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# Maleylacetoacetate *cis-trans* Isomerase: One-Step Double *cis-trans* Isomerization of Monomethyl Muconate and the Enzyme's Probable Role in Benzene Metabolism[1]

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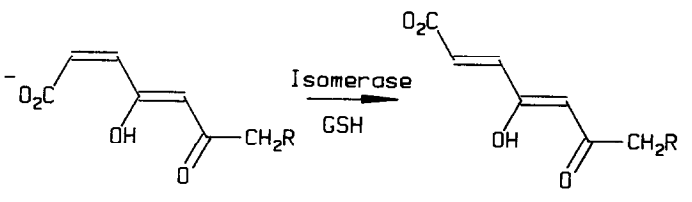
Maleylacetoacetate *cis-trans* isomerase together with glutathione has been found to isomerize *cis-trans* isomers of monomethyl muconate. Isomerization about a single double bond and concerted double isomerization of the diene unit occurs. In addition to the variations in substrate structure previously identified the current results demonstrate that a *cis,cis* diene skeleton and a conjugated ester function are accepted by the enzyme. The present work and the fiding of *trans,trans*-muconic acid in the urine of benzene-fed mice (M. M. Gad-El-Karim, V. M. Sadagopa Ramanujam, and M. S. Legator (1985) Xenobiotica **15,** 211) suggest that maleylacetoacetate *cis-trans* isomerase may be responsible for the geometrical isomerization. However, *cis,cis*-muconaldehydic acid rather than *cis,cis*-muconic acid is suggested to be the early intermediate in benzene metabolism capable of rapid enzyme-catalyzed *cis-trans* isomerization. 

Maleylacetoacetate *cis-trans* isomerase (EC 5.2.1.2) is found in mammalian liver (*1*) and in Vibrio 01 bacteria (*2, 3*). Its sole role is believed to be as a necessary catalyst in the metabolism of phenylalanine and tyrosine. Maleylacetoacetate, an intermediate in the process, is *cis-trans* isomerized to fumarylacetoacetate (both shown in their enol forms, $R = CO_2^-$):




[1]

a: $R = CO_2^-$

b: R = H

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The resulting C8 *trans* structure is required before enzymatic hydrolytic cleavage to two C4-fragments, fumarate and acetoacetate, can proceed.

β-Ketoacids are generally thermally unstable. Thus it would appear that some maleylacetone would be present as a natural product of maleylacetoacetate-uncatalyzed decarboxylation. Consequently, a parallel route for maleylacetone degradation would be anticipated. It is not surprising, therefore, that the enzyme also catalyzes the *cis-trans* isomerization of maleylacetone (Eq. [1], $R$ = H) to fumarylacetone. It does this with about the same efficiency as with maleylacetoacetate. Since most of our studies have been with maleylacetone, we have often referred to the enzyme as maleylacetone *cis-trans* isomerase (*4, 5*). The enzyme specifically requires glutathione (GSH)[4] for action (*3, 6*). Previous reports from this laboratory have discussed the role of GSH in the enzymatic reaction and the mechanism of reaction (*5, 7, 8*). The evidence strongly suggests that catalysis is achieved through nucleophilic attack of enzyme-bound GSH on the substrate to form a dienediol (or diolate) adduct capable of internal rotation about the isomerizing bond (vide infra).

We reported recently that the enzyme together with GSH isomerizes a deoxy analog of maleylacetone (6-oxo-2,4-hexadienoate) by the same mechanism it uses for maleylacetone (*9*). It was discovered in that study that the enzyme and GSH executed a *concerted double cis-trans isomerization on the conjugated diene system utilizing a bicycle pedal-rotation mechanism.* The enzyme system catalyzes the equlibration of 6-oxo-2*Z*,4*E*-hexadienoate with its 2*E*,4*Z*-isomer without evidence of a free intermediate before both isomers are converted to the most thermodynamically stable 2*E*,4*E*-isomer.

We report there that the enzyme together with GSH catalyzes the *cis-trans* isomerization of muconic acid monomethyl ester (2,4-hexadiendioic acid monomethyl ester). *A fraction of these substrate molecules proceed through enzyme-catalyzed one step double cis-trans isomerizations.* In identifying these reactions, the enzyme is shown to process an additional diene geometry and a different carbonyl function than that previously recognized. The enzyme system also *cis-trans* isomerizes *cis,cis*-muconic acid very slowly. These results have importance in benzene metabolism.

Although the toxic effects of benzene are known (*10*), the active metabolite(s), directly responsible for it being a human leukemogen, has (have) not been completely identified (*11, 12*). Benzene oxide, an early intermediate in enzyme-catalyzed benzene oxidation, has been suggested to react directly with nucleophilic constituents of tissue, resulting in the alteration of their chemical reactivity and biological function, thereby leading to cytotoxicity (*13*). Intermediates of more advanced benzene oxidation such as mucondialdehyde (*14*), semibenzoquinone, and hydroxybenzosemiquinone (*15*) have also been proposed as possible cancer causing intermediates.

In a recent paper, Gad-El-Karim *et al.* (*16*) have identified *trans,trans*-muconic acid as a metabolite of benzene. Aside from the oxidative steps, two double bond

[4] Abbreviations used: GSH, glutathione; ZZ, 6-methyl hydrogen 2Z,4Z-hexadiendioate; ZE, 6-methyl hydrogen 2Z,4E-hexadiendioate; EZ, 6-methyl hydrogen 2E,4Z-hexadiendioate; EE, 6-methyl hydrogen 2E,4E-hexadiendioate; Enz, enzyme.

isomerizations are required to reach *trans,trans*-muconic acid. No enzyme has previously been reported to carry out this isomerization.

## METHODS

*Materials.* Hypersil (C-18, 3 $\mu$m) was obtained from Phenomenex. Deionized (Milli Q) water was obtained by passing distilled water through a Milli Q (Millipore Corp.) resin. Tetrahydrofuran, HPLC grade, was obtained from Burdick and Jackson. Maleylacetone was prepared as previously described (*17*). Glutathione was a Sigma product. All other compounds were reagent grade chemicals.

Maleylacetoacetate *cis-trans* isomerase was isolated from Vibrio 01 bacteria which had been previously grown at The New England Enzyme Center. The enzyme was purified as previously described (*3*) through the affinity chromatography step (*4*). The specific activity with maleylacetone at this point was 23.1 $\mu$mol/min–mg of protein.

*HPLC analysis.* A 30 × 0.46-cm, 3-$\mu$m Hypersil (C-18) column was used. The eluting solvent was prepared in the following way. To 1 liter of Milli Q water was added 10 ml of reagent grade glacial acetic acid. This was partially neutralized with 0.67 ml of 10 N NaOH. The aqueous solution (pH 3.37, 938 ml) was mixed with 62 ml of HPLC grade tetrahydrofuran and the resulting solution degassed before use. Solvent flow rate was maintained at 1.0 ml/min. Detection was by an Altex Model 153 detector operating at 254 nm. HPLC peak areas were integrated by a Hewlett–Packard integrator, Model 3380S. A Nuclear Enterprises flow radioactivity detector ("Isoflo") was connected downstream from the Altex detector. A solid scintillant packed cell of 0.2 ml internal volume was used. Counting intervals of 30 s were utilized. Typical elution times were 19.5, 21.2, 23.6, and 29.9 min for the 2*E*,4*E*-, 2*Z*,4*E*-, 2*E*,4*Z*-, and 2*Z*,4*Z*-isomers of monomethyl muconate, respectively.

NMR spectra were obtained with a Bruker AM-300 spectrometer.

*6-Methyl hydrogen 2Z,4Z-hexadiendioate* (***1,*** *monomethyl cis,cis-muconate*). **1** was prepared essentially by the method of Tsugi and Takayanagi (*18*), whereby catechol is oxidized using a Cu(I) catalyst in pyridine solvent containing a small amount of methanol. CuCl, used in the catalyst preparation, was prepared by the method of Keller and Wycoff (*19*). Ultraviolet (95% EtOH): $\lambda_{max}$ 258 nm ($\varepsilon$ 18,870), $\varepsilon_{254}$ 18,530. $^1$H NMR ($CDCl_3$): $\delta$ 3.77 (singlet, 3H, methyl), 6.02 (multiplet, 1H, H-2), 6.03 (multiplet, 1H, H-5), 7.87 (td, 1H, H-4), 8.02 (td, 1H, H-3); see Table 1 for coupling constants.

*6-Methyl hydrogen 2Z,4E-hexadiendioate* (***2,*** *monomethyl cis,trans-muconate*). **1** (7.7 mg) was dissolved in 10 ml of water and heated at 50°C for 25 h. The aqueous solution was lyophilized and the residue purified by flash chromatography using chloroform/88% formic acid (330 : 2). Ultraviolet (95% EtOH): $\lambda_{max}$ 258 nm, $\varepsilon_{258}$ 19,200; $\varepsilon_{254}$ 18,800. $^1$H NMR ($CDCl_3$): $\delta$ 3.81 (s, 3H, methyl), 6.01 (d, 1H, H-2), 6.17 (d, 1H, H-5), 6.76 (t, 1H, H-3), 8.38 (dd, 1H, H-4); see Table 1 for coupling constants.

*6-Methyl hydrogen 2E,4Z-hexadiendioate* (***3,*** *monomethyl trans,cis-muconate*). **1** (12 mg) was dissolved in 25 ml of methanol contained in a quartz flask. The

TABLE 1

Chemical Shifts and Coupling Constants for 6-Methyl Hydrogen 2,4-Hexadiendioates

| | δ (ppm) | | | | *J* (Hz) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Isomer | H-2 | H-3 | H-4 | H-5 | 2,3 | 2,4 | 2,5 | 3,4 | 3,5 | 4,5 |
| 2*Z*,4*Z* | 6.02 | 8.02 | 7.87 | 6.03 | 11.6 | 0.4 | −0.4 | 12.0 | 0.4 | 11.6 |
| 2*Z*,4*E* | 6.01 | 6.76 | 8.38 | 6.17 | 11.5 | 0.0 | 0.0 | 11.7 | 0.0 | 15.7 |
| 2*E*,4*Z* | 6.13 | 8.49 | 6.68 | 6.03 | 15.5 | 0.0 | 0.0 | 11.7 | 0.0 | 11.5 |
| 2*E*,4*Z* | 6.21 | 7.35 | 7.39 | 6.25 | 15.5 | −1.2 | −0.2 | 11.7 | −1.2 | 15.6 |

contents of the flask were irradiated at 254 nm by three lamps in a Rayonet mini reactor. An aliquot, examined by HPLC after 56 min of irradiation, indicated that the mixture contained the following isomeric distribution: 33% 2*E*,4*E*, 25.9% 2*Z*,4*E*, 21% 2*E*,4*Z*, and 20.1% 2*Z*,4*Z*. The solvent was removed and the mixture was subjected to flash chromatography using chloroform : 88% formic acid (400 : 2). Flash chromatography was repeated resulting in a mixture which contained about 70% of 2*E*,4*Z*-, 10% of 2*E*,4*E*-, and 20% of the 2*Z*,4*E*-isomer. The $^1$H NMR of the enriched *EZ*-isomer was obtained and the spectra of the contaminants were subtracted digitally to obtain a sufficiently clean spectrum for fitting of spectral parameters and for spectrum simulation. $^1$H NMR ($CDCl_3$): δ 3.79 (s, 3H, methyl), 6.03 (d, 1H, H-5), 6.13 (dd, 1H, H-2), 6.68 (t, 1H, H-4), 8.49 (dd, 1H, H-3); see Table 1 for coupling constants.

*6-Methyl hydrogen 2E,4E-hexadiendioate* (***4**, monomethyl trans,trans-muconate*). A few crystals of the *cis,cis*-isomer were dissolved in ether and to this was added a small crystal of iodine. It was allowed to stand at ambient temperature under light of the hood for 72 h. The iodine and ether were removed by evaporation under reduced pressure. HPLC analysis indicated only one isomer present. $^1$H NMR ($CDCl_3$): δ 3.80 (s, 3H, methyl), 6.21 (dd, 1H, H-2), 6.25 (dd, 1H, H-5), 7.35 and 7.39 (irregular septet, 2H, H-3 and H-4); see Table 1 for coupling constants.

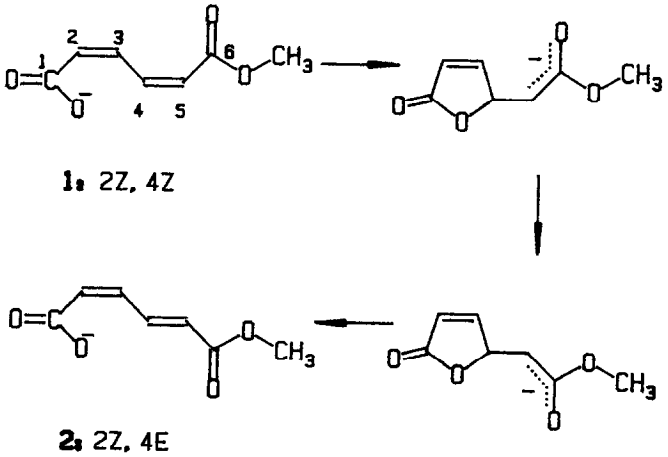
*6-Methyl-$^{14}$C hydrogen 2Z,4Z-hexadiendioate* (***3**-$^{14}$C, monomethyl-$^{14}$C cis,cis-muconate*). *cis,cis*-Muconic acid was esterified with [$^{14}$C]methyl iodide by a variation of the method of Heathcock *et al.* (*20*). [$^{14}$C]Methyl iodide (Amersham, 500 μCi, 56 mCi/mmol), in a breakseal tube, was attached to a vacuum line through one arm of a three-way stopcock. A small tube, containing 15.4 μl of unlabeled methyl iodide, was attached to the other arm. The attached tubes were evacuated while cooled in liquid nitrogen. The [$^{14}$C]methyl iodide was distilled over to the small tube and cooled in liquid nitogen, and the breakseal tube removed. A small reaction flask containing 38 mg of *cis,cis*-muconic acid, 81 mg of KF · $2H_2O$, 1 ml of dry dimethylformamide, and a magnetic stirrer was attached to the vacant arm of the three-way stopcock and the contents of the flask were degassed by several freeze–pump–thaw cycles. The [$^{14}$C]methyl iodide was then distilled over to the flask which was cooled in liquid nitrogen. The reaction mixture was stirred for 3.5 h at room temperature, at which time the flask and three way stopcock, still

attached, were removed to the hood. The reaction mixture was diluted with 6 ml of water and the solution extracted with ether (3 × 2 ml). One milliliter of glacial acetic acid was added to the aqueous layer and it was again extracted with ether (2 × 2 ml). The solution of combined ether layers was allowed to dry over sodium sulfate. The ether was evaporated in a stream of Ar and the residue flash chromatographed on silica gel using chloroform : 88% formic acid (350 : 2). The top 5 ml of the eluting solvent was first removed in a separatory funnel. The material applied to the column amounted to 272 $\mu$Ci. Monoester was separated from radioactive diester and unesterified acid. The monester fraction, which at this point was shown to contain about 15% of the 2*Z*,4*E*-isomer, was rechromatographed to yield the desired compound with less than 0.1% of the *Z*,*E*-isomer as judged by HPLC. Simultaneous uv detection and radiocounting during HPLC analysis indicated that the yield of the desired monoester was 0.28 mg with a specific activity of 11.0 $\mu$Ci/$\mu$mol.

## RESULTS

*Preparation and analysis of monomethyl muconates.* Monomethyl *cis,cis*-muconate **(1)** was synthesized directly by the Cu(I)-catalyzed oxidation of catechol in pyridine–methanol. The three other *cis-trans* isomers of monomethyl muconate were prepared from **1** by a variety of methods. The chemical shifts and coupling constants were calculated by PANIC[5] and these are shown in Table 1. The assignments of isomer configuration are based on the method of synthesis and the observed proton–proton vicinal coupling constants and supported by the identified configuration of the products of enzymatic isomerization.

**1** in neutral aqueous solution undergoes slow autoisomerization to form the 2*Z*,4*E*-isomer **(2)**. The observation that the only product is the 2*Z*,4*E*-isomer suggests that the reaction proceeds by formation of the lactonic intermediate,




[5] An NMR minicomputer simulation program adapted from LAOCOON by Bruker Instruments Co.

The half-time of this reaction at pH 7.4 at 22°C in the presence of 1.5 mM GSH, 13 mM mercaptoethanol in 10 mM phosphate buffer (the enzyme reaction mixture) is 31 h. A more detailed study of the effect of pH variation, buffer concentration, etc., was not carried out. A sample of the 2*Z*,4*E*-isomer required for the spectral and chromatographic analysis was obtained by simply allowing a dilute aqueous solution of the 2*Z*,4*Z*-isomer to isomerize at 50°C.

The *trans,trans*-monoester **(4)** was isolated in pure form after **1,** in ether, was treated under visible light with a trace of iodine (*21*).

Finally, the 2*E*,4*Z*-isomer **(3)** which is also formed enzymatically (vide infra) was prepared photolytically. **1,** irradiated at 254 nm where it exhibits an absorption maximum, isomerizes to a mixture of all four isomers. Repeated flash chromatography allowed the elimination of the 2*Z*,4*Z*-isomer and sufficient enrichment of the 2*E*,4*Z*-isomer in the remaining mixture to obtain a $^1$H NMR spectrum of high quality for calculation of its chemical shifts, coupling constants, and spectral simulation, necessary for structure identification.

*Enzymatic isomerizations.* Incubation of the enzyme and GSH with monomethyl 2*Z*,4*Z*-muconate leads initially to a mixture of all four isomers. At longer reaction times the 2*Z*,4*Z*- and the 2*Z*,4*E*-isomers disappear leaving a mixture of 2*E*,4*Z*- and 2*E*,4*E*-isomers. A typical plot of composition with time is shown in Fig. 1. It was of interest to find which isomerization reactions are enzyme catalyzed and which are not.

As already mentioned the 2*Z*,4*Z*-monoester undergoes autoisomerization to the 2*Z*,4*E*-isomer (Eq. [2]) and it was of interest to see whether this isomer is also

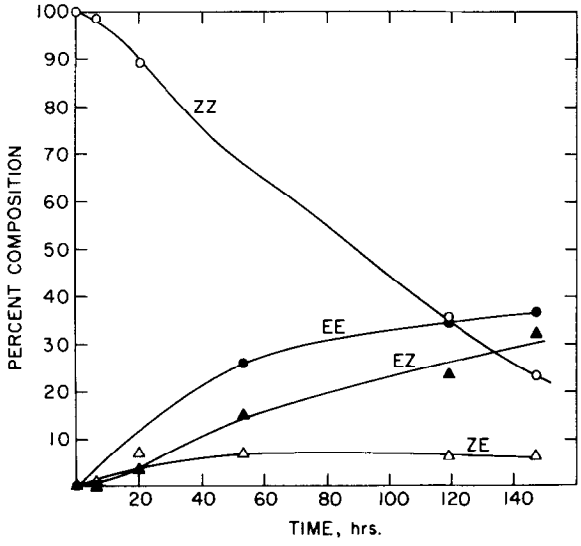



FIG. 1. Progress of maleylacetoacetate *cis-trans* isomerase-catalyzed isomerization of monomethyl muconate isomers at 0° as determined by HPLC analysis and detected at 254 nm. The assumption is made that all isomers have equal response factors over the bandwidth of the "254" nm detector. The initial reaction mixture contained 10 mM phosphate buffer, pH 7.4, 1.1 mM GSH, 6 mM mercaptoethanol, 0.11 units of isomerase (as assayed against maleylacetone), and 1.6 mM monomethyl 2*Z*,4*Z*-muconate in a total volume of 335 μl.

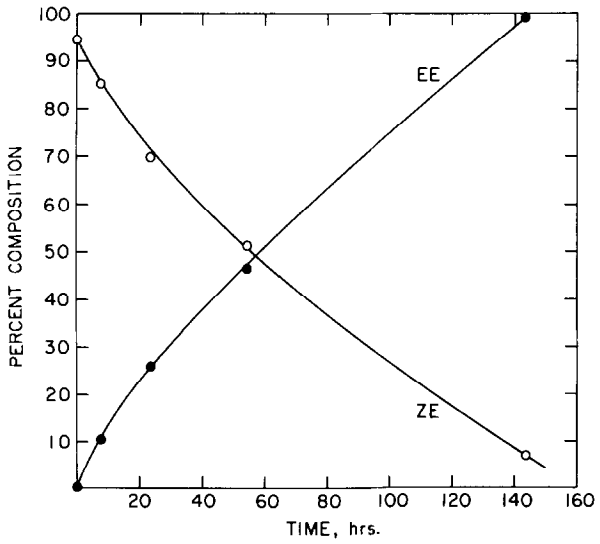
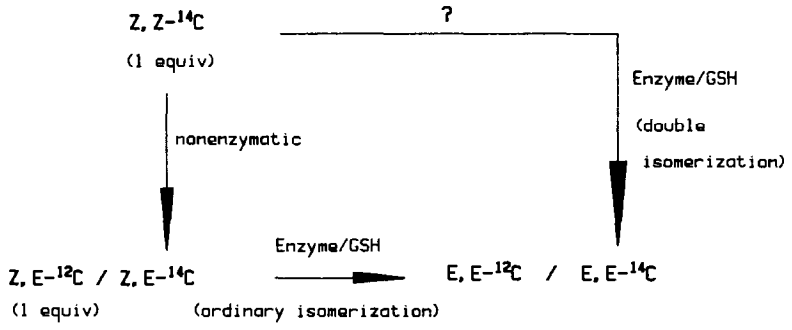



FIG. 2. Progress of maleylacetoacetate *cis-trans* isomerase-catalyzed isomerization of monomethyl 2*Z*,4*E*-muconate at 0° as determined by HPLC analysis and detection at 254 nm. The assumption is made that both isomers have the same response factor over the bandwidth of the "254" nm detector. The initial reaction mixture contained 10 mM phosphate buffer, pH 7.4, 1.1 mM GSH, 6 mM mercaptoethanol, 0.11 units of isomerase (as assayed against maleylacetone), and 1.6 mM monomethyl 2*Z*,4*E*-muconate in a total volume of 335 μl.

isomerized by the enzyme system. As shown in Fig. 2 the 2*Z*,4*E*-isomer is converted directly into the 2*E*,4*E*-isomer. No isomerization occurs in the absence of enzyme.

*Experiments with* $^{14}$*C-labeled substrates.* A study was made to see if any double *cis-trans* isomerization reactions operate in this system. Could the enzyme catalyze the direct isomerization of the 2*Z*,4*Z*- to the 2*E*,4*E*-isomer? The 2*E*,4*E*-isomer was already shown to be produced by one path. It is formed enzymatically from the 2*Z*,4*E*-isomer. The 2*Z*,4*E*-isomer, however, appears to be produced from the 2*Z*,4*Z* only in a slow nonenzymatic process. In order to monitor that amount of 2*E*,4*E*-isomer arising directly from the 2*Z*,4*Z*-isomer an isotope dilution method was used. $^{14}$C-Labeled monomethyl 2*Z*,4*Z*-muconate was mixed with an approximately equal amount of unlabeled monomethyl 2*Z*,4*E*-muconate and incubated with enzyme and GSH. The specific activity of the monomethyl 2*E*,4*E*-muconate being formed was monitored by HPLC by simultaneously measuring the concentration (uv detection) and the radioactivity of that isomer (radio flow counting). The 2*E*,4*E*-isomer coming directly from the 2*Z*,4*Z*-isomer would have a relatively high specific radioactivity while the 2*E*,4*E*-isomer coming from the 2*Z*,4*E*-isomer should have no activity except for that coming by way of the slow nonenzymatic isomerization of the 2*Z*,4*Z* to the 2*Z*,4*E*, dilution with unlabeled 2*Z*,4*E*, and then enzyme-catalyzed isomerization of the 2*Z*,4*E*- to the 2*E*,4*E*-isomer. This is shown in Scheme 1. Such experiments were performed and specific activities of the four isomers developed in a typical experiment are shown in Table 2. The very slow




SCHEME 1

appearance of radioactivity in the 2*Z*,4*E*-isomer affirms that there is little or no direct enzymatic isomerization of the 2*Z*,4*Z*- to the 2*Z*,4*E*-isomer. The fact that the instantaneous initial specific activities of the 2*E*,4*E*-isomer is always considerably higher than that of the 2*Z*,4*E*-isomer but lower than that of the 2*Z*,4*Z*-isomer indicates that some of the 2*E*,4*E*-isomer arises by one step double *cis-trans* isomerization. The fraction proceeding by this path is calculated below.

In Scheme 2 are presented the simplest possible kinetic reactions whereby the enzyme and GSH catalyze the isomerizations of the monomethyl muconates. The shortened notations, ZZ, ZE, EZ, and EE, refer to the 2*Z*,4*Z*-, the 2*Z*,4*E*-, the 2*E*,4*Z*-, and the 2*E*,4*E*-isomers of 6-methyl hydrogen 2,4-hexadiendioate, respectively. The vertical arrows represent the association–dissociation reactions between enzyme and the two substrates, *ZZ* and *ZE* monoesters, which are initially present. Thus Enz–ZE and Enz–ZZ refer to the enzyme–2*Z*,4*E* and enzyme–

TABLE 2

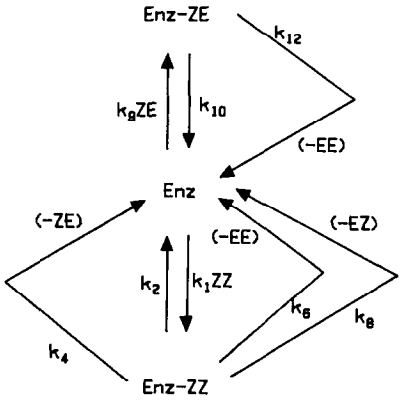
Isomer Distribution and Initial Specific Radioactivities in Enzyme-Catalyzed *cis-trans* Isomerization of Unlabeled 6-Methyl-2*Z*,4*E*-muconate and 6-Methyl-2*Z*,4*Z*-muconate-6-$^{14}$C[a]

| Time (min) | Composition (%)[b] | | | | Specific activity[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | EE | ZE | EZ | ZZ | EE | ZE | EZ | ZZ |
| 1 | 1.1 | 51.3 | 1.2 | 46.5 | 0 | — | — | 85 |
| 51 | 3.4 | 49.1 | 1.9 | 45.7 | 8.2 | 1.7 | 62 | 83 |
| 87 | 6.1 | 47.9 | 3.0 | 43.1 | 7.7 | 2.1 | 69 | 94 |

[a] The initial solution contained $7.3 \times 10^{-4}$ M ZZ, $8.1 \times 10^{-4}$ M ZE, $2 \times 10^{-3}$ M GSH, 0.01 M phosphate buffer, pH 7.4, $1.1 \times 10^{-2}$ M mercaptoethanol, and 0.45 units of isomerase (as assayed against maleylacetone) in a total volume of 130 μl.

[b] The simplifying assumption is made that all isomers show equal response factors with the Altex detector at 254 nm.

[c] Specific activity units are given as radioactivity counts divided by HPLC peak area counts recorded by the Hewlett–Packard integrator and have been multiplied by $10^5$.




SCHEME 2

2Z,4Z complexes, respectively. The angular arrows represent conversion of enzyme–substrate complexes to free enzyme (Enz) and product. Each product is denoted with a negative sign in parentheses indicating that it is being lost from the precursor enzyme–substrate complex. The EE-isomer is thermodynamically the most stable and therefore the long term end-product isomer. The specific activity of the early formed EE-isomer is to be compared with the specific activity of the ZZ-isomer. In the early part of the experiment only the 2Z,4Z- and the 2Z,4E-isomers are present in significant concentration and it is only these isomers which are considered in Scheme 2 to interact with the enzyme to form significant concentrations of enzyme substrate complexes. These two enzyme–substrate complexes are shown to lead to the formation of the four substrate isomers. The current experiment is being carried out to determine whether the path ($k_6$) from Enz-ZZ to Enz and EE-isomer is a viable route. The results here show that when the enzyme catalyzes the *cis-trans* isomerization of only one of the two double bonds, it is the 2,3- not the 4,5-double bond (see Eq. [2] for numbering of skeleton) which isomerizes. Thus $k_4$ is considered here to be zero.

Similarly, direct enzyme-catalyzed isomerization of the EZ- to the EE-isomer does not appear to be a productive route. This would also require enzyme-catalyzed isomerization of the 4,5-double bond which appears not to happen on a short-term time scale. The observation that both EE and EZ accumulate (Fig. 1) in enzyme-catalyzed isomerization of the ZZ-isomer suggests that the EZ product is an apparent dead end product on a short-term time scale. Enzyme-catalyzed double-isomerization, EZ → ZE (vide infra) followed by enzyme-catalyzed isomerization of one double bond ZE → EE, may be a path for EZ to EE, likely the most stable isomer. Judging from the data in Fig. 1, however, the putative conversion of EZ to ZE would appear to be too slow with respect to the productive reactions in Scheme 2.

King and Altman (*22*) have presented a simple algorithm which allows one to write down the equations for initial rates of an enzymatic reaction by essentially inspecting the kinetic scheme. Plowman (*23*) has amplified the discussion of this

technique. Using their method in conjunction with Scheme 2, an expression for the initial specific activity in the 2*E*,4*E*-isomer can be given as

$$(EE)/(EE^*) = \frac{k_6 \times K_{ZE} \times (ZZ) + k_{12} \times K_{ZZ} \times (ZE)}{k_6 \times K_{ZE} \times (ZZ^*) + k_{12} \times K_{ZZ} \times (ZE^*)}, \quad [3]$$

where $K_{ZZ} = (k_2 + k_6 + k_8)/k_1$ and $K_{ZE} = (k_{10} + k_{12})/k_9$. At the beginning of the experiment the concentration of ZE* (i.e., radioactive ZE) is negligible so the second term in the denominator can be considered negligible. The equation after rearrangement becomes

$$\left[\frac{(EE)}{(EE^*)} - \frac{(ZZ)}{(ZZ^*)}\right] \times \frac{(ZZ^*)}{(ZZ)} \times \frac{(ZZ)}{(ZE)} = \frac{k_{12}/K_{ZE}}{k_6/K_{ZZ}}, \quad [4]$$

where (EE)/(EE*) and (ZZ)/(ZZ*) are the reciprocals of the specific radioactivities of the designated isomers which are present at tracer levels. Application of this equation to the initially observed activities and concentrations indicate that starting with approximately equal concentration of ZZ and ZE isomers the ratio $(k_{12}/K_{ZE})/(k_6/K_{ZZ})$ equals about 13. *These results indicate that one-step direct, double cis-trans isomerization proceeds from the ZZ- to the EE-isomer.*

In Table 2 there is another interesting observation and that is the specific activity of the initially formed 2*E*,4*Z*-isomer. If this isomer were only to form directly from the 2*Z*,4*Z* starting material it should have the same specific activity as its precursor. It appears always to be formed with an activity lower than that of its precursor. One way for this to happen is for a fraction of the unlabeled 2*Z*,4*E*-isomer to isomerize in one step to the 2*E*,4*Z*-isomer to thereby dilute that coming from the 2*Z*,4*Z*-isomer. This double isomerization was looked for in another way. Starting with only the 2*Z*,4*E*-isomer, enzymatic isomerization leads to the 2*E*,4*E*-isomer. Careful examination of the reaction mixture by HPLC at various times, however, reveals the gradual formation of a small amount of the 2*E*,4*Z*-isomer (see Fig. 3).

## DISCUSSION

In previous reports we have presented evidence for the role of GSH in the enzyme catalyzed reaction as being that of a nucleophile attacking C2 of the maleylacetone system to generate an enzyme-bound dienediol intermediate which can undergo rotation about the C2–C3 bond (Eq. [5]). Reketonization with loss of GSH generates fumarylacetone. Recently we reported a one-step double *cis-trans* isomerization of analogs of maleylacetone catalyzed by the isomerase. Isomerase and GSH catalyze the interconversion between the 2*Z*,4*E*- and the 2*E*,4*Z*-isomers of 6-oxo-2,4-heptadienoate. The isomerization is best rationalized by suggesting a similar mechanism (Eq. [6]): enzyme-catalyzed addition of GS to C2 to generate an intermediate which undergoes rotation by bicycle-pedal motion followed by reketonization and loss of GSH. That study demonstrates that the enzyme accepts

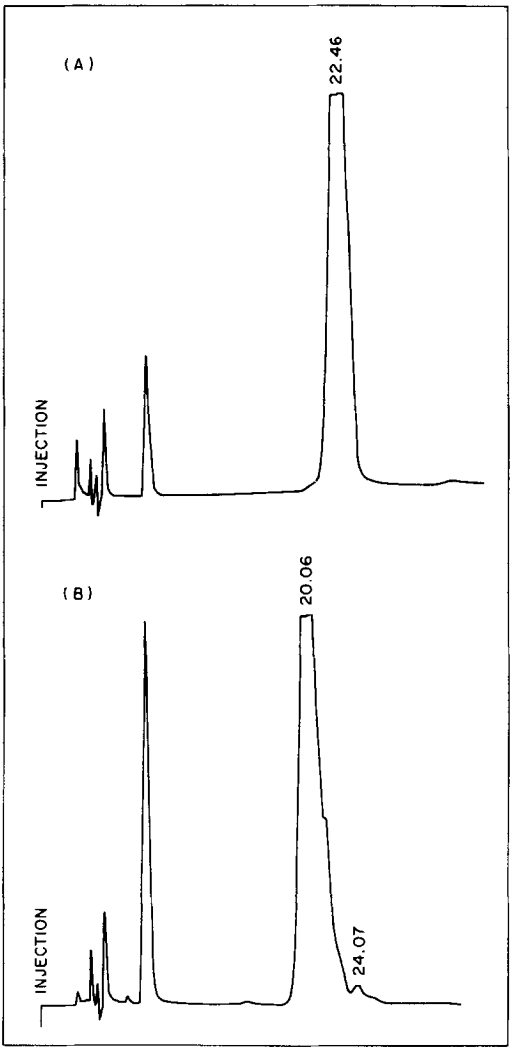
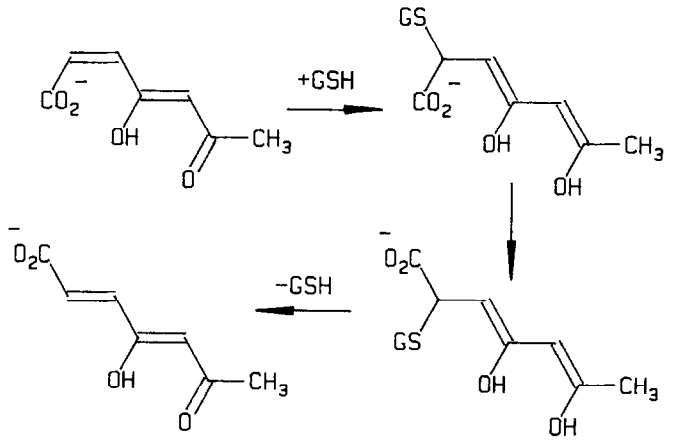


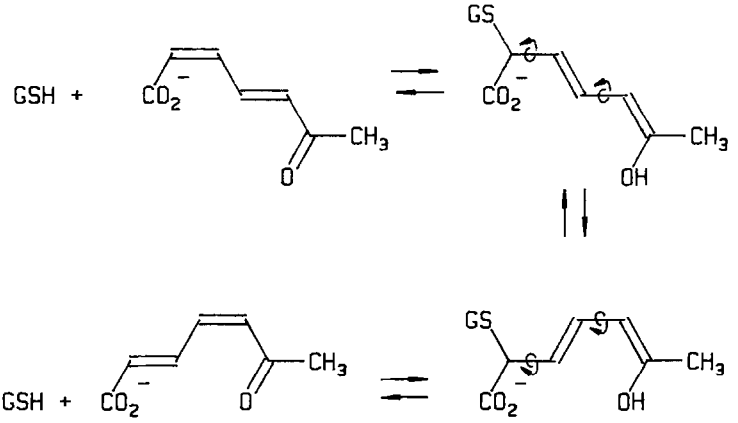

FIG. 3. Enzyme-catalyzed *cis-trans* isomerization of monomethyl 2*Z*,4*E*-muconate. The solution contained $5.5 \times 10^{-4}$ M 2*Z*,4*E*-monoester, $1.5 \times 10^{-3}$ M GSH, 0.01 M phosphate, pH 7.4, 6 mM mercaptoethanol, and $6.5 \times 10^{-3}$ units of isomerase (as assayed against maleylacetone) in a total volume of 125 $\mu$l. (A) HPLC trace of reaction mixture, 1 min after mixing enzyme–buffer–mercaptoethanol solution with glutathione and the 2*Z*,4*E*-monoester. (B) HPLC trace of reaction mixture after 7.4 h at ambient temperature in the dark. Peaks at 20.06 and 24.07 min correspond to the 2*E*,4*E*- and the 2*E*,4*Z*-isomers, respectively. A control experiment with all components except enzyme under the same conditions led to the formation of 2.5% of the 2*E*,4*E*-isomer after 19 h.

the 2*Z*,4*E* and the 2*E*,4*Z* structures but is silent about whether it also accepts the 2*Z*,4*Z*-structure. 6-Oxo-2*Z*,4*Z*-heptadienoate is unstable; it autoisomerizes instantaneously to the 2*Z*,4*E*-isomer. 6-Methyl 2*Z*,4*Z*-hexadiendioate, an analog with the same geometric configuration, however, is sufficiently stable and is shown here to undergo enzyme-catalyzed single and double *cis-trans* isomerization.



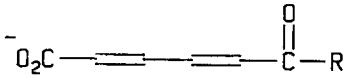

[5]

These results then suggest that maleylacetone and maleylacetoacetate molecules having 2*Z*,4*Z*, 2*Z*,4*E*, or 2*E*,4*Z* geometries are accepted at the binding site of the isomerase. Information about the binding of the 2*E*,4*E* product is not available at present.




[6]

In early experiments of this study the enzyme system was observed to catalyze isomerization of muconic acid itself (i.e., its dianion) at a very slow rate (data not shown). The three related derivatives of the 6-oxo-2,4-hexadienoate structure **(5)**, stand in decreasing order of isomerization rate as $R = CH_3 > OCH_3 > O^-$. Their relative rates are approximately 0.5, $3 \times 10^{-3}$, and $3 \times 10^{-7}$, respectively. This order of rates is to be expected if the rate of enzymatic reaction were dependent on the electrophilicity of the substituted carbonyl-carbon atom of the substrate analog as might be the case if the slow step involved attack of GS at the terminus of a diene system conjugated to a substituted carbonyl system. Measurements in two types of reactions, (1) acidity of $\alpha$-protons neighboring to substituted carbonyl groups and (2) rates of carbonyl-oxygen exchange, provide data on the relative electrophilicity of carbonyl groups in $CH_3COX$ **(6)** compounds, where $X = CH_3$, OAlkyl, or $O^-$. Unfortunately, a complete set of data is lacking for each of the two reactions but overlap of the two provides the desired information. The acidities of



5

–CH adjacent to COX in **6** are in order of $X = CH_3 > OAlkyl$. $pK_a$'s for these two are 20 and ~24.5, respectively (*24*). Rates of oxygen exchange are available in the older literature. Extrapolation of data of Cohn and Urey (*25*) to neutral pH suggest that acetone ($X = CH_3$) exchanges oxygen with water with a pseudo-first-order rate constant of $1 \times 10^{-5}$ min$^{-1}$ at 25°C. Data of Herbert and Lauder (*26*) on the oxygen exchange rate of acetate ion ($X = O^-$) in water with no added catalyst set the first-order rate constant at $\leq 7 \times 10^{-6}$ min$^{-1}$ at 25°C. With these two sets of data the carbonyl carbon electrophilicity for compounds of structure **5** would follow $R = CH_3 > OAlkyl > O^-$. With these parallels in these three reactions it allows one to predict that the enzyme-catalyzed isomerization of 6-oxo-2,4 hexadienoate (**5**, $R$ = H), i.e., muconaldehydic acid, would even be faster than the previous three measured since the gas phase activity of the $\alpha$-protons of acetaldehyde are about 3 kcal/mol more acidic than those of acetone (*27*). Moreover, the pseudo-first-order rate constant for oxygen exchange of acetaldehyde (**6**, $X$ = H) is $5.75 \times 10^{-3}$ min$^{-1}$ at 20°C (*28*), indicating that its carbonyl carbon is more electrophilic than those already considered above.

*The possible role of the isomerase in the detoxification of benzene metabolites.* There has been considerable interest concerning the carcinogenicity of benzene. As mentioned above *trans,trans*-muconic acid is found in the urine of mice when benzene is added to their diet. *trans,trans*-Muconic acid is not found when phenol or catechol is administered which suggests that these phenolic compounds are not on the oxidation pathway between benzene and muconic acid (*16*). Oxidative metabolism of benzene would be expected to lead to an acyclic six carbon *cis,cis* structure. How this *cis,cis* structure is isomerized to the *trans,trans* structure ultimately found in mouse urine is of interest in the present discussion.

It is tempting to suggest that maleylacetoacetate *cis-trans* isomerase also has the role of isomerizing this *cis,cis* six carbon structure to its *trans,trans*-isomer. What might be the oxidation level of the six carbon compound that it isomerizes? It is unlikely that this intermediate is *cis,cis*-muconic acid since it is isomerized very slowly by the enzyme system (vide supra). Based on (1) the enzyme activity toward maleylacetoacetate observed in a rat liver homogenate (*6*), (2) the assumption that mouse liver and rat liver contain about the same concentration of maleylacetoacetate *cis-trans* isomerase per gram of liver tissue, and (3) the relative rate for isomerization of muconic acid dianion vs maleylacetone, one can calculate that the total isomerase activity expected in mouse liver is about 150 times smaller than is necessary to isomerize the amount of *cis,cis*-muconic acid to the *trans,trans*-isomer found in mouse urine after 24 h of benzene metabolism. Neither phenol, catechol, nor *cis,cis*-muconate now appears to be an intermediate in the *in vivo* conversion of benzene to *trans,trans*-muconate. Consequently, it is reasonable to suggest that *cis,cis*-muconaldehydic acid is an intermediate on the dioxygenase-catalyzed pathway of benzene metabolism. *cis,cis*-Muconaldehydic

acid would be expected to undergo rapid single and double *cis-trans* isomerization catalyzed by maleylacetoacetate *cis-trans* isomerase. It would also be expected to undergo nonenzymatic isomerization to 6-oxo-2*Z*,4*E*-hexadienoate which the enzyme would be expected to isomerize rapidly to the 2*E*,4*E*-isomer. Thus enzymatic isomerization to *trans,trans*-muconaldehydic acid anion followed by oxidation is suggested here to be the path to *trans,trans*-muconate.

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