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Effect of 1,3-*Bis*-(2-Chloroethyl)-1-Nitrosourea on *Saccharomyces cerevisiae*.* (30361)

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Schabel *et al*(1) reported the effects on L1210 leukemia of a number of substituted nitrosoureas synthesized by Johnston *et al* (2). Of those tested, 1-(2-chloroethyl)-1-nitrosourea (NSC-47547) and 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (NSC-409962; BCNU) were markedly effective when the tumor was inoculated by the intraperitoneal or intracerebral route. Cross-resistance studies using plasmacytoma in hamsters suggested a biochemical mode of action similar to that of alkylating agents. The authors pointed out, however, that the 2-chloroethyl groups on these compounds would not be expected to form ethylenimonium ions *in vivo*, and are not essential for activity of the parent 1-methyl-1-nitrosourea. In clinical trials of BCNU, Rall *et al*(3) found that even though toxicity was rather severe, the drug decreased the number of circulating leukemic cells and was uniquely effective in clearing meningeal leukemia when given orally. More recently Goldin *et al*(4) confirmed the effectiveness of BCNU against a number of experimental leukemias, emphasizing its extensive activity over a wide range of treatment schedules, and suggested that studies of the mechanism of action of this drug be undertaken.

The data obtained previously on hydroxyurea in a microbial test system(5) were compatible with other studies on mechanism of action of that drug(6-9). Consequently a similar study was initiated with BCNU; a report follows.

Materials and methods. The 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (NSC-409962; BCNU) and 1-methyl-1-nitrosourea (NSC-23909; MNU) were supplied by Dr. Harry B. Wood, Jr. and Dr. J. A. R. Mead of the Cancer Chemotherapy National Service Center, and were stored at —20°C. Mechlorethamine hydrochloride (nitrogen mustard, HN2) was obtained from Merck and Co., Inc. All microorganisms used were stock laboratory strains maintained in this laboratory.

Initial tests of inhibition of microorganisms were done on agar plates on which a filter paper disc impregnated with a 1% solution of each compound was placed. Measurements of dose-response relationships were done in Sabouraud dextrose broth (Difco) dispensed in matched culture tubes. Growth was expressed as optical density at 640 λ after establishing linearity of optical density with cell population. Nephelo-culture flasks with 19 mm diameter side arms (Bellco Glass Co.) were used in measurements of antagonism of inhibition; growth was expressed as optical density at 640 λ as above. Nucleic acid and protein determinations were performed with minor modifications of the procedures described earlier(5); such modifications were used only to compensate for the relatively low concentration of deoxyribonucleic acid (DNA) in yeast. Measurements of carbon to chlorine bonds were done by a

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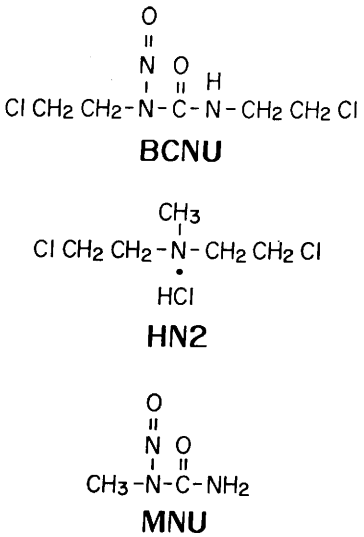


FIG. 1. Structural relationships of BCNU, HN2, and MNU.

variation of the procedure described by Witten *et al*(10); details of the variations are described in the *Results* section. Pyridine nucleotides were estimated with the Farrand fluorometer after alkali treatment(11). Manometric methods were described earlier(12). Purine and pyrimidine bases were chromatographed on paper with an isopropanol-HCl solvent(13) and detected with a short wave ultraviolet source (Mineralight; Ultra-Violet Products, Inc., San Gabriel, Calif.).

Results. Fig. 1 shows structural similarities among the 3 compounds. While the nitrosourea group is present in MNU and the two 2-chloroethyl groups are present in HN2, both groups occur in BCNU. Under the premise that correlations of antimicrobial spectra may yield some indication as to which functional group of BCNU contributes primarily to its pharmacological action, each compound was assayed against a variety of organisms. Table I shows that BCNU displayed no selectivity of action identical with either HN2 or MNU among the organisms tested. Indeed, HN2 and MNU, each containing a different functional group, appeared to possess more spectral similarities than either did with BCNU. In view of the greater activity of BCNU against *Saccharomyces cerevisiae* as compared with the other organisms, this yeast was chosen for further studies.

According to Finney(14) two agents with similar modes of action on a test organism, especially drugs of related chemical constitution, often show parallel dose-response regression lines. To compare characteristics of the regression induced by the 3 drugs, each was assayed in 6 experiments against *S. cerevisiae*. Optical density of each culture tube was measured while the control tubes were in mid log phase. Per cent of control growth was converted to probits(14) and the values obtained were fitted to the multiple regression equation

$$\text{Probit } Y = B_0 + B_1X + B_2X^2.$$

B_2 was tested for significance and the data then fitted to the usual regression line. All curves are shown in Fig. 2 along with the fiducial limits of each. Certain differences in the nature of the regressions are notable. In all 6 experiments with BCNU, B_2 in the equation above was not significant and the regression lines were linear when plotted against absolute drug concentration; *i.e.*, there was no skewedness of the response as is often encountered in biological assays. The regressions for HN2 were also linear with absolute dose in 2 of the 6 experiments; for MNU, in 4 of the 6 experiments. Lines which were not linear when plotted in this fashion were satisfactorily converted to linearity by the customary normalizing operation of using a logarithmic scale on the abscissa; however,

TABLE I. Inhibitory Spectra of BCNU, HN2, and MNU Against Certain Microorganisms.

	BCNU	HN2	MNU
Candida albicans	10	10	18
Saccharomyces cerevisiae	20	13	12
Aspergillus niger		7	6
Rhizopus oryzae		3	15
Penicillium sp.	7	20	20
Escherichia coli		2	
Bacillus subtilis			
Staphylococcus aureus		2	
Aerobacter sp.		11	
Pseudomonas aeruginosa			
Mycobacterium phlei			
M. smegmatis			

Numbers indicate zone of inhibition, in mm, from periphery of filter paper disc impregnated with each drug. No entry indicates no inhibition.

the absolute dose scale is used in Fig. 2 to illustrate the differences.

The 50% inhibitory concentration (LD_{50}) of each drug, with the range encountered over the 6 dose-response experiments with each given in parentheses, were as follows: BCNU, 3.0×10^{-5} M ($2.0\text{-}3.9 \times 10^{-5}$ M); HN2, 3.8×10^{-5} M ($3.0\text{-}5.0 \times 10^{-5}$ M); MNU, 17.0×10^{-5} M ($15.2\text{-}18.0 \times 10^{-5}$ M). Thus there was very little difference in regression lines and LD_{50} values between BCNU and HN2; however, MNU consistently yielded lower slopes with LD_{50} values almost 6 times higher than BCNU and HN2. It appears that the fundamental mechanism of action of MNU may differ from that of the two 2-chloroethyl compounds, the implication being that the nitrosourea moiety of BCNU is not primarily involved in its action mechanism in the yeast test system.

In view of the reactivity of mustards with functional groups in a number of compounds

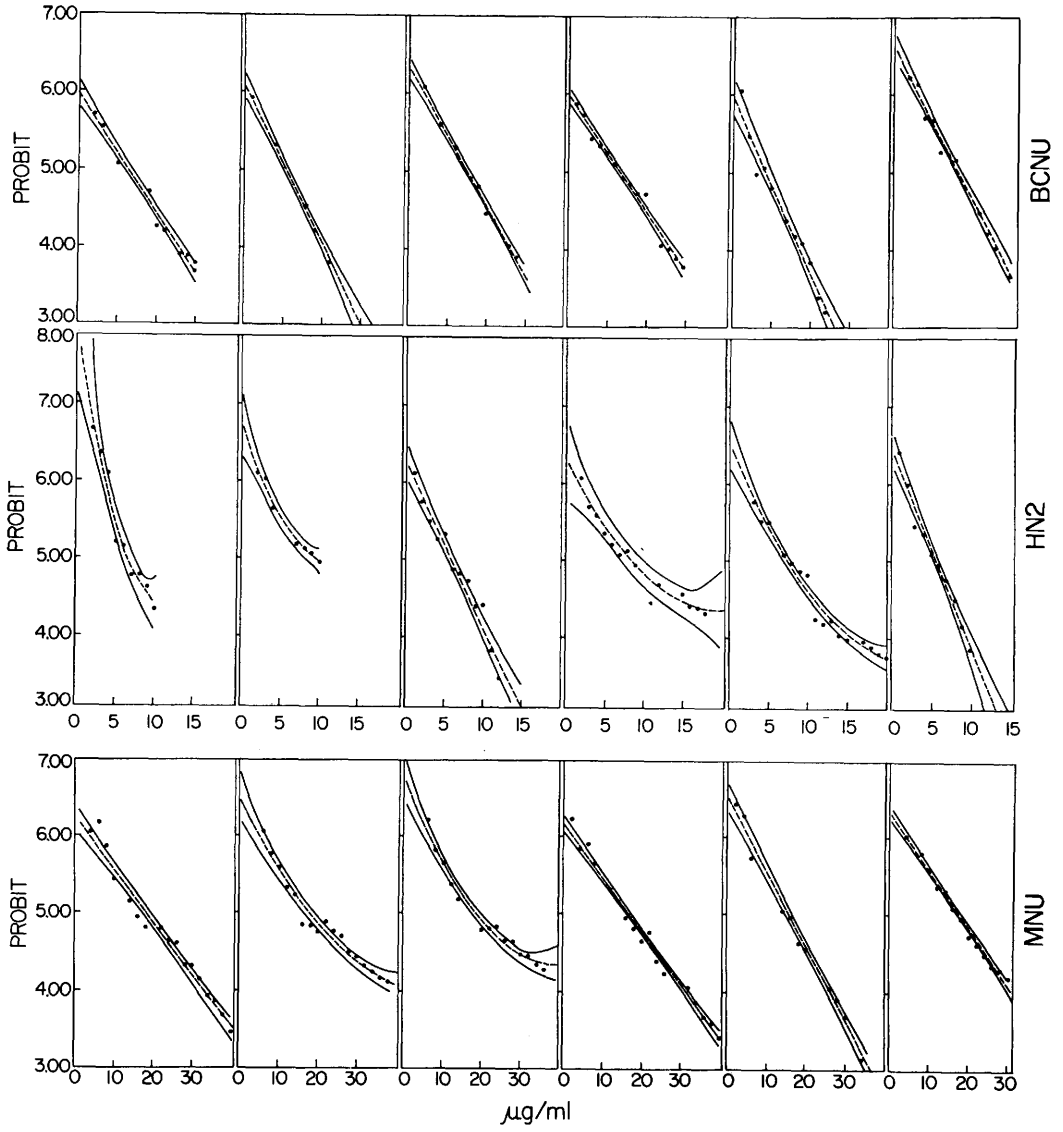


FIG. 2. Dose-response relationships of *S. cerevisiae* to BCNU, HN2, and MNU. Dotted lines are the calculated lines, solid lines are the 95% confidence limits, and solid points are experimental values.

TABLE II. Effect of Sulfhydryl Compounds on Inhibition of Growth of *S. cerevisiae* by BCNU, HN2, and MNU.

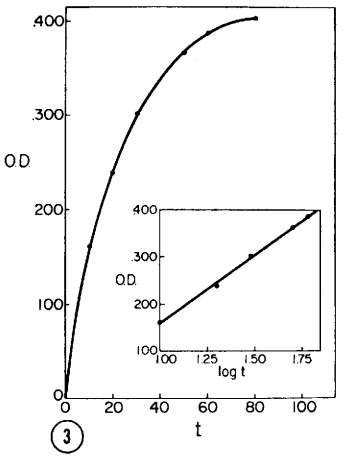
–SH source	No drug	% Difference in O.D. BCNU, 10 μg/ml	HN2, 10 μg/ml	MNU, 70 μg/ml
Cysteine, 100 mg/ml	−33	+1311	+155	+10
DTT, 10 "	− 4	+ 381	+ 6	−18
DTT, 20 "	− 9	+ 603	+ 1	−10
DTT, 30 "	−19	+ 352	− 2	−30

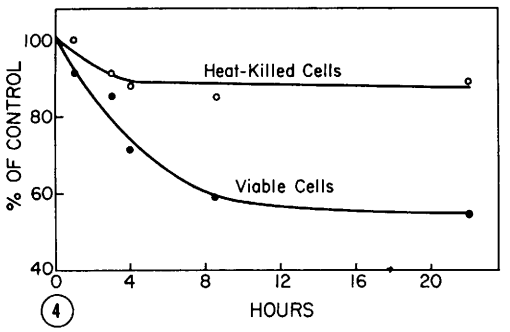
Each Nephelo-culture flask containing 100 ml Sabouraud broth was inoculated with 5 ml of an 18 hr culture of *S. cerevisiae*. Optical density was followed until cells were in early log phase. Additions were then made to flasks as indicated. Numbers in "% difference" columns were calculated on the ratio $\left(\frac{\text{O.D. at 24 hr of culture with –SH}}{\text{O.D. at 24 hr of culture with no –SH}}\right)$ − O.D. at time additions were made.

(15), the ability of one such group to antagonize the inhibition by each drug was investigated. For this, the sulfhydryl groups of cysteine and of dithiothreitol (DTT, Cleland's reagent) were used. The choice of compounds was made on the basis that cysteine is a physiological sulfhydryl compound, while DTT is a synthetic compound with no known natural occurrence. Table II shows considerable antagonism by both sulfhydryl compounds of the inhibitory action of BCNU. Some antagonism by cysteine of the action of HN2 was noted, but DTT over the concentration range employed was without effect in the presence of HN2. The value of +10% difference obtained with cysteine when the inhibitor was MNU is probably of little significance. Similarly, DTT appeared to have no antagonistic action with MNU, and there was actually a possible potentiation. Patterns of antagonism, by a specific functional group, of the inhibitory actions of the 3 compounds therefore differ, further indicating differences in modes of action of the 3 drugs.

The pharmacological action of nitrogen mustards is dependent upon cyclization of the compounds with formation of the ethylenimonium ion (see 15). This transformation occurs quite rapidly in HN2, its half-life being about 30 minutes in 0.1 M phosphate buffer at pH 7.4 and a temperature of 37°C. The secondary mustards such as nor-HN2 also cyclize in an aqueous medium; the charged species is reactive toward nucleophilic reagents, while the uncharged cyclized product is stable. In 0.1 M phosphate at pH 7.4 and a temperature of 37°C, nor-HN2 is virtually unchanged in 24 hr(16). To obtain data regarding the stability of the carbon-chlorine (C-Cl) bonds in BCNU, the 4-(4′-nitrobenzyl)pyridine (NBP) method for the colorimetric measurement of alkylating agents(10) was used. Preliminary studies showed that quite rigorous conditions were required for color development as compared with the conditions used by Witten *et al*(10). Whereas those authors found mild heating for relatively short periods gave best reproducibility with their sulfur mustards, in the test for BCNU maximum color development and reproducibility were obtained only after 60-70 minutes at 100°C. The time course of color development with heating at 100°C after addition of NBP to a solution of BCNU is shown in Fig. 3, and the apparent first order kinetics of the reaction are demonstrated in the inset in which the time axis is converted to a logarithmic scale. The low rate of reactivity of BCNU with NBP is thus indicative of a low order of either cyclization, or reaction of the cyclized species with the nucleophilic group of NBP.

Using the standardized procedure of heating at 100°C for 70 minutes for color development, the stability of BCNU in a number of systems was investigated. At room temperature, the concentration of BCNU decreased less than 1% per hour in water or 0.1 M phosphate buffer, pH 7.4; disappearance in a 0.2 mg/ml solution of cysteine was negligible, *e.g.*, considerably less than 1% per hour. In the presence of a 10% suspension





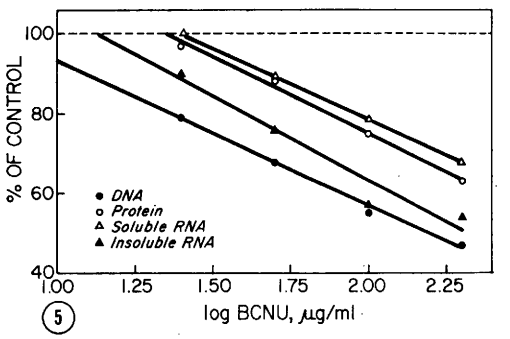


FIG. 3. Development of color with time (t) at 100°C in the 4-(4′-nitrobenzyl)pyridine (NBP) test for carbon to chlorine bonds in BCNU. Ordinate represents optical density at 575 λ. Inset shows apparent first order kinetics of color development upon conversion of time scale to logarithms. BCNU = 100 μg/ml and reagents were as described in reference(10).

FIG. 4. Decrease in concentration of carbon to chlorine bonds of BCNU in supernatant solutions of viable and non-viable 10% cell suspensions of *S. cerevisiae* in phosphate buffer; control was buffer alone, pH 7.4. Initial concentration of BCNU = 100 μg/ml; temp = 30°C.

FIG. 5. Effect of BCNU on concentration of protein, DNA, soluble RNA, and insoluble RNA in *S. cerevisiae*. Five hundred ml Erlenmeyer flasks containing 200 ml Sabouraud broth were inoculated with 5 ml of a 24-hr broth culture of cells. After incubation for 8 hr on a reciprocating shaker at 30°C, BCNU was added to a final concentration of 25, 50, 100, and 200 μg/ml. Control flasks received no drug. After further incubation for 14 hr analyses were performed as described in reference(5), with the minor modifications stated in text. Lines were placed by the method of least squares.

of washed cells of *S. cerevisiae* in 0.1 M phosphate buffer, however, there was a slow but significant disappearance of C-Cl bonds from the supernatant solution, and the extent of disappearance was depressed by heating the cell suspension to 100°C for 10 min prior to addition of BCNU (Fig. 4).

Attempts to prepare a cell-free system, by grinding cells with aluminum oxide followed by high speed centrifugation, which would promote a decrease in BCNU concentration, were not successful. In one such experiment there was a loss of 58% of the C-Cl bonds from the supernatant solution of intact cells, while only 12% was lost in the presence of cell-free extract. The data suggest that BCNU is bound or otherwise removed from the supernatant solution of viable cells, but to a lesser extent by heat-killed cells or a cell-free extract.

Although extensive studies were not done to determine a possible enzymatic mechanism for removal of BCNU from the supernatant solution of viable cells, the recent work of Williamson(16) should be mentioned at this point. Those authors reported the presence of a biochemical mechanism in fresh blood serum of humans and animals which causes a very rapid disappearance of secondary nitrogen mustards from *in vitro* blood, and this mechanism displayed a number of characteristics of an enzymatic reaction. It remains to be shown whether or not a similar enzymatic mechanism is present in viable yeast for decreasing the concentration of BCNU.

Anaerobic glycolysis in *S. cerevisiae* was inhibited by BCNU, but relatively high concentrations were required for complete inhibition. Concentrations of 50, 100, and 1000 μg/ml BCNU caused reductions in rate of CO_2 release of 18, 49, and 95% respectively. Per cent inhibition obtained was the same when cells were exposed to BCNU 15 min-

TABLE III. Effect of BCNU on Growth, DPN Synthesis, and Protein Synthesis of *S. cerevisiae.*

BCNU, μg/ml	% Inhibition		
	O.D.	DPN	Protein
10	15	0	0
50	34	6	24
100	47	36	63

Numbers in "% inhibition" columns indicate % reduction of each parameter as compared with control cultures. See text for details.

utes or 120 minutes prior to addition of substrate. Neither adenine nor nicotinamide antagonized the inhibition of glycolysis. This is in contrast to the report of Fraser(17) who found the inhibition by HN2 of glycolysis in ascites tumor cells was prevented by addition of nicotinamide.

Dold *et al*(18) found that a number of alkylating cytostatic agents lowered the concentration of pyridine nucleotides in ascites cells and prevented the viability of the cells after transplantation. No alterations of the DNA/RNA ratio were noted. They took these results to be further evidence that alkylating agents exert their antitumor action primarily by inhibiting the synthesis of diphosphopyridine nucleotide (DPN) and not by acting on nucleic acids. To determine if a similar action was exerted by BCNU, cultures of *S. cerevisiae* in Nephelo-culture flasks were permitted to grow to approximately mid log phase, at which time the optical density of each culture was measured and samples removed for protein and DPN analyses. BCNU was then added and the cells incubated for an additional 3.5 hours. Determinations of optical density, protein, and DPN were repeated, and the change in each during the 3.5-hour incubation period was compared to the changes in replicate parallel control cultures. Table III shows there was an apparent inhibition of DPN synthesis; however, in each case the % inhibition was less than % inhibition of either optical density or protein synthesis. Such a situation does not strongly indicate that the inhibition of DPN synthesis is the primary mechanism of action of BCNU.

The effects of BCNU on protein, DNA, soluble RNA and insoluble RNA in *S. cerevisiae* are shown in Fig. 5. Of these 4 parameters, it appears that the synthesis of DNA is affected to the greatest extent. However, the near parallelism of the 4 dose-response regression lines does not suggest a marked specificity for any single anabolic system to the exclusion of the others.

To determine if BCNU binds or otherwise alters purines and pyrimidines, BCNU was added at a final concentration of 0.5 mg/ml to solutions of adenine, guanine, cytosine, thymine, and uracil, each at a final concentration of 0.1 mg/ml in water. After incubation for 48 hours at 37°C, each mixture, along with the base solutions without BCNU, were chromatographed on Whatman No. 1 paper. No change in the Rf value of any base was induced by the drug, strongly indicating no binding.

Discussion. Although no definitive evidence is forthcoming that the fundamental mechanism of action of BCNU differs from that of HN2, some responses of the test system seem to differ under the influence of each compound. The consistently normal distribution of the dose-response curve of *S. cerevisiae* to BCNU in 6 experiments is in contrast to the 4 skewed responses and 2 normal ones obtained in the presence of HN2. In view of the current state of knowledge regarding the causes of skewedness in biological assay, a demonstration of the different responses to each compound must be considered only possible evidence of different action mechanisms. Further suggestions of differences regard antagonism by sulfhydryl groups of the inhibition mediated by each compound. The action of BCNU is overcome by sulfhydryl compounds to a much greater degree than that of HN2. No antagonism at all by DTT of the action of HN2 can be shown, and cysteine has only about 1/10 the activity against HN2 as against BCNU.

The studies indicate that BCNU must be considered as an alkylating agent in view of the reactivity of the carbon to chlorine (C-Cl) bonds with the nucleophilic NBP. Herein may lie at least a partial explanation of the effectiveness of the drug. Whereas the C-Cl bonds of HN2 are extremely labile, those of BCNU are relatively stable, requiring rather rigorous treatment to evoke reaction with NBP. This low order of reactivity with nu-

cleophilic groups may be reflected in maintenance of therapeutically effective blood levels of the drug for considerable periods, which, coupled with the lipid solubility of the compound and its resulting passage into the central nervous system, confers an unique effectiveness in meningeal leukemia.

Summary. The pharmacological actions of 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (BCNU) were investigated with a microbial test system. The antimicrobial spectrum of BCNU differed from that of the parent 1-methyl-nitrosourea (MNU) and that of nitrogen mustard (HN2). Computer analysis likewise showed certain differences in the dose-response relationships of *S. cerevisiae* to each of the 3 drugs; HN2 and BCNU yielded similar numerical slopes and LD_{50} values but the form of the curve at times differed between the 2 drugs. With MNU, much lower slopes and higher LD_{50} values were obtained. The inhibitory action of BCNU on yeast was antagonized by cysteine and by dithiothreitol (DTT); only cysteine annulled the action of HN2. Neither sulfhydryl compound antagonized the inhibitory action of MNU. BCNU was apparently more stable in solution than HN2. In the presence of viable yeast cells, there was a slow decrease in concentration of BCNU in the supernatant solution. This decrease was much less in the presence of nonviable cells. Glycolysis in yeast was limited by BCNU and this was not antagonized by nicotinamide or adenine. Addition of the drug to cultures of *S. cerevisiae* reduced the concentration of protein, DNA, insoluble RNA, and soluble RNA in the cells. These dose-response regression lines were almost parallel, but DNA was depressed to a somewhat greater extent than the others. Binding of BCNU to pyrimidines or purines could not be demonstrated.

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