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BIOTRANSFORMATION OF 4-DIMETHYLAMINOPHENOL IN THE ISOLATED PERFUSED RAT LIVER AND IN THE RAT

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Abstract—Isolated rat livers were perfused with various concentrations of 4-dimethylaminophenol-[^{14}C] (DMAP). During single pass perfusion with modified protein-free Krebs–Henseleit solution up to 70μM prehepatic 4-dimethylaminophenol (DMAP) were metabolized by the liver. The main route of biotransformation was conjugation. At steady state conditions glucuronide formation showed an apparent V_{max} of 8.5 μmoles × min^{-1} × g protein^{-1}, and K_m of 562 μM, whereas sulfate formation had an apparent V_{max} of 1.2 and a K_m of 35. Thus, at low substrate concentration the sulfate conjugation outweighed glucuronidation whereas at high substrate concentration the ratio of conjugates was reversed. In contrast to DMAP-sulfate, some DMAP-glucuronide was stored by the liver and was released with a half life of about 15 min which showed positive correlation with the dose of DMAP during the washout period. Perfusion with human or rat erythrocytes demonstrated the other important path of biotransformation of DMAP within erythrocytes, namely thioether formation with glutathione and SH-groups of hemoglobin. The pattern of DMAP-conjugation was affected depending on the time of prehepatic exposure to erythrocytes, and the species of red cells. The results obtained from the isolated metabolic system resemble the hepatic part of the overall metabolism under *in vivo* conditions.

4-Dimethylaminophenol (DMAP), an antidote used in cyanide poisoning to produce methemoglobinemia, has been shown to be metabolized substantially by erythrocytes [1–4].

DMAP-Thioethers (23 per cent) formed by erythrocytes, DMAP-glucuronide (15 per cent), and DMAP-sulfate (25 per cent) were eliminated by the urine of dogs within 24 hr after an i.v. injection of DMAP-hydrochloride, 3.25 mg/kg [5]. Thus, half the DMAP was recovered as the glucuronide or sulfate although DMAP was rapidly oxidized by hemoglobin *in vitro* [2] and no DMAP was found in the dogs' blood 10 min after i.v. administration [6]. This suggested quick conjugation of DMAP by the liver, competing with rapid biotransformation of DMAP within erythrocytes.

To investigate these competing metabolic pathways of DMAP separately, we perfused isolated rat livers with protein free medium in presence or absence of erythrocytes.

MATERIALS AND METHODS

4-Dimethylaminophenol hydrochloride and the radioactive compound, labeled [^{14}C] randomly in the ring with sp. act. of 5 mCi/m-mole, were prepared by Farbwerke Hoechst. β-Glucuronidase-arylsulfatase was purchased from Boehringer, Mannheim. All other reagents were analytical grade chemicals from Merck, Darmstadt.

Unstarved male Sprague–Dawley rats, weighing 190–220 g and fed with Altromin^R, were anesthetized by an i.p. injection of 50 mg/kg pentobarbital sodium (Nembutal^R).

The procedure for removal of the liver from the corpus, and the perfusion apparatus, suitable for both non-recirculating and recirculating organ perfusion, were described in an earlier paper [7].

The perfusion medium was Krebs–Henseleit-solution [8] with the following modification: 150 mM sodium, 6 mM sulfate, 5 mM glucose, 2 mM pyruvate, 0.3 mM lactate. The flow rate was about 4.6 ml/min/g liver at 37°. Prior to substrate addition to perfusion medium the liver was perfused with the recirculating buffer for 30 min.

In case of *non-recirculating perfusion* experiments, DMAP hydrochloride, dissolved in 0.9% sodium chloride solution, was infused for 30 min at a flow rate of 0.5 ml/min into the affluent tube (2 mm diameter) 15 cm before the liver to avoid autoxidation of DMAP in the perfusion medium, saturated with O_2–CO_2 (95:5, v/v).

Samples were removed from a small mixing chamber (3 ml) through a rubber septum to determine substrate concentration 5 cm before the portal entrance.

To avoid autoxidation of DMAP during posthepatic sampling the liver efflux was mixed with 4 N hydrochloric acid by continuous infusion with a rate of 0.3 ml/min. Hence, the pH of the effusate was lowered to about 3. Samples of about 16 ml were collected automatically for chemical analysis of 0.4 min intervals with a LKB Ultrorac^R and

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; t.l.c., thin layer chromatography; TCA, trichloroacetic acid; Hb, hemoglobin.

cooled on ice. After a 30 min washout period the liver was weighed and homogenized with an Ultra-Turrax® in twice the volume of 0.9% sodium chloride solution for the determination of radioactivity and protein content.

In *recirculating perfusion* experiments the substrate dissolved in 2 ml of 0.9% sodium chloride solution was infused during a 2-min period into the efflux either before or after passage through the oxygenator.

The mean liver wet wt after perfusion was 8.15 ± 0.32 g with a total protein content of 0.96 ± 0.03 g; the mean flow rate was 37.5 ± 0.5 ml/min. The pressure in the afflux was continuously measured. It varied between 10–24 cm of water among the experiments and was almost stable within an experiment.

For single pass perfusion experiments of the lung a catheter with a buffer flow rate of 10 ml/min was forced through the right ventricle of the heart into the pulmonary artery and fixed. The catheter for the efflux was pushed through the left ventricle near the septum and fixed in the pulmonary vein. The lung was removed together with the heart from the corpus and the broncheal tree was exposed to a constant positive air pressure of 30 cm water. The perfusion rate of the lung was 40 ml/min.

Erythrocytes from heparinized fresh blood were washed three times with 0.9% sodium chloride solution in presence of 10 mM glucose to keep glutathione in the reduced state. Packed red cells were added to the recirculating perfusion fluid after the liver was incorporated into the system to give a final hemoglobin concentration of 4 g per cent and a fluid volume of 80 ml.

In vivo experiments were carried out with two rats for each dose. 3.25 or 25 mg/kg DMAP[^{14}C] hydrochloride was i.v. injected within 1 min. The urine was collected for 24 hr on ice. In addition, two rats were sacrificed 5 min after 3.25 mg/kg DMAP[^{14}C]hydrochloride injection and the blood from the jugular vein collected. The biological material was analyzed as described in the preceding paper [5].

Radioactive DMAP was determined by an isotope dilution technique. 0.5 ml of a sample was quickly mixed with 5 mg of non-radioactive DMAP-HCl in 0.5 ml of 0.2 M phosphate solution, pH 7.4, and extracted with 2 ml of ether. Of the ether extract 0.5 ml was chromatographed on silica gel plates with chloroform–methanol (95:5). The DMAP containing band with a R_f value of 0.4 was eluted with ether. By reading the ultra violet (u.v.) absorbance at 247 nm (log E = 4.07) and determining the radioactivity the concentration of radioactive DMAP in the sample was calculated.

Radioactive metabolites were separated by column chromatography on Sephadex LH 20 as described elsewhere [5]. Prior to applying the material to the column, the sample was extracted with ether at pH 7.4 to remove DMAP which was found to be the only ether extractable radioactive compound. From the aqueous phase two radioactive compounds were separated by column chromatography with R_f values of DMAP-glucuronide and DMAP-sulfate, respectively [5]. At all DMAP concentrations used, both of these compounds yielded 97 ± 2 per cent of the radioactivity applied to the column. The homogeneity of the pooled fractions was confirmed by t.l.c. on silica gel with ethanol–water–acetic acid (76:19:5). In this system, the R_f values of the expected radioactive compounds were as follows: thioethers of DMAP = 0.0–0.05; DMAP-glucuronide = 0.50–0.55; DMAP = 0.70–0.75; DMAP-sulfate = 0.85–0.90. Radioactivity on the plates was measured with a scanner for t.l.c. (Berthold, Wildbad, FRG). Additionally, the pooled fractions of DMAP-glucuronide and DMAP-sulfate, respectively were enzymically hydrolyzed yielding more than 93 per cent of DMAP. The enzymic incubation was performed in 0.1 M sodium acetate, pH 5.0, at 37° under nitrogen over night in the presence of β-glucuronidase-aryl sulfatase, 0.2 U/ml of incubation medium. The concentration of DMAP-glucuronide and DMAP-sulfate were determined from the specific radioactivity after separation of the compounds by column chromatography.

Protein was determined with the Biuret method. After precipitation of the homogenate with an equal volume of 0.6 M TCA the protein-bound radioactivity was roughly estimated from the difference of radioactivity in the homogenate and the TCA supernatant.

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

All computations like mean estimates and regression lines were carried out by programs designed for a pocket computer Hewlett Packard 25. Mean values are presented with S.E.

RESULTS

Analysis of metabolites

For qualitative analysis of DMAP metabolites the liver was perfused with the recirculating fluid containing an initial DMAP-concentration of 400 μM. After a 30-min perfusion period the perfusate was analyzed. No intact DMAP was detected. After freeze drying the perfusate was chromatographed on Sephadex LH 20. Ninety per cent of the radioactivity was separated in two peaks.

Compound I (57%) exhibited the same R_f values as DMAP-glucuronide [5] on column chromatography and t.l.c. Enzymic hydrolysis yielded DMAP and glucuronic acid whose detection was described previously [5].

Compound II (33%) showed the same R_f values as DMAP-sulfate [5] on column chromatography and t.l.c. Enzymic hydrolysis yielded DMAP. The u.v. spectra were identical with synthetic DMAP-sulfate [5].

To elucidate possible biliary excretion of DMAP and/or its metabolites, the bile duct was cannulated in two single pass perfusion experiments, and the bile (about 1 ml in 1 hr) was collected during the DMAP-infusion and washout period. With initial DMAP concentrations of 22 or 164 μM, 8 or 1.5 per cent of the radioactivity infused was found in

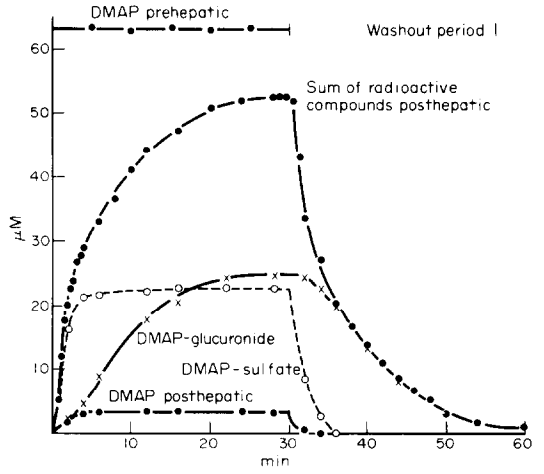

Fig. 1. Concentration of DMAP and its conjugates during protein-free single pass perfusion of rat liver. The liver was perfused with 63 μM DMAP[^{14}C] in the afflux for 30 min followed by a 30-min washout period.

the bile of which 93 or 96 per cent was DMAP-glucuronide.

S,S,S-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-glutathione (Tris-(GS)-DMAP) [3], which has been separated from the blood of different species including the rat, could not be detected in the perfusion fluid. To rule out rapid degradation to unknown products, authentic, radioactive Tris-(GS)-DMAP [5] was added to the recirculating perfusion fluid (90 mM). The bile (0.8 ml) collected for 60 min contained only 0.5 per cent of the total radioactivity. Analysis of the perfusion fluid revealed more than 80 per cent of unchanged Tris-(GS)-DMAP. Thus, glutathione conjugation of DMAP did not occur in substantial quantities in the isolating perfused rat liver.

DMAP-conjugation occurred only with O_2–CO_2 (95:5, v/v) saturated perfusion fluid. When the liver was perfused with buffer saturated with pure carbon monoxide for 20 min prior to DMAP addition and the following 30-min perfusion period, all of the radioactivity in the 80 ml perfusion fluid corresponded to intact DMAP.

Kinetics of the DMAP biotransformation

Single pass liver perfusion. Since autoxidation of DMAP was significant ($t/2$ of about 10 min in buffer saturated with O_2–CO_2 (95:5, v/v) at 37° and normal pressure) single pass perfusion was performed to determine the rates of DMAP-glucuronide and -sulfate formation.

Figure 1 shows the concentrations of DMAP and its metabolites vs time during such an experiment with 63 μM of DMAP. From the fourth min of DMAP infusion onward only 5 per cent of the infused DMAP concentration appeared in the effusate, and its concentration remained constant during the 30-min DMAP infusion period. Thus, about 95 per cent of the infused DMAP was eliminated during one passage through the liver.

The posthepatic concentration of radioactive compounds increased steadily and approached its maximum towards the end of the DMAP infusion period. Considering the conjugates, the DMAP-sulfate concentration quickly reached its steady state level and, after ending the DMAP infusion, returned quickly to zero concentration. In contrast, the DMAP-glucuronide concentration increased steadily during about 25 min of DMAP perfusion and the slow decrease in radioactivity in the efflux was due to a delayed release of DMAP-glucuronide. The "washout" of DMAP-glucuronide by the perfusion medium seems to follow first order kinetics with a half-life ($t/2$) of about 15 min.

When this "washout" was corrected for flow rate and the amount of liver protein [$t/2 \times$ ml/min $\times$ (g protein)$^{-1}$] a positive correlation was found vs steady state DMAP-glucuronide concentration in the efflux; $r^2 = 0.57$, $P < 0.01$, $n = 18$.

Figure 1 suggests some loss of radioactivity during liver perfusion. By comparing the total area under the curve of posthepatic radioactivity with the total area of prehepatic DMAP, a loss of 14 per cent of the infused radioactivity was calculated. Similarly, the posthepatic radioactivity at steady state conditions near the end of the DMAP infusion was also 16 per cent lower than the prehepatic. As indicated in Table 1 88 ± 4 per cent of the prehepatic radioactivity was found in the efflux. Single pass perfusion of the system without liver revealed 92 ± 4 per cent recovery which was independent on DMAP concentration (20 or 200 μM). Therefore, the loss in radioactivity of approximately 10 per cent could be attributed to adsorption on Plexiglas of the perfusion table and the plastic tubings rather than to binding to liver proteins (Table 1).

However, the percentage of bound radioactivity was higher ($P < 0.005$) at high DMAP concentrations with incomplete metabolic removal.

As shown in Table 1 the maximum capacity of the liver to conjugate DMAP was reached at concentrations of about 160 μM, at higher concentrations sulfate conjugation decreased. Whereas the glucuronide formation prevailed at high DMAP concentrations, sulfate formation was clearly favoured at low DMAP concentrations. From the data of Table 1 apparent V_{max} and K_m values were calculated by estimating the regression lines of the Lineweaver–Burk transformation shown in Fig. 2.

Since the DMAP-glucuronide data in experiments with low DMAP (10 μM) in the afflux showed significant deviation from the straight line, the regression line for DMAP-glucuronide was calculated only from those experiments where some DMAP was found in the efflux. This ensured that the regression line was not influenced by false low DMAP-glucuronide formation rates caused by total removal of the substrate by sulfotransferase(s). Statistical treatment of the regression lines revealed V_{max} estimates not overlapping each other at 95 per cent confidence intervals and slopes not likely to be similar at P 0.01 ($t > 4 \times 10^4$). Apparent V_{max} for DMAP-glucuronide formation was about 8.5 vs 1.2 μmoles $\times$ min^{-1} $\times$ g protein^{-1} for DMAP sulfate formation. In contrast, apparent K_m for sulfation (35 μM) was about a fifteenth of the K_m for glucuronidation.

Figure 3 shows the probit plot [9] of per cent DMAP remaining unchanged during single pass

Table 1. Experimental data of single pass perfusion of livers with DMAP

DMAP Prehepatic (μM)	RC* Post-hepatic (μM)	DMAP† Posthepatic (μM)	DMAP-Gluconide† posthepatic (μM)	DMAP-Sulfate† posthepatic (μM)	Rate of DMAP-glucuronide formation‡	Rate of DMAP-sulfate formation‡	Radioactivity remaining in the liver (%)	RC* Bound to liver protein (%)
390	330	270	40	20	1.80	0.87	2.30	0.33
354	300	236	44	20	1.92	0.87	0.45	0.16
170	145	74	43	28	2.02	1.31	0.20	0.14
164	130	59	43	28	1.50	1.00	0.51	0.27
155	123	58	40	25	1.82	1.14	0.15	0.13
90	72	10	36	26	1.18	0.85	0.31	0.14
63	50	3.3	26	21	1.07	0.79	0.77	0.28
37	28	2.5	13	13	0.49	0.48	0.56	0.32
37	29	0.4	15	14	0.60	0.57	0.09	0.09
22	19	0.0	8.6	10.4	0.42	0.51	0.05	0.05
22	21	0.0	8.7	11.9	0.35	0.50	0.25	0.09
22	20	0.2	7.8	12.2	0.32	0.51	0.32	0.14
21	20	0.0	9.2	11.2	0.34	0.42	0.16	0.08
21	19	0.0	6.7	12.4	0.24	0.44	0.15	0.08
17	14	0.0	4.8	9.3	0.19	0.36	0.11	0.08
11	10	0.0	2.2	8.1	0.08	0.30	0.12	0.08
11	9.5	0.0	1.7	7.8	0.06	0.25	0.17	0.11
10	9.5	0.0	2.0	7.5	0.09	0.33	0.11	0.09
10	9.3	0.0	2.5	6.8	0.09	0.24	0.12	0.10

* Sum of radioactive compounds.
† Concentrations at steady state conditions.
‡ μMoles × min^{-1} × g $protein^{-1}$.

perfusion vs prehepatic DMAP concentration. The data revealed a correlation coefficient of $r^2 = 0.86$ for the calculated line. Below 30 μM virtually all of the DMAP was eliminated by one liver passage.

Recirculating liver perfusion with red cells. Addition of red cells to the perfusion fluid provided the opportunity to investigate the other important metabolic pathway of DMAP, competing with the liver for the substrate [5]. DMAP was added to the livers efflux to give a final concentration of 105 μM in the recycling fluid. After 30 min the metabolites listed in Table 2 were found. The important role of the red cells on DMAP biotransformation is illustrated by variation of the time of prehepatic exposure of DMAP to erythrocytes. This was achieved by injection of DMAP into the recirculating fluid at different points in the perfusion apparatus. After 2 min of exposure to red cells nearly 90 per cent of DMAP had reacted within the red cells. Thus, the low DMAP concentration actually entering the liver caused a higher DMAP-sulfate production over DMAP-glucuronide.

Human and rat erythrocytes showed similar metabolic activity but differed widely in the ratio of Tris-(GS)-DMAP to protein-bound DMAP formation.

These results confirm the important metabolic activity of the red cells towards DMAP, but they seem to disagree with results obtained from concurrent in vivo studies.

In vivo *conjugation of DMAP*[^{14}C]. When DMAP[^{14}C] was administered i.v. to rats (25 mg DMAP-HCl/kg), 28 per cent of the radioactivity was excreted in 24 hr as DMAP-glucuronide and 32 per cent of DMAP-sulfate. At a lower dose (3.25 mg DMAP-HCl/kg), 27 per cent of the radioactivity was excreted as DMAP-glucuronide and 42 per cent as DMAP-sulfate in the 24-hr-urine. Analysis of the

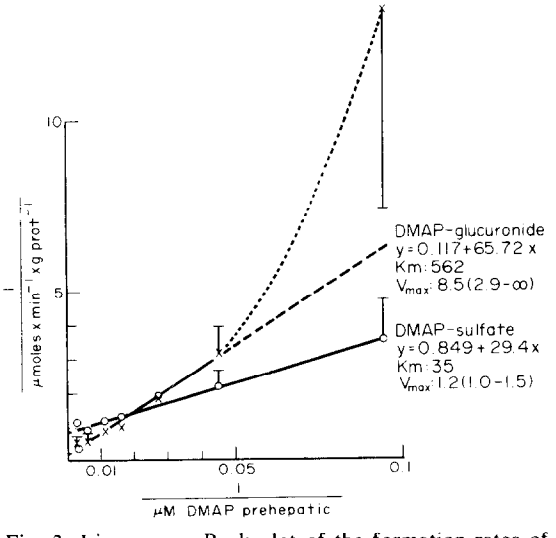

Fig. 2. Lineweaver–Burk plot of the formation rates of DMAP-glucuronide and -sulfate at steady state conditions following protein-free single pass perfusion of rat livers. Regression lines were calculated from data connected by solid line. The slopes are not equal at $P < 0.01$. Bars or brackets indicate 95 per cent confidence interval. Dimensions of kinetic data as indicated in the graph.

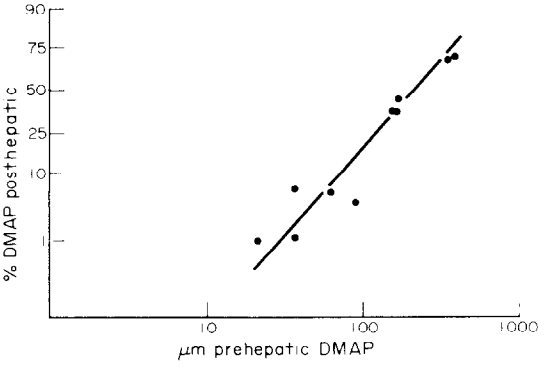

Fig. 3. Probit plot of per cent DMAP concentration appearing in the efflux vs prehepatic DMAP concentration after one passage through rat livers. DMAP concentrations were determined between 25 and 30 min following single pass perfusion.

Table 2. Metabolites of 4-dimethylaminophenol (DMAP) after recirculating liver perfusion with erythrocytes

Time (sec) of prehepatic exposure to red cells Source of red cells	45 Human	120 Human	120 Rat
Tris-(GS)-DMAP	26	29	5
DMAP-Glucuronide	32	3.3	5
DMAP-Sulfate	25	8.7	11
DMAP Covalently bound to hemoglobin	21	60	83

DMAP-Metabolites (nmoles/ml) in erythrocyte suspension 30 min after 105 nmoles/ml of DMAP.

blood, 5 min after an i.v. injection of 3.25 mg DMAP-HCl/kg in rats, showed that only 15 per cent of the radioactive compounds in the blood was covalently bound to hemoglobin and the content of glutathione thioethers in the blood was lower than 3 per cent. Surprisingly, 10 per cent of the blood radioactivity still corresponded to intact DMAP. Thus, the half life of DMAP in vivo seems to be longer than under the conditions of the *in vitro* experiments.

Incubation of DMAP[^{14}C] *with red cells.* DMAP (105 μM) was incubated with freshly drawn, heparinized rat blood or washed rat erythrocytes at 37°. The incubates were equilibrated either with pure oxygen or with a mixture of oxygen and nitrogen producing an oxygen pressure of 38 mm Hg. The pH was adjusted to 7.4 with 1 M phosphate buffer.

Figure 4 shows the dependence in DMAP elimination rate upon oxygen pressure and plasma content. The half life of DMAP in blood was about 4 min under 38 mmHg of oxygen. This half life was shortened in the absence of plasma to about 2 min. A more drastic reduction of half life to less than 30 sec was seen under pure oxygen. The dotted line in Fig. 4 shows the decrease in DMAP under conditions similar to the liver perfusion experiments (4 g hemoglobin/100 ml, high oxygen pressure). About 70 per cent of DMAP was eliminated within 2 min, resembling the average time DMAP could react with red cells before reaching the perfused liver.

Lung perfusion. Biotransformation of DMAP by the lung was investigated by isolated lung single pass perfusion without red cells. The DMAP concentration entering the lung was 110 μM, the concentration of radioactive compounds in the efflux was 100 μM. More than 98 per cent of the radioactivity in the efflux could be extracted with ether and was shown to be DMAP. The remaining aqueous phase was chromatographed.

About half of the radioactivity was eluted with a R_f value of DMAP-glucuronide; DMAP-sulfate was not detectable.

DISCUSSION

Single pass perfusion, initially chosen to overcome the consequences of rapid DMAP autoxidation during metabolic studies, revealed some advantages. This method allowed steady state concentration measurements without interference by

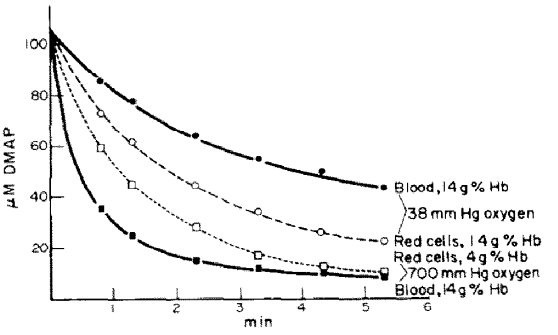

Fig. 4. Decrease in DMAP concentration in the presence of rat erythrocytes at pH 7.4 and 37° *in vitro* (means of three experiments).

● = Whole blood (14 g Hb/100 ml) at an oxygen pressure of 38 mmHg. ○ = Washed red cells (14 g Hb/100 ml) at an oxygen pressure of 38 mmHg. □ = Washed red cells (4 g Hb/100 ml) at an oxygen pressure of 700 mmHg. ■ = Whole blood (14 g Hb/100 ml) at an oxygen pressure of 700 mmHg.

product accumulation during recirculation, and easier kinetic evaluation of delayed release of metabolites from the liver cell, as shown for DMAP-glucuronide, both of which may lead to erronous interpretation of results obtained from other metabolizing systems. Since extensive covalent binding of DMAP to liver proteins and thioether formation with glutathione were not found, most likely the rapid conjugation of DMAP anticipates oxidation of DMAP by the endoplasmatic oxygenases to reactive compounds such as radicals or the quinonimine found in the presence of hemoglobin [2]. Thus, the values listed in Table 1 gave an opportunity to determine kinetic data for DMAP-glucuronidation and DMAP-sulfation in the isolated liver. The pattern of DMAP-conjugation varied with DMAP concentration in the liver. At low DMAP concentrations DMAP sulfation predominated whereas at high DMAP concentrations the glucuronidation was favoured. Such a dose-dependent ratio of glucuronide–sulfate formation was first reported by Bray *et al.* [10, 11] with phenol fed to rabbits. In addition, inorganic sulfate was found to be rate limiting for sulfate-conjugation. The latter observation had prompted us to raise the sulfate concentration of the original Krebs–Henseleit buffer to prevent possible limitation of sulfate conjugation even at high DMAP concentrations. Similar results were observed by Büch *et al.* [12] after administration of paracetamol into rats. Minck *et al.* [13] gave their results the interpretation of competition between the sulfotransferase and UDP-glucuronyl-transferase for the substrate and a possible "negative cooperativity" of the glucuronyltransferase with the acceptor substrate as discussed by Winsnes [14].

We can confirm in principle the results of Mulder *et al.* [15] in that the apparent V_{max} for glucuronidation was eight times larger than for sulfation and the value for the apparent K_m of glucuronidation 16 times larger than for sulfation (Fig. 2).

In the case of glucuronidation at low substrate concentration, and the deviation of experimental data from the straight line of the Lineweaver–Burk plot (Fig. 2), one might postulate allosteric com-

plexities (cf. the commentary of Dutton [16] (such as product activation [17]. Our experiments led to some other explanation. No DMAP was recovered in the efflux from 10 μM of prehepatic DMAP. Since DMAP had shown a lower K_m for sulfation than for glucuronidation most likely a large fraction of the DMAP was conjugated by the cytoplasmic soluble sulfotransferase before it had reached the UDP-glucuronyltransferase hidden in the microsomal membranes [18]. If about 5 μmoles/l had reached the UDP-glucuronyltransferase the experimental data would fit the straight line.

The important extrahepatic biotransformation of DMAP was demonstrated with erythrocytes in the recirculating perfusion-fluid. Even during 45 sec of exposure (Table 2) an appreciable fraction of DMAP was oxidized within red cells and had formed thioethers with glutathione and SH-groups of hemoglobin. The species differences between DMAP-glutathione- and DMAP-hemoglobin-thioether formation can be explained by the larger number of reactive SH-groups in rat hemoglobins [19] at equal concentration of glutathione in human and rat erythrocytes.

However, this extrahepatic biotransformation *in vivo* of DMAP was much less pronounced. Only 15 per cent of the radioactive compounds in the blood was covalently bound to hemoglobin whereas 2/3 of the dose was excreted as DMAP-conjugates. Since conjugation of DMAP in the lung was negligible this suggested a longer half life of DMAP in vivo than in the *in vitro* studies with recirculating perfusion fluid. Addition of DMAP to blood or washed red cells at different oxygen pressures (Fig. 4) confirmed this hypothesis. The half life of DMAP in red cell suspensions was shortened by the absence of plasma and in the presence of high oxygen pressure; at an oxygen pressure of 38 mmHg the half life of DMAP in blood was 10 times longer than under 700 mmHg oxygen. Therefore, assuming same time of exposure to red cells DMAP had its first pass through the liver *in vivo* at higher concentrations than in artificial liver perfusion.

In addition, the mean blood circulation time in the rat of 18 sec (calculated on the basis of a 200 g rat, 65 ml/min stroke volume of the heart [20], 14 ml/min liver blood flow [21]), allows the liver to clear about 70 per cent of DMAP from the circulating blood within 1.5 min. During that time only 25 per cent of DMAP is expected to be metabolized by the red cells (cf. the upper curve in Fig. 4). Thus, the higher amount of DMAP conjugates found in the rats' urine than during liver perfusion may be explained.

Comparison of the biotransformation of DMAP *in vivo* in rats and dogs (cf. preceding paper) reveals a higher amount of conjugates in the urine of rats. Although DMAP is metabolized at equal rates within rat and dog erythrocytes [19], the shorter mean blood circulation time in the smaller animal favours hepatic conjugation.

The use of this protein- and erythrocyte-free liver perfusion technique has proven its ability to furnish qualitative and quantitative data on liver metabolism resembling the hepatic part of the overall metabolism under *in vivo* conditions.

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