be considered carcinogenic.

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