tals with the same optical orientation when viewed in crossed nicols. In contrast, species A has very thin calcitic walls in which the mineralized crystals appear to be oriented parallel to the depositional surface.

In species of more than one mineralogy (Fig. 4, species B and C), spatial differences in Sr and Mg are most apparent in the frontal wall, although differences may also occur in lateral and end walls. The skeletal layer adjacent to the internal space is rich in Mg (calcite) (Fig. 4, d and g). Outside of this layer, a Sr-rich layer (aragonite) occurs in a crust up to 30 μm thick, which confirms spatial relationships suggested by staining and other methods (*4*, *5*). This aragonitic crust extends proximally, distally, and laterally downward over the proximal, distal, and lateral calcitic walls. It does not extend to the base but rather pinches out between individuals. Thus a sequence of calcite-aragonite followed by aragonite-calcite in a traverse across lateral walls indicates that the traverse was made in the middle to upper part of the wall where the aragonitic layers of adjacent individuals were wrapping around the calcitic skeletal boxes. This wrapping around of the aragonitic layer is most evident in the right-hand individual of Fig. 4h.

The structure of the calcified wall is similar in both species with calcite and aragonite. The calcitic inner layer consists of crystallites parallel to the wall, whereas the aragonitic outer layer is composed of fibers radiating from the wall. This pattern has also been found in a species of *Metrarabdotos* that had an aragonitic outer layer and a calcitic inner layer (*4*). Perhaps the pattern of a calcitic internal lamellar layer and an aragonitic external fibrous layer is typical of ectoproct species with more than one mineralogy.

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References and Notes

1. L. Hyman, *The Invertebrates*, vol. 5, *Smaller Coelomate Groups* (McGraw-Hill, New York, 1959); R. S. Boardman and A. H. Cheetham, *J. Paleontol.* **43**, 205 (1969).
2. T. J. M. Schopf and F. T. Manheim, *J. Paleontol.* **41**, 1197 (1967); *ibid.* **42**, 858 (1968).
3. J. B. Rucker, *Atti Soc. Ital. Sci. Natur. Mus. Civico Storia Natur. Milano* **108**, 101 (1968); P. A. Sandberg, M. C. Moore, R. D. Stieglitz, T. R. Worsley, *Mtg. Geol. Soc. Amer., Program 1969 Southeastern Sect.*, p. 73 (1969).
4. A. H. Cheetham, J. B. Rucker, R. E. Carver, *J. Paleontol.* **43**, 129 (1969).
5. J. B. Rucker and R. E. Carver, *ibid.* **43**, 791 (1969); T. J. M. Schopf and D. F. Travis, *Biol. Bull.* **135**, 436 (1968).
6. The analyses (percent) and species used were as follows. *Flustra foliacea*: CaO, 48.8; MgO, 3.1; SrO, 0.26; P_2O_5, 0.93; calcite, 100; aragonite, 0; *Cryptosula pallasiana*: CaO, 44.4; MgO, 3.0; SrO, 0.41; P_2O_5, 0.41; calcite, 70; aragonite, 30; *Schizoporella unicornis*: CaO, 51.4; MgO, 1.9; SrO, 0.62; P_2O_5, 0.37; calcite, 50; aragonite, 50. *Parasmittina nitida*: CaO, 53.3; MgO, 0.25; SrO, 1.0; P_2O_5, 0.19, calcite, 0; aragonite, 100. Carbon dioxide accounts for most of the rest. Wet chemical and mineralogic determinations are averages from the same lots of material used for our microprobe analyses (*2*). Percentages of oxides are given as percentage (by weight) of organic-free ash (500°C).
7. W. C. Banta, *J. Morphology* **125**, 497 (1968); *Atti Soc. Ital. Sci. Natur. Mus. Civico Storia Natur. Milano* **108**, 93 (1968).
8. We thank Dr. J. Goldstein and P. LaRussa for general advice and for technical assistance in operating the probe; Dr. F. T. Manheim, Dr. Alan Cheetham, and K. W. Kaufmann, Jr., for technical review. Support provided by the Office of Research, Lehigh University, where the data were mostly obtained.

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Alcohol Oxidation in Rats Inhibited by Pyrazole, Oximes, and Amides

Abstract. *Pyrazole, previously reported to inhibit ethanol oxidation in the rat, also effectively blocks the in vivo metabolism of methanol, propanol, isopropanol,* n-*butanol, and isobutanol. A variety of oximes and amides are also effective inhibitors of ethanol metabolism. These various inhibitors may prove important in the elucidation of several facets of alcohol metabolism and also may have application in the treatment of methanol poisoning and in the reduction of the sequelae of the disulfiram-ethanol reaction syndrome in man.*

In 1963 Theorell and Yonetani reported that pyrazole is an inhibitor of liver alcohol dehydrogenase (ADH) in vitro (*1*). Pyrazole was shown to form a ternary complex with ADH and nicotinamide-adenine dinucleotide (NAD) with pyrazole occupying the ethanol binding site (*2*, *3*). Later, pyrazole and various 4-substituted pyrazoles were shown to inhibit ethanol oxidation in vivo whereas substitution at other than the 4-position resulted in loss of the inhibitory effect (*3–5*). In several recent studies pyrazole has been shown to be an effective in vivo inhibitor of ethanol oxidation in rats and dogs (*6*, *7*). Amides form a ternary complex with ADH and reduced NAD (NADH) in vitro with the amide occupying the aldehyde binding site (*2*, *3*). Therefore, we studied the effect of the formation of this complex in vivo on ethanol metabolism and observed inhibition with various amides and with pyrazole.

Increased amounts of blood acetaldehyde were detected in men who had ingested ethanol after having been exposed to *n*-butyraldoxime (*8*). Koe and Tenen have found that *n*-butyraldoxime is a potent inhibitor of liver ADH both in vivo and in vitro and, in addition, that there is a large decrease in liver aldehyde dehydrogenase activity in mice pretreated with *n*-butyraldoxime, an inhibition not demonstrable in vitro (*9*). Various other oximes are shown here to be equally effective inhibitors of the oxidation of ethanol and other alcohols in the rat.

In the present study we demonstrate the inhibitory action of pyrazole on a series of alcohols and document the inhibition of alcohol metabolism by various amides and oximes. We determined the rate of alcohol metabolism in fasted Sprague-Dawley rats, each weighing 175 to 225 g, by measuring the rate of disappearance of alcohol (*10*) or the rate of excretion of ^{14}C-labeled CO_2 in expired air after administration of the ^{14}C-labeled alcohol (*11*). An attempt was made to compare the relative effectiveness of these inhibitors as well as the margin of safety.

The effectiveness of pyrazole (6.6 mmole/kg) as an inhibitor of the oxidation of a series of alcohols is shown in Table 1. Oxidation of the primary alcohols proceeds at a rate of 5.8 to 7.2 mmole kg^{-1} $hour^{-1}$, except for methanol which is oxidized at about one-tenth of this rate. Although these primary alcohols are oxidized nearly equally well, pyrazole is a more effective inhibitor of ethanol oxidation than of the oxidation of the other alcohols, a relationship expected from the competition between inhibitor and substrate for ADH and the decrease of the Michaelis constant (K_m) with increase in the chain length of the alcohols (*12–14*). That the inhibition occurs at the first stage of the oxidation of the alcohol is shown by the inhibition of ADH in vitro and the fact that acetate metabolism is not inhibited after administration of pyrazole. The rate of metabolism of [1-^{14}C]acetate (10 mmole/kg) was 4.19 mmole kg^{-1}

Table 1. Noninhibited rate and rate after maximum inhibition by pyrazole of the disappearance of a series of alcohols. Pyrazole (6.6 mmole/kg) was administered intraperitoneally in isotonic saline. Values are based on at least two animals for each alcohol.

Alcohol	Initial alcohol dose (mmole/kg)	Noninhibited rate (mmole kg^{-1} hr^{-1})	Rate after maximum inhibition (mmole kg^{-1} hr^{-1})
Methanol	31.3	0.7	0.3
Ethanol	21.7	7.2	0.7
Propanol	16.7	5.8	2.5
Isopropanol	16.7	0.8	0.5
n-Butanol	6.8	6.8	3.1
Isobutanol	6.8	6.9	3.5

hour^{-1} in rats given pyrazole at 2.2 mmole/kg and was 4.21 mmole kg^{-1} hour^{-1} in the controls.

In Table 2 is shown a comparison of the effectiveness of pyrazole and *n*-butyraldoxime as inhibitors of the oxidation of methanol, ethanol, and *n*-butanol. Both inhibitors appear to be equally effective in inhibiting the oxidation of methanol and ethanol but differ in their effects on the oxidation of *n*-butanol. The variation in the dosage of the inhibitors necessary for 50 percent inhibition may possibly be accounted for by the following factors: (i) The fact that the K_m of ADH for *n*-butanol is one-tenth as large as the K_m of ADH for ethanol (*12*) and the K_m of ADH for ethanol is one-third as large as the K_m of ADH for methanol (*13*), (ii) the major involvement of catalase (as well as ADH) in methanol metabolism (its role is insignificant in ethanol and *n*-butanol metabolism), (iii) the varying alcohol concentrations against which these inhibitors were tested, and (iv) the longer time course of action of pyrazole. Although these considerations may serve to explain the difference in dosages required for 50 percent inhibition of the oxidation of the alcohols with each inhibitor separately, they do not explain satisfactorily their comparative effects; whereas both inhibitors inhibit methanol and ethanol oxidation equally, *n*-butyraldoxime is a considerably more potent inhibitor of *n*-butanol oxidation than pyrazole. Thus, there appears to be a basic difference in their mechanism of action, a difference not surprising in view of the inhibition of aldehyde dehydrogenase and ADH by *n*-butyraldoxime in vivo (*9*), in contrast to the inhibition of only ADH by pyrazole (*4*, *7*).

Table 2. Dosages of pyrazole and *n*-butyraldoxime ($\times 10^{-4}$ mole/kg in isotonic saline) estimated to produce 50 percent inhibition in the rate of oxidation of alcohols to ^{14}C-labeled CO_2 after intraperitoneal injection of [^{14}C]methanol, [1-^{14}C]ethanol, or [1-^{14}C]*n*-butanol. Estimates are derived from rates of oxidation and varying doses of inhibitors.

Alcohol	Initial alcohol dose (mmole/kg)	Pyrazole	*n*-Butyraldoxime
Methanol	31.3	58	52
Ethanol	21.7	3	2
n-Butanol	6.8	30	1

Table 3. Comparative safety of inhibitors: the ratio of the intraperitoneal dose lethal to 50 percent of fasted Sprague-Dawley rats to the intraperitoneal dose required for 50 percent inhibition of ethanol oxidation.

Inhibitor	50% Inhibition ($\times 10^{-4}$ mole/kg)	LD_{50} ($\times 10^{-4}$ mole/kg)	LD_{50}/50% inhibition
Pyrazole	3	129	43
n-Butyraldoxime	2	49	25
n-Butyramide	7	580*	83

* Minimum estimate.

Ethanol oxidation was inhibited for prolonged periods after administration of a single dose (6.6 mmole/kg) of pyrazole. Oxidation of ethanol at ½, 18, 42, and 66 hours after the administration of pyrazole was inhibited by 88, 82, 64, and 34 percent, respectively. An estimate of 14 hours for the half-life of pyrazole was calculated from the rate of urinary excretion of pyrazole (*15*). The duration of the inhibitory effect of *n*-butyraldoxime has not been studied in detail but the period of alcohol inhibition is much shorter than after pyrazole.

The amides, *n*-butyramide and isobutyramide, inhibit the oxidation of ethanol by 50 percent at a dosage of 0.7 mmole/kg whereas acetamide is not inhibitory at a dosage of 10.2 mmole/kg. Acetaldoxime, isobutyraldoxime, and *n*-butyraldoxime are equally effective inhibitors of the oxidation of ethanol. Acetoxime, methyl ethyl ketoxime, and cyclohexanone oxime have inhibitory activity approximately equal to that of the aldoximes. Hydroxylamine at the highest tolerated dose (7.2 mmole/kg) is one-third as active as *n*-butyraldoxime, whereas pyridine-2-aldoxime and pyridine-4-aldoxime are not inhibitory.

The margin of safety for pyrazole, *n*-butyraldoxime, and *n*-butyramide is shown in Table 3 where the dose lethal to 50 percent of the rats (LD_{50}) is compared with the dose required to inhibit ethanol oxidation by 50 percent. It is apparent that greater "safety" was associated with the administration of *n*-butyramide, although all inhibitors were well tolerated in short-term experiments.

The availability of effective in vivo inhibitors of alcohol metabolism should be useful experimentally, and possibly therapeutically, in problems associated with alcoholism and the use of alcohol. They may prove useful in revealing the "normal" or "physiologic" role of ADH (*2*, *6*) and in the separation of the direct effects of ethanol from those associated with its metabolism (*16*). These inhibitors may also prove useful (i) in the management of methanol poisoning, especially if the human liver ADH is wholly responsible, as it is in the monkey (*17*), for methanol oxidation and (ii) in treatment of the disulfiram-ethanol reaction syndrome, attributable, at least in part, to the acetaldehyde arising from ethanol oxidation.

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References and Notes

1. H. Theorell and T. Yonetani, *Biochem. Z.* **338**, 537 (1963).
2. H. Theorell, *Experientia (Basel)* **21**, 553 (1965).
3. ———, T. Yonetani, B. Sjöberg, *Acta Chem. Scand.* **23**, 255 (1969).
4. D. Lester, W. Keokosky, F. Felzenberg, *Quart. J. Stud. Alc.* **29**, 449 (1968).
5. T. Li and H. Theorell, *Acta Chem. Scand.* **23**, 892 (1969).
6. H. Theorell, *The Harvey Lectures* (Academic Press, New York, 1967), p. 17.
7. L. Goldberg and U. Rydberg, *Biochem. Pharmacol.* **18**, 1749 (1969); M. Reynier, thesis, L'Université de Lyon, France (1969); ———, *Acta Chem. Scand.* **23**, 1119 (1969).
8. W. Lewis and L. Schwartz, *Med. Ann. D. C.* **25**, 485 (1956).
9. B. K. Koe and S. S. Tenen, *Fed. Proc.* **28**, 546 (1969).
10. Disappearance of alcohol was determined by monitoring the concentration of alcohol in an air bubble formed by the injection of 25 ml of air under the skin on the back of a rat. The alcohol in the air of the bubble equilibrates with the alcohol of the blood, and its concentration is determined by injecting a sample through the loop of an automatic gas-sampling valve attached to a gas-liquid chromatograph equipped with a hydrogen flame-ionization detector. The rate of metabolism was calculated by dividing the administered dose by the time taken for the alcohol concentration to reach zero, with the time determined by extrapolating to zero the characteristically linear portion of the decrease in alcohol concentration.
11. After the intraperitoneal injection of a ^{14}C-labeled alcohol the unmetabolized alcohol in expired air was removed by magnesium perchlorate; the respiratory labeled CO_2 was collected continuously and quantitatively in ethanolamine and methanol [1 : 4 (by volume)] a sample of which was assayed by liquid scintillation spectrometry. The rate of alcohol metabolism was calculated from the the linear phase of labeled CO_2 excretion.

12. H. Sund and H. Theorell, in *The Enzymes* (Academic Press, New York, ed. 2, 1963), vol. 7, chap. 2, p. 25.
13. A. H. Blair and B. L. Vallee, *Biochemistry* **5**, 2026 (1966).
14. C. S. Tsai, *Can. J. Biochem.* **46**, 381 (1968).
15. Pyrazole was extracted with ethylene dichloride from urine saturated with K_2HPO_4 and was returned to 0.12*N* HCl, and the absorption at 215 to 217 nm was determined [log ϵ (molar extinction coefficient) = 3.66 in 0.12*N* HCl]; only 10 percent of an ingested dose was recoverable as pyrazole in samples after 72 hours, 20 percent of this total being released by β-glucuronidase-sulfatase hydrolysis.
16. W. K. Lelbach, *Experientia (Basel)* **25**, 816 (1969).
17. W. D. Watkins, J. I. Goodman, T. R. Tephly, *Fed. Proc.* **28**, 546 (1969).
18. Presented in part at the meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., 16 April 1969 [*Fed. Proc.* **28**, 546 (1969)]; at the meeting of the American Association for the Study of Liver Disease, Washington, D.C., 16 May 1969; and at the Fourth International Congress on Pharmacology, Basel, Switzerland, 17 July 1969. Supported in part by grants MH-05655 and MH-11612 (D.L.) and AM-10150 and MH-18176 (G.D.B.)
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In vitro Lymphocyte Reactivity during Depression of Tuberculin Hypersensitivity by 6-Mercaptopurine

Abstract. *Administration of 6-mercaptopurine suppressed appearance of tuberculin skin test reactivity for up to 6 weeks after mycobacterial injection. Lymphocytes obtained during the period of suppressed tuberculin reactivity exhibited normal in vitro proliferative responses to tuberculin, suggesting that the drug may not be qualitatively affecting function of immunologically competent cells.*

Several studies (*1*, *2*) have demonstrated a suppressive effect of 6-mercaptopurine and its analogs on delayed hypersensitivity. The present study was undertaken to further elucidate the cellular mechanisms in this suppressive effect. In a previous study in our laboratory (*3*) we showed that in guinea pigs sensitized with mycobacteria, tuberculin skin test reactivity and in vitro proliferative responsiveness to tuberculin by peripheral blood lymphocytes develop concomitantly. In the present work we investigated the effect of 6-mercaptopurine treatment on the development of these reactivities in guinea pigs similarly injected with mycobacteria.

Male albino guinea pigs, 500 to 700 g, were sensitized by footpad and subcutaneous injections of 1.0 ml of complete Freund's adjuvant containing 1.0 mg of heat-killed H-37 Ra mycobacteria per milliliter. From the 4th to the 14th day after the injection, each animal received either (i) 6-mercaptopurine in daily intramuscular injections of 50 mg/kg or (ii) an equal volume of control solution of 1.0*N* NaOH, which had been used as the diluent for the 6-mercaptopurine. Each animal was skin tested at weekly intervals from the day before sensitization until termination of the study. Agents used for skin testing were PPD-S, 0.1 μg/0.1 ml (5 tuberculin units), and old tuberculin, 0.01 mg/0.1 ml (Koch's Old Tuberculin). Skin tests were expressed as the mean of two perpendicular diameters of induration at 24-hour readings.

Immediately before sensitization and at weekly intervals thereafter, sterile heparinized blood was obtained by cardiac puncture and sedimented by using the method of Hulliger and Blazkovec (*4*). The leukocyte-rich supernatant layer was centrifuged; the resultant cell button was washed twice with Hanks solution, and resuspended in a culture medium containing Eagle's minimal essential medium, 20 percent serum from newborn pre-colostrum calves (Colorado Serum Co.), glutamine (final concentration, 2 percent), and penicillin and streptomycin (final concentration, 100 units and 100 μg/ml, respectively). Each culture vial contained 1×10^6 lymphocytes in a total volume of 1.0 ml. To selected test vials were added 0.5 μg of Koch's Old Tuberculin for comparison with control vials containing no antigen. Replicate cultures were incubated at 37°C in CO_2–air (5 : 95) for 5 days. Proliferative responses of the lymphocytes were measured by incorporation of H^3-thymidine (specific activity, 6.7 c/mmole; New England Nuclear Co.) into DNA as previously described (*5*). The response was expressed as an Isotope Incorporation Index (I.I.I.) equal to the ratio of the mean counts per minute of replicate test vials to the mean counts per minute in replicate control vials for any particular experiment. In selected experiments, cytologic studies looking for blast cell transformation and mitotic division were carried out as described previously (*6*).

Some of the guinea pigs not treated with 6-mercaptopurine showed positive skin test and in vitro lymphocyte proliferative (I.I.I. > 3) reactivities to tuberculin starting on day 14 after sensitization. These positive responses were present to varying degrees in almost all these animals by day 21 (Table 1). The in vitro antigen-induced proliferative response was shown not only by increasing isotope uptake (I.I.I.) but by increased blast cell transformation and mitotic division in lymphocytes cultured with tuberculin.

Guinea pigs treated with 6-mercapto-

Table 1. Skin test and lymphocyte reactivities in control group on day 21 after complete Freund's adjuvant. Skin test = PPD skin test reactivity (mean diameter of induration at 24 hours). I.I.I. = lymphocyte proliferative response (see text). Percentage of blasts = blast cells/total lymphocytes, at time of harvest, × 100. M.I. (mitotic index) = cells in mitoses/1000 lymphocytes at time of harvest.

Animal No.	Skin test	I.I.I.	Blood lymphocytes per mm³	Percentage of blasts/ M.I.
12	7	4.4	6255	31/8
16	8	3.2	6930	31/6
18	8	29.4	4900	41/24
22	10	1.9	5495	20/4
23	6	6.4	5775	42/9
28	6	24.2	4800	39/21
29	6	22.8	5100	40/21
46	11	30.4	9500	55/24
47	12	10.4	6000	24/12
52	6	5.0	2500	
53	7	21.0	3000	
67	6	31.5	3400	
68	10	1.5	3100	
75	6	3.2	2000	
76	5	1.5	4700	
Means ±S.E.	7.6± 2.1	12.4± 10.6	4963± 1701	38±9.1/ 14±7.7

Table 2. Skin test and lymphocyte reactivities in 6-mercaptopurine group on day 21 after complete Freund's adjuvant.

Animal No.	Skin test	I.I.I.	Blood lymphocytes per mm³	Percentage of blasts/ M.I.
17	0	4.3	1850	20/5
19	2	6.5	2000	31/11
21	2	2.6	2145	25/6
24	3	11.5	2700	54/18
25	1	16.0	2775	72/15
31	1	15.5	4100	30/12
32	2	42.1	2100	40/36
42	0	1.0	950	15/0
43	0	5.5	1560	38/10
44	2	4.0	1000	28/5
45	0	6.0	2100	35/9
49	3	16.0	2400	
51	2	4.1	2600	
61	1	16.0	4900	
62	0	2.8	3900	
64	1	38.0	3600	
65	1	37.0	2500	
66	2	4.5	2800	
71	1	3.1	1640	
72	0	3.0	1940	
74	1	7.0	2130	
Means S.E.±	1.2± 0.94	11.7± 3.18	2490± 985	35±15/ 13±9