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DEPLETION OF MITOCHONDRIAL COENZYME A AND GLUTATHIONE BY 4-DIMETHYLAMINOPHENOL AND FORMATION OF MIXED THIOETHERS

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Abstract—4-Dimethylaminophenol (DMAP), an antidote in cyanide poisoning, has been shown to produce kidney lesions in rats, to damage isolated rat kidney tubules and to impair mitochondrial functions as already described for 4-aminophenol. Since DMAP upon oxidation forms bis- and tris-substituted thioethers with GSH, it was anticipated that mitochondrial toxicity of DMAP might result from CoA depletion. In a model reaction DMAP was oxidized by oxyhemoglobin in the presence of CoA and GSH resulting in formation of tris-(CoA-S-yl)-DMAP, tris-(GSH-S-yl)-DMAP and two mixed thioethers, namely, (CoA-S-yl)-bis-(GSH-S-yl)-DMAP and (GSH-S-yl)-bis-(CoA-S-yl)-DMAP. The compounds were isolated by HPLC and identified spectroscopically, by amino acid analysis and Raney-Nickel desulfuration. Rat liver mitochondria (5 mg protein/ml) incubated under state IV conditions with 20 and 50 μM DMAP were depleted of GSH and total coenzyme A with formation of GSSG and the above-mentioned thioethers which were quantified by isotope dilution techniques using [^{14}C]-labelled DMAP and the isolated, inactive thioethers. The results confirm earlier suggestions that part of the cytotoxicity of DMAP may result from depletion of vital mitochondrial thiols, particularly CoA. Since 4-aminophenol reacts analogously, similar cytotoxic effects can be expected from compounds which on (aut)oxidation form quinoid systems capable of 1.4-addition reactions with nucleophilic thiols.

Aminophenols and congeners are cytotoxic agents which after activation to electrophilic intermediates react with cellular nucleophiles [1–5]. Differences in the organotropy of these compounds have been related to differences in the balance of cellular toxication and detoxication reactions. Thus, *o*-aminophenols with primary amino groups are more easily activated by oxyhemoglobin than the *p*-isomers and produce more rapidly ferrihemoglobin [6], but are significantly less nephrotoxic compared to the *p*-isomers [7]. This phenomenon has been attributed in part to the ability of *o*-aminophenols to condense to phenoxazones after their oxidation. Hence, condensation of the reactive *o*-quinonimines competes with addition to cellular nucleophiles and has been regarded as a self-detoxication reaction [6].

Among *p*-aminophenols, great differences in reactivity have been observed, too. Comparing the toxicities of 4-aminophenol, 4-methylaminophenol and 4-dimethylaminophenol in isolated perfused rat kidneys, Elbers [8] found minimal toxic doses of > 100 μmol/g, 19 μmol and 2 μmol, respectively. These differences in cellular toxicity corresponded with the ferrihemoglobin forming activities of the above-mentioned homologous compounds. Hence, the concept arouse that cytotoxicity of *p*-aminophenols might be directly correlated with steady-state levels of the *p*-quinonimines [2, 9] as postulated also for the hepatotoxic action of acetaminophen [5].

The most active compound, 4-dimethylaminophenol (DMAP) which is therapeutically used as an antidote in cyanide poisoning [10–12] has been shown to produce kidney lesions in rats *in vivo* [13] and to damage isolated rat kidney tubules [14]. Covalent binding of *N,N*-dimethylquinonimine to tubular proteins [14], particularly in the proximal convoluted tubules [15] has been demonstrated. DMAP‡ inhibiting gluconeogenesis and a variety of glycolytic enzymes led to GSH depletion in isolated kidney tubules [16]. The reduction in the rate of ketogenesis (sum of 3-hydroxybutyrate and acetoacetate) pointed to depletion also of the mitochondrial CoA-pool [9]. In fact, cellular CoA of freeze-clamped, isolated perfused rat livers was reduced to 35% of the controls after perfusion with toxic doses of DMAP [17]. Since CoA is distributed mainly in the mitochondrial fraction [18] it appeared that part of the DMAP toxicity might be due to thioether formation of *N,N*-dimethylquinonimine with intramitochondrial CoA. Formation of thioethers of DMAP with GSH has been found *in vivo* in dogs and in man [19–21]. Since the primary thioethers of DMAP with GSH [22, 23] and with the cysteine residues of hemoglobin [3] are highly autoxidizable and can add other thiols up to tris-substituted compounds, it was suggested that DMAP might produce mixed thioethers consisting of GSH, CoA and other cellular thiols.

The present study was undertaken to identify such compounds. The first part was concerned with model

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‡ Abbreviations used; CoA, reduced coenzyme A; DMAP, 4-dimethylaminophenol; GSH, reduced glutathione; GSSG, glutathione disulfide; NEM, *N*-ethylmaleimide.

reactions where DMAP was activated by purified oxyhemoglobin with formation of phenoxyl radicals and *N,N*-dimethylquinonimine [24]. Addition of known amounts of GSH and CoA produced these thioethers in quantities sufficient for identification. In the second part of the study DMAP was allowed to react with isolated liver mitochondria. Alterations of the thiol status of GSH and CoA were determined and formation of thioethers was established and quantified by isotope dilution techniques making use of [^{14}C]-DMAP and isolated thioethers from the model experiments. Since previous studies [25] have shown that micromolar concentrations of cytochrome *c*, as present in liver cytosol [26], markedly enhanced the toxic actions of DMAP on mitochondrial functions (increase in covalent binding, decrease in CO_2 production and ATP synthesis, loss of respiratory control) all mitochondrial experiments were performed in the presence of 7 μM cytochrome *c*.

MATERIALS AND METHODS

Chemicals. DMAP (4-dimethylaminophenol hydrochloride) and [U-^{14}C-phenyl]-DMAP (specific activity 9 mCi/mmol, radiochemical purity > 98%) were prepared by Farbwerke Hoechst (Frankfurt, F.R.G.) Radioactive coenzyme A [^{3}H(G)], (specific activity 1.2 Ci/mmol, radiochemical purity 97%) and GSH [glycine-2-^{3}H] (specific activity 1.8 Ci/mmol, radiochemical purity 95%) were purchased from NEN (Dreieich, F.R.G.). L-[4-^{14}C]-Aspartic acid (specific activity 54 mCi/mmol, radiochemical purity 99%) was procured from Amersham–Buchler (Braunschweig, F.R.G.).

Biochemicals were obtained from Sigma-Chemie (Deisenhofen, F.R.G.), and from Boehringer (Mannheim, F.R.G.). Cation exchange resin, AG 50X8, was purchased from Bio-Rad, (München, F.R.G.) and Sephadex G 25 fine from Pharmacia (Freiburg, F.R.G.). All other reagents were from Merck (Darmstadt, F.R.G.) at the purest grade available.

Purified human hemoglobin was prepared as described earlier [27].

Incubation of mitochondria. Unstarved male Sprague–Dawley rats, weighing 250–350 g and fed with Altromin® with free access to food and water, were stunned, decapitated and exsanguinated. Mitochondria were prepared from rat livers as described by Cain and Skilleter [28]. The protein content of the suspended mitochondria (usually 60 mg/ml) was quickly determined by the biuret method. Typically, incubation experiments began about 2 hr after death. These mitochondria exhibited a respiratory control ratio of about 8 as determined from the oxygen consumption in state III vs state IV (in the presence of malate and glutamate, 10 mM each, ± 0.2 mM ADP, Elbers, unpublished).

The mitochondria isolation medium consisted of 200 mM mannitol, 20 mM sucrose, 10 mM triethanolamine, 2.5 mM potassium dihydrogenphosphate, 2 mM magnesium chloride and 1 mM EDTA, pH 7.2. The mitochondria incubation medium contained (final concentration) 140 mM mannitol, 10 mM sucrose, 60 mM potassium dihydrogenphosphate, 10 mM triethanolamine, 2 mM magnesium chloride, 1 mM EDTA, 0.5 mg/ml desferrioxamine, 10 mM glutamate, 10 mM malate, 3 mM ADP, 50 mM fructose-6-phosphate, 2 μM fructose-2,6-bisphosphate, phosphofructokinase (5 U/ml) and 7 μM cytochrome *c*, pH 7.4. Incubation of the mitochondria (3.2 ml, 5 mg protein/ml) was performed in 10-ml Erlenmeyer flasks which were gently shaken in a gyrotory water bath at 37° under a stream of carbogen (95% oxygen, 5% carbon dioxide, 20 ml/min). After equilibration for 5 min, DMAP (20 or 50 μM) or saline was added.

Analytical procedures. GSH and GSSG were determined according to Tietze [29] in the modification previously described [30]. To avoid autoxidation of GSH during sample processing, GSH was alkylated by *N*-ethylmaleimide (NEM). Prior to analysis excess NEM was absorbed on Sep-Pak C_{18} cartridges (Waters, Eschborn, F.R.G.). The whole procedure has been previously described in detail [31]. Briefly, 0.75 ml of the mitochondrial suspension was mixed with 0.75 ml ice-cold perchloric acid (1 M) containing 1 mM EDTA and 40 mM NEM followed by centrifugation. Immediately afterwards, 1.3 ml of the supernatant was brought to pH 6.0 with 1.1 ml 0.65 M dipotassium hydrogenphosphate. After standing at room temperature for 30 min and cooling on ice for 5 min, potassium perchlorate was spun off and the supernatant passed through a Sep-Pak C_{18} cartridge, which had been previously rinsed with 5 ml of water. Of the eluent the fraction between 1.4 and 1.9 ml containing GSSG was used for the assay. NEM and alkylated glutathione were removed from Sep-Pak C_{18} by elution with 3 ml methanol. After rinsing the cartridge with 5 ml water the next sample could be applied (up to ten cycles). The whole procedure lasted no longer than 3 min and is much more convenient than the removal of excess NEM by ethyl acetate extraction as proposed earlier [30].

The mitochondrial GSH content of fed rat livers was determined by Jocelyn [32] who found 4.9 nmol/mg in washed mitochondria, a value consistent with our data. Wahlländer *et al.* [33] using freeze-clamped livers which were further processed by a non-aqueous fractionation procedure found 8.8 ± 1.2 nmol/mg mitochondrial protein.

Total coenzyme A (reduced, disulfides and acetyl-CoA) was determined radiometrically. The principle as described by Decker [34] includes reduction of the disulfides by dithiothreitol, enzymic conversion of CoA to acetyl–CoA with acetyl phosphate by phosphate acetyltransferase (EC 2.3.1.8.) from Cl. Kluyveri (Sigma) and formation of radioactive citrate by enzymic condensation of acetyl–CoA with radioactive oxaloacetate by means of citrate synthase (EC 4.1.3.7) from procine heart (Sigma). After decomposition of excess [4-^{14}C]-oxaloacetate with liberation of [$^{14}CO_2$], the remaining radioactivity in the synthesized citrate corresponds to total coenzyme A. Due to a variety of sources of error under our conditions (inactive oxaloacetate in the sample leads to "dilution" of the specific activity of the radioactive preparation, the high phosphate concentration in the mitochondria incubates shifts the equilibrium of the phosphate transacetylase reaction to the left, CoA liberated in the condensing reaction might recycle,

if transacetylase activity is present) some modifications of the method are described in detail: 0.5 ml of the mitochondrial suspension was mixed with 0.5 ml of ice-cold perchloric acid (1 M) containing 1 mM ascorbic acid, and the proteins were spun off. To 0.8 ml of the supernatant 0.2 ml potassium acetate (1 M) and 0.165 ml potassium carbonate (1 M) were added to give a final pH of about 4.5. Fifty μl dithiothreitol (20 mM) and 5 μl $CuSO_4$ (100 mM) were added, and the mixture was allowed to react at room temperature for 30 min (at pH 4.5 cupric ions catalyse oxaloacetate decomposition [35]). To the mixture 0.155 ml of 1 M Tris was added under stirring to bring the pH to 7.2. After centrifugation of the potassium perchlorate, 0.1 ml of the supernatant was mixed in an Eppendorf cup with 0.2 ml Tris–HCl (0.2 M, pH 7.2) and 177 μl water, 20 μl of dithiothreitol (20 mM), 100 μl acetyl phosphate Li-salt (40 mM) and 5 μl of phosphate acetyltransferase (2 U/ml in the mixture). After reaction at 30° for 15 min, the enzyme was inactivated by immersing the cup in boiling water for 5 min. Then EDTA (1 mM) was added to complex the copper ions followed by addition of citrate synthase (3 U/ml), [4-^{14}C]-oxaloacetate (0.4 μM) and NEM (4 mM) to prevent possible cycling [35]. After 15 min reaction at 30°, the content of the cup was quantitatively transferred to a plastic scintillation vial and oxaloacetate was decarboxylated by addition of $CuSO_4$ (5 mM) and potassium acetate (0.2 M) and boiling for 30 min. Radioactivity was measured in Bray's solution (10 ml). Under these conditions, controls without mitochondria gave low background radioactivity (due to radiochemical impurities of the oxaloacetate preparation) not exceeding 5 pmol per assay. The recovery of CoA (mitochondria ± known amounts of added CoA) was throughout 35 ± 2% for which the results have been corrected.*

[4-^{14}C]-Oxaloacetate was prepared from [4-^{14}C]-aspartate as described [34] and quantified by the malate dehydrogenase reaction.

HPLC was performed on Novapak C_{18} (Waters) by gradient elution with methanol: water in the presence of 1% of PIC® A-reagent (Waters). Thioethers were eluted between 34 and 46%, methanol (v/v). Solvent gradients were achieved by a Gynkotek gradient former M 250 B, Gynkotek (Germering, F.R.G.) peaks were detected at 325 and 260 nm, respectively. Acid hydrolysis of the thioethers was carried out in 6 N hydrochloric acid at 110° for 24 hr under nitrogen with norvaline as internal standard. Amino acid analysis was performed with a Multichrom M amino acid analyser (Beckman, München), using the lithium picobuffer system of Pierce, 3260 BA Oud-Beijerland (Netherlands).

* The consistently low recovery of CoA in mitochondrial suspensions deserves comment. Recovery of authentic CoA in plain water was > 95%. In the incubation medium without mitochondria, however, recovery fell to 41 ± 2%. Part of this decrease is due to phosphate in the medium which under the processing conditions outlined above reduces the recovery by about 20% (phosphate acetyltransferase equilibrium). An additional reduction of recovery is due to the high salt content introduced by the sample (various pH adjustments). We are unable at present to reasonably explain this phenomenon. Due to the good reproducibility in recovery of known amounts of CoA added to mitochondrial suspensions, we feel the method to be reliable enough under standardized conditions. Our control values of the mitochondrial CoA content (0.85 nmol/mg) are consistent with literature data (1.2 nmol/mg [36]; 0.5 nmol/mg [18]; 1.3 nmol/mg [37]).

RESULTS

1. *Formation of tris-(CoA-S-yl)-DMAP*

Purified human oxyhemoglobin (1.2 mM Fe) was incubated with [^{3}H]-CoA (2 mM) and [^{14}C]-DMAP (0.3 mM) at pH 7.4, 37° for 30 min. After precipitation of the protein by 0.6 M TCA, the supernatant containing 88% of [^{14}C] and 72% of [^{3}H] was applied to HPLC. Besides [^{3}H]-CoA and the radioactive disulfide, a major peak was eluted containing both isotopes at a ratio corresponding to 3.04 CoA per DMAP. Only traces of other material with both labels were detected. The UV spectrum of the major product showed maximal extinction at 259 nm ($\varepsilon_{mM} = 51.1$) and an α-band at 324 nm ($\varepsilon_{mM} = 5.6$) at pH 6.5. For preparative scale, 10 ml of a similarly prepared supernatant using radioactive DMAP and inactive CoA was chromatographed on Sephadex G 25 fine (2.5 cm i.d. × 80 cm) with 10 mM acetic acid. The radioactive high-molecular fraction was identical with the above-mentioned product (HPLC, UV). After Raney–Nickel desulfuration [19], DMAP (HPLC, TLC, UV) was the only ether-extractable product. After acid hydrolysis the amino acid analysis yielded 3.07 mol β-alanine per mol of DMAP. These data indicated that the adduct was a thioether, tris-(CoA-S-yl)-DMAP.

2. *Formation of mixed thioethers of CoA and GSH with DMAP*

Purified human oxyhemoglobin (1.2 mM Fe) was incubated with [^{14}C]-DMAP (0.3 mM), [^{3}H]-CoA (1 mM) and inactive GSH (1 mM) or with [^{3}H]-GSH and inactive CoA, 1 mM each, at pH 7.4, 37° for 30 min. After precipitation of hemoglobin by 0.6 M TCA, the supernatant contained about 87% of [^{14}C] and about 75% of [^{3}H] in both experiments. HPLC analyses of the supernatants revealed four major adducts containing the [^{14}C]- and [^{3}H]-labels (Fig. 1).

The first eluting adduct I contained [^{3}H] only from [^{3}H]-GSH at a ratio of 3 GSH per DMAP and was identical with authentic tris-(glutathione-S-yl)-DMAP [19] as revealed by HPLC (sample spiking) and UV spectroscopy. Adduct II contained tritium both from [^{3}H]-GSH and [^{3}H]-CoA at a ratio of 2 GSH and 1 CoA per DMAP. Adduct III exhibiting also both tritium labels showed a ratio of 1 GSH and 2 CoA per DMAP. Both compounds were considered to be mixed thioethers. Adduct IV contained the [^{3}H]-label only from [^{3}H]-CoA and was identical with tris-(CoA-S-yl)-DMAP (HPLC, UV; see above).

For further analyses, 10 ml of a similarly prepared supernatant using radioactive DMAP and inactive thiols was desalted on Sephadex G 25 as described above. The high-molecular radioactive material was lyophilized, and the adducts II and III separated by several HPLC runs. Raney–Nickel desulfuration of

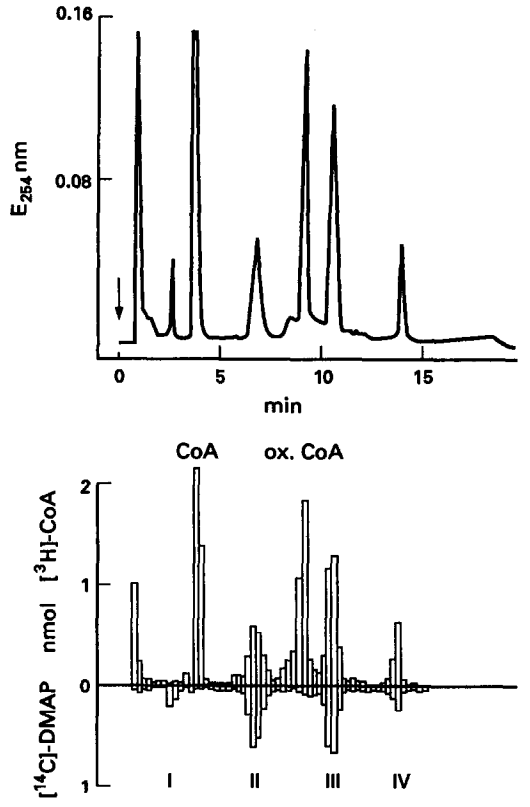

Fig. 1. Formation of thioethers of CoA and GSH with DMAP. Oxyhemoglobin (1.2 mM Fe) was incubated with [^{14}C]-DMAP (0.3 mM), GSH (1 mM) and [^{3}H]-CoA (1 mM) in 0.2 M phosphate buffer, pH 7.4, at 37° for 30 min. The deproteinized supernatant was analysed by HPLC with gradient elution. The upper panel shows the elution profile as monitored at 254 nm. The lower panel indicates the distribution of both isotope labels corresponding to [^{3}H]-CoA and [^{14}C]-DMAP amounts in 0.25 min fractions. Besides CoA and its disulfide (ox. CoA) four major adducts (I–IV) were observed.

both adducts liberated DMAP as the only ether-extractable compound. After acid hydrolysis and amino acid analysis, adduct II was confirmed to be composed of 2 mol glutathione and 1 mol CoA, whereas adduct III consisted of 2 mol CoA and 1 mol GSH per DMAP. Table 1 summarizes the amino acid composition and the spectroscopical data of all four adducts of DMAP containing three thiol residues via a thioether linkage. No further efforts have been undertaken to elucidate the position of the different substituents at the aromatic ring.

The pattern of distribution of the four adducts depended on the thiol ratio. At a ratio of 1:1 (cf. Fig. 1) about 10% each of adduct I and IV were formed whereas adduct II and III amounted to about 40% each. These data indicated a similar reactivity of both thiols in thioether formation. When 0.3 mM DMAP was incubated with 0.3 mM CoA and 1 mM GSH, the distribution of the adducts changed, 47% I, 39% II, 12% III and 2% IV.

3. *Influence of DMAP on the content of GSH and CoA in isolated rat liver mitochondria*

Freshly prepared rat liver mitochondria (5 mg protein/ml) were incubated under state IV conditions (see Methods) in the presence of 20 and 50 μM DMAP, respectively. After precipitation of the proteins, the supernatants were assayed for GSH, GSSG and total CoA (CoA, CoA disulfide and acetyl–CoA). As shown in Fig. 2 incubation of the mitochondria with DMAP resulted in an increase of GSSG and a decrease in GSH + GSSG. Figure 3 illustrates the influence of DMAP on the CoA content of the mitochondria as determined in separate experiments.

4. *Formation of CoA-thioethers with DMAP in isolated rat liver mitochondria*

The decrease in both the CoA and GSH content of the mitochondria pointed to thioether formation as observed in the model reactions described above. Freshly prepared rat liver mitochondria (5 mg/ml) were incubated under the above-described conditions with either 20 or 50 μM [^{14}C]-DMAP for 30 min. After precipitation of the proteins with 0.5 M perchloric acid containing 1 mM ascorbic acid, the supernatant was neutralized with solid potassium carbonate and extracted three times with three vol. of ether to remove DMAP and lipophilic metabolites (less than 10%). After lyophilization the hydrophilic material was desalted by chromatography on Sephadex G 25 fine (see above). The radioactive high-molecular fractions containing the CoA-thioethers were combined, lyophilized, spiked with known amounts of authentic, inactive DMAP–CoA-thio-

Table 1. Characterization of the tris-substituted DMAP thioethers

	Millimolar extinction (260 nm, pH 7) found	calc.	Amino acid composition (per DMAP) β-alanine	glycine	glutamate
I tris-(GSH-S-yl)-DMAP	6.7	6.7	0	2.81	2.92
II (CoA-S-yl)-bis-(GSH-S-yl)-DMAP	22.5	21.5	0.94	2.05	2.15
III(GSH-S-yl)-bis-(CoA-S-yl)-DMAP	35.9	36.3	2.02	1.10	1.20
IV tris(CoA-S-yl)DMAP	51.1	51.1	3.07	0	0

The millimolar extinctions of the CoA containing thioethers were calculated as the sum of the absorbance of the CoA moiety (ε_{mM} = 14.8) and DMAP (ε_{mM} = 6.7) at 260 nm and pH 7. Amino acid residues were determined after acid hydrolysis of the isolated thioethers and referred to DMAP.

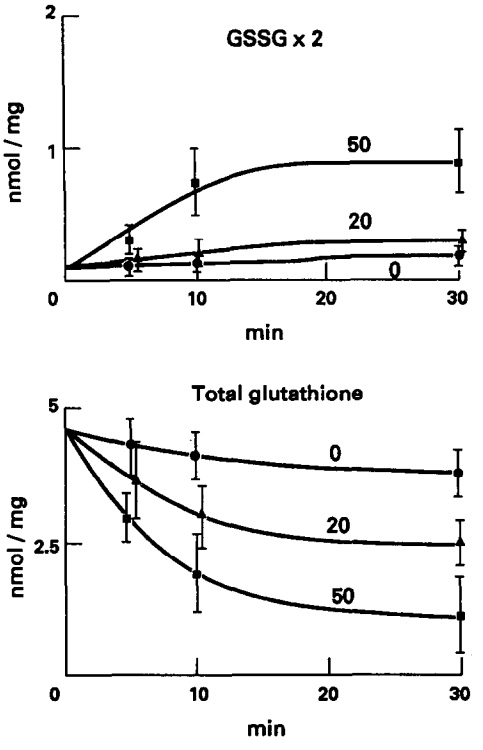

Fig. 2. Influence of DMAP on the glutathione status of isolated rat liver mitochondria. Mitochondria (5 mg/ml) were incubated under state IV conditions with 0, 20 and 50 μM DMAP at 37° for 30 min. The upper panel shows formation of GSSG, the lower panel the decrease in GSH plus GSSG (in glutathione equivalents each); (means ± SE, N = 5).

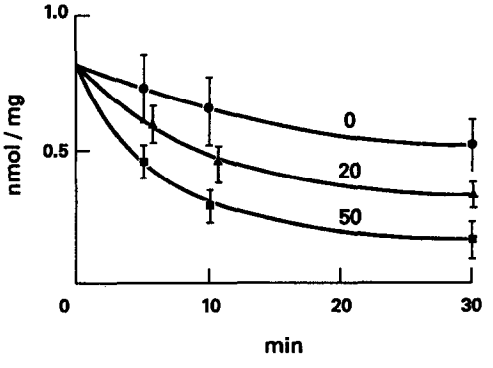

Fig. 3. Influence of DMAP on the content of coenzyme A in isolated rat liver mitochondria. Mitochondria were incubated with DMAP (0, 20 and 50 μM) as described in Fig. 2. The amount of coenzyme A represents the sum of CoA, its acid-soluble disulfide(s) and acetyl–CoA (means ± SE, N = 7).

ethers and separated by HPLC until constant ratios of radioactivity and inactive tracers were established (three consecutive HPLC separations). Table 2 summarizes the results from three mitochondria preparations.

DISCUSSION

When DMAP reacted with GSH in the presence of oxyhemoglobin, transient formation of bis-3,5-(GSH-S-yl)-DMAP has been observed while tris-2,3,5-(GSH-S-yl)-DMAP remained as the stable end-product [23]. Besides, part of GSH was oxidized to GSSG, presumably due to phenoxyl radical formation [24]. Similar reactions had been expected when GSH was replaced by CoA. In fact, at CoA concentrations exceeding DMAP about three-fold tris-(CoA-S-yl)-DMAP was the main product. Since part of the [^{3}H]-label of CoA was covalently bound to hemoglobin, formation of mixed disulfides and/or mixed thioethers with cysteine residues of hemoglobin may have additionally occurred. The position of the three CoA residues at the aromatic ring of DMAP has not been established, but we assume a similar substitution as observed with GSH, namely at position 2, 3 and 5 [22].

When GSH was present together with CoA, mixed thioethers were formed. Their composition was analysed by isotope ratios, molar extinctions and amino acid composition. Assuming substitution at position 2, 3 and 5, three isomers have to be expected for CoA-S-yl)-bis-(GSH-S-yl)-DMAP and for (GSH-S-yl)-bis-(CoA-S-yl)-DMAP. We were not able to detect such isomers unequivocally, although we observed sometimes "shoulders" of the peaks in HPLC chromatograms (cf. adduct II in Fig. 1). Efforts to improve resolution of those peaks mostly led to a marked tailing of the later eluting compounds. Nevertheless, analysis of such "twins" gave identical UV spectra and identical isotope ratios. Hence, we feel justified to assume isomers in those peaks.

Interestingly, CoA and GSH appeared to be equieffective as nucleophiles under our conditions. At equimolar concentrations the distribution of the four adducts was at random (1/8 I, 3/8 II, 3/8 III, 1/8 IV; cf. Fig. 1). At three-fold excess of GSH over CoA the pattern was about 47% (46%) I, 39% (41%) II, 12% (12%) III, and 2% (1%) IV, as calculated (values in brackets) for at random distribution. Since the pK_{sh} values of GSH (8.61) and CoA (9.6) differ markedly, such a result is surprising, if the thiolate is the reactive nucleophile [38].

When isolated rat liver mitochondria were incubated with subtoxic (20 μM) and toxic (50 μM) concentrations of DMAP, marked effects on the mitochondrial contents of GSH and CoA were observed. At 50 μM DMAP, the GSH content fell from 4.6 to 0.23 nmol/mg protein within 30 min. Part of the missing glutathione was found in GSSG (1.15 nmol/mg), the other part (2.7 nmol/mg as compared with controls) may have formed thioethers with DMAP. In fact, 2.9 nmol/mg of acid soluble products of DMAP consisted of the high-molecular fraction (MW > 1 kD). Similar to GSH, the content of total CoA (reduced, disulfides and acetyl–CoA) fell markedly upon incubation with DMAP. The remaining CoA, conceivably composed of (mixed) disulfides (e.g. with glutathione), is expected to be useless for metabolic functions, i.e. to sustain the citrate cycle. Under such conditions, $^{14}CO_2$ formation from 2-[^{14}C]-pyruvate completely ceased

Table 2. Formation of mixed thioethers of CoA and GSH with DMAP in rat liver mitochondria

	Products (nmol/mg protein)	
DMAP applied	4	10
Radioactive products in extracted acid supernatant	2.4 ± 0.2	5.0 ± 0.3
High molecular fraction	1.2 ± 0.2	2.9 ± 0.4
(CoA-S-yl)-bis-(GSH-S-yl)-DMAP	0.004 ± 0.001	0.04 ± 0.01
(GSH-S-yl)-bis-(CoA-S-yl)-DMAP	0.017 ± 0.003	0.04 ± 0.02
Tris-(CoA-S-yl)-DMAP	0.004 ± 0.001	0.008 ± 0.002
Sum of CoA-thioethers (CoA equivalents)	0.05	0.15
Loss of CoA (c.f. Fig. 3)	0.18	0.35

Rat liver mitochondria (5 mg/ml) were incubated in the presence of 20 and 50 μM [^{14}C]-DMAP for 30 min. The acid-soluble supernatant was analysed for thioethers by isotope dilution techniques as described in Results.

(Elbers, to be published). About half the missing CoA was found in thioethers of DMAP. Since we were scrupulous in obtaining rather narrow cuts of the radioactive peaks during isotope dilution analysis to avoid false-positive results, we may have lost some isomers, particularly (CoA-S-yl)-bis-(GSH-S-yl)-DMAP (see above). Hence, the amounts of CoA thioethers may have been underestimated.

In conclusion, the results show that cytotoxic effects of DMAP are due to a loss of vital intracellular thiols by thioether formation. Although GSH is at 1000-fold excess over CoA in the cytosol, the mitochondrial ratio is only 5–10 [18]. When GSH of this pool is being depleted a parallel depletion of CoA occurs. It appears reasonable to assume that such electrophiles may affect also other vital low-molecular thiols like dihydroliponamide resulting in impaired mitochondrial function. These events may be of major contribution to the various cytotoxic actions observed with DMAP and other *p*-aminophenols which produce mitochondrial lesions as observed by electron microscopy [1].

Usually, electrophiles will be inactivated by nucleophiles to give stable adducts as shown for CoA-adducts with haloethylenes [39]. Poorly substituted, autoxidizable aromatics, like aminophenols, however, may deplete intracellular thiols more effectively. As recently shown for 4-aminophenol, subsequent oxidation–addition reactions may result in formation of up to four-fold substituted compounds [40]. Of course, such compounds can be composed of a variety of thiols. As to our knowledge formation of mixed thioethers in biological systems, as shown in this study, has not been reported hitherto.

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