tems (*2, 10, 11*). The curiosities reported here provide further evidence of the complexity of patterned precipitation.

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References and Notes

1. S. Kai, S. C. Müller, J. Ross, *J Chem. Phys.* **76**, 1392 (1982).
2. S. C. Müller, S. Kai, J. Ross, *ibid.*, in press; in preparation.
3. E. S. Hedges, *Liesegang Rings and Other Periodic Structures* (Chapman & Hall, London, 1932); K. H. Stern, *Chem. Rev.* **54**, 79 (1954); *A Bibliography of Liesegang Rings* (Government Printing Office, Washington, D.C., ed. 2, 1967).
4. The term "secondary Liesegang structures" frequently appears in the literature; it usually refers to the separation of Liesegang bands into several thinner bands, mostly observed for $Ag_2Cr_2O_7$ [K. S. Ramaiah, *Proc. Indian Acad. Sci. Sect. A* **9**, 467 (1939)].
5. The preparation of Liesegang experiments is described in detail in (*1, 2*).
6. Rings of PbI_2 are also formed in the iodide solution, provided that the lead concentration sufficiently exceeds the iodide concentration (compare Fig. 2B).
7. We confirmed that the gaps are not due to fractures in the gel.
8. For example, see *Sci. News* **120**, 107 (1981).
9. A different type of radial structure is a set of radially aligned dislocations, observed for Ag_2CrO_4 rings [C. V. Raman and K. S. Ramaiah, *Proc. Indian Acad. Sci. Sect. A* **9**, 455 (1939)].
10. M. Flicker and J. Ross, *J. Chem. Phys.* **60**, 3458 (1974).
11. D. Feinn, P. Ortoleva, W. Scalf, S. Schmidt, M. Wolff, *ibid.* **69**, 27 (1978).
12. R. E. Liesegang, *Z. Phys. Chem.* **88**, 1 (1914); *Kolloid Z.* **87**, 57 (1939).
13. Formation of $Mg(OH)_2$ spirals in gelatin and agar gel has been briefly mentioned [R. Fricke and O. Suwelack, *Z. Phys. Chem.* **124**, 359 (1926); E. S. Hedges and R. V. Henley, *J. Chem. Soc.* **1928**, 2714 (1928)]. On a photograph of a cobalt hydroxide spiral in gelatin the helicoidal structure is barely recognizable [W. Ostwald, *Kolloid Z.* **36**, 380 (1925)]. Another photograph has been presented of a "screwlike" form of calcium phosphate in specially treated gelatin [E. Hatschek, *Biochem. J.* **14**, 418 (1920)].
14. R. Lovett, P. Ortoleva, J. Ross, *J. Chem. Phys.* **69**, 947 (1978); G. Venzl and J. Ross, in preparation.
15. S. Kai, S. C. Müller, J. Ross, unpublished results.
16. The presence of $Ba(NO_3)_2$ affects the details of the patterns but is not necessary for the appearance of radial features.
17. Supported in part by the National Science Foundation and by the Air Force Office of Scientific Research. A fellowship from the Deutsche Forschungsgemeinschaft for S.C.M. is gratefully acknowledged.
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Human Aldehyde Dehydrogenase: Mechanism of Inhibition by Disulfiram

Abstract. *Disulfiram labeled with carbon-14 reacts specifically with human liver aldehyde dehydrogenase E_1 with loss of catalytic activity and no incorporation of label. Carbon-14–labeled diethyldithiocarbamate is formed and the number of enzyme sulfhydryl groups decreases from 34 to 30 during this process. Activity is recovered by 2-mercaptoethanol but not by glutathione, the physiological reducing agent.*

Disulfiram (tetraethylthiuram disulfide) is used therapeutically in the treatment of alcoholism. Its administration before drinking of alcoholic beverages results in unpleasant symptoms such as blurred vision, nausea, and flushing of the face and neck. Disulfiram apparently acts by inhibiting aldehyde dehydrogenase (E.C. 1.2.1.3) (*1*), causing elevation of blood acetaldehyde after consumption of ethanol (*2*). The mechanism by which disulfiram inhibits hepatic aldehyde dehydrogenase has been investigated for the past 20 years. In 1966 disulfiram was shown by Neims *et al.* (*3*) to be a sensitive, general reagent for the modification of protein sulfhydryl groups by the reaction shown in Fig. 1A.

Studies with purified cytoplasmic and mitochondrial aldehyde dehydrogenases from various species (*4–7*) indicate that cytoplasmic enzyme is more susceptible to disulfiram inhibition than mitochondrial enzyme. Activity lost after treatment with disulfiram is recovered with 2-mercaptoethanol or 1,4-dithiothreitol but not after prolonged dialysis, suggesting formation of a covalent bond between disulfiram and enzyme by a mechanism similar to that described in Fig. 1A. There are, however, numerous enzymes with essential sulfhydryl groups that are not inhibited by disulfiram; in addition, Strömme (*8*) has reported that soluble rat liver proteins incorporate little radioactivity after in vivo treatment with [^{35}S]disulfiram. The mechanism by which disulfiram specifically inhibits aldehyde dehydrogenase activity is therefore poorly understood.

We report on the mechanism by which human liver cytoplasmic aldehyde dehydrogenase is inhibited by disulfiram. Our results indicate that disulfiram binds at a specific site and oxidizes essential enzyme sulfhydryl groups to form internal S–S bonds.

The E_1 isoenzyme of human aldehyde dehydrogenase, purified to homogeneity by a modification of the procedure of Greenfield and Pietruszko (*9*), was dialyzed against ten changes of N_2-saturated 30 m*M* phosphate buffer (*p*H 7.0) containing 1 m*M* EDTA at 4°C. Also used were glutathione, 2-mercaptoethanol, and 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) (Sigma); disulfiram (Ayerst); ^{14}C-labeled disulfiram (tetraethylthiuram disulfide) (Amersham); sodium arsenite (Baker); and nicotinamide adenine dinucleotide (NAD^+) (Boehringer-Mannheim).

Aldehyde dehydrogenase activity was determined spectrophotometrically at 25°C and 340 nm in 100 m*M* sodium pyrophosphate buffer (*p*H 9.0) containing 1 m*M* EDTA, 900 μ*M* NAD^+, and 260 μ*M* propionaldehyde. Protein concentrations were determined by the method of Lowry *et al.* (*10*) with bovine serum albumin (Sigma) used as a standard. Enzyme was inactivated by mixing with buffer containing disulfiram. Incubations (in 30 m*M* sodium phosphate, *p*H 7.0, containing 1 m*M* EDTA) were kept under nitrogen at room temperature for the desired length of time; portions were drawn to determine enzyme activity and for counting of radioactivity on an Intertechnique Liquid Scintillation Counter. Control and disulfiram-treated enzyme

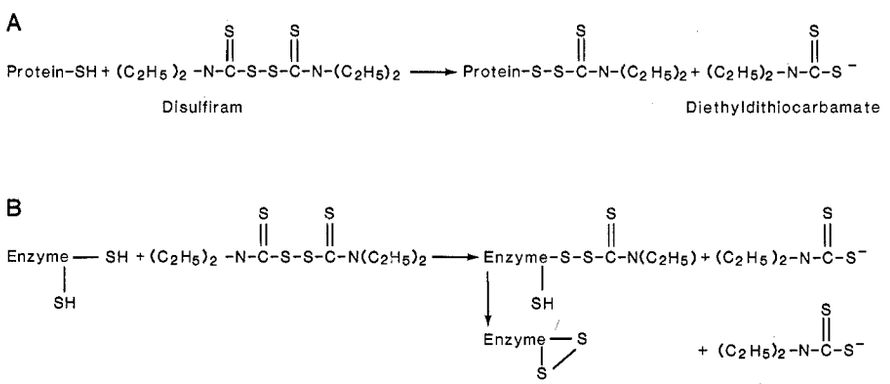

Fig. 1. (A) Reaction proposed by Neims *et al.* (*3*) as the general mechanism by which protein sulfhydryl groups are covalently modified by disulfiram. (B) Proposed mechanism of inhibition of aldehyde dehydrogenase by disulfiram.

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Table 1. Stoichiometry of inhibition of activity and incorporation of label from [^{14}C]disulfiram by human cytoplasmic aldehyde dehydrogenase E_1.

Ratio of disulfiram to E_1 tetramer	Disulfiram (μM)	Counts per minute	Activity (percent of control)		Dialyzate (count/min)	Retentate (count/min)	Recovery of activity* (%)	Radioactive dialyzate
			Initial	After 3 to 6 minutes				
0	0	0	100	100	0	0	100	
0.93	4.1	9,100	53	53	9,690	0		
1.99	8.7	19,400	0	11	18,850	600	92	Diethyldithiocarbamate
2.98	13.0	29,100	0	3	27,000	1,500		
4.1	17.9	59,600	0	1.6	49,840	8,760		

*2-Mercaptoethanol (20 mM) was added into the assay cuvette.

samples were routinely dialyzed against three changes of the dialysis buffer described above under nitrogen at 4°C in order to remove any radioactivity not bound to the enzyme. The volume ratio of enzyme to dialyzate was 1:1000.

After dialysis the labeled species was identified by thin-layer chromatography (Whatman K6 silica plate) with a solvent of methanol, pyridine, and water (8:4:16), followed by autoradiography. [^{14}C]Disulfiram and [^{14}C]diethyldithiocarbamate, formed by mixing [^{14}C]disulfiram with excess 2-mercaptoethanol, were used as controls. Enzyme activity was regained either by direct addition of 2-mercaptoethanol into the assay cuvette or by preincubation with 2-mercaptoethanol under nitrogen. Sulfhydryl group content was determined by the method of Habeeb (*11*). Fluorescence polarization experiments were done with a Perkin-Elmer MPF-2 spectrofluorometer equipped with a polarizer accessory. Emission spectra were recorded at 10°C in 30 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and approximately 0.07 mg of enzyme per milliliter at an excitation wavelength of 288 nm. Circular dichroism spectra were obtained on a Cary model 61 spectropolarimeter with an enzyme concentration of 0.1 mg/ml at 200 to 260 nm and 0.9 mg/ml at 260 to 320 nm range.

Initial experiments were designed primarily to determine the stoichiometry of disulfiram binding to enzyme as related to catalytic activity. Incubations containing 0, 1, 2, 3, and 4 molar equivalents of disulfiram per enzyme tetramer were tested with unlabeled and [^{14}C]disulfiram. Inhibition was virtually complete in 5 minutes after mixing in the incubations with 3 and 4 equivalents per tetramer but proceeded more slowly in incubations with less inhibitor. At 20 hours after mixing, inhibition was complete at all incubation stoichiometries. When initial velocity (expressed as the percent of original activity) was plotted against equivalents of disulfiram used during incubation, a straight line was obtained, which was extrapolated to approximately 2 equivalents of disulfiram per enzyme necessary for 100 percent inhibition of dehydrogenase activity (Fig. 2 and Table 1). However, when a sample containing the enzyme and 2 or more equivalents of inhibitor remained in the assay cuvette for 3 to 6 minutes, a small residual enzyme activity appeared (Fig. 2). Presumably, two types of inhibitory processes occur; one is reversible upon dilution in the assay cuvette and the other is not. Further investigation indicated that aldehyde has a role in the reversal of a small part of disulfiram inhibition, since increased concentrations appear to reduce the lag time.

Unmodified and [^{14}C]disulfiram-inhibited enzyme was then dialyzed to remove any unbound radioactivity and to determine the amount of the incorporated label. It can be seen in Table 1 that the amount of incorporation was significant only at disulfiram concentrations greater than two molecules per molecule of enzyme; below this concentration essentially no incorporation occurred, although the enzyme was found to be fully inhibited. Full catalytic activity could be recovered by preincubation with 2-mercaptoethanol (23 mM) or by adding 2-mercaptoethanol (20 mM) to the cuvette. The dialyzable radioactive material was identified by thin-layer chromatography as diethyldithiocarbamate. The sulfhydryl content of the enzyme treated with 2 equivalents of disulfiram and of the control was determined by using Ellman's reagent. In two separate experiments done in duplicate we observed 34 sulfhydryl groups in the untreated enzyme but only 30 in the modified enzyme. These results indicate that disulfiram first combines with essential sulfhydryls to form diethyldithiocarbamate adducts, as suggested by Neims *et al.* (*3*). The diethyldithiocarbamate adduct then combines with another suitably positioned enzyme sulfhydryl to form a disulfide according to the scheme shown in Fig. 1B.

Since this scheme requires a sulfhydryl adjacent or close to the diethyldithiocarbamate-modified sulfhydryl, we tested E_1 enzyme for inhibition with arsenite. This compound was noninhibitory at concentrations up to 10 mM and only slightly inhibitory (29 percent) at 25 mM. The possibility that disulfiram induces conformational adjustment within the enzyme, which causes otherwise nonadjacent sulfhydryl groups to move closer together, was examined by fluorescence polarization and by circular dichroism; however, no evidence for a conformational change could be detected.

Deitrich and Erwin (*1*) demonstrated

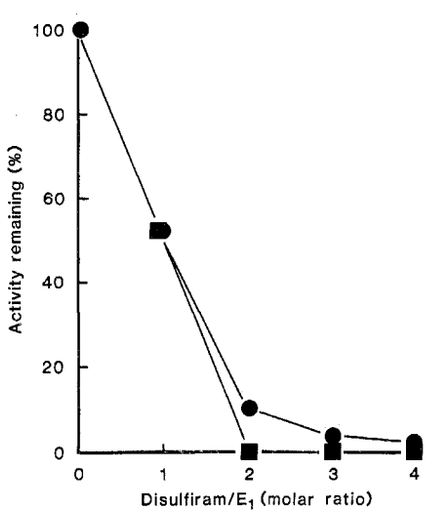

Fig. 2. Relation between catalytic activity and mole fraction of disulfiram used during incubation: initial activity (■) and maximum activity (after several minutes) (●). All tests were conducted after 20 hours of incubation at 25°C.

that administration of disulfiram to rats in vivo decreases aldehyde dehydrogenase activity in the liver and that recovery of activity is dependent on synthesis of new enzyme. (Activity was not regained when cycloheximide, an inhibitor of protein synthesis, was also administered.) This observation appears to be inconsistent with our proposed mechanism (Fig. 1B), especially on consideration of the fact that glutathione, the physiological reducing agent, is present in the liver at concentrations ranging from 2 to 12 m*M*, depending on the species (*12*). However, when we incubated the unlabeled, inhibited enzyme with 9 m*M* glutathione (*p*H 7) for up to 18 hours, we found that only about 5 percent of the catalytic activity was restored, compared to 100 percent when 2-mercaptoethanol was used. These results could explain why synthesis of new enzyme is required for return of aldehyde dehydrogenase activity in vivo, even though the modification is reversible in vitro in the presence of the nonphysiological reducing agent 2-mercaptoethanol. The reason why glutathione is inefficient in returning oxidized aldehyde dehydrogenase to its reduced form is not clear, but will probably be found in the primary structure of the enzyme surrounding the points of disulfiram interaction.

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References and Notes

1. R. A. Deitrich and V. G. Erwin, *Mol. Pharmacol.* **7**, 301 (1971).
2. T. E. Reed *et al.*, *Can. Med. Assoc. J.* **115**, 851 (1976).
3. A. H. Neims, D. S. Coffey, I. Hellerman, *J. Biol. Chem.* **241**, 3036 (1966).
4. R. A. Deitrich, *Arch. Biochem. Biophys.* **119**, 253 (1967).
5. ——— and I. Hellerman, *J. Biol. Chem.* **238**, 1683 (1963).
6. T. M. Kitson, *Biochem. J.* **151**, 407 (1975).
7. J. Eckfeldt, L. Mope, K. Takio, T. Yonetani, *J. Biol. Chem.* **251**, 236 (1976).
8. J. H. Strömme, *Biochem. Pharmacol.* **14**, 393 (1965).
9. N. J. Greenfield and R. Pietruszko, *Biochim. Biophys. Acta* **193**, 265 (1966).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. A. F. S. A. Habeeb, *Methods Enzymol.* **25**, 25 (1972).
12. C. Long, Ed., *Biochemists' Handbook* (Van Nostrand, Princeton, N.J., 1961), p. 682.
13. Supported by National Institute of Alcohol Abuse and Alcoholism grants AA 00186 and K02 00046.

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Phosphoglucomutase: Evidence for a New Locus Expressed in Human Milk

Abstract. *Electrophoretic study of phosphoglucomutase (PGM) in human milk revealed different patterns that can be explained by the existence of a locus distinct from the common PGM1, PGM2, and PGM3. One hundred and forty samples were tested and the results showed four different alleles of PGM4 whose frequencies were under Hardy-Weinberg equilibrium.*

Phosphoglucomutase (PGM), α-D-glucose 1,6-bisphosphate: α-D-glucose-1-phosphate phosphotransferase (E.C. 2.7.5.1), is known to be the product of three loci: PGM1 (*1*), PGM2, and PGM3, each determining a characteristic set of two or three isozymes. Electrophoretic variants attributable to alleles have been identified for the three genes (*2*). In most tissues, PGM1 isozymes account for 85 to 95 percent of the total PGM activity, PGM2 for 2 to 5 percent, and PGM3 for 1 to 2 percent. In erythrocytes, however, PGM1 and PGM2 isozymes are found in almost equal amounts, with practically no PGM3 activity (*3*).

We used electrophoresis to determine, by the method of Spencer *et al.* (*4*), the PGM phenotypes in 140 samples of human milk from Mexican *mestizas* who had given birth 3 to 10 days previously. We also examined samples of erythrocytes from the same women. Milk samples were treated with CCl_4 to remove fat, which produces an unspecific stained background in this electrophoretic system. The PGM patterns in human milk were different and independent from those observed in the erythrocytes (Figs. 1 and 2). Eight phenotypes were easily distinguishable, and were explained on the basis of a distinct PGM4 locus with four alleles conventionally designated here as PGM4*1, PGM4*2, PGM4*3, and PGM4*4. The homozygotes PGM4*1/PGM4*1, PGM4*2/PGM4*2, and PGM4*3/PGM4*3 showed three bands with activities decreasing from the central region to the anode. The phenotypes in the heterozygotes PGM4*1/PGM4*2, PGM4*1/PGM4*3, and PGM4*2/PGM4*3 showed the combination of the expected bands. PGM4*4 could not be found in homozygosis but only combined with alleles *1 and *2; it migrates as a single intense band with mobility similar to the slowest band produced by PGM4*1/PGM4*1 homozygotes. The distribution of the PGM phenotypes in the 140 samples is presented in Table 1. The gene frequencies of each of these alleles were: 0.346 for PGM4*1, 0.475 for PGM4*2, 0.114 for PGM4*3, and 0.065 for PGM4*4. The statistical analysis of these frequencies under Hardy-Weinberg equilibrium showed good agreement between the observed and the expected [χ^2 (9) = 10.5].

Tests for phosphopentomutase (PPM) activity, present in PGM2 (*5*), were carried out in ten milk samples, giving nega-

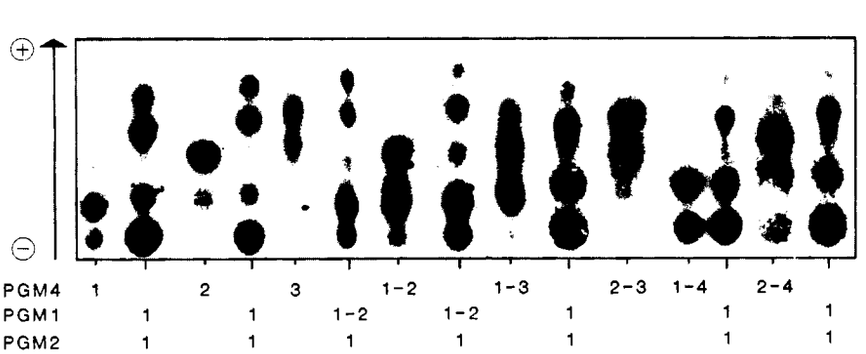

Fig. 1. Photograph showing the PGM4 phenotype in human milk compared with the phenotypes of PGM1 and PGM2 in erythrocytes.

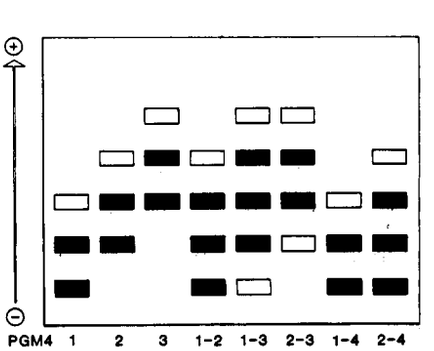

Fig. 2. Diagram of the eight different observed phenotypes of PGM4.

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