Inhibition of Glyceraldehyde-3-phosphate Dehydrogenase by Pentalenolactone. 2. Identification of the Site of Alkylation by Tetrahydropentalenolactone†

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ABSTRACT: Incubation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the antibiotic pentalenolactone (**3**) results in time-dependent, irreversible inhibition of GAPDH by modification of a single Cys residue in each subunit of the homotetrameric enzyme. Reduction of pentalenolactone with tritium gas gave [2,3,7,8-$^3H_4$]tetrahydropentalenolactone (**7**), which also exhibited time-dependent, irreversible inactivation of GAPDH. The site of covalent attachment of **7** was determined. Tryptic digestion of inactivated GAPDH and purification of the resultant products by reverse-phase HPLC gave a single labeled peptide. Amino acid sequence analysis of the radioactive peptide gave Ile-Val-Ser-Asn-Ala-Ser-X-Thr-Thr-Asn-(...). This sequence is identical to the highly conserved region from Ile-143 to Asn-152 in pig muscle GAPDH, except for the active site Cys-149 to which the tetrahydropentalenolactone was covalently bound. Molecular modeling was used to compare both pentalenolactone (**3**) and heptelidic acid (**4**), a mechanistically related inactivator of GAPDH, with the normal substrate, glyceraldehyde 3-phosphate (**1**). Finally, pentalenolactone was shown by reaction with model thiols to undergo epoxide ring opening exclusively by nucleophilic attack at the primary carbon, C-10.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH[1]) catalyzes the reversible NAD^+-dependent oxidative phosphorylation of glyceraldehyde 3-phosphate (G-3-P, **1**) to 1,3-diphosphoglycerate (1,3-DPG, **2**). GAPDH was first purified and crystallized from yeast (Warburg & Christian, 1939) and shortly thereafter from rabbit skeletal muscle (Cori et al., 1945; Caputto & Dixon, 1945). High-resolution structures have been determined for the crystalline enzyme from lobster tail muscle (Buehner et al., 1974; Moras et al., 1975) and *Bacillus stearothermophilus* (Biesecker et al., 1977; Skarzynski et al., 1987). Complete amino acid sequences were established for each of these enzymes (Davidson et al., 1967; Walker et al., 1980), as well as from pig muscle (Harris & Perham, 1968) and yeast (Jones & Harris, 1972). More recently, several genes coding for GAPDH have been cloned and sequenced, including those from *Escherichia coli* and *B. stearothermophilus* (Branlant et al., 1983), *Bacillus megaterium* (Schlapfer et al., 1990), *Thermotoga maritima* (Schultes et al., 1990), and *Arabidopsis thaliana* (Shih et al., 1992). The various enzymes have sequence identities of 50–60%. The enzymes from each source appear to have four identical polypeptide chains and molecular weights of 145 000 (Harris & Walters, 1976). In addition to its well-known glycolytic activity, GAPDH has recently been reported to bind to transfer RNA in a sequence-specific fashion (Singh & Green, 1993) and to bind to single-stranded DNA (Grosse et al., 1986).

The currently accepted mechanism for GAPDH, first proposed in 1953 (Segal & Boyer, 1953), is supported by extensive pre-steady-state and steady-state kinetic studies (Figure 1) (Orsi & Cleland, 1972; Harrigan & Trentham, 1974; Duggleby & Dennis, 1974; Meunier & Dalziel, 1978; Canellas & Cleland, 1991; Liu & Huskey, 1992). The enzyme

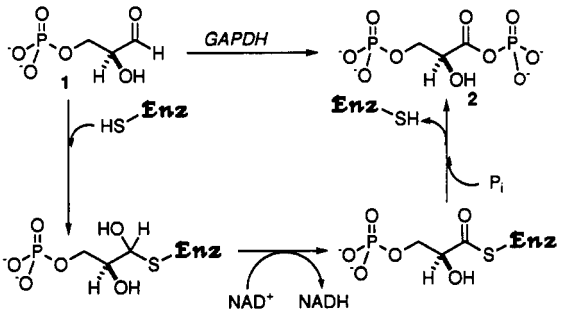

FIGURE 1: Mechanism of the conversion of G-3-P (**1**) to 1,3-DPG (**2**) by GAPDH.

has an active site cysteine residue, which reacts with the aldehyde of G-3-P to form a hemiacetal. Transfer of a hydride to tightly bound NAD^+ then generates the reduced coenzyme, NADH, and an acyl thioester. After dissociation of NADH and binding of another NAD^+, inorganic phosphate then attacks the thioester to form 1,3-DPG. Consistent with this mechanism is the observation that the thioester can also be generated by the reaction of GAPDH with acyl phosphates. A crucial aspect of the formation of 1,3-DPG from G-3-P is that a thermodynamically unfavorable reaction, the formation of an acyl phosphate from a carboxylate, is driven by a thermodynamically favorable reaction, the oxidation of an aldehyde.

Although there are no crystal structures of GAPDH with bound G-3-P, various investigators have used model building to infer the mode of binding of the substrate G-3-P, on the basis of the observation that there are two apparent anion-binding sites within 7 Å of Cys-149 in the *Bacillus stearothermophilus* and lobster GAPDH structures (Moras et al., 1975; Skarzynski et al., 1987). The active site is thought to be a cleft between the coenzyme-binding domain and the catalytic domain, with the essential thiol group of Cys-149 close to the nicotinamide C-4 atom of NAD^+. In one model (Moras et al., 1975), the aldehyde group of the substrate forms

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[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-3-P, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; TFA, trifluoroacetic acid.

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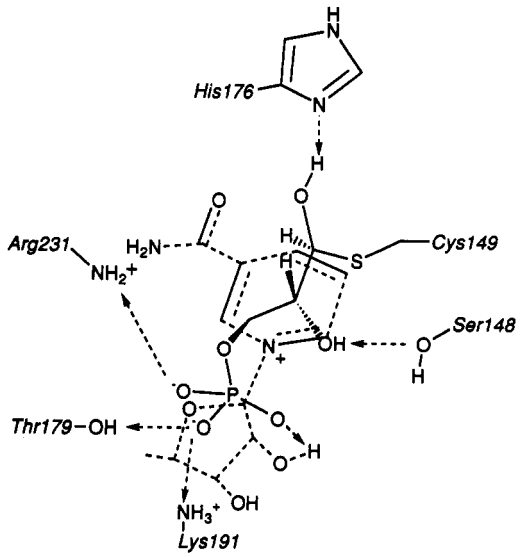
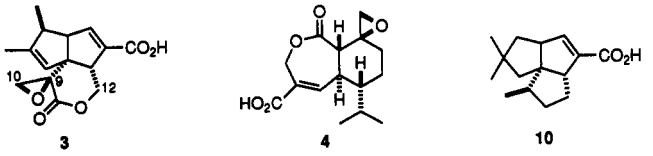


FIGURE 2: Active site model for GAPDH, illustrating the hemithioacetal between G-3-P and Cys-149 and showing proposed hydrogen bonds and ionic interactions with amino acid side chains and NAD^+ cofactor [based on Moras et al. (1975)].



3 4 10

FIGURE 3: Structures of pentalenolactone (**3**), heptelidic acid(**4**), and pentalenic acid (**10**).

a hemiacetal with Cys-149 with the 3-phosphate placed so as to make hydrogen bonds with the hydroxyl of Thr-179 and the 2′-hydroxyl of the ribose ring of NAD^+, with additional stabilization arising from ionic interactions with the positively charged side chains of Arg-231 and Lys-191 (Figure 2). The C-2 hydroxyl of the substrate can itself form a hydrogen bond with Ser-148, while the C-1 hydroxyl of the hemithioacetal can form a hydrogen bond with a nitrogen of His-176, suggesting that His-176 may act as a general-base catalyst. Rossman has suggested a somewhat different model, in which the 3-phosphate moiety is bound in the alternate anion-binding site (Skarzynski et al., 1987).

The sesquiterpene antibiotic pentalenolactone (**3**) (Figure 3) has been shown to be active against a range of microorganisms, including Gram-positive and Gram-negative bacteria, pathogenic and saprophytic fungi, and protozoa (Koe et al., 1957). Pentalenolactone has also been found to inhibit the replication of DNA viruses, including HSV-1 and HSV-2, the causal agents of herpes simplex (Nakagawa et al., 1985). Pentalenolactone blocks glycolysis in both prokaryotic and eukaryotic species by the selective inhibition of GAPDH (Hartmann et al., 1978; Mann & Mecke, 1979; Duszenko & Mecke, 1986; Duszenko et al., 1982; Nagle et al., 1985). On the other hand, pentalenolactone had no significant effect on the enzyme from spinach cytosol. The pentalenolactone-producing organism, *Streptomyces arenae*, contains two GAPDH isozymes: a pentalenolactone-sensitive form and an inducible, pentalenolactone-insensitive form (Fröhlich et al., 1989; Maurer et al., 1983). The pentalenolactone-sensitive enzyme was found to be a tetramer of four identical subunits of apparent M_r 43 000, which is similar to most other known GAPDH proteins, whereas the antibiotic-resistant form appeared to be an octamer consisting of M_r 37 000 subunits.

We have previously reported that incubation of pentalenolactone with rabbit muscle GAPDH results in time-dependent, irreversible inactivation of GAPDH (Cane & Sohng, 1989). The kinetics of inactivation was biphasic, exhibiting an initial rapid phase (k_{inact} = 3.3 min^{-1}, K_I = 11.8 μM) and a slower, but higher affinity, second phase (k_{inact} = 1.3 min^{-1}, K_I = 6.12 μM). The substrate G-3-P protected the enzyme against inactivation by pentalenolactone, whereas the nicotinamide cofactor actually stimulated the inactivation by increasing the apparent affinity of GAPDH for pentalenolactone, without a significant effect on the maximal rate of inactivation. Thiol titrations revealed that pentalenolactone inactivated GAPDH by reaction with four Cys-SH residues of the tetrameric enzyme. Pentalenolactone methyl ester was also an irreversible inactivator of GAPDH, but at a 10-fold lower rate and a 10-fold greater K_I.

In the report here, we identify the active site Cys in GAPDH that is covalently modified by tetrahydropentalenolactone. We also report the results of reaction of pentalenolactone with model thiols, as well as molecular modeling comparisons of pentalenolactone with both G-3-P and a second, structurally similar inhibitor, heptelidic acid (**4**).

MATERIALS AND METHODS

General instrumental and spectroscopic methods, as well as sources of common reagents, buffers, and enzymes, have been described previously (Cane & Sohng, 1989; Cane et al., 1992). Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was purchased from Sigma (St. Louis, MO) as a crystalline suspension in 2.6 M $(NH_4)_2SO_4$ (80–120 units/mg of protein). Inactivation and assay of GAPDH, as well as data analysis, were as previously described (Cane & Sohng, 1989). Pentalenolactone was isolated from cultures of *Streptomyces* UC5319 and converted to pentalenolactone methyl ester (**5**), also as previously described (Cane et al., 1981). Heptelidic acid was a gift from Professor D. Arigoni of the the Eidgenössische Technische Hochschule in Zürich, Switzerland. Synthetic (±)-heptelidic acid and (±)-epiheptelidic acid were gifts from Prof. S. Danishefsky of Yale University (New Haven, CT).

2,3,6,7-Tetrahydropentalenolactone Methyl Ester (**6**). Pentalenolactone methyl ester (**5**) (12 mg) in 10 mL of methanol was added along with 2 mg of 5% Pd/C to a 50-mL two-neck flask connected to a hydrogen-filled balloon, and the mixture was stirred for 24 h at room temperature. The Pd/C was removed by filtration through Celite, methanol was removed at aspirator pressure, and the crude reduction product (11 mg) was purified by flash column chromatography (silica gel, benzene/ethyl acetate, 10:1) to give tetrahydropentalenolactone methyl ester (**6**) (benzene/ethyl acetate, 5:1, R_f 0.32), accompanied by minor amounts of 6,7-dihydropentalenolactone methyl ester (benzene/ethyl acetate, 5:1, R_f 0.5) and 9,10-deoxy-9,10-dihydro-9-hydroxy-2,3,6,7-tetrahydropentalenolactone methyl ester (benzene/ethyl acetate, 5:1, $R_f < 0.2$). Each of the recovered products was purified by preparative thin-layer chromatography (silica gel, benzene/ethyl acetate = 20:1, four developments) to give pure **6** (solid, 7 mg), dihydropentalenolactone methyl ester (oil, 1.1 mg), and a trace of 9,10-deoxy-9,10-dihydro-9-hydroxy-2,3,6,7-tetrahydropentalenolactone methyl ester. 2,3,6,7-Tetrahydropentalenolactone methyl ester (**6**): ^1H NMR ($CDCl_3$) δ 4.63 (dd, J = 4.7, 11.8 Hz, H-12a, 1 H), 4.21 (dd, J = 4.7, 11.8 Hz, H-12b, 1 H), 3.69 (s, OCH_3, 3 H), 3.16 (d, J = 4.7 Hz, H-10a, 1 H), 2.92 (d, J = 4.7 Hz, H-10b, 1 H), 2.45–2.7 (m, H-5 and H-6, 2 H), 2.2 (m, H-8, 1 H), 1.98–1.3

(m, H-7, H-3, H-2, and H-1, 6 H), 0.92 (d, J = 5.9 Hz, H-14, 3 H), 0.81 (d, J = 6.8 Hz, H-15, 3 H); ^{13}C NMR ($CDCl_3$) δ 173.6, 169.9, 67.8, 59.0, 52.2, 52.1, 51.5, 51.2, 48.0, 46.6, 44.1, 43.8, 36.4, 31.5, 16.8, 12.9; mp 102–105 °C; R_f = 0.32 (benzene/ethyl acetate, 5:1). 6,7-Dihydropentalenolactone methyl ester: ^{1}H NMR ($CDCl_3$) δ 5.08 (s, H-3, 1 H), 4.70 (dd, J = 3.8, 11.8 Hz, H-12a, 1 H), 4.35 (dd, J = 1.8, 11.8 Hz, H-12b, 1 H), 3.69 (s, OCH_3, 3 H), 3.17 (d, J = 5.27 Hz, H-10a, 1 H), 2.90 (m, H-5, 1 H), 2.57 (d, J = 5.25 Hz, H-10b, 1 H), 2.71–2.51 (m, H-1, H-6, and H-8, 3 H), 2.0–1.6 (m, H-7, 2 H), 1.63 (s, H-14, 3 H), 0.93 (d, J = 7.25 Hz, H-15, 3 H); ^{13}C NMR ($CDCl_3$) δ 174.0, 169.4, 146.3, 123.8, 67.4, 58.5, 57.8, 52.0, 51.5, 47.8, 45.9, 45.7, 43.0, 31.2, 14.4, 12.4; R_f = 0.48 (benzene/ethyl acetate, 5:1). 9,10-Deoxy-9,10-dihydro-9-hydroxy-2,3,6,7-tetrahydropentalenolactone methyl ester: ^{1}H NMR ($CDCl_3$) δ 4.88 (dd, J = 6.4, 11.8 Hz, H-12a, 1 H), 4.23 (dd, J = 6.0, 11.8 Hz, H-12b, 1 H), 3.67 (s, OCH_3, 3 H), 2.75 (s, OH, 1 H), 2.65 (m, H-5, 1 H), 2.45 (m, H-6, 1 H), 2.38 (m, H-8, 1 H), 1.98–1.52 (m, H-6, H-3, H-2, and H-1, 6 H), 1.44 (s, H-10, 3 H), 0.94 (d, J = 6 Hz, H-14, 3 H), 0.88 (d, J = 6.8 Hz, H-15, 3 H); R_f = 0.5 (benzene/ethyl acetate, 5:1).

Inactivation of GAPDH with [2,3,6,7-$^3H_4$]-2,3,6,7-Tetrahydropentalenolactone. Pentalenolactone methyl ester (**5**) was reduced with carrier-free tritium gas over 5% Pd/C in methanol by New England Nuclear Co. by the procedure described above for unlabeled material to give, after purification, [2,3,6,7-$^3H_4$]-2,3,6,7-tetrahydropentalenolactone methyl ester (**6**) (specific activity 136 mCi/mg). The structure and purity of the tritiated sample of **6** were confirmed by ^{1}H NMR. After dilution with unlabeled **6**, the product was hydrolyzed to [2,3,6,7-$^3H_4$]-2,3,6,7-tetrahydropentalenolactone (**7**) and converted to the benzylamine salt, as previously described for pentalenolactone (Cane & Sohng, 1989). Glyceraldehyde-3-phosphate dehydrogenase (1 mg) was incubated with [^{3}H]-tetrahydropentalenolactone benzylamine salt (specific activity 20 μCi/mg, 0.2 mg) for 30 min in 0.1 M ammonium bicarbonate (pH 7.8). The inactivated enzyme was passed through a Sephadex G-25 column to remove the unbound inactivator. The residual enzyme activity (4%) and the bound radioactivity were determined on 50-μL aliquots of the eluent.

The labeled protein was digested overnight at 37 °C with trypsin (trypsin:protein = 2:100 (w/w)). The total tryptic digest was first fractionated by filtration through a Diaflow PM 10 (Amicon) ultrafiltration membrane to remove the high molecular weight fraction (M_r >10 000). The low molecular weight pool of tryptic peptides was then fractionated further by reverse-phase HPLC on an Altex Ultrasphere octyl ODS column using a 180-min linear gradient from 100% solvent I (0.1% TFA) to 40% solvent II (TFA/water/acetonitrile, 0.1:9.9:90, v/v/v), with a flow rate of 0.8 mL/min. Fractions (50 drops) were collected and monitored at λ = 214 nm. Aliquots (10 μL) from each fraction were counted for ^{3}H radioactivity.

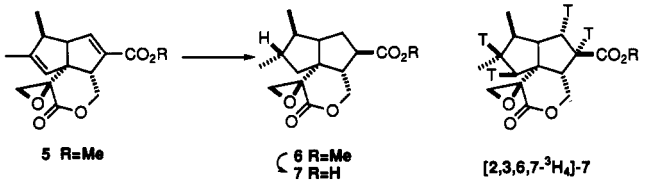
Two sets of fractions for which the aliquots showed ^{3}H activity significantly (at least 10×) above the background of 16–18 dpm were combined separately. Combined fraction A was from HPLC fractions 73 and 74 (94–95 min), while fraction B was from HPLC fractions 93 and 94 (116–117 min), as indicated in Figure 4. Both fractions were each separately purified further on a Vydac C-18 peptide/protein reverse-phase column using a 120-min linear gradient from solvent I (0.1% TFA) to solvent II (TFA/water/acetonitrile, 0.1:9.9:90, v/v/v), with an 0.8 mL/min flow rate. Fractions of 50 drops each were collected and monitored at λ = 214 nm, and 10-μL aliquots from each fraction were counted for ^{3}H radioactivity. Fraction A gave rise to two peptide peaks that were visible in the HPLC, only one of which (A-1) was radioactive. Fraction A-1 was applied once more to the Vydac C-18 column to confirm the presence of only a single component. Analysis of fraction B on the Vydac C-18 column showed three major peptide components, none of which were radioactive. More than 90% of the applied radioactivity eluted 1–2 min before the major peptide components in a region containing only minor peaks (<2% of total integrated intensity). It was concluded that fraction B most likely contained non-peptide-bound material, perhaps minor amounts of hydrolytic or other degradation products of tetrahydropentalenolactone, that had not been removed by the Sephadex G-25 treatment of inactivated GAPDH. Due to the extremely low amounts of (apparently) radioactive peptide, fraction B was not investigated further.

Sequencing of Labeled Tryptic Peptide A-1. The purified radioactive peptide A-1 was analyzed by automated gas-phase Edman degradation on an ABI Model 477A protein sequencer by Dr. William Lane of the Harvard Microchemistry Facility.

*Reaction of Pentalenolactone Methyl Ester (**5**) with Sodium Thiobutoxide.* Pentalenolactone methyl ester (**5**) (10 mg, 0.034 mmol) was added to a 50-mL flask containing 10 mL of ethanol. Sodium thiobutoxide (4 mg, 0.035 mmol, prepared from 1-butanethiol and an equimolar portion of finely dispersed sodium in diethyl ether) was added, and this mixture was stirred at room temperature for 1 h. The reaction mixture was transferred to a separatory funnel, and 30 mL of chloroform and 10 mL of water were added. After extraction, the organic layer was drawn off and the aqueous layer was extracted with an additional 20 mL of $CHCl_3$. The combined organic layers were washed with saturated sodium chloride. The organic layer was dried over anhydrous sodium sulfate, and the product was purified by preparative thin-layer chromatography (benzene:ethyl acetate = 20:1, three developments). The yield of **8** was 67% (9 mg, 0.023 mmol): ^{1}H NMR ($CDCl_3$) δ 6.69 (s, H-7, 1 H), 5.35 (s, H-3, 1 H), 5.21 (dd, J = 5.24, 11.3 Hz, H-12a, 1 H), 4.43 (d, J = 11.28 Hz, H-12b, 1 H), 4.17 (s, OH-9, 1 H), 3.72 (s, OCH_3, 3 H), 3.33 (d, J = 13.8 Hz, H-10a, 1 H), 3.18 (m, H-5, 1 H), 3.10 (m, H-8, 1 H), 2.73 (m, H-1, 1 H), 2.54 (m, H-1′, 2 H), 2.43 (d, J = 13.8 Hz, H-10b, 1 H), 1.61 (s, H-15, 3 H); 1.51 (m, H-2′, 2 H), 1.38 (sextet, J = 7.52 Hz, H-3′, 2 H), 1.01 (d, J = 7.30 Hz, H-14, 3 H), 0.89 (t, J = 7.3 Hz, H-4′, 3 H); ^{13}C NMR ($CDCl_3$) δ 44.8 (C-1), 146.2 (C-2), 124.7 (C-3), 64.8 (C-4), 49.8 (C-5), 134.1 (C-6), 145.3 (C-7), 55.1 (C-8), 76.1 (C-9), 36.2 (C-10), 171.6 (C-11), 68.6 (C-12), 164.4 (C-13), 15.3 (C-14), 14.5 (C-15), 51.6 (OCH_3), 13.6 (C-4′), 21.8 (C-3′), 31.9 (C-2′), 34.1 (C-1′); IR (neat) 3350, 2920, 2840, 1745, 1710, 1640, 1600, 1460, 1440, 1350, 1370 (cm^{-1}); CIMS m/z (NH_3) found, 380.1645 (calcd for $C_{20}H_{28}O_5S$, 380.1650); R_f = 0.34 (benzene/ethyl acetate, 5:1).

Reaction of Pentalenolactone Methyl Ester with L-Cysteine Methyl Ester. Pentalenolactone methyl ester (**5**) (5 mg, 0.017 mmol) was added to a 50-mL flask containing 5 mL of THF, and 10 mL of 0.135 M Tris base (pH 8.5) was added. After addition of L-cysteine methyl ester (0.085 mmol, 5 equiv), the reaction mixture was stirred at room temperature. After 8 h, the reaction product was extracted with successive 30-, 20-, and 20-mL portions of $CHCl_3$. The combined organic extracts were washed with saturated sodium chloride solution and then dried over anhydrous sodium sulfate. The product was purified by preparative thin-layer chromatography (benzene/ethyl acetate, 1:1, two developments) to give 4 mg of **9** (0.01 mmol,

Scheme 1: Preparation of [2,3,6,7-$^3H_4$]-7 by Reduction of Pentalenolactone Methyl Ester (5) with Tritium Gas over Pd/C Followed by Base-Catalyzed Hydrolysis



60% yield): ^1H NMR ($CDCl_3$) δ 6.68 (bs, H-7, 1 H), 5.4 (s, H-3, 1 H), 5.26 (dd, J = 11.1, 5.28 Hz, H-12a, 1 H), 4.40 (d, J = 11.08 Hz, H-12b, 1 H), 3.73 (s, OCH_3, 3 H), 3.72 (s, OCH_3, 3 H), 3.61 (dd, J = 4.32, 3.9 Hz, CHN, 1 H), 3.26 (d, J = 14.5 Hz, H-10a, 1 H), 3.15 (m, H-5, 1 H), 3.07 (dt, J = 2.76, 8.56 Hz, H-8, 1 H), 2.95 (dd, J = 4.36, 14.44 Hz, SCH_2a, 1 H), 2.78 (dd, J = 8.2, 14.44 Hz, SCH_2b, 1 H), 2.73 (m, H-1, 1 H), 2.56 (d, J = 14.5 Hz, H-10b, 1 H), 1.66 (s, H-15, 3 H), 1.0 (d, J = 7.36 Hz, H-14, 3 H); ^{13}C NMR ($CDCl_3$) δ 44.6 (C-1), 145.8 (C-2), 125.2 (C-3), 65.3 (C-4), 49.6 (C-5), 134.1 (C-6), 145.0 (C-7), 52.6 (C-8), 77.5 (C-9), 38.2 (C-10), 172.5 (C-11), 68.8 (C-12), 164 (C-13), 15.4 (C-14), 14.4 (C-15), 51.6 (OCH_3), 54.5 (CHN), 174.5 (CO_2), 37.7 (CH_2S), 55.3 (OCH_3); IR (neat) 3400, 2950, 2860, 1740, 1640, 1430, 1350 (cm^{-1}); CIMS m/z (NH_3) found, 425.1531 (calcd for $C_{20}H_{28}O_7NS$, 425.1501); R_f = 0.45 (benzene/ethyl acetate, 5:1).

RESULTS AND DISCUSSION

In order to establish the site of inactivation of GAPDH by pentalenolactone, we required a labeled sample of **3** or a simple derivative. We chose to use 2,3,6,7-tetrahydropentalenolactone (**7**), which could be readily prepared by catalytic hydrogenation of pentalenolactone methyl ester (**5**) followed by mild basic hydrolysis (Scheme 1). The tetrahydro derivative **6** was obtained as a single stereoisomer and its structure confirmed by ^1H and ^{13}C NMR spectroscopy. Both the characteristic broad triplet at δ 6.88 corresponding to H-3 in pentalenolactone methyl ester and the H-7 olefinic signal at δ 5.1 were absent from the spectrum of **6**. Similarly, the distinctive olefinic carbon resonances were replaced by four corresponding higher field signals in the ^{13}C NMR spectrum of **6**. The stereochemistry of **6** was assigned on the basis of the configuration of the corresponding bromohydrin, which has previously been established by X-ray crystallography (Martin et al., 1970). In the event, tetrahydropentalenolactone (**7**), as the benzylamine salt, proved to be a time-dependent, irreversible inactivator of GAPDH with k_{inact} = 0.66 ± 0.06 min^{-1} and K_I = 44 ± 7 μM. Inactivation was monophasic. Although slightly less effective than pentalenolactone itself, tetrahydropentalenolactone was assumed to inactivate GAPDH by the same mechanism.

To prepare tritiated **7**, pentalenolactone methyl ester (**5**) was treated with carrier-free tritium gas in the presence of a catalytic amount of Pd/C to give [2,3,6,7-^3H]-2,3,6,7-tetrahydropentalenolactone methyl ester (**6**). The methyl ester was diluted with unlabeled material, hydrolyzed to the free acid, and converted to the benzylamine salt.

Rabbit muscle GAPDH (1.5 mg) was incubated with [2,3,6,7-^3H]-2,3,6,7-tetrahydropentalenolactone (**7**) benzylamine salt (specific activity 20 μCi/mg, 0.2 mg) for 30 min at room temperature in 1 mL of 0.1 M ammonium bicarbonate (pH 7.8). The unbound inhibitor was removed by gel filtration, and the eluent was digested with trypsin at 37 °C overnight (Chen et al., 1986). Ultrafiltration of the trypsin-digested protein removed peptides of molecular weight >10 000. The resulting sample was purified by reverse-phase HPLC on an Altex Ultrasphere octyl column, using an increasing gradient of acetonitrile in 0.1% aqueous TFA. Aliquots from each fraction were counted for ^3H activity. Two groups of radioactive peaks were detected (Figure 4a). One peak (A) had a large absorbance at 214 nm and higher radioactivity and was chosen for further examination. Peak A was further purified on a Vydac C-18 peptide/protein column using the same gradient as above. Of the two peaks, A-1 was radioactive while A-2 was not (Figure 4b). A-1 was applied once more to a Vydac C-18 peptide/protein column to confirm the presence of only one peak (Figure 4c). The second radioactive peak (B) had considerably lower activity than peak A. Moreover, upon further purification on the C-18 column, the radioactivity did not coelute with any of the major peptide components.

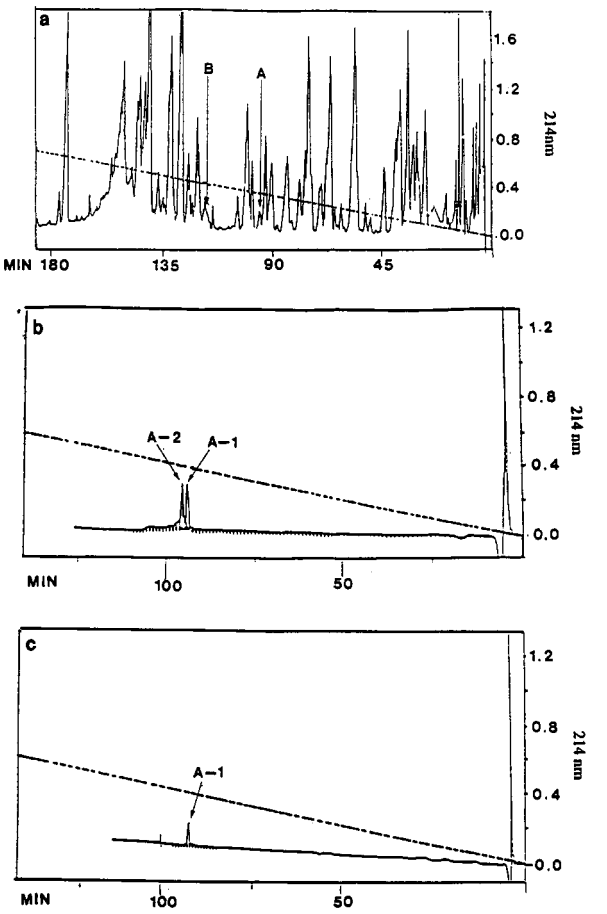


FIGURE 4: (a) Reverse-phase HPLC (Altex Ultrasphere octyl ODS) of tryptic peptides derived from rabbit muscle GAPDH inactivated with [2,3,6,7-$^3H_4$]-**7b** using a 180-min linear gradient from 100% solvent I (0.1% TFA) to 40% solvent II (TFA/water/acetonitrile, 0.1:9.9:90, v/v/v) (see Materials and Methods for experimental details). (b) Reverse-phase HPLC (Vydac C-18) purification of fraction A using a 120-min linear gradient from solvent I (0.1% TFA) to solvent II (TFA/water/acetonitrile, 0.1:9.9:90, v/v/v). (c) Reverse-phase HPLC (Vydac C-18) of peptide A-1.

Analysis of the purified radioactive peptide A-1 by automated gas-phase amino acid sequence analysis gave the sequence Ile-Val-Ser-Asn-Ala-Ser-X-Thr-Thr-Asn-(...). (Table 1). This sequence is identical to the region from Ile-143 to Asn-152 in both pig muscle and yeast GAPDH, save for the absence of the normal Cys-149 at the site of modification. The region from Ser-145 to Asn-152 is in fact completely conserved in GAPDH from numerous species (Harris & Perham, 1968; Walker et al., 1980; Branlant et al., 1983;

Table 1: Amino Acid Sequence Analysis of Peptide A-1 Labeled by [2,3,6,7-$^3H_4$]-**7**

cycle	amino acid released	pig muscle GAPDH
1	Ile	Ile-143
2	Val	Val-144
3	Ser	Ser-145
4	Asn	Asn-146
5	Ala	Ala-147
6	Ser	Ser-148
7	X	Cys-149
8	Thr	Thr-150
9	Thr	Thr-151
10	Asn	Asn-152

Jones & Harris, 1972; Davidson et al., 1967; Schlapfer et al., 1990; Schultes et al., 1990; Shih et al., 1992). Indeed, this same sequence has also been located at the active site of *B. stearothermophilus* and lobster tail muscle GAPDH by X-ray diffraction studies (Buehner et al., 1974; Moras et al., 1975; Biesecker et al., 1977; Skarzynski et al., 1987). It can therefore be concluded that [2,3,6,7-$^3H_4$]tetrahydropentalenolactone labels rabbit muscle GAPDH at Cys-149.

Several groups have reported the time-dependent inactivation of GAPDH by synthetic epoxide-containing inhibitors (McCaul & Byers, 1976; Bruice et al., 1978; Stallcup & Koshland, 1973). The majority of the latter inhibitors have relatively poor affinity for GAPDH, with inhibition constants in the 100 mM range or greater, and exhibit rates of inactivation 1–2 or more orders of magnitude slower than does pentalenolactone. On the other hand, the fungal antibiotic heptelidic acid (**4**) (avocettin, koningic acid), which bears a number of intriguing structural similarities to pentalenolactone, is also an effective, time-dependent inhibitor of GAPDH. We have previously reported that the inactivation of GAPDH by heptelidic acid exhibits biphasic pseudo-first-order kinetics with $k^1_{inact} = 5.75 \pm 1.82$ min^{-1}, $K^1_I = 31.7 \pm 11.4$ μM and $k^2_{inact} = 3.12 \pm 0.94$ min^{-1}, $K^2_I = 19.3 \pm 6.5$ μM (Cane & Sohng, 1989). We have also found that synthetic (±)-heptelidic acid (Danishefsky & Mantlo, 1988) shows the same pattern of inactivation of GAPDH and almost the same rate constant k_{inact} as heptelidic acid ($k^1_{inact} = 6.53 \pm 1.52$ min^{-1} and $k^2_{inact} = 3.4 \pm 0.72$ min^{-1}), but the dissociation constants, K_I, of the racemate were 2-fold higher than those of the natural product. On the other hand, the epimeric epoxide isomer, (±)-epiheptelidic acid (Danishefsky & Mantlo, 1988), has only a very weak inhibitory action against GAPDH. This result establishes that the stereochemistry of the epoxide in heptelidic acid is very important in the mechanism of inactivation of GAPDH.

Endo and co-workers have reported more extensive studies of the inactivation of GAPDH by heptelidic acid (Sakai et al., 1988; Kato et al., 1992). Like pentalenolactone, **4** could act as a competitive inhibitor with respect to G-3-P, whereas inhibition with respect to NAD$^+$ was uncompetitive. Similarly, the presence of NAD$^+$ had a marked stimulatory effect on the inactivation. In contrast to the behavior of pentalenolactone, they found that heptelidic acid modified a maximum of two sulfhydryl residues per tetramer. These workers did not observe the biphasic inhibition behavior (subsequently) reported by us for both pentalenolactone and heptelidic acid. Although the K_I that they measured (1.6 μM) was more than a factor of 10 smaller than that found by us, the rate of inactivation by **4** ($k_{inact} = 3.4$ min^{-1}) was in good agreement with the value found in our independent study. The reasons for these small differences are unclear, but most likely reflect differences in buffer composition and assay methods used in the two studies. Endo has also used microsequencing and fast atom bombardment mass spectrometry to analyze a labeled tryptic peptide resulting from the inactivation of rabbit muscle GAPDH with [^{3}H]heptelidic acid (Sakai et al., 1991). They found that **4** specifically modifies the active site cysteine corresponding to Cys-149 in the homologous pig muscle enzyme.

Arylazido-β-alanyl-NAD$^+$, an analog of NAD$^+$, has been used for photoaffinity labeling of rabbit muscle GAPDH, also resulting in preferential modification of the active site Cys-149 (Chen et al., 1986). Methylglyoxal is yet another time-dependent irreversible inhibitor of GAPDH (Leoncini et al., 1981). Although inactivation by methylglyoxal was competitively prevented by G-3-P and stimulated by NAD$^+$,

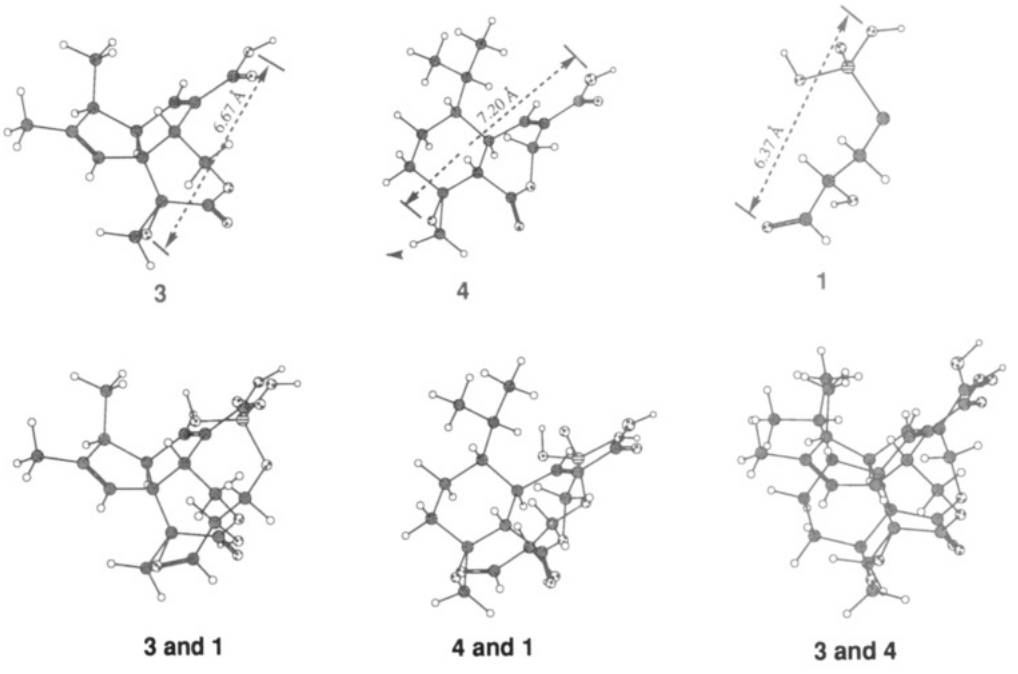
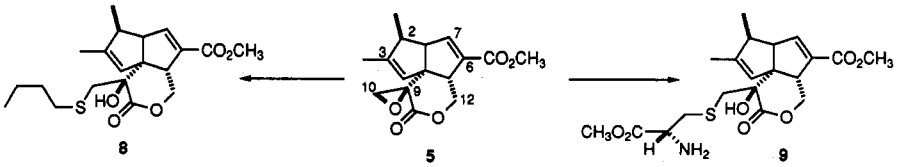


FIGURE 5: Conformations of pentalenolactone (**3**), heptelidic acid (**4**), and G-3-P (**1**) calculated by molecular mechanics and pairwise superpositions of **3** and **1**, **4** and **1**, and **3** and **4**.

Scheme 2: Reaction of Pentalenolactone Methyl Ester with Model Thiols, *n*-Butanethiol, and Cysteine Methyl Ester To Give **8** and **9**, Respectively



methylglyoxal apparently acts by modifying an arginine residue rather than the active site cysteine-SH.

Reactions with Model Thiols. Pentalenolactone (**3**) has three functional groups that potentially might react with an active site Cys-SH residue. The fact that tetrahydropentalenolactone (**7**) is an effective irreversible inhibitor of GAPDH rules out a direct role for the 6,7-double bond. Of the other two reactive groups, both the lactonic carbonyl and the epoxide could conceivably react with the active site thiol. To explore these possibilities further, we examined the reaction of pentalenolactone with model thiols.

Pentalenolactone methyl ester (**5**) was reacted with n-BuSNa in ethanol (Scheme 2). The structure of the resulting epoxide ring-opened product (**8**) was assigned by ^{1}H, ^{13}C, and ^{1}H COSY NMR spectra. The peaks for the two H-10 protons in **8** were both shifted (δ 3.28 and 2.72 to δ 3.33 and 2.43, 2 H) and the new OH group at C-9 appeared at δ 4.17. The C-10 and C-9 resonances in the ^{13}C NMR spectrum of **8** showed large shifts compared to pentalenolactone [C-10: δ 47.1 (CH_2) to δ 36.2 (CH_2); C-9: δ 59.1 (C) to δ 76.1(C)]. The doublet of doublets centered at δ 5.26 (dd, J = 11.1, 5.28 Hz, H-12, 1 H) and the doublet centered at δ 4.40 (d, J = 11.1 Hz, H-12, 1 H) corresponded to the H-12 methylene protons of the intact lactone. The combined data supported the assigned structure and ruled out the alternative mode of epoxide ring opening.

In a second model reaction, L-cysteine methyl ester was reacted with pentalenolactone methyl ester (**5**) under mild basic conditions. The ^{1}H NMR spectrum of the resulting adduct **9** displayed upfield-shifted resonances at δ 3.26 and 2.56 for the two H-10 protons and a signal at δ 4.17 corresponding to the C-9 hydroxyl proton. The signals for C-10 and C-9 in the ^{13}C NMR spectrum showed the expected chemical shift changes [C-10: δ 47.1 (CH_2) to δ 38.2 (CH_2); C-9: δ 59.1 (C) to δ 77.5 (C)], confirming attack of the sulfhydryl group of the cysteine methyl ester at the primary carbon, C-10, of the epoxide moiety. Interestingly, Endo has also reported that reaction of heptelidic acid with L-cysteine results in the formation of a thioether by exclusive attack at the primary carbon of the epoxide (Sakai et al., 1991).

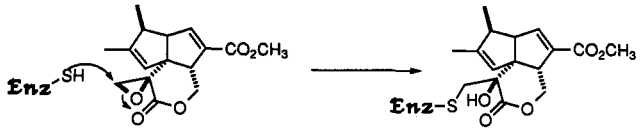
That pentalenolactone binds at the G-3-P site of GAPDH is supported by several lines of evidence, including the facts that G-3-P afforded protection against inactivation by pentalenolactone, that pentalenolactone could act as a competitive inhibitor with respect to G-3-P, and that pentalenolactone reacted specifically with the active site Cys-149. It is also evident that pentalenolactone and heptelidic acid inhibit GAPDH by closely related mechanisms, as borne out by close parallels in both the kinetics of inactivation and the site of protein modification and consistent with their similar reactivities toward simple thiols. On the other hand, neither pentalenolactone nor heptelidic acid bears any immediately obvious structural resemblance to either the natural substrate for GAPDH, G-3-P, or the coenzyme, NAD^+. Pentalenolactone (**3**) and heptelidic acid (**4**) share several structural features, including the presence of epoxide, lactone and α,β-unsaturated carboxylic acid groups as well as lipophilic, hydrocarbon moieties. G-3-P (**1**) has three polar groups, the aldehyde, hydroxyl, and phosphate moieties. In light of the fact that pentalenolactone and heptelidic acid both bind to the G-3-P binding site of GAPDH, it appeared useful to compare the structures of these two inhibitors with that of G-3-P.

Toward this end, energy minimization calculations were carried out on G-3-P (**1**), pentalenolactone (**3**), and heptelidic acid (**4**), using an MM-2 force field in conjunction with the MacroModel molecular modeling program (Mohamadi et al., 1990). For these calculations, we assumed an extended conformation for the G-3-P substrate.[2] The MacroModel program was also used to calculate the expected vicinal ^{1}H–^{1}H coupling constants for the minimized structures of both pentalenolactone and heptelidic acid, and these values were in good agreement with the experimental ^{1}H NMR data for each compound (Cane et al., 1992). On the basis of the lowest energy extended structure for G-3-P, the distance between the aldehyde oxygen atom and one of the phosphate oxygen atoms in G-3-P was calculated to be 6.37 Å. Interestingly, the calculated distance between the epoxide oxygen and one of the carboxylate oxygen atoms in pentalenolactone was 6.67 Å, while the corresponding distance in heptelidic acid was 7.20 Å. As illustrated in Figure 4, superposition of G-3-P with either pentalenolactone or heptelidic acid suggests that the carboxylate and epoxide oxygen atoms of the individual inhibitors are readily superimposed on the anionic phosphate and aldehyde oxygen atoms, respectively, of the substrate G-3-P. According to the active site model proposed by Moras et al. (1975), the sulfhydryl residue of Cys-149 attacks the aldehyde carbonyl of G-3-P from the *si* face. This same SH group would therefore be correctly positioned for nucleophilic attack on the back side of the epoxide moiety of either pentalenolactone or heptelidic acid. Moreover, in each case, the lactonic carbonyl oxygen is well-placed to occupy the site normally occupied by the G-3-P 2-hydroxyl group, which is believed to be hydrogen-bonded to Ser-148. Intriguingly, comparison of **3** and **4** indicates that the additional carbocyclic rings must occupy similar sites in GAPDH, in a region not normally filled by G-3-P, but oriented away from the nicotinamide and ribose rings of the NAD^+ cofactor. In this regard, it is interesting to note that the K_I's for both inhibitors (1–10 μM) are 1–2 orders of magnitude smaller than the K_m for G-3-P (90–100 μM).

On the basis of these comparisons, the carboxylate moiety of pentalenolactone might be expected to interact with one or more of the anion-binding sites of GAPDH (for example, Arg-231, Thr-179, and the 2′-OH of the ribose moiety of NAD^+) (Buehner et al., 1974; Moras et al., 1975; Biesecker et al., 1977). The diminished binding of pentalenolactone methyl ester is consistent with this hypothesis. Although the methyl ester inactivates GAPDH with a k_{inact}/K_I only 1–1.5% that of pentalenolactone and with monophasic rather than

[2] This is similar, but not identical, to the conformation previously used by Moras et al. (1975) in model-building experiments of the active-site hemithioacetal between G-3-P and Cys-149.

Scheme 3: Proposed Mechanism of Inactivation of GAPDH by Pentalenolactone by Reaction of Epoxide with Cys-149



biphasic kinetics, the apparent stoichiometry and the site of inactivation remain the same. The proposed interaction with the nicotinamide ribose may explain the observed effect of NAD^+ in stimulating inactivation by pentalenolactone by lowering the K_I for the inactivator as well as the observed uncompetitive inhibition with respect to NAD^+. Pentalenolactone and heptelidic acid contain three functional groups that potentially might react with the active site Cys-SH residue. The fact that tetrahydropentalenolactone is itself a potent irreversible inactivator of GAPDH rules out a direct role for the conjugated 6,7-double bond, a conclusion consistent with our earlier observation that pentalenic acid (**10**) is completely ineffective as an inhibitor of GAPDH (Cane & Sohng, 1989). Of the other two reactive functionalities, the lactonic carbonyl could conceivably occupy the site normally occupied by the C-2 hydroxyl of G-3-P. In reactions with model thiols, pentalenolactone undergoes epoxide ring opening by nucleophilic attack exclusively at the primary carbinyl carbon. The epoxide moiety of pentalenolactone could therefore act as a potent alkylating agent for the active site thiol of GAPDH, Cys-149, as illustrated in Scheme 3. Nucleophilic attack of Cys-149 on the primary epoxide carbon, C-10, would result in epoxide ring opening and the generation of a covalent adduct. Further characterization of the GAPDH–pentalenolactone adduct will require direct spectroscopic or X-ray crystallographic analysis.

REFERENCES

Biesecker, G., Harris, J. L., Thierry, J. C., Walker, J. E., & Wonacott, A. J. (1977) *Nature (London) 266*, 328–333.

Branlant, G., Flesch, G., & Branlant, C. (1983) *Gene 25*, 1–7.

Bruice, P. Y., Wilson, S. C., & Bruice, T. C. (1978) *Biochemistry 17*, 1662–1669.

Buehner, M., Ford, E. E., Moras, D., Olsen, K. W., & Rossman, M. G. (1974) *J. Mol. Biol. 90*, 25–49.

Cane, D. E., & Sohng, J.-K. (1989) *Arch. Biochem. Biophys. 270*, 50–61.

Cane, D. E., Rossi, T., Tillman, A. M., & Pachlatko, J. P. (1981) *J. Am. Chem. Soc. 103*, 1838–1843.

Cane, D. E., Sohng, J.-K., & Williard, P. G. (1992) *J. Org. Chem. 57*, 844–851.

Canellas, P. F., & Cleland, W. W. (1991) *Biochemistry 30*, 8871–8876.

Caputto, R., & Dixon, M. (1945) *Nature (London) 156*, 630–631.

Chen, S., Lee, T. D., Legesse, K., & Shively, J. E. (1986) *Biochemistry 25*, 5391–5395.

Clasper, S., Easterby, J. S., & Powls, R. (1991) *Eur. J. Biochem. 202*, 1239–1246.

Clermont, S., Corbier, C., Mely, Y., Gerard, D., Wonacott, A., & Branlant, G. (1993) *Biochemistry 32*, 10178–10184.

Cori, G. T., Stein, M. W., & Cori, C. F. (1945) *J. Biol. Chem. 159*, 565–566.

Danishefsky, S. J., & Mantlo, N. (1988) *J. Am. Chem. Soc. 110*, 8129–8133.

Davidson, B. E., Sajgo, M., Noller, N. F., & Harris, J. I. (1967) *Nature (London) 216*, 1181–1185.

Duggleby, R. G., & Dennis, D. T. (1974) *J. Biol. Chem. 249*, 167–174.

Duszenko, M., & Mecke, D. (1986) *Mol. Biochem. Parasitol. 19*, 223–229.

Duszenko, M., Balla, H., & Mecke, D. (1982) *Biochim. Biophys. Acta 714*, 344–350.

Fröhlich, K.-U., Wiedmann, M., Lottspeich, F., & Mecke, D. (1989) *J. Bacteriol. 171*, 6696–6702.

Grosse, F., Nasheuer, H.-P., Scholtissek, S., & Schomburg, U. (1986) *Eur. J. Biochem. 160*, 459–467.

Harrigan, P. J., & Trentham, D. R. (1974) *Biochem. J. 143*, 353–363.

Harris, J. I., & Perham, R. N. (1968) *Nature (London) 219*, 1025–1028.

Harris, J. L., & Walters, M. (1976) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 13C, pp 1–49, Academic Press, New York.

Hartmann, S., Neeff, J., Heer, U., & Mecke, D. (1978) *FEBS Lett. 93*, 339–342.

Jones, G. M. T., & Harris, J. I. (1972) *FEBS Lett. 22*, 185–189.

Kato, M., Sakai, K., & Endo, A. (1992) *Biochim. Biophys. Acta 1120*, 113–116.

Koe, B. K., Sobin, B. A., & Celmer, W. D. (1957) *Antibiot. Annu. 1956–1957*, 672–675.

Leoncini, G., Marescxa, M., Ronchi, S., & Bonsignore, A. (1981) *Experientia 37*, 443–444.

Liu, L., & Huskey, W. P. (1992) *Biochemistry 31*, 6898–6903.

Mann, K., & Mecke, D. (1979) *Nature 282*, 535–536.

Martin, D. G., Slomp, G., Mizsak, S., Duchamp, D. J., & Chidester, C. G. (1970) *Tetrahedron Lett.* 4901–4904.

Maurer, K. H., Pfeiffer, F., Zehender, H., & Mecke, D. (1983) *J. Bacteriol. 153*, 930–936.

McCaul, S., & Byers, L. D. (1976) *Biochem. Biophys. Res. Commun. 72*, 1028–1034.

Meunier, J. C., & Dalziel, K. (1978) *Eur. J. Biochem. 82*, 483–492.

Mohamadi, F., Richards, N. G. J., Guida, W. C., Liskamp, R., Lipton, M., Caufield, C., Chang, G., Hendrickson, T., & Still, W. C. (1990) *J. Comput. Chem. 11*, 440–467.

Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., & Rossman, M. G. (1975) *J. Biol. Chem. 250*, 9137–9162.

Nagle, W. A., Moss, A. J., Jr., & Henle, K. J. (1985) *Int. J. Radiat. Biol. 48*, 821–835.

Nakagawa, A., Tomoda, H., Hao, M. V., Okano, K., Iwai, Y., & Omura, S. (1985) *J. Antibiot. 38*, 1114–1115.

Orsi, B. A., & Cleland, W. W. (1972) *Biochemistry 11*, 102–109.

Sakai, K., Hasumi, K., & Endo, A. (1988) *Biochim. Biophys. Acta 952*, 297–303.

Sakai, K., Hasumi, K., & Endo, A. (1991) *Biochim. Biophys. Acta 1077*, 192–196.

Schlapfer, B. S., Portmann, W., Branlant, C., Branlant, G., & Zuber, H. (1990) *Nucleic Acids Res. 18*, 6422.

Schultes, V., Deutzmann, R., & Jaenicke, R. (1990) *Eur. J. Biochem. 192*, 25–31.

Segal, H. L., & Boyer, P. D. (1953) *J. Biol. Chem. 204*, 265–281.

Shih, M. C., Heinrich, P., & Goodman, H. M. (1992) *Gene 119*, 317–319.

Singh, R., & Green, M. R. (1993) *Science 259*, 365–368.

Skarzynski, T., Moody, P. C. E., & Wonacott, A. J. (1987) *J. Mol. Biol 193*, 171–187.

Stallcup, W. B., & Koshland, D. E. (1973) *J. Mol. Biol. 80*, 41–62.

Walker, J. E., Carne, A. F., Runswick, M. J., Bridgen, J., & Harris, J. I. (1980) *Eur. J. Biochem. 108*, 549–565.

Warburg, O., & Christian, W. (1939) *Biochem. Z. 303*, 40–68.

Zwickl, P., Fabry, S., Bogedain, C., Haas, A., & Hensel, R. (1990) *J. Bacteriol. 172*, 4329–4338.