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ALTERATIONS IN CELLULAR INTERMEDIARY METABOLISM BY 4-DIMETHYLAMINOPHENOL IN THE ISOLATED PERFUSED RAT LIVER AND THE IMPLICATIONS FOR 4-DIMETHYLAMINOPHENOL TOXICITY

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Abstract—To evaluate the influence of 4-dimethylaminophenol (DMAP)‡ on cellular intermediary metabolism, isolated rat livers were single pass perfused with subtoxic (0.3 mM) and toxic (1 mM) concentrations of DMAP. The rate of glycolysis and oxygen consumption both increased with biphasical kinetics immediately after the onset of DMAP infusion. After a transient reduction, the cytosolic NAD system was oxidized by DMAP; the mitochondrial NAD system, except for a brief initial oxidation, remained almost unaffected. DMAP caused an intracellular alkalinization. At 0.3 mM, this alkalinization was confined to the cytosol (+0.6 pH units); at 1 mM the mitochondria were alkalized in addition (+0.8 pH units). In freeze-clamped and lyophilized tissue, following 0.3 mM DMAP, the ATP/ADP ratio was lowered by two-thirds and 2-oxoglutarate decreased by one-half; citrate, malate and the sum of adenine nucleotides were unchanged. After 1 mM DMAP, all metabolites were decreased, the ATP/ADP ratio was lowered by three-quarters. Subcellular fractionation revealed inhibition of the citric acid cycle by DMAP, resulting in lowered mitochondrial/cytosolic gradients for Krebs cycle intermediates. The cellular content of CoA was unchanged at 0.3 mM but diminished by 65 per cent at 1 mM, in accordance with the unchanged rate of ketogenesis at 0.3 mM and inhibition at 1 mM. We conclude that mitochondrial CoA depletion with subsequent inhibition of the citric acid cycle and of oxidative energy metabolism suffices to explain DMAP toxicity.

4-Dimethylaminophenol (DMAP) has been used successfully in the treatment of cyanide poisoning [1] by exploiting its ability to form more than a stoichiometric amount of ferrihaemoglobin. In this reaction, DMAP is oxidized by oxyhaemoglobin to the phenoxyl radical or to *N*,*N*-dimethylquinonimine [2, 3]. Oxidized DMAP reacts with ferrohaemoglobin yielding ferrihaemoglobin and DMAP. Since oxidized DMAP is also reduced by NADH, electrons are transferred from NADH to molecular oxygen, bypassing the respiratory chain [4, 5]. In addition, oxidized DMAP binds rapidly to thiol groups from glutathione or cellular proteins [6].

In kidney tubules, the capability of cellular haemproteins other than haemoglobin to catalyse DMAP oxidation has been demonstrated by an inhibition of this reaction in the presence of CO or CN^- [7]. Like other aminophenols, injected DMAP produced kidney lesions in rats, while no hepatotoxicity has been observed [8]. These findings are in accordance with results obtained in single pass perfused rat kidney [9] and rat liver [10]. Nevertheless, at higher concentrations, rat liver showed the same signs of intoxication as rat kidneys. The nearly two orders of magnitude higher DMAP tolerance of the liver as compared to the kidney has been attributed to correspondingly high conjugation rates with glucuronide and sulfate [10]. This faster conjugation presumedly exceeds DMAP oxidation and prevents its binding to thiol groups [11].

In spite of the wealth of information on DMAP biotransformation, only little is known about the effects of DMAP on cellular intermediary metabolism and whether such effects play any part in DMAP toxicity. This communication is concerned with alterations of endogenous metabolic rates, subcellular redox ratios and pH values, as well as mitochondrial and cytosolic concentrations of citric acid cycle intermediates at subtoxic and toxic DMAP concentrations. The results will be discussed for their consequences in the interpretation of DMAP toxicity.

‡ Non-standard abbreviations used: CoA, coenzyme A and derivatives in general; Acetyl-CoA, thiol esters of coenzyme A; CoA-SH, thiol form of coenzyme A; CoA-SS-CoA, coenzyme A disulfide; DMAP, 4-dimethylaminophenol; DMO, 5,5'-dimethyloxazolidine-2,4-dione; L, lactate; P, pyruvate; B, 3-hydroxybutyrate; A, acetoacetate; m/c, concentration gradient between mitochondria and cytosol; AN, sum of ATP, ADP and AMP.

MATERIALS AND METHODS

Perfusion. Livers from male Wistar rats of 150–200 g body wt, fed *ad lib.* on Altromin rat chow were perfused for 30 min with Krebs–Henseleit bicarbonate buffer, equilibrated at 37° with moistened oxygen and carbon dioxide (95%: 5%, v/v), at a flow rate of 5 ml/min/g in a single pass system [12], followed

Table 1. Contents of cellular metabolites in freeze-dried liver tissue (μmoles/g dry wt)

DMAP (mM)	–.–	0.3		1.0	
Experiment	controls	1	2	1	2
ATP	11.7 ± 0.7	7.6	7.7	4.0	3.4
ADP	2.9 ± 0.1	5.2	5.2	3.5	3.9
AMP	1.0 ± 0.1	2.7	2.9	2.1	2.3
AN	15.1 ± 1.4	15.5	15.8	9.6	9.6
ATP/ADP	4.0 ± 0.1	1.5	1.5	1.1	0.90
Citrate	0.67 ± 0.07	0.74	0.86	0.20	0.28
Malate	0.74 ± 0.06	1.2	1.3	0.31	0.43
2-Oxoglutarate	0.87 ± 0.08	0.35	0.40	0.37	0.48
Acetyl-CoA	1 2	0.23	0.21	0.056	0.087
	0.20 0.19				
+ CoA-SH					
+ CoA-SS-CoA					

by a 15 min period of perfusion with 0.3 or 1 mM DMAP in the perfusate. Thereafter, the livers were freeze-clamped and freeze-dried. For the determination of the intracellular distribution of malate, citrate and 2-oxoglutarate, the method of subcellular fractionation of freeze-dried tissue in non-aqueous solvents [13, 14] was applied.

Assays. Samples of effusate were collected at intervals of 1 min and were analysed for lactate, pyruvate, 3-hydroxybutyrate and acetoacetate by standard enzymatic procedures [15]. In the fractionated liver [14], marker enzymes for the mitochondrial matrix (citrate synthase EC 4.1.3.7), the cytosolic compartment (3-phosphoglycerate kinase, EC 2.7.2.3) and protein were determined [15–17]. ATP, ADP, AMP, CoA, 2-oxoglutarate, citrate and malate were measured after extraction with 0.6 M perchloric acid by enzymatic analysis [15, 18–20].

Calculations. Mitochondiral and cytosolic contents of 2-oxoglutarate, citrate and malate obtained from subcellular fractionation [14] were converted into concentrations assuming a free water content of 0.8 and 3.8 μl H_2O/mg mitochondrial or cytosolic protein, respectively [21].

All experiments were carried out as duplicates and individual results are listed in Tables 1–3; for the controls the means of 4–6 experiments ± S.E.M. are presented. For additional information about the controls the reader is referred to ref. 22.

Determination of the subcellular pH values. The subcellular pH values were determined by measuring the distribution of 2-^{14}C-5,5'-dimethyloxazolidine-2,4-dione (^{14}C-DMO) ([23] and ref. therein). DMO infusion at rates of 5 μCi/min, together with [^{3}H]-inulin at rates of 20 μCi/min, was started 5 min prior to the freeze stop. In the fractionated liver tissue, subcellular DMO concentrations were determined by liquid scintillation spectroscopy. These concentrations have been corrected for extracellular space by the simultaneous determination of the [^{3}H]-inulin radioactivity. Concentration gradients of DMO between cytosol and perfusate or mitochondria and cytosol together with the pH value of 7.4 in the effusate served for the calculation of mitochondrial and cytosolic pH values by using the equation:

$$\log \frac{DMO^- \text{ in}}{DMO^- \text{ out}} = pH_{in} - pH_{out} = \Delta\, pH.$$

Chemicals and biochemicals. All chemicals (analytical grade) were purchased from Merck (Darms-

Table 2. Concentration (mM) of di- and tricarboxylic acids (malate, citrate, 2-oxoglutarate) in mitochondria and cytosol after freeze stop and subcellular fractionation [13] of the perfused liver

DMAP (mM)	–.–	0.3		1.0	
Experiment	controls	1	2	1	2
Mitochondria					
Malate	1.1 ± 0.28	1.39	1.88	0.09	0.07
Citrate	1.6 ± 0.21	0.60	0.79	0.16	0.31
2-Oxoglutarate	1.4 ± 0.28	0.15	0.20	0.34	0.48
Cytosol					
Malate	0.29 ± 0.02	0.37	0.37	0.14	0.22
Citrate	0.20 ± 0.04	0.30	0.31	0.08	0.10
2-Oxoglutarate	0.35 ± 0.05	0.15	0.17	0.14	0.18
Concentration ratios (m/c)					
Malate	3.7	3.8	5.0	0.6	0.3
Citrate	7.8	2.0	2.5	2.0	3.1
2-Oxoglutarate	3.9	1.0	1.2	2.4	2.7

Table 3. Subcellular pH values calculated from the distribution of ^{14}C-DMO in cytosol and mitochondria after freeze stop and subcellular fractionation [13] of the perfused liver

DMAP (mM)	–.–	0.3		1.0	
Experiment	controls	1	2	1	2
pH perfusate	7.4	7.4	7.4	7.4	7.4
pH cytosol	7.0	7.7	7.6	7.9	7.9
pH mitochondria	7.3	7.3	7.3	8.2	8.1
Δ pH cyt.–perf.	−0.41 ±0.004	0.30	0.20	0.51	0.50
Δ pH mit.–cyt.	0.30 ±0.001	−0.40	−0.30	0.30	0.20

tadt), biochemicals and enzymes from Boehringer (Mannheim) or Sigma (München). ^{14}C-DMO, 11 mCi/mole, and ^{3}H-inulin, 84 mCi/g were obtained from New England Nuclear (Boston, MA). 4-dimethylaminophenol hydrochloride was prepared by Farbwerke Hoechst (Hoechst).

Terminology. Without further specification, the words "metabolism", "metabolic (rates)" or "metabolite(s)" are used in their biochemical sense and refer to endogenous cellular metabolism or to its metabolites, but not to the biotransformation of DMAP.

RESULTS

Effects of DMAP on metabolic rates and redox ratios. Single pass perfusion with 0.3 mM prehepatic DMAP (Fig. 1) stimulated both oxygen consumption and the rate of glycolysis (sum of lactate and pyruvate). With the onset of DMAP infusion, both rates increased rapidly within 30 sec followed by a slower increase thereafter. The lactate/pyruvate ratio, reflecting the cytosolic NADH/NAD system, increased briefly, fell during the next 2 min to half the starting value and then slowly returned to the

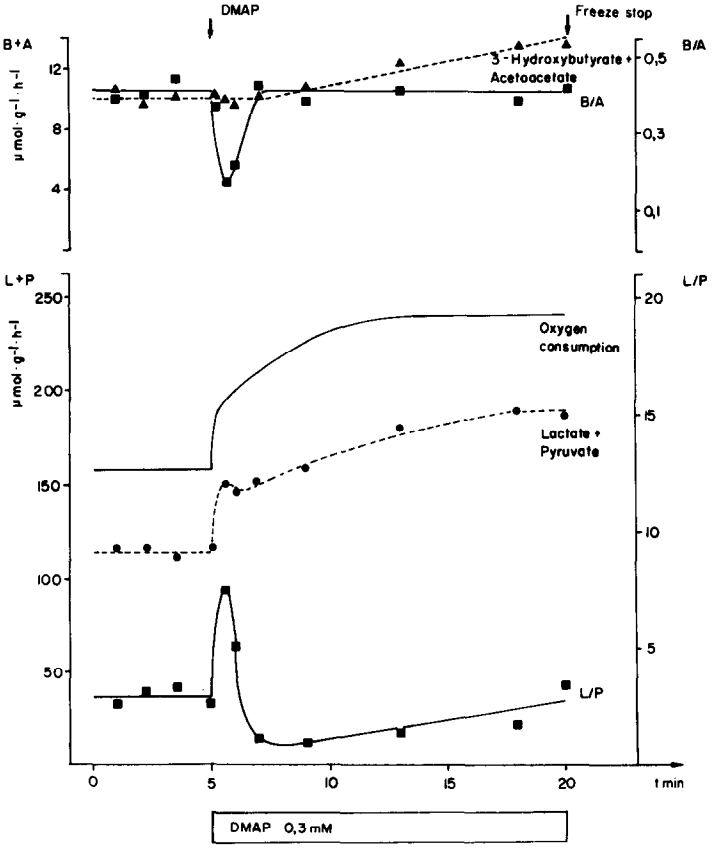

Fig. 1. Time course of metabolic rates (oxygen consumption, L + P, B + A, scale on left side of figure) and redox ratios (L/P, B/A, scale on right side of figure) during single pass perfusion with 0.3 mM DMAP (single experiment shown).

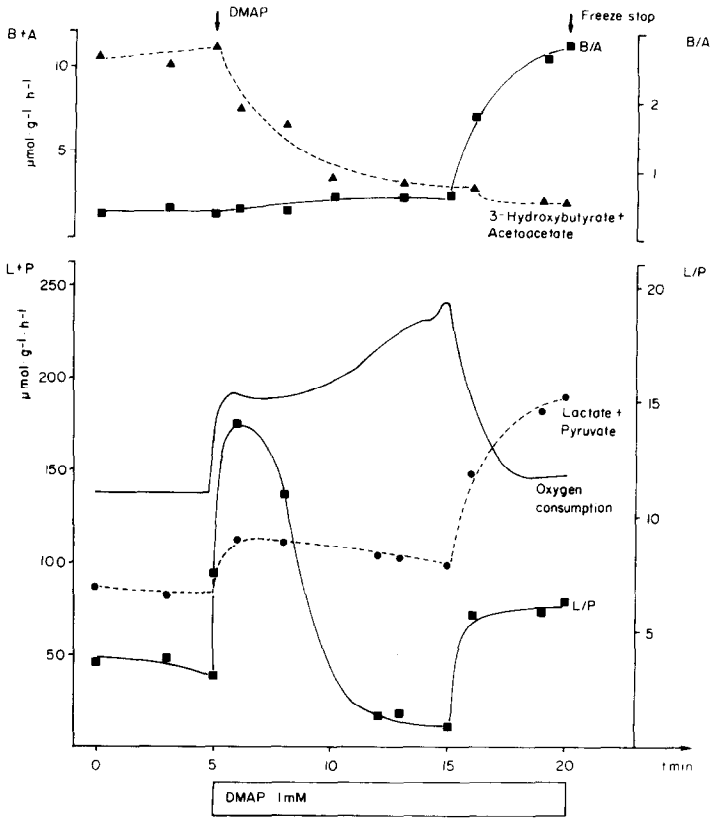

Fig. 2. Time course of metabolic rates (oxygen consumption, L + P, B + A, scale on left side of figure) and redox ratios (L/P, B/A, scale on right side of figure) during single pass perfusion with 1 mM DMAP (single experiment shown).

initial condition. The only effect of DMAP on the mitochondrial NADH/NAD redox potential, indicated by the 3-hydroxybutyrate/acetoacetate ratio, was a brief, transitory decrease of this ratio. Ketogenesis (sum of 3-hydroxybutyrate and acetoacetate) was unaffected by this concentration of DMAP.

The metabolic effects of DMAP were more pronounced at 1 mM (Fig. 2). Oxygen consumption showed a fast initial increase, reaching a plateau after 30 sec with subsequent slower increase until a maximum was reached after 10 min. At this time the oxygen consumption fell rather abruptly. The rate of glycolysis increased twice, first at the onset of DMAP infusion by 35 per cent and again 10 min thereafter by about 100 per cent of the initial value. Changes in the L/P ratio were similar to those found with 0.3 mM DMAP up to 10 min after the start of DMAP infusion, then there was a 6-fold increase in this ratio within 60 sec. The B/A ratio remained initially constant for 10 min, then it increased from 0.7 to 3 at the end of perfusion (note the different scaling for B/A in Fig. 1 and Fig. 2). With the addition of DMAP, there was a steady decrease in the rate of ketogenesis until this rate was inhibited by 80 per cent after 10 min.

Overall cellular content of metabolites. In Table 1 the contents of metabolites in lyophilized rat livers, freeze-clamped after control perfusion or perfusion with 0.3 or 1 mM DMAP, are shown. The contents of adenine nucleotides and of coenzyme A were the same in controls and in livers perfused with 0.3 mM DMAP. The ATP/ADP ratio, however, was lowered from 4 to 1.5. Citrate and malate contents were enhanced, while the 2-oxoglutarate content was half the amount measured in controls. After perfusion with 1 mM DMAP, the contents of all the metabolites were lowered. Only 65 per cent of the adenine nucleotides and only 35 per cent of coenzyme A was found relative to the controls. The ATP/ADP ratio was 1; citrate, malate and 2-oxoglutarate contents were about half the controls.

Effects of DMAP on subcellular distribution of citric acid cycle intermediates. Table 2 shows the mitochondrial and cytosolic concentrations of citrate, malate and 2-oxoglutarate. In livers perfused with 0.3 mM DMAP, the concentration of malate was slightly higher in both compartments compared to the controls. The subcellular concentration ratios for citrate and 2-oxoglutarate were decreased due to lower intramitochondrial concentrations. The cytosolic concentration of citrate was slightly higher and the cytosolic concentration of 2-oxoglutarate slightly lower than the corresponding control values. After perfusion with 1 mM DMAP, the concentrations of all three metabolites were lowered drastically in the mitochondria and somewhat less pronounced in the cytosol. In the case of malate, this effect resulted in an inversion of the concentration ratio.

Effect of DMAP on subcellular pH values. In Table 3 the mitochondrial and cytosolic pH values and the corresponding pH gradients are listed. DMAP (0.3 mM) caused an alkalinization of the cytosol by 0.6 pH units without affecting the intramitochondrial pH, thus reversing the mitochondrial and cellular transmembrane pH gradients. After perfusion with 1 mM DMAP, the alkalinization was observed in both compartments with mitochondria 0.8 and cytosol 0.9 pH units more alkaline than the controls. The pH gradient between mitochondria and cytosol was therefore similar to the controls, while the gradient across the cell membrane was inverted.

DISCUSSION

It has been suggested [7] that DMAP toxicity is caused by the inhibition of various enzymes due to the formation of DMAP-thioethers with thiol groups once the cellular glutathione pool has been depleted by DMAP conjugation. Nevertheless, the possibility was not ruled out that deleterious effects of DMAP are caused by more specific disturbances in endogenous metabolism. In the following we shall discuss the changes in metabolic rates and concentrations of endogenous metabolites due to DMAP, which will foster some insight into the regulation of the cellular intermediary metabolism and into the mechanism of DMAP toxicity.

Changes in intracellular pH. The cellular alkalinization induced by DMAP (Table 3) can be explained by the assumption that only DMAP but not DMAP-H^+ is able to diffuse into the hepatocytes. DMAP is a weak base with a pK of 6.2 at the dimethylamino group (the pK of the phenolic group is above 10 and can be neglected at physiological pH). Thus, DMAP binds protons which have to be supplied by the intracellular buffer. Since this buffer has a limited capacity, this reaction will lead to an intracellular alkalinization. Metabolic consequences due to this alkalinization will be discussed below.

At 0.3 mM DMAP only cytosolic alkalinization has been observed; at 1 mM DMAP the mitochondrial compartment was alkalized in addition. This observation in connection with the differences in ketogenesis (see below) leads to the conjecture that the inner mitochondrial membrane is less permeable for DMAP relative to the cell membrane, necessitating higher concentrations of DMAP for observable intramitochondrial metabolic effects.

Changes in metabolic rates. The rapid onset of the biphasically enhanced O_2 consumption is explained by the chemiosmotic hypothesis [24]. According to this hypothesis, respiration is coupled to ATP synthesis by a protonmotive force acting across the inner mitochondrial membrane. The observed cytosolic alkalinization by DMAP reverses the Δ pH term of the protonmotive force. This means at least partial uncoupling of oxidative phosphorylation with subsequent stimulation of oxygen consumption and inhibition of mitochondrial ATP synthesis, until coupling is restored by an increase of the $\Delta\psi$ term due to further transport of protons by the respiration.

The second phase in the increase in oxygen consumption seems to be due to a reaction in which DMAP catalyses the transport of electrons from

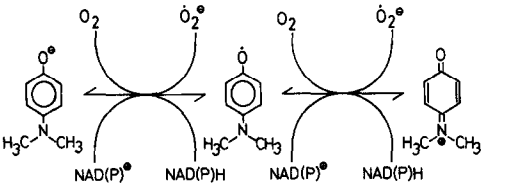

Fig. 3. Oxidation of DMAP by oxygen and reduction of the phenoxylradical and the quinonimine by NAD(P)H according to Eyer *et al.* [2–4].

NADH to oxygen as shown in Fig. 3. Additional experiments (not shown in Results) where the respiratory chain was inhibited by 0.23 mg Antimycin A/g liver, 10 min prior to the perfusion with 0.3 mM DMAP, support this notion. In these experiments 95 μmoles extra oxygen were consumed per gram liver per hour at steady state *ca.* 10 min after DMAP addition; half of this rate was reached within 90 sec in a monophasic time course similar to the 'second phase' mentioned above. A simultaneous drop in the lactate/pyruvate ratio from 17 to 5.5 revealed NADH oxidation. For the interpretation of the time course of oxygen consumption at 1 mM DMAP (Fig. 2), a superposition of increasing DMAP autoxidation with progressive inhibition of the mitochondrial respiration has to be considered.

The rate of glycolysis, represented in our experiments by the sum of lactate and pyruvate, is regulated (among other factors) by the cytosolic pH [25], by the cytosolic redox potential of NAD [26], and by the cytosolic phosphorylation potential [27]. The increase in the rate of glycolysis immediately with DMAP addition seems to be caused by changes at all of these regulation sites. Alkalinization brings the phosphofructokinase reaction closer to its pH optimum at pH 8.2 and diminishes allosteric inhibition by ATP and other endogenous metabolites (i.e. citrate [28]), thus increasing the turnover of this regulatory enzyme. Since the redox potential of the NAD system is defined by $10^{-pH} \times [NADH]/[NAD^+]$, alkalinization will lower this potential. In addition, it will be lowered further in the presence of oxidized DMAP (Fig. 3), thus facilitating glycolysis. The transitory uncoupling of oxidative phosphorylation mentioned above interrupts the cytosolic supply with mitochondrially synthesized ATP and thereby stimulates glycolysis. The redox state of the cytosolic and mitochondrial NAD system, represented in our experiments by the lactate/pyruvate (L/P) and the 3-hydroxybutyrate/acetoacetate (B/A) ratios, reflect this uncoupling effect, clearly recognizable, especially in Fig. 1. In Fig. 2, this effect is obscured by the coarser scale used for B/A. Similar to livers perfused with classical uncouplers [27], infusion of DMAP reduced the cytosolic NAD system while it was oxidized simultaneously in the mitochondrial compartment, suggesting that rates of cytosolic NADH production, via glycolysis, and mitochondrial consumption, via respiration at the increased rate during uncoupling, exceed the rate of hydrogen transport into the mitochondria [29].

DMAP toxicity. In perfusion experiments with 0.3 mM DMAP, the metabolic rates approached a

new steady state within 10 min, in contrast to 1 mM DMAP where the metabolic state of the liver changed abruptly at about this time (Fig. 2), depicting an inhibition of oxidative energy metabolism. This toxic effect has been observed reproducibly at different metabolic conditions in liver (unpublished results) and in rat kidney [9].

Analysis of the freeze-stopped liver tissue revealed an unchanged sum of adenine nucleotides (which should be invariant to metabolic conditions) at 0.3 mM, but a depletion by one-third at 1 mM DMAP. This indicates partial loss of these compounds due to a damage of the liver cell membrane at high DMAP concentrations. The overall ATP/ADP ratio, which mainly reflects the cytosolic phosphorylation potential [22], was lowered at both concentrations of DMAP, pointing to a partial inhibition of mitochondrial ATP synthesis at 0.3 mM DMAP which cannot be derived from the metabolic rates.

It is known that di- and tricarboxylates are not equally distributed within the liver cell [21]. The differentiation of mitochondrial and cytosolic metabolite concentrations has revealed some inhibition of the citric acid cycle even at low concentrations of DMAP not observable by other means. At 0.3 DMAP, the mitochondrial malate content was slightly enhanced compared to the control, whereas citrate was lowered less than 2-oxoglutarate. This pattern of concentrations points to some inhibition of the citric acid cycle at the side of the citrate synthesis without decreased activity of the malate–asparate shuttle system. The method for the determination of subcellular metabolite distribution requires the measurement of the citrate synthase activity [13]. No differences in the specific activity of citrate synthase compared to controls have been observed at 0.3 mM DMAP, therefore citrate cycle inhibition has to be due to some indirect factors.

Whereas 0.3 mM DMAP did not affect ketogenesis, 1 mM DMAP reduced B+A immediately. Since formation of ketone bodies depends on the availability of mitochondrial CoA, depletion in the intramitochondrial CoA pool may be expected. In fact, cellular CoA (90 per cent intramitochondrial [21]) was identical with the controls at 0.3 mM DMAP but diminished by 65 per cent at 1 mM DMAP. Since the method used for the determination of CoA does not differentiate between CoA-SH and CoA-SS-CoA, the amount of CoA available for metabolism may be even lower. Because of *N,N*-dimethylquinonimine quickly forms thioethers with GSH [6], thioether formation with CoA-SH has also to be assumed.

In conclusion, in single pass perfused rat liver the effects of 0.3 mM DMAP seem to be confined to the cytosolic compartment. In spite of the futile oxidation of cytosolic NADH, the metabolic disturbance induced by DAMP at this concentration is compensated by the cell and DMAP exhibits no acute toxicity. If higher concentrations of DMAP are infused into the liver, however, the capacity of the cytosolic metabolism to lower the cytoplasmic concentration of either free DMAP (i.e. sulfate and glucuronide conjugation [10]) or oxidized DMAP (i.e. reduction by NADH [4, 5]), and thus to prevent the penetration of DMAP (in metabolic relevant amounts) into mitochondria, is exhausted.

Oxidation of intramitochondrial DMAP to *N,N*-dimethylquinonimine and subsequent conjugation with CoA-SH will deplete the mitochondrial CoA pool, resulting in a complete inhibition of all energy yielding processes within the mitochondria. For any aerobic cell, this is a deleterious metabolic condition, leading to cellular necrosis. Therefore, the ultimate cause of DMAP toxicity is most likely a depletion of the mitochondrial key metabolite, coenzyme A.

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