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Effects of Vitamin E and Selenium on Copper-Induced Lipid Peroxidation *in Vivo* and on Acute Copper Toxicity[1] (41332)

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Abstract. Copper sulfate injected intraperitoneally at a dose of 2 mg Cu/kg into vitamin E- and selenium-deficient rats caused a sixfold increase in the formation *in vivo* of the lipid peroxidation product ethane, and caused acute mortality in 4/5 rats. Selenium supplementation of the diet at 0.5 ppm Se largely prevented the increase in ethane production caused by copper injection and reduced mortality to 1/5 rats. Vitamin E supplementation of the diet at 200 IU/kg fully eliminated the increase in ethane production caused by copper injection, and completely prevented mortality. Vitamin E-deficient rats injected with copper sulfate at 5 mg Cu/kg produced over 10 times the ethane produced by rats injected with sodium sulfate or left uninjected. The ethylene produced by the rats injected with copper sulfate was 5% of the ethane produced, and did not differ significantly from the ethylene produced by the controls. Adding copper sulfate at 5 ppm Cu to a liver homogenate stimulated the production of ethane but not of ethylene. The correlation of increased ethane production with increased mortality suggests that lipid peroxidation may be important in the increased toxicity of copper in vitamin E- and selenium-deficient rats.

The tissues of animals deficient in selenium and vitamin E have a greater tendency to peroxidize both *in vitro* (1) and *in vivo* (2) than those of animals adequate in these nutrients. Moreover, agents such as iron and carbon tetrachloride which stimulate peroxidation *in vitro* (3, 4), are much more toxic to rats deficient in vitamin E and selenium (5, 6) than to rats fed adequate amounts of these nutrients. Toxic doses of these agents stimulate peroxidation *in vivo* in vitamin E- and selenium-deficient rats, and dietary supplements of vitamin E or selenium reduce both peroxidation *in vivo* and acute toxicity (5, 7). In addition, both iron toxicity and carbon tetrachloride toxicity can be diminished by synthetic antioxidants (6, 8).

Copper salts have been shown to catalyze the peroxidation of fatty acids (9), phospholipids (10), and microsomes (11) *in vitro*. Copper is also capable of catalyzing the oxidation of sulfhydryl groups of proteins (12) and of low-molecular-weight sulfhydryl compounds such as glutathione (13). Oxidation of protein sulfhydryls might promote lipid peroxidation by disrupting membrane structure or by inhibiting enzymes which protect against peroxidation. Oxidation of glutathione could reduce the protection against peroxidation afforded by the glutathione peroxidase system.

That copper may cause peroxidation *in vivo* is suggested by the accumulation of copper-containing lipofuscin pigments in the livers of sheep subjected to chronic copper toxicity (14). Similar pigments have also been found in the livers of patients suffering from Wilson's Disease, a genetic abnormality which causes excessive copper accumulation in the liver (15).

It was reported recently that rats fed a vitamin E- and selenium-deficient diet were more vulnerable to acute copper toxicity than rats raised on a diet adequate in selenium (16). The present experiments were designed to determine whether copper injections increase lipid peroxidation *in vivo* in vitamin E- and selenium-deficient and adequate rats, and whether vitamin E

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and selenium could reduce both copper-induced peroxidation and mortality. Lipid peroxidation was estimated by measuring expired ethane (17), a volatile product of the peroxidative decomposition of ω-3-unsaturated fatty acids (18, 19).

Materials and Methods. *Experimental animals and diets.* Male weanling Sprague–Dawley rats (40–50 g) were housed individually in wire mesh cages. Food and water were provided *ad libitum*. The Torula yeast-based diet (Table I) was deficient in vitamin E and selenium. Where appropriate, 200 IU vitamin E/kg diet was supplemented as *dl*-α-tocopherol dissolved in ethanol. Selenium was supplemented to the diet where appropriate at 0.5 ppm Se as sodium selenite in aqueous solution.

Cod liver oil was the primary source of ω-3-unsaturated fatty acids in this diet. To reduce the possible yield of ethane from diet peroxidizing in the gut, all rats were shifted to diets lacking cod liver oil 2 days before ethane collection. During diet preparation, all ingredients except cod liver oil were thoroughly mixed, then a portion of this mixture was removed and cod liver oil was mixed with the remainder. So each cod liver oil-free diet contained nearly the same concentration of vitamin E, selenium, and other components as the corresponding complete diet.

TABLE I. COMPOSITION OF BASAL DIET

Ingredient	Percentage
Torula yeast[a]	30.0
Sucrose	58.5
Cod liver oil	5.0
Salt mix[b]	5.0
Vitamin mix[c]	0.9
DL-Methionine	0.5
Choline chloride	0.1

[a] Lake States Yeast Corp., Rhinelander, Wisc.

[b] Salt mix (g/kg): $CaCO_3$, 543; KH_2PO_4, 212; KCl, 112; NaCl, 69; $MgCO_3$, 25; ferric ammonium citrate (brown), 20.5; $MgSO_4$, 16; NaF, 1.0; $CuSO_4$, 0.9; $MnSO_4 \cdot H_2O$, 0.39; $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$, 0.17; KI, 0.08.

[c] Vitamin mix (per kg of diet): glucose monohydrate, 8.85 g; thiamin–HCl, 4.0 mg; riboflavin, 2.5 mg; pyridoxine–HCl, 2.0 mg; calcium D-pantothenate, 20.0 mg; niacin, 100 mg; menadione, 1.0 mg; folic acid, 2.0 mg; biotin, 1.0 mg; B-12 triturate (0.1% B12), 10.0 mg; vitamin A palmitate (500,000 IU/g), 6.0 mg; vitamin D_2 (500,000 IU/g), 3.2 mg.

Injections. Rats were anesthetized briefly with diethyl ether, injected intraperitoneally with copper sulfate solutions, and placed in ethane collection chambers. Uninjected controls were also anesthetized.

Estimation of carbon dioxide production. Each ethane collection system contained a carbon dioxide trap. This consisted of 350 ml of a 15% potassium hydroxide solution. Air was bubbled through this at the rate of 400 ml/min. To estimate CO_2 production, 50-ml aliquots of KOH solution were removed and brought to pH 10.0 by adding 2 *N* HCl. Trapped CO_2 was then estimated by titrating with 0.2 *N* HCl and measuring the volume required to go from an inflection point near pH 8 to one near pH 4. The background CO_2 level was estimated by titrating traps from collection systems run without rats. Background values averaged less than 10% of the values measured in chambers containing rats.

Ethane collection chambers. The ethane collection system used was described by Hafeman and Hoekstra (5). Briefly, rats were sealed in a 2.5-liter desiccator. A pump circulated air from the desiccator through traps for ammonia, carbon dioxide, and water. Oxygen entered the system from a boiling liquid oxygen source to replace the oxygen consumed by the rat. Rats were placed in the desiccators with water but no food immediately after injection of copper sulfate. The initial ethane content of each collection system was measured 5 min after the animals were sealed in the chambers. This value was subtracted from all subsequent ethane readings.

The recovery of ethane standards in these systems after 5 hr averaged 98 ± 3%. Ethylene recovery was 97 ± 2%.

Analysis of ethane and ethylene. One-milliliter samples of collection system air were taken in plastic syringes and injected into a gas chromatograph equipped with a flame ionization detector. The column used was 3-ft × 0.075-in. porous polymer beads (Poropak R, Waters Associates Inc., Framingham, Mass.) in a 1/8th in. outer diameter aluminum tubing. Gas flow rates were: nitrogen, 50 ml/min; hydrogen, 25

ml/min; and oxygen, 250 ml/min. The column temperature was 50°C. In experiments involving ethylene, a 6-ft × 0.075-in. Poropak R column at 50°C was used. Gas flow conditions in these experiments were: nitrogen, 35 ml/min; hydrogen, 35 ml/min; and oxygen, 350 ml/min.

For experiments involving ethylene, gas samples were taken in glass disposable syringes and metal needles sealed with an epoxy glue. Ethane and ethylene standards were purchased from Matheson.[3]

Tissue homogenate. Male weanling rats were fed for 8 days a vitamin E- and selenium-deficient Torula yeast-based diet. Rats were killed by decapitation and livers were homogenized in 150 m*M* KCl, 25 m*M* Tris buffer (pH 7.5 at 25°C). The homogenate was used immediately after preparation. Four-milliliter aliquots of 10% homogenate were placed in 25-ml Ehrlenmeyer flasks on ice. Copper sulfate at 5 ppm copper was supplemented to four flasks and four were unsupplemented. Flasks were sealed with serum stoppers and transferred to a Dubnoff shaker at 37°C. Air samples were taken after 3 min at 37°C and 0.5, 1.0, and 2.0 hr later. The 3-min readings, which were close to the background levels of ethane and ethylene in room air, were subtracted from subsequent readings.

Statistics. All confidence intervals given are standard errors of the means. Differences between groups of data were tested for significance using Student's two-tailed *t* test.

Results. *Effect of copper on lipid peroxidation in vivo as assessed by ethane production.* The first experiment was designed to find whether copper stimulated ethane production in vitamin E-deficient rats. Male weanling rats were fed for 54 to 55 days the diet supplemented with selenium and deficient in vitamin E. Two days before injection they were shifted to diets lacking cod liver oil. They were injected intraperitoneally with either 5 mg copper/kg as copper sulfate or equivalent sulfate as sodium sulfate, or were left uninjected. Immediately after treatment, they were placed in collection chambers. Cumulative ethane production was measured at 3, 6, and 18 hr after the injection with the results shown in Fig. 1. Copper sulfate caused a rapid rise in ethane production while sodium sulfate did not.

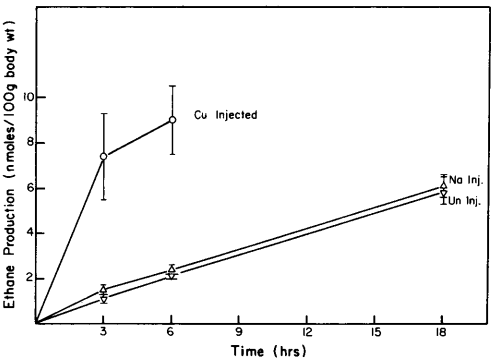

FIG. 1. Effects of intraperitoneally injected copper sulfate at 5 mg Cu/kg or equivalent sodium sulfate on the ethane production of vitamin E-deficient rats. Two of three copper sulfate-injected rats (○) died between 3 and 6 hr after injection. All four sodium sulfate-injected rats (△) and all four uninjected rats (▽) survived.

Effects of dietary vitamin E and selenium on peroxidation in vivo and survival of rats injected with copper sulfate. Male weanling rats were fed for 61 to 71 days the following Torula yeast-based diets: one group was fed a basal diet; one was supplemented with selenium; one was supplemented with vitamin E; and one was supplemented with selenium and with vitamin E. Two days before injection the rats were shifted to diets lacking cod liver oil. Rats were injected with 2 mg copper/kg as copper sulfate or with sodium sulfate to give the same dose of sulfate. They were placed immediately in collection chambers and survival and ethane production (Fig. 2) were followed. Three of five copper-injected rats fed the basal diet died between 5 and 9 hr after injection; one more died before 24 hr. One of five selenium-supplemented rats died between 24 and 48 hr after copper injection. All other rats survived until the end of the experiment 5 days later.

All rats fed the basal diet and injected with copper exhibited haematuria and showed kidney congestion. Lungs were mildly congested to hemorrhagic. The abdominal walls and small intestines were

[3] Joliet, Ill. 60434.

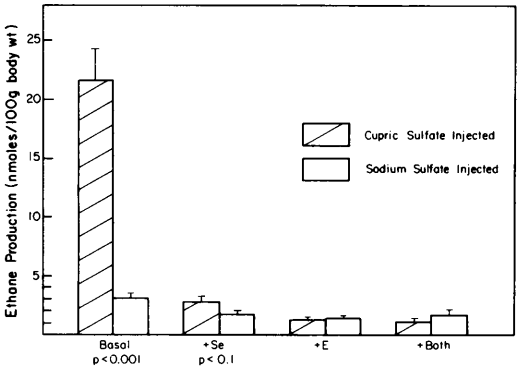

FIG. 2. Ethane production during 5 hr following injection of 2 mg/kg copper as copper sulfate or of equivalent sulfate as sodium sulfate. According to Student's two-tailed *t* test, copper caused a highly significant ethane rise ($P < 0.001$) in basal rats, a marginally significant ($P < 0.1$) rise in selenium-supplemented rats, and no change in either of the vitamin E-supplemented groups.

reddish, and bloody fluid was frequently found in the peritoneal cavity.

Ethane production at 5 hr, the last reading before deaths began, is presented in Fig. 2. Basal rats injected with copper sulfate produced seven times as much ethane as their sodium sulfate-injected controls. In rats fed the selenium-supplemented diet, copper sulfate appeared to cause a slight rise in ethane production, but this was only marginally significant. Copper injection did not significantly alter ethane production in either of the vitamin E-supplemented groups. Thus, vitamin E completely prevented copper-induced ethane rises and protected completely against copper toxicity. Selenium was only slightly less effective in preventing copper-caused ethane increases. Selenium also appeared to protect against copper toxicity, though it should be noted that according to the χ^2 test 4/5 surviving differs from 4/5 dying only at the level $0.1 > P > 0.05$. This pattern is consistent with the hypothesis that lipid peroxidation is important in the increased toxicity of copper in vitamin E- and selenium-deficient rats.

In the above experiments, ethylene appeared as a small peak on the leading shoulder of the ethane peaks. Copper injection had no apparent effect on ethylene production, but this will receive further attention later.

Effect of injected copper sulfate on rat respiration. Some fraction of the increased ethane production caused by copper sulfate might have been caused by an overall increase in respiration. Eight rats fed for 75 to 76 days a selenium- and vitamin E-deficient diet were used to check the effect of injected copper on respiration. The rats were shifted for 2 days to cod liver oil-free diets. Four were injected with 2 mg Cu/kg as copper sulfate. Four others were injected with the same dose of sulfate as sodium sulfate. Ethane and carbon dioxide production were measured for 1 hr. None of the rats died during this period. Copper sulfate-injected rats produced 4.9 ± 1.2 nmole ethane/100 g and 5.1 ± 0.6 mmole carbon dioxide/100 g. Sodium sulfate-injected rats produced 0.6 ± 0.2 nmole ethane/100 g and 5.2 ± 0.2 mmole carbon dioxide/100 g. Clearly, copper caused a large increase in ethane production without changing respiration.

Effect of copper sulfate injection on ethylene production. In the previous two experiments, the ethane and ethylene peaks were not completely separated. When the ethane peak was a large one it was impossible to accurately measure ethylene. Since ethylene has been detected after the copper-catalyzed decomposition of lipid peroxides (20), it was decided to check for ethylene production by rats after an acutely toxic dose of copper sulfate. For this experiment gas chromatography was done using a column which clearly separated ethane and ethylene.

Rats were fed for 37 to 38 days a basal diet and then shifted for 2 days to diet lacking cod liver oil. They were injected with 5 mg copper/kg as $CuSO_4$ or with an equivalent dose of Na_2SO_4, and ethane and ethylene production were measured during the next 4 hr. Two of five copper sulfate-injected rats died between 2 and 4 hr after injection. The rest died overnight. Ethane and ethylene production values at 2 and 4 hr after injection are presented in Table II. Copper injection caused a large rise in ethane production and did not significantly alter ethylene production.

TABLE II. ETHANE AND ETHYLENE PRODUCTION BY RATS FED A VITAMIN E AND SELENIUM DEFICIENT DIET AND INJECTED WITH 5 mg Cu/kg AS $CuSO_4$[a]

Substance injected	Number of rats	2 hr		4 hr	
		Ethane (nmols/100 g)	Ethylene (nmols/100 g)	Ethane (nmols/100 g)	Ethylene (nmols/100 g)
Copper sulfate	5	19.6 ± 11.8[b]	0.9 ± 0.3	24.5 ± 11.6[b]	1.3 ± 0.3
Sodium sulfate	5	0.6 ± 0.4[b]	1.0 ± 0.4	1.5 ± 0.6[b]	1.6 ± 0.5

[a] Two of five $CuSO_4$-injected rats died between 2 and 4 hr after injection.

[b] $P < 0.01$. Differences between copper sulfate- and sodium sulfate-injected rats were analyzed using Student's t test. Data were subjected to a log $(X + 1)$ transformation to make variances homogenous and independent of the means (21).

Effect of copper sulfate on ethane production by liver homogenates in vitro. The effects of 5 ppm copper on ethane and ethylene production of a liver homogenate from a vitamin E- and selenium-deficient rat are shown in Fig. 3. Copper clearly accelerated ethane production, apparently in large part by removing a lag period before rapid peroxidation. However, it did not appear to stimulate ethylene production. In the copper-supplemented flasks ethane production at 1 and 2 hr was 40–50 times greater than ethylene production.

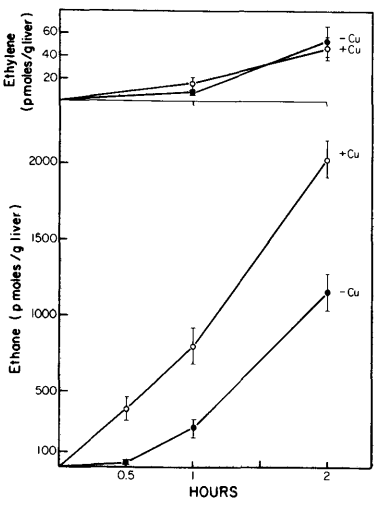

FIG. 3. Ethane and ethylene production by liver homogenates incubated at 37°C. Copper (○) was supplemented at 5 ppm as copper sulfate.

Discussion. Injection of vitamin E- and selenium-deficient rats with 2 mg Cu/kg as copper sulfate greatly increased *in vivo* production of the lipid peroxidation product ethane and caused acute mortality in 4/5 rats. Among rats fed a diet supplemented with the biological antioxidant vitamin E, copper sulfate caused no increased ethane production and no acute mortality. Among rats fed diets supplemented with selenium, copper sulfate caused a small and marginally significant rise in ethane production, and 4/5 rats survived. The effects of selenium may be explainable in terms of the selenium-containing enzyme glutathione peroxidase which is capable of metabolizing hydrogen peroxide and lipid peroxides (22, 23). Whatever the mechanisms by which selenium and vitamin E protect, the correlation of increased ethane production with increased mortality in these experiments supports the hypothesis that lipid peroxidation may be important to the acute toxicity of copper in vitamin E- and selenium-deficient rats.

It is worth noting that copper chloride was shown to inhibit the NADPH-dependent peroxidation of rat liver microsomes (24). Copper inhibited peroxidation and the enzyme NADPH-cytochrome *c* reductase to about the same extent. Richter *et al.* (24) suggested that the inhibition of peroxidation could be explained by the ability of copper to inhibit this enzyme and thus inhibit electron transfer from NADPH to oxygen and cytochrome *P*-450. In another experiment the injection of rats with

copper sulfate was shown to inhibit paracetamol-induced ethane production *in vivo* (25). Thus, in two systems involving the microsomal drug metabolizing system, copper acted to inhibit peroxidation. No attempts were made to induce antioxidant deficiencies in these experiments.

In our experiments the rats showing increased ethane production were those deficient in vitamin E and selenium. Moreover, there is no theoretical need for an involvement of the liver microsomal drug metabolizing system in the increased ethane production we observed.

It is well known that copper and a number of other transition metals can catalyze lipid peroxidation. The most important role of these catalysts is believed to be the decomposition of lipid peroxides to initiate new free radical chains (26).

$$Cu^{+} + ROOH \rightarrow Cu^{2+} + RO\cdot + OH- \quad [1]$$

$$Cu^{2+} + ROOH \rightarrow Cu^{+} + RO_2\cdot + H^{+} \quad [2]$$

Tissue constituents such as ascorbic acid are capable of reducing the copper in $CuSO_4$ to the Cu^{+} state (20). In tissues there are other ways in which metal catalysts might influence peroxidation. Copper is an excellent catalyst for the oxidation of glutathione (13). Reduced glutathione appears to play an important role in the body's protective system against peroxidation (27) by acting as a substrate for the selenium-dependent (22) and selenium-independent (28) glutathione peroxidases. Copper might also act on hydrogen peroxide in a reaction analogous to [1] above to generate the very reactive hydroxyl radical (26).

Ethane is a product formed by the peroxidation of ω-3-unsaturated fatty acids (18). Its volatility and its lack of reactivity have made it a useful tool for the estimation of peroxidation *in vivo* (2, 17). In the studies reported here, the injection of antioxidant-deficient rats with copper sulfate caused large increases of ethane production and little change in the production of ethylene. Similarly, the addition to a tissue homogenate from a vitamin E- and selenium-deficient rat of 5 ppm copper stimulated ethane production and had no apparent effect on ethylene production.

However, in several previous studies the copper-catalyzed decomposition of lipid hydroperoxides had been found to cause more ethylene production than ethane production. The incubation of copper sulfate and ascorbic acid with linolenic acid hydroperoxide or with methyl linolenate hydroperoxide caused the formation of much more ethylene than ethane (20, 29). Moreover, the cuprous ion catalyzed the formation of ethylene from methionine; only minute amounts of ethane were formed in this reaction (30). Lieberman and Hochstein (31) found that adding cupric ion and ascorbic acid to microsomes undergoing NADPH-dependent peroxidation stimulated ethylene production. No mention was made of ethane. Dumelin and Tappel (29) found that adding ascorbate and 0.7 m*M* copper sulfate to preformed methyl linolenate hydroperoxide caused a rapid release of hydrocarbon gases and little further peroxidation. Under these conditions 12 times as much ethylene as ethane were formed. However, when ascorbic acid and 0.7 m*M* copper sulfate were incubated with methyl linolenate to catalyze peroxidation, and more ascorbic acid was added later to decompose the accumulated peroxides, twice as much ethane as ethylene was detected. The reason that ethylene formation predominated when copper was added to preformed peroxides and ethane formation predominated when copper was present during peroxidation was not apparent (29).

In comparing these results to ours it should be noted that we worked with whole animals or with tissue homogenates. The systems yielding predominantly ethylene were purified lipids or microsomes. So part of the differences in the observed ethane to ethylene ratios might involve interactions of copper or conceivably of ethylene with factors in the cytosol. Also, the systems of Dumelin and Tappel (29) and of Lieberman and his collaborators (20, 31) contained high concentrations of ascorbic acid. Ascorbic acid can chelate metals, and chelators can suppress or increase the reactivity of the chelated metals (26). In addition, a copper–ascorbate chelate might show an altered ability to penetrate nonaqueous environments.

The proposed mechanisms for the formation of ethane and ethylene from peroxides point to another factor which might influence the ratio of ethane to ethylene. Evans (18) has proposed that formation of saturated hydrocarbons like ethane involves decomposition of an alkoxy radical near the ω end of an unsaturated fatty acid by β-scission to form a fatty acid aldehyde and an ethyl radical.

$$CH_3-CH_2-\underset{\underset{O.}{|}}{CH}-CH=CH-R \rightarrow CH_3-CH_2\cdot + H-\underset{\underset{O}{||}}{C}-CH=CH-R \quad [3]$$

The ethyl radical could form ethane by abstracting a hydrogen from another fatty acid.

In systems containing copper, the ethyl radical may react with copper to form ethylene (32):

$$Cu^{2+} + CH_3-CH_2\cdot \rightarrow Cu^{+} + H^{+} + CH_2 = CH_2 \quad [4]$$

Note that ethane formation by abstraction of a hydrogen from an unsaturated lipid would lead to propagation of the free radical chain reaction. Ethylene formation from an ethyl radical would constitute chain termination.

In view of these mechanisms one might expect relatively more ethane to be formed at lower concentrations of Cu^{2+}. This supposition has not been systematically tested. The concentration of $CuSO_4$ added to the tissue homogenate in these experiments was 1/8th that used in the experiments of Dumelin and Tappel (29) and 1/12th that used in the experiments of Lieberman and his collaborators (20, 31). However, the concentrations of Cu^{2+} in the different systems, which would depend also on the reducing and oxidizing agents in those systems, are not known.

It is apparent that there are important differences between the hydroperoxide decomposition systems of Lieberman (20, 31) which yielded more ethylene than ethane, and the tissue homogenate and whole animal studies reported here which yielded primarily ethane. In view of Lieberman's results, we expected to see increased ethylene production when we commenced these experiments. In retrospect, realizing that the systems we investigated differed considerably from the systems studied by Lieberman and his collaborators and by Dumelin and Tappel (29), we do not feel that our failure to see elevated ethylene production directly conflicts with their results.

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