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STUDIES ON METHANOL TOXICITY AND FORMATE METABOLISM IN ISOLATED HEPATOCYTES

THE ROLE OF METHIONINE IN FOLATE-DEPENDENT REACTIONS*

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Abstract—Following the administration of methanol to monkeys, formate accumulates and produces a marked metabolic acidosis and ocular toxicity. Formate does not accumulate in the rat, a species not sensitive to methanol toxicity. When isolated rat hepatocytes were incubated with methanol, the rate of methanol oxidation to carbon dioxide was reduced compared to the rate of oxidation observed in the rat *in vivo*. Furthermore, there was an accumulation of formate similar to that seen in the monkey *in vivo*. The enzymes in isolated hepatocytes which mediate methanol oxidation to formaldehyde, formate and carbon dioxide are similar to those reported for the rat *in vivo*. Thus, formate is oxidized to carbon dioxide by folate-dependent reactions. The addition of methionine to hepatocyte incubations containing methanol enhanced the oxidation rate of methanol to carbon dioxide by stimulating the oxidation of formate. Thus, in isolated hepatocytes the accumulation of formate during methanol oxidation was due to the development of a methionine deficiency which produced an apparent functional folate deficiency leading to a reduction in the rate of oxidation of formate to carbon dioxide. The importance of methionine in formate oxidation to carbon dioxide is also apparent in hepatocyte preparations obtained from folate-deficient rats. In these hepatocytes, the addition of 5-formyltetrahydrofolate alone was ineffective in restoring formate oxidation, but it was effective when methionine was also added. Isolated hepatocytes may be a useful model for studying the factors involved in regulating the metabolism of methanol to carbon dioxide, since it is possible to regulate each step in the pathway of methanol oxidation to carbon dioxide.

Methanol toxicity in man is characterized by a 12–24 hr latent period, followed by metabolic acidosis, ocular toxicity and death [1–3]. Studies in our labaratory [4–6] and other labaratories [7, 8] have demonstrated that rhesus and pigtail monkeys may serve as appropriate models for the study of methanol poisoning in man. In contrast, rodents do not exhibit the methanol poisoning syndrome seen in man and monkey [1, 2]. The metabolic acidosis and ocular toxicity appear to be due to a metabolite or metabolities of methanol and not to the alcohol *per se*. This is indicated by the latent period before signs of toxicity develop and by the observations of Roë [9, 10] that the administration of ethanol soon after the ingestion of methanol significantly alters the time course and pattern of methanol toxicity. The species differences in susceptibility to methanol toxicity suggests that there is a qualitative or quantitative difference between the metabolism of methanol in a sensitive species, such as the monkey, and its metabolism in an insensitive species, such as the rat. Methanol is metabolized to carbon dioxide through the intermediates, formaldehyde and formic acid. Recent studies indicate that formate accumulates in monkeys given methanol and that its accumulation correlates with the development of metabolic acidosis [4, 5, 7] and ocular toxicity [6]. Formate does not accumulate after methanol administration to an insensitive species, such as the rat [11]. Ocular toxicity, identical to that seen after methanol ingestion, has been produced in monkeys by the administration of sodium formate [12].

The accumulation of formate in monkeys appears to be due to its slower rate of metabolism in monkeys than in rats [13]. Two pathways have been suggested for the disposition of formate. One involves the reaction of formate with catalase–peroxide complex I [14]. This pathway appears to predominate when formate oxidation is measured *in vitro* [15, 16]. However, the involvement of this pathway *in vivo* has not been demonstrable [17]. An alternative mechanism for the metabolism of formate involves folate-dependent reactions [18, 19]. It has been shown recently that formate is metabolized in both the rat and the monkey by folate-dependent reactions [13, 17]. Since the rate of formate metabolism is slower in the monkey than in the rat, the accumulation of formate may relate to a difference in the qualitative or quantitative nature of the folate-dependent system. Indeed, Makar and Tephly [20] have observed accumulation of formate in methanol-treated folate-deficient rats, and these rats developed severe metabolic acidosis.

Further studies on factors which regulate formate metabolism by folate-dependent reactions would be greatly facilitated by an *in vitro* model system. Recently, Krebs *et al.* [21] reported that hepatocytes isolated from untreated rats exhibit certain characteristics of folate deficiency. They observed that hepatocytes incubated with L-histidine accumulated *N*-formiminoglutamate (FIGLU), an intermediate of histidine degradation which is metabolized through folate-dependent reactions. The accumulation of FIGLU was abolished by addition of L-methionine to the incubation medium. The effect of methionine on FIGLU metabolism was also reflected as stimulation of the conversion of histidine to carbon dioxide. The oxidation of formate to carbon dioxide was also stimulated by methionine.

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The present studies with isolated rat hepatocytes were initiated with the idea that this system might be an appropriate model for studying the regulation of formate metabolism by folate-dependent reactions. Our results show that, in isolated rat hepatocytes, formate is metabolized by folate-dependent reactions. Furthermore, as in the monkey which is a methanol-sensitive species, formate accumulates from methanol in isolated rat hepatocytes. Methionine abolishes formate accumulation by stimulating the metabolism of formate to carbon dioxide. The results provide additonal evidence for the importance of methionine in maintaining the functional integrity of folate 1-carbon pool reactions.

MATERIALS AND METHODS

Chemicals. [^{14}C]-Methanol, [^{14}C]-sodium formate and [^{14}C]-formaldehyde were obtained from New England Nuclear (Boston, MA). L-[ring-2-^{14}C]Histidine was purchased from the Amersham-Searle Corp. (Arlington Heights, IL). Collagenase, Type IV or V, was obtained from the Sigma Chemical Co. (St. Louis, MO). It was selected for use on the basis of the ratio of collagenase to protease activity, which was determined by the procedure of Mandl *et al.* [22]. Preparations with a collagenase to protease ration of about 5:1 were most satisfactory in the preparation of isolated hepatocytes. All other chemicals were of reagent quality and they were purchased from commercial suppliers.

Animals. Male Sprague–Dawley rats (220–280 g) were obtained from Bio-Labs (Madison, WI). They were maintained on standard Purina Laboratory Chow.

For the folate-deficiency study, weanling rats (25 days old) were divided into two groups. One group was fed a folate-deficient diet which also contained 2% succinylsulfathiazole to inhibit folate synthesis by intestinal bacteria, and the other group was fed the same diet supplemented with 2.25 mg folic acid/kg diet. The diets were obtained from Bio-Serv, Inc. (Frenchtown, NJ). Rats were maintained on these diets for 8–10 weeks. Folate-deficiency was then assessed by measuring the urinary excretion of FIGLU by the method of Tabor and Wyngarden [23], using kits obtained from the Sigma Chemical Co. Rats on the folate-deficient diet excreted an average of 69 mg FIGLU/kg body wt/day (range 23–124), while animals on the control diet excreted an average of 0.29 mg/kg body wt/day (range 0.10–0.54). FIGLU excretion was determined without administering a histidine load.

Preparation of isolated hepatocytes. Hepatocytes were isolated by a modification of a method described previously [24]. The liver was perfused *in situ* for 5 min at 37° with Ca^{2+}-free Krebs–Henseleit buffer containing 0.5 mM EGTA.* The liver was then removed and perfused at 37° for 5 min with 150 ml of recirculating Ca^{2+}-free Krebs–Henseleit buffer containing 1% bovine serum albumin (Sigma, Fraction V). Collagenase, 15,000 units, was then added in 3.75 ml of 0.1 M $CaCl_2$ and the perfusion was contained for 8 min. One unit of collagenase is defined as the amount of enzyme which releases amino acids from native collagen equivalent in ninhydrin color to 1 μmole of leucine in 2 hr at pH 7.4 at 37°. The liver was then placed in a petri dish and the capsule was removed with forceps. The hepatocytes were released by gently stirring the liver in 40–50 ml of Krebs–Henseleit buffer. The cell suspension was filtered through a single layer of size 220 nylon mesh and the filtrate was centrifuged at 50 *g* for 45 sec. The cell pellet was collected, and washed twice by resuspending in buffer and repeating the centrifugation. The washed, isolated hepatocytes were then suspended in Krebs–Henseleit buffer containing 2% bovine serum albumin (Sigma, crystalline, fatty acid free). The final suspension contained 4–8 $\times$ 10^6 cells/ml. All preparations used in these experiments were greater than 90 per cent viable, as judged by trypan blue exclusion. In addition, viability was periodically assessed by lactate dehydrogenase release, and it was found that less than 20 per cent of the cellular lactate dehydrogenase was released during a 2 hr incubation.

Incubation of hepatocytes. One-ml aliquots of the isolated hepatocyte suspension were placed in 25-ml Erlenmeyer flasks containing both a center well and a side arm. The stoppered flasks were then gassed through the side arm for 5 min with CO_2:O_2 (5:95), while shaking at 80 rev/min in an orbital shaker operated at 37°. The side arm was then stoppered and the reaction was initiated by the addition of the substrate in 50 μl H_2O. For most experiments, the concentration of [^{14}C]-methanol was 10 mM with a specific activity of 100,000 dis./min/μmole, and the concentration of [^{14}C]-sodium formate was 2 mM with a specific activity of 25,000 dis./min/μmole. The concentration of histidine was 2 mM (specific activity, 100,000 dis./min/μmole). The incubation was stopped by the addition of 0.1 ml of 1 N HCl. At the same time, 0.5 ml of 2 N NaOH was injected through the stopper into the center well in order to trap $^{14}CO_2$. The incubations were allowed to stand at room temperature for 2 hr to collect CO_2.

Determination of reaction products. $^{14}CO_2$ which was formed from [^{14}C]-sodium formate or [^{14}C]-histidine was determined by directly counting 0.2-ml aliquots of the center well contents in 15 ml of 3a70B liquid scintillating mixture (Research Products Corp., Elk Grove Village, IL) containing 4ml H_2O. With incubations containing [^{14}C]-methanol, it was necessary to precipitate the carbonate as the barium salt since significant methanol distilled into the center well solution. This was accomplished by diluting a 0.2-ml aliquot of the center well contents to 2 ml with H_2O and adding 2 ml of a 2 N solution of $BaCl_2$ containing 2 N NH_4Cl. $BaCO_3$ was collected in Millipore filters (0.45 μm) and the radioactivity was determined by liquid scintillation spectrometry. The recovery of $^{14}CO_2$ in both procedures was essentially 100 per cent.

Following removal of $^{14}CO_2$, protein was precipitated from the hepatocyte incubation mixture by the addition of 0.2 ml of 7.5% $ZnSO_4$ and 0.2 ml of 0.4 N NaOH; the protein was removed by centrifugation. For the determination of [^{14}C]-formaldehyde, the dimedon precipitation method was used [25]. A 0.5-ml aliquot of the protein-free supernatant fraction was added to 1 ml of 1 M sodium acetate buffer (pH 4.5), 10 ml of 0.5% dimedon and 5 μl of unlabeled 37% formaldehyde. The reaction mixture was heated in a boiling water bath for 5 min, allowed to cool in ice for 30 min,

* EGTA = ethyleneglycolbis(aminoethylether)tetraacetate.

and then the precipitate was collected on filter paper. The precipitate was counted by liquid scintillation spectrometry.

[^{14}C]-Formate, formed from methanol, was measured by conversion to $^{14}CO_2$ with formate dehydrogenase isolated from cultures of *Pseudomonas oxalaticus* [26, 27]. A 0.5-ml aliquot of the protein-free supernatant fraction of the hepatocyte incubation mixture was incubated for 2 hr with 0.5 mM NAD and 0.03 units formate dehydrogenase in a total volume of 1 ml of 0.25 M potassium phosphate buffer, pH 7.4. Under these conditions, [^{14}C]-formate was quantitatively converted to $^{14}CO_2$. The incubations were conducted in stoppered flasks with a center well containing 0.5 ml of 2 N NaOH. The reactions were terminated by injecting 0.5 ml of 25% perchloric acid into the flasks. The flasks were allowed to stand for 2 hr at room temperature to allow the absorption of $^{14}CO_2$ into the NaOH. $^{14}CO_2$ in the center well contents was then precipitated as the barium salt, as described previously.

Standard curves for the determinations of [^{14}C]-formate and [^{14}C]-formaldehyde were constructed by adding known amounts of metabolite to unincubated hepatocyte suspensions and carrying the samples through the entire procedure. These new methods were developed because previously used methods [26–28] were not sufficiently sensitive to measure the concentrations of formate and formaldehyde present in incubation samples containing small numbers of isolated hepatocytes.

Methanol concentrations were determined by the gas chromatographic method of Baker *et al.* [29].

RESULTS

Pattern of methanol metabolism and rate of metabolism. Figure 1 shows that incubation of [^{14}C]-methanol (10 mM) with isolated hepatocytes resulted in the formation of substantial amounts of [^{14}C]-carbon dioxide. In addition, there was a significant accumulation of [^{14}C]-formate, although [^{14}C]-formaldehyde was detectable in only trace amounts through the course of incubation. Very little radioactivity was incorporated into other, inidentified metabolites. For example, only about 7 per cent of the total methanol metabolism which occurred in a 2-hr incubation was to radioactive products which precipitated from 6% trichloroacetic acid. In addition, about 10 per cent of the radioactive metabolites were unidentified water-soluble, non-volatile

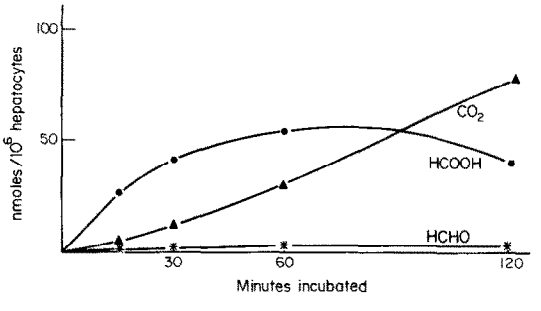

Fig. 1. Accumulation of formate from methanol in isolated hepatocytes. [^{14}C]-Methionine (10 mM) was incubated with isolated hepatocytes as described in Materials and Methods. Data given are representative of three experiments.

metabolites. This was determined by incubating [^{14}C]-methanol for 120 min with isolated hepatocytes, and then extracting, with diethylether, the [^{14}C]-formic acid in the incubation sample. The extraction procedure was followed by distillation of the aqueous phase to remove remaining unmetabolized methanol and other volatile metabolites such as formaldehyde.

Incubation of varying amounts of [^{14}C]-methanol for 30 min showed that a concentration of 50 mM was necessary to approach maximum velocity. At this concentration the overall rate of methanol oxidation, as indicated by the sum of formaldehyde, formate and carbon dioxide, was found to be 3.6 ± 0.81 nmoles/min/10^6 hepatocytes. Assuming that there are 120×10^6 hepatocytes/g of liver, this rate is equivalent to 432 nmoles/g of liver/min, a rate comparable to the rate of methanol oxidation obtained in the intact rat. The maximum rate of carbon dioxide formation *in vivo* was reported to be 390 nmoles/g of liver/min [30]. However, formate does not accumulate in the intact rat as it does in isolated hepatocytes [11, 20]. The metabolic pattern seen in isolated hepatocytes does resemble the pattern seen in the monkey *in vivo* where formate accumulates after methanol treatment [4, 5, 7].

Effects of inhibition and stimulation of the catalase–peroxidative pathway. To explore the involvement of the catalase-peroxidative pathway in the oxidation of methanol and of formate, two experiments were designed. In the first experiment, rats were treated with 3-amino-1,2,4-triazole (AT), an irreversible catalase inhibitor [31, 32], prior to the preparation of hepatocytes. As expected, this treatment results in about a 90 per cent inhibition of hepatic catalase activity (Table 1). In cells from AT-treated rats, the rate at which formate accumulated was reduced by about 50 per cent and formaldehyde concentrations were undetectable. The rate of CO_2 formation was unchanged by catalase inhibition, a result which would be expected if the formation of CO_2 from formate was independent of catalase activity. This was shown by incubation of formate with hepatocytes from AT-treated rats (Table 1).

Glycolate is known to stimulate the oxidation of substrates of the catalase–peroxidative pathway by generating hydrogen peroxide, the rate-limiting component of the overall reaction [34]. Table 2 shows that addition of glycolate to the incubation medium containing [^{14}C]-methanol resulted in a concentration-dependent increase in the rate of accumulation of [^{14}C]-formaldehyde and [^{14}C]-formate, but there was only a slight increase in the rate of formation of $^{14}CO_2$. These results indicate that glycolate stimulated the rate of methanol oxidation about 4– to 5-fold. Identical experiments using [^{14}C]-formate as the substrate showed that there was very slight stimulation of formate oxidation (Table 2) compared to the increase seen in methanol oxidation.

Effect of folate deficiency. To investigate the role of folate-dependent reactions in the oxidation of formate, hepatocytes were prepared from folate-deficient rats and from pair-fed control rats. The mean rate ($\pm$ S.E.) of formate oxidation was reduced from 20 ± 2.6 nmoles CO_2/10^6 hepatocytes/30 min in control rats to 9.1 ± 1.5 nmoles/10^6 hepatocytes/30 min in folate-deficient rats.

The only known route of L-[ring-2-^{14}C]histidine oxi-

Table 1. Effects of aminotriazole on methanol and formate metabolism*

	Methanol metabolism				Formate Metabolism
	HCHO	HCOOH	CO_2	Total	CO_2
Control	1.7 ± 0.19	41 ± 8.1	11 ± 3.2	54 ± 11	30 ± 6.8
AT-Treated†	‡	16 ± 1.0	13 ± 1.9	29 ± 1.2	36 ± 9.9

* Results are expressed as nmoles/10^6 hepatocytes/30 min ± S.E. (control, n = 7; AT, n = 6).

† Rats were given 1 g/kg of 3-amino-1,2,4-triazole (AT) 1 hr prior to hepatocyte preparation. Catalase activity (Kat f) was determined by the method of Feinstein [33] and it was found to be 0.87 ± 0.13/10^6 cells in control hepatocytes and 0.068 ± 0.013/10^6 cells in hepatocytes from AT-treated rats.

‡ Not detectable.

dation to $^{14}CO_2$ depends upon tetrahydrofolate [35]. For this reason, the effect of folate deficiency on histidine oxidation was also determined. In hepatocytes from control rats, the rate of $^{14}CO_2$ formation from L-[ring-2-^{14}C-]histidine was 3.0 ± 0.56 nmoles/10^6 hepatocytes/30 min but in hepatocytes from folate-deficient rats the rate was 1.5 ± 0.05 nmoles/10^6 hepatocytes/30 min. These results show that folate deficiency reduces formate and histidine oxidation to about the same extent, an indication that both substrates depend upon folate derivatives for their conversion to CO_2.

Effect of L-*methionine.* The addition of L-methionine stimulated the rate of formate oxidation to CO_2 (Fig. 2). This kinetic experiment indicates that the degree of stimulation is dependent upon the concentration of formate, and, most importantly, that methionine stimulates the maximum velocity of formate oxidation to CO_2.

The methionine-stimulated maximum rate (± S.E.) of formate oxidation was 4.53 ± 0.48 nmoles/min/10^6 hepatocytes (n = 3). Assuming a cell density of 120 × 10^6 hepatocytes/g of liver, this rate (544 nmoles/g of liver) is the same order of magnitude as the maximal rate of formate oxidation previously found *in vivo* (430 nmoles/g of liver/min) [17].

The addition of L-methionine to hepatocyte incubation mixtures containing methanol abolished the accumulation of formate (Table 3) and stimulated CO_2 formation. There was no effect on the accumulation of formaldehyde. That methionine had no effect on the first step of methanol metabolism was shown by measuring methanol disappearance. Upon incubation on 1.0 μmole [^{14}C]-methanol with 1 ml of isolated hepatocytes for 120 min, 0.33 μmole was metabolized, both in the presence and absence of 1 mM methionine. In this experiment, 88 nmoles of formate were present at the end of the incubation period but only 6 nmoles were found when methionine was added to the incubation. The rest of the metabolized methanol was accounted for as $^{14}CO_2$.

Once formate has entered the folate-dependent 1-carbon pool via formation of 10-formyltetrahydrofolic acid, it can be incorporated into a variety of metabolic products, such as serine, purines and proteins, as well as being oxidized to CO_2 [36]. The extent of formate metabolism by these alternate metabolic pathways was investigated and found to be minor (Table 4). About 85 per cent of the formate metabolized is converted to CO_2. Furthermore, the addition of methionine does not alter the proportion of formate oxidized to CO_2, but rather it stimulates formate disappearance in addition to CO_2 formation.

To explore the possibility that there might be a lack of tetrahydrofolate in the hepatocyte system, 5-formyltetrahydrofolate, a compound which has been shown to restore folates *in vivo* [37–39], was added. However, the addition of 5-formyltetrahydrofolate did not stimulate formate or histidine metabolism to CO_2 in hepatocytes from either control rats of folate-deficient rats (Table 5). On the other hand, methionine stimulated CO_2 formation in folate-deficient cells as well as in control cells. In the presence of methionine, the addition of 5-formyltetrahydrofolate stimulated further the rate of formate oxidation in folate-deficient cells, but had no effect in control cells. Similar results were obtained when an equimolar amount of folate was substituted for 5-formyltetrahydrofolate. These results indicate that 5-formyltetrahydrofolate and folate cannot restore tetrahydrofolate concentrations in the absence of methionine. It is further apparent from this

Table 2. Effects of glycolate on methanol and formate metabolism*

Glycolate	Methanol metabolism				Formate metabolism
(mM)	HCHO	HCOOH	CO_2	Total	CO_2
0	5	23	28	56	25
0.05	5	38	26	69	33
0.5	17	111	46	174	45
5.0	73	156	37	266	45
50	70	145	45	260	40

* Results are expressed as nmoles product/10^6 hepatocytes/30 min. Sodium glycolate and methanol (10 mM) or formate (2 mM) were added to hepatocyte incubations after gassing the incubation flasks with O_2:CO_2 (95:5) for 5 min. The incubations were conducted for 30 min. Data given are for one experiment which is representative of three separate experiments.

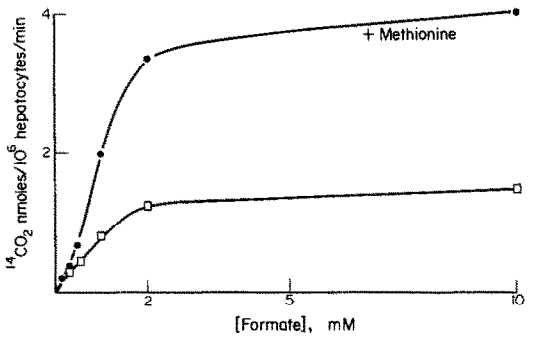

Fig. 2. Effect of methionine on [^{14}C]-formate oxidation to $^{14}CO_2$. Hepatocytes were incubated with methionine (1 mM) for 15 min prior to the addition of formate. The incubation was continued for 30 min. The rate of formate oxidation, both in the presence and absence of methionine, was linear for this time period. The concentration of methionine was determined to achieve maximal stimulation. Data given are representative of three experiments.

Table 4. Effects of methionine on formate metabolism to CO_2 and other metabolites*

	Formate remaining	CO_2	TCA insoluble	Other†
Control	3.67	2.01	0.06	0.27
Methionine (1 mM)	2.00	2.99	0.04	0.66

* Results are expressed as μmoles metabolite after 6 μmoles [^{14}C]-formate was incubated for 2 hr with 3 ml (24×10^6) hepatocytes. $^{14}CO_2$ was determined as described in Materials and Methods. An aliquot of the incubation mixture was assayed for formate as described in Materials and Methods. The remaining incubation mixture was deproteinized with trichloroacetic acid (TCA) and then extracted with diethyl ether to remove formic acid. Formic acid was added to zero time incubation samples and carried through the procedure in order to correct for its contamination in each fraction.

† Unextractable metabolites.

experiment that folates are not rate-limiting components in formate or histidine oxidation in hepatocytes from control rats in the presence of adequate methionine.

DISCUSSION

Isolated rat hepatocytes are an appropriate model for studying methanol toxicity which results from formate accumulation in methanol-sensitive species. Formate accumulates in isolated rat hepatocytes as it does in the monkey, a species which develops metabolic acidosis [4, 7] and visual disturbances [2, 6, 8] following methanol administration. It has been found in the present study that the key steps in the metabolism of methanol are catalyzed by the same enzyme systems in isolated hepatocytes as they are *in vivo*. Thus, methanol is metabolized to formaldehyde by the catalase–peroxidative system whereas formate is oxidized to CO_2 by folate-dependent reactions. The involvement of the folate pathway as the primary route of formate metabolism is indicated by the observation that the oxidation rate of formate and histidine to CO_2 is reduced to the same extent in hepatocytes from folate-deficient rats. That the catalase–peroxidative system is of minor importance in formate oxidation in isolated hepatocytes was shown by methods which stimulate and inhibit this system. These manipulations dramatically affected methanol oxidation to formaldehyde but had little or no effect on formate oxidation to CO_2. Jones *et al.* [40] recently reported that formate will form a complex with catalase compound I in isolated hepatocytes, but these investigators did not attempt to demonstrate that formate is metabolized through this system. In accordance with our observations, Waydhas *et al.* [41] have also concluded that formate is not appreciably oxidized in isolated hepatocytes by the catalase–peroxidative system.

Although formate is metabolized by folate-dependent reactions in isolated hepatocytes as well as *in vivo*, it does not accumulate in the rat after methanol treatment *in vivo* as it does in isolated rat hepatocyte preparations. The accumulation is due to a slower rate of formate metabolism in the isolated hepatocytes than that obtained in the rat *in vivo*. This indicated by the observation that methionine stimulates formate metabolism in isolated hepatocytes and abolishes the accumulation of formate which results from methanol metabolism. Recent studies in this laboratory have established that formate accumulates in monkeys administered methanol due to a slower rate of formate metabolism in the monkey than in the rat [13]. Thus, the metabolism of methanol is isolated hepatocytes resembles the pattern of methanol metabolism in a methanol-sensitive species, such as the monkey, *in vivo*. This is an extremely useful observation and allows for other studies *in vitro* on the regulation of formate metabolism, folate biochemistry, and the mechanism of species differences in the rate of formate metabolism.

Krebs *et al.* [21] observed that in isolated hepatocytes histidine oxidation to CO_2 is impaired, and that

Table 3. Effects of L-methionine on formate accumulation and CO_2 formation from methanol*

Incubation (min)	HCOOH		CO_2	
	Control	Methionine	Control	Methionine
15	30 ± 1.9	8.2 ± 1.9	4.8 ± 1.6	16 ± 1.1
30	41 ± 5.4	6.4 ± 1.8	11 ± 3.2	31 ± 1.8
60	54 ± 11	3.3 ± 6.7	29 ± 3.0	58 ± 7.2
120	41 ± 3.2	1.2 ± 0.12	78 ± 2.0	83 ± 2.8

* Results are expressed as nmoles product/min/10^6 hepatocytes ± S.E. (n = 3). Hepatocytes were incubated for 15 min. with methionine (1 mM) prior to the addition of methanol (10 mM).

Table 5. Effects of methionine and 5-formyltetrahydrofolic acid on formate and histidine oxidation*

Addition	Formate oxidation		Histidine oxidation	
	Control cells	Folate-deficient cells	Control cells	Folate-deficient cells
None	20 ± 2.6	9.1 ± 1.5	3.0 ± 0.56	1.5 ± 0.05
5-formyl-THF	19 ± 1.5	9.9 ± 1.8	2.8 ± 0.42	1.7 ± 0.04
Methionine	36 ± 6.7	15 ± 2.2	12 ± 2.9	6.9 ± 1.6
Methionine + 5-formyl-THF	40 ± 6.9†	27 ± 2.1†‡	13 ± 2.6†	14 ± 2.5†‡

* Hepatocytes were incubated for 15 min with methionine (1 mM) and/or 5-formyl-THF (0.1 mM) prior to the addition of formate (2 mM) or histidine (2 mM). The incubations were continued for 30 min. The concentrations of methionine and 5-formyl-THF were previously found to be maximally effective. Results are expressed as nmoles $^{14}CO_2/10^6$ hepatocytes/30 min ± S.E. (n = 3).

† Not significantly different from the rate when methionine alone was added to hepatocytes from control rats.

‡ Significantly different from methionine alone. $P < 0.05$.

this is accompanied by the accumulation of FIGLU. Since the metabolism of both formate and FIGLU depends upon the integrity of the folate system, the isolated hepatocytes appear to display a functional folate deficiency. Krebs *et al.* [21] and Waydhas *et al.* [41] reported, and we have confirmed, that methionine restores the function of the folate system to normal. Thus, methionine abolishes the accumulation of FIGLU from histidine and the accumulation of formate from methanol. Neither of these metabolites accumulates *in vivo* in the rat. Krebs *et al.* [21] observed further that the concentration of methionine in their isolated hepatocyte perparation was only about 10 per cent of that found in intact livers. Our preparation contains similar concentrations of methionine. That methionine plays a role in folate metabolism in vitamin B_{12} of folate-deficient animals is well known [42]. However, the importance of methionine in maintaining the normal operation of folate-dependent reactions in the presence of normal concentrations of folate and vitamin B_{12} was not appreciated until the work of Krebs *et al.* [21]. The experiments described here extend the work of Krebs *et al.* [21]. For example, we have observed that, in hepatocytes from folate-deficient rats, neither 5-formyltetrahydrofolate nor folate stimulates formate or histidine metabolism unless methionine is also supplied. The additional observation that in the presence of methionine supplementary folates do not stimulate formate or histidine oxidation in control hepatocytes indicates that tetrahydrofolate does not limit the rate of either reaction. Whether tetrahydrofolate is rate-limiting in the absence of methionine cannot be discerned from the experiment. Other experiments must be done to determine whether methionine facilitates the generation of more tetrahydrofolate. Figure 3 shows the metabolism of histidine and formate by the folate system. We have observed that methionine stimulates the "maximum velocity" of formate oxidation to CO_2. This effect could be due to (1) stimulation of one of the enzymes, (2) an increase in the proportion of 10-formyltetrahydrofolate which is oxidized to CO_2 of (3) an increase in the concentration of a co-factor such as tetrahydrofolate (THF), ATP or NADP$^+$. We have observed* that 10-formyl-THF synthetase activity in rat liver cytosol is not altered by or dependent upon either methionine of *S*-adenosyl methionine (SAM). Krebs *et al.* [21] noted no effects on 10-formyl-THF dehydrogenase. We have observed that formate disappearance, as well as CO_2 formation from formate, in hepatocytes is enhanced by methionine. This result indicates that methionine is producing its stimulatory effect on 10-formly-THF synthetase rather than on the 10-formyl-THF dehydrogenase step. Since L-[ring-2-

* R. E. Billings, P. E. Noker and T. R. Tephly, manuscript submitted for publication.

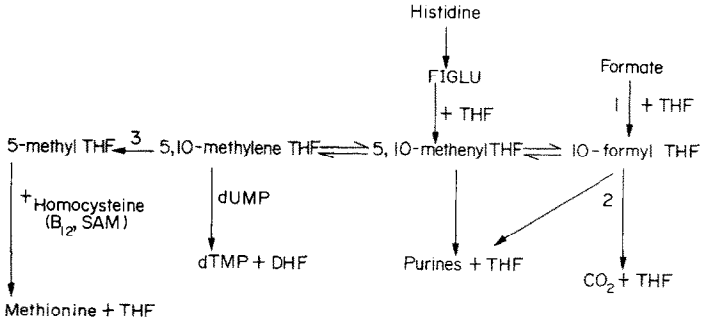

Fig. 3. Metabolism of formate and histidine by folate-dependent reactions. Reaction 1 is catalyzed by 10-formyl-THF synthetase and requires prior activation of formate with ATP. Reaction 2 involves 10-formyl-THF dehydrogenase and utilizes NADP$^+$ for hydrogen transfer. Reaction 3 is catalyzed by 5,10-methylene-THF reductase, is essentially irreversible, and is inhibited by SAM [43, 44]. Reaction 4 leads to the regeneration of THF and it is catalyzed by methionine synthetase, which is a vitamin B_{12} associated-enzyme. This reaction also requires catalytic amounts of SAM.

[^{14}C]histidine oxidation to $^{14}CO_2$ is stimulated by methionine, as is formate utilization, and since different enzymes catalyze these steps, the most likely explanation linking these events is the regulation by methionine of tetrahydrofolate concentrations.

Krebs *et al.* postulated that the mechanism of methionine stimulation of formate and histidine oxidation to CO_2 was to enhance the activity of 10-formyl-THF dehydrogenase due to an increase in the concentration of 10-formyl-THF. This increased concentration was proposed to be due to an inhibition of 5,10-methylene-THF reductase (Fig. 3, reaction 3), resulting in an increased concentration of 5,10-methylene-THF which is in equilibrium with 10-formyl-THF. That 5,10-methylene-THF reductase is inhibited by SAM, the concentration of which is dependent upon the concentration of methionine [45], is known from the work of Kutzbach and Stokstad [43, 44]. The additional information obtained in our studies is not consistent with the idea that 10-formyl-THF dehydrogenase activity is stimulated by methionine. Rather our results indicate that an increased methionine concentration leads to an increased concentration of THF. Of course, an increased concentration of 10-formyl-THF, and thus enhanced activity of 10-formyl-THF dehydrogenase, would result secondarily from the increased THF concentration.

An increased THF concentration could be the result of methionine stimulation of any of the reactions which generate THF (Fig. 3). However, it has been suggested that THF is generated primarily through the methionine synthetase reaction (Fig. 3, reaction 4), a reaction which requires catalytic amounts of SAM [46–48]. Additional experiments are in progress to determine the involvement of SAM and methionine synthetase in the effect of methionine on folate-dependent reactions.

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