Chapter 6

The Biochemical Toxicology of Methanol

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I. INTRODUCTION

Methanol poisoning in man, though its occurrence is rare, presents a rather unique problem in toxicology in that its syndrome has not been described in other species. In man, methanol poisoning is a complicated clinical entity best described by consideration of a sequential series of events *(1)*. First, there is a mild central nervous system depression not unlike that produced by other aliphatic alcohols. Second, there is a quiescent period of 12–24 hours during which time no signs or symptoms have been recognized. Finally, after this latent period, a severe metabolic aci-

dosis and ocular toxicity results with possible blindness even if the patient recovers. Lower animals exhibit only the first phase of the toxic syndrome seen in man *(2)*, although acidosis and retinal edema have been described in monkeys *(3)*.

The special susceptibility of man to methanol toxicity is generally accepted to be due to a metabolite or metabolites of methanol and not to the alcohol per se *(1)*. This is indicated by the latent period before signs of toxicity develop, and by the observation of Röe *(4,5)* that the administration of ethanol soon after the ingestion of methanol significantly alleviates methanol toxicity in humans. At the present time it is not known whether a unique toxic metabolite of methanol is formed in man or whether man is highly sensitive to one of the metabolites of methanol commonly formed in lower animals.

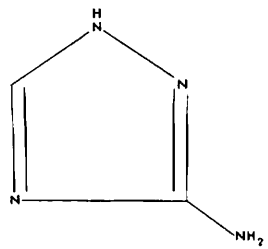
II. THE METABOLIC DISPOSITION OF METHANOL

A. Peroxidative Oxidation

1. Role of Catalase

In 1936, Keilin and Hartree *(6)* described a reaction system whereby catalase mediated the oxidation of a variety of alcohols to their corresponding aldehydes in the presence of a hydrogen peroxide-generating source. This mechanism was surprising in view of the fact that catalase had been thought for years to be mainly a protective enzyme involved only in the decomposition of hydrogen peroxide. These workers suggested *(6,7)* that the physiological function of catalase might be concerned with the mediation of such oxidative reactions *in vivo*. The hydrogen peroxide in the system had to be supplied slowly in low concentrations which could be accomplished by (1) adding peroxide-generating enzymes, such as xanthine oxidase, glucose oxidase, or D-amino acid oxidase with appropriate substrates; (2) the slow decomposition of barium or cesium peroxide; or (3) the direct addition of hydrogen peroxide itself. This last method was accomplished by adding hydrogen peroxide in small drops with sufficient stirring, by volatilizing hydrogen peroxide into the action vessel at 39°C or by supplying the hydrogen peroxide in the form of a fine mist. A study of the specificity of this reaction showed that methanol and ethanol were oxidized at an equal rate which was more rapid than for other higher molecular weight alcohols tested.

Employing rapid-reaction techniques, Chance *(8)* found that catalase and hydrogen peroxide combined to form a complex, catalase–peroxide complex I, which was shown to react rapidly with alcohols, such as



3-AMINO-1,2,4-TRIAZOLE

FIG. 1

ethanol and methanol, to yield the corresponding aldehydes. Hydrogen peroxide was shown to react with this complex at a rate much greater than that observed with methanol or ethanol (3.5×10^4). This would explain why an alcohol such as ethanol or methanol does not react appreciably with catalase in the presence of high concentrations of hydrogen peroxide. Upon analyzing the data obtained by Agner and Belfrage *(9)* on the rate of methanol disappearance from the blood of rabbits, Chance *(8)* calculated that the peroxidatic activity of the intermediate compound was adequate to account for the disappearance of methanol *in vivo*. Jacobsen later considered the peroxidative system to be the important mechanism for methanol oxidation *in vivo (10)*, although he assumed with Theorell and Bonnichsen *(11)* that ethanol was oxidized *in vivo* by alcohol dehydrogenase.

The first direct test of the role of catalase in the oxidation of methanol came as a result of studies performed by Heim *et al. (12)*. These workers showed that the intraperitoneal injection of 1 gm/kg of 3-amino-1, 2,4-triazole (Fig. 1) produced a 90% inhibition in hepatic and renal catalase activity in rats within the first 3 hours after injection. It is interesting to note, however, that there was no effect of aminotriazole on erythrocytic catalase activity. Furthermore, there was no effect on liver cytochrome *c* content, blood hemoglobin levels, or urobilinogen excretion. It was shown later *(13,14)* that there was a requirement for hydrogen peroxide in the aminotriazole inhibition of catalase activity. Substrates for complex I, such as methanol and ethanol, were capable of preventing the aminotriazole-induced inhibition of catalase activity in a manner predictable from a knowledge of their reactivity as substrates for this complex *(15)*. When aminotriazole was reacted with complex I prior to the addition of ethanol or methanol, catalase activity was irreversibly inhibited. Therefore, aminotriazole has proved to be a valuable compound for

studying the role of catalase and for detecting the presence of hydrogen peroxide in biological systems.

One of the first applications of this information was the use of aminotriazole in studies on ethanol metabolism. It was shown by Nelson *et al. (16)* that aminotriazole had no effect on ethanol elimination in the dog, although catalase activity was markedly reduced. This information served to strengthen the concept that catalase was not involved in ethanol metabolism *in vivo.*

Mannering and Parks *(17)* first used aminotriazole to assess the role of catalase in the metabolism of methanol *in vitro.* They found that the administration of aminotriazole to rats (3 gm/kg) inhibited about 95% of the hepatic catalase activity and 70% of the methanol oxidizing capacity of rat liver homogenates. When the homogenates from aminotriazole-treated animals were supplemented with crystalline beef liver catalase, the methanol oxidizing capacity of hepatic homogenates was restored to normal. They further established that hydrogen peroxide generation was the rate-limiting process *in vitro* in the oxidation of methanol by liver homogenates from untreated rats. When hepatic homogenates from untreated rats were fortified with an exogenous peroxide-generating source, such as glucose plus glucose oxidase, the oxidation rate of methanol was substantially enhanced. In order to show that catalase was limiting in homogenates of aminotriazole-treated rats, glucose and glucose oxidase were added. No increase in methanol oxidation was observed in systems containing enzymes derived from aminotriazole-treated rats.

Later, Smith *(18)* showed that liver slices from aminotriazole-pretreated rats exhibited a methanol oxidation rate that was reduced to 30% of values obtained with liver slices from untreated rats. The rate of ethanol disappearance and acetaldehyde formation was not affected under the conditions of these experiments.

Mannering and Parks *(17)* showed that aminotriazole administration to rats had no effect on the disappearance of methanol from the blood, although a marked effect was observed *in vitro.* This apparent paradox was resolved by Tephly *et al. (19)* when it was shown that at high doses of methanol (3 gm/kg), a considerable percentage of the administered dose is excreted unchanged. These experiments demonstrated that with volatile substances that are metabolized slowly, disappearance of the substrate from the blood represents a complex of excretion and metabolism and requires that one study metabolism exclusive of the total disappearance of the substrate from the organism. Therefore, in recent years, methanol metabolism has been studied *in vivo* by measuring the rate of carbon dioxide-^{14}C formation from methanol-^{14}C.

It was shown by Tephly *et al. (19)* that pretreatment of rats with 1

gm/kg of aminotriazole 1 hour before methanol administration led to a decrease in the rate of conversion of methanol-^{14}C to carbon dioxide-^{14}C to 50% of control values. Furthermore, it was shown that a dose versus metabolism curve could be constructed for methanol in the untreated rat which could be used to calculate an "apparent Michaelis constant" (K_m). Aminotriazole pretreatment produced an increase in the K_m value. These data suggest that catalase occupies a major role in the oxidation of methanol *in vivo* as well as *in vitro* in the rat. Aebi *et al. (20)* presented evidence which substantiated this hypothesis. They found that when rats were fed allylisopropylacetureide, an inhibitor of catalase synthesis, a 50% reduction in the rate of methanol oxidation occurred *in vivo*. When aminotriazole was injected 4 hours after methanol administration, a slight drop in the oxidation rate of methanol was noted. That a more dramatic decrease in the methanol oxidation rate was not seen may be explained by the fact that methanol, if injected prior to aminotriazole, protects catalase from aminotriazole inhibition *in vivo (15)*.

Further evidence demonstrating the importance of the catalase-dependent peroxidative mechanism for methanol metabolism comes from studies employing competing substrates *in vivo (19)*. Ethanol and methanol have equal reactivity for catalase peroxide complex I *(8)*, but 1-butanol has negligible reactivity. When ethanol and methanol were injected in equimolar concentrations in the rat, the methanol oxidation rate was reduced to about 50% of control values. Although 1-butanol had only a very slight inhibitory effect on the methanol oxidation rate, it produced a profound inhibition of ethanol metabolism in the rat. 1-Butanol has greater reactivity with alcohol dehydrogenase than ethanol. It was suggested *(19)* that these results were consistent with a major role of the catalase peroxidative system for methanol oxidation in the rat and a predominant role for the alcohol dehydrogenase system for ethanol oxidation in this species. It was shown that methanol had no effect on the oxidation of ethanol in the rat even when the molar ratio of ethanol to methanol administered was 8 to 1 *(19)*. These results were also observed in experiments with the isolated, perfused rat liver *(21)*.

Although these data clearly show the role of a catalase peroxidative oxidation of methanol in the rat, results obtained with monkeys revealed a different pattern. Makar *et al. (22)* showed that pretreatment of monkeys with aminotriazole at 1 or 3 gm/kg of body weight 1 hour prior to methanol injection yielded no inhibition in the rate of methanol oxidation, although catalase activity in the liver was reduced to 10% of control values. Whereas equimolar concentrations of ethanol to methanol produced a 50% inhibition of the rate of methanol oxidation in the rat, an 80% inhibition of the rate of methanol oxidation was observed in the

monkey *(22)*. 1-Butanol which produced only a slight effect on methanol oxidation in the rat, produced a 90% inhibition of methanol oxidation in the monkey. These results strengthened the view that the catalase peroxidative system is not functional for methanol oxidation in the primate, and that the metabolism of methanol in the monkey depends upon alcohol dehydrogenase.

2. Role of Peroxide Generation

Although a requirement for hydrogen peroxide generation was established by Keilin and Hartree in 1936, the importance of peroxide generation as a limiting factor in the peroxidative oxidation of methanol was not recognized until 1957. Mannering and Parks *(17)* showed that when a purified peroxide-generating system, such as glucose and glucose oxidase, was added to hepatic homogenates from untreated rats, a marked stimulation of methanol oxidation occurred *in vitro*. In hepatic homogenates from aminotriazole-treated rats, where catalase activity had been made the limiting factor, addition of this peroxide-generating system was without effect. Later it was shown by Koivusalo *(23)* and Tephly *et al. (24)* that treatment of animals with sodium tungstate lowered hepatic xanthine oxidase activity as well as the rate of methanol oxidation in hepatic homogenates. When a purified preparation of xanthine oxidase was added to hepatic homogenates from tungstate-treated animals, complete restoration of methanol oxidation resulted *(24)*. Animals treated with sodium molybdate in combination with sodium tungstate had levels of xanthine oxidase intermediate between control and sodium tungstate-treated rats. Methanol oxidation in liver homogenates displayed activities consistent with the level of xanthine oxidase in hepatic homogenates. Since liver catalase activity in these animals was unchanged, these results indicated that the rate-limiting factor in peroxidative methanol oxidation was the rate of hydrogen peroxide generation.

Further evidence that the rate of methanol oxidation depended upon the rate of hydrogen peroxide generation was provided by Smith *(18)*. Xanthine addition to incubation mixtures containing rat liver slices doubled the rate of methanol oxidation to formaldehyde, whereas the rate of ethanol oxidation to acetaldehyde was unaffected.

Rat erythrocytes contain an abundance of catalase but are incapable of oxidizing methanol. Furthermore, erythrocytic catalase is insensitive to aminotriazole *(12,25)*. Methods used to supply hydrogen peroxide to these cells sensitize erythrocytic catalase to aminotriazole inhibition and induce erythrocytes to metabolize alcohols *(25)*. These results explain why alcohol is normally not metabolized by erythrocytes, i.e. there is not

enough hydrogen peroxide generation in normal, nonnucleated erythrocytes.

In 1966, Blair and Vallee *(26)* isolated human hepatic alcohol dehydrogenase and showed that ethylene glycol was capable of reacting with this enzyme. Van Harken *(27)* began a series of experiments in order to examine the effect of ethylene glycol on methanol oxidation *in vivo*. It was expected that ethylene glycol would inhibit methanol metabolism if its metabolism was dependent upon hepatic alcohol dehydrogenase. Rather than observing a decrease in methanol oxidation, ethylene glycol stimulated the rate of methanol oxidation in the rat *(27,28)*. A review of the pathway of ethylene glycol metabolism quickly revealed that ethylene glycol was providing substrate for a peroxide-generating enzyme in the rat liver, and that it was likely that increases in peroxide generation led to an increase in the oxidation of methanol in this species. Glycolic acid is one of the metabolites of ethylene glycol, and in its reaction with glycolic acid oxidase, hydrogen peroxide is formed in addition to glyoxylic acid *(29,30)*. This would lead to an increase in the steady state level of catalase peroxide complex I and an elevated rate of methanol oxidation in the rat. Glycolic acid was capable of stimulating methanol oxidation to the same degree that was observed with ethylene glycol *(28)*. These experiments further demonstrate the rate-limiting role imposed by peroxide generation in the peroxidative oxidation of methanol.

Whereas the peroxidative system appears to be important for the oxidation of methanol in the rat, its role in the primate appears to be minimal. Experiments demonstrating the role of this system in the rat have also been performed in the monkey. These will be reviewed more extensively relative to the alcohol dehydrogenase system (Section II, B).

The question of why the peroxidative system does not function in the monkey has been studied by Makar and Mannering *(31)* and Goodman and Tephly *(32)*. One possible explanation was that monkey liver contained less catalase activity than rat liver. However, the monkey liver contains more catalase activity than does rat liver *(31)*. Makar and Mannering *(31)* have examined the distribution of hepatic catalase and evaluated its peroxidative activity in a number of species in which methanol appears to be oxidized peroxidatively and in which methanol seems not to be metabolized by this system.

Catalase and oxidases capable of generating hydrogen peroxide exist in both the soluble and particulate fraction of the hepatic cell *(33)*, and within the particulate fraction, catalase and several oxidases reside in the microbody (peroxisome) *(34)*. In the rat, mouse, and guinea pig, in which methanol is metabolized primarily through a catalase peroxidative

TABLE I

OXIDASE-DEPENDENT METHANOL OXIDATION IN HEPATIC HOMOGENATES[a]

Oxidase	Rat	Monkey	Human
Urate oxidase	95.5 ± 5.5[b]	11.7 ± 1.2	0
Glycolate oxidase	53.0 ± 4.8	21.0 ± 2.0	22.0 ± 3.4
Xanthine oxidase	5.4 ± 1.0	0	0
D-Amino acid oxidase	2.7 ± 0.5	1.6 ± 0.4	0.9 ± 0.2
L-α-Hydroxy acid oxidase	0	0	0

[a] From Goodman and Tephly *(35)*, reprinted from *Res. Commun. Chem. Pathol. Pharmacol.*, by permission of Publications Ltd., Westbury, New York.

[b] Micromoles of methanol oxidized per gram of liver per hour. Each value represents the mean of four livers ± SEM.

system, methanol metabolism *in vivo* correlated well with the catalase activity in the particulate fraction of the liver homogenate *(31)*. In the monkey, no correlation between methanol oxidation and particulate catalase was observed. It was found that the peroxidative capacity of hepatic particulate catalase activity for the monkey was about 20% of that observed for the rat liver particulate catalase, and it was suggested that these findings account at least in part for the low peroxidative oxidation of methanol in the monkey.

Another possibility is one that has been discussed previously in this section, namely that the rate of hydrogen peroxide generation regulates the rate of methanol oxidation. Goodman and Tephly *(32)* studied a number of peroxide-generating enzymes and evaluated their capacity to provide hydrogen peroxide for the catalase-dependent peroxidative oxidation of methanol in the rat, monkey, and human (Table I). Their investigations included systems associated with the microbody fraction of liver homogenates as well as certain soluble peroxide-generating enzymes. Since peroxide generation was limiting for methanol oxidation in the rat, where the peroxidative system for methanol oxidation was shown to play a major role, they reasoned that these enzymes might play an even more limiting role in the monkey, and, perhaps even in human liver *(35)*. It was shown *(32,35)* that urate oxidase and glycolate oxidase were the most active hepatic hydrogen peroxide-generating enzymes in the rat, and that much less activity was found in preparations obtained from monkey and human liver. It has long been known that urate oxidase is absent in human liver, and that urate is the end product of purine catabolism in man. D-Amino acid oxidase and L-hydroxy acid oxidase activity were low in each species studied. The addition of crystalline catalase to systems containing hepatic oxidases did not alter the rate of methanol ox-

idation *in vitro (32)*. This further substantiated the proposal that hydrogen peroxide generation was rate limiting in the peroxidation of methanol in the rat, monkey, and human liver. The low activity of oxidases in monkey and human liver may explain the lack of peroxidative oxidation of methanol in these species and may relate to its toxicity. This is discussed further in Section IV on formate metabolism.

B. Role of Hepatic Alcohol Dehydrogenase

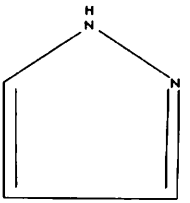
For many years hepatic alcohol dehydrogenase (ADH) was generally accepted as the catalyst for the oxidation of aliphatic alcohols. This concept was supported experimentally in 1938 by Lutwak-Mann *(36)*, who first studied a partially purified preparation of hepatic ADH. She showed the requirement of nicotinamide adenine dinucleotide (NAD) as cosubstrate, and the reactivity of this preparation with ethanol and methanol. In 1948, Bonnichsen and Wassen prepared crystalline horse hepatic ADH *(37)*. Reports followed which showed that horse liver ADH was reactive with ethanol and higher aliphatic alcohols but was unreactive with methanol under the conditions of their experiments *(11,38–40)*. That methanol failed to serve as a substrate for hepatic ADH redirected the attention of many investigators to another enzyme system which was known to mediate this oxidative reaction, the catalase peroxidative system. Its role has been discussed in the previous section.

Although the report of Theorell and Bonnichsen *(11)*, which showed that horse liver ADH is unreactive with methanol, may be credited with having directed attention to the peroxidative route of methanol metabolism, the observations of Kini and Cooper brought attention back to ADH *(41)*. They showed that methanol in relatively high concentrations (about 0.4 *M)* had appreciable reactivity with crystalline horse liver ADH. It is now known that purified liver ADH from rat *(31)*, rhesus monkey *(41)*, and man *(42,43)* reacts with methanol.

Once it became apparent that methanol oxidation to carbon dioxide in the intact mammal could be initiated by hepatic ADH and catalase peroxidative systems, a number of experimental approaches were employed in order to define the degree to which these systems contribute to methanol metabolism in the rat and monkey.

1. Use of Inhibitors of Hepatic Alcohol Dehydrogenase

A differential susceptibility of methanol oxidation to alternate alcohol substrates between the rat and monkey *(19,22)* has been shown. In a homologous series of straight chain aliphatic alcohols, relative activity (maximal velocity) and affinity for ADH *(44)* increase with increases in molecular weight. The reverse is true for alcohol interactions with the cata-



PYRAZOLE

FIG. 2

lase peroxidative system *(8)*. Tephly *et al. (19)* and Makar *et al. (22)* demonstrated that, whereas ethanol produced a 50% inhibition of methanol oxidation in the rat, an inhibition of 80% was observed in the monkey. 1-Butanol, which inhibited methanol oxidation to only an insignificant degree in the rat, effected a more dramatic inhibition of methanol oxidation in monkey than did ethanol. These results are in accord with the concept that the catalase peroxidative system serves as the major metabolic route for methanol in the rat, while ADH is the primary catalyst for methanol oxidation in the monkey.

Although aminotriazole served to demonstrate the role of the catalase peroxidative system in methanol and ethanol oxidation in the rat and monkey, Watkins *et al. (45)* have shown that pyrazole (Fig. 2) dramatically inhibits the oxidation of methanol in the rhesus monkey. These results demonstrate directly that methanol is oxidized primarily by the ADH system in the monkey. Furthermore, it was shown by Watkins *et al. (45)* that pyrazole produced a significant inhibition of methanol oxidation in the rat but, as expected, not to the same degree as observed in the monkey. These findings revealed that, although the catalase peroxidative system plays a major role for methanol oxidation in the rat, the ADH system also contributes to methanol metabolism in this species.

The possibility that pyrazole might influence catalase peroxidative activity has been a source of particular concern to many investigators. This stems in part from the chemical similarity between pyrazole and aminotriazole and from studies which have shown that hepatic catalase activity is decreased following the treatment of rats with pyrazole *(46)*. Lieber *et al. (46)* measured hepatic catalase activity 23 hours after the oral administration of pyrazole (300 mg/kg of body weight) and observed a marked inhibition of hepatic catalase activity. This inhibition is probably due to a metabolite of pyrazole formed *in vivo,* since pyrazole is not an inhibitor of catalase activity. Watkins *et al. (45)* studied the interaction of pyrazole with hepatic catalase seven ways: (1) as a direct inhibitor of

crystalline beef liver catalase activity; (2) as an inhibitor of crystalline beef liver catalase in the presence of hydrogen peroxide; (3) as a substrate for catalase–peroxide complex I; (4) as an inhibitor *in vitro* of the catalase peroxidative oxidation of methanol to formaldehyde; (5) as a substrate which protects against the aminotriazole inhibition of catalase peroxidative oxidation of methanol to formaldehyde *in vitro;* (6) as an inhibitor of hepatic catalase activity *in vivo* over the time course used to study methanol metabolism; and (7) as a substrate for catalase–peroxide complex I *in vivo.* Because pyrazole exerts an almost immediate inhibition of methanol or ethanol metabolism *(45) in vivo,* any effect of a pyrazole metabolite on these processes appears to be unlikely. Furthermore, conditions are obtainable in which methanol or ethanol metabolism is inhibited by pyrazole in the rat and in the monkey, without evidence of hepatotoxicity *(45).* Therefore, it would seem unlikely that the alcohol inhibitory effects of pyrazole are due to some hepatotoxic effect.

Much investigation has been devoted to studying the mechanism by which pyrazole exerts its inhibitory effect on equine hepatic ADH *(47).* Recent publications suggest that the same inhibitory mechanism exists for liver ADH from the human *(48)* and rat *(49).* Pyrazole competes with ethanol for the alcohol binding site on liver ADH, forming an inactive ternary complex composed of pyrazole–NAD–ADH. The formation of this complex has been ascribed to bond formation between the negatively charged pyrazole ion and the 4-carbon of the pyridinium ring of NAD, occurring at or near the alcohol binding site of the enzyme. Zinc atoms present at the active centers of liver ADH are currently considered to promote the dissociation of H^+ from pyrazole. The resulting pyrazole anion then interacts with NAD *(47).*

The relative inhibitory specificity of pyrazole has been the subject of considerable recent study. The influence of pyrazole upon the activities of a number of zinc-containing enzymes has been examined by Reynier *(49).* In these studies, pyrazole in concentrations 100- to 1000-times that required to inhibit liver ADH exerted no inhibitory effects on a number of mammalian enzyme activities. The possible effect of pyrazole on aldehyde dehydrogenase has been examined due to the intimate relationship of these enzymes to the metabolism of alcohols in the intact mammal. Partially purified rat hepatic aldehyde dehydrogenase activity is unaffected by 10,000 times (10 m*M)* the concentration of pyrazole which effectively inhibits hepatic ADH *(50).* Studies on the effect of pyrazole on hepatic formaldehyde dehydrogenase will be discussed in Section III.

A number of compounds other than pyrazole and its derivatives have been reported to serve as effective inhibitors of hepatic ADH, including

various amides and oximes. Koe and Tenen *(51)* have reported that *n*-butyraldoxime effectively inhibits mouse hepatic ADH activity *in vivo* and *in vitro*. In addition, they reported that *n*-butyraldoxime exerted an inhibitory effect on aldehyde dehydrogenase (AldDH) *in vivo*, but not *in vitro*. These investigators suggest that *n*-butyraldoxime inhibits ADH activity directly, while AldDH is probably inhibited by a metabolite of this inhibitor. We have recently shown that *n*-butyraldoxime can serve as a good substrate for AldDH and may inhibit aldehyde oxidation *in vivo* on the basis of competitive substrate interaction. Recently it has been shown *(52)* that *n*-butyraldoxime is rapidly hydrolyzed to hydroxylamine and *n*-butyraldehyde, a substrate for AldDH and ADH. This process occurs at a rapid rate in aqueous solutions as well as in enzyme incubation reactions. The observed inhibition of AldDH activity *in vivo* which appears to be associated with *n*-butyraldoxime is probably a function of the degree of its hydrolysis to *n*-butyraldehyde, which can compete with acetaldehyde. *In vitro*, the substrate character of *n*-butyraldoxime is probably due to *n*-butyraldehyde. "Disulfiram-like" side effects associated with environmental exposure to *n*-butyraldoxime *(53)* may also be explained on this basis. Variable results observed during kinetic analysis of *n*-butyraldoxime inhibition of ADH activity may also be explained in part by the variable amounts of *n*-butyraldehyde which are available and which influence the enzyme. Also, the hydrolytic formation of hydroxylamine, a relatively modest inhibitor of hepatic ADH, further complicates the kinetic analysis of ADH inhibition by *n*-butyraldoxime.

In order for peroxidative or ADH reactions to occur, relatively high concentrations of methanol must be achieved. However, the concentration of methanol can reach a sufficient magnitude to permit these reactions to assume significance *in vivo*. If a man drank a pint of 20% methanol, he would achieve a concentration (in total body water) *in vivo* of approximately 0.05 *M*. This concentration would be sufficient to allow either the peroxidative or the ADH system to metabolize methanol to formaldehyde at significant rates.

It is likely that the ADH system predominates for methanol oxidation in man as well as in the rhesus monkey. The effectiveness of pyrazole and its derivatives in inhibiting alcohol oxidation *in vivo* suggests that perhaps such inhibitors might possess some clinical value in modifying the pharmacology and toxicology of substances such as methanol. Although 4-methylpyrazole has already been administered to the human with no apparent adverse effects *(54)*, the use of such agents would wisely be limited until more definitive information is available with regard to their biologic disposition and the influence of these agents and their metabolites upon systems other than hepatic ADH. Although all the

pertinent information is not available concerning the disposition and toxicity of pyrazole and its derivatives, when we consider the consequences of methanol poisoning, the use of these substances alone or in combination with ethanol *(45)* may be better than the alternative.

C. Reactions of Methanol with Retinal Dehydrogenases

Until recently it was generally believed that retinol–retinaldehyde interconversion in the retina was mediated by alcohol dehydrogenase (E.C. 1.1.1.1). This concept was based and sustained primarily on observations made using either purified hepatic ADH *(49,55,56)* or crude retinal preparations *(57–60)*. However, evidence has been obtained which suggests that the "hepatic type" ADH is absent from the retina, and that retinol is oxidized in the retina and liver by specific and distinct enzymes *(61)*. Retinal ADH's are of particular bearing in this review for two reasons: (a) few details are presently known of the role of "retinal ADH" in retinopathies associated with methanol or its metabolites; and (b) retinal ADH activity has been only partially characterized with regard to its relative susceptibility to inhibitors of liver ADH, substrate specificities, or physical characteristics. Since the metabolites of methanol appear to be responsible for the unique toxic manifestations of poisoning by this alcohol, and since inhibitors of liver ADH may be of value in preventing the formation of these metabolites, two additional toxicological considerations relative to the retinal ADH activity have been explored *(62)*. First, if the enzymatic oxidation of methanol proceeds more favorably in the retina than in the liver, it is possible that methanol retinopathies could be a result of exposure to relatively high concentrations of formaldehyde generated *in situ*. Secondly, if retinal ADH participates in visual pigment metabolism, and if the retinal enzyme is similar to hepatic ADH, inhibitors of hepatic ADH could interrupt normal visual pigment generation. Watkins and Tephly *(62)* have studied the kinetics of retinal ADH and AldDH activities purified from the rat and monkey. They showed that NAD-dependent oxidation of ethanol (RADH) is catalyzed by an enzyme purified from the retinal cytosol fraction and is inhibited by pyrazole and *n*-butyraldoxime. In contrast to the hepatic enzyme, this possesses no reactivity with respect to retinol or methanol, is a poor catalyst of acetaldehyde reduction, and is relatively insensitive to the zinc-chelating effects of 1,10-phenanthroline. These authors conclude that although RADH activity was inhibited by inhibitors of hepatic ADH, it is unlikely that these substances could exert retinotoxicity on the basis of inhibition of oxidation of retinol. This view was substantiated by direct measurements of oxidation of retinol to retinaldehyde in homogenates of

TABLE II

OXIDATION OF RETINOL IN HOMOGENATES OF RAT RETINAE[a]

	Retinol oxidation	
	I	II
Control	1.7[b]	1.8
Pyrazole	1.8	1.8
Ethanol	1.6	1.7
Methanol	1.9	2.2

[a] From Watkins and Tephly *(62)*, reprinted from *J. Neurochem*, by permission of Pergamon, Oxford and New York.

[b] Micromoles of retinaldehyde oxidized per gram of retinae per hour. Retinal homogenates were incubated with 1.1 m*M* retinol, 2 m*M* NADP, and when used, 1 m*M* pyrazole, 100 m*M* ethanol, or 100 m*M* methanol in a final volume of 1.5 ml with 0.1 *M* potassium phosphate buffer (pH 7.6). Reactions were carried out in air at 37°C for 60 minutes, during which time product formation was linear. Each set of values represents an individual experiment.

rat retina, where neither methanol, ethanol, nor pyrazole inhibited the oxidation of retinol (Table II). While studies to date have not revealed the mechanism by which methanol exerts its retinotoxic effects, these investigations may have disqualified certain time-honored concepts about the role of retinal ADH in the metabolism of methanol and retinol.

III. THE METABOLIC DISPOSITION OF FORMALDEHYDE

A. Introduction

Although our knowledge of the first step in the oxidation of methanol to carbon dioxide has increased considerably in the last ten years, the second step in this process, the disposition of formaldehyde, has received relatively little attention. An assessment of this step is extremely important to the study of methanol poisoning, since formaldehyde is the first metabolic product from this alcohol and has been suggested as the toxic metabolite responsible for methanol poisoning *(2)*. However, the presence of formaldehyde has not been demonstrated in blood, urine, or expired air after methanol administration *(9,63,64)*. An unconfirmed study by Kesser *(65)* reported the presence of formaldehyde in the cerebrospinal fluid, vitreous humor of the eye, and peritoneal fluid of rats given methanol.

These findings are not surprising in view of the high degree of reactivity of formaldehyde with proteins and other compounds containing active hydrogen atoms *(66)*. Formaldehyde can combine with any of a number of functional groups found in proteins. The most frequently encountered

reaction of formaldehyde is its addition to a compound containing an active hydrogen atom with the formation of a hydroxymethyl compound:

$$R\text{-}H + CH_2O \rightleftarrows R\text{-}CH_2OH$$

When steric relations are favorable, the hydroxyl group is usually reactive and may condense with a functional group containing an active hydrogen atom to form a methylene bridge:

$$R\text{-}CH_2\text{-}OH + H\text{-}R' \rightleftarrows R\text{-}CH_2\text{-}R' + H_2O$$

Reactions of this type are likely to modify the structural properties of the protein *(66)*. One result of such interaction between formaldehyde and protein is interruption of the normal physiological role of that protein.

For example, formaldehyde (2.5 μmoles/mg protein) has been shown to inhibit the phosphate dependent respiration of mitochondria *(67)*, and it was proposed that this inhibition was due to the inhibition by formaldehyde of phosphate transport into the mitochondria. In view of the high degree of reactivity of formaldehyde, it may be very difficult to pinpoint a specific toxic receptor for this substance.

B. Formaldehyde Dehydrogenase

In 1955, Strittmatter and Ball isolated a formaldehyde-specific, NAD-dependent formaldehyde dehydrogenase from beef liver *(68)*. This enzyme activity exhibited a requirement for reduced glutathione (GSH) which was suggested to participate in its catalytic mechanism. The enzyme activity was purified 4-fold but was never completely freed of ADH or AldDH. The pH optimum was found to be 8.0 which is in contrast to the pH optima of pH 9–10 for human liver AldDH, a factor which will be discussed shortly. Formate was shown to be formed enzymatically in that reaction, and it was demonstrated that 1 mole of NAD was reduced for each mole of formate formed. In addition, the presence of sodium formate did not reduce the rate of NAD reduction, which indicated that the formaldehyde dehydrogenase reaction was not readily reversible. Kinetic data *(8)* indicated that the initial step in the reaction is the nonenzymatic, nonrate-limiting formation of the thiohemiacetal, *S*-hydroxymethylglutathione, which in turn acts as the actual substrate for the enzyme. The proposed reaction scheme was *(68)*:

$$HCHO + GSH \rightarrow HO\text{-}CH_2\text{-}SG$$
$$HO\text{-}CH_2\text{-}SG + FDH \rightarrow \{HO\text{-}CH_2\text{-}SG\}FDH$$
$$\{HO\text{-}CH_2\text{-}SG\}FDH + NAD \rightarrow \{HCO\text{-}SG\}FDH + NADH + H^+$$
$$\{HCO\text{-}SG\}FDH + H_2O \rightarrow FDH + GSH + HCOOH$$

That GSH participates directly in the reaction, rather than nonspecifically to protect protein sulfhydryl groups, was indicated by the following observations: (1) the GSH requirement was quite specific, i.e., high concentration of other sulfhydryl compounds (thioglycolate, ergothionine, CoA, thiolhistidine, DL-cysteine, DL-homocysteine, or cysteinylglycine) which might also protect protein sulfhydryl groups, could not substitute for GSH; (2) an excess of either formaldehyde or oxidized glutathione, two compounds which might inactivate protein sulfhydryl groups, did not affect the rate of formaldehyde oxidation; (3) GSH was active in catalytic amounts and required no preincubation with the enzyme for maximal effect; and (4) the rate of formaldehyde oxidation was dependent on the relative concentrations of GSH and formaldehyde. These observations would be difficult to explain on the basis of an indirect protective mechanism for GSH.

Rose and Racker *(69)* isolated a formaldehyde, NAD-, and GSH-dependent formaldehyde dehydrogenase from Bakers yeast and provided evidence in support of the view that the substrate of the dehydrogenase reaction is formaldehydeglutathione thiohemiacetal, and that *S*-formylglutathione is an intermediate in the reaction. If the substrate for the reaction is the postulated thiohemiacetal, the oxidation product would be a thiol ester. *S*-Formylglutathione was synthesized chemically, and in the presence of the enzyme it caused a rapid oxidation of NADH but not of NADPH. *S*-Acetylglutathione and *S*-lactylglutathione, in the presence of the enzyme, did not result in NADH oxidation, and neither compound was an inhibitor of the formaldehyde dehydrogenase reaction. These results help to explain why Strittmatter and Ball *(68)* were unable to reverse the formaldehyde dehydrogenase reaction by the addition of sodium formate.

Kinoshita and Masurat *(70)* have demonstrated that the addition of GSH to bovine, rabbit, and human retinal preparations resulted in a marked increase in NAD-dependent formaldehyde oxidation. The pH optimum of this reaction was shown to be 8.0. These studies indicate that the retina possesses an enzyme similar to the hepatic and yeast formaldehyde dehydrogenase previously described.

Recent studies by Goodman and Tephly *(71)* have shown the presence of a specific formaldehyde dehydrogenase (FDH) requiring NAD and GSH in rat and human liver. A 102-fold purification of FDH from rat liver and a 225-fold purification of FDH from human liver were achieved.

Rat and human hepatic FDH were found to have certain similarities *(71)*. Both enzymes were unreactive toward acetaldehyde and benzaldehyde, substrates which are known to react favorably with human liver

AldDH *(72,73)*. In addition, the GSH requirement of the enzyme obtained from both species could not be replaced by dithiothreitol, CoA, or cysteine. NADP was not capable of replacing NAD. The K_m of formaldehyde was similar for both rat and human liver enzymes: 7.1×10^{-6} *M* and 8.7×10^{-6} *M*, respectively. The K_m for NAD was 10^{-5} *M* and 7×10^{-6} *M* for the rat and human liver enzyme, respectively, and the pH optimum of rat hepatic FDH was between 7.5 and 8.4. The human liver enzyme had a pH optimum which was slightly broader and higher, 7.6–8.9, which contrasts with the sharp pH optimum between 9 and 10 reported for human liver AldDH *(72,73)*.

Several qualitative differences exist between rat and human liver FDH (Table III). 1,10-Phenanthroline had no effect on the rat liver enzyme but did inhibit human liver FDH activity. Since 1,10-phenanthroline is a chelating agent, these data suggest that the enzyme from human liver is a metalloprotein. However, a rather high concentration (1 m*M*) of 1,10-phenanthroline was required to inhibit the enzyme and, therefore, the inhibition may be due to a mechanism other than metal chelation. Folic acid inhibited rat liver FDH, but had no effect on the human liver enzyme, whereas methotrexate, an inhibitor of numerous dehydrogenases *(74)*, had no effect on either enzyme.

Pyrazole, a potent inhibitor of liver ADH *(75)* was examined for its effect on FDH activity *(71)*. Unexpectedly, pyrazole stimulated purified rat liver FDH activity, and it was capable of substituting for GSH in the reaction. It is explained that formaldehyde might react with pyrazole to form *N*-hydroxymethylpyrazole, analogous to the reaction of formaldehyde with amino and imino groups *(66)*, which then serves as a substrate for rat liver FDH. In contrast to the results obtained with the rat liver enzyme, pyrazole had no effect on five out of six human liver FDH prepa-

TABLE III

EFFECTS OF VARIOUS INHIBITORS ON PURIFIED RAT AND HUMAN FORMALDEHYDE DEHYDROGENASE[a]

	Rat	Human
Formaldehyde	0.12[b]	0.10
Formaldehyde + 1,10-phenanthroline (0.1 m*M*)	0.12	0.07
Formaldehyde + 1,10-phenanthroline (1.0 m*M*)	0.12	0
Formaldehyde + folic acid (0.1 m*M*)	0	0.10
Formaldehyde + methotrexate (0.5 m*M*)	0.12	0.10

[a] From Goodman and Tephly *(71)*, reprinted from *Biochem. Biophys. Acta*, by permission of Elsevier, Amsterdam.

[b] Rates are expressed as micromoles of NAD reduced per milligram per minute.

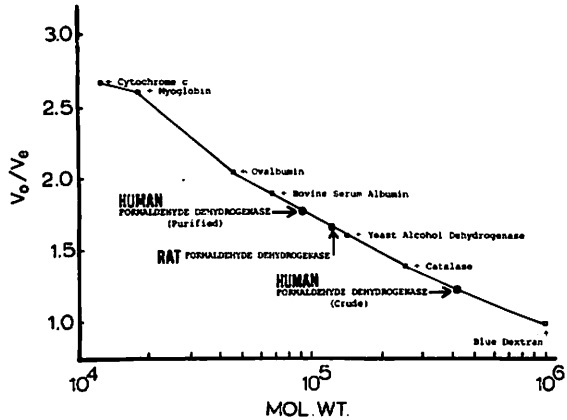


FIG. 3. Estimation of the molecular weight of rat and human liver formaldehyde dehydrogenase. A Sephadex G–200 column (2 cm × 50 cm) was calibrated with six crystalline proteins. The elution volume of a 4-fold purified rat liver formaldehyde dehydrogenase preparation was measured, and the molecular weight was estimated to be 110,000. The elution volume of a purified human liver formaldehyde dehydrogenase preparation and a crude human liver formaldehyde dehydrogenase preparation were measured. The molecular weights of the enzyme in purified and crude preparations were estimated at 90,000 and over 250,000, respectively. V_e = elution volume; V_o = void volume (62 ml). From Goodman and Tephly *(71)*, reprinted from *Biochem. Biophys. Acta,* by permission of Elsevier, Amsterdam.

rations *(71)*. However, at a concentration of 1 m*M*, pyrazole did produce a 20% inhibition of FDH purified from one human liver. This may be due to the existence of an atypical human liver FDH in certain individuals. Von Wartburg and Schurch *(75)* have reported the existence of an atypical human liver ADH in 20% of a Swiss and 4% of a London population. The typical and atypical ADH enzymes differed in their susceptibility to inhibition by pyrazole. The human liver FDH preparation inhibited by pyrazole was as sensitive to inhibition by 1,10-phenanthroline as was the FDH preparation which was not inhibited by pyrazole *(71)*. However, at this point one cannot state that an atypical human liver FDH exists.

Pyrazole was shown to be useful in the study of AldDH when this enzyme is contaminated by ADH *(71)*. In the presence of ADH, AldDH activity is masked due to the cycling of pyridine nucleotides. Pyrazole inhibits ADH without affecting human hepatic AldDH and, thus, provides a means of determining the actual amount of AldDH in preparations containing high levels of ADH activity.

FDH, AldDH, and ADH can be considered to be functionally related

enzymes. Aldehydes serve as substrates for these enzymes, and a product of the ADH reaction, acetaldehyde, is a substrate for AldDH. It is interesting that in the human liver, these dehydrogenases remain inseparable throughout a variety of purification procedures *(71)*. Only by including a procedure involving adsorption on calcium phosphate gel was FDH freed from ADH and AldDH *(71)*. The molecular weight of the purified human liver FDH was estimated at 90,000 (Fig. 3). However, when a relatively crude preparation of human liver FDH which also contained ADH and AldDH was applied to a Sephadex G-200 column, FDH was eluted in a volume indicating its molecular weight to be over 250,000 (Fig. 3). The AldDH and about 10% of the ADH present in these preparations were also eluted in the same volume of FDH. The remainder of the ADH was eluted in a volume indicating its molecular weight to be about 80,000. The molecular weight of purified human liver ADH has been reported to be 87,000. These data *(71)* suggest the possibility that FDH, ADH, and AldDH exist in a multifunctional enzyme complex within the cytosol of the human hepatic cell. This would explain the difficulty encountered in isolating these dehydrogenases, and the observation that in a purified preparation the molecular weight of FDH was estimated at 90,000, while in relatively crude preparations in which ADH and AldDH were also present, the molecular weight of FDH was estimated at over 250,000. The physical association of enzymes located in the cytosol as multifunctional enzyme complexes has been reported to exist in bacteria *(76–78)*. We believe that this occurs in mammals, also. However, it is also possible that FDH dissociates into subunits during the final stages of the purification procedure, and that this accounts for the discrepancy in molecular weight between crude and purified preparations. The molecular weight of rat liver FDH, in both crude and purified preparation, was estimated at 110,000 (Fig. 3).

A low capacity of human liver to oxidize formaldehyde would at least partially explain the unique susceptibility of man to methanol poisoning. However, this was not found to be the case *(71)*. The total amount (units per gram of liver) of formaldehyde dehydrogenase activity in human liver was found to be about 50% higher than in rat liver. In addition, the capacity for NAD-linked formaldehyde oxidation which is not dependent upon GSH was severalfold higher (in terms of units per gram of liver and specific activity) in human liver than rat liver. While it is possible that there are other as yet undescribed pathways for formaldehyde disposition which are operative to a greater extent in rat liver, it appears that the conversion of formaldehyde to formate can proceed more readily in human liver than in rat liver *(71)*.

C. Aldehyde Dehydrogenases

A number of AldDH's have been reported to employ formaldehyde as substrate, but most of these studies have not been performed with emphasis directed toward formaldehyde per se. Careful kinetic studies are lacking, and analysis of the properties of these enzymes have been performed primarily with substrates other than formaldehyde *(79)*.

An NAD-dependent AldDH has been isolated by Kraemer and Deitrich *(72)* from the soluble fraction of human liver and purified 23-fold. The enzyme activity was found to increase with increasing pH, but at pH 10 and above, irreversible inactivation occurred. Both aromatic and aliphatic aldehydes served as substrates, and of the aldehydes studied, benzaldehyde exhibited the lowest K_m. Formaldehyde was shown to be a relatively poor substrate for the enzyme, and reactions were not performed in the presence of GSH. The molecular weight of AldDH was estimated to be about 90,000 *(72)*. Other investigators have studied AldDH from various species, and although they have provided physical and chemical data for these catalysts, none have evaluated the substrate potential of formaldehyde *(73,80,81)*.

D. Additional Reactions Involving Formaldehyde

Abeles and Lee *(82)* reported that crystalline horse liver ADH catalyzes the dismutation of formaldehyde to formic acid and methanol in the presence of NAD. The following reaction scheme was proposed:

$$\begin{array}{r} CH_2O + NAD \rightleftarrows HCOOH + NADH \\ CH_2O + NADH \rightleftarrows CH_3OH + NAD \\ \hline 2CH_2O \rightarrow HCOOH + CH_3OH \end{array}$$

They also noted that when methanol was initially present, the products included methylformate and suggested that this ester was formed by the oxidation of formaldehyde–methylhemiacetal by way of the following reaction sequence:

$$\begin{array}{r} CH_2OHOCH_3 + NAD \rightleftarrows HCOOCH_3 + NADH \\ CH_2O + NADH \rightleftarrows CH_3OH + NAD \\ \hline CH_2OHOCH_3 + CH_2O \rightarrow HCOOCH_3 + CH_3OH \end{array}$$

Hift and Mahler *(83)* described an enzyme isolated from beef liver which condenses formaldehyde and pyruvate in equimolar amounts to yield α-keto-γ-hydroxybutyric acid. The enzyme was concentrated in the mitochondrial fraction and appeared highly specific for both substrates.

Neither formaldehyde nor pyruvate disappeared when incubated singly with the enzyme. The presence of a similar enzyme could be demonstrated in whole tissue homogenates of rat kidney and liver but not of heart, diaphragm, spleen, or brain.

Neither methylformate nor α-keto-γ-hydroxybutyric acid have been thoroughly evaluated for their toxic potential.

E. The One Carbon Pool

Formaldehyde occupies, with formic acid, a central role in the metabolism of the one carbon pool. In 1950, DuVigneaud and Verly *(84)* demonstrated that the carbon atom of methanol was incorporated into the methyl groups of choline isolated from the tissues of the rat after the administration of methanol-^{14}C. It was also shown that the carbon atoms of formaldehyde and formate are incorporated into the methyl groups of choline *(85)*. That methanol and formate could serve as precursors of the methyl groups of choline was confirmed in a study by Arnstein *(86)*. Jonsson and Mosher *(87)* also demonstrated that formaldehyde was incorporated into the methyl groups of choline, and these investigators indicated that pteroylglutamic acid and vitamin B_{12} may be involved in this process.

The appearance of the carbon atom from methanol in choline isolated from animal tissues allows several interpretations: (1) it may be serving as a donor of intact methyl groups; (2) it is possible that methanol is a metabolic intermediate in the transfer of methyl groups; or (3) methanol may serve as a precursor of the methyl group through oxidation and subsequent reduction. This question was answered in a study by DuVigneaud *et al. (88)* (4) which employed methanol labeled in the methyl group with both deuterium and ^{14}C. The deuterium to ^{14}C ratio in the methyl groups of the choline isolated was considerably lower than that of the methanol injected. These experiments indicated that the methyl group of methanol was not being utilized directly in transmethylation, but was being utilized through oxidation and subsequent reduction. A similar study was performed with formatedeutero-^{14}C *(89)*. The lability of the formate hydrogen atom was determined and found to be extremely low and thus warranted the use of formate labeled with deuterium and ^{14}C as a biological tool. If the hydrogen atom of formate is retained in the conversion of the latter to the methyl group, the deuterium to ^{14}C ratio in the methyl group of choline would be expected to be one-third of that existing in the formate administered to the animal. This value is based upon the fact that formate would necessarily be diluted with two additional hydrogen atoms upon its conversion to the methyl group. The results of this study indicated that formate enters the methyl group with

no detectable loss of its hydrogen. It is thus clear, that in the conversion of formate into a labile methyl group, any intermediate possessing no hydrogen atom, such as carbon dioxide, cyanide, or isocyanate, and any intermediate possessing readily exchangeable hydrogen atoms under biological conditions are precluded. The results of these studies also indicate that it is at the oxidation level of formaldehyde that hydrogen is lost from the methyl group of methanol during the synthesis of labile methyl groups from the latter.

There are numerous reactions in which formaldehyde and formate enter into biosynthetic pathways. The primary single carbon transformations in which folic acid is directly or indirectly involved have been reviewed extensively *(90)*.

IV. THE METABOLIC DISPOSITION OF FORMIC ACID

Though formic acid may appear to be a prime candidate in the production of methanol toxicity, attempts to correlate formate accumulation with the acidosis of methanol poisoning have failed. Formic acid was found to represent a very small proportion of the total amount of organic acid excreted in the urine by monkeys poisoned with methanol *(2)*. The pyruvate and lactate levels in the urine of these animals were also very low *(2)*. Therefore, the major organic acids excreted by the monkey after methanol poisoning have yet to be identified. In 31 lethal cases of methanol poisoning in man, a marked variation appeared in blood levels of formate (5.7–274 mg/100 ml) *(1)*. The most likely explanation for variabilities in formate measurements following methanol ingestion resides in the lack of a sensitive and specific assay for formate.

The central role occupied by formate in the metabolism of the one carbon pool and reactions of formate in the presence of crystalline ADH have been discussed previously.

Formate has been shown to be oxidized in the intact animal *(91)* and in tissue homogenates *(92,93)*. Chance *(94)* demonstrated that catalase–hydrogen peroxide complex I reacts with formate and methanol at about the same rate. In 1953, Nakada and Weinhouse *(92)* presented evidence which indicated that the catalase peroxidative system is responsible for the oxidation of formate *in vivo*. Oro and Rappoport *(93)* demonstrated that formate oxidation *in vitro* could be inhibited with ethanol and presented evidence that inhibition was due to interactions of catalase–hydrogen peroxide complex I.

Since evidence has been presented that both methanol and formate can be oxidized by the peroxidative pathway, the question arises as to which step may be rate-limiting if both substances are oxidized in this manner *in*

vivo. In view of the demonstration by Chance *(94)* that formate and methanol are about equally reactive with the catalase peroxidative system, it would seem that the oxidation of methanol would be rate-limiting. During the initial period following the administration of methanol, the relative concentration of methanol to formate would be very high and methanol should inhibit the oxidation of formate by the catalase peroxidative system. However, it is obvious that there are more mechanisms available to the animal organism for the oxidation of formate to carbon dioxide than the catalase peroxidative pathway. The rate of oxidation of methanol to carbon dioxide in the monkey, a species in which the catalase peroxidative system has been shown to be functioning only minimally *(22,28),* is much faster than the rate of methanol oxidation to carbon dioxide in the rat *(19),* a species in which the catalase peroxidative system has been shown to be important *(19,28).* If the catalase peroxidative pathway were solely responsible for the oxidation of formate, one would expect the rate of oxidation of methanol to carbon dioxide to be lower in the monkey than in the rat and not higher as has been demonstrated *(19,22,28).*

Although the presence of an NAD-dependent formate dehydrogenase in *Pseudomonas oxalaticus* has been demonstrated by Johnson *et al. (95),* the existence of a similar enzyme in mammalian tissues has not been shown.

Leiberman *(96)* demonstrated that extracts of *Clostridium kluyveri* catalyze a decomposition of acetyl phosphate that is dependent upon the presence of formate and coenzyme A (CoA). This observation led Sly and Stadtman *(97,98)* to a series of studies in which they demonstrated two enzymatic pathways for the synthesis of formyl-CoA.

$$\text{acetyl-P} + \text{CoA-SH} \xrightleftharpoons{\text{phosphate acetyltransferase}} \text{acetyl-CoA} + P_i$$

$$\text{acetyl-CoA} + \text{formate} \xrightleftharpoons{\text{CoA-transferase}} \text{formyl-CoA} + \text{acetate}$$

$$\text{formyl-CoA} + H_2O \xrightleftharpoons{\text{formyl-CoA hydrolase}} \text{formate} + \text{CoA-SH}$$

$$\text{Net: acetyl-P} + H_2O \rightarrow \text{acetate} + P_i$$

In addition, the presence of two enzymes (one rather nonspecific) catalyzing the synthesis of formyl–CoA from formyl phosphate and CoA was identified from *Clostridium cylindrosporum (98).* However, the existence of this system in a mammalian species has not been demonstrated.

Therefore, the major system responsible for formate disposition and the impact of the presence of high levels of this acid has yet to be determined.

V. SUMMARY

Our knowledge of the metabolism of methanol and its metabolite, formaldehyde, has expanded over the last 10 years. We have learned of a species difference with respect to the relative importance of certain pathways for the metabolism of methanol to formaldehyde. In the rat, a catalase-dependent peroxidative oxidation of methanol is important while it appears to have no established function in methanol metabolism in the monkey. Hepatic ADH appears to be relatively important in the monkey for the conversion of methanol to formaldehyde, although it plays a lesser role in the rat. Even though the exact mechanism of this is still unexplained, it is likely that within the primate there is a deficiency of peroxide generation and a possible lack of peroxidative activity for catalase. These taken together could explain why the primate resorts to the ADH system for the metabolism of methanol to formaldehyde.

Formaldehyde metabolism has been examined in detail recently and the importance of a specific NAD- and GSH-dependent FDH has been evaluated in several species including man. While certain qualitative differences exist between the rat and human liver enzymes, there appears to be more activity in human liver than in rat liver for FDH as well as for other dehydrogenases that participate in the disposition of formaldehyde. However, because of the reactivity of formaldehyde, it still remains as one of the leading candidates for an explanation of methanol toxicity. Its presence has not been verified in an animal organism after the administration of methanol. This may well be due, however, to the high reactivity of this substance from which one could predict that a great deal of formaldehyde is nonspecifically bound to many types of endogenous substances, thereby altering their function.

Despite our increase in information on methanol and formaldehyde metabolism, we have very little new information available on formic acid utilization. This has been partly due to the lack of a specific assay which has been a plague to investigators in this area. This has been overcome to some degree with the development of sensitive fluorometric analysis for reduced pyridine nucleotides and the utilization of a specific formate dehydrogenase induced in cultures of *Pseudomonas oxalaticus*. It is hoped that this development will contribute to an improved understanding of the disposition of formate in the animal organism. The limitations of a catalase-dependent pathway were expressed in Section IV.

Perhaps our greatest dearth of information lies in an understanding of how metabolites contribute to the acidosis provoked by methanol in man. It may be that metabolites of methanol indirectly affect normal metabolic

pathways, whereby intermediates of those pathways accumulate and exert an effect on the organism. On the other hand, formic acid itself may contribute to the acidosis. Information currently available does not allow us to answer this question completely, but it is in this area that investigation currently is being intensified.

REFERENCES

1. Röe, O. (1959). The metabolism and toxicity of methanol. *Pharmacol. Rev.* **7**, 399–412.
2. Gilger, A. P., and Potts, A. M. (1955). The visual toxicity of methanol. V. The role of acidosis in experimental methanol poisoning. *Amer. J. Ophthalmol.* **39**, 63–86.
3. Potts. A. M. (1955). The visual toxicity of methanol. VI. The clinical aspects of experimental methanol poisoning treated with base. *Amer. J. Ophthalmol.* **39**, 86–92.
4. Röe, O. (1943). Clinical investigations of methyl alcohol poisoning with special reference to the pathogenesis and treatment of amblyopia. *Acta Med. Scand.* **113**, 558–608.
5. Röe, O. (1946). Methanol poisoning. Its clinical course, pathogenesis and treatment. *Acta Med. Scand.* **126**, Suppl. 182.
6. Keilin, D., and Hartree, E. F. (1936). Coupled oxidation of alcohol. *Proc. Roy. Soc., Ser. B* **119**, 141–159.
7. Keilin, D., and Hartree, E. F. (1945). Properties of catalase. Catalysis of coupled oxidation of alcohols. *Biochem. J.* **39**, 293–301.
8. Chance, B. (1947). An intermediate compound in the catalase hydrogen peroxide reaction. *Acta Chem. Scand.* **1**, 236–267.
9. Agner, K., and Belfrage, K. E. (1947). A specific micro-method for colorimetric determination of methanol in blood. *Acta Physiol. Scand.* **13**, 87–94.
10. Jacobsen, E. (1952). The metabolism of ethyl alcohol. *Pharmacol. Rev.* **4**, 107–135.
11. Theorell, H., and Bonnichsen, R. (1951). Studies on liver alcohol dehydrogenase. I. Equilibria and initial reaction velocities. *Acta Chem. Scand.* **5**, 1105–1126.
12. Heim, W. G., Appleman, D., and Pyfrom, H. T. (1956). Effects of 3-amino-1,2,4-triazole (AT) on catalase and other compounds. *Amer. J. Physiol.* **186**, 19–23.
13. Margoliash, E., and Novogrodsky, A. (1958). A study of the inhibition of catalase by 3-amino-1,2,4-triazole. *Biochem. J.* **68**, 468–475.
14. Margoliash, E., Novogrodsky, A., and Schejter, A. (1960). Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem. J.* **74**, 339–348.
15. Tephly, T. R., Mannering, G. J., and Parks, R. E., Jr. (1961). Studies on the mechanism of inhibition of liver and erythrocytic catalase activity by 3-amino-1,2,4-triazole (AT). *J. Pharmacol. Exp. Ther.* **134**, 147–151.
16. Nelson, G. H., Kinard, F. W., Aull, J. C., and Hay, M. G. (1957). Effect of aminotriazole on alcohol metabolism and hepatic enzyme activities in several species. *Quart. J. Stud. Alc.* **18**, 343–348.
17. Mannering, G. J., and Parks, R. E., Jr. (1957). Inhibition of methanol metabolism with 3-amino-1,2,4-triazole. *Science* **126**, 1241–1242.
18. Smith, M. E. (1961). Interrelations in ethanol and methanol metabolism. *J. Pharmacol. Exp. Ther.* **134**, 233–237.

19. Tephly, T. R., Parks, R. E., Jr., and Mannering, G. J. (1964). Methanol metabolism in the rat. *J. Pharmacol. Exp. Ther.* **143**, 292–300.
20. Aebi, H., Kiblet, H., and Wartburg, J. P., von. (1957). Uber den mechanismus der biologischen methanoloxydation. *Helv. Physiol. Pharmacol. Acta* **15**, 384–399.
21. VanHarken, D. R., Tephly, T. R., and Mannering, G. J. (1965). Methanol metabolism in the isolated perfused rat liver. *J. Pharmacol. Exp. Ther.* **149**, 36–42.
22. Makar, A. B., Tephly, T. R., and Mannering, G. J. (1968). Methanol metabolism in the monkey. *Mol. Pharmacol.* **4**, 471–483.
23. Koivusalo, M. (1959). Studies on the metabolism of methanol and formaldehyde *in vitro* in molybdenum deficiency induced by tungstate feeding. *Acta Physiol. Scand.* **45**, 109–115.
24. Tephly, T. R., Parks, R. E., Jr., and Mannering, G. J. (1961). The effects of 3-amino-1,2,4-triazole (AT) and sodium tungstate on the peroxidative metabolism of methanol. *J. Pharmacol. Exp. Ther.* **131**, 147–151.
25. Tephly, T. R., Atkins, M., Mannering, G. J., and Parks, R. E., Jr. (1965). Activation of a catalase peroxidative pathway for the oxidation of alcohols in mammalian erythrocytes. *Biochem. Pharmacol.* **14**, 435–444.
26. Blair, A. H., and Vallee, B. L. (1966). Some catalytic properties of human liver alcohol dehydrogenase. *Biochemistry* **5**, 2026–2034.
27. Van Harken, D. R. (1965). Role of the hepatic catalase-peroxidative system in the metabolism of methanol by the rat. Thesis submitted to the University of Minnesota for partial fulfillment of the requirements for the degree of Doctor of Philosophy.
28. Mannering, G. J., Van Harken, D. R., Makar, A. B., Tephly, T. R., Watkins, W. D., and Goodman, J. I. (1969). Role of the intracellular distribution of hepatic catalase in the peroxidative oxidation of methanol. *Ann. N. Y. Acad. Sci.* **168**, 265–280.
29. Kun, E. J. (1952). A study on the metabolism of glyoxal *in vitro*. *J. Biol. Chem.* **194**, 603–611.
30. Kun, E. J., Dechary, J. M., and Pitot, H. H. (1954). The oxidation of glycolic acid by a liver enzyme. *J. Biol. Chem.* **210**, 269–280.
31. Makar, A. B., and Mannering, G. J. (1968). Role of the intracellular distribution of hepatic catalase in the peroxidative oxidation of methanol. *Mol. Pharmacol.* **4**, 484–491.
32. Goodman, J. I., and Tephly, T. R. (1968). The role of hepatic microbody and soluble oxidases in the peroxidation of methanol in the rat and monkey. *Mol. Pharmacol.* **4**, 492–501.
33. Feinstein, R. N., Hampton, M., and Cotter, G. J. (1953). Species specificity of catalase. *Enzymologia* **16**, 219–225.
34. deDuve, C. (1965). The separation and characterization of subcellular particles. *Harvey Lect.* **59**, 49–87.
35. Goodman, J. I., and Tephly, T. R. (1970). Peroxidative oxidation of methanol in human liver: The role of hepatic microbody and soluble oxidases. *Research Commun. Chem. Pathol. Pharmacol.* **1**, 441–450.
36 Lutwak-Mann, C. (1938). Alcohol dehydrogenase of animal tissue. *Biochem. J.* **32**, 1364–1374.
37. Bonnichsen, R. K., and Wassen, A. M. (1948). Crystalline alcohol dehydrogenase from horse liver. *Arch. Biochem. Biophys.* **18**, 361–363.
38. Theorell, H., and Chance, B. (1951). Studies on liver alcohol dehydrogenase. II. The kinetics of the compound of horse liver alcohol dehydrogenase and reduced diphosphopyridine nucleotide. *Acta Chem. Scand.* **5**, 1127–1144.

39. Winer, A. D. (1958). A note on the substrate specificity of the horse liver alcohol dehydrogenase. *Acta Chem. Scand.* **12**, 1695–1696.
40. Wratten, C. C., and Cleland, W. W. (1965). Kinetic studies with liver alcohol dehydrogenase. *Biochemistry* **4**, 2442–2451.
41. Kini, M. M., and Cooper, J. R. (1961). Biochemistry of methanol poisoning. III. The enzyme pathway for conversion of methanol to formaldehyde. *Biochem. Pharmacol.* **8**, 207–215.
42. von Wartburg, J. P., Bethune, J. L., and Vallee, B. L. (1964). Human liver-alcohol dehydrogenase. Kinetic and physicochemical properties. *Biochemistry* **3**, 1775–1782.
43. Blair, A. H., and Vallee, B. L. (1966). Some catalytic properties of human liver alcohol dehydrogenase. *Biochemistry* **5**, 2026–2034.
44. Sund, H., and Theorell, H. (1963). Alcohol dehydrogenases. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd rev. ed., Vol. 7, pp. 25–83. Academic Press, New York.
45. Watkins, W. D., Goodman, J. I., and Tephly, T. R. (1970). Inhibition of methanol and ethanol oxidation by pyrazole in the rat and monkey *in vivo*. *Mol. Pharmacol* **6**, 567–572.
46. Lieber, C. S., Rubin, E., DeCarli, L. M., Misra, P., and Gang, H. (1970). Effects of pyrazole on hepatic function and structure. *Lab. Invest.* **22**, 615–621.
47. Theorell, H., and Yonetani, T. (1963). Liver alcohol dehydrogenase-DPN-pyrazole complex: A model of a ternary intermediate in the enzyme reaction. *Biochem. Z.* **338**, 537–553.
48. Li, T. K., and Theorell, H. (1969). Human liver alcohol dehydrogenase: Inhibition by pyrazole and pyrazole analogs. *Acta Chem. Scand.* **23**, 892–902.
49. Reynier, M. (1969). Pyrazole inhibition and kinetic studies of ethanol and retinol oxidation catalyzed by rat liver alcohol dehydrogenase. *Acta Chem. Scand.* **23**, 1119–1129.
50. Goodman, J. I., and Watkins, W. D. Unpublished observations.
51. Koe, B. K., and Tenen, S. S. (1970). Inhibiting action of n-butyraldoxime on ethanol metabolism and on natural ethanol preference of C57BL mice. *J. Pharmacol. Exp. Ther.* **174**, 434–449.
52. Watkins, W. D. Unpublished observations.
53. Lewis, W., and Schwartz, L. (1956). An occupational agent (n-butyraldoxime) causing reaction to alcohol. *Med. Ann. D. C.* **25**, 485–490.
54. Blomstrand, R., and Theorell, H. (1970). Inhibitory effect on ethanol oxidation in man after administration of 4-methyl pyrazole. *Life Sci.* **9**, 631–640.
55. Bliss, A. F. (1951). The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. *Arch. Biochem. Biophys.* **31**, 197–204.
56. Grangaud, R., and Nicol, M. (1966). Action de retinal sur la transformation *in vitro* de la transdéhydroepiandrosterone en androsténedione par le cortex surrénalien du rat blanc. *C. R. Acad. Sci.* **263**, 446–452.
57. Wald, G., and Hubbard, R. (1949). The reduction of retinene to Vitamin A_1 *in vitro*. *J. Gen. Physiol.* **32**, 367–389.
58. Wald, G., and Hubbard, R. (1960). Enzymic aspects of the visual processes. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd rev. ed., Vol. 3, pp. 369–386. Academic Press, New York.
59. Reading, H. W., and Sorsby, A. (1966). Alcohol-dehydrogenase activity of the retina in the normal and dystrophic rat. *Biochem. J.* **99**, 3c–5c.
60. Muirhead, J. J. (1967). Drug effects on retinal oxidation: Retinal alcohol: NAD^+ oxidoreductase. *Invest. Ophthalmol.* **6**, 635–641.

61. Koen, A. L., and Shaw, C. (1966). Retinal and alcohol dehydrogenases in retina and liver. *Biochim. Biophys. Acta* **128**, 48–54.
62. Watkins, W. D., and Tephly, T. R. (1971). Studies on the properties of retinal alcohol dehydrogenase from the rat. *J. Neurochem.* **18**, 2397–2406.
63. Bartlett, G. R. (1950). Inhibition of methanol oxidation by ethanol in the rat. *Amer. J. Physiol.* **163**, 619–621.
64. Koivusalo, M. (1956). Studies on the metabolism of methanol and formaldehyde in the animal organism. *Acta Physiol. Scand.* **39**, Suppl., 9.
65. Kesser, E. (1931). Ueber die ursache der giftigkeit des methyl-alkohols. *Deut. Med. Wothenschr.* **57**, 398–399.
66. French, D., and Edsall, J. T. (1945). The reactions of formaldehyde with amino acids and proteins. *Advan. Protein Chem.* **2**, 277–335.
67. Tyler, D. D. (1968). The inhibition of phosphate entry into rat liver mitochondria by organic mercurials and by formaldehyde. *Biochem. J.* **107**, 121–123.
68. Strittmatter, P., and Ball, E. G. (1955). Formaldehyde dehydrogenase, a glutathione-dependent enzyme system. *J. Biol. Chem.* **213**, 445–461.
69. Rose, Z. B., and Racker, E. (1962). Formaldehyde dehydrogenase from baker's yeast. *J. Biol. Chem.* **237**, 3279–3281.
70. Kinoshita, J. H., and Masurat, T. (1958). The effect of glutathione on the formaldehyde oxidation in the retina. *Amer. J. Ophthalmol.* **46**, 42–46.
71. Goodman, J. I., and Tephly, T. R. (1971). A comparison of rat and human liver formaldehyde dehydrogenase. *Biochim. Biophys. Acta* **252**, 489–505.
72. Kraemer, R. J., and Deitrich, R. A. (1968). Isolation and characterization of human liver aldehyde dehydrogenase. *J. Biol. Chem.* **243**, 6402–6408.
73. Blair, A. H., and Bodley, F. H. (1969). Human liver aldehyde dehydrogenase: Partial purification and properties. *Can. J. Biochem.* **47**, 265–272.
74. Snyder, R., Vogel, W., and Schulman, M. P. (1965). Effect of folic acid and analogues on the dehydrogenase and isomerase activities of liver alcohol dehydrogenase. *J. Biol. Chem.* **240**, 471–474.
75. von Wartburg, J. P., and Schurch, P. M. (1968). Atypical human liver alcohol dehydrogenase. *Ann N. Y. Acad. Sci.* **151**, 936–946.
76. Krerich, N. M., and Tomkins, G. M. (1967). *In* "Organizational Biosynthesis" (H. J. Vogel, J. O. Lampen, and V. Bryson, eds.), p. 189. Academic Press, New York.
77. Gaertner, F. H., and DeMoss, J. A. (1969). Purification and characterization of a multienzyme complex in the tryptophan pathway of Neurospora crassa. *J. Biol. Chem.* **244**, 2716–2725.
78. Smith, D., and Bauerle, R. H. (1969). The anthranilate synthetase-5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase complex of the tryptophan pathway in *Salmonella typhimurium*. Purification by the *in vitro* assembly of its subunits. *Biochemistry* **8**, 1451–1459.
79. Jakoby, W. B. (1963). Aldehyde dehydrogenases. *In* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd rev. ed., Vol. 7, pp. 203–221. Academic Press, New York.
80. Raison, J. K., Henson, G., and Rienits, K. G. (1966). The oxidation of gentisaldehyde by nicotinamide adenine dinucleotide-specific, aromatic aldehyde dehydrogenase from rabbit liver. *Biochim. Biophys. Acta* **118**, 285–298.
81. Feldman, R. I., and Weiner, H. (1972). Horse liver aldehyde dehydrogenase. I. Purification and characterization. *J. Biol. Chem.* **247**, 260–266.
82. Abeles, R. H., and Lee, H. A. (1960). The dismutation of formaldehyde by liver alcohol dehydrogenase. *J. Biol. Chem.* **235**, 1499–1503.

83. Hift, H., and Mahler, H. R. (1952). The enzymatic condensation of pyruvate and formaldehyde. *J. Biol. Chem.* **198**, 901–914.
84. DuVigneaud, V., and Verly, W. G. L. (1950). Incorporation *in vivo* of C^{14} from labeled methanol into the methyl groups of choline. *J. Amer. Chem. Soc.* **72**, 1049.
85. DuVigneaud, V., and Verly, W. G. L. (1950). Incorporation of the carbon of formaldehyde and formate into the methyl groups of choline. *J. Amer. Chem. Soc.* **72**, 2819–2820.
86. Arnstein, H. R. V. (1951). The biosynthesis of choline methyl groups by the rat. *Biochem. J.* **48**, 27–32.
87. Jonsson, S., and Mosher, W. A. (1950). The *in vivo* synthesis of labile methyl groups. *J. Amer. Chem. Soc.* **72**, 3316.
88. DuVigneaud, V., Verly, W. G. L., Wilson, J. E., Rachele, J. R., Ressler, C., and Kinney, J. M. (1951). One-carbon compounds in the biosynthesis of the "biologically labile" methyl group. *J. Amer. Chem. Soc.* **73**, 2782–2785.
89. Ressler, C., Rachele, J. R., and DuVigneaud, V. (1952). Studies *in vivo* on labile methyl synthesis with deuterio-C^{14}-formate. *J. Biol. Chem.* **197**, 1–5.
90. Stokstad, E. L. R., and Koch, J. (1967). Folic acid metabolism. *Physiol. Rev.* **47**, 83–116.
91. Weinhouse, S., and Friedman, B. (1952). Study of precursors of formate in the intact rat. *J. Biol. Chem.* **197**, 733–740.
92. Nakada, H. I., and Weinhouse, S. (1953). Studies of glycine oxidation in rat tissues. *Arch. Biochem. Biophys.* **42**, 257–270.
93. Oro, J., and Rappoport, D. A. (1959). Formate metabolism by animal tissues. II. The mechanism of formate oxidation. *J. Biol. Chem.* **234**, 1661–1665.
94. Chance, B. (1950). On the reaction of catalase peroxides with acceptors. *J. Biol. Chem.* **182**, 649–658.
95. Johnson, P. A., Mortimer, M. C. J., and Quayle, J. R. (1964). Use of a purified bacterial formate dehydrogenase for the micro-estimation of formate. *Biochim. Biophys. Acta* **89**, 351–353.
96. Lieberman, I. (1954). The influence of formate and coenzyme A on the decomposition of acetyl phosphate by extracts of *Clostridium kluyverii*. *Arch. Biochem. Biophys.* **51**, 350–366.
97. Sly, W. S., and Stadtman, E. R. (1963). Formate metabolism. I. Formyl coenzyme A, an intermediate in the formate-dependent decomposition of acetyl phosphate in *Clostridium kluyverii*. *J. Biol. Chem.* **238**, 2632–2638.
98. Sly, W. S., and Stadtman, E. R. (1963). Formate metabolism. II. Enzymatic synthesis of formyl phosphate and formyl coenzyme A in *Clostridium cylindrosporum*. *J. Biol. Chem.* **238**, 2639–2647.

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