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SHORT COMMUNICATIONS

Effect of pregnenolone-16α-carbonitrile on the metabolism of dimethylnitrosamine and binding to rat liver macromolecules

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Many of the simple dialkyl *N*-nitrosamines are quite toxic and carcinogenic [1–3], and most available evidence indicates that the biological actions of these compounds are due, not to the parent compounds *per se*, but to their metabolic products which spontaneously decompose and thereby elicit their effects [1, 4–6]. The exact nature of the active metabolites and the mechanisms through which they act remain unclear. Several nitrosamines have been shown to give rise to alkylating agents [4, 7–9], and it is thought that such reactions are the cause of their toxicity and carcinogenicity [5, 6].

Dimethylnitrosamine (DMN) is one of the most toxic and carcinogenic nitrosamines known [1, 3, 5], and insofar as being a potential human health hazard, it is probably the most important [10–12]. It is possible to interrupt or reduce the toxicity and hepatocarcinogenicity of DMN by certain agents, such as aminoacetonitrile [13, 14], polycyclic aromatic hydrocarbons [15, 16] or protein-free diet [17, 18]. Although they produce an interesting effect on DMN metabolism, these agents can also have an extremely deleterious effect on the cells.

Pregnenolone-16α-carbonitrile (PCN), a hormonally inactive steroid, inhibits the acute toxicity of DMN, yet does not affect its overall metabolism *in vivo* (as judged by the rate of disappearance from the blood). PCN, however, does greatly reduce *N*-demethylation *in vitro* [19], a step considered essential for conversion of DMN to carcinogenic and toxic metabolites [1, 5]. PCN is also the first nontoxic agent capable of counteracting mortality and hepatic necrosis induced by DMN. These unique properties indicate a need for further elucidation of the relationship of PCN to the toxicity and metabolism of DMN.

Female Sprague Dawley rats (Eppley Colony), weighing 100–120 g, were maintained on Wayne Lab-Blox and water *ad lib*. All animals were treated according to the method of Somogyi *et al.* [19]. To determine the time of maximum incorporation of radioactivity into liver DNA, an initial group of 18 animals was given five oral doses (at 12-hr intervals) of 5 mg PCN (Searle, Chicago, Ill.) as a microcrystal suspension (5 mg/ml) stabilized with "Tween 80" (1 drop/10 ml). Twenty-four hr after the last gavage of the steroid, the animals were lightly anesthetized with ether and injected in the jugular vein with 35 mg/kg body wt DMN [Eastman Organic Chemicals, Rochester, N.Y.; 3.5 mg/ml in 0.9% saline; 5 μCi DMN (New England Nuclear, Boston, Mass.)]. Three animals were killed after different time intervals, the livers were extracted and the DNA was isolated, according to a modified method of Kirby and Cook [20]. The isolated and purified DNA was quantitated by the diphenylamine method [21] and assayed for radioactivity by scintillation counting.

After about 8 hr, radioactive incorporation was maximized and this time was used in subsequent experiments. Therefore, another group of eight animals was treated with PCN, as above, while an additional group of eight rats, as controls, was given the vehicle only and subsequent DMN treatment. Each animal (in treated and control groups) received 35 mg/kg body wt DMN (30 μCi). The rats were then housed separately in air-tight glass metabolism cages. Expired carbon dioxide (CO_2) was collected in a series of three flasks containing 5 N NaOH. All animals were killed after 8 hr and the liver DNA, RNA and protein were isolated, using the previously stated technique [20]. Protein was quantitated by the method of Lowry *et al.* [22] and RNA by the orcinol method [23]. All purified macromolecular fractions were assayed for radioactivity by scintillation counting. Expired $^{14}CO_2$ in this single time period was precipitated as $BaCO_3$, washed exhaustively with cold water and assayed, as a suspension, for radioactivity by scintillation counting.

Purified DNA was hydrolyzed in 1 N HCl for 1 hr at 100° (final concentration was 10 mg/ml) and the hydrolysates were chromatographed first on a Dowex 50 X8 column (hydrogen form, 50 × 0.9 cm) with a linear gradient of 1 N to 4 N HCl. Early eluting peaks were pooled, concentrated and re-chromatographed on an Aminex Q-150S column (30 × 0.9 cm, ammonium form) with distilled water. Mild acid hydrolysis was carried out by incubating samples of liver DNA in 0.1 M HCl for 16 hr at 37°. Chromatography of this hydrolysate was done on a 50 × 0.9 cm column of Aminex Q-150S (NH_4^+ form) equilibrated and eluted with 0.3 M ammonium formate, pH 7.0. Aliquots of each 3-ml fraction were assayed for radioactivity by scintillation counting using Aquasol (New England Nuclear, Boston, Mass.) as scintillant.

Table 1 gives the specific activity of liver DNA, RNA and protein, and total expired $^{14}CO_2$ from PCN-treated rats and untreated controls at 8 hr after receiving [^{14}C]DMN (30 μCi, 35 mg/kg body wt). For all the liver macromolecules, there was no appreciable difference in the gross amount of incorporated radioactivity. Also, similar analysis of these molecules 10 hr after receiving DMN from previously untreated controls led to similar results (Table 1), indicating that radioactive incorporation for these animals is also maximal after about 8 hr. Similar metabolic rates and hence DMN incorporation from PCN-treated and control animals are further reflected in the total amount of expired $^{14}CO_2$ in 8 hr. It appears that the total expired by PCN-treated animals is slightly less than that of controls; however, this difference is not statistically significant.

Although the overall extent of binding from PCN-treated and control animals is similar, the nature of the

Table 1. Specific activity of rat liver DNA, RNA and protein after an 8-hr exposure to [^{14}C]-DMN*

Sample	Specific activity† (dis./min/mg) Untreated	PCN-treated
DNA	22131 ± 1204	20371 ± 3672
RNA	10342 ± 706	8392 ± 969
Protein	704 ± 101	915 ± 142
CO_2‡	$3.51 \pm 16 \times 10^7$ (52.70% ± 2.40)	$2.98 \pm 19 \times 10^7$ (44.74% ± 6.82)

*Each animal received 35 mg/kg body wt DMN (30 μCi).
†Values represent mean of eight determinations ± S. D.
‡Values are total des./min in expired air and represent eight determinations ± S. D.

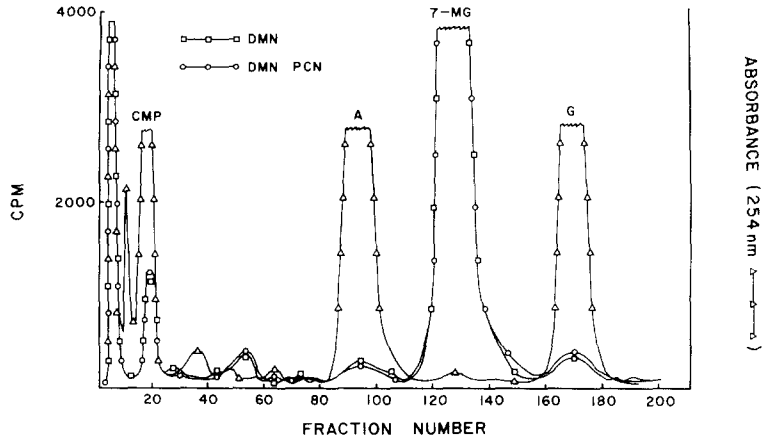

Fig. 1. Elution patterns of DNA hydrolysates from untreated and PCN-treated rats after an 8-hr exposure to [^{14}C]-DMN (35 mg/kg body wt, 30 μCi). The column (50 × 0.9 cm) packed with Dowex 50 X8 (H^+ form) was developed with two column volumes of distilled H_2O, followed by a linear gradient of 1 N to 4 N HCl. Abbreviations: CMP = cytidine monophosphate, G = guanine, and A = adenine.

incorporation may be different. Therefore, liver DNA isolated from both control and treated rats after an 8-hr exposure to DMN was hydrolyzed in 1 N HCl for 1 hr at 100° and the hydrolysate was chromatographed (Fig. 1); after elution with two column volumes of distilled water, followed by a linear gradient of 1 N to 4 N HCl, there was no qualitative difference between the two chromatograms, nor quantitative variance in the extent of methylation of guanine. That is, after pooling the fractions between adenine and guanine and counting an aliquot, it was demonstrated that 79.6 (± 4.3) and 81.4 (± 3.9) per cent of the radioactive sample applied to the column eluted as 7-methylguanine from control and PCN-treated animals respectively.

Further analyses of the early eluting fractions (1–10) on Aminex Q-150S and of the mild hydrolytic products on Aminex Q-150S (NH_4^+ form) again showed no qualitative differences between the DNA hydrolysates from PCN-treated or control animals.

Samples of liver DNA from PCN-treated and control animals exposed to DMN for 2–7 hr were analyzed chromatographically, as above, and the results were the same as for the 8-hr samples. Also, DNA samples from animals that received only 5 mg/kg body wt DMN (10 μCi) for 8 hr showed the same pattern as those that received 35 mg/kg body wt.

It has been postulated that the cause of the toxicity and carcinogenicity of nitrosamines is due to the formation of alkylating agents [5, 6]. Pretreatment of rats with PCN inhibits the toxicity of DMN [19], yet our results show that such pretreatment does not alter the overall rate of metabolism of DMN to CO_2 (Table 1), nor does it affect the extent of methylation of macromolecules, either quantitatively or qualitatively (Table 1 and Fig. 1). The unique protective action of PCN, which is drastically different from other agents [13, 16], remains unknown. Many possibilities still exist which could explain this protective action against toxicity and possibly against carcinogenicity. Studies are now underway to further elucidate PCN's mechanism of action.

Addendum—After this paper was submitted for publication, a report by Kleihues, Margison and Margison appeared [*Cancer Res.* **35**, 3667 (1975)] showing, in agreement with our data, the lack of effect of PCN on the methylation of hepatic DNA.

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