8. Bessman, M. J., Lehman, I. R., Simms, E. S., Kornberg, A., *J. Biol. Chem.*, 1958, v233, 171.
9. Bollum, F. J., Potter, V. R., *ibid.*, 1958, v233, 478.
10. Bollum, F. J., *ibid.*, 1960, v235, 2399.
11. Davidson, J. N., Smellie, R. M. S., Keir, H. M., McArdle, A. H., *Nature*, 1958, v182, 589.
12. Furlong, N. B., Griffin, A. C., *Fed. Proc.*, 1960, v19, 306.
13. Furlong, N. B., *Arch. Biochem. and Biophys.*, 1960, v87, 1954.
14. Gornal, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.
15. Furlong, N. B., *Analyt. Biochem.*, 1963, v5, 515.

Received October 24, 1963. P.S.E.B.M., 1964, v115.

Inhibition of Alcohol Dehydrogenase by Folic Acid and Several of Its Analogs.* (28963)

WOLFGANG H. VOGEL, ROBERT SNYDER AND MARTIN P. SCHULMAN
(Introduced by K. R. Unna)
Department of Pharmacology, University of Illinois College of Medicine, Chicago

The biochemical function of folic acid as a necessary cofactor in one-carbon metabolism has been well documented. While studying the oxidation of ethanol in homogenates of rat liver, it was observed that folic acid arrested ethanol metabolism. This finding led to the discovery that folic acid inhibited alcohol dehydrogenase. Since 4-amino analogs of folic acid exert actions antagonistic to the vitamin, the effect of these substances on alcohol dehydrogenase was studied. It was found that aminopterin and amethopterin share with folic acid the property of inhibiting alcohol dehydrogenase. The common inhibition of alcohol dehydrogenase by folic acid and analogs cannot be explained on the basis of the known chelating properties of folic acid with zinc.

To determine whether alcohol dehydrogenase could be inhibited in intact animals, the influence of folic acid was studied (a) on oxidation of ethanol and acetate in the rat, and (b) on rate of ethanol disappearance from the plasma of dogs. Large amounts of folic acid decreased ethanol but not acetate metabolism which is evidence that alcohol dehydrogenase was inhibited *in vivo* by folic acid.

Materials and methods. Folic acid and aminopterin were obtained from the California Corp. for Biochemical Research. 1,10-Phenanthroline was purchased from Eastman Organic Chemicals. Folvite, calcium leucovorin, amethopterin and 4-dimethylamino-4-deoxy-N^{10}-methylpteroylglutamic acid (4-dimethylamethopterin) were kindly supplied by Lederle Labs. Radioactive ethanol, acetaldehyde and acetate were obtained from Volk Radiochemical Corp. Crystalline alcohol dehydrogenase derived from horse liver was purchased from Worthington Biochemical Corp.

Studies with rat liver homogenates. Each incubation vessel contained 0.01 M K phosphate (pH 7.4), 0.0032 M $MgCl_2$, 0.088 M KCl, 0.0015 M K_2 ATP, 8.8 mg cytochrome c, 1 g of rat liver (Sprague-Dawley strain), 100 μmoles of ethanol-1-C^{14} or 50 μmoles of acetaldehyde-1-C^{14} plus inhibitors (Table I) in a total volume of 40 ml. Each vessel was incubated for 2 hours in air at 37°C before the reaction was stopped with $CuSO_4$ and

TABLE I. Effect of Folic Acid or Analogs (3×10^{-5}M) on Ethanol Metabolism in Homogenates of Rat Liver.

Inhibitor	μmoles per vessel: Ethanol metabolized	μmoles per vessel: Acetaldehyde present
None	27	15.5
Folic acid	0	0
Aminopterin	0	0
Amethopterin	2	.6
Leucovorin	24	12.5

* This study was supported in part by U. S. Public Health Service Grant. Preliminary reports of several aspects of this work have been presented(1,2).

$Ca(OH)_2$. The centrifugate and washings were combined and made up to 100 ml. For ethanol-1-C^{14} determination absolute ethanol (0.4 ml) was added to a 10 ml aliquot of the combined supernatant extracts followed by sufficient K_2CO_3 to separate ethanol. The ethanol layer was removed and converted to ethyl-3,5-dinitrobenzoate, which was recrystallized and counted in a flow counter. To another aliquot (5 ml) 1 ml of 0.2 M acetaldehyde was added. Acetaldehyde was precipitated as acetaldehyde-2,4-dinitrophenylhydrazone, recrystallized and counted. Whether acetaldehyde was precipitated directly from the extract as described or aerated into 2,4-dinitrophenylhydrazine before precipitation of the derivative, identical specific activities resulted.

Studies with horse liver alcohol dehydrogenase. Alcohol dehydrogenase activity was determined spectrophotometrically based on the absorption of NADH at 340 mu(3).

Studies on intact animals. Male rats (150 g) were injected intraperitoneally with either 1 ml of 5.7 M ethanol-1-C^{14} (2.8×10^6 cpm) or 1 ml of 0.016 M Na acetate-1-C^{14} (2×10^6 cpm) and placed in metabolic tracer units (4). Respiratory CO_2 was absorbed in 1 N NaOH. Folic acid (200 mg/kg) was injected intraperitoneally 90 and 30 minutes after administration of labeled ethanol and acetate, respectively. Radioactive CO_2 was assayed as $BaCO_3$.

An indwelling catheter was placed in a branch of the femoral vein of female dogs (6-9 kg). One or two grams of ethanol/kg were infused as a 20% isotonic solution. Blood samples were withdrawn every half hour for determination of ethanol(5). After 4 hours folic acid (Folvite) was injected (80 mg/kg). Because Folvite contains ethylenediamine tetra-acetic acid and benzyl alcohol, control dogs received these compounds at the same concentrations present in the commercial preparation.

Results. Studies with rat liver homogenates and extract. Folic acid, aminopterin and amethopterin inhibited the metabolism of ethanol in the homogenate. The data presented in Table I are derived from the averages of 4 experiments, each of which showed the same results. Increasing ethanol concentration did not alter the inhibition. Leucovorin, a reduced folate derivate, was essentially ineffective as an inhibitor at 3×10^{-5} M; however, 80% inhibition was observed at 3×10^{-4} M leucovorin.

The disappearance of labeled acetaldehyde in homogenates was unaffected by the presence of folic acid. The action of folic acid could therefore be attributed to inhibition of alcohol dehydrogenase. This was confirmed directly by measuring the alcohol dehydrogenase activity of the supernatant fraction of the homogenate; folic acid or aminopterin inhibited enzymic activity.

Studies with horse liver alcohol dehydrogenase. The instantaneous inhibition of horse liver alcohol dehydrogenase was studied at varying concentrations of folic acid, amethopterin, aminopterin and 4-dimethylamethopterin. 1,10-Phenanthroline, a known inhibitor of alcohol dehydrogenase(6), was included for comparison. Folic acid, amethopterin, 4-dimethylamethopterin and aminopterin inhibited the enzyme at concentrations as low as 3.5×10^{-5} M; inhibition was complete at 2×10^{-4} M (Fig. 1). Identical inhibitions were obtained with commercial samples of folic acid and chromatographically purified folic acid(7), which eliminated the possibility that some impurity in folic acid could be responsible for enzymic inhibition. The folates were more potent than 1,10-phenanthroline. Leucovorin caused no significant inhibition at concentrations as high as 8×10^{-4} M. Another reduced folate, 5-methyltetrahydrofolic acid (kindly supplied by Dr. J. M. Buchanan of M.I.T.) also did not inhibit alcohol dehydrogenase.

Studies in intact animals. Fig. 2 illustrates the typical time course of the oxidation of ethanol-1-C^{14} to $C^{14}O_2$ in rats in presence and absence of folic acid. In the control, the exhaled $C^{14}O_2$ increased for 90 minutes, remained constant for 90 minutes and then decreased. After 4 hours about 65% of the label was recovered at $C^{14}O_2$, which agrees with the findings of Bartlett and Barnett(8). After folic acid administration the rate of $C^{14}O_2$ production decreased as depicted. Statistical analysis of 7 experiments showed that

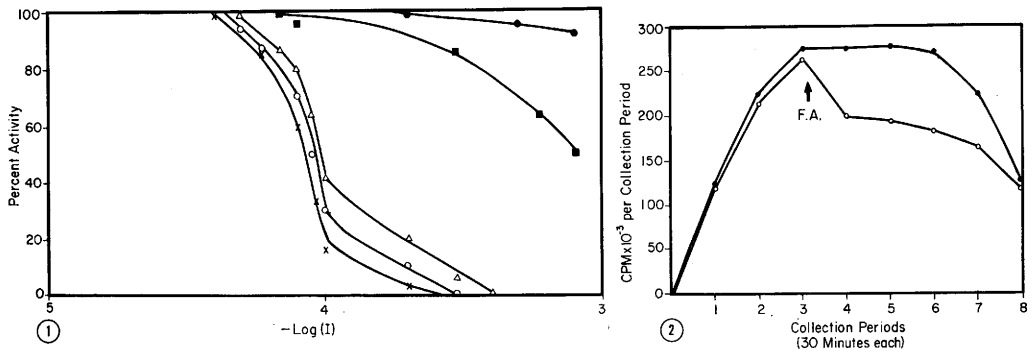

FIG. 1. Instantaneous inhibition of LADH by folic acid (×), amethopterin or 4-dimethylamethopterin (○), aminopterin (△), 1,10-phenanthroline (■), and leucovorin (●) at room temperature.

FIG. 2. Effect of folic acid on conversion of ethanol-1-C^{14} to respiratory $C^{14}O_2$ in the rat. (●) is the curve for a control animal while (○) represents a rat injected with folic acid (F.A.) intraperitoneally.

there was no difference in $C^{14}O_2$ production between both groups during the 90 minute period before administering folic acid (524 000 cpm *vs* 596 000 cpm). In the next 90 minutes, the controls expired 776 000 cpm which was significantly greater than the recovery in the first 90 minute period ($p=<0.01$). After treatment with folic acid, there was a 24% decrease in $C^{14}O_2$ production (587 000 cpm) as compared with the second 90 minutes in the control animals ($p=<0.01$). In contrast, the oxidation of acetate-1-C^{14} was not altered by folic acid; $C^{14}O_2$ recoveries were the same as with the controls. Since acetate-1-C^{14} was oxidized more rapidly than ethanol-1-C^{14} and reached peak specific activity in respiratory CO_2 after 30 minutes, folic acid was administered at this time and not after 90 minutes as with labeled ethanol. Since folic acid did not inhibit the oxidation of acetate-1-C^{14} to $C^{14}O_2$, the effect of folate on ethanol metabolism could not be attributed to a metabolic step following formation of an acetyl unit from ethanol.

The data presented in Table II represent the disappearance of ethanol from the plasma of dogs before (hours 1-4) and after folic acid (hours 5-8). In these experiments the rate of ethanol metabolism was somewhat higher than those reported by others(9-11). The control animals showed a negligible decrease in rate of ethanol metabolism after 4 hours while the dogs treated with folic acid showed a 50% decrease in rate of disappearance of plasma ethanol.

Discussion. Alcohol dehydrogenase was implicated as the site of inhibition of ethanol metabolism by folic acid and its analogs, since ethanol was recovered quantitatively and acetaldehyde was not formed in homogenates containing added folic acid. Further support came from the finding that the disappearance of labeled acetaldehyde in the homogenate was not hampered by the presence of folic acid. The direct determination of alcohol dehydrogenase in the supernatant fluid of rat liver homogenates showed indeed that added folic acid inhibited the enzymic activity. It was also demonstrated that folic acid and its analogs were potent inhibitors of purified horse liver alcohol dehydrogenase. The effect of folic acid and its analogs was tested on other dehydrogenases. Folic acid, aminopterin and amethopterin inhibited glutamic, glucose-6-phosphate, malic and lactic dehydrogenases(12).

TABLE II. Effect of Folic Acid on Rate of Plasma Alcohol Disappearance in the Dog.

		Decrease in plasma ethanol concentration	
		Hours 1-4	Hours 5-8
		mg % per hr	
Control	1	36	30
"	2	31	27
Folic acid	1	36	16
" "	2	40	20

To explain the inhibition of alcohol dehydrogenase by folic acid, attention was drawn to the observation of Vallee that alcohol dehydrogenase contained zinc(13) and to that of Albert that zinc ions were chelated by pteridines(14). These findings suggested that pteridines may chelate enzymic zinc and render zinc unavailable for reaction with its substrate. The results obtained in our study, however, do not confirm this hypothesis. First, while 4-hydroxypteridines (*e.g.* folic acid) readily form chelates with metal ions, 4-aminopteridines (*e.g.* aminopterin and amethopterin) do not(14); despite this difference, amino- and hydroxypteridines inhibited alcohol dehydrogenase approximately to the same degree. Second, 4-dimethylamethopterin, whose methyl substituted amino group would greatly restrict or prevent chelation with metals(15), was as effective as the other folate derivatives in inhibiting alcohol dehydrogenase.

1,10-Phenanthroline is assumed to inhibit alcohol dehydrogenase by chelating the enzymic zinc(6). The inhibition curve obtained with 1,10-phenanthroline was quite different from that obtained with folic acid. While both inhibitions were immediate, the striking feature of the inhibition with folic acid was the narrow concentration range required for initiation and completion of enzymic inhibition. This is in contrast to the wide range of concentrations required with 1,10-phenanthroline to complete inhibition. It has been shown for 1,10-phenanthroline that one molecule reacted with each zinc atom to form an enzyme-zinc-1,10-phenanthroline complex(16). In contrast, more than one folate reacted per active site as calculated from the steepness of the inhibition curves(17). Therefore, one is led to the conclusion that folic acid and its analogs may inhibit the enzyme by reacting at some site other than the zinc. For example, the folates may inhibit by altering the conformation of the active site of the enzyme.

The experiments in which large doses of folic acid decreased ethanol metabolism in the intact rat and dog by a possible inhibition of alcohol dehydrogenase may be of interest as an experimental tool to evaluate an enzymic activity in the intact animal. Because of the large dosages used, these experiments obviously cannot imply any biological significance to enzyme inhibition by folic acid. The large dose was selected merely to assure enzyme inhibition *in vivo*.

It was found recently that the action of folic acid on alcohol dehydrogenase appeared similar to the allosteric inhibition observed with glutamic dehydrogenase(18). Diethylstilbesterol inhibited the glutamic dehydrogenase activity of purified glutamic dehydrogenase but stimulated the alanine dehydrogenase activity of the same molecule. With alcohol dehydrogenase, folic acid completely inhibited the alcohol dehydrogenase activity of the enzyme but stimulated the isomerase activity 400%(19).

Summary. Folic acid as well as the antifolates, aminopterin and amethopterin, arrested ethanol metabolism in homogenates of rat liver by inhibiting alcohol dehydrogenase. Acetaldehyde metabolism was unaffected. In the rat, the conversion of ethanol-1-C^{14} to $C^{14}O_2$ was slowed by large amounts of folic acid while that of acetate-1-C^{14} was not. In dogs, folic acid decreased the rate of ethanol disappearance from the plasma. Thus, folic acid inhibited alcohol dehydrogenase *in vivo*. Purified horse liver alcohol dehydrogenase was inhibited similarly by folic acid, aminopterin, amethopterin, and 4-dimethylamino-4-deoxy-N^{10}-methylpteroylglutamic acid. For these compounds, inhibition started at 3.5×10^{-5} M and was completed at 2×10^{-4} M. Leucovorin did not inhibit the enzyme.

The authors wish to thank Dr. Satoshi Murayama for preparing the dogs with indwelling catheters and to acknowledge the technical assistance of Mrs. Sheela George and Mr. John A. Robinson.

1. Vogel, W. H., Schulman, M. P., *Fed. Proc.*, 1962, v21, 173.

2. Vogel, W. H., Snyder, R., Schulman, M. P., *The Pharmacologist*, 1962, v4, 155.

3. Racker, E., *J. Biol. Chem.*, 1950, v184, 313.

4. Weinhouse, S., Friedmann, B., *ibid.*, 1951, v191, 707.

5. Bücher, T., Redetski, H., *Klin. Wochschr.*, 1951, v29, 615.

6. Hoch, F. L., Williams, R. J. P., Vallee, B. L., *J. Biol. Chem.*, 1958, v232, 453.

7. Johns, D. G., Plenderleith, I. H., Cooper, B. A., *Biochem. Pharmacol.*, 1963, v12, 388.
8. Bartlett, G. R., Barnett, H. N., *Quart. J. Studies Alcohol.*, 1949, v10, 381.
9. Westerfeld, W. W., Schulman, M. P., *J. Am. Med. Assn.*, 1959, v170, 197.
10. Smith, M., Newmann, W., *J. Biol. Chem.*, 1959, v234, 1544.
11. Marshall, E. K., Fritz, W. F., *J. Pharmacol. Exp. Therap.*, 1953, v109, 431.
12. Vogel, W. H., Snyder, R., Schulman, M. P., *Biochem. Biophys. Res. Commun.*, 1963, v10, 97.
13. Vallee, B. L., Hoch, F. L., *J. Biol. Chem.*, 1957, v225, 185.
14. Albert, A., *Biochem. J.*, 1953, v54, 646.
15. Sidgewick, N. V., *J. Chem. Soc.*, 1941, 433.
16. Vallee, B. L., Coombs, T. L., *J. Biol. Chem.*, 1959, v234, 2615.
17. Snyder, R., Vogel, W. H., Schulman, M. P., *Fed. Proc.*, 1963, v22, 183.
18. Tomkins, G. M., Yielding, K. L., Currain, J., *Proc. Nat. Acad. Sci.*, 1961, v47, 270.
19. Snyder, R., Vogel, W. H., Schulman, M. P., *Biochem. Biophys. Res. Commun.*, 1963, v13, 335.

Received October 25, 1963. P.S.E.B.M., 1964, v115.

Isolation of *Histoplasma capsulatum* from Soil by Direct Culture Methods. (28964)

COY D. SMITH AND ROBERT J. WEEKS (Introduced by Michael L. Furcolow)
Communicable Disease Center, Public Health Service, U. S. Department of HEW, Kansas City Field Station, Kansas City, Kans.

The isolation of *Histoplasma capsulatum* from the soil is a fairly common practice. The methods used by many investigators(1-3) are modifications of the flotation technique described by Stewart and Meyer(4) used to isolate *Coccidioides immitis* from the soil. These methods involve injection of mice with the test material to isolate *H. capsulatum*. Only one report is available in which investigators used another method of isolating the organism from soil without the use of animals. This was one isolation by direct culture obtained by Furcolow and Larsh(5).

This paper reports the use of direct culture to isolate *H. capsulatum* from 2 separate soil specimens.

Methods. Two soil specimens, labeled #1, and #2, were collected from a starling (*Sturnis vulgaris*) roost, in one gallon ice cream cartons. Immediately upon arrival in the laboratory the 2 specimens were tested by an indirect mouse inoculation technique modified from the procedure previously described by Larsh(2). Six weeks later *H. capsulatum* was cultured from the mouse tissues. During this 6 weeks period the remainder of the 2 soil specimens was stored at room temperature in the closed collection cartons. The specimens were still slightly moist after 6 weeks of storage, and various kinds of fungi were visible growing on the surfaces.

When the 2 soils were found to contain *H. capsulatum* by the indirect method, a direct culture method was tried. The medium used to culture the soil suspensions was recently described by Smith(6). The concentration of yeast extract used was 6.0 ml of a 15% stock solution per liter of medium. In addition, antibiotics were added in the following concentrations: penicillin 20,000 units, streptomycin 40 mg, polymyxin B 10 mg, and cycloheximide 1.0 g per liter of medium. All of the above ingredients were added to the warm 2% agar suspension after it was autoclaved. The pH of the medium was 6.0.

Ten grams of each soil specimen were diluted 1:10 by placing into a 250 ml stoppered Erlenmeyer flask containing 90 ml of sterile distilled water. The soil suspensions were mixed thoroughly by hand shaking and further dilutions of 1:100 and 1:1000 were made in sterile distilled water using serial dilution techniques. One-tenth ml of the 1:100 dilution of the suspension of soil #1 was inoculated onto 40 plates of the media described above. The inoculum was spread evenly over the surface using curved glass