Biochemical Pharmacology, Vol. 27, pp. 2215–2221.

0006-2952/78/0915-2215$02.00/0

BIOTRANSFORMATION OF 4-DIMETHYLAMINOPHENOL IN THE DOG

PETER EYER and HARALD GABER

Pharmakologisches Institut der Universität München, Nussbaumstrasse 26, 8000 München 2, FRG

(*Received* 11 *November* 1977; *accepted* 7 *February* 1978)

Abstract—4-Dimethylaminophenol (DMAP), after an i.v. injection, quickly forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen. This reaction is rapidly terminated by covalent binding of oxidized DMAP to the reactive SH-groups of hemoglobin and to reduced glutathione within the red cells, and by conjugation with glucuronic or sulfuric acid presumably in the liver. Fifteen min after i.v. injection of DMAP, 3.25 mg/kg, ^{14}C-labeled in the ring, no intact DMAP was detected in the blood. The concentrations of metabolites in the blood were as follows: 33 μM DMAP covalently bound to hemoglobin, 30 μM *S,S,S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-glutathione(Tris-(GS)-DMAP) 90 per cent of it located within the red cells, 5 μM DMAP-glucuronide, and 22 μM DMAP-sulfate. Within 3 days, 90 per cent of the radioactivity was excreted in the urine, 4 per cent in the faeces. In the 24 hr urine, 25 per cent of the DMAP injected was excreted as DMAP-sulfate, 15 per cent as DMAP-glucuronide, and 23 per cent as DMAP-thioethers, mainly as *S,S,S*-(dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-cysteine. When DMAP, ^{14}C-labeled in the methyl groups, was administered 11 per cent of the radioactivity was excreted in the urine as dimethylamine. It is concluded that most of the thioethers found in the urine derived from Tris-(GS)-DMAP which had been produced within the red cells indicating an important role of the red cells on biotransformation of DMAP.

4-Dimethylaminophenol (DMAP) in dogs and humans quickly forms ferrihemoglobin. This effect differs from ferrihemoglobin production by other substances in that it takes only 5–10 min after an i.v. injection to raise the ferrihemoglobin content of the blood to the maximal level corresponding to the dose applied. DMAP, therefore, has been used successfully in the treatment of cyanide poisoning [1–3].

Previous studies with purified human hemoglobin have shown that oxyhemoglobin rapidly oxidizes DMAP, and the oxidation product, i.e. a radical or the quinonimine, oxidizes ferrohemoglobin to ferrihemoglobin. DMAP thus catalytically transfers electrons from ferrohemoglobin to oxygen [4]. Quick binding of oxidized DMAP to the globin moiety, in particular to cysteine β 93 terminates this reaction [5] and greatly disturbs the physiological functions of hemoglobin. It could be shown that these important changes in hemoglobin function were due to a lack of allosteric transition upon ligation of the modified hemoglobin which seems to be frozen in the quaternary R-state [6].

Reduced glutathione (GSH) in high concentrations, as found in red cells, substantially diminished covalent binding of oxidized DMAP to hemoglobin by formation of *S,S,S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-glutathione(Tris-GS-DMAP) [7]. DMAP disappeared more rapidly from hemoglobin solutions in the presence of GSH and consequently ferrihemoglobin formation was diminished. This thioether formation was also observed to occur within the red cells in suspensions of human and ox blood where the thioether slowly penetrated the red cell membrane, $t/2$ about 60 min [8].

Therefore, it seemed of interest to study the biotransformation of DMAP *in vivo*, especially to elucidate the influence of the red cells upon biotransformation of DMAP *in vivo*.

MATERIALS AND METHODS

Male beagles, body wt 10–17 kg, were fed the usual diet and received water *ad lib*. The animals were kept in stainless steel metabolic cages. Urine was collected on ice and filtered through cellulose acetate membranes with 0.45 μm pore diameter (Sartorius).

4-Dimethylaminophenol hydrochloride and the compounds, $^{14}CH_3$-labeled, sp. act. 1.2 Ci/mole, or ^{14}C-labeled randomly in the ring, sp. act. 5 Ci/mole, were prepared by Farbwerke Hoechst. For i.v. injections the DMAP preparations were mixed with inactive DMAP to give a final sp. act. of 0.4 Ci/mole.

4-Dimethylaminophenyl sulfate, ^{14}C-labeled in the ring, sp. act. 22 mCi/mole, was synthetized by treatment of radioactive DMAP with chlorosulfonic acid in dry pyridine at 0°. After neutralization with sodium hydroxide pyridine and unreacted DMAP were extracted with ether. After evaporation of the solvent the residue was dissolved with acetone and crystallized by addition of ether, after recrystallization m.p. 187–189°, yield 50 per cent. The struc-

Abbreviations—DMAP, 4-dimethylaminophenol; DMAP-glucuronide, 4-dimethylaminophenyl glucuronide; DMAP-sulfate, 4-dimethylaminophenyl sulfate; Tris-(GS)-DMAP, *S,S,S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-glutathione; Tris-(Cys)-DMAP, *S,S,S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-cysteine; GSH, reduced glutathione; TCA, trichloroacetic acid; t.l.c. thin layer chromatography.

ture was proved by nuclear magnetic resonance spectroscopy; the compound showed infra red (i.r.) absorption at 1066 and 1077 cm^{-1} and yielded DMAP and sulfate after hydrolysis with 1 N hydrochloric acid at 100° for 1 hr.

Tris-(GS)-DMAP, ^{14}C-labeled in the ring, sp. act. 78 mCi/mole, was synthetized from radioactive DMAP and GSH in the presence of hemoglobin [7]. 1.2 mM DMAP and 7 mM GSH reacted in the presence of human hemoglobin, 12 g Hb/100 ml, at pH 7.4 and 37° for 10 min. After deproteination with TCA the mixture was chromatographed on Sephadex LH 20 with 10 mM acetic acid which separated two radioactive compounds. The major compound was rechromatographed with the same system and proved to be Tris-(GS)-DMAP, yield 40 per cent. Purity of the compound was checked by t.l.c., ultra violet (u.v.) and amino acid analysis [7].

Tris-(*N*-acetylcysteyl)-DMAP and Tris-(Cys)-DMAP were prepared similarly to Tris-(GS)-DMAP by use of *N*-acetylcysteine or cysteine, respectively, in place of GSH. After desulfuration with Raney nickel followed by acid hydrolysis 3 moles of alanine/DMAP were found from both compounds.

β-Glucuronidase-arylsulfatase solution from helix pomatia was purchased from Boehringer, Mannheim. Enzymic digestion was performed in 0.1 M sodium acetate, pH 5, at 37° under nitrogen for 24 hr.

Glucuronic acid was purchased from Sigma Chemie GmbH, München; *N*-acetylcysteine from Fluka AG, Buchs, Switzerland. All other reagents were analytical grade chemicals from Merck, Darmstadt.

Amino acid analysis after hydrolysis of the sample in 6 N hydrochloric acid under nitrogen at 110° for 20 hr was carried out with a Beckman Multichrom M amino acid analyzer using the lithium Pico-Buffer-System of Pierce, Eurochemie, Rotterdam.

Raney nickel desulfuration was performed according to Kuss [9].

4-Dimethylaminophenol in ether extracts after t.l.c. on silica gel with chloroform–methanol (95:5) was determined by reading the u.v. absorbance at 247 nm (log E = 4.07).

Plasma was quickly prepared by centrifugation of blood for 2 min in an Eppendorf centrifuge 3200 at 12000 rpm (8000 *g*).

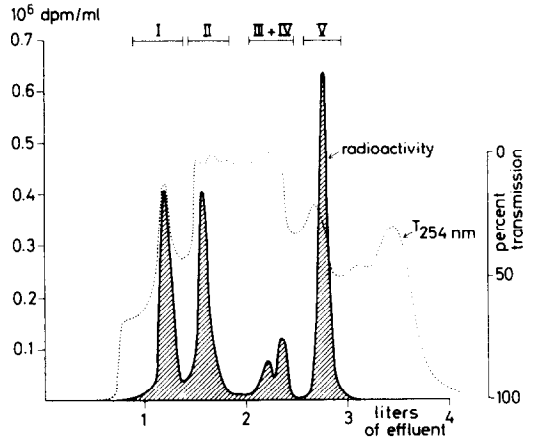

Fig. 1. Chromatography of the pooled urine from three dogs collected for 24 hr after an i.v. injection of DMAP hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring. (Sephadex LH 20 (7 × 80 cm), 10 mM acetic acid)

Glutathione, reduced and oxidized, were determined as described elsewhere [7].

Radioactivity was measured in Bray's solution with a Packard Tricarb scintillation spectrometer using an external standard. All results have been corrected for recovery and background radiation.

Covalent binding. As it was shown that DMAP was irreversibly bound to the SH-groups of hemoglobin [4, 6] the protein bound radioactivity was roughly estimated from the difference of radioactivity in the sample and in its supernatant after precipitation of proteins with an equal volume of 0.6 M TCA.

RESULTS

Urinary metabolites. 4-Dimethylaminophenol hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring, was i.v. injected within 1 min into three beagles. The spontaneously voided 24 hr urine was pooled from the three dogs. It contained about 2/3 of the radioactivity injected.

After lyophilization of the urine the residue was chromatographed on Sephadex LH 20 (7 × 80 cm) with 10 mM acetic acid. Figure 1 shows the elution profile. Fractions were pooled as indicated. They

Table 1

Compound	Yield %	Amino acid composition after hydrolysis GLU	GLY	Amino acid composition after desulfuration and hydrolysis GLU	GLY	ALA	Amino acid composition presumed GLU	GLY	CYS
Ia	63	0.0	0.0	0.0	0.0	2.74	0	0	3
Ib	16	0.0	0.71	0.0	0.35	1.66	0	1	3
Tris-(GS)-DMAP	—	2.92	2.81	0.35	0.33	0.23	—	—	—
GSH	—	0.98	0.93	0.08	0.06	0.06	—	—	—

Amino acid composition of the two main compounds of the thioether fraction (peak I, Fig. 1) excreted with the urine of dogs 24 hr after an i.v. injection of DMAP hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring. The amounts were corrected for losses in the analytical procedure and expressed as moles/mole of DMAP. Yield = per cent of radioactivity of the analyzed sample.

contained the following proportions of the radioactivity applied to the column: peak I 25 per cent, peak II 23 per cent, peak III + IV 9 per cent, peak V 36 per cent.

Isolation of thioethers of 4-dimethylaminophenol. Peak I was further purified by column chromatography on DE_{52}-cellulose (Whatman), (2.5 × 30 cm) equilibrated with 10 mM NH_4-acetate, pH 6.0. Chromatography with a linear salt gradient (300 ml 10 mM NH_4-acetate, pH 6.0, 300 ml 100 mM NH_4-acetate, pH 6.0) separated two radioactive compounds (Ia = 63 per cent of the total radioactivity; Ib = 16 per cent). Compound Ia showed the same chromatographic behaviour as the synthetic Tris-(Cys)-DMAP. The remaining radioactivity was found in several minor peaks after elution with acetic acid. Compound Ia and compound Ib were lyophilized and desalted by chromatography on Sephadex LH 20.

The u.v. spectra of both compounds were nearly identical. They showed maxima at 320 and 265 nm in acid solution and at 355 nm in alkaline solution. They were very similar to the spectrum of Tris-(GS)-DMAP [7], and identical with the spectrum of Tris-(Cys)-DMAP.

Both compounds reacted with ninhydrin and showed the same molar absorbance coefficient at 570 nm as Tris-(GS)-DMAP. Table 1 presents the results of amino acid analyses after acid hydrolysis of the isolated compounds or after their desulfuration with Raney nickel and acid hydrolysis.

In the ether extracts after Raney nickel treatment DMAP was identified as the only radioactive compound (t.l.c., u.v.). As the yields of amino acids were unusually low after Raney nickel treatment the results of amino acid analyses of Tris-(GS)-DMAP and GSH are also presented in Table 1.

These results pointed to *S,S,S*-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-cysteine for compound IA and to *S,S,S*-(2-dimethylamino-5-hydroxy - 1,3,4 - phenylene) - bis - cysteine - monocysteyl glycine for compound Ib.

N-Acetylated thioethers of DMAP were not found in the urine. Tris-(*N*-acetylcysteyl)-DMAP did not react with ninhydrin and was eluted from DE_{52}-cellulose only with high acetic acid concentrations. Thus, the excretion of *N*-acetylcysteine derivatives of DMAP in the dog was unlikely. To make sure, a sample of the urine of the dog treated with radioactive DMAP was mixed with 10 mg synthetic Tris-(*N*-acetylcysteyl)-DMAP and chromatographed on Sephadex LH_{20} and DE_{52}-cellulose as described above. No radioactivity could be detected in the fractions containing Tris-(*N*-acetylcysteyl)-DMAP. When a sample of the urine was mixed with 10 mg of synthetic Tris-(Cys)-DMAP and chromatographed with the above procedure 16 per cent of the radioactivity was eluted with the Tris-(Cys)-DMAP containing fractions the ratio of radioactivity and u.v. absorbance being constant.

Since Tris-(GS)-DMAP was shown to be formed within the red cells of human and ox blood [7] the excretion of Tris-(Cys)-DMAP in the urine of the dog points to a peptic decomposition of Tris-(GS)-DMAP, the glycine containing urinary metabolite being an intermediate degradation product. Therefore the fate of Tris-(GS)-DMAP in the dog was studied. ^{14}C-Labeled Tris-(GS)-DMAP, 10 mg/kg, was i.v. injected into a beagle within 1 min. The dog showed no unusual reactions, formation of ferrihemoglobin was not detected.

The urine was obtained by a catheter 0.5, 1, 2, and 4 hr after injection. Half the radioactivity was excreted within 30 min. After 4 hr about 98 per cent of the radioactivity was found in the urine.

The urine collected from the 0–2 hour period containing 85 per cent of the radioactivity was chromatographed for isolation of the thioethers as described above. Figure 2 shows the elution pattern from DE_{52}-cellulose and the buffers used. The radioactive fractions were pooled (cuts as indicated) and lyophilized. After desulfuration with Raney nickel DMAP could be extracted with ether from each compound with an average 90 per cent yield. After acid hydrolysis the amino acids were analyzed. The yield of the metabolites and the amino acid composition is shown in Table 2.

These results indicate that Tris-(GS)-DMAP was

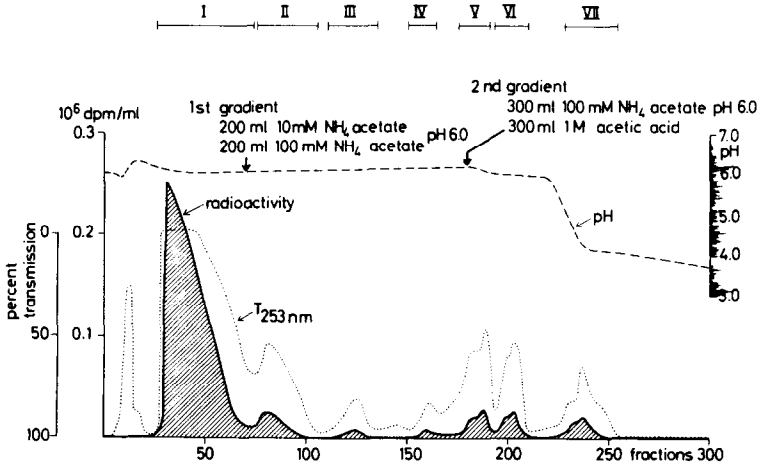

Fig. 2. Chromatography of the thioether fraction of the urine of a dog collected for 24 hr after an i.v. injection of Tris-(GS)-DMAP, 10 mg/kg, ^{14}C-labeled in the ring. (DE_{52}-cellulose (1.5 × 30 cm) equilibrated with 10 mM NH_4-acetate, pH 6.0.)

Table 2

Compound	Yield %	Amino acid composition after hydrolysis GLU	GLY	Amino acid composition after desulfuration and hydrolysis GLU	GLY	ALA	Amino acid composition presumed GLU	GLY	CYS
I	73	0.0	0.14	0.0	0.18	2.73	0	0	3
II	5	0.22	1.08	0.0	0.88	2.44	0	1	3
III	2	0.24	1.87	0.0	1.70	2.70	0	2	3
IV	1	0.69	2.02	0.45	1.07	1.73	1	2	3
V	4	1.11	2.04	0.26	0.51	1.13	1	2	3
VI	3	1.71	1.88	0.36	0.45	0.62	2	2	3
VII	3	3.03	2.70	0.34	0.31	0.35	3	3	3

Amino acid composition of radioactive compounds (cf. Fig. 2) isolated from the urine of a dog 2 hr after an i.v. injection of Tris-(GS)-DMAP, 10 mg/kg, ^{14}C-labeled in the ring. The amounts were corrected for losses in the analytical procedure and expressed as moles/mole of DMAP. Yield = per cent of radioactivity of the analyzed sample.

rapidly metabolized in the dog by peptic cleavage of the glutathione residues, Tris-(Cys)-DMAP being the major urinary metabolite.

Isolation of 4-dimethylaminophenyl glucuronide. The material isolated with peak II by chromatography of the pooled urines was rich in salts. Therefore, the freeze dried material was extracted three times with 70 ml of methanol. The methanol extract which contained 85 per cent of the radioactivity was evaporated, the residue dissolved in a small volume of water and chromatographed on silica gel plates with ethanol–water (90:10). The radioactive material was found in a single band ($R_f = 0.6$), eluted with methanol, and evaporated (80 per cent of the radioactivity of peak II). A sample was incubated with β-glucuronidase-arylsulfatase. Ninety five per cent of the radioactivity was extracted with ether and proved to be DMAP (t.l.c., u.v.) with the same sp. act. as the DMAP preparation used for the i.v. injection. As the 4-dimethylaminophenyl-sulfate (DMAP-sulfate) showed a R_f value of 0.9 in the t.l.c.-system described above, the isolated compound was supposed to be 4-dimethylaminophenyl glucuronide (DMAP-glucuronide). To identify the glucuronic acid an aliquot of the aqueous phase remaining after enzymic hydrolysis was chromatographed on silica gel with *n*-butanol–glacial acetic acid–water (50:25:25), different amounts of authentic glucuronic acid serving as reference. After spraying the plate with resorcinol (0.2% solution in 20% sulfuric acid) and heating for 15 min at 100° a purple spot revealed glucuronic acid with the same R_f value of 0.37 as the authentic reference substance. The color intensity corresponded to a glucuronic acid quantity as expected from DMAP-glucuronide.

Isolation of 4-dimethylaminophenyl sulfate. Peak V from the chromatography of the pooled urines (Fig. 1) was further purified on a DE_{52}-cellulose column (2.5 × 13 cm) equilibrated with 100 mM NH_4-acetate, pH 6.0. Ninety two per cent of the radioactive material was eluted in a single peak by use of a linear gradient (200 ml 100 mM NH_4-acetate, pH 6.0, 200 ml 1 M acetic acid). After lyophilization the radioactive compound was chromatographed on silica gel plates with ethylacetate–methanol–water (77:13:10). The radioactive material formed a single band ($R_f = 0.4$), was extracted with acetone, and crystallized on the addition of ether. R_f Value, u.v. and i.r. spectra were identical with those of synthetic DMAP-sulfate. Incubation of the isolated compound with β-glucuronidase-arylsulfatase yielded quantitatively DMAP (t.l.c., u.v.).

Peaks III and IV, containing 9 per cent of the urinary radioactivity, could not be identified. Analytical t.l.c. pointed to more than five different compounds. Enzymic digestion with β-glucuronidase-arylsulfatase yielded less than 10 per cent of ether extractable radioactive material. It was neither 4-dimethylaminophenol nor 4-methylaminophenol.

Isolation of dimethylamine as N,N-*dimethyl-3,5-dinitrobenzamide.* As dimethylamine was formed in large quantities on reaction of DMAP with human red cells [4] and was easily identified as *N,N*-dimethyl-3,5-dinitrobenzamide this compound was expected in urine.

DMAP ^{14}C-Labeled in the methyl groups, 3.25 mg/kg, was i.v. injected into a dog. Ten ml of the 24 hr urine was alkalized with 0.5 g of sodium hydrogen carbonate and shaken with a solution of 0.1 g of 3,5-dinitrobenzoyl chloride in 5 ml of benzene. The benzene phase was reduced to a small volume and chromatographed on a silica gel plate with benzene–ethanol (95:5). Only one radioactive band appeared. It had the same R_f value as authentic *N,N*-dimethyl-3,5-dinitrobenzamide [4]. The band was excised and its radioactivity determined directly in the liquid scintillation spectrometer. After correction for recovery (83 per cent [4]) the proportion of radioactivity excreted as dimethylamine in the 24-hr-urine was calculated to be 11 per cent.

Kinetics of the elimination of 4-dimethylaminophenol. 4-Dimethylaminophenol hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring, was i.v. injected into a hind leg of a dog as in the above experiments. Blood samples were taken from a catheter in the jugular vein. One min after the end of the i.v. injection of DMAP only 25 per cent of the radioactivity in the blood corresponded to unchanged DMAP. After 10 min no DMAP could be detected.

Figure 3 shows the distribution of the radioactive metabolites in the blood on the assumption of a hematocrit of 40 per cent and a volume of distribution for water in the blood of 85 per cent [10].

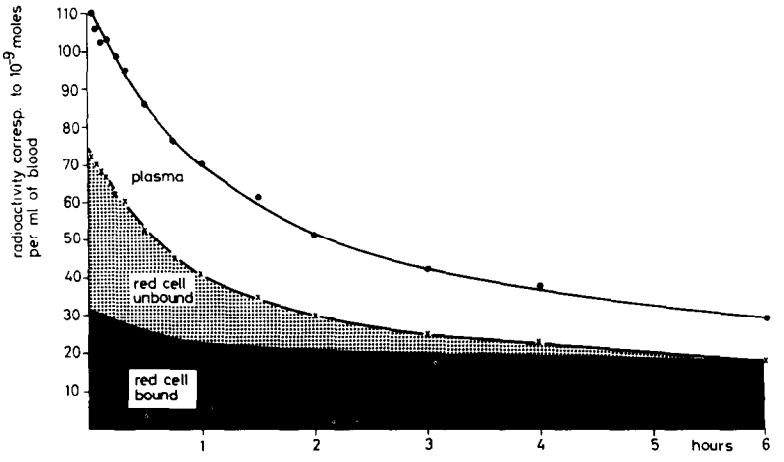

Fig. 3. Distribution of radioactivity in the blood of a dog after an i.v. injection of DMAP hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring.

About 25 per cent of the initial radioactivity in the blood was found to be bound covalently to hemoglobin. This proportion decreased very slowly and amounted still 8 per cent after 2 weeks. About 40 per cent of the initial radioactivity in the blood was located within the red cells but was not bound to hemoglobin. This proportion decreased with apparent first order kinetics, $t/2$ being about 70 min.

To identify the metabolites in the blood, in a similar experiment, 15 min after the injection of radioactive DMAP, 100 ml of blood was taken from the jugular vein (19 μCi).

After centrifugation of the heparinized blood the cells were washed once with ice-cold saline and the supernatants were combined: plasma fraction (7.5 μCi). The red cell sediment was mixed with an equal volume of water and, after stirring for 10 min on ice, proteins were precipitated with an equal volume of ice-cold 0.6 M TCA. To remove TCA the colorless supernatant was extracted with ether. This intracellular "unbound" radioactivity amounted to 6 μCi. After addition of TCA to the plasma about 90 per cent of the radioactivity remained in the supernatant.

The intracellular "unbound" radioactive material was lyophilized and chromatographed on Sephadex LH 20 (3 × 100 cm) with 10 mM acetic acid. Ninety five per cent of the radioactivity applied to the column was eluted with a R_f value corresponding to Tris-(GS)-DMAP.

Ultra violet spectra were identical with Tris-(GS)-DMAP. After Raney nickel desulfuration 92 per cent of the radioactivity could be extracted with ether as DMAP (t.l.c., u.v.). After acid hydrolysis, 2.92 moles of glutamic acid and 2.81 moles of glycine/mole of DMAP were found. Thus, the "unbound" material within the red cell was essentially Tris-(GS)-DMAP. The plasma fraction was lyophilized and chromatographed on Sephadex LH 20 as described above for the fractionation of the urine. Nine per cent of the radioactivity was found in a peak I with the same R_f value as the thioether fraction of the urine; 15 per cent in a peak II with the same R_f value as DMAP-glucuronide, and 69 per cent in a peak III with the same R_f value as DMAP-sulfate.

Peak I was identified as Tris-(GS)-DMAP by u.v. and amino acid analysis which yielded 2.94 moles of glutamic acid and 2.91 moles of glycine/mole of DMAP. Peak II was identified as DMAP-glucuronide (t.l.c.) and yielded 90 per cent of DMAP (t.l.c., u.v.) after enzymic hydrolysis with β-glucuronidase-arylsulfatase. Peak III was identified as DMAP-sulfate (t.l.c.) which yielded 92 per cent of DMAP (t.l.c., u.v.) after enzymic hydrolysis with β-glucuronidase-arylsulfatase.

Figure 4 summarizes the distribution of radioactive metabolites in the blood of a dog 15 min after an i.v. injection of DMAP. Surprisingly, the ratio of the glucuronide and sulfate conjugates did not parallel the ratio which was found in the urine. Determination of the content of total glutathione in the blood showed a decrease of the glutathione content from 1.08 mM before DMAP injection to 0.96 mM after 15 min, indicating a consumption of 0.12 mM.

The cumulative excretion of radioactive metabolites in the urine and faeces is presented in Fig. 5.

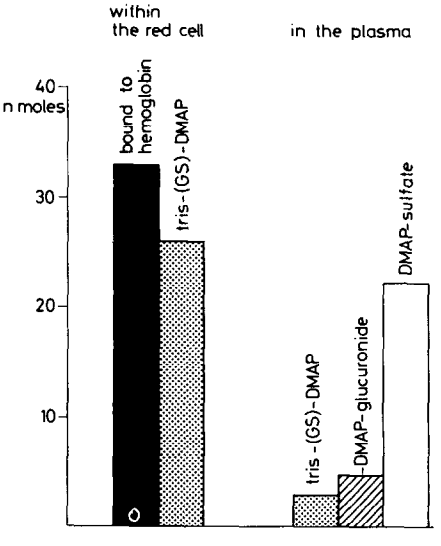

Fig. 4. Distribution of metabolites in the blood of a dog 15 min after an i.v. injection of DMAP hydrochloride, 3.25 mg/kg, ^{14}C-labeled in the ring. Nanomoles in 1 ml of blood.

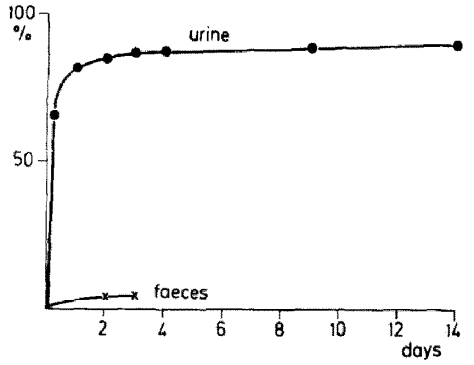

Fig. 5. Cumulative excretion of radioactive substances after an i.v. injection of DMAP hydrochloride in the dog, 3.25 mg/kg, ^{14}C-labeled in the ring. Per cent of the dose applied.

After 2 weeks 4 per cent of the radioactivity was found to be bound to hemoglobin on the assumption that blood amounts to 8 per cent of the body wt.

The time course of the urinary excretion of the three major metabolites is presented in Fig. 6. Radioactive DMAP was injected i.v. into four beagles as usual. One, 2, 4, 7, and 24 hr after injection the urine was collected through a catheter, the bladder being rinsed with saline. The samples were chromatographed on Sephadex LH 20 and the radioactive fractions combined as described in Fig. 1. The uniformity of the radioactive cuts were confirmed by t.l.c. on silica gel. The thioether fraction was not further differentiated. As early as 1 hr after injection is consisted to more than 70 per cent of Tris-(Cys)-DMAP. The urine voided on the second day contained mainly DMAP-sulfate.

Whereas elimination of the DMAP-glucuronide followed apparent first order kinetics ($t/2 = 70$ min) the elimination curve of the thioethers indicated a lag phase probably due to the relatively slow penetration of Tris-(GS)-DMAP across the red cell membrane. The substantially slower excretion of the DMAP-sulfate was surprising and its elimination curve seemed to deviate from first order kinetics.

To allow distinction between slow formation of the sulfate conjugate and slow elimination, radioactive DMAP-sulfate, 3 mg/kg, was i.v. injected in a beagle. The dog showed no unusual reactions,

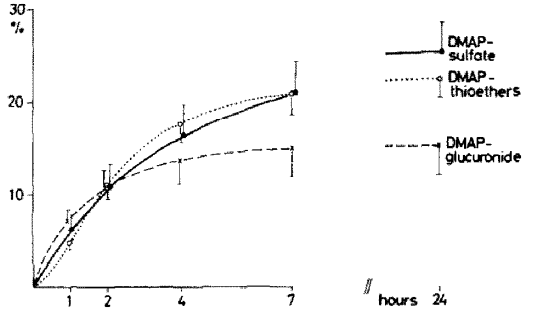

Fig. 6. Urinary excretion of radioactive metabolites after an i.v. injection of DMAP hydrochloride in dogs, 3.25 mg/kg, ^{14}C-labeled in the ring. Per cent of the dose applied, means of four experiments, ± S.D.

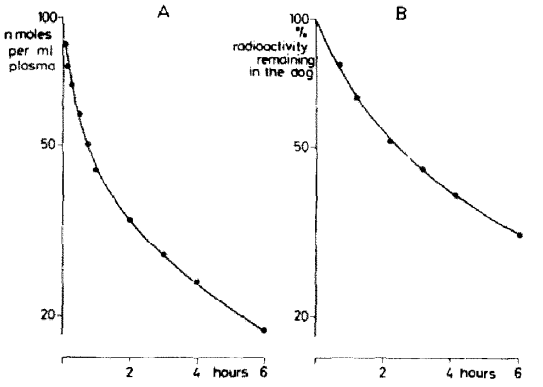

Fig. 7. Elimination of ^{14}C-DMAP-sulfate after an i.v. injection of ^{14}C-DMAP-sulfate, 3 mg/kg, in the dog. Left panel A, shows the decline of radioactivity in the plasma in logarithmic scale; right panel B, the decrease in radioactivity remaining in the dog, i.e. applied radioactivity minus radioactivity excreted with the urine, ordinate in log scale.

ferrihemoglobin formation could not be detected. Figure 7 shows the decline in plasma concentration of DMAP-sulfate and the decrease in the radioactivity in the whole body, i.e. administered radioactivity minus radioactivity excreted in the urine. Both curves deviated from first order kinetics. Ninety three per cent of the radioactivity injected was recovered in the urine after 2 days, 2.6 per cent were found in the faeces. The radioactive material in the urine consisted essentially of DMAP-sulfate. Thus, the elimination kinetics of the injected DMAP-sulfate after its biotransformation from DMAP corresponded to that observed for DMAP-sulfate.

DISCUSSION

The results of our experiments confirm the importance of the reaction of DMAP with glutathione even *in vivo*. Fifteen min after an i.v. injection of DMAP the proportion of Tris-(GS)-DMAP in the red cells was nearly ten times higher than in the plasma indicating a formation of the thioether within the red cells. The consumption of 120 μM of glutathione in the blood supports this idea. As the amount of Tris-(GS)-DMAP found within the red cells was nearly equal to the sum of the thioethers of DMAP excreted with the urine, a substantial thioether formation in other organs is improbable. As observed with other glutathione conjugates [11] this thioether was not expected to be excreted unchanged in the urine. Its major metabolite was found to be Tris-(Cys)-DMAP. The peptic cleavage of the glutathione residue occurred quite rapidly as shown in the experiment when Tris-(GS)-DMAP synthetized *in vitro* was i.v. injected in a beagle. In 30 min, half of the injected radioactivity was excreted in the urine—two thirds as Tris-(Cys)-DMAP. From the pattern of the minor metabolites a preferential peptic cleavage of the γ-glutamyl bond can be supposed. The same degradation sequence was proposed by Meister [12] for the metabolism of glutathione in the γ-glutamyl cycle. Of course, the amino acid composition of these metabolites as presumed in Table 2 cannot give any information

about the different possible isomers. As the yields of amino acids, after Raney nickel desulfuration and acid hydrolysis, decreased markedly with the increase in the remaining side chains of Tris-(Cys)-DMAP, an adsorption of the liberated tri-peptide, γ-glutamylalanylglycine to the nickel particles had to be taken into account. This could be confirmed by comparison of the analyses of GSH and authentic Tris-(GS)-DMAP.

N-Acetyl-derivatives, mercapturic acids in the strict sense, could not be detected in the urine of the dogs. Boyland *et al.* [13] isolated (2-amino-1-naphthyl)mercapturic acid from the urine of the dog after administration of 2-naphthylamine, and Colucci and Buyske [14] detected mercapturic acid formation after administration of benzothiazole-2-sulfonamide to dogs. In contrast, Jagenburg *et al.* [15] reported the excretion of 5-(1-acetamido-4-hydroxyphenyl)-cysteine in the urine of man after administration of *p*-acetamidophenol. These authors found only traces of *N*-acetylated derivatives.

In the 24 hr urine about 40 per cent of the DMAP dose was excreted as glucuronide and sulfate conjugates. This amount corresponded to earlier findings in man when 30 per cent of the administered DMAP was found in the 8 hr urine as glucuronide and sulfate conjugates [16]. This high proportion was the more surprising as the reaction of DMAP within the red cells occurs so quickly. In suspensions of washed human red cells (15 g of hemoglobin/100 ml) incubated with 0.23 mM [^{14}C]DMAP at pH 7.4 and 37°, 85 per cent of the radioactivity was found to be located within the red cells after 2 min [17]. These findings are consistent with those in the dog when 1 min after the end of an i.v. injection of DMAP only 25 per cent of the blood radioactivity could be accounted for as intact DMAP. The high amount of glucuronide and sulfate conjugates in the urine thus indicated a very high conjugation capacity of the liver. As demonstrated in the accompanying paper this assumption could be confirmed by experiments with isolated, protein-free perfused rat livers [18].

The delayed excretion of the DMAP-sulfate conjugate with its deviation from first order kinetics obviously was not due to a slow conjugate formation but rather due to distribution phenomena, as seen in the experiment when synthetically prepared DMAP-sulfate was i.v. injected into a dog. Significant enterohepatic circulation of DMAP-sulfate seems to be less probable since the bile of the bladder of a dog 24 hr after DMAP injection contained only traces of radioactivity. This finding was confirmed by experiments with isolated rat liver (cf. accompanying paper [18]) when the bile contained only small amounts of DMAP-glucuronide but no DMAP-sulfate as would be predicted from the low mol. wt of DMAP-sulfate.

The excretion of radioactive dimethylamine after administration of DMAP, labeled in the methyl groups, pointed to hydrolysis of *N*,*N*-dimethyl-quinonimine. Dimethylamine was formed by autoxidation of DMAP and after binding of DMAP to hemoglobin [4, 14]. Furthermore, dimethylamine was liberated upon autoxidation of *S*,*S*-(2-dimethylamino-5-hydroxy-1,3-phenylene)-bis-glutathione [7]. Since about 14 per cent of the administered DMAP was covalently bound to hemoglobin in the dog, and dimethylamine has been reported to be excreted unchanged in the urine of man [19], the urinary excretion of 11 per cent of the administered methyl radioactivity as dimethylamine suggests prevailing dimethylamine formation from DMAP within the red cells.

Acknowledgements—This study was supported by the Deutsche Forschungsgemeinschaft. The authors are grateful to Miss Gabriele Dietrich and to Mrs. Mira Strosar for excellent technical assistance.

REFERENCES

1. M. Kiese and N. Weger, *Eur. H. Pharmac.* **7**, 97 (1969).
2. W. Lörcher and N. Weger, *Arch. exp. Path. Pharmack.* **270**, R88 (1971).
3. D. Christel, P. Eyer, M. Hegemann, M. Kiese, W. Lörcher and N. Weger, *Arch. Tox.* **38**, 177 (1977).
4. P. Eyer, M. Kiese, G. Lipowsky and N. Weger, *Chem. Biol. Interact.* **8**, 41 (1974).
5. P. Eyer, *Arch. Pharm.* **293**, R218 (1976).
6. P. Eyer, *Arch. Pharm.* **297**, R83 (1977).
7. P. Eyer and M. Kiese, *Chem. Biol. Interact.* **14**, 165 (1976).
8. P. Eyer, in *Industrial and Environmental Xenobiotics* (eds J. R. Fouts and I. Gut), p. 290. Excerpta medica, AM (1978).
9. E. Kuss, *Z. Physiol. Chem.* **352**, 817 (1971).
10. H. U. Bergmeyer, in *Methoden der enzymatischen Analyse* (ed. H. U. Bergmeyer), Vol. 1, p. 278. Verlag Chemie, Weinheim (1970).
11. E. Boyland and L. F. Chasseaud, *Adv. Enzymol.* **32**, 175 (1969).
12. A. Meister, *Science, N.Y.* **180**, 33 (1973).
13. E. Boyland, D. Manson and R. Nery, *Biochem. J.* **86**, 263 (1962).
14. D. F. Colucci and D. A. Buyske, *Biochem. Pharmac.* **14**, 457 (1965).
15. O. R. Jagenburg and K. Toczko, *Biochem. J.* **92**, 639 (1964).
16. P. Eyer, M. Kiese, G. Lipowsky and N. Weger, *Arch. exp. Path. Pharmak.* **270**, R29 (1971).
17. P. Eyer, unpublished experiments, Munich (1975).
18. P. Eyer and H. Kampffmeyer, *Biochem. Pharmac.* **27**, 2223 (1978).
19. J. Rechenberger, *Z. Physiol. Chem.* **265**, 275 (1940).