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Developmental Expression of Cocaine Hepatotoxicity in the Mouse

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SMOLEN, T. N. AND A. SMOLEN. *Developmental expression of cocaine hepatotoxicity in the mouse*. PHARMACOL BIOCHEM BEHAV **36**(2) 333–338, 1990.—Cocaine may be metabolized either by ester hydrolysis to inactive products or by oxidation via a cytochrome P-450 and FAD-monooxygenase pathway to a hepatotoxic metabolite, presumably norcocaine nitroxide. Mice are the species most susceptible to cocaine-induced hepatotoxicity (CIH), and marked strain differences in response have been found. Female mice are very resistant to CIH, whereas males are susceptible, indicating that hormonal factors may be involved. We treated mice of 5 inbred strains with cocaine at three ages: 20 days (weanling), 30 days (adolescent) and 60 days (adult). The CIH response was assessed by measurement of plasma alanine aminotransferase (ALT) activity 18 hours later. For each of the strains females of all three age groups were resistant to CIH, and males did not begin to develop CIH until approximately 30 days of age. The degree of CIH in 30-day-old males was intermediate between the levels found in 20-day-old males and adult males. These data suggest that the enzyme, or enzymes, responsible for the production of the toxic metabolite are absent, or at very low levels, in female and immature male mice, and that they are either inducible by androgens or are repressed by estrogens or progestins. It is possible that these enzymes may be involved in the production of toxic metabolites of compounds other than cocaine.

Cocaine Hepatotoxicity Mice Development Drug metabolism Alanine aminotransferase

THE metabolism of cocaine occurs by two separate pathways. The major pathway involves hydrolysis of cocaine to ecgonine methyl ester and benzoic acid by serum cholinesterase and nonspecific tissue esterases (24, 25, 28). The other hydrolytic metabolite of cocaine, benzoyl ecgonine, produced by hydrolysis of the methyl ester group, is apparently formed by spontaneous hydrolysis of cocaine, since no specific enzyme responsible for the formation of benzoyl ecgonine has yet been identified (24). The second route of cocaine metabolism involves a hepatic cytochrome P-450 and FAD-containing monooxygenase pathway (15–17). Although this pathway is of only minor importance in humans under normal circumstances, it is of interest because it appears to be responsible for production of a hepatotoxic metabolite (16, 17, 26). The resulting cocaine-induced periportal necrosis has been studied most extensively in mice (5, 16, 26), but recent reports indicate that it may also occur in human cocaine abusers (19,22).

Mice are the species most susceptible to cocaine-induced hepatotoxicity (CIH). There are marked differences in susceptibility to CIH among inbred mouse strains (2,27) with DBA/2 mice being very susceptible and C57BL/6 mice being rather resistant to the development of CIH. In addition, females are much more resistant than males to the development of CIH.

Cocaine use among the young has increased greatly in the U.S. over the last several years (7), nearly doubling from 9% of the adolescent population in 1975 to 17% in 1983. Approximately 5.8% of all U.S. high school students have used cocaine, a figure which has never been higher (10,11). Abuse of cocaine has spread to all ages and socioeconomic groups, which has also led to an increased incidence of acute cocaine poisoning and sudden death (12,18). With the increasing use of cocaine among the young, studies of age-related effects of cocaine become of greater interest. In this paper we describe our studies of age dependency of CIH in 5 inbred mouse strains. We found that young males of all 5 strains were very resistant to CIH compared to adults, and that females were uniformly resistant to CIH at all ages.

METHOD

Animals

Male and female A/Ibg, BALB/cByIbg, C3H/Ibg, C57BL/6Ibg and DBA/2Ibg mice 20–21, 30 ± 1 and 65 ± 5 days of age were used in these studies. Mice were born and raised at the Institute for Behavioral Genetics, maintained on a 12-hour light cycle (lights on 0700–1900) and allowed free access to food (Wayne Lab Blox) and water. Animals were used only once. All procedures described in this paper were reviewed and approved by the University of Colorado Animal Care and Use Committee as being consistent

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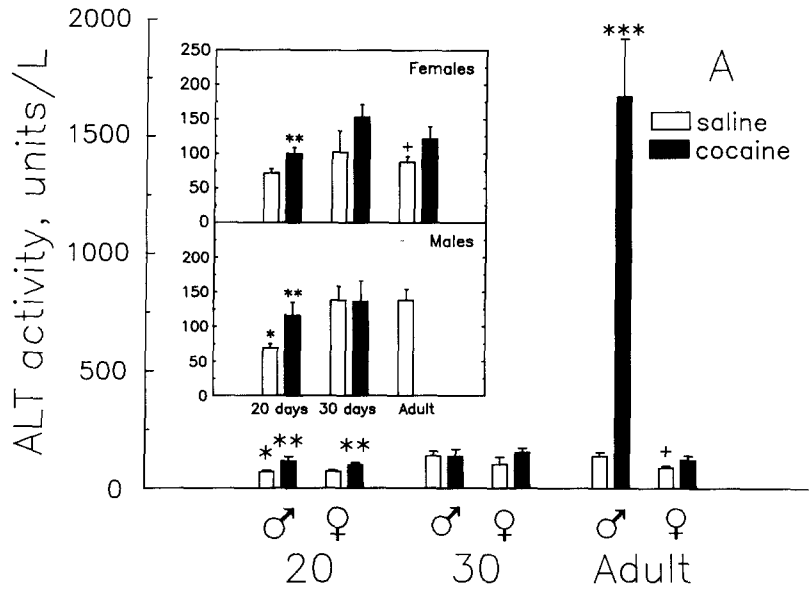

FIG. 1. Cocaine-induced hepatotoxicity in A mice. Mice were injected with saline (open bars) or 50 mg/kg cocaine (shaded bars), and ALT activity was measured 18 hours later. Values are mean ± SEM of 5–10 mice per age and treatment group. Main effect of age on ALT activity for: males, saline treated: $F(2,17) = 6.4$, $p<0.05$; males, cocaine treated: $F(2,22) = 68.3$, $p<0.05$; females, saline treated: $F(2,16) = 0.2$, NS; females, cocaine treated: $F(2,18) = 2.6$, NS. *Twenty-day-old males significantly lower than 30-day-old males or adults. **Significantly higher than respective saline control group. ***Adult males significantly higher than any other group. + Saline-treated adult females significantly lower than saline-treated adult males.

with USPHS standards of humane care and treatment of laboratory animals.

Drug Administration

Mice were injected intraperitoneally with doses of cocaine-HCl (Sigma Chemical Co., St Louis, MO) prepared in saline, ranging from 50 to 70 mg/kg as indicated in the figures. Doses were prepared such that mice received 0.01 ml of solution per gram of body weight. Controls received an equal volume of saline.

Blood Collection

Blood was collected from the retroorbital sinus 18 ± 2 hours after cocaine (or saline) administration. Animals were anesthetized with pentobarbital (60 mg/kg) prior to blood collection. Anesthesia did not affect subsequent analyses. Blood was collected into two heparinized microhematocrit tubes (approximately 150 μl). Plasma was prepared by centrifuging the tubes in a table top IEC clinical centrifuge fitted with a hematocrit rotor at room temperature for 10 min at the maximum setting (approximately 7150 rpm, 5125 × g). The tubes were scored with a file, snapped apart just above the buffy coat, and the clear plasma was expelled into a clean microcentrifuge tube. This procedure results in plasma devoid of any detectable hemolysis as indicated by measurement of hemoglobin (6).

Measurement of Alanine Aminotransferase Activity

Hepatotoxic responses to cocaine were measured by increases in plasma alanine aminotransferase (ALT, also called SGPT) activity using a commercial test kit (ALT No. 59-UV, Sigma) (14,29). A 5–25 μl aliquot of plasma was assayed.

Data Analysis

Data were analyzed by analysis of variance using strain, sex, age and drug treatment as between-subjects factors. A natural log transformation was used to correct for nonhomogeneous variances among groups. Differences in individual sample means were detected using the Tukey B post hoc test. A p value of 0.05 was considered significant, and is the only level reported.

RESULTS

Figures 1–5 show the effects of a single injection of cocaine on CIH as measured by increases in plasma ALT activity 18 ± 2 hours after cocaine administration. Data for females, immature males and saline-treated adult males are shown in the insets to the figures on an expanded scale since it is difficult to display the data meaningfully using the large scale required for the cocaine-treated adult males. The results for all of the strains are clear: females of all three age groups were resistant to the development of significant CIH compared to adult males, and males do not begin to develop CIH until approximately 30 days of age. The degree of CIH in 30-day-old males was intermediate between that found in 20-day-old males and adult males.

Data were analyzed by four-way analysis of variance using strain, sex, age and treatment (saline or cocaine) as between-subjects factors. There were significant main effects of strain, $F(4,398) = 15.8$, sex, $F(1,398) = 179.9$, age, $F(2,398) = 120.1$, and treatment, $F(1,398) = 238.1$, $p<0.05$, for all effects. All of the two- and three-way interactions were significant: strain-by-sex, $F(4,398) = 5.8$, strain-by-age, $F(8,398) = 6.5$, strain-by-

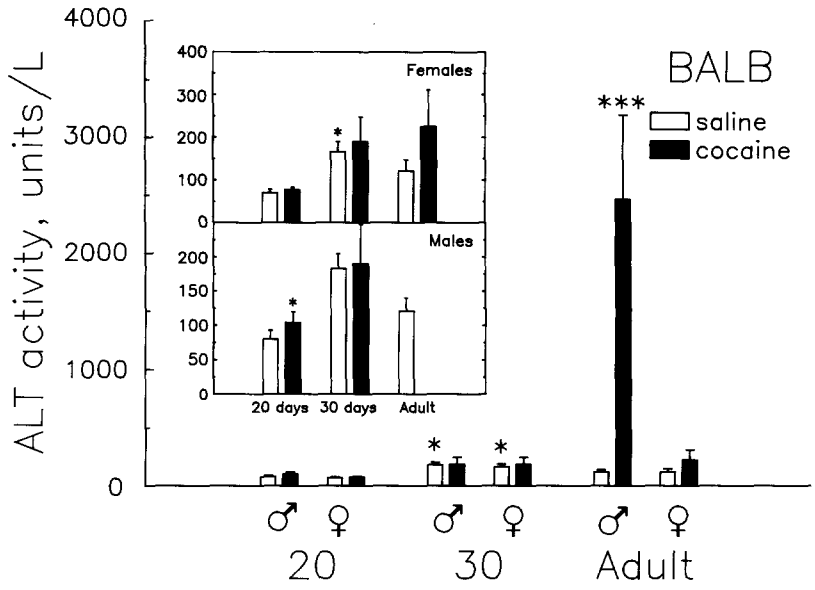

FIG. 2. Cocaine-induced hepatotoxicity in BALB mice. Mice were injected with saline (open bars) or 60 mg/kg cocaine (shaded bars), and ALT activity was measured 18 hours later. Values are mean ± SEM of 6–9 mice per age and treatment group. Main effect of age on ALT activity for: males, saline treated: $F(2,18)=7.8$, $p<0.05$; males, cocaine treated: $F(2,13)=29.8$, $p<0.05$; females, saline treated: $F(2,19)=7.7$, $p<0.05$; females, cocaine treated: $F(2,19)=1.3$, NS. *Thirty-day-old saline-treated males significantly greater than 20-day-old or adult saline-treated males. ***Adult males significantly higher than any other group.

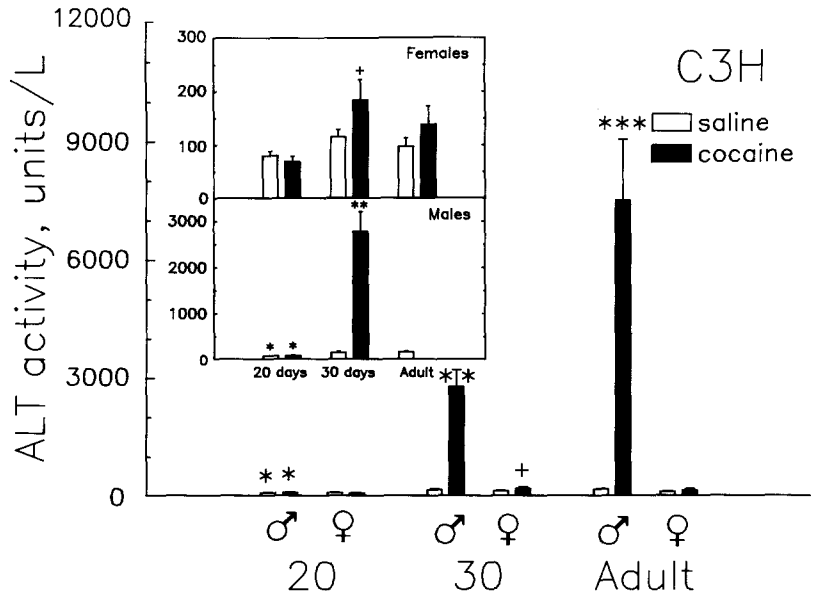

FIG. 3. Cocaine-induced hepatotoxicity in C3H mice. Mice were injected with saline (open bars) or 50 mg/kg cocaine (shaded bars), and ALT activity was measured 18 hours later. Values are mean ± SEM of 5–10 mice per age and treatment group. Main effect of age on ALT activity for: males, saline treated: $F(2,23)=8.3$, $p<0.05$; males, cocaine treated: $F(2,22)=126.2$, $p<0.05$; females, saline treated: $F(2,22)=1.5$, NS; females, cocaine treated: $F(2,23)=6.2$, $p<0.05$. *Twenty-day-old saline- or cocaine-treated males significantly lower than 30-day-old or adult saline- or cocaine-treated males, respectively. **Significantly higher than respective saline control group. ***Adult males significantly higher than any other group. +Thirty-day-old cocaine-treated females significantly lower than 30-day-old cocaine-treated males.

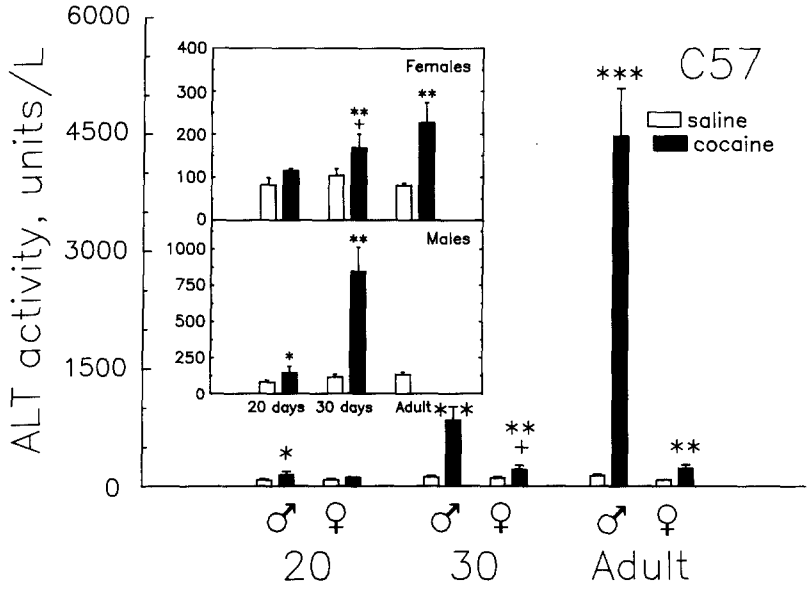

FIG. 4. Cocaine-induced hepatotoxicity in C57BL mice. Mice were injected with saline (open bars) or 70 mg/kg cocaine (shaded bars), and ALT activity was measured 18 hours later. Values are mean ± SEM of 5–12 mice per age and treatment group. Main effect of age on ALT activity for: males, saline treated: $F(2,19)=3.5$, NS; males, cocaine treated: $F(2,24)=77.7$, $p<0.05$; females, saline treated: $F(2,18)=1.1$, NS; females, cocaine treated: $F(2,18)=2.0$, NS. *Twenty-day-old cocaine-treated males significantly lower than 30-day-old males or adults. **Significantly higher than respective saline control group. ***Adult males significantly higher than any other group. +Thirty-day-old cocaine-treated females significantly lower than 30-day-old cocaine-treated males.

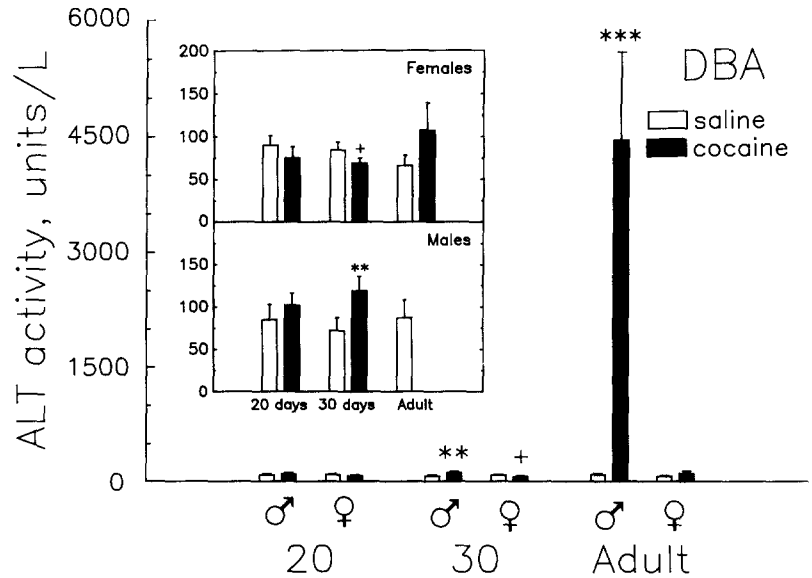

FIG. 5. Cocaine-induced hepatotoxicity in DBA mice. Mice were injected with saline (open bars) or 50 mg/kg cocaine (shaded bars), and ALT activity was measured 18 hours later. Values are mean ± SEM of 7–13 mice per age and treatment group. Main effect of age on ALT activity for: males, saline treated: $F(2,21)=0.3$, NS; males, cocaine treated: $F(2,25)=19.9$, $p<0.05$; females, saline treated: $F(2,21)=1.5$, NS; females, cocaine treated: $F(2,20)=0.4$, NS. **Significantly higher than respective saline control group. ***Adult males significantly higher than any other group. +Thirty-day-old cocaine-treated females significantly lower than 30-day-old cocaine-treated males.

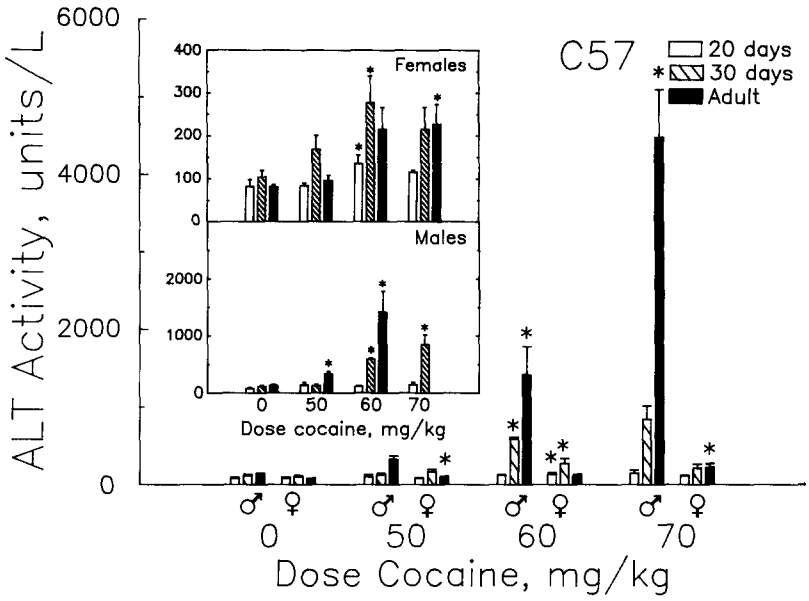

FIG. 6. Dose-response relationship for cocaine-induced hepatotoxicity in male and female C57BL mice 20 days old (open bars), 30 days old (hatched bars) or adult (shaded bars). Mice were injected with varying doses of cocaine and ALT activity was measured 18 hours later. Values are mean ± SEM of 3–10 mice per age and treatment group. *Significantly greater than respective saline control.

treatment, $F(4,398)=7.4$, sex-by-age, $F(2,398)=71.9$, sex-by-treatment, $F(1,398)=119.3$, age-by-treatment, $F(2,398)=66.5$, strain-by-sex-by-age, $F(8,398)=2.5$, strain-by-sex-by-treatment, $F(4,398)=5.4$, strain-by-age-by-treatment, $F(8,398)=4.3$, and sex-by-age-by-treatment, $F(2,398)=45.2$, as was the four-way interaction, strain-by-sex-by-age-by-treatment, $F(8,398)=2.3$, $p<0.05$ for all interactions. Results of one-way analyses of variance broken down by sex and strain, and post hoc tests are given in the legends to the figures.

Strain differences in CIH responses have been reported previously (2,27) and are evident in Figs. 1–5. Adult male A, C3H and DBA mice show a marked response at 50 mg/kg, whereas the BALB and C57BL mice require doses of 60 and 70 mg/kg, respectively. The strain sensitivity of the females is generally similar to the males, but the magnitude of the response is very small in comparison. Elevations of ALT were found in females of all five strains, but these differences are often nonsignificant. At the doses used, there was no significant elevation of plasma ALT in the BALB, C3H or DBA females.

The cocaine doses used for the data in Figs. 1–5 are the minimum, or threshold, doses required to elicit a CIH response in the adult males (2). A dose-response curve illustrating this point is shown in Fig. 6 for C57BL mice. A dose-response relationship for CIH was found in the 30-day-old and adult males, but not the 20-day-old males. At all dose levels 20-day-old males failed to respond to cocaine, and the response of the 30-day-old males was intermediate between the 20-day-old and the adult males. A similar dose-response relationship was found for the females, but the magnitude of the response was very small compared to the males.

DISCUSSION

The results of this study show a clear developmental influence on the production of CIH in male, but not female mice. Young, preweanling male mice are extremely resistant to cocaine-induced hepatotoxicity, adult males are very susceptible to CIH, and 30-day-old males are intermediate in their response. Females are resistant to CIH at all ages. These data suggest that there may be age- and sex-related differences in the metabolism of cocaine to hepatotoxic intermediates which may be mediated by hormonal influences. Previous studies have suggested that the sex difference observed is under androgenic control (27). Since the development of CIH in the males appears to coincide with sexual maturation, it is likely that hormonal changes also mediate the age-related hepatotoxic response to cocaine in the males.

It is known that a metabolite of cocaine, not cocaine itself, is the ultimate hepatotoxin (4, 5, 8, 20, 21, 23, 26). The oxidative pathway of cocaine metabolism requires the participation of FAD-containing monooxygenase as well as cytochrome P-450 (15–17). This pathway leads sequentially from cocaine to norcocaine, to N-hydroxynorcocaine, and finally to norcocaine nitroxide. Most investigators now believe that norcocaine nitroxide is the actual toxic metabolite of cocaine, although opinions vary on its mechanism of action. It may be involved in futile oxidative cycling (16, 20, 21), production of lipid peroxides (15), or it may bind to cellular macromolecules thereby affecting their function (4). Recently, norcocaine nitroxide has been identified in liver and brain of cocaine-injected mice pretreated with metabolic inhibitors (1).

Development of CIH represents a balance between degradation of cocaine to nontoxic products, metabolic conversion of cocaine to a hepatotoxic metabolite, and efficacy of cellular protective mechanisms [glutathione peroxidase activity, reduced glutathione (GSH) levels, etc.]. Changes in any of these parameters could mediate age- and sex-dependent changes in CIH. We have found no age-related differences in the major detoxifying enzyme, serum cholinesterase (in preparation), and although cocaine is known to cause decreases in GSH levels and significant lipid peroxidation, studies in isolated hepatocytes suggest that these two mechanisms may not be critical determinants of cocaine-induced cytotoxicity (3).

Our data suggest that the enzyme, or enzymes, responsible for the production of the toxic metabolite are absent, or at very low level, in female and immature male mice. These developmental and age-dependent patterns indicate that this enzyme is either inducible by androgens, or repressed by estrogens or progestins (9,13). Whether the sex hormones act directly on the liver or through the hypothalamic-pituitary axis is not known. Our studies of CIH are concentrating on the enzymes potentially involved in the production of the toxic metabolite of cocaine. In particular, we are exploring the possibility that these enzymes may be involved in the production of toxic metabolites of compounds other than cocaine. The use of cocaine-induced hepatotoxicity as a model to study hormonal regulation of expression of drug metabolizing systems may have implications exceeding the investigation of cocaine metabolism alone.

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