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COVALENT INTERACTION OF REACTIVE METABOLITES WITH CYTOSOLIC COENZYME A AS MECHANISM OF HALOETHYLENE-INDUCED ACETONEMIA

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Abstract—Previous experiments have shown that a number of xenobiotics such as halogenated ethylenes cause an experimental acetonemia. In addition, under exposure of rats to vinylidene fluoride (one of the agents producing this effect), the urinary excretion rates of acetoacetate and 3-hydroxybutyrate are enhanced. The enhanced formation of ketone bodies is theoretically explained by a covalent interaction of reactive metabolites of the applied xenobiotic with hepatic cytosolic coenzyme A. This theory is further corroborated by the following experiments: (a) Microsomal incubations of [^{14}C]vinyl chloride and [^{3}H]coenzyme A lead to one metabolite containing 2 moles vinyl chloride/mole coenzyme A and two other with equimolar ratios of both components. (b) Exposure of rats to vinyl chloride leads to a progressive depletion of hepatic cytosolic CoASH, but not of CoASH in mitochondria. In the cytosol acetyl-CoA is also diminished after vinyl chloride exposure. These changes may cause secondary effects in lipid metabolism which are regarded as responsible for the enhancement of ketone bodies.

Based on studies with different halogenated hydrocarbons [1, 2], the theory has developed that biologically reactive metabolites, under conditions *in vivo*, may cause acetonemia [2, 3]. As an explanation we have proposed [4] that alkylation of hepatic cytosolic coenzyme A by reactive metabolites should lead to an increased production of acetone in the liver (see Discussion). The xenobiotic-induced acetonemia, according to pharmacokinetic studies [5], is accompanied by an increase in the endogenous acetone production rate.

The present investigation was designed to provide additional evidence for the concept that the xenobiotic-induced acetonemia is the consequence of a depletion of cytosolic coenzyme A due to reactive metabolites. As test compounds, which lead to formation of reactive metabolites and cause acetonemia [2, 3], we selected the haloethylenes vinyl chloride (chloroethylene) and vinylidene fluoride (1,1-difluoroethylene). Vinylidene fluoride was used in some experiments *in vivo*: it can be handled easily in closed (static) exposure chambers because of its slow decline in the system's atmosphere [6].

The experiments were focused on the following issues: (a) to provide evidence for chemical alkylation of coenzyme A by vinyl chloride metabolites on incubation with vinyl chloride and the hepatic microsomal metabolizing system; (b) to provide evidence for a (selective) depletion of hepatic cytosolic coenzyme A (and acetyl coenzyme A) in rats exposed to vinyl chloride; and (c) to demonstrate, under exposure to vinylidene fluoride, an increase in the two other "ketone bodies", acetoacetate and 3-hydroxybutyrate, in addition to acetone.

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MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats (from Ivanovas, Kisslegg) were fed a standard laboratory diet (Altromin®). [1,2-^{14}C]Vinyl chloride, sp. act. 12 mCi/mmole, was synthesized by the Radiochemical Department of Farbwerke Hoechst AG, Frankfurt. [^{3}H]Coenzyme A, sp. act. 1.48 Ci/mmole, was supplied by NEN Chemicals, Dreieich; it contained dithiothreitol as an antioxidant.

Unlabeled vinyl chloride was purchased from Linde AG, and vinylidene fluoride as a gift of Dynamit-Nobel AG, Troisdorf. Both compounds showed no apparent impurities on gas-liquid chromatography (GLC) (Tenax-GC). Biochemicals were obtained from the sources specifically indicated.

Incubation experiments

Rat liver microsomes were obtained according to a standard procedure [7]. Protein determinations followed the method of Lowry *et al.* [8]. The microsomal stock suspension contained 5 mg protein/ml. For microsomal incubations with [1,2-^{14}C]vinyl chloride which was added to the gas phase we used the apparatus described by Kappus *et al.* [9]. Each incubation vessel (1 ml) contained: 0.1 ml 5 mM $MgCl_2$, 0.1 ml 8 mM DL-Na-isocitrate, 0.3 ml microsomal suspension (as above), 0.3 ml Tris–HCl buffer (0.25 M, pH 7.4), 0.1 ml 1 mM NADP Boehringer, 5 μl isocitric dehydrogenase solution (Boehringer) and 0.1 ml Tris–HCl buffer (as above) containing 0.1 mM of tritiated coenzyme A.

The reaction was started by addition of [1,2-^{14}C]-vinyl chloride to the gas phase of the incubation system. The partial pressure of vinyl chloride in the gas phase was 187 Pa which assured a development

of oxidative vinyl chloride metabolites under v_{max} conditions [9]. Incubation time was 90 min at 37°.

The reaction was stopped by addition of ethanol (3 ml). The protein precipitate was centrifuged and repeatedly washed with 70% ethanol; the combined supernatants were evaporated (at 27°) to dryness. Pilot experiments to separate doubly labeled material from these residues with sephadex-G 15 columns were unsuccessful. Hence the following high-performance liquid chromatography (HPLC)-separation method was employed.

The residues were dissolved in phosphate buffer (0.1 M, pH 6.0) of which 20 µl was applied to a commercial Lichrosorb RP 18 column. The column was eluted with (A): 40 mM KH_2PO_4/60 mM K_2HPO_4, set to pH 6 with HCl and (B): methanol/water, 80:20 (v/v). The following gradient was used (Altex programmer): 0–1 min, 0% B; 1–15 min, 0–35% B; 15–20 min, 35–100% B; 20–30 min, 100% B. Elution rate was 1.5 ml/min. The effluent from the column passed a UV detector (254 nm) and was fractionated in 0.75 ml fractions. Of each fraction 0.5 ml was counted for ^3H and ^{14}C radioactivity in 10 ml Aquasol®.

Exposure experiments

Rats were exposed to unlabeled vinyl chloride or vinylidene fluoride in closed static all-glass exposure chambers as previously described [1–6]. Usually two animals (250 g each) were placed in a chamber of 6.4 liter gas volume; the system contained soda lime as the CO_2 absorbent. The initial concentration of the haloethylene added was 2.500 ppm vinyl chloride or 1.500 ppm vinylidene fluoride. The concentration of the chemical in the gas phase was monitored in short intervals by GLC (for GLC conditions, see ref. [6]). When the declining concentration of vinyl chloride approached a level of 1.000 ppm further doses of vinyl chloride were injected to assure that vinyl chloride was always metabolized at the constant (v_{max}) rate (110 µmoles/hr/kg). Because of the slow decline of vinylidene fluoride repeated injections of this haloethylene were not necessary to ensure metabolism at the v_{max} of 1.1 µmoles/hr/kg (for further kinetic details, see ref. [6]).

Exposure experiments during which urine was collected over a prolonged period (ketone body excretion under vinylidene fluoride exposure; see Results) were performed in a closed all-glass metabolic cage as previously described [10]. The urine collection vessels were cooled with methanol/dry ice.

Determination of hepatic cytosolic and mitochondrial coenzyme A

Mitochondria. The crude mitochondrial pellets obtained from the rat livers at 16.300 × *g* according to a standard procedure [7] were resuspended in Tris–HCl buffer (0.02 M, pH 7.4, containing 0.25 M sucrose and 5.5 mM EDTA) and again sedimented at 16.300 × *g*. This pellet was resuspended in 0.1 M phosphate buffer, pH 6.0 (35 mg pellet/ml). Mitochondrial membranes were then destroyed by adding 0.1 ml 4% Triton X-100/ml suspension and stirring at 0° for 15 min. For removal of proteins to each millilitre of this lysate 0.2 ml cold 11% perchloric acid was given. The whole was chilled on ice for 2 min and the protein precipitate was removed by centrifugation. The clear supernatant was brought to pH 6.5 with cold 10 N KOH, and dithiothreitol (10 µl/ml supernatant) was added to protect the free SH-group of coenzyme A (CoA) from oxidation. After removing the $KClO_4$ by centrifugation (20 min; 16.300 × *g*) the supernatant was used for the enzymatic and HPLC analyses (*vide infra*).

Cytosol. Homogenization of the livers was done very carefully in Tris–HCl buffer (containing sucrose and EDTA as above and 1.5 mg dithiothreitol/100 ml), according to general standard procedures [7]. The 105.000 × *g* supernatants were percolated through a piece of cloth to remove fat. The subsequent removal of proteins by perchloric acid and the neutralization were performed as described for the mitochondrial lysates.

The concentrations of CoA, as determined by enzymatic and HPLC analyses, were finally related to cytosolic (or mitochondrial) protein concentrations as determined by the method of Lowry *et al.* [8]. The mitochondrial matrix enzyme glutamate dehydrogenase [11] was determined in all samples for CoA analysis to ensure complete removal of mitochondrial particles (or complete lysis of membranes in the mitocondrial lysates). For determination of this enzyme the commercial "monotest GLDH aktiviert" (Boehringer, Mannheim) was used.

Enzymatic determination of CoA and acetyl-CoA. The first method for analysis of CoA in the mitochondrial lysates and in cytosol used α-ketoglutarate dehydrogenase which was isolated from pig heart according to Massey [12]. The enzymic oxidation of α-ketoglutarate (CoA-SH + α-ketoglutarate + $NAD^+ \rightarrow$ succinyl-CoA + NADH + H^+ + CO_2) was spectrophotometrically followed at 340 nm and 25° for determination of the purification factors. The original homogenate contained 0.019 enzyme units/mg protein, the final preparation was at 4.1 U/mg.

The low K_m of the enzyme (about 0.1 µM) allows enzymic determinations of CoA even in nmole quantities when a fluorimetric NADH detection is used [13]. Acetyl-CoA can be determined from parallel samples after arsenolysis (with arsenate and phosphortransacetylase) to CoA. In these samples (arsenolysis) the enzymic CoA determination gives the total of CoA plus acetyl-CoA. The details of these analytical procedures have been described by Williamson *et al.* [13].

High performance liquid chromatography determination of CoA and acetyl-CoA. It was attempted to use two different methods, independent of each other, for CoA analysis. The alternative method was HPLC. An Altex HPLC system with a gradient programmer was used. Of the protein-free supernatants to be analysed, 100 µl was applied to an analytical Lichrosorb RP 18 column. The gradient programme was the same as already described (see: "Incubation experiments"). A UV-detector at 254 nm was used. Calibration was done with known amounts of CoA and acetyl-CoA. An example of a chromatogram (rat liver cytosol) is given in Fig. 1(a). Chromatograms of lysates of hepatic mitochrondria (see Fig. 1b) also showed the presence of both CoA

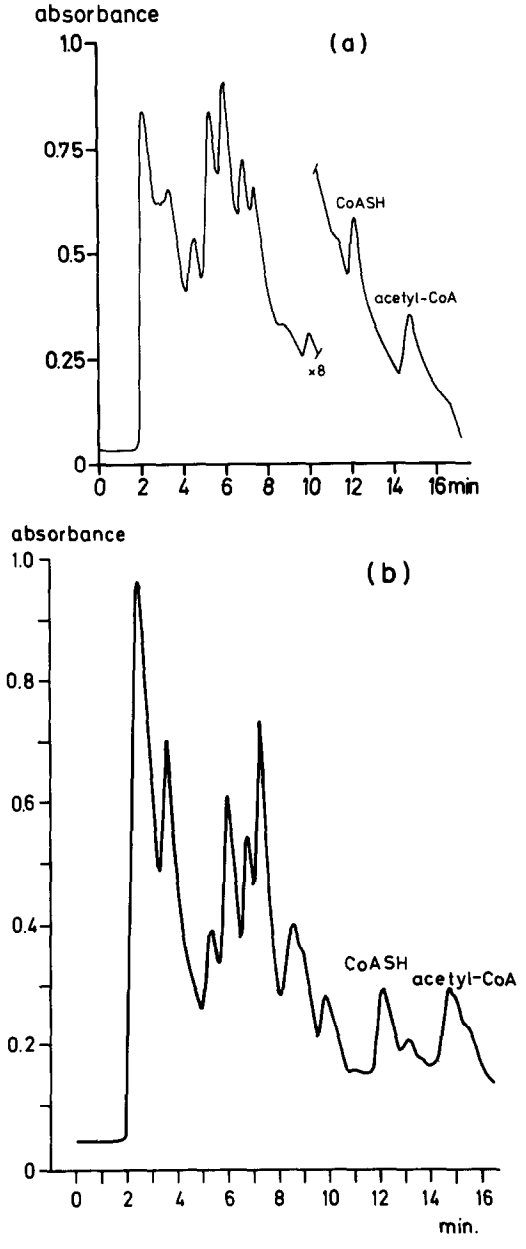

Fig. 1. (a) Chromatogram (HPLC) of the acid-soluble extract from cytosol of rat liver with optical (254 nm) peaks of coenzyme A (CoASH) and acetyl coenzyme A (acetyl-CoA). (b) Chromatogram (HPLC), as in (a), from mitochondrial lysate. Unknown compounds interfere with the determination of acetyl-CoA.

and acetyl-CoA but the acetyl-CoA peak was superimposed by those of other (unknown) compounds. For this reason it was decided to calculate the CoA levels in cytosol as well as in mitochondria, but the acetyl-CoA levels only in cytosol. A postulate was that both independent analytical methods led to comparable results.

Excretion of "ketone bodies" in urine

In some experiments the haloethylene-induced excretion of the "ketone bodies" 3-hydroxybutyrate and acetoacetate was quantitated. 3-Hydroxybutyrate was enzymatically determined using 3-hydroxybutyrate-dehydrogenase and photometric NADH detection at 340 nm [14]. Acetoacetate was reacted with resorcine to 4-methyl-umbelliferone which was fluorimetrically analysed [15].

RESULTS

Alkylation of coenzyme A by vinyl chloride metabolites. Pilot incubation experiments with rat liver microsomes, CoA and vinyl chloride confirmed that vinyl chloride, in the presence of NADPH, reduced the content of free CoA in the incubations (data not shown). Therefore attempts were made to separate doubly labeled reaction products from rat liver microsomal incubations with [^{3}H]coenzyme A and [^{14}C]vinyl chloride (see Materials and Methods). After precipitation of proteins (perchloric acid) and neutralization, a separation of different products was achieved on HPLC. Three doubly labeled reaction-products appeared when active microsomes, in the presence of NADPH (regenerating system) and O_2, were incubated with both labeled substrates (Fig. 2). In absence of NADPH these products did not occur. Control experiments in which only one substrate (either [^{3}H]coenzyme A or [^{14}C]vinyl chloride) was incubated showed no radioactivity elution in those fractions (Fig. 2) in which the doubly labeled compounds appeared when the incubation was "complete".

From the amounts of radioactivity, according to the specific activity of both labeled substrates, the absolute amounts (in nmoles) of eluted metabolites derived from each substrate were calculated. A quantitation of peaks I–III (Fig. 2) is given in Table 1. The major reaction-product containing both ^{3}H and ^{14}C radioactivity was composed of 2 molecules of vinyl chloride metabolites per molecule CoA. Two other products contained both substrates at molar ratios close to 1 : 1. This shows that CoA has (at least) two different sites for alkylation by vinyl chloride metabolites.

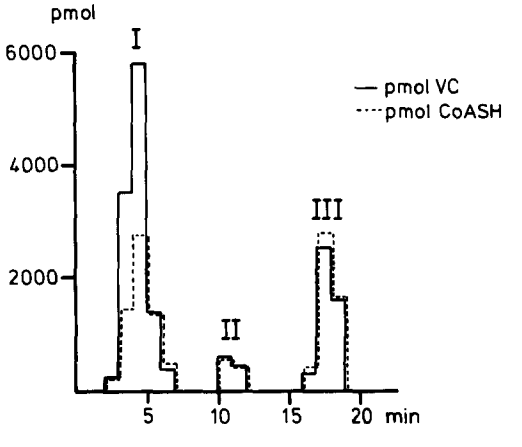

Fig. 2. Chromatogram (HPLC) of an extract of a microsomal reaction mixture after coincubation of [^{14}C]vinyl chloride (VC) and [^{3}H]coenzyme A (CoASH). The radioactivity amounts eluted in each fraction were transformed into the pmol metabolite(s) originating from both precursors.

Table 1. Quantitation of the doubly labeled peaks (I–III, see Fig. 2) obtained from incubations (37°; 90 min; 1 ml) of rat liver microsomes, NADPH-regenerating system, [^{3}H]coenzyme A (100 nmoles) and [^{14}C]vinyl chloride (present in the gas phase, see Materials and Methods)

Peak	[^{3}H]Coenzyme A	[^{14}C]Vinyl chloride	Ratio (^{14}C/^{3}H)
I	6.2 nmoles	12.6 nmoles	2.032
II	0.9 nmoles	1.1 nmoles	1.222
III	5.0 nmoles	4.7 nmoles	0.940

Depletion of CoA by vinyl chloride in vivo. It has previously been suggested [4], but not proven, that reactive metabolites of xenobiotics ought to preferentially deplete cytosolic CoA, as opposed to CoA in mitochondria. As a consequence acetyl-CoA was also postulated, in the cytosol, under such conditions to be at a level lower than normal.

An experimental confirmation of this theory was difficult because of the very low physiological levels of CoA (and acetyl CoA) in the cytosol. Hence it was decided to employ two different analytical methods relying on different analytical principles and being independent of each other.

Both methods should lead to a similar result. A conventional standard method used was enzymic analysis using fluorometry for endpoint determination; acetyl-CoA, in parallel experiments, was converted to CoA by arsenolysis and then determined in the same way (see Materials and Methods). Moreover, a HPLC separation for CoA and acetyl-CoA was developed (Fig. 1).

Rats were exposed for 14 hr to concentrations of vinyl chloride which assured a constant metabolism at v_{max}. Livers from these and untreated controls served for preparation of the mitochondria and cytosol. Because a contamination of cytosol by mitochondrial constituents (with a much higher level of CoA than the cytosol) had to be avoided, determinations of GLDH were performed in all preparations. In the initial homogenate GLDH was 176 ± 28.5 U/g liver ($\bar{x}$ ± S.D.; N = 11). Of this (= 100%) 81.2 ± 13% remained in the (isolated) mitochondria and 3.7 ± 1.7% in the cytosol used for the CoA determinations. This was considered satisfactory.

Table 2 compiles the results of both analytical methods. Because acetyl-CoA from mitochondria could not be clearly separated by HPLC (Fig. 1b), values for acetyl-CoA in this compartment are not given. The treatment of rats with vinyl chloride did not affect the (relatively high) mitochondrial CoA levels, but it lowered those of both CoA and acetyl-CoA in the cytosol, as suggested in the underlying hypothesis [4]. Due to the larger standard deviations obtained with the enzymic method (Table 2b) the differences in cytosolic CoA levels between control and vinyl chloride-treated animals were not statistically significant (*t*-test). However, the more precise HPLC analyses demonstrate that in fact vinyl chloride exposure causes a significant reduction of free cytosolic CoA (Table 2a).

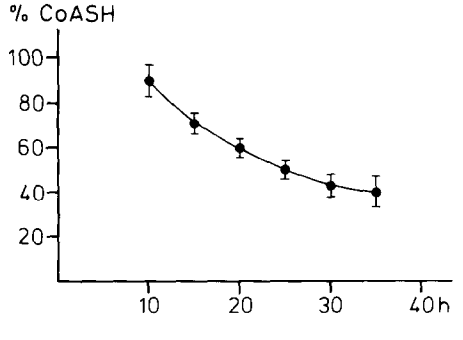

Fig. 3. Time dependence of depletion of cytosolic coenzyme A (CoASH; control values of Table 2 = 100%) upon exposure to high (≥ 1000 ppm) concentrations of vinyl chloride. The bars represent ± S.D.; N = 3.

Table 2. Coenzyme A (CoASH) and acetyl coenzyme A (acetyl-CoA) in hepatic cytosol and mitochondria of control rats (C) and rats exposed to vinyl chloride for 14 hr (VC; see Materials and Methods). Figures are given in nmoles/mg protein ($\bar{x}$ ± S.D.; N = 4)

(a) HPLC analysis

	Cytosol	Mitochondria
CoASH	C 0.39 ± 0.04	2.3 ± 0.4
	VC 0.28 ± 0.03	2.2 ± 0.4*
Acetyl CoA	C 0.81 ± 0.08	
	VC 0.52 ± 0.06	

(b) Enzymic determinations

	Cytosol	Mitochondria
CoASH	C 0.37 ± 0.06	2.0 ± 0.5
	VC 0.27 ± 0.04*	2.0 ± 0.5*
Acetyl CoA	C 0.79 ± 0.12	
	VC 0.53 ± 0.08	

* Not statistically significant (*t*-test, $P > 0.05$) vs controls.

In a further series of experiments rats were exposed in a similar way of vinyl chloride, but for different periods of time before sacrifice. Figure 3 shows the time-course of depletion of hepatic cytosolic CoA (as determined by HPLC).

The observation [2] that acetonemia on exposure to haloethylenes starts after a distinct lag period is consistent with this time course. A prolonged vinyl chloride exposure depresses free cytosolic CoA to less than 50% of its original level.

Excretion of acetoacetate and 3-hydroxybutyrate. For methodological reasons (see Materials and Methods) vinylidene fluoride was used as an acetonemia-causing haloethylene in experiments on the excretion of the "ketone bodies" acetoacetate and 3-hydroxybutyrate.

Figure 4(a, b) shows the cumulative urinary excretion of these two compounds by rats on

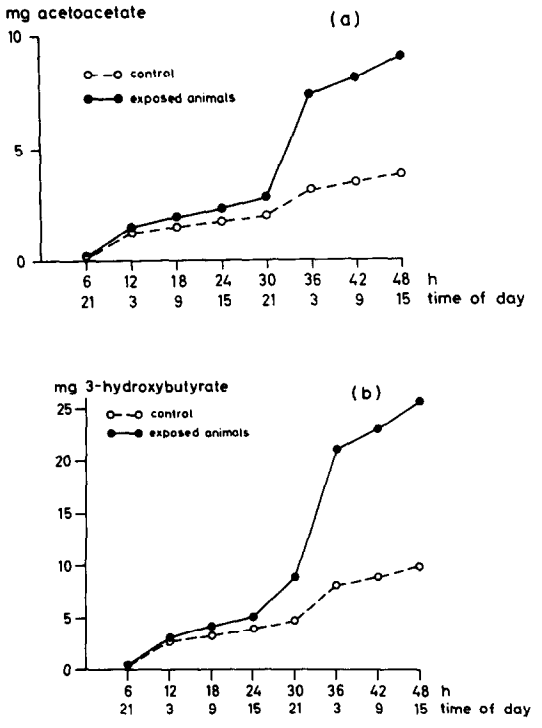

Fig. 4. (a) Cumulative urinary excretion of acetoacetate by rats exposed to vinylidene fluoride and by untreated controls. (b) Cumulative urinary excretion of 3-hydroxybutyrate by rats exposed to vinylidene fluoride and by untreated controls.

exposure to vinylidene fluoride. These data show that under conditions for which development of acetonemia is well established [2], also the other "ketone bodies" are excreted at an elevated rate, compared to untreated controls.

DISCUSSION

Subsequent to the discovery that several halogenated xenobiotics which lead to reactive metabolites cause acetonemia in experimental animals [1–3] we have proposed a mechanism to explain this effect [4]. The theory was based on the established role of CoA in the intermediary metabolism [16]. If, according to Fig. 5, cytosolic CoA is progressively depleted by covalent binding of reactive metabolites of a xenobiotic, the sequence in which enzymes requiring CoA will be affected by this depletion will depend on their affinity (K_m) for CoA. The K_m of the acyl-CoA synthetases (for medium- and long-chain fatty acids) is about 10^{-4} M whilst the ATP-citrate lyase (catalysing splitting of citrate to oxalacetate and acetyl-CoA) has a K_m of 2×10^{-6} M [17]. This means that changes in the low level of cytosolic CoA would, in a first step, preferentially lead to diminished acetyl-CoA levels. That this is true is demonstrated in the present experiments.

The further consequence (Fig. 5) of a diminished formation of acetyl-CoA is a lower level of malonyl-CoA which (as a negative allosteric effector) causes increase of transport of activated fatty acids through the mitochondrial membrane.

The absolute magnitude of reduction of cytosolic CoA is small (about 0.1 nmoles/mg protein). However, this means a 30% reduction in this particular compartment whilst a similar absolute change in the mitochondria, because of the much larger size of the CoA pool there, would not be significant.

Inside the mitochondria (where CoA is not significantly depleted according to the present data) the excess of activated fatty acids is transformed to ketone bodies (acetone and, as demonstrated here, acetoacetate and 3-hydroxybutyrate).

In the case of vinyl chloride reactive metabolites are well known. The primary metabolite, chloroethylene oxide, alkylates DNA *in vivo*; it undergoes rearrangement to chloroacetaldehyde which is able to alkylate proteins [18, 19]. Chloroacetaldehyde is oxidized to chloroacetic acid; haloacetic acids are also of known reactivity towards SH-groups [20, 21].

Alkylation of CoA by electrophilic metabolites is possible in presence of an excess of glutathione [22]. This has been demonstrated *in vitro* [4] and is supported by electrochemical data [4]. Hence, the

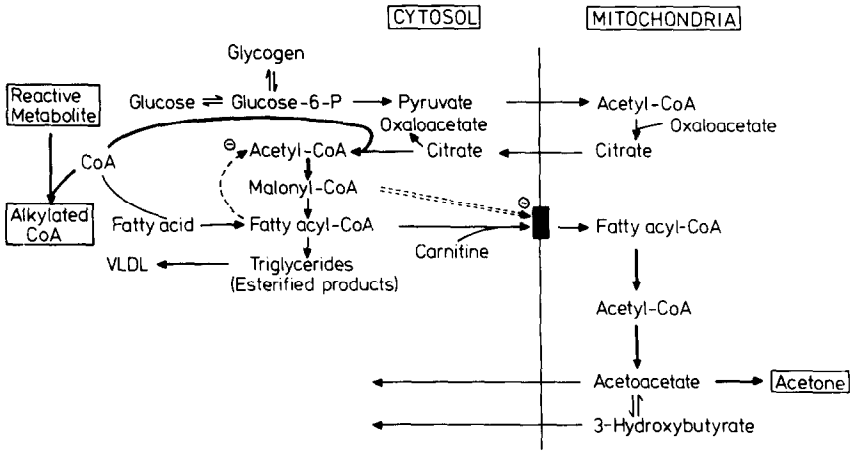

Fig. 5. Possible influences of alkylation of cytosolic CoA by reactive metabolites upon fatty acid metabolism, according to ref. [4]. The different steps are explained in the text.

physiological 10-fold excess of glutathione over CoA (cytosolic plus mitochondrial) does not prevent CoA from reaction with electrophiles.

The present data show that at least two different alkylation sites of the CoA molecule exist for vinyl chloride metabolites. It is likely (*vide supra*) that the free SH-group represents one of these sites; this is also supported by the conjugation of metabolites with the SH-group of glutathione [23]. The second site is probably the adenine moiety of CoA. Adenine is readily alkylated by vinyl chloride metabolites (chloroethylene oxide, chloroacetaldehyde) under formation of $1,N^6$-ethenoadenine [24].

After exposure of rats to radioactive vinyl chloride $1,N^6$-ethenoadenosine is present as an alkylation product in RNA [25, 26]. As both these sites of possible alkylation in the CoA molecule are distinct from each other the occurrence of two alkylation products with a 1:1 molar ratio and of one product with a 2:1 ratio (doubly alkylated) (Table 1) can easily be explained.

In parallel with the present investigation attempts were made to establish conditions under which an increased acetone production could probably serve as an indicator for formation of reactive metabolites. A pilot study in humans occupationally exposed to perchloroethylene [27] has revealed a tendency of an increase in acetone exhalation during exposure. However, high interindividual variations and confounding factors such as alcohol intake made it impossible to use this parameter under field conditions. By contrast, under well-controlled conditions of animal experiments the situation is different. A study in rats [28] has demonstrated that the reactive metabolite vinyl oxirane (1,2-epoxybutene-3) is formed from 1,3-butadiene. Under exposure to 1,3-butadiene, the reactive metabolite builds up a stationary concentration in the body. Simultaneously, a huge increase in acetone exhalation is observed which returns to normal when exposure ceases [28]. Hence, it seems that under experimental conditions an increased acetone exhalation may be indicative of reactive metabolites of a xenobiotic acting upon the organism.

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