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ENZYMIC *cis-trans* ISOMERIZATION OF NITROFURAN DERIVATIVES

ISOMERIZING ACTIVITY OF XANTHINE OXIDASE, LIPOYL DEHYDROGENASE, DT-DIAPHORASE AND LIVER MICROSOMES

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Summary

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) supplemented with an electron donor could catalyze the *cis-trans* isomerization of 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide, 3-(5-nitro-2-furyl)-2-phenylacrylamide and 3-(5-nitro-2-furyl)-2-(2-furyl)acrylonitrile. The direction of isomerization (*cis* → *trans*, *cis* ⇌ *trans* or *trans* → *cis*) is dependent on the chemical structure of these nitrofuran derivatives. Lipoyl dehydrogenase (NADH:lipoamide oxidereductase, EC 1.6.4.3), DT-diaphorase (NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2) and liver microsomes could also catalyze the conversion of *cis*-3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide to its *trans* isomer in the presence of an appropriate electron donor. Such isomerizing activity of these enzymes is much higher than their nitro-reducing activity. In addition, the *cis-trans* isomerization of some nitrofuran derivatives was demonstrated with the liver slices and the small intestines of rats. A new *cis-trans* isomerization mechanism which is based on transfer of a single electron by an enzyme system to a nitrofuran derivative to give the radical-anion was proposed. This postulated mechanism was supported by the preliminary experiments using pulse radiolysis technique.

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Introduction

Enzymes capable of catalyzing *cis-trans* isomerization about carbon-carbon double bonds have not been studied as extensively as enzymes catalyzing other types of reactions. However, such *cis-trans* isomerases which have been characterized so far play an important role in intermediary metabolism of animals and bacteria; maleate *cis-trans* isomerase [1—3], maleylpyruvate *cis-trans* isomerase [4—6], maleylacetoacetate *cis-trans* isomerase [7], maleylacetone *cis-trans* isomerase [8] and all-*trans*-retinene 11-*cis-trans* isomerase [9].

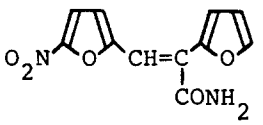
Nitrofuran derivatives with olefinic double bonds in the side chain at the 2-position of the furan ring (some of which have mutagenic and carcinogenic activities [10] in addition to powerful antibacterial activity) form geometrical *cis* and *trans* isomers. In a preliminary communication [11], we provided the evidence for *cis-trans* isomerization of 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (I) and 3-(5-nitro-2-furyl)-2-(5-bromo-2-furyl)acrylamide (II) by xanthine oxidase supplemented with an electron donor such as xanthine.

We wish to describe in detail the *cis-trans* isomerization of I, II, 3-(5-nitro-2-furyl)-2-phenylacrylamide (III) and 3-(5-nitro-2-furyl)-2-(2-furyl)acrylonitrile (IV) by xanthine oxidase (xanthine:oxygen oxidoreductase, EC, 1.2.3.2), lipoyl dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3), DT-diaphorase (NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2) and liver microsomal enzymes which are known as nitroreductases, liver slice and everted small intestine. In addition, we propose a possible mechanism of such enzymic *cis-trans* isomerization.

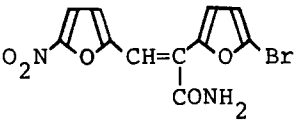
Materials and Methods

Chemicals. *Cis*-I (m.p. 151—152°C), *trans*-I (m.p. 176—177°C) and *cis*-III (m.p. 169—170°C) were kindly donated by Ueno Pharmaceutical Co. *Cis*-II (m.p. 181°C) and its *trans* isomer (m.p. 173—174°C) were prepared by the method of Hirao et al. [12], and *cis*-IV (m.p. 186—187°C) and its *trans* isomer (m.p. 132—133°C) by the method of Kato et al. [13]. *Trans*-III (m.p. 173—175°C) was prepared as follows: methanolic solution of *cis*-III was irradiated with ultraviolet light (3650 Å) for 1 h, the solution was evaporated to dryness under vacuum and the residue was purified by preparative thin-layer chromatography (Kieselgel G Merck, 0.25 mm thick) with benzene/acetone (7:3, v/v). IRν_{max}^{KBr} cm^{-1}: 3440, 3165, 1678, 1575, 1520. High mass spectrum: calculated for $C_6H_9O_2N_4$ (M^+, *m/e*) 258.0639. Found 258.0163. UV$\lambda_{max}^{ethanol}$ nm: 375. NADPH, NADH, NADP, NAD and glucose 6-phosphate were purchased from Sigma Chemical Co. Nicotinamide was obtained from Wako Pure Chemical Industries, bovine serum albumin (Fraction V from bovine plasma) from Armour Pharmaceutical Co., xanthine from Yoneyama Chemical Industries, and hypoxanthine from Nakarai Chemicals, respectively.

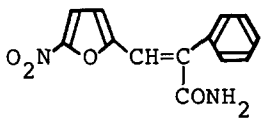
The *cis* or *trans* form described in this paper means the isomer in which the furan rings, or the furan and benzene rings attached to the olefinic double bond lie on the same or opposite sides of the molecule, respectively. Such geometrical configurations of all nitrofuran derivatives tested were determined from their NMR spectra. Namely, when one isomer reveals a signal of olefinic



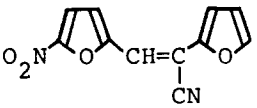
I



II



III



IV

Fig. 1. Structures of nitrofuran derivatives tested.

proton in a lower magnetic field and another isomer as a result of the magnetic anisotropy of the C ≡ N or $CONH_2$ bond, the configuration of this isomer was determined to be *cis*, and that of another to be *trans*.

Enzymes and tissue preparations. Purified buttermilk xanthine oxidase, pig heart lipoyl dehydrogenase and alcohol dehydrogenase were purchased from Sigma Chemical Co. For preparation of enzymes, male Donryu-strain rats weighing 160—180 g were used. Partially purified DT-diaphorase was prepared from rat liver by the procedure of Ernster et al. [14]. Rat liver microsomes were prepared as follows: rats were killed by cervical fracture and exsanguinated. The livers were removed and homogenized with a Teflon-glass homogenizer in four volumes of 1.15% KCl. The homogenate was centrifuged for 20 min at 9000 $\times g$ in a Kubota KR-6L-D centrifuge, and the supernatant fraction was centrifuged for 60 min at 105 000 $\times g$ in a Hitachi 65P ultracentrifuge. The microsomes were suspended in a volume of 1.15% KCl, equivalent to twice the volume of the original liver. The protein concentration in the suspension was 16 mg/ml. In tissue preparations, rat livers were sliced to 0.5 mm thickness with a sharp blade, and small intestines were everted over a glass rod and cut into pieces of 1 cm in length (about 0.1 g).

Enzyme unit. The enzyme activities of xanthine oxidase, lipoyl dehydrogenase and DT-diaphorase were measured by the methods of Kalckar [15], Savage [16] and Ernster et al. [14], respectively. One unit of xanthine oxidase is that quantity which will convert 1.0 μmol of xanthine to uric acid/min at pH 7.5 at 25°C. One unit of lipoyl dehydrogenase and DT-diaphorase is defined as that amount which causes an initial rate of change of absorbance at the absorption maximum of 2,6-dichlorophenolindophenol (600 nm) of 0.01/min at 25°C.

Enzyme assays for isomerizing activity. A Thunberg tube was used for all anaerobic experiments. A typical incubation mixture consisted of 0.075 μmol of substrate, 0.15 μmol of electron donor and enzyme in a final volume of 2.5 ml of 0.03 M phosphate buffer (pH 7.4). After the tube was evacuated with an aspirator for 3 min and then prewarmed for 2 min at 37°C, the reaction was

started by addition of the enzyme solution from the side arm to the tube and continued for 10 min in the dark. In aerobic experiments, the above incubation was carried out in open vessels. In the experiments with liver slice or everted small intestine, an incubation mixture consisted of 0.2 μmol of substrate, 0.1 g of tissue and 5 ml of 0.03 M phosphate buffer (pH 7.4). The incubation was carried out for 10 min at 37°C under aerobic conditions. As a control, a substrate was incubated under above conditions with enzyme or slice which had been heat inactivated at 100°C for 5 min.

After incubation, the mixture was immediately extracted twice with an equal volume of ethyl acetate, and the combined extract was evaporated to dryness under vacuum. The residue was then applied to a silica gel plate (Kiesel gel G Merck, 0.25 mm thick) and developed with benzene/acetone (7:3, v/v) or chloroform/hexane (4:1, v/v). After removal of solvents, the spots corresponding to the *cis* and *trans* isomers of a nitrofuran derivative, which were visualized under ultraviolet light (3650 Å), were scraped and eluted separately with 2.5 ml of 95% ethanol. Table I shows the R_F values of *cis-trans* isomers of nitrofuran derivatives in thin-layer chromatography.

The amount of each isomer in these ethanolic extracts was determined spectrophotometrically at 386 nm for *cis*-I, 402 nm for *trans*-I, 395 nm for *cis*-II, 400 nm for *trans*-II, 357 nm for *cis*-III, 375 nm for *trans*-III, 400 nm for *cis*-IV and 396 nm for *trans*-IV.

Protein was determined by the method of Lowry et al. [17] with crystalline bovine serum albumin as standard.

Enzyme assays for nitroreducing activity. The reduction of I by xanthine oxidase/xanthine, lipoyl dehydrogenase/NADH, DT-diaphorase/NAD(P)H or liver microsomes/NAD(P)H system was investigated using the same incubation mixture as that in above isomerization reaction. In these cases, the *trans* isomer of I was used as a substrate, because *cis* type I is isomerized rapidly to the *trans* type almost completely by above enzyme systems before the initiation of reduction as described later. Prior to incubation, the Thunberg type cuvette containing *trans*-I and an electron donor in the phosphate buffer was fitted with side arm containing the enzyme preparation and then evacuated with an aspirator for 5 min. The cuvette was placed in the cuvette chamber of a

TABLE I

R_F VALUES OF *cis-trans* ISOMERS OF NITROFURAN DERIVATIVES IN THIN-LAYER CHROMATOGRAPHY

The compounds were developed on silica gel thin-layer plates with the solvents described below and the spots were visualized under ultraviolet light (3650 Å). Solvent A, benzene/acetone (7:3, v/v); solvent B, chloroform/hexane (4:1, v/v). I = 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide; II = 3-(5-nitro-2-furyl)-2-(5-bromo-2-furyl)acrylamide; III = 3-(5-nitro-2-furyl)-2-phenylacrylamide; IV = 3-(5-nitro-2-furyl)-2-(2-furyl)-acrylonitrile.

Compound	*cis* isomer	*trans* isomer	Solvent
I	0.25	0.31	A
II	0.28	0.39	A
III	0.38	0.47	A
IV	0.52	0.45	B

spectrophotometer and prewarmed for 2 min at 37°C. The reaction was started by addition of the enzyme preparation and continued for 10 min. The amount of reduced I was calculated from the observed decrease in the absorbance at 417 nm (the absorption maximum of *trans*-I in 0.03 M phosphate buffer (pH 7.4)). The reduction of *trans*-III or *cis*-IV by xanthine oxidase/xanthine system was also carried out by using a Thunberg tube and the same incubation mixture as described in isomerization reaction. After incubation, *cis* and *trans* isomers of the nitrofuran derivative were extracted with ethyl acetate from the solution, separated by thin-layer chromatography and then determined spectrophotometrically as described above. The amount of reduced III or IV was calculated from the recoveries of *cis* and *trans* isomers of each nitrofuran derivative.

Identification of isomerization products. All isomerization products were identified by comparing their thin-layer chromatographic R_F values and ultraviolet absorption spectra with those of authentic samples of expected isomers. In addition, 3 μmol of *cis*-I, *cis*-III, *trans*-III or *trans*-IV in 250 μl of ethanol or acetone was incubated with 0.02 unit of xanthine oxidase and 0.6 μmol xanthine in 150 μl of 0.1 N NaOH in a final volume of 7 ml of 0.03 M phosphate buffer (pH 7.4) under anaerobic conditions. After 10 min incubation, the mixture was extracted twice with two volumes of ethyl acetate and the combined extract was evaporated to dryness under vacuum. The residue was thin-layer chromatographed preparatively by the same procedures as described above. The isomerization products obtained from the plates after elution were further identified by mass spectrometry.

Incubation of I in deuterium oxide. Phosphate buffer was prepared by using deuterium oxide (^2H_2O concentration: 98%). The incubation mixture contained 0.75 μmol of *cis*-I in 250 μl of methanol, 0.5 unit of xanthine oxidase and 1.5 μmol of xanthine in 150 μl of 0.1 N NaOH, in a final volume of 6.25 ml of 0.03 M deuterium phosphate buffer (pH 7.4). The mixture was incubated for 10 min at 37°C under anaerobic conditions and then extracted twice with two volumes of ethyl acetate. The combined extract was evaporated to dryness under vacuum and then the residue was subjected to mass spectrometric examination.

γ-Radiolysis. Aqueous solutions of I for radiolysis experiments were made up in 50 mM sodium formate and 2 mM phosphate buffer (pH 7.4). γ-Radiolysis was performed using ^{60}Co source and the pulse radiolysis equipment [18].

Analytic procedures. Ultraviolet spectra were taken on a Hitachi 124 UV and a Unicam SP8000 spectrophotometers. Mass spectra were recorded on a Japan Electron Optics (JEOL) JMS-01SG mass spectrometer.

Results

Isomerization of I, III and IV by xanthine oxidase

Table II shows the *cis-trans* isomerization of some nitrofuran derivatives by xanthine oxidase. In the case of I, it was easily isomerized from the *cis* to the *trans* form, but little in the reverse direction, by the enzyme supplemented with xanthine. As reported previously, II was also converted exclusively from the *cis* to the *trans* isomer [11]. In the case of III, *cis-trans* interconversion was

TABLE II

cis-trans ISOMERIZATION OF NITROFURAN DERIVATIVES BY MILK XANTHINE OXIDASE

The incubation mixture consisted of 0.075 μmol of an isomer of 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (I), 3-(5-nitro-2-furyl)-2-phenylacrylamide (III) or 3-(5-nitro-2-furyl)-2-(2-furyl)acrylonitrile (IV) in 20 μl ethanol or acetone, 0.15 μmol canthine in 30 μl 0.1 M NaOH, and 0.005 (for I and IV) or 0.05 units (for III) milk xanthine oxidase in a final volume of 2.5 ml 0.03 M phosphate buffer (pH 7.4). Data indicate percent isomerization and each value represents mean ± S.D. of four experiments.

	I (%)		III (%)		IV (%)	
	cis → *trans*	*trans* → *cis*	*cis* → *trans*	*trans* → *cis*	*cis* → *trans*	*trans* → *cis*
Enzyme/xanthine	86.9 ± 1.5	11.3 ± 1.0	36.9 ± 1.4	40.2 ± 2.5	1.9 ± 0.4	91.9 ± 2.1
Boiled enzyme/xanthine	5.5 ± 1.1	5.4 ± 0.9	2.7 ± 0.5	6.2 ± 1.1	2.9 ± 0.5	15.2 ± 2.0
Enzyme only	7.2 ± 1.1	7.7 ± 0.6	3.7 ± 0.7	7.7 ± 1.9	2.2 ± 0.2	34.5 ± 6.1
Enzyme/xanthine/O_2 *	41.5 ± 4.5	7.6 ± 1.4	—	—	—	—
Phosphate buffer (pH 7.4)	3.4 ± 0.5	4.8 ± 0.5	3.1 ± 0.7	9.7 ± 0.5	2.2 ± 0.6	5.9 ± 0.5

* The incubation was carried out under aerobic conditions.

observed, and the isomerizations of the *cis* to the *trans* form and in the reverse direction are equally rapid. Contrary to I, IV was easily isomerized from the *trans* to the *cis* form, but not in the reverse direction.

Thus it was found that the direction of isomerization of nitrofuran derivatives is dependent on their chemical structures. When the enzyme in this system was inactivated by boiling or when xanthine as an electron donor was omitted from the system, the isomerization rates decreased markedly in all cases, indicating that these reactions were catalyzed by xanthine oxidase supplemented with an electron donor, namely by the enzymic oxidoreduction system. In the case of IV, even though the enzyme was inactivated or the electron donor was omitted, its *trans* → *cis* isomerization was observed at some extents, indicating its instability in protein-containing medium. In fact, when 0.075 μmol of *trans*-IV was incubated for 10 min at 37°C in 0.03 M phosphate buffer (pH 7.4) containing bovine serum albumin at a concentration of 0.07%, about 30% of the compound was converted to its *cis* isomer non-enzymatically. Under aerobic conditions, the isomerization rate of I decrease to about a half compared with anaerobic conditions.

Isomerization of I by lipoyl dehydrogenase

Table III shows the *cis-trans* isomerizing activity of lipoyl dehydrogenase toward I. The enzyme supplemented with NADH could catalyze the conversion of *cis*-I to its *trans* isomer, but little the reverse reaction, similar to xanthine oxidase/electron donor system described above. In this case, the inactivation of the enzyme, the omission of the electron donor or the presence of oxygen also reduced the *cis* → *trans* isomerization rate markedly.

Isomerization of I by DT-diaphorase

As shown in Table IV, DT-diaphorase could also catalyze anaerobically the *cis* → *trans* isomerization of I in the presence of NADH. However, the isomeri-

TABLE III

cis-trans ISOMERIZATION OF 3-(5-NITRO-2-FURYL)-2-(2-FURYL)ACRYLAMIDE (I) BY LIPOYL DEHYDROGENASE

The incubation mixture consisted of 0.075 μmol *cis-* or *trans*-I in 20 μl ethanol, 0.15 μmol NADH and 0.005 unit pig heart lipoyl dehydrogenase in a final volume of 2.5 ml 0.03 M phosphate buffer (pH 7.4). Each value represents mean ± S.D. of four experiments.

	cis → *trans* (%)
Enzyme/NADH	85.9 ± 1.9
Boiled enzyme/NADH	8.4 ± 2.5
Enzyme only	5.5 ± 0.8
Enzyme/NADH/O_2 *	16.0 ± 1.9
	trans → *cis* (%)
Enzyme/NADH	10.9 ± 0.4

* The incubation was carried out under aerobic conditions.

zation rate was lower than that observed in xanthine oxidase (Table II) or lipoyl dehydrogenase (Table III). When bovine serum albumin, which is known as a stimulator of the enzyme [14,19], was added to the incubation mixture, the isomerization rate increased up to about 70%. NADPH also served as a cofactor of the enzyme in such conversion of I.

Isomerization of I by liver microsomes

Table V shows the *cis-trans* isomerizing activity of rat liver microsomes to I.

TABLE IV

cis-trans ISOMERIZATION OF 3-(5-NITRO-2-FURYL)-2-(2-FURYL)ACRYLAMIDE (I) BY DT-DIAPHORASE

The incubation mixture consisted of 0.075 μmol *cis-* or *trans*-I in 20 μl ethanol, 0.15 μmol NADH or NADPH, and 0.05 unit rat liver DT-diaphorase in a final volume of 2.5 ml 0.03 M phosphate buffer (pH 7.4). Each value represents mean ± S.D. of four experiments. BSA, bovine serum albumin.

	cis → *trans* (%)
Enzyme/NADH	44.5 ± 4.4
Boiled enzyme/NADH	6.6 ± 0.8
Enzyme/BSA/NADH	71.3 ± 13.4
Boiled enzyme/BSA/NADH	12.9 ± 4.5
Enzyme/BSA/NADPH	43.9 ± 1.5
Enzyme/NADH/O_2 *	6.3 ± 0.6
Enzyme/BSA/NADH/O_2	6.2 ± 0.5
Enzyme/BSA/NADPH/O_2	3.6 ± 0.5
Enzyme only	3.2 ± 0.8
	trans → *cis* (%)
Enzyme/NADH	7.1 ± 0.5
Enzyme/BSA/NADH	8.5 ± 0.7
Enzyme/BSA/NADPH	7.1 ± 1.1

* The incubation was carried out under aerobic conditions.

TABLE V

cis-trans ISOMERIZATION OF 3-(5-NITRO-2-FURYL)-2-(2-FURYL)ACRYLAMIDE (I) BY LIVER MICROSOMES

The incubation mixture consisted of 0.075 μmol *cis-* or *trans*-I in 20 μl ethanol, NADPH-generating system (0.15 μmol NADP, 2.25 μmol glucose 6-phosphate and 3 units glucose-6-phosphate dehydrogenase) or NADH-generating system (0.15 μmol NAD and 10 units alcohol dehydrogenase), 2.25 μmol $MgCl_2$, 5 μmol nicotinamide and rat liver microsomes (12 μg protein) in a final volume of 2.5 ml 0.03 M phosphate buffer (pH 7.4). Each value represents mean ± S.D. of four experiments.

	cis → *trans* (%)
Microsomes/NADPH	81.8 ± 8.0
Boiled microsomes/NADPH	4.7 ± 0.4
Microsomes/NADH	86.5 ± 2.6
Microsomes/NADPH/O_2*	3.4 ± 0.5
Microsomes/NADH/O_2*	3.4 ± 0.5
Microsomes only	5.1 ± 3.2
	trans → *cis* (%)
Microsomes/NADPH	8.7 ± 0.9
Microsomes/NADH	7.3 ± 2.9

* The incubation was carried out under aerobic conditions.

The microsomes supplemented with NADPH or NADH could exclusively converted *cis*-I to its *trans* form, like other enzyme systems described above. In this case, NADPH and NADH were effective to similar degrees as an electron donor. This enzymic reaction was also sensitive to oxygen.

Isomerization of nitrofuran derivatives by liver slice and everted small intestine

As shown in Table VI, rat liver slice and everted small intestine, like xanthine oxidase described above, could catalyze the *cis* → *trans* isomerization of I and II, and the *trans* → *cis* conversion of IV. When the tissues were boiled, the

TABLE VI

cis-trans ISOMERIZATION OF NITROFURAN DERIVATIVES BY RAT LIVER SLICE AND EVERTED SMALL INTESTINE

The incubation mixture consisted of 0.2 μmol of an isomer of 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (I), 3-(5-nitro-2-furyl)-2-(5-bromo-2-furyl)acrylamide (II) or 3-(5-nitro-2-furyl)-2-(2-furyl)acrylonitrile (IV) in 50 μl ethanol or acetone, and 0.1 g rat liver slice or everted small intestine in a final volume of 5 ml 0.03 M phosphate buffer (pH 7.4). Data indicate percent isomerization and each value represents mean ± S.D. of four experiments.

	I		II		IV	
	cis → *trans*	*trans* → *cis*	*cis* → *trans*	*trans* → *cis*	*cis* → *trans*	*trans* → *cis*
Liver slice	86.1 ± 2.0	10.0 ± 0.5	85.3 ± 4.0	12.7 ± 0.9	2.2 ± 1.1	82.1 ± 6.4
Boiled liver slice	10.4 ± 1.3	3.5 ± 0.3	7.0 ± 0.6	2.6 ± 0.3	1.7 ± 0.6	27.9 ± 1.2
Everted small intestine	40.9 ± 1.9	9.7 ± 0.9	76.2 ± 2.8	12.5 ± 0.8	1.3 ± 0.4	82.4 ± 3.7
Boiled everted small intestine	7.3 ± 0.3	4.4 ± 0.8	5.8 ± 1.0	4.0 ± 0.4	2.0 ± 0.6	34.5 ± 6.4
Phosphate buffer (pH 7.4)	1.8 ± 0.1	4.9 ± 0.3	4.3 ± 0.2	4.6 ± 1.6	1.0 ± 0.2	5.9 ± 0.5

isomerization rates were decreased significantly in all cases. These results indicate that the *cis-trans* isomerization of nitrofuran derivatives by these tissue preparations proceeds enzymatically and that such isomerization might occur in vivo when these drugs are given to animals.

Isomerization of I in deuterium oxide medium

It is well established that fumaric acid can reversibly added deuterium oxide to form maleate [20]. Accordingly, in order to explore the isomerization mechanism of nitrofuran derivatives, large scale incubation of *cis*-I with xanthine oxidase and xanthine was run in a medium containing deuterium oxide. After incubation, the reaction product, *trans*-I was isolated and examined mass spectrometrically. As a result, the mass spectrum of the *trans* isomer showed a molecular ion at *m/e* 248 *, indicating that in the course of enzymic isomerization of *cis*-I no deuterium was incorporated into the ethylene position of *trans*-I, namely that no labilization of the olefinic hydrogen atom took place during the *cis-trans* conversion.

Relation between isomerizing and nitroreducing activities

Xanthine oxidase [21—24], lipoyl dehydrogenase [25], DT-diaphorase [26] and liver microsomes [24,27] described above are known to act as nitroreductases for some nitro compounds. For example, I is reduced to 2-(2-furyl)-3-(5-oxo-2-pyrrolin-2-yl)acrylamide, and 5-nitro-2-furfural semicarbazone to the aminofuran derivative by xanthine oxidase or rat liver microsomes, respectively [24]. These reduction products are not autoxidized in air, unlike hydroxylamine derivatives.

An attempt was made to compare the relative activities of these enzymes for isomerization and nitroreduction. When 0.005 unit or 0.05 unit of xanthine oxidase, which was enough for isomerization of I and IV, or III (see Table II), was used with xanthine, only 3.1% of I, 5.0% of III and 5.1% of IV were reduced, respectively. However, when the enzyme unit increased up to ten-fold, a significant nitroreducing activity to these nitro compounds was observed (reduction rates: 52.6% for I, 43.1% for III and 31.6% for IV). Also, lipoyl dehydrogenase (0.005 unit)/NADH, DT-diaphorase (0.05 unit)/bovine serum albumin/NADH and microsomes (12 μg protein)/NAD(P)H systems could isomerize *cis*-I to its *trans* isomer significantly as shown in Table III, IV and V, while these enzyme systems exerted little nitroreducing activity to the nitro compound (reduction rates: 1.0—7.5%). When the amount of enzyme was increased to ten-fold in lipoyl dehydrogenase/NADH system and to 25-fold in microsomes/NADPH system, respectively, these enzyme systems could reduce the nitro compound to a significant extent (reduction rates: 22.5% with lipoyl dehydrogenase (0.05 unit) and 27.8% with microsomes (300 μg protein)). However, in both cases of DT-diaphorase/bovine serum albumin/NADH and microsomes/NADH, the increased nitroreducing activity of these systems was not observed significantly in spite of the increased enzyme amounts (reduction rates: 6.7% with DT-diaphorase (1.0 unit) and 7.3% with microsomes (300 μg protein)). These results indicate that with a nitrofuran derivative, the

* If a vinyl proton of I is replaced by an ^{2}H atom, its molecular weight (248) changes to 249.

isomerizing activity of the several enzyme systems described above is much higher than their nitroreducing activity, and that isomerization can occur whenever nitroreduction does.

γ-Radiolysis of cis-I in the presence of oxygen

Spectrum (a) in Fig. 2 is that of *cis*-I (40 μM) in a solution containing formate and oxygen (continuously bubbled with 0.68% O_2; $[O_2] \approx 9.8$ μM). γ-Radiolysis for 2 min at a dose rate of 0.84 Gy/min (1 gray = 1 J · kg^{-1} = 100 rad) resulted in spectrum (b); spectrum (c) was obtained after 6 min irradiation, after which it did not change. Spectrum (c) is identical to that of *trans*-I; intermediate irradiation times resulted in curves with highly reproducible isobestic point at 270, 314 and 372 nm. This behaviour is characteristic of no net loss of I. The efficiency of isomerization was calculated to be G (isom) ≈ 80, an order of magnitude higher than the total radiation-chemical yield G ($R\dot{N}O_2^- + O_2^-$) ≈ 6. Almost complete isomerization to the *trans* form was confirmed using high performance liquid chromatography. Similar experiments at an increased dose-rate (14.4 Gy/min) gave a similar value for G (isom). Saturation with air ($[O_2] \approx 300$ μM) decreased G (isom) to ≈1.6. γ-Radiolysis of *trans*-I in the presence of formate and oxygen using the conditions described did not result in any change in the absorption spectrum.

γ-Radiolysis of I in the absence of oxygen

Saturation of the formate/I solution with N_2O results in the reduction of the single reducing species $\dot{C}O_2^-$ which then produces $R\dot{N}O_2^-$. Radiolysis of *trans*-I (45 μM) resulted in the transformation to a species with λ_{max} 305 nm. Radiolysis of a similar solution of *cis*-I gave spectra indicating that isomerization to the *trans* form was occurring simultaneously to reduction, with the efficiency of isomerization being higher than that of reduction similar to the enzymic reaction described above, since the spectrum changed initially to give the absorption at 418 nm characteristic of the *trans* isomer followed by the transformation to the spectrum of the reduced species with λ_{max} 305 nm.

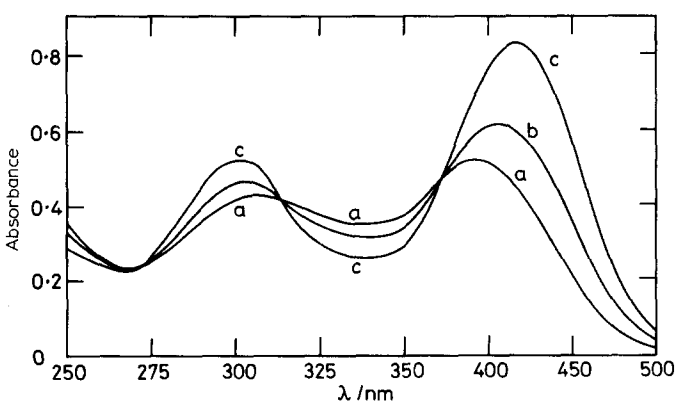


Fig. 2. Change in ultraviolet absorption curve during the isomerization of *cis*-3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (I) to its *trans* isomer by γ-radiolysis. Curves, a, b and c represent the spectrum of *cis*-I before irradiation, and that after 2 min and 6 min irradiation, respectively.

Discussion

So far it is known that isomerases which catalyze the isomerization of maleyl derivatives to fumaryl derivatives have been reported to require reduced glutathione or cysteine for their full activity [28]. From these facts, it is postulated that the mechanism of such isomerization involves the formation of an unstable transition complex between glutathione and a maleyl derivative [5]. On the contrary, the present study showed that the *cis-trans* isomerization of a nitrofuran derivative by xanthine oxidase, lipoyl dehydrogenase, DT-diaphorase and liver microsomes, which are known to have nitroreductase activity, proceeds in the absence of glutathione, unlike the isomerization of maleyl derivatives described above. In these cases, however, the presence of electron donors was an absolute requirement for catalysis to occur. In addition, the data from deuterium experiments demonstrated that no labilization of the olefinic hydrogen atoms takes place during the *cis-trans* conversion. Previously, we showed that when *cis-* or *trans*-3-(2-furyl)-2-(2-furyl)acrylamide lacking the nitro group was incubated with xanthine oxidase and xanthine, no enzymic isomerization

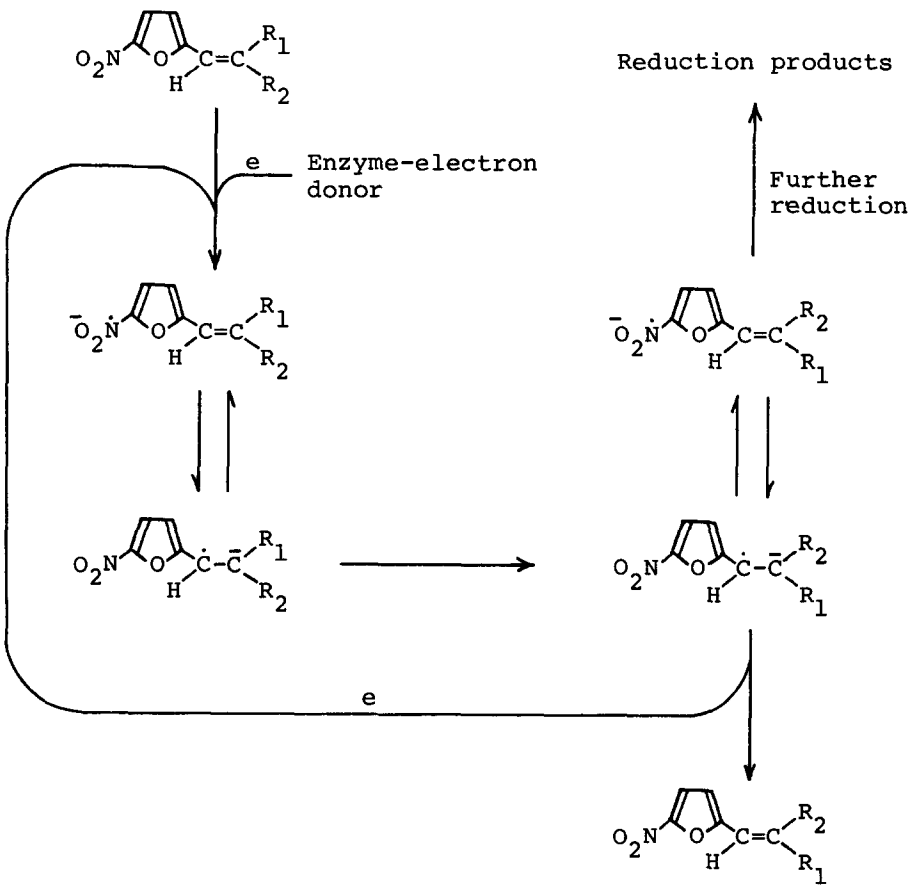


Fig. 3. Postulated mechanism for enzymic *cis-trans* isomerization of nitrofuran derivatives.

could be observed in either case, indicating the importance of the nitro group in such isomerization [11]. These results strongly suggest that the reduction of the nitro group of nitrofuran derivatives by the enzyme systems presented in this paper is an important first step in isomerization. Based on these findings, a possible mechanism of the isomerization is depicted schematically in Fig. 3.

It is postulated that a single electron derived from an enzyme system attacks first the nitro group of a nitrofuran derivative to form the radical-anion. Spin density on the olefinic double bond results in free rotation between the olefinic carbons followed by conversion to its thermodynamically more stable isomer until equilibrium. In some cases whether the radical formed receives more electrons or not is dependent on the amount of enzyme used. The preliminary experiments using pulse radiolysis technique prove that *cis-trans* isomerization of I occurs when the nitro radical-anion is produced, supporting above postulated mechanism. If one irradiates a solution of a nitrofuran derivative in the presence of formate and oxygen, only the radical-anion, RNO_2^- is formed as follows [29]:

$$H_2O \text{ — } \sim\!\!\!\!\rightarrow e_{aq}^-, H, \cdot OH$$

$$e_{aq}^- + R\text{-}NO_2 \rightarrow R\text{-}\dot{N}O_2^-$$

$$H \text{ (or } \cdot OH) + HCO_2^- \rightarrow H_2 \text{ (or } H_2O) + \dot{C}O_2^-$$

$$\dot{C}O_2^- + R\text{-}NO_2 \rightarrow CO_2 + R\text{-}\dot{N}O_2^-$$

Oxygen is not necessary for isomerization, but facilitates the study by completely preventing net loss of a nitro compound. The important observation in the present study is that irradiating *cis*-I in aerated solution quickly converted it to the *trans* isomer. The isomerization could only arise via the radical-anion and this isomerization could only arise via the radical-ion, and this isomerization must occur before electron transfer to O_2 takes place.

Mason and Holtzman [30] demonstrated by electron spin resonance examination that nitrofurantoin is reduced anaerobically by microsomal reductase to form its one-electron reduction product, i.e nitrofurantoin radical-anion, which may rapidly react with O_2 to give the original nitrofuran derivative and superoxide under aerobic conditions.

Thus, the present study provides the involvement of various nitroreductase in *cis-trans* isomerization of nitrofuran derivatives. Sugimura et al. [31] also reported briefly a similar isomerization of *cis*-I to its *trans* form by a rat liver microsomal enzyme(s) with NADPH or by rat liver cytosol enzyme, xanthine oxidase, with NADH or hypoxanthine. On the other hand, Tomoeda and Kitamura [32] have found that in *Escherichia coli* K-12 JE 2100, the soluble enzyme fraction exhibited NADH-dependent *cis-trans* isomerizing activity toward I in addition to NADH- and NADPH-dependent reductase activity, while the particulate fraction derived from the bacterial cells reveals only an isomerizing activity toward I in the presence of NADH. They suggest that isomerizing and nitroreducing activities of the bacteria toward I may be due to enzymes which are different in nature. However, considering from the facts that the bacterial *cis-trans* isomerase requires NADH as a electron donor for its isomerizing activity toward I, like the mammalian enzymes shown in the

present study, and that *E. coli* contains not only NADPH- and NADH-dependent nitrofuran reductase, but also NADH-dependent reductase [33,34], there is a possibility that the mechanism of nitrofuran *cis-trans* isomerization by the bacterial enzyme is similar to that by mammalian enzymes shown in Fig. 3.

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