4-fold. If the catecholamines were to achieve their inhibitory effects by altering the specific binding we should reasonably expect to see this reflected in the K_{Ca} value for ^{45}Ca binding in our studies. However in our experiments the K_{Ca} for ^{45}Ca binding to synaptic membranes in the presence of 10^{-6} M adrenaline was $2.01 \pm 0.4 \times 10^{-5}$ M and $1.89 \pm 0.27 \times 10^{-5}$ M in the presence of noradrenaline. It is reasonable to assume that specific calcium binding can take place on either surface but from a functional point of view it is likely that the affinity for binding would be greater on the inside surface of the membrane.

If noradrenaline were to increase calcium binding to synaptic membrane it could on one hand inhibit calcium efflux from synaptosomes. On the other hand, enhanced binding of calcium to the inside surface and decreased binding to the external surface could induce an increased calcium efflux from the synaptosomes.

However, in a series of experiments to investigate the extrusion of calcium from synaptosomes, the rate of efflux was insensitive to noradrenaline at concentrations up to 5×10^{-5} M (Logan, unpublished data).

Therefore it is our view that the inhibition of Na–Ca-ATPase is not the consequence of an enhancement of calcium binding to membranes.

To summarise, the catecholamines inhibit Na–Ca-ATPase by a mechanism which may be receptor-mediated and which is most probably a result of direct interaction with the enzyme rather than by some modulation of the free calcium ion concentration.

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On the interaction of diazepam with human, rat and mouse plasma proteins and erythrocytes

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The binding of a drug to plasma proteins usually affects its activity, distribution, rate of metabolism, and glomerular filtration [1–4]. Drugs bind to plasma proteins to different degrees depending upon their physico-chemical characteristics. Binding of drugs occurs mainly in the albumin fraction of plasma proteins [5]. Some lipophilic drugs, however, are also dissolved in the lipid phase of serum lipoproteins to an extent almost equal to that bound to serum albumin. To predict changes in protein binding of a specific drug in plasma of an individual patient, the extent of binding to the different protein fractions of plasma should be known because of the variations in the plasma protein pattern. In the comparison with albumin, other proteins are often subject to greater changes. Consequently, if proteins other than albumin contribute to the binding of drugs, more frequent variations in the binding are to be expected [6].

Although the possible influence of the erythrocytes and other cellular blood components in pharmacokinetics has been neglected by most authors, high binding of some lipophilic drugs to erythrocytes has recently been demonstrated [7–9].

The binding of diazepam to serum albumin and whole plasma of different species has been evaluated by others [10–12]. This communication is concerned with the binding of diazepam to human, rat and mouse plasma protein fractions and erythrocytes.

Human blood was obtained from healthy volunteers, fasted overnight, 18–30 years of age; rat blood from male, albino Wistar rats fasted 18 hr, weighing 200–250 g; mouse blood from male mice, strain H Konárovice, fasted 18 hr, weighing 18–30 g. Plasma was obtained by centrifugation of heparinized (approx. 5000 IU·l^{-1}) blood at 1000 *g* for 30 min.

[*N*-$^{14}CH_3$]Diazepam (sp. act. 4.92 GBq/g) was supplied by ÚVVVR (Prague, Czechoslovakia). The radiochemical purity of [*N*-$^{14}CH_3$]diazepam verified by TLC on silica gel (in the system heptane–choloroform–ethanol 5:5:2) was found to be greater than 98%. Diazepam was dissolved in acetone and mixed with unlabelled drug to achieve suitable concentrations. The required amount of diazepam was dissolved, after evaporation of acetone, in the blood, plasma or erythrocytic suspension in the period of 30 min. In the case of incubation of blood or erythrocytic suspension the samples were subsequently centrifuged and the drug was determined in the centrifugate and supernatant.

The binding of diazepam to whole plasma was studied by a method of equilibrium dialysis [13]. The samples were dialysed against 0.15 M NaCl solution buffered with 0.01 M phosphate (pH 7.4) for 20 hr at 37°.

The interaction between diazepam and various plasma proteins by molecular exclusion chromatography was studied by applying 1 ml fresh plasma, containing diazepam of a suitable concentration, to 11 × 350 mm column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 0.15 M NaCl solution buffered with 0.01 M phosphate (pH 7.4) containing diazepam, 0.1

Table 1. Erythrocytic and plasmatic binding of diazepam in different species

Species	Diazepam concn ($mg \cdot l^{-1}$)	C_{EB}	C_{ES}	Plasma protein binding (%)
Man	1	13.6 ± 1.7	75.5 ± 1.6	97.5 ± 0.5
	10	14.7 ± 0.1	71.3 ± 0.7	97.5 ± 0.2
	50	16.5 ± 0.5	70.2 ± 0.3	97.3 ± 0.4
Rat	1	55.2 ± 0.4	94.3 ± 1.6	89.9 ± 0.7
	10	50.5 ± 0.5	93.6 ± 0.7	90.1 ± 0.8
	50	51.9 ± 0.7	93.1 ± 0.5	88.7 ± 1.0
Mouse	1	30.0 ± 1.2	83.3 ± 1.5	89.9 ± 0.4
	10	26.6 ± 0.8	81.1 ± 0.2	89.5 ± 0.8
	50	25.6 ± 0.4	80.6 ± 0.4	89.3 ± 0.9

C_{EB}—Diazepam content in erythrocytes in whole blood (% total amount).
C_{ES}—Diazepam content in erythrocytes in erythrocytic suspension (% total amount).
Results are expressed in the form of averages ± S.D.
6–12 Samples in each experimental group.

$mg \cdot l^{-1}$. Diazepam was dissolved in plasma in the concentration equal to $0.1/f$ ($mg \cdot l^{-1}$), where f is fraction of free drug determined previously. The proteins were eluted with the same buffer with which the column was equilibrated, at a flow rate 10 $ml \cdot hr^{-1}$ and at 37°, collecting 1 ml fractions of the eluate. Samples of the eluate were taken for liquid scintillation counting and for the determination of protein. Bound diazepam appeared as peaks of increased radioactivity over the background activity (equal to 0.1 $mg \cdot l^{-1}$ of diazepam). Protein was determined by Bio-La-test (Lachema, Brno, Czechoslovakia) by the method of Chromý and Fischer [14].

Radioactivity of diazepam in tested material was counted after mixing of samples with 10 ml of scintillation cocktail of Bray (Spolana, Neratovice, Czechoslovakia) with an automatic spectrometer Nuclear Enterprises NE 8312.

In the concentration range used the proportion of diazepam in the erythrocytic component is relatively high, but there are great interspecies differences, as shown in Table 1. Erythrocytes binding is most marked in rat (more than 50% of total amount of the drug) and relatively low in man (13–15%). The binding of drug with erythrocytes suspended in buffer, pH 7.4 (Table 1), is highest in rats (more than 90%) and lowest in man (70–75%). The binding of diazepam with erythrocytes slightly decreases with increasing concentration of the drug in all species.

The distribution of diazepam in whole blood, i.e., between erythrocytes and plasma, shows that the binding of this drug to erythrocytic mass is relatively high, but does not inform us about the binding of erythrocytes themselves. Thus the binding of erythrocytes alone without the influences of plasma proteins, i.e., erythrocytes suspended in buffer, was determined. In these experiments similar interspecies relationships in the binding of diazepam with erythrocytes were found to those in the experiments with whole blood. This means that erythrocytic binding of diazepam and its marked interspecies differences are evidently connected with binding specifity of erythrocytes in the individual species.

The binding of diazepam to the whole plasma of man, rat and mice (Table 1) indicates that binding shows little dependence on the total drug concentration in solution within the measured concentration range. The highest binding was found for human plasma (97–98%), binding of rat

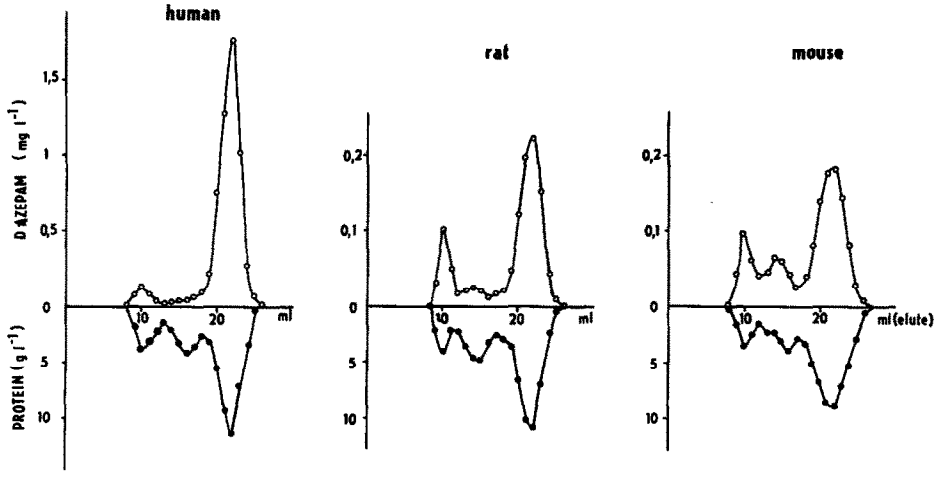

Fig. 1. Binding of diazepam (○——○) to plasma protein (●——●) fractions of human, rat and mouse plasma. One ml of plasma containing diazepam of suitable concentration was applied to an 11 × 350 mm column of Sephadex G-200 previously equilibrated with 0.15 M NaCl solution buffered with 0.01 M phosphate (pH 7.4) containing diazepam, 0.1 $mg \cdot l^{-1}$. The proteins were eluted with the same buffer at a flow rate of 10 $ml \cdot hr^{-1}$ and at 37°.

Table 2. Binding of diazepam to plasma protein fractions at concentration of free drug 0.1 mg·l^{-1}

	Protein fraction							
	Peak I		Peak II		Peak III		Total plasma (sum of fractions)	
	Bound diazepam (mg·l^{-1})	% Bound	Bound diazepam (mg·l^{-1})	% Bound	Bound diazepam (mg·l^{-1})	% Bound	Bound diazepam (mg·l^{-1})	% Bound
Human plasma	0.332	76.8	0.122	54.9	5.467	98.2	5.921	98.3
Rat plasma	0.195	66.1	0.083	45.4	0.815	89.1	1.093	91.6
Mouse plasma	0.242	70.8	0.235	70.2	0.777	88.6	1.254	92.6

and mouse plasma is similar (about 89–90% of bound diazepam). Although in analysis of the interaction between diazepam and albumin only limiting number of binding sites was reported (1.09 binding sites for human serum albumin [10] and 5.56 binding sites for bovine serum albumin [15]), in our experiments only little dependence of binding on diazepam concentration was found. Similar findings (no or little dependence on diazepam concentration) have been reported for binding of diazepam to dog plasma and bovine serum albumin [12]. Extremely low solubility of diazepam, however, made experiments possible only in dilute solutions and saturation of all first-class binding sites does not occur in our experiments.

The binding of diazepam to various plasma proteins when fractionated by molecular exclusion chromatography (Fig. 1 and Table 2) indicates that diazepam is bound to fractions of plasma in a different degree. Proteins in the first protein peak eluted where found to be lipoproteins (VLDL and LDL) and α_2- and β_2-macroglobulins, in the middle protein peak γ-globulin, ceruloplasmin and lipoprotein (HDL), and in the final protein peak albumin, transferrin and α-globulin, as observed by others [16]. Although diazepam is bound to all three fractions of plasma in studied species, the main binding fraction is third peak; yet only small differences were found between the binding of diazepam to the whole plasma and to this third fraction.

In comparison of binding for diazepam found by a method of equilibrium dialysis and by molecular exclusion chromatography on Sephadex G-200, in all cases higher binding was found by the latter method. This is probably due to an increase in binding affinity (i.e., association binding constant) of albumin molecule for diazepam with decreasing albumin concentration, as has been determined for some other drugs [17].

The binding affinity of human serum albumin to diazepam determined by Müller and Wollert [10] is nearly the same as that in the third fraction of human plasma in our experiments. It means that albumin is responsible for a great part for the binding of diazepam in this third fraction of plasma.

In interspecies comparison of plasma protein binding of diazepam, differences are both in the amount of diazepam bound to albumin and in the binding to the other components of plasma, respectively.

Few differences between binding of diazepam to the whole plasma and to albumin (or to the third protein fraction) mean that changes in other proteins than albumin result in no or little changes in diazepam binding. Interspecies differences in plasma protein binding of diazepam are mainly due to differences in albumin binding affinity of different species.

The example of the model drug diazepam thus shows that in the drugs in which affinity to blood elements occurs, the fate of drug in blood must be judged in a complex way from the viewpoint of continuous renewal of the equilibrium between the free drug and the drug bound both to erythrocytes and plasma proteins. None of these components can be changed independently of the others. In such a case, for example, a decrease in binding to plasma proteins does not necessitate a marked increase in the concentration of free drug, as long as the binding of erythrocytes is sufficient and vice versa.

In summary, binding of diazepam to the blood fractions (erythrocytes and plasma proteins) in man, rat and mouse was studied. Only little dependence of binding on total drug concentration was found. The main binding fraction of plasma in studied species is albumin, but interspecies differences are both in the amount of diazepam bound to albumin and in that bound to other components of plasma. The determination of the binding of diazepam with erythrocytic mass and with blood plasma demonstrates the proportion of these bonds in total distribution of the drug under study in blood and the importance of thus experimentally followed interspecies comparison.

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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