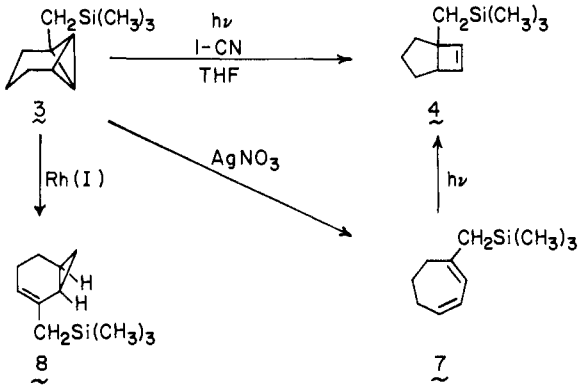
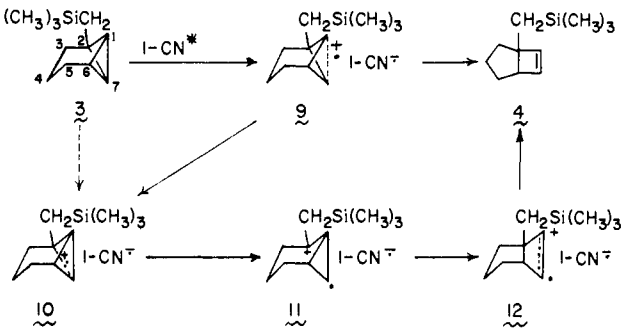


of **4** in C_6D_6 showed δ 5.91 (1 H, d, J = 2.7 Hz), 5.80 (1 H, d, J = 2.7 Hz), 2.64 (1 H, d, J = 6.9 Hz), 1.6-1.2 (6 H, m), 1.00 (2 H, m), and 0.04 (9 H, s), while the ^{13}C NMR showed δ 141.64 (d), 134.57 (d), 57.48 (s), 56.42 (d), 34.56 (t), 26.56 (t), 26.30 (t), 25.04 (t), and 0.29 (q).

In order to obtain an independent synthesis of **4**, we desired **7**. Treatment of **3** with (triphenylphosphine)carbonylrhodium chloride dimer failed to produce **7**, giving instead **8**. Fortunately, the use of silver nitrate in benzene readily converted **3** into a 3:2 mixture of **7** and **8**, respectively,[13,14] The diene **7** was purified by chromatography and showed the expected NMR properties, in addition to λ_{max} cyclohexane 265 nm (log ϵ 3.48). Direct (nonsensitized) irradiation of **7** gave a 96% yield of **4**.

The ease with which **7** was converted to **4** raised the question of whether **7** was an intermediate in the photosensitized conversion of **3** into **4**. Several lines of evidence indicate that **7** did not play such a role. Although **7** was readily oxidized ($E_{1/2}$ = 0.86 V vs. SCE) and reacted with excited state 1-CN at a diffusion controlled rate to quench the fluorescence of 1-CN the sensitized irradiation of **7** gave only low yields of **4**. While this would appear to rule out **7** as an intermediate, the evidence was not unequivocal, since it could not be rigorously established that **7** would not be converted to **4** in higher yield if **7** were present in only trace amounts at any instant. More definitive evidence against the intermediacy of **7** was obtained via a study of the quantum yields for the formation of **4** from both **3** and **7** under 1-CN-sensitized conditions in Pyrex and soft glass. The limiting quantum yields for the formation of **4** from **3** were 0.19 and 0.18 in Pyrex and soft glass, respectively. The corresponding values for the conversion of **7** to **4** in the presence of 1-CN were 0.05 and 0.09. Lastly, in soft glass, the quantum yield for the unsensitized formation of **4** from **7** was ca. 0.01 for 1.1×10^{-3} M **7** in tetrahydrofuran. No buildup of **7** could be detected in any of the reactions involving the photosensitized conversion of **3** into **4**. Thus, the cumulative evidence indicated that **7** was not an intermediate in the photoconversion of **3** into **4**.

Mechanistically, all evidence pointed to a single electron transfer process. No photoreaction occurred in the absence of 1-CN. In addition, the easily oxidized ($E_{1/2}$ = 1.37 V vs. SCE) hydrocarbon **3** quenched the fluorescence of 1-CN at a diffusion-controlled rate ($k_q = 1.4 \times 10^{10}$ L/mol/s) in tetrahydrofuran. On the basis of earlier precedent,[3,4] we would propose that a tight cation radical-anion radical pair involving **9** and the radical anion of 1-CN was formed. "Leakage" of **9** to **10**,[15] followed by opening of **10**, would give **11**.[16] Ample precedent for the closed and open forms of a cation radical exists in the recent work of Williams and co-workers.[17] Cyclopropylcarbinyl to cyclobutyl rearrangement of **11** to **12**, followed by back electron transfer from 1-CN anion radical to **12** would then produce **4**.[18]



We are continuing to explore the chemistry of cation radicals generated from highly strained ring systems.

Acknowledgment. We are indebted to the National Science Foundation for support of this investigation.

Registry No. **3**, 96503-08-1; **4**, 96503-09-2; **5**, 56432-00-9; **6**, 96532-34-2; **7**, 96503-10-5; **8**, 96503-11-6; $[Rh(CO)Cl(PPh_3)]_2$, 34676-63-6; $AgNO_3$, 7761-88-8; 1-CN, 86-53-3; [(trimethylsilyl)methyl]magnesium chloride, 13170-43-9; 3-bromocyclohexene, 1521-51-3; dichlorocarbene, 1605-72-7; 2-methylbicyclo[4.1.0]hept-2-ene, 53262-14-9.

(13) Acid-catalyzed rearrangement of **3** gave 2-methylbicyclo[4.1.0]-hept-2-ene.

(14) A nonphotochemical, Lewis acid catalyzed isomerization of the tricyclo[4.1.0.0^{2,7}]heptyl ring system to the bicyclo[3.2.0]hept-6-enyl ring system has previously been reported: Gassman, P. G.; Atkins, T. J. *J. Am. Chem. Soc.* **1972**, *94*, 7748.

(15) The possibility of the direct conversion of **3** into **10** must also be considered. Theoretical calculations on simple bicyclo[1.1.0]butane derivatives indicate that carbocation stabilizing groups in the 2-position of a bicyclo-[1.1.0]butane do not result in the HOMO shifting from its association with the central bond.[2] Comparison of the ease of oxidation of **3** ($E_{1/2}$ = 1.37 V vs. SCE) to that of **1** ($E_{1/2}$ = 1.50 V vs. SCE)[3] indicated that the addition of the (trimethylsilyl)methyl group of **1** had relatively little effect on the energy of the HOMO.

(16) We cannot rule out the possible conversion of **9** to an "open" cation radical followed by a cyclobutyl to cyclpropylcarbinyl rearrangement of the open form of **9** to produce **11**.

(17) Qin, X.-Z.; Snow, L. D.; Williams, F. *J. Am. Chem. Soc.* **1984**, *106*, 7640. See also: Roth, H. D.; Schilling, M. L. M. *J. Am. Chem. Soc.* **1980**, *102*, 7956; **1983**, *105*, 6805.

(18) Back electron transfer could also occur prior to the rearrangement of **11** to **12**. However, this would require that the intermediate zwitterion (or diradical) rearrange rather than reform the C_2-C_7 bond.

Prooxidant Effects of Glutathione in Aerobic Hemoglobin Solutions. Superoxide Generation from Uncoordinated Dioxygen

Vijaya Sampath and Winslow S. Caughey*

Department of Biochemistry, Colorado State University Ft. Collins, Colorado 80523

Received February 1, 1985

Glutathione (GSH), the tripeptide Glu-Cys-Gly, is found widely in nature at relatively high concentrations.[1,2] Intracellular concentrations range from 0.5 to 10 mM in mammalian cells.[3] In human red blood cells the thiol GSH is normally ca. 2 mM and the disulfide GSSG 0.0035 mM.[4] The GSH redox cycle (GSH peroxidase–GSSG reductase) provides cells with important protection against oxidant damage from H_2O_2.[5] GSH generally limits chemically induced injury to cells and tissues[6] and is also considered highly effective as an antioxidant, e.g., by reacting directly with free radicals.[7] However, we find that the addition

(1) Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711-760.

(2) "Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects"; Larsson, A., Orrenius, S., Holmgren, A., Mannervik, B., Eds.; Raven Press: New York, 1983.

(3) Kosower, N. S.; Kosower, E. *Int. Rev. Cytol.* **1978**, *54*, 109-160.

(4) Beutler, E. In "Hematology"; Williams, W. J., Beutler, E., Erslev, A., Lichtman, M., Eds.; McGraw-Hill: New York, 1983; pp 280-287.

(5) Harlan, J. M.; Levine, J. D.; Callahan, K. S.; Schwartz, B. R.; Harker, L. A. *J. Clin. Invest.* **1984**, *73*, 706-713.

(6) Reed, D. J.; Fariss, M. W. *Pharmacol. Rev.* **1984**, *36*, 25S-33S.

(7) Moldeus, P.; Jernström, B., ref 2, pp 99-108. Meister, A. *Hepatology (Baltimore)* **1984**, *4*, 739-742. Siems, W.; Mielke, B.; Müller, M.; Heumann, C.; Räder, L.; Gerber, G. *Biomed. Biochim. Acta* **1983**, *42*, 1079-1089.

0002-7863/85/1507-4076$01.50/0

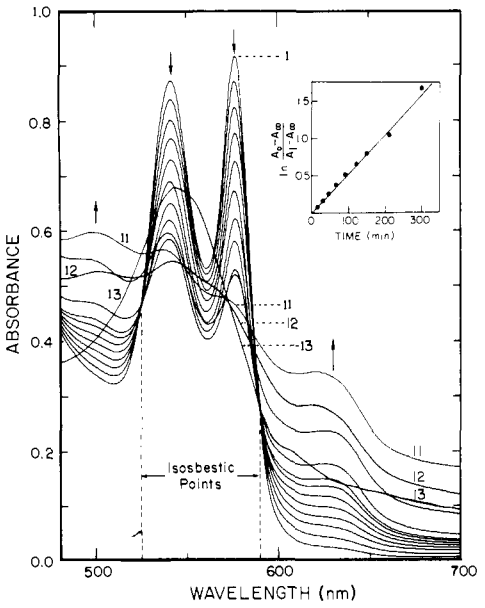


Figure 1. Visible spectra at various times after the addition of glutathione to an aerobic hemoglobin solution. The experimental conditions are given in the text. After the reactants were mixed, the cuvette was sealed to prevent entry of more oxygen during the reaction. Times after mixing for scans 1–13 were 0, 15, 30, 45, 65, 90, 120, 150, 210, 300, 1305, 1720, and 4205 min, respectively. The arrows apply for scans 1–11, but not for 12 and 13. Absorbances for the insert were measured at 577 nm.

of GSH to aerobic solutions of highly purified hemoglobin A (HbA) at a pH and temperature found in vivo *causes* both heme oxidation and oxidant damage to protein. An investigation of the reactions involved in this prooxidant effect of GSH is reported here.

An air-saturated solution of extensively purified human $HbAO_2$[8] (60 μM) in 0.05 M Na phosphate buffer pH 7.2 at 37 °C when treated with GSH[9] (0.6 mM) exhibits the spectral changes with time shown in Figure 1. These visible spectra indicate an initial conversion of HbO_2 to metHb (isosbestic points at 590 and 524 nm);[10] the insert demonstrates the pseudo-first-order character of the reaction without a lag phase[11] with an initial rate constant (k_i) of 5.1×10^{-3} min^{-1}. After the first 58% of the reaction course absorbances at 590 and 524 nm increase. Absorbances at 480 and 700 nm first increase and then decrease, accompanied by precipitate formation. Finally metHb is reduced to deoxyHb[12] as excess GSH depletes the system of O_2. About 15% of the total Hb is lost from solution as precipitate. GSH greatly accelerates both heme oxidation and protein degradation under these conditions. Addition of chloride (125 mM) affects neither the course nor the magnitude of the reactions. Increasing GSH concentration increases both k_i and precipitation; e.g., from 0.06 to 1.9 mM GSH k_i increases linearly with [GSH]. At [GSH] < 0.6 mM the reduction of metHb to deoxyHb at the final stage is not complete.

Decreasing pO_2 alters both k_i and the reaction course. An air-saturated solution of Hb (60 μM) and GSH (60 μM) exhibits $k_i = 7 \times 10^{-4}$ min^{-1} whereas with Hb only 90% saturated with O_2 $k_i = 7.3 \times 10^{-3}$ min^{-1} followed by a slow phase with a $k = 1.7 \times 10^{-3}$ min^{-1}. With Hb only 55% O_2 saturated, deoxyHb results without detection of metHb or precipitate. The direct conversion of HbO_2 to deoxyHb exhibits isosbestic points at 585, 568, 550, 522, and 500 nm ($k = 9.8 \times 10^{-3}$ min^{-1}). Without GSH, conversion of HbO_2 to metHb (autoxidation) occurs with $k = 2.2 \times 10^{-3}$ min^{-1}. Although at low pO_2 GSH may seem to act as an antioxidant because no metHb is seen, O_2 is rapidly lost and protein modification may occur without evidence of precipitation.

Addition of superoxide dismutase (SOD, 1 μM) to an air-saturated Hb (60 μM) solution increases k_i by ca. 25%. Thus, $O_2^{-}\cdot$ is produced and reduces the apparent metHb formation (presumably due to metHb + $O_2^{-}\cdot \rightarrow HbO_2$ or to higher H_2O_2 levels due to $O_2^{-}\cdot$ disproportionation). Addition of catalase (1 μM) reduces metHb formation to less than the autoxidation rate and stops precipitation. Therefore H_2O_2 is formed and increases both k_i and precipitation. HO· scavengers (e.g., EDTA, thiourea) also reduce both k_i and precipitation.

MetHb production cannot be primarily due to the autoxidation reaction (eq 1) since the rate is too small and catalase would not

$$HbO_2 \rightarrow \rightarrow \text{metHb} + O_2^{-}\cdot \quad (1)$$

stop the reaction.[13] Nor can GSH react directly with HbO_2 as a one-electron donor as in eq 2 because catalase could not stop

$$HbO_2 + e^- + 2H^+ \rightarrow \text{metHb} + H_2O_2 \quad (2)$$

this reaction.[14,15] Furthermore, metHb formation would also occur with reaction 2, albeit more slowly, under conditions of low O_2 saturation. Present evidence does support the reduction of O_2 by GSH as shown in eq 3 to yield radical products which lead to the

$$O_2 + GSH \rightarrow O_2^{-}\cdot + GS\cdot + H^+ \quad (3)$$

formation of H_2O_2, HO·, and other products. The reaction (eq 3) is also supported by NMR studies. An air-saturated solution of GSH (2 mM) (without Hb present) in 0.05 M Na phosphate buffer pH 7.2 in D_2O at 37 °C forms GSSG ($t_{1/2} \approx 10$ h).[16] Metal ion catalysis of reaction 3 was proposed earlier.[17] However, we conclude that reaction 3 may not occur solely due to such catalysis.[18] $O_2^{-}\cdot$ disproportionation or reaction with GSH[19] can yield H_2O_2. H_2O_2, but not $O_2^{-}\cdot$, causes heme oxidation and protein modification.[20] DeoxyHb, and not HbO_2, is rapidly oxidized by H_2O_2 (eq 4), which explains a greater k_i for solutions with 90%

(8) $HbAO_2$ was isolated from fresh human blood and purified extensively to remove all other proteins (e.g., catalase, SOD, and GSH peroxidase) as well as organic phosphates and other anions. Caughey, W. S.; Watkins, J. A. In "Handbook of Methods for Oxyradical Research"; Greenwald, R., Ed.; CRC Press: West Palm Beach, FL in press.

(9) GSH (reduced) 98–100% from Sigma exhibited only the expected ^1H and ^{13}C NMR spectra and microanalyses (C, H, N, and S). A 1 mM solution in water contained <0.005 μg/mL of both Cu and Fe.

(10) Van Kampen, E. J.; Zijlstra, W. G. *Adv. Clin. Chem.* **1983**, *23*, 199–257.

(11) An initial lag phase has been reported in a solution of Hb, GSH, and EDTA. Eyer, P.; Hertle, H.; Kiese, M.; Klein, G. *Mol. Pharmacol.* **1975**, *11*, 326–334.

(12) The initial rate for the reduction of metHb (87 μM) in 0.05 M Na phosphate buffer pH 7.2 at 37 °C by GSH (0.86 mM) under N_2 was calculated to be 1.31×10^{-2} min^{-1}.

(13) Wallace, W. J.; Houtchens, R. A.; Maxwell, J. C.; Caughey, W. S. *J. Biol. Chem.* **1982**, *257*, 4966–4977.

(14) Wallace, W. J.; Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1975**, *62*, 561–567. Caughey, W. S.; Choc, M. G.; Houtchens, R. A. In "Biochemical and Clinical Aspects of Oxygen"; Caughey, W. S., Ed.; Academic Press: New York, 1979; pp 1–18. Kawanishi, S. Caughey, W. S. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 1203–1208. Caughey, W. S.; Kawanishi, S. In "Oxy Radicals and Their Scavanger Systems"; Cohen, G., Greenwald, R. A., Eds.; Elsevier: Amsterdam, 1983; Vol. 1, pp 105–110. Kawanishi, S.; Caughey, W. S. *J. Biol. Chem.* **1985**, *260*, 4622–4631.

(15) It had been suggested that thiols can participate in the reaction shown by eq 2. Strömme, J. H. *Biochem. Pharmacol.* **1963**, *12*, 937–948.

(16) Resonances for GSH at δ 1.95, 2.35, and 3.57 (H_2O at δ 4.6) shift to δ 2.09, 2.47, and 3.7; the δ 2.73 resonance for GSH dissappears as resonances at δ 2.91 and 3.24 for GSSG appear. Line broadening seen during the initial stage suggests the presence of paramagnetic species (e.g., $O_2^{-}\cdot$ and GS·). Under N_2 no changes occur.

(17) Misra, H. P. *J. Biol. Chem.* **1974**, *249*, 2151–2155.

(18) Reaction mixtures typically contain <0.4 μM of Cu (range 0.2–1.0 μM) and comparable amounts of non-heme Fe; However, the variations in rates observed for different hemoglobin preparations do not correlate with the amounts of Cu and non-heme Fe present. Additions of $CuSO_4$ and $FeSO_4$ to levels up to 14 μM result in only small rate enhancements that are not proportional to metal concentration. A saturation effect of these metals with a maximum 3-fold rate enhancement under somewhat similar conditions was reported very recently (Rigo, A.; Scarpa, M.; Argese, E.; Ugo, P.; Viglino, P. In "Oxygen Radicals in Chemistry and Biology"; Bors, W., Saran, M., Tait, D., Eds.; deGruyter: Berlin, 1984; pp 171–176). Additions of desferrioxamine (up to 2 mM) or EDTA (up to 2 mM) do not stop heme oxidation, although rates may be reduced somewhat. Treatment of reaction solutions with Chelex, a metal ion chelating resin, did not alter reaction rates significantly.

(19) Asada, K.; Kanematsu, S. *Agric. Biol. Chem.* **1976**, *40*, 1891–1892. Wefers, H.; Sies, H. *Eur. J. Biochem.* **1983**, *137*, 29–36.

(20) Lemberg, R.; Legge, J. W.; Lockwood, W. H. *Biochem. J.* **1941**, *35*, 339–352. Tomoda, A.; Yoneyama, Y. *Dev. Biochem.* **1980**, *10* (Front. Protein Chem.), 407–417. Rice, R. H.; Lee, Y. M.; Brown, W. D. *Arch. Biochem. Biophys.* **1983**, *221*, 417–427.

$$Hb + H_2O_2 + H^+ \rightarrow metHbH_2O + HO\cdot \quad (4)$$

HbO_2-10% Hb than for air-saturated solutions. The HO·, which may form via eq 4,[21] can also promote oxidation and protein damage.[22]

In summary, GSH serves effectively as an electron donor to free O_2 but not to O_2 as a ligand to heme iron. Furthermore, in the absence of GSH peroxidase GSH does not prevent oxidative damage to protein by H_2O_2 or H_2O_2 reaction products. GSH must be added to the list of Hb,[13,14] ascorbate, and flavins as likely electron donors for $O_2^{-}\cdot$ generation in red cells.[23,24] This reaction of GSH is undoubtedly disadvantageous to the red cell. However, the hemoglobin remains only partially saturated with O_2 during normal O_2 transport and can maintain the free O_2 concentration at low levels. It is clearly advantageous to red cell function for GSH to be a much better electron donor to metHb than to HbO_2.

Acknowledgment. This work was supported by USPHS Grant HL-15980. The assistance of M. M. Miller, Department of Chemistry, and Stephen M. Workman, Soil Testing Laboratory of Colorado State University, for determinations of NMR spectra and ICP-AES metal analyses, respectively, is gratefully acknowledged.

Registry No. GSH, 70-18-8; O_2, 7782-44-7; HbA, 9034-51-9; superoxide, 11062-77-4; hydroxyl radical, 3352-57-6.

(21) Sadrzadeh, S. M. H.; Graf, E.; Panter, S. S.; Hallaway, P. E.; Eaton, J. W. *J. Biol. Chem.* **1984**, *259*, 14354-14356.
(22) Girotti, A. W.; Thomas, J. P. *J. Biol. Chem.* **1984**, *259*, 1744-1752.
(23) Scarpa, M.; Viglino, P.; Contri, D.; Rigo, A. *J. Biol. Chem.* **1984**, *259*, 10657-10659.
(24) Preliminary results indicate that ascorbate also promotes heme oxidation and precipitation similar to GSH since catalase stops both the reactions.

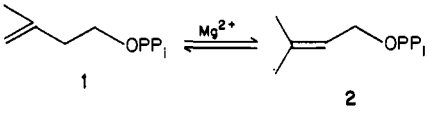
Time-Dependent Inhibition of Isopentenyl Pyrophosphate Isomerase by 2-(Dimethylamino)ethyl Pyrophosphate

John E. Reardon and Robert H. Abeles*

Contribution 1556
Graduate Department of Biochemistry
Brandeis University
Waltham, Massachusetts 02254

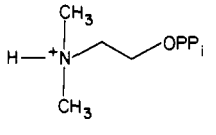
Received February 7, 1985

Isopentenyl pyrophosphate isomerase[1] (IPPI) (EC 5.3.3.2) catalyzes the isomerization shown in eq 1.



1 2 (1)

The available evidence indicates that a two-base mechanism is involved but is consistent with either a carbonium or carbanion mechanism.[2] To distinguish between the two mechanisms, we synthesized 2-(dimethylamino)ethyl pyrophosphate[3] (**3**) and examined its effect on the reaction catalyzed by IPPI. If the reaction proceeds by a carbonium ion mechanism, then **3** could be a transition-state analogue.



3

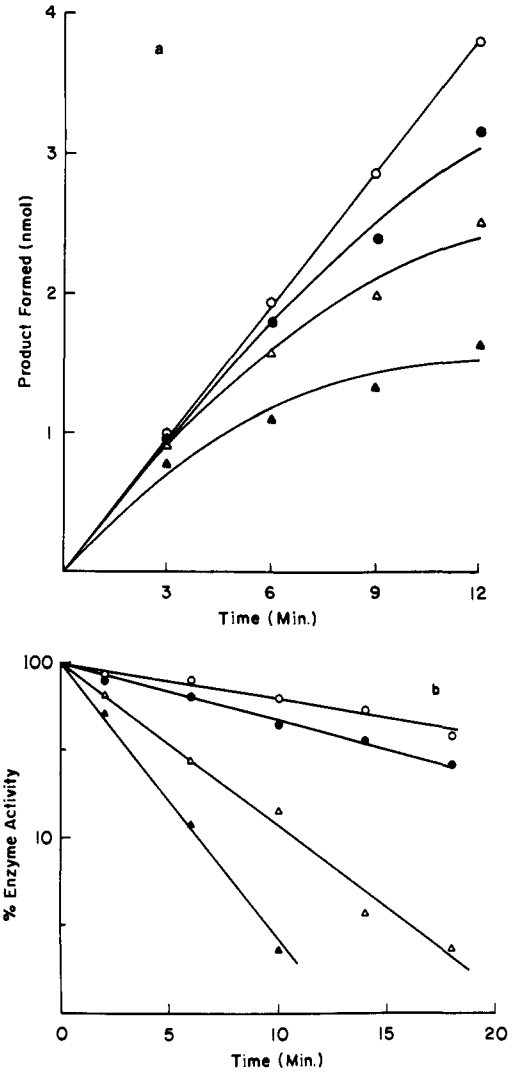


Figure 1. Time-dependent inactivation of isopentenyl pyrophosphate isomerase by 2-(dimethylamino)ethyl pyrophosphate. (a) Assay mixtures contained 0.1 M sodium maleate, pH 6.3, 10 mM $MgCl_2$, 100 μM [4-^{14}C]isopentenyl pyrophosphate, and **3** [(○) 0.0; (●) 0.67; (△) 1.33; (▲) 5.67 μM] in a final volume of 1.5 mL at 37 °C. The assays were started by addition of isomerase (1.7 units). Aliquots, 250 μL, were removed at time intervals and quenched with 15 μL 5 N HCl, and the amount of product formed was determined.[8] (b) Incubation mixtures contained 0.1 M sodium maleate, pH 6.3, 10 mM $MgCl_2$, isomerase (23 units), and **3** [(○) 1.33 μM + 500 mM isopentenyl pyrophosphate; (●) 0.67 μM; (△) 1.33 μM; (▲) 2.0 μM]. At time intervals 4-μL aliquots were diluted into an assay mixture (250 μL) containing 0.1 M sodium maleate, pH 6.3, 10 mM $MgCl_2$, and 350 μM [4-^{14}C]isopentenyl pyrophosphate. After 6 min at 37 °C, 15 μL of 5 N HCl was added and the amount of product formed was determined.[8]

The effect of **3** on the isomerization of **1** is shown in Figure 1a. The data show that the onset of inhibition is slow, suggesting that either combination of enzyme and inhibitor is slow or that

(1) Isopentenyl pyrophosphate isomerase was isolated from Bakers yeast (specific activity 6.5 nmol/min/mg).[7] Enzyme purified in this manner was essentially free of prenyl transferase activity and was assayed as described.[8] A unit of enzyme activity is defined here as 1 nmol/min.
(2) (a) Shah, D. H.; Cleland, W. W.; Porter, J. W. *J. Biol. Chem.* **1965**, *240*, 1946. (b) Rose, I. A. "The Enzymes", 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1970; Vol. II, p 301.

(3) **3** was synthesized from *N*-CBZ-ethanolamine pyrophosphate.[11,12] The CBZ group was removed by catalytic hydrogenation over 10% Pd–carbon. Reductive methylation with formaldehyde/$NaBH_4$ (Means, G. E.; Feeney, R. E. *Biochemistry* **1968**, *7*, 2192) gave the desired product. **3** was purified by chromatography on QAE Sephadex A25 using a linear triethylammonium bicarbonate gradient from 0.0 to 0.45 M. Fractions containing product were pooled and the buffer was removed by rotary evaporation in vacuo. A samaple for NMR analysis was prepared by passage through Dowex 50·Na^+ form. ^{1}H NMR (D_2O) δ 4.24 multiplet (2 H), 3.43 triplet (2 H), 2.92 singlet (6 H). A sample was further purified by paper chromatography in methanol/water/28% (w/w NH_3) ammonium hydroxide (60:30:10) for use in enzyme assays.

0002-7863/85/1507-4078$01.50/0