Cellular Injury and Carcinogenesis

INHIBITION OF METABOLISM OF DIMETHYLNITROSAMINE BY AMINOACETONITRILE

By L. FIUME,* GABRIELLA CAMPADELLI-FIUME,* P. N. MAGEE AND J. HOLSMAN
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W.1, U.K.

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1. Aminoacetonitrile, a lathyrogenic agent known to decrease the hepatotoxic action of dimethylnitrosamine, inhibited the metabolism of this compound by rats *in vivo* and by rat liver slices *in vitro*. 2. Methylation of nucleic acids in rat liver and kidney by dimethylnitrosamine *in vivo* was inhibited by treatment of the animals with aminoacetonitrile. 3. These findings are discussed in relation to the hypothesis that dimethylnitrosamine requires metabolism to exert its hepatotoxic and carcinogenic action.

Aminoacetonitrile, a lathyrogenic compound (Wawzonek, Ponseti, Shepard & Wiedenman, 1955), was shown to inhibit the production of cirrhosis of the liver in rats by chronic administration of carbon tetrachloride (Fiume & Favilli, 1961). It prevents the necrosis of the liver induced by this hepatotoxin (Fiume, 1962a; Fiume & Laschi, 1963) and also the necrosis caused by allylamine (Fiume, 1962b), bromobenzene (Fiume, 1963), chloroform and dimethylnitrosamine (Fiume, 1962c).

The available evidence suggests that dimethylnitrosamine is not itself hepatotoxic but that it is metabolized into a methylating intermediate compound which is the actual toxic agent (see Magee & Barnes, 1967; Magee & Swann, 1969). Among the earliest biochemical changes after a dose of dimethylnitrosamine sufficient to cause liver necrosis is inhibition of protein synthesis in the liver (Magee, 1958). Aminoacetonitrile administration was shown to prevent this early inhibition of hepatic protein synthesis (Fiume, 1964; Mager, Halbreich & Bornstein, 1965) suggesting that its protective action might be mediated by inhibition of the metabolism of the nitrosamine. The finding by Fiume & Roffia (1965) that the concentration of dimethylnitrosamine in the livers of rats treated with aminoacetonitrile was higher than that when the animals received the nitrosamine alone gave support to the hypothesis that metabolism of dimethylnitrosamine was inhibited. However, the lack of data on the extent of this inhibition made it impossible to ascertain whether the entire protec-

tive action of aminoacetonitrile could be attributed to this mechanism.

The experiments reported in the present paper were undertaken to elucidate this point and to gain further information on the inhibition of metabolism of dimethylnitrosamine by aminoacetonitrile.

MATERIALS AND METHODS

Animals. Female Wistar albino rats were maintained on Rowett diet 81.

Chemicals. Dimethylnitrosamine was obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.) and purified by distillation (b.p. 151°C). Aminoacetonitrile bisulphate was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.).

Radioactive material. [^{14}C]Dimethylnitrosamine was prepared by Dr D. F. Heath, by the method of Dutton & Heath (1956). The radioactive material (4.8 mCi/mmol; 3.06 mg/ml of water) was diluted with unlabelled dimethylnitrosamine before use. The labelled starting material was [^{14}C]methyl iodide (30 mCi/mmol) purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Measurement of radioactivity. A Packard Tri-Carb model 3320 liquid-scintillation spectrometer was used. Corrections from c.p.m. to d.p.m. were made either by the addition of standard [^{14}C]toluene (Packard Instrument Co., La Grange, Ill., U.S.A.) or by the channel-ratios method (Baillie, 1960). Three scintillation solutions were used: 0.6% 2,5-diphenyloxazole in toluene for samples that did not contain water; 1% 2,5-diphenyloxazole, 0.05% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 8% naphthalene dissolved in dioxan-Cellosolve-toluene (3:3:1, by vol.) (Bruno & Christian, 1961) was used for aqueous samples; this second scintillation solution plus 4% Cab-O-Sil (Packard Instrument Co.) was used to suspend $\text{Ba}^{14}\text{CO}_3$ precipitates. Nucleic acids and proteins were dissolved in Hyamine (Packard Instrument Co.).

Metabolism of dimethylnitrosamine in vivo. (a) With non-radioactive dimethylnitrosamine. Rats were given

* On leave of absence from Department of General Pathology, University of Bologna, Italy. Present address: Max-Planck-Institut für Medizinische Forschung, Abteilung Chemie, Heidelberg, Germany.

dimethylnitrosamine (30mg/kg body wt.) dissolved in 0.9% NaCl in a volume of 5ml/kg body wt. by intraperitoneal injection. At the time of dimethylnitrosamine administration some rats were injected subcutaneously with aminoacetonitrile, 200mg/kg body wt., as a 20% (w/v) solution of the bisulphate neutralized with NaOH. At increasing time-intervals the rats were anaesthetized with sodium pentobarbital and blood was withdrawn from the abdominal aorta into a syringe moistened with heparin solution. Dimethylnitrosamine was determined in blood samples by the polarographic method of Heath & Jarvis (1955).

(b) With [^{14}C]dimethylnitrosamine. Rats received [^{14}C]dimethylnitrosamine, (30mg and 20 μCi /kg body wt.) in 5ml of 0.9% NaCl/kg body wt., by intraperitoneal injection. Aminoacetonitrile was administered subcutaneously either as one dose (200mg/kg body wt.) simultaneously with the dimethylnitrosamine or as repeated doses (100mg/kg body wt.) on the preceding 2 days and at the same time as the dimethylnitrosamine. Each rat was placed individually in a metabolism cage and air was drawn through the cage and passed through three cylinders, each containing 400ml of 4M-NaOH, which was replaced every 8–9h. Samples of the alkaline solution were taken at various times and the radioactive carbonate was precipitated with BaCl_2 after the addition, when necessary, of carrier Na_2CO_3 . The precipitate of BaCO_3 was washed repeatedly with water and assayed for radioactivity.

Metabolism of dimethylnitrosamine in vitro. Liver and kidney slices were cut with a hand microtome. About 150mg of slices was incubated at 37°C for 60 min in 2.2ml of Krebs–Ringer phosphate buffer (Umbreit, Burris & Stauffer, 1964) containing 20 μmol of glucose in a conventional Warburg apparatus. [^{14}C]Dimethylnitrosamine (542nmol, 0.01 μCi) dissolved in 0.2ml of the phosphate buffer was added from the side arm after the flasks had been allowed to equilibrate at 37°C. The gas phase was O_2 and $^{14}\text{CO}_2$ was trapped in 4M-NaOH in the centre wells of the flasks. The radioactive carbonate, after addition of carrier Na_2CO_3 , was precipitated with BaCl_2 , washed repeatedly with water, and assayed for radioactivity.

Methylation of nucleic acids and isolation of labelled proteins. Groups of eight rats were starved overnight and injected intraperitoneally with [^{14}C]dimethylnitrosamine (30mg and 20 μCi /kg body wt.) in 0.9% NaCl. Four rats received dimethylnitrosamine alone and the other four rats were injected subcutaneously on the previous 2 days and at the time of dimethylnitrosamine administration with aminoacetonitrile (100mg/kg body wt.). The animals were killed by exsanguination and the livers and kidneys were rapidly transferred to liquid N_2 . Total nucleic acids were extracted from about 1g of liver and from both kidneys of each animal by the method of Schneider (1945). DNA and RNA were isolated separately from pooled livers from each group of rats as described by Kidson, Kirby & Ralph (1963), as modified by Swann & Magee (1968). The nucleic acids were hydrolysed in M-HCl for 1 h at 100°C and chromatographed as described by Magee & Farber (1962). The extent of methylation was measured by the amount of 7-methylguanine expressed as a percentage of the total guanine (Swann & Magee, 1968). Proteins were isolated by the method of Rabinovitz, Olson & Greenberg (1954).

Presentation of results. The results are presented as means $\pm$ s.d., with Student's *t* test for significance of difference between means where appropriate.

RESULTS

Effect of aminoacetonitrile on the metabolism of dimethylnitrosamine. The rate of disappearance of dimethylnitrosamine from the circulating blood of rats to which it had been administered was greatly decreased by treatment of the animals with aminoacetonitrile (Fig. 1). The rate of clearance of dimethylnitrosamine from the blood has been shown by Heath (1962) to correspond closely to the rate of its metabolism, which therefore appeared to be strongly inhibited.

The inhibition of metabolism of dimethylnitrosamine by aminoacetonitrile was confirmed by measurement of the rate of exhalation of $^{14}\text{CO}_2$ by rats treated with [^{14}C]dimethylnitrosamine. Animals receiving aminoacetonitrile simultaneously with the nitrosamine, with or without treatment on the preceding 2 days, showed marked inhibition in their output of $^{14}\text{CO}_2$ (Fig. 2).

Dimethylnitrosamine is metabolized by rat liver slices and, at a slower rate, by rat kidney slices *in vitro* (Magee & Vandekar, 1958; Montesano & Magee, 1970). Addition of aminoacetonitrile to liver and kidney slices *in vitro* inhibited metabolism of [^{14}C]dimethylnitrosamine, as shown by the lower production of $^{14}\text{CO}_2$ (Table 1).

Effect of aminoacetonitrile on methylation of liver

Fig. 1. Effect of aminoacetonitrile on the disappearance of injected dimethylnitrosamine from the blood. All rats received dimethylnitrosamine (30mg/kg body wt.) by intraperitoneal injection. Those treated with aminoacetonitrile received 200mg/kg body wt. subcutaneously at the same time. Rats were killed after increasing time-intervals and the concentration of dimethylnitrosamine in the blood was measured. ●, Dimethylnitrosamine alone; ○, dimethylnitrosamine + aminoacetonitrile. The calculated regression lines are shown.

Fig. 2. Effect of aminoacetonitrile on the exhalation of ¹⁴CO₂ after injection of [¹⁴C]dimethylnitrosamine. All rats received [¹⁴C]dimethylnitrosamine (30 mg, 20 μ Ci/kg body wt.). Each point represents the mean of values from two rats. ○, [¹⁴C]Dimethylnitrosamine alone; ▲, [¹⁴C]dimethylnitrosamine + aminoacetonitrile (200 mg/kg body wt.) simultaneously; △, [¹⁴C]dimethylnitrosamine + aminoacetonitrile (100 mg/kg body wt.) on the preceding 2 days and at the same time as the nitrosamine.

and kidney nucleic acids by dimethylnitrosamine in vivo. The marked inhibitory effect of aminoacetonitrile on metabolism of dimethylnitrosamine suggested that it might also inhibit methylation of nucleic acids by the nitrosamine. Rats were given [¹⁴C]dimethylnitrosamine at a dosage (30 mg/kg body wt.) that would induce necrosis of the liver in all and tumours of the liver in some (about 20%) of the surviving treated animals. Nucleic acids and proteins were extracted from the livers and kidneys at increasing time-intervals after the injections and assayed for radioactivity. Aminoacetonitrile inhibited incorporation of the label into proteins of both organs at 4 h and 12 h after injection of the dimethylnitrosamine and the inhibition was still apparent in the kidney at 18 h, but the incorporation in the liver was not significantly different from that in the control at this time (Table 2). Incorporation into total plasma proteins was also measured at 12 h, when the mean specific radioactivity in four rats after dimethylnitrosamine alone was 332 $\pm$ 31 d.p.m./mg and that in four rats that also received aminoacetonitrile was 147 $\pm$ 34 d.p.m./mg, indicating an inhibition comparable with that observed in the liver and kidney proteins. Since these inhibitions might have been due to an effect of aminoacetonitrile on the distribution of the dimethylnitrosamine in the bodies of the animals the radioactivities of the acid-soluble fractions of the liver and kidney were measured at 12 h. The mean acid-soluble

Table 1. Effect of aminoacetonitrile added in vitro on the metabolism of [¹⁴C]dimethylnitrosamine by liver and kidney slices

The metabolism of [¹⁴C]dimethylnitrosamine was measured by measuring the amounts of ¹⁴CO₂ produced during incubation with tissue slices in a conventional Warburg apparatus as described in the Materials and Methods section. The number of flasks used are given in parentheses.

	Production of ¹⁴ CO ₂ (d.p.m./min per g wet wt. of tissue)	
	Liver	Kidney
Control	44.8 $\pm$ 8.8 (8)	11.7 $\pm$ 1.6 (5)
Aminoacetonitrile (0.5 mM)	15.3 $\pm$ 3.0 (8) (66% inhibition)	8.1 $\pm$ 0.6 (<i>P</i> < 0.01) (5) (31% inhibition)
Aminoacetonitrile (5 mM)	6.1 $\pm$ 1.06 (6) (86% inhibition)	

Table 2. Effect of aminoacetonitrile on the specific radioactivity of proteins from livers and kidneys of rats given [¹⁴C]dimethylnitrosamine

All rats received [¹⁴C]dimethylnitrosamine (30 mg, 20 μ Ci/kg body wt.) by intraperitoneal injection and those treated with aminoacetonitrile received 100 mg/kg body wt. of this compound subcutaneously at the same time as the injection of the nitrosamine and on the previous 2 days. There were four animals per group.

Time after injection of [¹⁴ C]dimethylnitrosamine (h)	Liver proteins (d.p.m./mg)		Kidney proteins (d.p.m./mg)	
	-Aminoacetonitrile	+Aminoacetonitrile	-Aminoacetonitrile	+Aminoacetonitrile
4	120 $\pm$ 10	48 $\pm$ 7	97 $\pm$ 13	24 $\pm$ 2
12	298 $\pm$ 19	112 $\pm$ 26	196 $\pm$ 29	62 $\pm$ 16
18	117 $\pm$ 28	112 $\pm$ 18	107 $\pm$ 11	74 $\pm$ 8

radioactivities in the livers and kidneys of the rats receiving dimethylnitrosamine alone were 20830 ± 3683 d.p.m./g wet wt. of tissue and 10200 ± 2180 d.p.m./g respectively and in the animals receiving dimethylnitrosamine plus aminoacetonitrile the corresponding values were 39500 ± 4124 d.p.m./g and 40620 ± 3277 d.p.m./g respectively. These results indicate that there was probably no failure of the nitrosamine to reach the two organs. The higher amounts of total acid-soluble radioactivity in the organs of the rats receiving aminoacetonitrile is consistent with the greatly lowered rate of metabolism of the nitrosamine (Table 1; Figs. 1 and 2).

The incorporation of radioactivity from labelled dimethylnitrosamine into cellular components probably follows two different pathways (Magee & Farber, 1962). After initial demethylation formaldehyde is produced, which enters the normal metabolic pool of C_1 intermediates and becomes incorporated into nucleic acids and proteins by normal metabolic processes. In addition to this pathway the active methylating intermediate that is formed metabolically reacts with proteins, nucleic acids and probably other cellular components. In the nucleic acids of organs of rats treated with dimethylnitrosamine the main site of methylation is on the 7-position of guanine (Magee & Farber, 1962; Lawley, Brookes, Magee, Craddock & Swann, 1968) and the extent of methylation has been expressed as the percentage of the nucleic acid guanines converted into 7-methylguanine (Swann & Magee, 1968). Methylation of nucleic acids of liver and kidney was measured in groups of rats killed at intervals after injection with [14 C]dimethylnitrosamine with or without aminoacetonitrile. In some cases DNA plus RNA was extracted with hot trichloroacetic acid by the Schneider (1945) method and in others DNA and RNA were prepared separately. Aminoacetonitrile clearly inhibited methylation of both nucleic acids at all times studied (Table 3).

DISCUSSION

The experiments reported above clearly show that aminoacetonitrile inhibits the metabolism of dimethylnitrosamine *in vivo* and *in vitro*. It seems probable that the inhibition of metabolism of dimethylnitrosamine *in vivo* is strong enough to account for the protective action of aminoacetonitrile against the liver lesions induced by the nitrosamine. The metabolic inhibition may also explain the ability of aminoacetonitrile to prevent the appearance of liver tumours in rats fed on a diet containing a carcinogenic concentration of dimethylnitrosamine (E. Bonetti & L. Fiume, unpublished work) since the available evidence

Table 3. *Effect of aminoacetonitrile on methylation of nucleic acids by dimethylnitrosamine in vivo*

Time after injection of [14 C]dimethylnitro- samine (h)	DNA and RNA in liver						Total nucleic acids			
	-Aminoacetonitrile			+Aminoacetonitrile			Liver		Kidney	
	DNA		RNA	DNA		RNA	-Aminoacetonitrile	+Aminoacetonitrile	-Aminoacetonitrile	+Aminoacetonitrile
	0.87	0.95	0.87	0.15	0.13	0.34	0.85 $\pm$ 0.09 (4)	0.12 $\pm$ 0.01 (4)	0.21	0.04
4	0.74	1.17	—	0.25	—	—	—	—	—	—
12	—	—	—	—	—	—	0.77 $\pm$ 0.06 (4)	0.50 $\pm$ 0.03 (4)	0.10 $\pm$ 0.003 (4)	0.07 $\pm$ 0.01 (4)
18	—	—	—	—	—	—	—	—	—	—

Total tissue nucleic acids were extracted by the Schneider (1945) method; RNA and DNA were prepared separately from the pooled livers of four rats by a modification of the method of Kidson *et al.* (1963). Methylation is expressed as the amount of 7-methylguanine formed as a percentage of the total guanine, calculated as described by Swann & Magee (1968). The results are given as the mean ($\pm$ s.d. where applicable) and the numbers of rats are in parentheses.

indicates that dimethylnitrosamine requires metabolic activation for its carcinogenic as well as for its hepatotoxic activity (see Magee & Barnes, 1967; Magee & Swann, 1969).

If the protective action of aminoacetonitrile against the toxicity of dimethylnitrosamine is due to inhibition of metabolism of the latter, it is possible that a similar mechanism may operate when aminoacetonitrile modifies the action of other hepatotoxins. There is evidence largely from the work of McLean and his colleagues that carbon tetrachloride must be metabolized to exert its hepatotoxic action. Rats maintained for short periods on protein-deficient diets become resistant to carbon tetrachloride poisoning and metabolism of the compound *in vivo* and by liver microsomal preparations *in vitro* is depressed (Seawright & McLean, 1967). Pretreatment with DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane], a known inducer of metabolism of foreign compounds, greatly increases the sensitivity of rats to carbon tetrachloride toxicity (Seawright & McLean, 1967) and a similar increase in susceptibility to this hepatotoxin in rats treated with phenobarbitone was reported by Garner & McLean (1969). The protection by aminoacetonitrile against carbon tetrachloride toxicity may therefore be mediated by inhibition of metabolism of the latter. It is possible, however, that aminoacetonitrile may modify hepatotoxicity by mechanisms other than inhibition of metabolism of the poison since Mager & Halbreich (1965) found that aminoacetonitrile decreases the inhibition of hepatic protein synthesis induced in rats by ethionine. Ethionine is thought to inhibit protein synthesis by a decrease in the amount of available ATP in the liver through the formation of *S*-adenosylethionine (Farber, Shull, Villa-Treviño, Lombardi & Thomas, 1964). Since there is no reason to believe that ethionine is metabolized in a similar way to carbon tetrachloride and other foreign compounds, it seems probable that a different mechanism is involved in the modifying effect of aminoacetonitrile on the action of ethionine on hepatic protein synthesis.

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