

EVALUATION OF THE GENETIC ACTIVITY OF INDUSTRIALLY PRODUCED CARBON BLACK

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Commercially produced oil furnace carbon black (Chemical Abstract Service Registry No. 1333-86-4) has been evaluated by five different assay for genetic activity. These were the Ames Salmonella typhimurium reverse mutation test, sister chromatid exchange test in CHO cells, mouse lymphoma test, cell transformation assay in C3H/10T $\frac{1}{2}$ cells, and assay for genetic effects in Drosophila melanogaster. Limited cellular toxicity was exhibited but no significant genetic activity was noted.

INTRODUCTION

Carbon black is a generic term applied to a large family of colloidal, nongraphitic carbons made under carefully controlled manufacturing conditions (Johnson and Eberline, 1978). This finely divided material is produced from gas or oil by thermal decomposition when brought in direct or indirect contact with heat from the products of combustion of fuel and air. Temperatures of decomposition vary up to a maximum of about 1650°C. The decomposition is most often controlled by quenching with water, after which the reaction gases are usually filtered to remove the carbon black product.

Carbon black is made by three different processes: channel, thermal, and furnace. It is no longer made by the channel process in the United

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States, but some operations still exist in other countries. The furnace process is the preferred method of manufacture and, with heavy oil used as feedstock, accounts for over 97% of carbon black production in Western nations.

Unfortunately, many involved in biological evaluation do not distinguish carbon black from soot (Sax, 1975; Eisenstadt, 1979; NIOSH, 1978; Falk et al., 1958; Kaden et al., 1979). Carbon black and soot differ in quantity and chemical content of inorganic ash, particulate makeup, and organic extract (carbon black has less). Organic extract and aqueous extract are chemically different in carbon black and soot (D. Rivin, personal communication). On the other hand, the Chemical Abstract Service has assigned the number 1333-86-4 to carbon black, considering it an identifiable classification of materials, but no attempt has been made to classify soot. The definitions of carbon black and soot in standard technical dictionaries and encyclopedias indicate that they are synonymous but technically this is unwarranted (Johnson and Eberline, 1978; Hampel and Hawley, 1976; Considine, 1976; Lapedes, 1974; Smith and Bean, 1964).

Occupational exposure of carbon black production workers has been studied in epidemiologic surveys over 40 yr (Ingalls, 1950; Ingalls and Riskey-Iribarren, 1961; Robertson and Ingalls, 1980). No increased incidence of cancer mortality has been noted. Animal studies with various types of carbon black were reviewed by Rigdon (1975) and NIOSH (1978). No tumor-forming potential in animals was described for carbon black. All available evidence indicates that carbon black is not tumorigenic and does not present a carcinogenic hazard to employees involved in its production. Nevertheless, because of the increased emphasis in recent years on the possible hazards of all chemicals, a study of the mutagenic potential of carbon black was undertaken. There is a general lack of information on genetic activity for industrial carbon black.

METHODS AND MATERIALS

A single sample of N-339, oil furnace carbon black, was used. Properties of the sample are shown in Table 1. The polynuclear aromatic hydrocarbon (PAH) content was determined by suspending carbon black in benzene for 48 h. The extract was analyzed by gas chromatography-mass spectrometry. PAHs having a molecular weight above 330 are not included in the quantitative value. The PAH content is approximately 20% of the total toluene extractables. The cyclohexane procedure was not as efficient as the toluene procedure for total extractables.

All the short-term procedures employed were considered to be standard methods. In the mammalian microsome plate test five tester strains of *Salmonella typhimurium* were used: TA98, TA100, TA1535, TA1537, and TA1538. The procedure (Ames et al., 1975) was conducted with a nonactivated system and an activated system, using Aroclor 1254

TABLE 1. Characterization Tests of N-339 Carbon Black

Test	Units	Procedure
Nitrogen surface area	100 m ² /g	ASTM D3037-A
CTAB ^a surface area	100 m ² /g	ASTM D3765
Iodine number	90 mg/g	ASTM D1510
Dibutyl phthalate (DBP) absorption	124 cm ³ /100 g	ASTM D2414
Compressed DBP	104 cm ³ /100 g	ASTM D3493
Tint strength	109%	ASTM D3265
Heat loss at 125°C	0.76 wt %	ASTM D1509
Volatiles at 950°C	1.80 wt %	Phillips method
Ash at 550°C	0.61 wt %	ASTM D1506
pH	7.5	ASTM D1512
Toluene discoloration	84%	ASTM D1618
Toluene extractables	0.15 wt %	48 h (Phillips)
Cyclohexane extractables	0.06 wt %	48 h (Phillips)
Polynuclear aromatic hydrocarbons	294 ppm	Gas chromatography-mass spectrometry (Phillips)

^aCetyltrimethylammonium bromide.

induced liver microsomal enzymes from Sprague-Dawley male rats. All control and test compound dilutions were plated in triplicate.

In the activated system, the S9 mix was prepared from Aroclor 1254 (500 mg/kg) induced liver microsomal enzymes from Sprague-Dawley male rats. Liver was homogenized in 0.15 M KCl at 3 ml per gram of wet liver and centrifuged at 9000 X g for 10 min. The supernatant, referred to as the S9 fraction, was decanted and stored in liquid N₂. A 1-ml portion of the microsomal enzyme reaction mixture (S9 mix) added to the soft agar overlay contained the following:

S9	0.05 ml
0.4 M MgCl ₂	0.02 ml
1.65 M KCl	0.02 ml
0.04 M NADP	0.10 ml
0.05 M glucose-6-phosphate	0.10 ml
1.00 M NaH ₂ PO ₄ , pH 7.4	0.10 ml
H ₂ O	0.61 ml
Total	1.00 ml

Chinese hamster ovary (CHO) cells were used to evaluate sister chromatid exchange. The procedure (Brusick, 1980) was conducted with a nonactivated system and an activated system, using liver microsomal enzymes from Fischer 344 male rats. Due to its insolubility, the N-339 carbon black was handled in dimethyl sulfoxide (DMSO) as a stock suspension of 0.1 g/ml. Four replicate cultures per dose were employed in this assay. The CHO biopsy cells were obtained from the American Type

Culture Collection (Rockville, Md.; Repository No. CCL61). Cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum, split back to 3×10^5 cells per 75-cm^2 plastic flask, and fed 24 h before treatment with 10 ml fresh medium. Approximately 10^6 cells were treated with growth medium with the test material and then incubated at 37°C for 2 h on a rocker. Exposure was terminated by washing the cells twice with saline. After addition of 5-bromo-2'-deoxyuridine to a final concentration of $20\text{ }\mu\text{M}$, incubation was continued in the dark for 24 h. Colcemid at a final concentration of $2 \times 10^{-7}\text{ M}$ was added for the final 3 h of incubation. Metaphase cells were collected by mitotic shake-off. These cells were swollen with 0.075 M KCl, hypotonic solution, then washed 3 times with methanol and acetic acid (3:1) fixation, dropped onto slides, and air-dried.

For the activated system, Fischer 344 rat livers were induced with Aroclor 1254. Five days later, the animals were killed by decapitation, bled, and the livers were excised and placed in ice-cold 0.25 M sucrose buffered with Tris at pH 7.4. A collection of livers were washed with buffered sucrose and homogenized. The homogenate was centrifuged for 10 min at $9000 \times g$, and the S9 supernatant acted as the core reaction mixture in NADP (Na salt) (2.4 mg/ml) and isocitric acid (4.5 mg/ml). The S9 preparation contained 24.2 mg protein per milliliter and the aryl hydrocarbon hydroxylase (AHH) specific activity was 0.45, which is an indirect measure of the cytochrome P-450 and P-448 activity of the preparation. The S9 preparation, which included $0.15\text{ }\mu\text{l}$ S9 and 0.60 ml cofactors, was used at 0.75 ml per 10-ml test volume.

Slides were stained for 10 min with Hoechst 33258 ($5\text{ }\mu\text{g/ml}$) in phosphate buffer (pH 6.8) and exposed to ultraviolet light. They were then stained with 10% Giemsa for 10 min, rinsed in deionized water, and air-dried.

Results for the solvent control and the test material or positive control were compared by Student's t-test and the standard error (SE) of the mean.

The mouse lymphoma assay was a modification of the procedure of Clive and Spector (1975). Toxicity was evaluated to determine the optimum dose levels for mutagenesis assay of carbon black. Test material was sterilized by suspending it in a single glass centrifuge tube with 0.5 ml acetone for each dose level; the same volume of acetone was used for the two control tubes. The acetone was evaporated in a hood for 4 d. Test cultures were started by adding 4.0 ml of Fischer's medium supplemented with streptomycin and Pluronic S68 (F_0P) to tubes not receiving metabolic activation and 4.0 ml S9 plus cofactors to those receiving metabolic activation. A 6-ml portion of a L5178Y TK+/- cell suspension (clone 3.7.2c from D. Clive, Burroughs Wellcome, Research Triangle Park, N.C.) containing 1×10^6 cells per milliliter was then added to each tube, and the tube was gassed with 5% CO_2 . The tubes were placed on a Bellco roller drum at 25 rpm for a 4-h exposure period. The preparation was conducted under

amber lights and kept in darkness during the exposure period. Because of the difficulty of separating the insoluble test material from the cells, the 4-h contact time was extended. Cells were counted with hemacytometers. Cell population density was determined 24, 48, and 72 h after compound exposure by making 1:10 dilutions of 1-ml samples of 0.1% trypsin, incubating at 37°C for 10 min to disperse the cells, and counting them in a hemacytometer.

In preparation of cells before use in the assay, the growing and cleansing procedures of Clive and Spector (1975) were employed to reduce the frequency of spontaneously occurring TK-/- cells. Dose levels for the assay were chosen so that the highest nonactivated dose approximated an LD90.

To establish the background level of TK-/- colonies, two control tubes containing acetone solvent only were run. The concentrations of ethylmethane sulfonate (EMS) and 7,12-dimethylbenz[*a*]anthracene (DMBA) were used as positive controls for direct-acting mutagens and promutagens, respectively. The 4-h exposure conditions were as described above; however, at the end of the exposure period the cells were washed twice with 10 ml Fischer's medium supplemented with streptomycin, 10% horse serum and Pluronic S68 (F₁₀P) and centrifuged at 1000 X g for 10 min. After the supernatant was decanted, the cells were resuspended in 20 ml F₁₀P, gassed with 5% CO₂ in air, and placed on a roller drum at 37°C.

After initial exposure to the test material, the cells were incubated for 3 d with an adjustment each day to yield a cell population of 0.3×10^6 cells per milliliter. At the end of this expression period, cells were placed in selective medium containing 1.0 µg/ml trifluorothymidine (TFT) and 0.35% noble agar. The selective cloning medium kills the TK+/- cells and allows growth of discrete colonies of TK-/- cells.

The general cloning preparation included two flasks per control and two flasks per test concentration for both activated and nonactivated systems. One flask was labeled selective medium and one was labeled viable count (VC). Each flask was warmed to 37°C, filled with 100 ml cloning medium, and shaken until used.

From each culture 3×10^6 cells were removed, centrifuged, and resuspended in 2 ml medium. This suspension was placed in a selective medium flask. A 5×10^{-4} dilution was made by adding 1 ml selective medium to 9 ml F₁₀P, adding 1 ml of this to 4 ml F₁₀P, and adding 1 ml of that dilution to the appropriate VC flask containing 100 ml cloning medium. After the dilution, 1 ml stock solution of TFT (100 µg/ml) was added to the selective medium flask, and both the selective medium flask and the VC flask were placed on a shaker at 125 rpm at 37°C.

After 15 min the flasks were removed and 33 ml cell suspension was placed in each of three appropriately labeled petri dishes. The plates were incubated at 37°C in a humidified 5% CO₂ atmosphere for 10 d.

The activated test system was derived from Sprague-Dawley rats. The

S9 mix for the L5178Y mouse lymphoma assay contained 15.49 mg/ml DL-isocitric acid, 6.0 mg/ml NADP, 0.25 ml S9 supernate per milliliter of mix, and 0.75 ml FoP per milliliter of mix. The concentrations in the cultures during exposure were

Isocitric acid	6.2 mg/ml
NADP	2.4 mg/ml
S9 supernatant	0.1 ml/ml
FoP	0.3 ml/ml

The supernatant was prepared by homogenizing rat liver induced with Aroclor 1242 and 1254 suspended in 3 ml 0.25 M sucrose per gram of liver. The homogenized material was centrifuged at 9000 X g for 10 min and the microsomal enzyme fraction stored at -195°C.

The cell transformation study was conducted with modifications of the procedure of Bertram (1977) and Reznikoff et al. (1973a, 1973b). C3H/10T1/2 CL8 cells were obtained at the sixth passage from C. Heidelberger (McArdle Laboratory for Cancer Research, University of Wisconsin). Stock inoculum was examined for typical growth characteristics and freedom from spontaneous transformation before use. Stock cultures were grown in antibiotic-free Eagle's basal medium (BME) containing 10% heat-inactivated fetal bovine serum (HIFBS). Cultures were subpassaged weekly at a 1:5 split ratio and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ in air.

Cells in late logarithmic phase were trypsinized with 0.1% trypsin for 4 min and plated at a concentration of 1×10^3 cells per petri dish in 5 ml BME with 10% HIFBS, penicillin (50 U/ml), and streptomycin (50 µg/ml). A second series of replicate cultures containing 200 cells per plate were set up from the same cell suspension for toxicity determinations.

Cell toxicity was determined by suspending the test material in 0.5 ml acetone and adding the desired dose to tissue culture medium. The material was tested in duplicate with 200 cells per plate grown in 5 ml BME with 10% HIFBS for 24 h. After 18 h of exposure, the cells were washed, refed with medium, and incubated for 10 d. Toxicity plates were then washed, fixed for 20 min with absolute methanol, air-dried, and stained with Giemsa stain for 30 min. The number of colonies per plate was determined from:

$$\text{Cloning efficiency} = \frac{\text{average no. of colonies per plate}}{\text{no. of cells per plate}} \times 100$$

At 24 h after seeding the cells, the transformation cultures were treated with appropriate concentrations of test material suspended in acetone. Solvent control cultures received an equal amount of solvent. Twelve cultures per dose were used. At 18 h after treatment with the test compound, the

plates were refed with growth medium and reincubated. This refeeding schedule was repeated weekly for 35 d after removal of the test material. All plate cultures were washed, fixed, stained, examined microscopically and macroscopically, and scored for transformation as type II and type III foci.

Various *Drosophila melanogaster* stocks were used to conduct assays for the detection of point mutations, chromosomal aberrations, and chromosome loss.

Mosaics Males with X chromosomes carrying five copies of the white-ivory (w^i) mutant and marked with the recessive mutants yellow body (y) and echinus eyes (ec) were mated to attached-X females with normal red eyes, yellow body, and forked bristles (f). The stock was placed on bakers' yeast for egg-laying periods of up to 15 h and then removed. Three days later larvae were collected by washing through a 60-mesh screen. These larvae were treated with the test substance at a concentration of 1% in the food until pupation. The flies were scored for mosaic spots after eclosion. Mutagenic activity is detected by a significant increase in the frequency of red mosaic spots in the eyes of the males.

Y Chromosome Loss Treated males that carry a duplication of the gene for normal (red) eyes on the Y chromosome and a mutant allele white (w) on the X chromosome were fed with 1% test material for 6-h and then mated to white-eyed (w/w) females. Male offspring are expected to carry the genes for red eyes. The occurrence of a white-eyed male signals loss of the Y chromosome from the spermatozoon.

Dominant Lethal Treated Canton-S males were mated with Canton-S females in nylon net cages on Welch's grape juice solidified with 2% agar. After an egg-laying period of not more than 12 h, the plates were stored at 25°C for approximately 30 h. Any genetic change that blocks development prior to hatching from the egg may be termed a dominant lethal mutation. The agar plates were scored to determine the fraction from which larvae were not hatched.

Bithorax Test of Lewis (Lewis, 1954; Mendelson, 1976). The occurrence of rearrangements with a breakpoint between the centromere of chromosome 3 and the locus of bithorax (bx) can be determined by scoring the offspring of treated bithorax-34e males and Ultrabithorax (Ubx) females. These Ubx/bx^{34e} offspring, which have their halteres modified to form a second set of wings, develop a band of hairy tissue, the metanotum, between the thorax and abdomen if a chromosomal aberration between bx and the centromere has been induced.

Sex-linked Lethals Females homozygous for the X-chromosome balancer *Basc* were mated to Canton-S (wild-type) males that had been fed 1% test material. All males from this parental cross carry the *Basc* X chromosome. All daughters carry one *Basc* chromosome and a treated X from their fathers. In the F_1 , individual daughters were mated to their brothers. If the treatment applied to the parental males induced a lethal in a sperm, the F_1 female that inherited that chromosome will produce no

TABLE 4. Chinese Hamster Ovary Cells, Sister Chromatid Exchange (SCE) Frequencies, Nonactivated

Observations	Negative control	DMSO control	N-399 carbon black						EMS (0.5 μ l/ml)
			0.32 μ g/ml	1.60 μ g/ml	8.00 μ g/ml	40.00 μ g/ml	200.00 μ g/ml	1000.00 μ g/ml	
No. of chromosomes	399	481	383	388	367	401	393	405	424
SCE per chromosome	328	303	317	297	298	309	342	252	1736
$\pm$ SE	0.822	0.630	0.828	0.765	0.812	0.771	0.870	0.622	4.094
SCE per cell	± 0.045	± 0.036	$\pm 0.046^a$	± 0.044	$\pm 0.047^a$	± 0.044	$\pm 0.047^a$	± 0.036	$\pm 0.098^a$
	16.49	12.60	16.55 ^a	15.30	16.24 ^a	15.42	17.40 ^a	12.44	81.88 ^a

^aSignificantly greater than solvent control value, $p < 0.01$ (Student's t -test).

TABLE 5. Chinese Hamster Ovary Cells, Sister Chromatid Exchange (SCE) Frequencies, Activated

Observations	Negative control	DMSO control	N-339 carbon black						DMN (0.3 μ l/ml)
			0.32 μ g/ml	1.60 μ g/ml	8.00 μ g/ml	40.00 μ g/ml	200.00 μ g/ml	1000.00 μ g/ml	
No. of chromosomes	383	412	376	412	400	502	382	389	495
SCE per chromosome	334	291	383	355	345	373	265	363	774
$\pm$ SE	0.872	0.706	1.019	0.862	0.863	0.743	0.694	0.933	1.564
SCE per cell	± 0.048	± 0.041	$\pm 0.052^a$	± 0.046	± 0.046	± 0.038	± 0.043	$\pm 0.049^a$	$\pm 0.056^a$
	17.44	14.12	20.38 ^a	17.24	17.26 ^a	14.86	13.88 ^a	18.66 ^a	31.28 ^a

^aSignificantly greater than solvent control value, $p < 0.01$ (Student's t -test).

TABLE 6. Mouse Lymphoma, L5178Y TK+/- Locus Cells, Mutant Frequency, Nonactivated

Observations	Acetone control	N-339 carbon black (μg/ml)								EMS (μl/ml)	
		10,000	15,000	20,000	25,000	30,000	35,000	40,000	Acetone control	0.50	1.00
Cell counts (×10 ⁻⁶)											
Day 1	1.07	0.98	0.63	0.58	0.41	0.47	0.50	0.30	0.96	1.05	0.52
Day 2	0.84	1.17	1.18	0.99	0.64	0.84	0.55	0.52	1.13	0.99	0.66
Day 3	0.93	1.01	1.14	1.14	0.82	1.17	0.52	0.52	0.94	0.95	0.49
Suspension growth											
Total	29.7	42.8	31.3	24.2	8.7	17.1	5.3	3.2	37.0	36.3	6.1
Percent	100	144	105	81	29	58	18	11	100	98	17
Cloning data											
VC	215	250	262	266	277	260	87	264	147	106	28
Selective media	212	246	242	266	244	267	92	235	114	94	26
	229	251	244	258	228	298	84	226	140	94	24
	71	83	75	96	87	85	26	53	125	540	422
	84	91	65	68	97	90	18	45	128	539	455
	86	90	80	76	82	101	39	49	135	529	414
Cloning VC											
Percent	219	249	249	263	250	275	88	242	133	98	26
Percent growth	100	114	114	120	114	126	40	111	100	72	17
	100	164	164	98	34	72	7	12	100	72	3
Mutant frequency	0.7	0.7	0.6	0.6	0.7	0.7	0.0	0.4	1.4	10.9	33.1
Induced mutant frequency	—	0.0	-0.1	-0.1	0.0	0.0	-0.1	-0.3	—	8.9	31.1

induce any significant increase in mutant frequencies in the L5178Y mouse lymphoma assay. This was true for both nonactivated and S9 activated preparations. Results are presented in Tables 6 and 7. The cloning data for 35,000 $\mu\text{g/ml}$ carbon black in Table 6 include average colonies per plate that are less than lower or higher dose levels. This is probably due to loss of cells in the supernate during the cloning process, not to toxicity. It does not affect the mutant frequency value, since the reduction is evident in both the selective medium plates and the VC plates, and the mutant frequency is based on the ratio of the average number of colonies in these plates.

The reduction in the average number of colonies per plate for the VC plates does, however, affect on the total compound toxicity for the culture treated with 35,000 $\mu\text{g/ml}$. The cloning growth, expressed as percent of control, should probably be around 100 rather than 40%, which would change the percent growth from 7 to approximately 18%.

The positive control for the nonactivated system was EMS and that for the activated system was DMBA.

Because of the wide spread of doses in the first evaluation, a second assay was conducted with a narrower range of doses. The results are presented in Table 8. These data corroborate those of the first evaluation. The compound did not cause significant increases in mutant frequencies of

TABLE 7. Mouse Lymphoma, L5178/ TK+/- Locus Cells, Mutant Frequency, Activated

Observations	Acetone control	N-339 carbon black ($\mu\text{g/ml}$)		Acetone control	DMBA ($\mu\text{g/ml}$)	
		10,000	15,000		25	50
Cell counts ($\times 10^{-6}$)						
Day 1	0.86	0.64	0.40	0.62	0.25	0.25
Day 2	1.26	1.10	0.06	1.37	0.62	0.62
Day 3	1.25	0.95	0.61	1.06	1.0a	1.01
Suspension growth						
Total	52.2	24.7	0.6	33.3	62	5.7
Percent	100	47	1	100	19	17
Cloning data						
VC	204	257	183	128	154	125
	233	263	198	131	138	117
	237	271	1a7	133	128	115
Selective media	59	74	72	88	260	295
	74	76	58	90	213	288
	67	71	66	86	257	298
Cloning						
VC	225	264	189	131	140	119
Percent	100	117	a4	100	107	91
Percent growth	100	56	1	100	20	15
Mutant frequency	0.6	0.6	0.7	1.4	35	4.9
Induced mutant frequency	—	0.0	0.1	—	2.1	3.5

TABLE 8. Mouse Lymphoma, L5178Y TK⁺/— Locus Cells, Mutant Frequency, Activated

Observations	N-399 carbon black (μg/ml)												DMBA (μg/ml)		
	Acetone control	5,000	7,000	9,000	11,000	13,000	15,000	Acetone control			5.0	7.5			
Cell counts (×10 ⁻⁶)															
Day 1	0.70	0.87	0.84	0.65	0.60	0.58	0.43	0.52			0.42	0.33			
Day 2	1.30	1.23	1.28	1.48	1.23	0.94	0.69	0.92			1.06	0.65			
Day 3	0.50	0.70	0.83	0.91	1.12	1.07	0.88	0.62			0.54	0.59			
Suspension growth															
Total	15.5	27.1	32.8	32.6	30.7	21.3	9.7	11.0			9.0	4.8			
Percent	100	175	211	210	198	138	63	100			82	43			
Cloning data															
VC	77	113	118	139	117	153	135	36			44	13			
Selective media	85	111	109	137	127	131	118	36			29	28			
	90	125	137	124	137	163	125	41			35	26			
	44	43	36	35	15	20	19	19			75	98			
	39	43	31	40	13	23	21	25			64	106			
	35	41	31	28	15	22	20	16			66	98			
Cloning															
VC	84	116	121	133	127	149	126	38			36	22			
Percent	100	139	144	159	151	178	150	100			95	58			
Percent growth	100	239	305	333	296	245	95	100			78	25			
Mutant frequency	0.9	0.7	0.5	0.5	0.3	0.3	0.3	1.1			3.8	9.0			
Induced mutant frequency	—	−0.2	−0.4	0.4	−0.6	−0.6	−0.6	—			2.7	7.9			

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