that of the 24 enzymes listed in their paper, 12 are within ±1 log of the break point; thus, estimating their specificity should be difficult. It is curious that for the reactions with values of -log E_{eq} near 11.2, where according to their hypothesis the energy difference between binding modes should be minimal, there is still complete conservation of stereochemistry for a given reaction; e.g., all glycerol-3-phosphate dehydrogenases (-log E_{eq} = 11.1) are *pro-S* specific and all glycerol-2-dehydrogenases (-log E_{eq} = 11.3) are *pro-R* specific.

The seven *pro-S*-specific enzymes with the smallest values of -log E_{eq} can be grouped into two broad categories on the basis of their substrate specificity: steroid dehydrogenases and 3-hydroxycarboxylic acid dehydrogenases. Further limitations arise because the prokaryotic steroid dehydrogenases from *P. testosteroni*, β-hydroxysteroid dehydrogenase (1.1.1.51) and 3α-hydroxysteroid dehydrogenase (1.1.1.50),[17] are adaptive enzymes. They are derived from a prokaryotic source that has no intrinsic role for sterols other than in this case the fortuitous battery of degradative enzymes necessary to grow on testosterone as a sole carbon source. The bacterial β-hydroxysteroid dehydrogenase is also quite nonselective, oxidizing 3β-, 17β-, and even 16β-hydroxy sterols. Therefore the "natural substrates" for these two enzymes must be considered as undefined. Exclusion of the two bacterial enzymes and inclusion of the two *pro-R* α-hydroxysteroid dehydrogenases dispels the correlation for reactions with -log E_{eq} < 11.2.

Finally, the range of reactions considered for enzymes with -log E_{eq} >12 (*pro-R*) is limited, with the general category of dehydrogenases utilizing α-hydroxy carboxylic acids predominating. All these observations suggest that within the various groups of enzymes the sterochemical preference may derive from some aspect of their evolutionary origin. Such relationships, if true, would greatly diminish the significance of any apparent mechanism-based correlation.

Without a unique correlation the mechanistic proposals of Benner and co-workers become no longer pertinent. Note that the essential features of their proposal regarding the mechanistic implication of distorting the dihydronicotinamide ring have been discussed previously[18] as have the relationship between stereochemistry of hydrogen transfer and torsional conformation around the nicotinamide–ribosidic bond.[19] Finally, the activated intermediate they propose leads, in the extreme, to a valence tautomer with a planar imminium ion at the anomeric position, a form that can be viewed as deactivating the dihydronicotinamide ring for hydrogen transfer.

In conclusion, discerning ordered patterns from chaos is a noble goal for scientists. The dehydrogenases, however, remain as intractable as ever and have yet to yield the secrets of their underlying principles.

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Registry No. NADH, 58-68-4; NADPH, 53-57-6; EC 1.1.1.149, 9040-08-8; EC 1.1.1.50, 9028-56-2; EC 1.1.1.150, 37250-75-2; dehydrogenase, 9035-82-9.

(17) Marcus, P. I.; Talalay, P. *J. Biol. Chem.* **1956**, *218*, 661–674.

(18) The potential influence of puckering on the stereospecificity of dehydrogenases was first proposed by Levy and Vennesland (Levy, H. R.; Vennesland, B. *J. Biol. Chem.* **1957**, *228*, 85–96) and more recently the results of NMR experiments have been discussed in terms of the specific activation of the axial N4 proton in a puckered conformation (Oppenheimer, N. J.; Arnold, L. J., Jr.; Kaplan, N. O. *Biochemistry* **1978**, *17*, 2613–2619).

(19) Rossman and co-workers were the first to discuss seriously the effects of syn vs. anti binding on the stereospecificity of dehydrogenases (Rossman, M. G.; Liljas, A.; Branden, C. I.; Banaszak, L. J. *in* "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975: Vol. XI, pp 61–102. See also ref 1e). Note, however, implicit in the argument that syn yields *pro-S* and anti yields *pro-R* is the assumption that the coenzyme binds first, with the substrate on top. If, instead, the coenzyme were to bind on top of the substrate, then the opposite correlation would hold. Glutathione reductase represents such an example (Pai, E. F.; Schulz, G. E., *J. Biol. Chem.* **1983**, *258*, 1752–1757). It is a *pro-S*-specific enzyme that binds the dihydronicotinamide ring anti, on top of the flavin ring to be reduced.

Mechanism of Methylthiolation of the Fungicide Pentachloronitrobenzene by *Tetrahymena thermophila*

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Pentachloronitrobenzene (**1**) is a fungicide in widespread use, and residues of **1** and its transformation products have been found in a variety of foodstuffs.[1] Major metabolites of **1** are pentachloro(methylthio)benzene (**2**) and pentachloroaniline.[2] We have been investigating the biotransformation of **1** to **2** in the free living protozoan *Tetrahymena thermophila*[2] and have suggested that the pathway for replacement of a nitro group by a SCH_3 group (i.e., methylthiolation) is as follows, via the thiol pentachlorobenzenethiol (**3**):

1 → *S*-(pentachlorophenyl)glutathione →→ *S*-(pentachlorophenyl)cysteine → **3** → **2**

Methylthiolation has recently been described in several biological systems with a variety of drugs and foreign compounds,[3] and it has been proposed that the SCH_3 group can be derived from methionine or methionine derivatives (R_2–SCH_3) by a pathway involving sulfonium intermediates (path a) or direct transfer of a SCH_3 group (path b),[4] where R_1–X is a foreign compound with an electrophilic center (Scheme I). Pathway c is the suggested route for conversion of **1** to **2** in *T. thermophila*[2] and onion[5] and for certain drugs in rat liver,[6] where an intermediate thiol (R_1–SH) is methylated by *S*-adenosylmethionine (AdoMet).

To distinguish unequivocally between these pathways, we have used dual isotopic labeling with [*methyl*-^{3}H]methionine and [^{35}S]methionine to label the methionine and AdoMet pools of *T. thermophila*. In addition, since this organism converts methionine to cysteine via transsulfuration,[7] cells exposed to [^{35}S]methionine contain [^{35}S]cysteine and [^{35}S]glutathione (GSH); the later thiol is responsible for initiating metabolism of **1** in *T. thermophila*.[2] If cells, radiolabeled to constant specific activity, are exposed to **1** and then **2** is isolated, the ^{3}H/^{35}S ratio of **2** should equal that of the methionine pool if pathway a or b is operative. If pathway c is used exclusively, the ^{3}H/^{35}S ratio of **2** should be equal to the ratio of cellular [^{3}H]AdoMet/cellular [^{35}S]GSH, assuming single pools of these compounds.[8]

T. thermophila was grown in defined medium[9] in the presence of L-[*methyl*-^{3}H]methionine (10.9 mCi/mmol) and L-[^{35}S]-methionine (3.5 mCi/mmol)[10] and transferred 3 times to assure uniform labeling of cellular pools. Labeled cells were incubated with **1**, and the resulting **2** and cellular methionine were isolated by a procedure developed to give pure **2** and methionine, each

(1) Dejonckheere, W.; Steurbaut, W.; Kips, R. H. *Bull. Environ. Contam. Toxicol.* **1975**, *13*, 720. Quirijns, J. K.; Van Der Paauw, C. G.; Ten Noever De Branw; Devvos, R. H. *Sci. Total Environ.* **1979**, *13*, 225. Heikes, D. L. *Bull. Environ. Contam. Toxicol.* **1980**, *24*, 338.

(2) Murphy, S. E.; Drotar, A.; Fall, R. *Chemosphere* **1982**, *11*, 33.

(3) Bergman, A.; Brandt, I.; Larsson, Y.; Wachtmeister, C. A. *Chem. Biol. Int.* **1980**, *31*, 65. Kaul, R.; Kiefer, G.; Hempel, B. *Chemosphere* **1981**, *10*, 929.

(4) Stillwell, W. G. *Trends Pharmacol. Sci.* **1981**, *2*, 250.

(5) Lamoureux, G. L.; Rusness, D. G. *Pestic. Biochem. Physiol.* **1980**, *14*, 50.

(6) Tateishi, M.; Shimiju, H. "Enzymatic Basis of Detoxication"; Jakoby, W. B. Ed.; Academic Press: New York, 1980; Vol. II, Chapter 6.

(7) Murphy, S. E. Ph.D. Thesis, University of Colorado, Boulder, 1982.

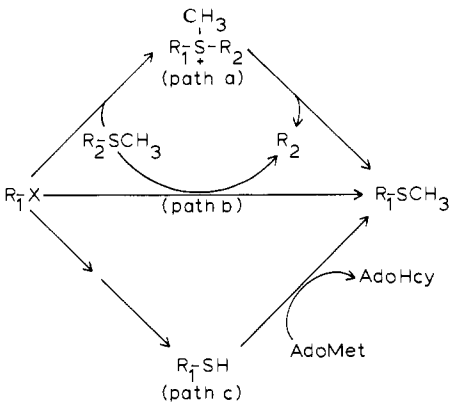
(8) Meredith, M. J.; Reed, D. J. *J. Biol. Chem.* **1982**, *257*, 3747.

(9) Holz, G. G.; Erwin, J.; Rosenbaum, N.; Aaronson, S. *Arch. Biochem. Biophys.* **1982**, *98*, 312. Sanford, Y. M.; Orias, E. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 7614. The methionine content was routinely reduced to 0.4 mM, and L-cysteine was added at 0.8 mM. Under these growth conditions approximately 25% of the intracellular cysteine pool is derived from methionione via transsulfuration.

(10) Radiolabeled methionine was obtained from Amersham, Arlington Heights, IL. Cells were transferred 3 times in medium containing [^{3}H, ^{35}S]-methionine to assure uniform labeling of cellular pools.

0002-7863/84/1506-3033$01.50/0

Scheme I



resolved from other radiolabeled metabolites.[11] The resulting $^3H/^{35}S$ ratio of **2** and methionine are shown in Table I (experiment A). The $^3H/^{35}S$ ratio of **2** was significantly higher than that of the methionine pool, suggesting that methionine is not a direct precursor of the SCH_3 group of **2**. In separate experiments cell extracts of *T. thermophila*, dialyzed to remove GSH, were incubated with **1** and [*methyl*-^3H, ^{35}S]methionine; carrier **2** was added and **2** isolated as described above. No significant amounts of ^3H or ^{35}S could be isolated in the purified **2** fraction, ruling out paths a and b as major contributions to the methylthiolation of **1**.[16]

To establish the source of the S atom and methyl group of **2**, we modified the isolation of radiolabeled metabolites (above) to allow the simultaneous determination of the specific radioactivity of **2**, methionine, AdoMet, and GSH.[17] Each of the four metabolites was quantitated, and the specific radioactivity (and $^3H/^{35}S$ ratio, where appropriate) was calculated.

Table I. [^{3}H] and [^{35}S] Contents of Metabolites from *T. thermophila* Cells Dual Labeled with [^{3}H]- and [^{35}S]Methionine

metabolite	product analysis	[^{3}H]/[^{35}S]
	Experiment A	
2	first crystals	19.51 ± 0.09
	second crystals	18.18 ± 0.11
	third crystals	18.21 ± 0.12
methionine	enzymatic release of methanethiol	2.35 ± 0.09[a]

metabolite	concentration, nmol/10^6 cells	[^{3}H], dpm/nmol	[^{35}S], dpm/nmol	[^{3}H]/[^{35}S]
		Experiment B		
2	1.6	18 215 (±4.6%)	1016 (±7.3%)	17.92 ± 0.14
methionine	0.44	20 370 (±4.6%)	7517 (±6.5%)	2.71 ± 0.12[a]
AdoMet	0.78	19 071 (±4.9%)	7363 (±6.5%)	2.59 ± 0.12
GSH	3.47		1036 (±7.3%)	
[^{3}H]AdoMet/[^{35}S]GSH				18.4 ± 0.3

[a] The [^{3}H]/[^{35}S] ratio of the dual labeled methionine added to the growth medium was 3.08 ± 0.08. In different experiments the [^{3}H]/[^{35}S] ratio of methionine recovered from the cell varied from 2.27 to 2.71 depending on the age of the culture. This decrease in the ratio is probably due to methionine recycling,[21] where the ^{3}H labeled methyl group of methionine is transferred to cellular acceptors (via AdoMet) and the resulting homocysteine is remethylated via a methyl transfer from nonradiolabeled precursors such as serine (via methyltetrahydrofolate-homocysteine methyltransferase[22]). L-Serine was present at 1.4 mM in the defined growth medium.[9]

Table I experiment B summarizes the specific radioactivity of **2**, methionine, AdoMet, and GSH. When $^3H/^{35}S$ ratios were calculated, again the ratio of **2** was higher than that of methionine, consistent with the results of experiment A. The calculated ratio of [^{3}H]AdoMet/[^{35}S]GSH was 18.4 compared to a similar ratio, 17.92, for **2**. This is consistent with a multistep origin of the SCH_3 group of **2** as in path c: (1) the S atom is contributed by GSH via a GSH transferase reaction, followed by a processing of the resulting GSH conjugate to **3** and (2) the methyl group of **2** is contributed by AdoMet in a specific thiol methyltransferase reaction. Additional evidence for this scheme has been obtained in our laboratory with the isolation of two key enzymes from *T. thermophila*.[18,19] These include a GSH transferase that is active with **1**, producing an *S*-(pentachlorophenyl)glutathione conjugate[7,19] and an AdoMet-dependent thiol methyltransferase that catalyzes the conversion of **3** to **2**.[18]

While the exact mechanism of methylthiolation of xenobiotics in other biological systems remains to be established, it seems likely that the multistep mechanism described here is operative in many eukaryotes, given the widespread distribution of cysteine conjugate β-lyases, enzymes that produce xenobiotic thiols,[6] and xenobiotic thiol methyltransferases, enzymes that detoxify such thiols by methylation, yielding much less reactive methylthio ether derivatives.[20]

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Registry No. 1, 82-68-8; **2**, 1825-19-0.

(11) The experimental procedure was as follows: Labeled cells were washed with 10 mM Tris-Cl (pH 7.4) and incubated with **1** for 3 h as previously described.[2] Cells were harvested by centrifugation and homogenized in cold 5% (w/v) trichloroacetic acid, and the acid extract was extracted 5 times with diethyl ether to remove **2** and trichloroacetic acid (organic phase); the aqueous phase was fractionated as described below. The organic phase was partitioned with 5% (w/v) Na_2CO_3 and hexane, separating **2** into the hexane layer; the hexane layer was applied to a TLC plate (silica gel HF264; E. Merck, Darmstadt, Germany) that was developed with hexane–chloroform (9:1)[12] to yield purified **2**, eluted with ether. Analysis of purified **2** by gas chromatography, as described previously,[2] revealed a single peak with a retention time identical with that for authentic **2**. Carrier **2** was added, **2** recrystallized from ethanol, 3 times, and an aliquot of the crystals counted by liquid scintillation.[13] The aqueous phase was applied to a column of (*p*-mercuribenzoyl)agarose[14] to remove thiols; the nonadsorbed fraction was retained for methionine analysis. The nonadsorbed fraction was treated with purified methionine γ-lyase[15] in order to release methanethiol (with the ^{3}H and ^{35}S radiolabel from methionine), and the resulting methanethiol was rapidly trapped on (mercuribenzoyl)agarose, eluted, and counted.

(12) Rusness, D. G.; Lamoureux, G. L. *J. Agric. Food Chem.* **1980**, *28*, 1070.

(13) Scintiverse (Fisher Chemicals) scintillation fluid was used. Samples were counted on a Beckman LS-3145 counter, measuring ^{3}H and ^{35}S by the methods for dual isotope counting that include quench correction, ^{35}S decay, and ^{35}S activity appearing in the ^{3}H window.

(14) Bio-Rad, Richmond, CA; column run as described in: Fahey, R. C.; Newton, G. L.; Dorian, R.; Kosower, E. M. *Anal. Biochem.* **1980**, *107*, 1.

(15) Tanaka, H.; Imahara, H.; Esaki, N.; Soda, K. *J. Appl. Biochem.* **1980**, *2*, 439.

(16) As pointed out be a reviewer, this experiment does not completely rule out the possibility that other potential methylthio donors, (methylthio)-adenosine, for example, could be involved.

(17) After the mercuribenzoyl-agarose step,[11] GSH was isolated from material bound to the column and AdoMet (and methionine) was resolved from the nonadsorbed fraction. GSH and other thiols were eluted by washing with dithiothreitol. The thiol fraction was freeze-dried, oxidized with GSH peroxidase, and applied to a column of Bio-gel P-2 (200-400 mesh) equilibrated with 0.05 M sodium phosphate, pH 7.4, and the peak of oxidized glutathione (GSSG) isolated. The pooled peak was treated with NADPH and GSH reductase for 20 min and the resulting thiol trapped on (mercuribenzoyl)agarose, eluted, and counted. The nonabsorbed fraction from above was adjusted to pH 3 and chromatographed on cellulose phosphate as previously described yielding pure AdoMet and a nonabsorbed fraction containing methionine. The latter fraction was freeze dried, resuspended in buffer, and treated with methionine γ-lyase; the resulting methanethiol was trapped on (mercuribenzoyl)agarose, eluted, and counted.[13]

(18) Drotar, A-M. Ph.D. Thesis, University of Colorado, Boulder, 1982.

(19) Overbaugh, J. Ph.D. Thesis, University of Colorado, Boulder, 1983.

(20) Larsen, G. L.; Bakke, J. E. *Xenobiottca* **1983**, *13*, 115.

(21) Barak, A. J.; Beckenhauer, H. C.; Tuma, D. J. *Anal. Biochem.* **1982**, *127*, 372.

(22) Finkelstein, J. D.; Kyle, W. E; Harris, B. J. *Arch. Biochem. Biophys.* **1971**, *146*, 84.