incorporation stems from a transport system activation or even from a modification of this carrier.

In summary, quaternary ammonium-containing compounds were assayed for their inhibiting capacities on *P. falciparum* growth *in vitro*. Hemicholinium 3, decamethonium and decyltrimethylammonium were efficient inhibitors, with IC_{50} of 4×10^{-6}, 10^{-6} and 7×10^{-7} M, respectively. They specifically inhibited phosphatidylcholine biosynthesis without altering the biosynthesis of other phospholipids, proteins, or nucleic acids. This effect was clearly related to an impairment of choline entry into erythrocytes.

Acknowledgements—This work was supported by the Institut National de la Santé et de la Recherche Médicale (CRL 811052), the UNDP/World Bank/WHO special program for Research and Training in Tropical diseases (T 16-181-M2-15A) and the Ministère de la Recherche et de l'Industrie (82-L-0785).

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Biochemical Pharmacology, Vol. 34, No. 22, pp. 4071–4073, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

In vitro DNA methylation by methylnitrosourea in isolated copper- or silver-preloaded rat liver nuclei

(*Received* 21 *September* 1984; *accepted* 8 *May* 1985)

The administration to rats of copper in the diet has been reported to suppress tumorigenesis in the liver [1] and kidney [2] by dimethylnitrosamine (DMN). However, the mechanism of action of copper against tumorigenesis in DMN-treated rats has not been studied adequately. We recently reported [3] that the subcutaneous injection of cupric acetate has a suppressive effect on tumorigenesis in the liver of DMN-treated rats. Under our experimental conditions, no tumor was observed in the kidney. We also showed that the methylation of liver DNA in rats given a single dose of DMN is suppressed significantly by copper administration. It was reported that the cytochrome P-450 content of liver microsomal fractions is decreased by the administration of cupric ions [4]. Therefore, the possibility exists that the administration of copper decreases DMN *N*-demethylase activity, leading to a decrease of DNA methylation and tumorigenicity in the liver of rats given DMN. We are studying whether the administration of copper alters DMN *N*-demethylase activity in the liver. Furthermore, we presume that the *in vivo* binding of copper to DNA protects the modification of DNA by DMN as an initiation step of carcinogenesis, because cupric ions and DMN as the methylating compound, which are recognized as electrophilic reactants, are known to react with nucleophilic sites in DNA *in vitro* and *in vivo*. Cupric ions are also known to prefer the GC regions of DNA [5]. There is the possibility, therefore, that the binding of cupric ions to guanine sites in DNA interacts with the modification of guanine by DMN. A selective reaction of silver ions with the GC regions of DNA has also been described [6]. By the administration of silver to rats, the binding of silver ions to the guanine sites in DNA may interact with the modification of guanine by DMN, although there is no convincing evidence that administration of silver to rats suppresses liver tumorigenesis by DMN. The methylation by DMN or methylnitrosourea (MNU) of guanine in DNA in copper- or silver-preloaded rat liver nuclei has not been clarified.

Jensen [7] has reported that the rate of DNA methylation *in vitro* with DMN activated by microsomal fractions correlates well with the rate of formaldehyde formation, indicating DMN *N*-demethylase activity. He also found 7-methylguanine (7MeG), 3-methyladenine and O^6-methylguanine (O^6MeG) in the DNA hydrolysates in the same relative proportions as observed in DNA isolated from similar incubation mixtures containing MNU as the methylating compound.

In this paper, we examine the *in vitro* methylation of guanine in DNA in isolated copper-preloaded or silver-preloaded rat liver nuclei by MNU, instead of DMN which requires activation by microsomes.

Materials and methods

Chemicals. MNU containing ^3H in the methyl group was purchased from the New England Nuclear Corp., Boston, MA (1.0 Ci/mmole). 7MeG was from the Sigma Chemical Co., St. Louis, MO. O^6MeG was synthesized in our laboratory according to the method of Balsiger and Montgomery [8]. All other reagents used in this study were of special grade.

Animals and administration of metal compounds. Male Wistar rats were obtained from the Matsumoto Labo-Animals Laboratory, Kimitsu, Japan, and maintained on the commercial diet CE-2 (CLEA Japan Inc., Tokyo). Rats weighing 200–220 g were used for the experiments. Metal compounds were dissolved in sterile water. Rats were killed at 24 hr after the subcutaneous injection of cupric acetate (2–10 mg Cu^{2+}/kg body wt) or silver nitrate (5–30 mg Ag^{+}/kg body wt).

Determination of copper and silver. The nuclei obtained from 4 g liver were decomposed by the ashing method using sulfuric and nitric acids. After decomposition, the samples for determination of copper were prepared by the diethyldithiocarbamate–methylisobutylketone extraction method, and the samples for the determination of silver were diluted with an appropriate volume of water. The concentrations of metals were determined by atomic absorption spectrophotometry [9].

In vitro *DNA methylation by MNU in isolated rat liver nuclei.* Nuclei were prepared as described by Widnell and Tata [10]. Liver was perfused with 0.32 M sucrose–3 mM $MgCl_2$. The homogenate was centrifuged at 700 *g* for 10 min. The pellet was resuspended in 25 ml of 2.2 M sucrose–1 mM $MgCl_2$ and centrifuged at 50,000 *g* for 60 min. The nuclear pellet was resuspended in 0.25 M sucrose–1 mM $MgCl_2$ at a concentration of 1 g liver per 0.25 ml solution.

Alkylation of nuclear DNA by MNU was carried out as follows. Nuclear pellet suspension containing about 200 μg DNA was reacted with 0.56 μmole [^{3}H]MNU (sp. act. 10.7 mCi/mmole) in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.4) at 37° for 60 min in the dark. The nuclear fraction was recovered by centrifuging at 800 *g* for 10 min, and isolation of nuclear DNA was carried out by the method of Boffa *et al.* [11]. Histones and other acid-soluble nuclear proteins were extracted with 0.25 N HCl. The nuclear non-histone proteins remaining after acid extraction were solubilized in 0.1 M sodium phosphate buffer (pH 7.2)–6 M urea–0.1% 2-mercaptoethanol–0.4 M guanidine · HCl and extracted by centrifuging at 9000 *g* for 20 min. The pellet, which contained DNA, was washed with 5% perchloric acid (PCA), hydrolyzed in 10% PCA at 75° for 20 min, and centrifuged. Aliquots of the supernatant fraction were analyzed for DNA content by the method of Burton [12] and fractionated by high performance liquid chromatography (HPLC). The DNA solution was co-chromatographed with authentic 7MeG and O^6MeG. HPLC conditions were as follows: ISC-07/S1504 cation-exchange column (Shimazu Co., 4 mm 150 mm), 0.5 M ammonium phosphate–NH_4OH (pH 5.0)–10% methanol, 0.6 ml/min, 60° and 280 nm (0.08 AUFS). Each fraction (0.6 ml) was dissolved in 10 ml of dioxane scintillator, and the radioactivity was measured by a liquid scintillation counter (Beckman LSC-5800). Amounts of 7MeG and O^6MeG were represented as dpm/mg DNA.

Results and discussion

The concentrations of copper and silver in the liver nuclei of rats increased with the increase in dose of cupric acetate and silver nitrate, as shown in Figs. 1 and 2. The copper concentration in the nuclei at 24 hr after the administration of 5 mg Cu^{2+}/kg was 8.86 ± 1.81 nmoles/g liver and that of silver in the nuclei at 24 hr after the administration of 30 mg Ag^{+}/kg was 6.30 ± 1.01 nmoles/g liver. The copper concentration in the liver nuclei after the copper administration was much higher than the silver concentration in the liver nuclei after administration of the same dose of silver.

The amounts of 7MeG and O^6MeG in the DNA hydrolysates after *in vitro* methylation by MNU of the nuclei isolated from livers of copper-treated rats were measured. As shown in Fig. 3, the suppressive rate of formation of both 7MeG and O^6MeG increased with the increase of copper concentration in the nuclei in the dose range of 2 to 10 mg Cu^{2+}/kg. The suppressive rate of O^6MeG formation by 5 or 10 mg Cu^{2+}/kg appeared to be a little higher than that of 7MeG. These results may indicate that cupric ions were bound to both the O-6 and N-7 sites of guanine, and that the binding of copper to these sites protects the methylation of guanine at these sites.

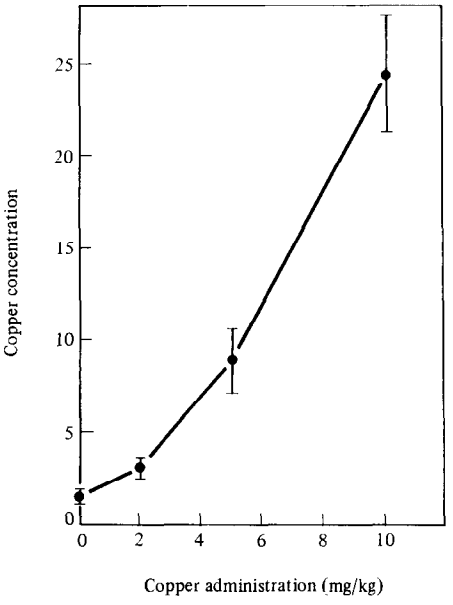

Fig. 1. Copper concentration in rat liver nuclei after the administration of cupric acetate. The copper concentration of the nuclei is shown as nmoles copper per the nuclei isolated from 1 g liver. Rats were subcutaneously injected with 2, 5 or 10 mg Cu^{2+}/ml of sterile water/kg of body weight and killed 24 hr after the injection. Each value represents the mean ± S.D. of six rats.

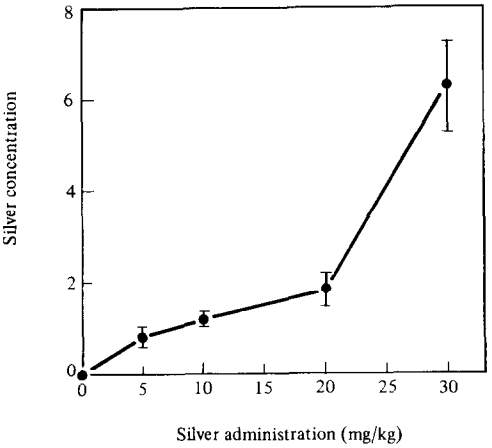

Fig. 2. Silver concentration in rat liver nuclei after the administration of silver nitrate. The silver concentration of the nuclei is shown as nmoles silver per the nuclei isolated from 1 g liver. Rats were subcutaneously injected with 5, 10, 20 or 30 mg Ag^{+}/ml of sterile water/kg of body weight. The experimental groups for doses of 20 and 30 mg Ag^{+}/kg received 10 mg Ag^{+}/kg of body weight/day once a day for 2 and 3 days respectively. These rats were killed 24 hr after the last administration. Each value represents the mean ± S.D. of five rats.

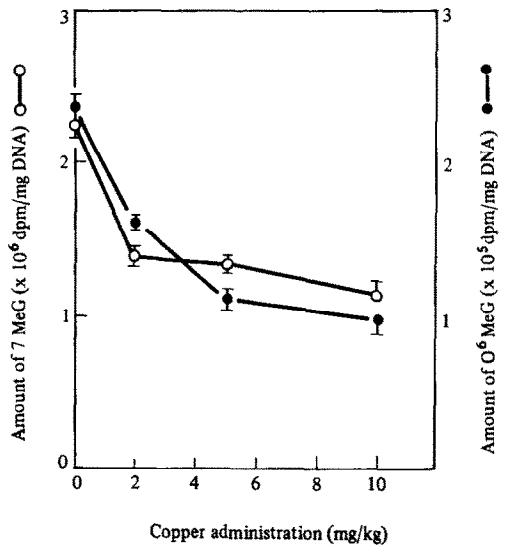

Fig. 3. *In vitro* DNA methylation by methylnitrosourea in isolated copper-preloaded rat liver nuclei. Rats were subcutaneously injected with 2, 5 or 10 mg Cu^{2+}/ml of sterile water/kg of body weight and killed 24 hr after the injection. The amounts of 7MeG (○) and O^6MeG (●) in the DNA isolated were determined as described in Materials and Methods. Each value represents the mean ± S.D. of six rats.

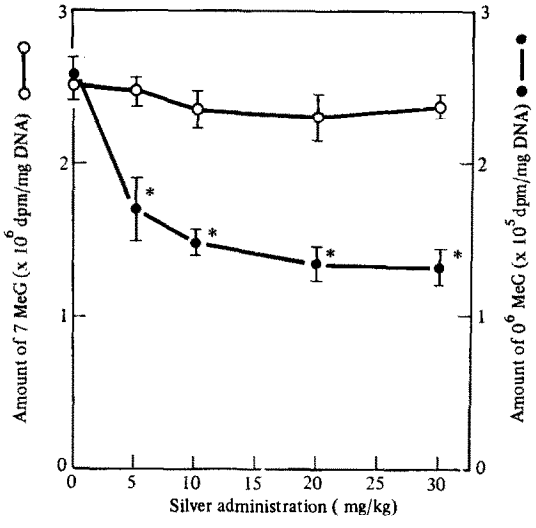

Fig. 4. *In vitro* DNA methylation by methylnitrosourea in isolated silver-preloaded rat liver nuclei. Rats were subcutaneously injected with 5, 10, 20 or 30 mg Ag^+/ml of sterile water/kg of body weight and killed 24 hr after the last injection. The experimental groups for doses of 20 and 30 mg Ag^+/kg received 10 mg Ag^+/kg of body weight/day once a day for 2 and 3 days respectively. The amounts of 7MeG (○) and O^6MeG (●) in the DNA isolated were determined as described in Materials and Methods. Each value represents the mean ± S.D. of five rats. Asterisks indicate a significant difference ($P < 0.05$) from control, as determined by Student's *t*-test.

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The amounts of 7MeG and O^6MeG in the DNA hydrolysates after the *in vitro* methylation by MNU of the nuclei isolated from livers of silver-treated rats were also measured. As shown in Fig. 4, the rate of 7MeG formation was not affected significantly by administration of 5–30 mg Ag^+/kg, whereas the rate of O^6MeG formation was decreased significantly by the same dose of silver. The suppressive rate of O^6MeG formation increased with the increase of silver concentration in the nuclei in the dose range of 5 to 20 mg Ag^+/kg. Although the amount of silver in the nuclei was increased much more by the administration of 30 mg Ag^+/kg than by 20 mg Ag^+/kg, the suppressive rate of O^6MeG formation by the administration of both doses was almost the same. This result may indicate that the action of silver was at a plateau beyond the administration of 20 mg Ag^+/kg under the experimental conditions tested. That the rate of O^6MeG formation was decreased by the administration of silver, but the rate of 7MeG was not affected, may indicate that silver ions are strongly bound to the O-6 site of guanine, but loosely or not bound to the N-7 site of guanine, and that the binding of silver to the O-6 site of guanine protects the methylation of the O-6 site of guanine. If it is true that the formtion of O^6MeG in DNA of rat liver nuclei by DMN leads to hepatocarcinogenesis, the *in vivo* binding of copper and silver may protect the modification of DNA by DMN as an initiation step of hepatocarcinogenesis in rats.

In summary, *in vitro* DNA methylation by MNU in nuclei isolated from livers of copper- or silver-treated rats was examined. In the copper-preloaded nuclei, the rate of formation of both O^6MeG and 7MeG was suppressed. In the silver-preloaded nuclei, the rate of O^6MeG formation was suppressed but the rate of 7MeG formation was not affected significantly.

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