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Enzymatic release of 7-methylguanine from methylated DNA by rodent liver extracts

(3-methyladenine/DNA repair/glycosylases/alkylating agents)

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ABSTRACT **Rat and hamster liver extracts were found to contain DNA glycosylases capable of removing 3-methyladenine and 7-methylguanine from methylated DNA. The activity of 7-methylguanine-DNA glycosylase was greater in hamster than in rat liver extracts. This finding is consistent with previous reports that the half-life of 7-methylguanine in DNA after treatment with the carcinogen dimethylnitrosamine is longer in rats than in hamsters. These enzymes may, therefore, play an important role in the removal of abnormal alkylation products from mammalian cell DNA. Rodent liver extracts also contained a DNA glycosylase able to remove from alkylated DNA the imidazole-ring-opened form of 7-methylguanine which is produced by treatment with alkali. Although this product may occur *in vivo* after treatment with alkylating agents to only a very small extent, the enzyme may be needed to minimize its potentially harmful biological effects.**

A major product of the interaction of carcinogenic and mutagenic methylating agents with DNA is 7-methylguanine (1–4). When radioactive methylating agents are applied to bacteria or cells in culture or intact animals *in vivo*, the amount of labeled 7-methylguanine formed in DNA decreases with time (2–4). However, 7-methylguanine is lost from DNA by chemical hydrolysis at a significant rate at neutral pH. Although the rates of loss *in vivo* are consistently greater than the rate expected from spontaneous depurination, uncertainty about the pH and ionic strength of the nuclear environment has precluded any definitive demonstration of the enzymatic nature of the removal process. In contrast, there is clear evidence that another minor methylation product, 3-methyladenine, is removed by the action of a glycosylase present in bacteria and human cells (5–7). A glycosylase acting on the ring-opened form of 7-methylguanine (rom^{7}G) produced by treating methylated DNA with alkali has been found in extracts of *Escherichia coli* (8).

In the present experiments we have tested the abilities of rat and hamster liver extracts to catalyze the loss of methylated purines from alkylated DNA. Both extracts contained 3-methyladenine-DNA glycosylase activity, 7-methylguanine-DNA glycosylase activity, and rom^{7}G-DNA glycosylase activity. These enzymes may play an important role in removal of alkylation products from DNA, and their activities in various species and tissues may determine the persistence of these abnormal bases.

MATERIALS AND METHODS

Preparation of Methylated DNA Substrates. Calf thymus DNA (32 mg) was dissolved in 40 ml of 0.2 M sodium cacodylate, pH 7.2/1 mM EDTA and treated with 5.0 mCi of [*methyl*-^{3}H]dimethylsulfate (2.4 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) at 37°C for 1.5 hr. The DNA was precipitated by addition of 2 vol of cold ethanol and washed by centrifugation with cold 80% (vol/vol) ethanol. The washed DNA was redissolved, dialyzed against 10 mM NaCl, and stored at −20°C. Sephadex G-10 chromatography of 0.1 M HCl hydrolysates of this DNA (see below) showed that it contains mainly (>95% of the products) 7-methylguanine and 3-methyladenine in the molar ratio 5:1.

A sample (0.5 ml) of this DNA was thawed, dialyzed against 100 ml of 50 mM Na_2HPO_4/NaOH, pH 11.4, for 36 hr at 4°C, and then allowed to stand at room temperature for 24 hr. This solution was dialyzed against 20 mM Tris·HCl/1 mM EDTA, pH 7.5, and stored at −20°C. Analysis of acid hydrolysates of this DNA by Sephadex G-10 chromatography showed that alkali treatment had caused imidazole-ring opening of about 50% of the 7-methylguanine residues.

Preparation of Tissue Extracts. A 45–70% ammonium sulfate fraction from extracts of *E. coli* B (frozen cell paste from late logarithmic growth phase purchased from Miles) was prepared as described (8). Female Sprague–Dawley rats (≈200 g) or Syrian golden hamsters (≈160 g) were anesthetized with Nembutal; the livers were removed and homogenized in an equal volume of ice-cold 50 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM 2-mercaptoethanol with a Polytron homogenizer (Brinkmann). The homogenate was centrifuged at 5000 rpm for 10 min at 5°C in a Sorvall SS-34 rotor, and the supernatant was saved. The precipitate was rehomogenized with an equal volume of extraction buffer, combined with the supernatant, and centrifuged at 105,000 × *g* at 5°C for 1 hr. The supernatant, which contained ≈30 mg of protein per ml, was removed and stored in batches at −20°C for use as enzyme preparation. Dialysis of the extract overnight against 50 vol of the extraction buffer did not affect the activity. In some experiments, the initial homogenates were sonicated for 30 sec, but this procedure did not improve the extraction of enzymatic activity.

Kinetics of Release of Ethanol-Soluble Material from Methylated DNA. Methylated DNA (3 μg) or alkali-treated methylated DNA (5 μg) was incubated with extraction buffer or with extracts (25 μl) of *E. coli* or of rat or hamster livers in 0.1 M KCl/70 mM Hepes·KOH, pH 7.8/1 mM EDTA/1mM dithiothrei-

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Abbreviation: rom^{7}G, ring-opened 7-methylguanine (2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine).

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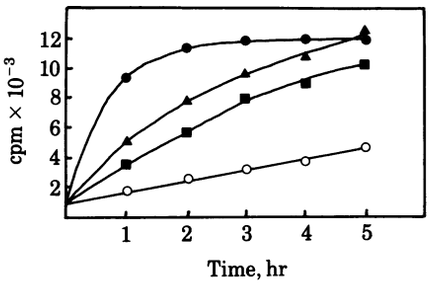

FIG. 1. Release of ethanol-soluble radioactivity from [^{3}H]methylated DNA. Extracts from *E. coli* (●), hamster liver (▲), or rat liver (■) or an equivalent amount of buffer solution (control, ○) was incubated for the time shown with [^{3}H]methylated calf thymus DNA. Addition of bovine serum albumin to the buffer solution did not increase the release of ethanol-soluble radioactivity.

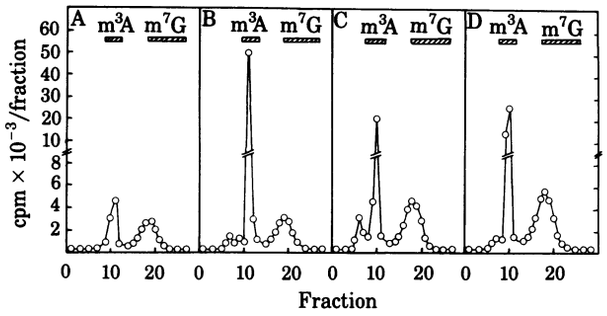

FIG. 2. Chromatographic separation of methylated purines released from DNA. The ethanol-soluble material present after incubation of [^{3}H]methylated DNA with no protein addition (control) (*A*) or with protein from *E. coli* (*B*), rat liver (*C*), or hamster liver (*D*) as described in Table 1 was analyzed by chromatography on Sephadex G-10. Shaded areas indicate the fractions corresponding to authentic markers of 3-methyladenine (m^{3}A) and 7-methylguanine (m^{7}G).

tol in a total volume of 50 μl at 37°C. After various periods, samples were cooled in ice, and carrier DNA (10 μl of a solution of heat-denatured calf thymus DNA, 2 mg/ml), NaCl (5 μl of a 2 M solution), and cold ethanol (150 μl) were added. After 10 min in ice, samples were centrifuged at maximal speed in an Eppendorf 3400 microcentrifuge for 10 min at 4°C, and 150 μl of the supernatant was assayed for radioactivity after addition of 10 ml of Formula 947 scintillation cocktail (New England Nuclear). Counting efficiency was approximately 35%.

Analysis of Ethanol-Solubilized Radioactivity. In order to generate material for chromatography, the above assay was scaled up to a total incubation volume of 0.3 ml. After 5 hr at 37°C, the appropriate volumes of DNA, NaCl, and ethanol were added and the samples were centrifuged as above. The alcohol supernatants were removed and evaporated to dryness. For the methylated DNA substrate, the precipitates were hydrolyzed in 0.5 ml of 0.1 M HCl by incubation at 37°C for 2 days. These hydrolysates and the supernatant residues (redissolved in 0.5 ml of 0.1 M HCl) were subjected to Sephadex G-10 chromatography or high-pressure liquid chromatography as described below.

Protein Dependence of Glycosylase Activity. Protein was determined in rat or hamster liver extracts by the method of Bradford (9) with reagents from Bio-Rad and bovine serum albumin as standard. Various amounts of protein were then incubated with methylated DNA (15 μg containing 35,000 cpm) in a total volume of 0.3 ml for 2 hr at 37°C. After addition of the appropriate amounts of carrier DNA, NaCl solution, and ethanol, followed by centrifugation, 150 μl was taken for determination of total ethanol-soluble radioactivity. The remainder was dried and subjected to Sephadex G-10 chromatography as described below.

Chromatographic Analysis of Methylated Purines. Samples for determination of 3-methyladenine and 7-methylguanine were applied in 0.1 M HCl to columns (28 × 1 cm) of Sephadex G-10 after addition of marker compounds (obtained as described in refs. 8 and 10–12). The column was eluted with 50 mM ammonium formate/0.02% sodium azide, pH 6.75, at a flow rate of 40 ml/hr. Fractions (2 ml) were collected and assayed for radioactivity at a counting efficiency of 31%.

For alcohol supernatants from enzymic digests of methylated DNA containing rom^{7}G residues, samples in 0.1 M HCl were chromatographed on columns (90 × 1 cm) of Sephadex G-10 at a flow rate of 30 ml/hr. Because rom^{7}G eluted in the same region as 3-methyladenine, additional samples were adjusted to pH 5.0 before chromatography. Under these conditions, 3-methyladenine eluted just before 7-methylguanine and away from the rom^{7}G region. In both cases, fractions (5 ml) were collected and assayed for radioactivity.

The alcohol supernatants and DNA hydrolysates were also analyzed by high-pressure liquid chromatography by using a Micromeritics solvent delivery system, injector, column oven, and microprocessor. The extract was applied to a column (25 × 0.46 cm) of Partisil 10SCX cation-exchange medium (Whatman) and eluted at a flow rate of 2.0 ml/min with 20 mM ammonium formate (pH 4.0) at 50°C. Fractions were monitored at 254 nm with a Waters model 440 absorbance monitor, and those corresponding to the appropriate marker bases were collected directly into scintillation vials and assayed for radioactivity. The approximate elution times for the methylated purines were as follows: 7-methylguanine, 6.5 min; 3-methylguanine, 9 min; 1-methyladenine, 26 min; 7-methyladenine, 17

Table 1. Release of 3-methyladenine and 7-methylguanine from methylated DNA

	3-Methyladenine			7-Methylguanine		
	cpm × 10^{-3}			cpm × 10^{-3}		
Extract added	In supernatant	In precipitate	% released	In supernatant	In precipitate	% released
None	1.20	6.84	15.2	1.84	30.9	5.5
E. coli	6.54	1.09	85.7	1.65	30.8	5.1
Rat liver	3.46	4.70	42.4	2.86	28.4	9.1
Hamster liver	5.09	2.68	65.5	3.80	27.8	12.0

Extracts were incubated with methylated DNA, and the radioactivity present in the ethanol-soluble fraction and in the residual DNA was analyzed. The incubation conditions were as given for the scaled-up assays; the tubes contained 18 μg of DNA (43,000 cpm) and 4.5 mg of rodent liver protein.

min; and 3-methyladenine, 38 min. rom^{7}G gave a double peak eluting at 3 min and 8 min.

RESULTS

When [^{3}H]methylated DNA was incubated with extracts from *E. coli*, rat liver, or hamster liver, radioactivity was released into the ethanol-soluble fraction (Fig. 1). This release was more rapid than that occurring by spontaneous depurination when no protein, bovine serum albumin, or extracts inactivated by heating for 10 min at 75°C were added instead of the active enzyme extracts. The radioactivity released was analyzed by chromatography on Sephadex G-10 (Fig. 2) or by high-pressure liquid chromatography (data not shown). Both methods gave similar results. Fig. 2 shows that approximately equal amounts of 3-methyladenine and 7-methylguanine were released by spontaneous depurination. Addition of extracts from *E. coli* increased the release of 3-methyladenine as expected, because it contains a 3-methyladenine-DNA glycosylase (5), but had no significant effect on 7-methylguanine release. Extracts of rat and hamster liver, however, brought about an increase in both 3-methyladenine and 7-methylguanine in the ethanol-soluble fraction, showing the presence of both a 3-methyladenine-DNA glycosylase and a 7-methylguanine-DNA glycosylase. Although there was a small peak of unidentified radioactivity that preceded 3-methyladenine in all of the chromatograms from the active extracts (particularly from that of rat liver), no other ethanol-soluble products were released. This suggests that the extracts did not have any significant nuclease activity under the assay conditions that included 1 mM EDTA. Analysis of the residual ethanol-precipitable DNA confirmed this conclusion; more than 95% of the starting radioactivity for 3-methyladenine and 7-methylguanine was present in the alcohol supernatant plus the acid-hydrolyzed alcohol precipitate (Table 1). Incubation for 5 hr with the *E. coli* extract released 86% of the 3-methyladenine, whereas incubation with rat and hamster liver extracts released 42% and 65%, respectively (Table 1). The rat and hamster extracts removed 9% and 12% of the initial 7-methylguanine, respectively, which was about twice the rate of spontaneous depurination.

A more detailed comparison of the removal of these methylated purines by the rat and hamster liver extracts is given in Fig. 3, in which the effect of protein concentration is shown. Under the assay conditions, release of 7-methylguanine was proportional to the protein added and the hamster extracts were

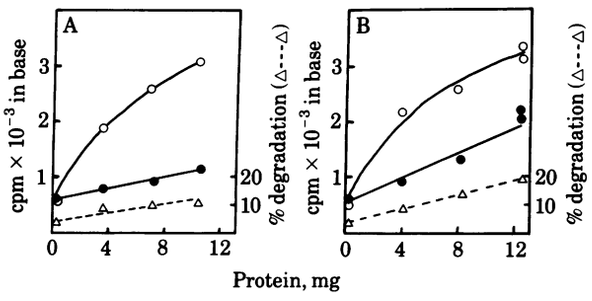

FIG. 3. Protein dependence of release of methylated purines by rodent liver extracts. [^{3}H]Methylated calf thymus DNA (15 μg, 35,000 cpm) was incubated for 2 hr in the presence of various amounts of rat liver (*A*) or hamster liver (*B*) protein. The amount of 3-methyladenine (○) or 7-methylguanine (●) released into the ethanol-soluble fraction was then determined. The total amount of radioactivity converted to ethanol-soluble components is also shown as percent degradation (△---△). More than 95% of this radioactivity was accounted for as free 7-methylguanine and 3-methyladenine.

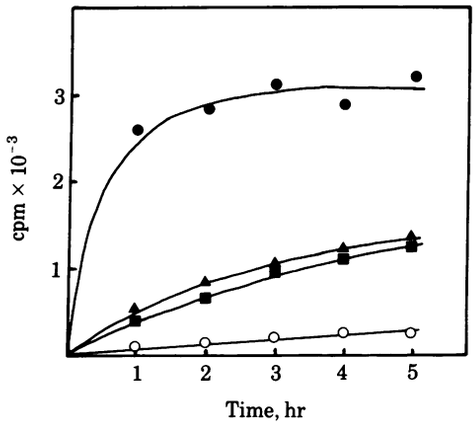

FIG. 4. Release of ethanol-soluble radioactivity from alkali-treated, [^{3}H]methylated DNA. Extracts from *E. coli* (●), hamster liver (▲), and rat liver (■) or an equivalent amount of buffer solution (control, ○) was incubated for the time shown with alkali-treated, [^{3}H]methylated calf thymus DNA.

about 1.7 times more active. Although too great a proportion of the available 3-methyladenine was released for the reaction to be directly proportional to the protein added, the rat and hamster extracts were approximately equally active in removal of 3-methyladenine.

When alkali-treated [^{3}H]methylated DNA was used as a substrate, extracts from *E. coli*, rat liver, and hamster liver were again able to catalyze the release of substantially more radioactivity into the supernatant fraction than occurred with the DNA alone (Fig. 4). Analysis of the material released was complicated by the following facts: (*i*) The DNA contained 3-methyladenine, rom^{7}G, and 7-methylguanine. (*ii*) The rom^{7}G consistently appeared both on chromatography on Sephadex G-10 and on high-pressure liquid chromatography as a double peak, suggesting that it was an equilibrium mixture of two or more components. This behavior has been noted by others using other analytical systems (13). (*iii*) The rom^{7}G peaks cochromatographed with 3-methyladenine on Sephadex G-10 without adjustment of the pH. When the pH was adjusted to 5 prior to chromatography, the peak for 3-methyladenine overlapped that for 7-methylguanine (Fig. 5). Therefore, chromatography under both conditions was needed to analyze the products released by the extracts. As shown in Fig. 5, rom^{7}G was not lost from the DNA by spontaneous hydrolysis, but its release was catalyzed by both the *E. coli* extract, as reported by Chetsanga and Lindahl (8), and by the rat and hamster extracts. A summary of these analyses is shown in Table 2. As found with the untreated [^{3}H]methylated DNA substrate, all three extracts released 3-methyladenine whereas only the rodent liver samples released 7-methylguanine. All three extracts catalyzed the release of rom^{7}G; with the substrate in which about 50% of the 7-methylguanine residues had been converted to the ring-opened form, the rodent liver extracts were 4–9 times more active with rom^{7}G than with 7-methylguanine itself. In all cases, the amount of the bases released from the alkali-treated substrate was equivalent to that lost from the alcohol-precipitable DNA (data not shown), implying that the reaction catalyzed was of the glycosylase type rather than the further metabolism of small oligonucleotides.

DISCUSSION

The present results indicate that rat and hamster livers contain glycosylase activity that can release 7-methylguanine from

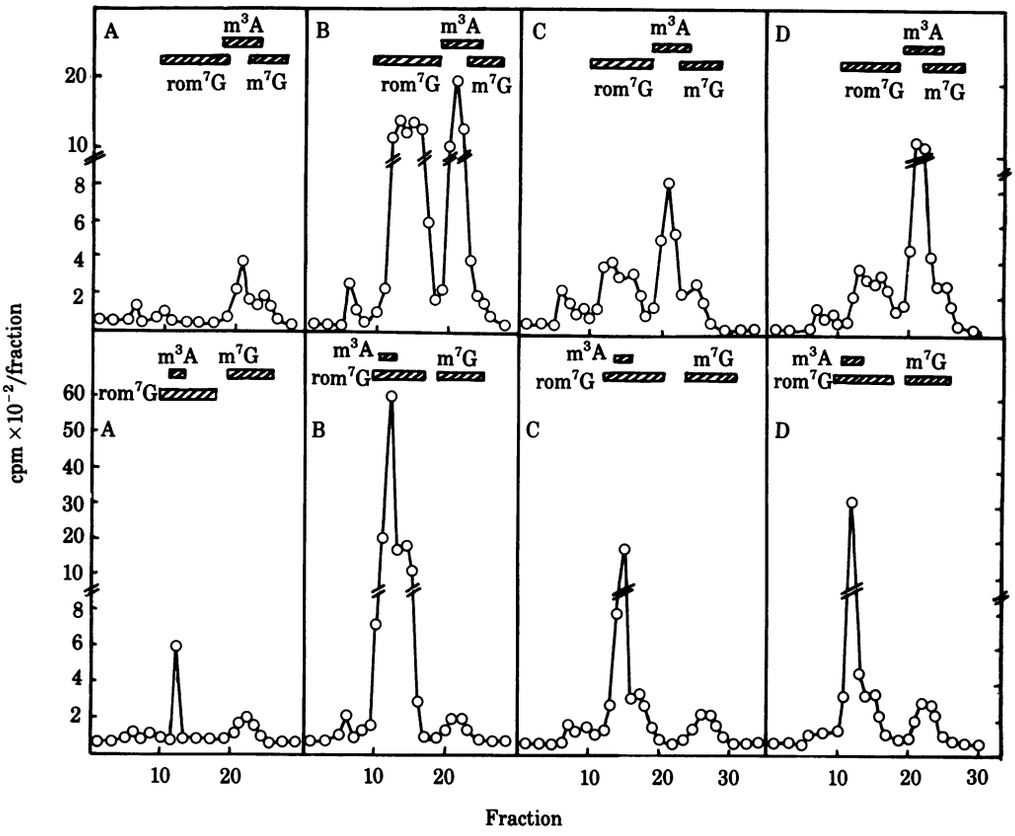

FIG. 5. Chromatographic separation of methylated products released from alkali-treated, methylated DNA. The ethanol-soluble material present after incubation of alkali-treated, [³H]methylated DNA without addition of protein (control) (*A*) or with protein from *E. coli* (*B*), rat liver (*C*), or hamster liver (*D*) as described in Table 2 was analyzed by chromatography on Sephadex G-10. (*Upper*) Analysis of samples adjusted to pH 5 prior to application; (*Lower*) analysis of samples applied at pH 1. Shaded areas indicate the fractions corresponding to authentic markers of rom⁷G, 3-methyladenine (m³A), and 7-methylguanine (m⁷G).

methylated DNA. The presence of this enzyme accounts for the more rapid loss of this product from DNA *in vivo* than would be expected from the half-life for spontaneous depurination. The existence of such an enzyme is strongly suggested by the observations that the persistence of 7-methylguanine in DNA of rat tissues is severalfold greater than in DNA of hamster tissues (10–12, 14); it seems unlikely that there would be substantial species differences in the pH or ionic environment of the nuclear DNA. The present results are consistent with the *in vivo* data; hamster liver extracts were more active than those from rats in catalyzing release of 7-methylguanine. 7-Methylguanine-DNA glycosylase appears to be widespread among mammalian species; an accompanying paper describes its presence in human lymphoblasts (15). This enzyme has also been observed in bacteria (16), although our extracts from *E. coli* did not catalyze this reaction in agreement with an earlier report (8). It is probable that freezing of the cells or a step in the fractionation procedure used here inactivated the enzyme from *E. coli*.

Table 2. Release of methylated products from alkali-treated methylated DNA

Extract added	Ethanol-soluble radioactivity, cpm × 10^{-2}			
	rom⁷G*	m⁷G†	m³A/rom⁷G†	m³A/m⁷G*
None	0.3	3.9	5.4	11.5
E. coli	75.0	4.5	131.0	52.1
Rat liver	19.1	6.1	35.9	28.1
Hamster liver	18.0	8.2	45.2	40.7

Extracts were incubated with alkali-treated methylated DNA, and the ethanol-soluble radioactivity was analyzed by chromatography on Sephadex G-10 (see Fig. 5). Incubation conditions were as given for the scaled-up assays; the tubes contained 30 μg of DNA (45,000 cpm) and 4.5 mg of rodent liver protein. m⁷G, 7-methylguanine; m³A, 3-methyladenine.

* Samples adjusted to pH 5 before chromatography.

† Samples adjusted to pH 1 before chromatography.

The preparations from *E. coli* were used in our experiments to provide a positive control for the removal of rom⁷G from methylated DNA. Our results confirm the existence of this glycosylase in bacteria (8) and show that it is also present in rodent liver, in which it was several times more active than the 7-methylguanine-DNA glycosylase under the assay conditions used. Its role in the loss of 7-methylguanine from DNA is doubtful because at physiological pH the expected rate of ring opening would be very slow (17, 18). Quantitatively, spontaneous hydrolysis and glycosylase-catalyzed release of 7-methylguanine would be much faster. However, even a small degree of ring opening may generate a potentially much more harmful product, and the rom⁷G-DNA glycosylase may protect against this. As previously suggested, it may also play a role in repair of other DNA lesions such as those induced by x-irradiation (8).

Finally, our results indicate that the 3-methyladenine-DNA glycosylase known to be present in bacteria (5, 7) and human cells (6) is also found in rodents. The 3-methyladenine-DNA glycosylases purified from bacteria and human cells do not act on 7-methylguanine (5–7). Because the ratio of 3-methyladenine removal to 7-methylguanine removal is different in rat and hamster livers, our results suggest that these are separate enzymes in these species. The family of glycosylases known to

exist in mammalian cells now includes those for uracil, 3-methyladenine, 7-methylguanine, and rom^{7}G.

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