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Action of ammonium *meta* vanadate on the activities of hepatic drug-metabolizing enzymes *in vitro*

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Vanadium compounds are known to interfere with the metabolism of biological phosphorus compounds [1–3], because of the similarity of the vanadate ion with the phosphate ion and because of the tendency of the vanadate ion to form complexes with phosphate. Because of their easy changes in valency [4], vanadium compounds interfere with biological redox reactions such as those of the mitochondrial respiratory chain [5–7]. As far as hepatic drug metabolism is concerned, vanadium (III) chloride is reported to inhibit benzo(a)pyrene hydroxylation *in vitro* [8] but vanadium pentoxide has been shown to have no clear-cut effect on aminopyrine *N*-demethylation and aniline hydroxylation *in vivo* [9]. So we studied the action of ammonium *meta* vanadate (NH_4VO_3) on a series of microsomal enzymic activities with guinea pig and rat liver preparations, *in vitro*. Part of our findings have been reported previously [10].

Materials and methods

Chemicals. The following chemicals were used: biochemicals from Boehringer (Mannheim, F.R.G.), coumarin from Merck AG (Darmstadt, F.R.G.), ammonium *meta* vanadate, biphenyl, and neotetrazolium chloride from Riedel-De Haën (Seelze, F.R.G.), aminopyrine, anisic acid *N,N*-diethylaminoethyl ester hydrochloride ('anisic ester'), 7-ethoxycoumarin, 4-methoxybiphenyl, and 7-methoxy-4-methylcoumarin ('methylayapanine') from Hoechst AG. (Frankfurt am Main, F.R.G.), papaverine hydrochloride from Serva (Heidelberg, F.R.G.) and all other inorganic and organic salts, reagents, buffer substances, and solvents from Riedel-De Haën and Merck.

Liver preparations. Livers of untreated guinea pigs and rats of either sex were used as the enzyme sources. Enzyme activities were assayed in the preparations as stated below, both in the absence and in the presence of ammonium vanadate (in concentrations between 0.01 and 1.0 mM). Rat livers were homogenized in ice-cold isotonic KCl solution with a glass/PTFE homogenizer of the Potter–Elvehjem type [11], and from these crude homogenates, 13,000 *g* supernatants were prepared by centrifugation. Rat and guinea pig liver microsomes were prepared by the $CaCl_2$ precipitation technique [12]. All preparations were diluted with isotonic KCl solution in such a way that 1.0 ml of each preparation was equivalent to 0.10 g of liver wet weight (in the case of 13,000 *g* supernatants) or to 0.20 g of liver wet wt (in the case of microsomes).

Enzyme determinations. In rat liver 13,000 *g* supernatants, aminopyrine *N*-demethylation [13], anisic ester *O*-demethylation [14], microsomal NADPH-dependent cytochrome *c* reductase [15], and microsomal NADPH-dependent neotetrazolium reductase [16, 17] were assayed. We have shown (unpublished results) that all these microsomal enzymic activities can be measured in 13,000 *g* supernatants without interference of the cytosolic enzymes present in this kind of preparation. In rat liver microsomes, uridine-diphosphate glucuronyltransferase (with phenolphthalein as the substrate) was assayed as described below; glucose-6-phosphatase by the method of Norseth [18] (with phosphate determination by the method of Burch *et al.* [19]), biphenyl 4-hydroxylation [20], papaverine *O*-demethylation [14], and 4-methoxybiphenyl *O*-demethylation [14] were measured. In guinea pig liver microsomes, methylayapanine *O*-demethylation [21], 7-ethoxycoumarin *O*-deethylation [22], and coumarin 7-hydroxylation [22] were tested.

The assay mixture for glucuronyltransferase contained, in a total volume of 1.50 ml, 250 μmoles of

tris(hydroxymethyl)aminomethane–HCl buffer (pH 8.0), 0.25 μmoles of phenolphthalein, 25 μmoles of uridinediphosphate glucuronic acid (as the disodium salt), and 0.25 ml of microsomal suspension in isotonic KCl solution (equivalent to 50 mg of liver wet wt). After preincubation of the mixture in the absence of uridinediphosphate glucuronic acid at 37° for 5 min, the enzymic reaction was started with uridinediphosphate glucuronic acid and, after incubation at 37° for 5 min (where reaction rates were linear), was terminated by the rapid addition of 3.0 ml of ice-cold 0.2 M glycine–NaOH buffer (pH 10.8) [23]. After centrifugation, absorbance was read at 550 nm, in a Zeiss spectrophotometer PMQ II. In reference incubations, uridinediphosphate glucuronic acid was replaced by water (for volume constance and for the reason of saving material) and the glycine–NaOH buffer was added before the addition of the microsomal suspension to the reaction mixture. It turned out to be necessary to include, into each series of incubations, a blind value in which both microsomes and uridinediphospate glucuronic acid had been replaced by water, and to subtract this blind value from the difference between sample and reference incubation.

In each of the enzyme assays, a certain volume of either water (in the case of uninhibited control) or ammonium *meta* vanadate solution (in the adequate concentration to yield the desired final concentration) was included. For each concentration of vanadate, each enzyme determination was run in duplicate, and in triplicate for the uninhibited control (water present), in at least two different liver preparations at different days and from different animals.

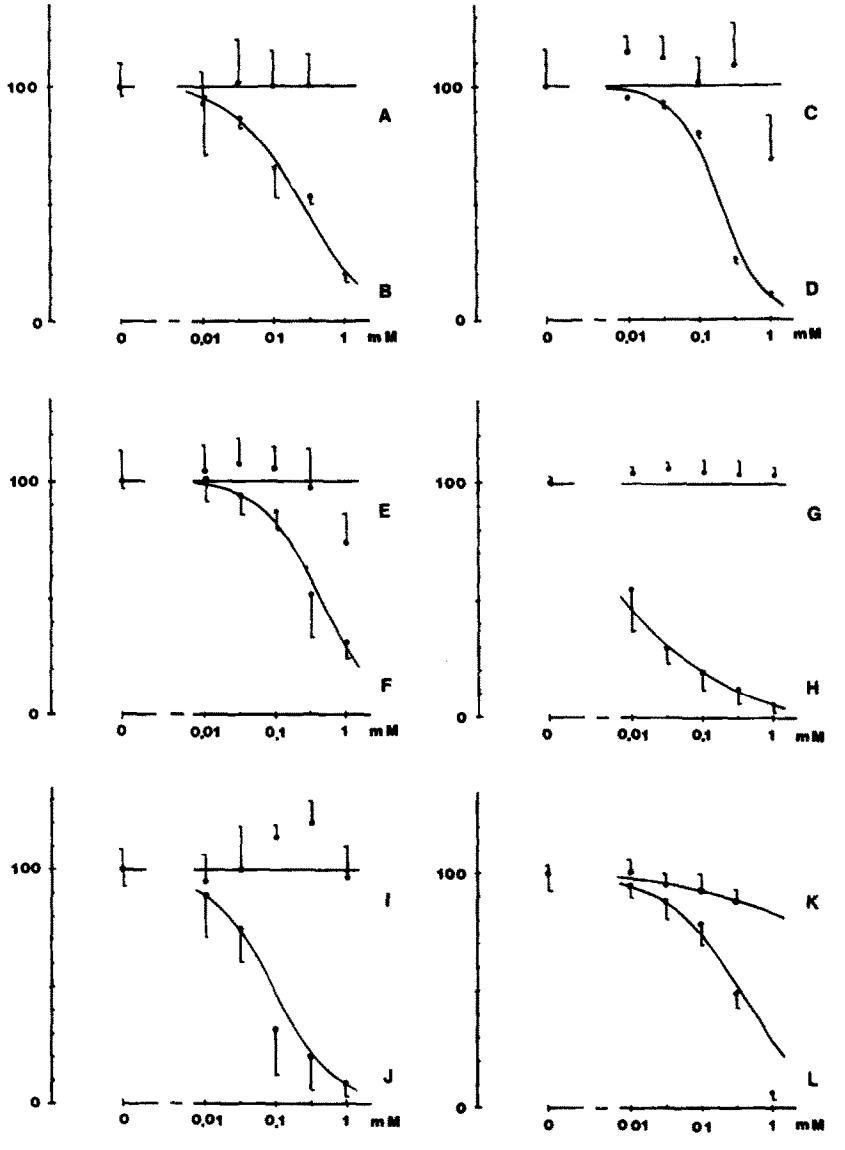

Fig. 1. Action of ammonium *meta* vanadate on hepatic microsomal enzymic activities *in vitro*. (A) Aminopyrine *N*-demethylation (rat); (B) 4-methoxybiphenyl *O*-demethylation (rat); (C) coumarin 7-hydroxylation (guinea pig); (D) biphenyl 4-hydroxylation (rat); (E) glucuronyltransferase (rat); (F) 7-ethoxycoumarin *O*-deethylation (guinea pig); (G) cytochrome *c* reductase (rat); (H) anisic ester *O*-demethylation (rat); (I) neotetrazolium reductase (rat); (J) papaverine *O*-demethylation (rat); (K) 4-methylayapanine *O*-demethylation (guinea pig); (L) glucose-6-phosphatase (rat). Abscissa: NH_4VO_3 concentration, in a logarithmic scale; ordinate: enzymic activity, in percent of uninhibited control. Means ± S.D.

Table 1. IC_{50} Values and Hill coefficients for the inhibition of microsomal enzymes by ammonium *meta* vanadate

Enzymic activity	IC_{50} (mM)	Hill coefficient
Anisic ester *O*-demethylation	0.0076	0.56
Methylayapanine *O*-demethylation	26	0.46
Methoxybiphenyl *O*-demethylation	0.26	0.94
Papaverine *O*-demethylation	0.0087	0.98
Ethoxycoumarin *O*-deethylation	0.44	1.08
Biphenyl 4-hydroxylation	0.21	1.42

Results and discussion

The results of this inhibition study are given graphically in Fig. 1. In the abscissae of the panels, ammonium vanadate concentration is plotted in a logarithmic way, as well as the concentration of 0 (uninhibited control where water had been present). In the ordinates, enzymic residual activities are plotted as percentage of the uninhibited control. Mean values ± S.D. are depicted. Curves were calculated by linear regression of the appropriately transformed mean values [24]. By this procedure, IC_{50} values (i.e., concentrations with 50% enzyme inhibition) and Hill coefficients [25] were also calculated (Table 1).

Aminopyrine *N*-demethylation is not inhibited by ammonium vanadate. This fits with the lack of *in vivo* effects on this enzyme activity as reported before [7]. There is also no effect on coumarin 7-hydroxylation. All other mixed-function oxidase reactions of this study are inhibited by ammonium vanadate, but to various degrees, with Hill coefficients ranging from about 0.5 (as is the case with anisic ester and methylayapanine *O*-demethylations) to about 1.0 (with 4-methoxybiphenyl and papaverine *O*-demethylations and 7-ethoxycoumarin *O*-deethylation) and to about 1.5 (in the case of biphenyl 4-hydroxylation). If Hill coefficients reflect by some way the interactions between protein and ligand [25, 26] our findings can be interpreted to suggest that the interactions between vanadate ions and the enzyme proteins are different in the three groups of enzymic activities. There are also marked differences in the IC_{50} values which range from about 8 nM (as is the case with anisic ester and papaverine *O*-demethylations) to more than 10 mM (with methylayapanine *O*-demethylation). The last-mentioned IC_{50} value means that this kind of enzymic activity is inhibited only weakly if at all by ammonium vanadate. In contrast to this enzyme activity, the two first-mentioned *O*-demethylation reactions are very susceptible to vanadate inhibition as judged from the small IC_{50} values. Ammonium *meta* vanadate seems therefore to be a good tool in order to discriminate between different microsomal, cytochrome P-450-dependent, mixed-function oxidation activities.

The way that ammonium *meta* vanadate acts on these mixed-function oxidases remains unclear at the moment. The lack of inhibition of the two microsomal, NADPH-dependent reductases suggests that the inhibitory interaction does not occur at the flavoprotein level of the microsomal respiratory chain. Studies on the kind of interaction between vanadate and cytochrome P-450-dependent mixed-function oxidase systems are going on which will lead to a better understanding of the kind of action and of the selectivity of this enzyme inhibitor.

Ammonium vanadate has no effect on glucuronyltransferase *in vitro* (as measured with phenolphthalein as the substrate); it only inhibits oxidative drug biotransformation reactions. The inhibition by ammonium *meta* vanadate of glucose-6-phosphatase, a microsomal enzyme not involved in drug metabolism, has to be seen in context with the property of the vanadate ion to inhibit other phosphate-metabolizing enzymes as mentioned above [1–3].

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Effect of alcohol on tumor folate supply*

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During periods of sustained alcohol ingestion, rapidly proliferating tissues such as the erythroid marrow demonstrate a reversible defect in DNA synthesis and cell replication which appears to result from an alteration of folate homeostasis [1–4]. With acute alcohol ingestion serum folate levels fall dramatically, and megaloblastic erythropoiesis may occur even when liver folate stores are still present [4, 5]. Recovery is prompt upon withdrawal of alcohol without folate supplementation, suggesting a reversible *in vivo* sequestration of folate. Hillman *et al.* [6] have shown that levels of bile folate fall dramatically with acute alcohol ingestion and suggest a specific toxic effect of alcohol on the folate enterohepatic cycle. However, direct studies of the effects of alcohol on peripheral tissue folate supply have not been available, since normal tissues do not take up sufficient amounts of folate. In this study, we have used a new animal model where multiple subcutaneous fibrosarcoma implants permit kinetic measurements of folate transport by serial sampling of the tumor nodules [7].

Female Wistar–Furth rats weighing 150–200 g were used for all experiments. In each animal, two 2–5 g non-metastasizing fibrosarcoma tumor nodules were produced by injections of 0.5 ml of a homogenate of a tumor cell line infected with polyoma virus DW-7410 obtained from Dr. Peter Wright (Swedish Hospital Tumor Institute). Three groups of animals were compared: (1) normal animals maintained on standard Purina rat chow containing 30 μg/g of *Lactobacillus casei* active folate; (2) folate/nutrient deprived animals (F/ND) fed a liquid diet of 25% sucrose in water with 1 mg/ml of succinyl sulfathizole (added to suppress intestinal production of folates) for 3 days; and (3) alcohol-treated animals (EtOH) fed 100 ml/kg per day of a solution of 10% ethanol (in divided doses) by stomach tube for 3 days, together with the succinyl sulfathizole/sugar water diet. Animals from each of the three dietary groups were anesthetized with ether and injected with 5 μCi (100 ng) of [^{3}H]PteGlu$_1$ by tail vein. The isotope was obtained from the Amersham Corp. (Arlington Heights, IL) and had a specific activity of 20 Ci/mmole with greater than 90–95% purity when chromatographed on DEAE-A-25 Sephadex just before use [8]. No significant increase in serum folate levels was observed with this dose. After 3 hr, the animals were again anesthetized, and a tumor nodule was removed. The nodule was immediately weighed, chopped, and homogenized in a solution of cold 1% ascorbate in 0.1 M phosphate buffer, pH 6.0, boiled for 7 min to inactivate gamma-glutamyl carboxypeptidase, autoclaved at 110° for 10 min, and centrifuged to precipitate remaining protein. A 1-ml aliquot of the supernatant fraction was pipetted into Aquasol for counting. An aliquot was also used for chromatography on a 0.9 by 120 cm column of Sephadex G-15, eluted with 0.1 M potassium phosphate buffer, pH 7.0, containing 200 mM 2-mercaptoethanol [9]. The fluid was collected in 1.6-ml fractions at a flow rate of 15 ml/hr, and 1 ml of each fraction was pipetted into Aquasol for counting, PteGlu$_1$, CH_3H_4PteGlu$_1$ and polyglutamate markers were used to verify peak positions. After 6 hr, each animal was again anesthetized, the portal vein was cannulated, and the liver was flushed with cold saline. The liver and remaining tumor nodule were then removed, weighed and immediately homogenized in cold 1% ascorbate in 0.1 M phosphate buffer, pH 6.0, and then prepared for counting and chromatography as above. Serum folate levels were determined by the aseptic *L. casei* method of Herbert [10]. Counting of tissue extracts and chromatographic eluates was carried out in a Packard liquid scintillation counter, and correction for quenching was made using the external automatic standard. The amount of polyglutamyl folate present was calculated as the percentage of total labeled folate in the tissue. Statistical analysis was performed by Student's *t*-test.

The serum folate levels of EtOH animals (41 ± 3 ng/ml) were significantly lower than in F/ND animals (63 ± 8 ng/ml; $P < 0.05$) which were, in turn, lower than normals (92 ± 9 ng/ml; $P < 0.02$). The uptakes of labeled folate per g of tumor tissue for the three groups are shown in Table 1. After 3 hr, tumor uptake in EtOH animals was only slightly less than that observed in F/ND animals but significantly less than in normals ($P < 0.05$). By 6 hr, tumor uptake in EtOH animals was significantly less than that seen in both normal and F/ND animals ($P < 0.05$ and $P < 0.05$ respectively). When hepatic uptakes were examined at 6 hr, F/ND animals appeared to take up more label than either normal ($P < 0.01$) or EtOH animals ($P < 0.02$).

Chromatographic analysis of labeled folate taken up by tumor tissue (Table 2) demonstrated that significantly more

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