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Studies on the Isomerization of 2,4-Dinitrophenylhydrazones of Some Aliphatic α-Keto Acids and the Preparation of Their Geometrical Isomers

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Cis-trans isomerization of 2,4-dinitrophenylhydrazones of some aliphatic α-keto acids takes place in water and some organic solvents by the catalysis of hydrogen ions. *Trans* isomer is more stable than *cis* isomer for lower keto acids and the reverse is the case for higher keto acids. The fraction of *cis* isomer in isomeric mixture at equilibrium in organic solvents such as ethyl acetate and ethyl ether, saturated with 6N hydrochloric acid, was larger than that in aqueous solution. The two isomers could be separated by means of the difference in their solubility characteristics in 1N sodium carbonate except for some keto acids with tertiary or quaternary β-carbon. Melting points of some pairs of the hydrazone isomers were found to be the same when measured by the usual method. This is due to the isomerization of one isomer to the other which takes place during the course of heating.

For the separation, identification and determination of carbonyl compounds, the 2,4-dinitrophenylhydrazine method is most often used in the fields of organic and biological chemistry. Studies have been carried out on physical and chemical properties of the 2,4-dinitrophenylhydrazones. However, only a limited number of studies has been reported on their geometrical isomers and isomerization.

Separation and assignment of *cis* and *trans* isomers of the hydrazones of the following α-keto acids and the related compounds were reported after the discovery of double spots on paper chromatograms of 2,4-dinitrophenylhydrazones of some α-keto acids:[1,2] pyruvic acid,[3,4] α-oxo-β,β-dimethyl-γ-butyrolactone,[5] and phenylglyoxylic acid.[6]

Although separation of the isomers has been conveniently performed by the use of paper or thin layer chromatography, preparation of a pure sample of the isomer or its identification was difficult due to the isomerization of the hydrazone during its development or elution in chromatography.[4] The first large scale separation of pure isomers has become feasible by the combination of salting-out method[3,6] with the isomerization treatment. Studies on the spectrographic properties and the isomerization with the pure hydrazone isomers thus prepared resulted in the detection[7,8] and

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determination[9,10] of some aliphatic α-keto acids which are important intermediates in metabolism, irrespective of the presence of geometrical isomers in their hydrazones.

This paper deals with the isomerization of 2,4-dinitrophenylhydrazone isomers of some aliphatic α-keto acids and separation of both isomers.

Keto acids used are classified into two groups, **A**(I-VI) and **B**(VII-IX). The hydrazones of keto acids in group **A** gave *cis* and *trans* isomers. *Cis* and *trans* hydrazones are represented by notations *c* and *t*, respectively, *e.g.* I*c*, I*t*. On the other hand, the presence of the two isomers of the hydrazones in group **B** could not be demonstrated and gave two different forms by recrystallization from different solvents. The hydrazones of VII, for example, are represented by VII*x* and VII*y*, respectively.

R-CO-COOH

Group **A**

	I	II	III	IV
R:	H–,	CH_3–,	CH_3CH_2–,	$CH_3CH_2CH_2$–,

	V	VI
R:	$CH_3(CH_2)_2CH_2$–,	$(CH_3)_2CHCH_2$–

Group **B**

	VII	VIII	IX
R:	$(CH_3)_2CH$–,	$CH_3CH_2(CH_3)CH$–,	$(CH_3)_3C$–

The assignment of the geometrical isomers based on their infrared and visible spectra and other properties was described in the preceeding paper.[11]

Experimental

Preparation of Keto Acids. Most of the keto acids used were synthesized by the condensation of fatty acid esters with diethyl oxalate, followed by hydrolysis with hydrochloric acid.[12] I, II, and IX were prepared by the methods of Radin and Metzler,[13] Haward and Fraser,[14] and Glucksmann,[15] respectively.

Preparation of the Isomers and Forms of the Hydrazones. The crude hydrazone of keto acid in group **A** obtained from aqueous solution by the usual method was first mixed with sufficient 1 N sodium carbonate to dissolve the *trans* isomer. After stirring the mixture for about half an hour, the insoluble hydrazone was filtered off. The filtrate was cooled in an ice bath. Hydrochloric acid (1 N) was then added dropwise until the solution became slightly acidic. After standing it for a few minutes, the precipitate was filtered and washed several times with 0.2 N hydrochloric acid, then with cold water. When necessary, especially in the case of higher keto acid hydrazones, the separation procedure with sodium carbonate was repeated. The hydrazone thus obtained was dried in a vacuum desiccator. It was recrystallized from acetone–benzene (1:3). Paper chromatography was effectively used to confirm complete purification.

The *cis* isomer of the hydrazone of keto acid in group **A** was prepared by utilizing the isomerization treatment as follows: The crude hydrazone was dissolved in ethyl acetate saturated with 6 N hydrochloric acid. After being left to stand at 10°C for 1 hr, 1 N sodium carbonate was added and the mixture was agitated vigorously in a separatory funnel. The resulting insoluble sodium salt of the *cis* isomer was collected by filtration. The treatment of the filtrate with sodium carbonate was repeated until insoluble hydrazone no longer separated out. When necessary, the sodium salt of *cis* hydrazone was dissolved in water and the salting-out separation was repeated. The sodium salt of *cis* hydrazone was washed with 1 N sodium carbonate until the color of the filtrate became yellow and it was then suspended in a small amount of cold water. Hydrochloric acid (1 N) was added until the mixture became slightly acidic. The free *cis* hydrazone produced was immediately collected by filtration,

Table 1. Elementary analyses of the hydrazones

Hydrazones of keto acids	Formulae	C (%)		H (%)		N (%)	
		Calcd	Found	Calcd	Found	Calcd	Found
I*t*	$C_8H_6O_6N_4$	37.81	37.98	2.38	2.47	22.05	22.12
I*c*			38.07		2.51		22.26
II*t*	$C_9H_8O_6N_4$	40.31	40.15	3.01	3.08	20.89	20.61
II*c*			40.12		3.24		20.72
III*t*	$C_{10}H_{10}O_6N_4$	42.56	42.79	3.57	3.83	19.85	19.72
III*c*			42.82		3.69		20.08
IV*t*	$C_{11}H_{12}O_6N_4$	44.59	44.45	4.08	4.32	18.92	18.86
IV*c*			44.30		4.25		19.16
V*t*	$C_{12}H_{14}O_6N_4$	46.46	46.56	4.58	4.75	18.06	18.12
V*c*			46.63		4.65		18.18
VI*t*	$C_{12}H_{14}O_6N_4$	46.46	46.26	4.58	4.82	18.06	18.21
VI*c*			46.22		4.86		17.90
VII*x*	$C_{11}H_{12}O_6N_4$	44.59	44.78	4.08	4.05	18.92	18.62
VII*y*			44.90		4.03		18.95
VIII*x*	$C_{12}H_{14}O_6N_4$	46.46	46.29	4.58	4.83	18.06	18.22
VIII*y*			46.59		4.76		18.21
IX*x*	$C_{12}H_{14}O_6N_4$	46.46	46.75	4.58	4.80	18.06	17.82
IX*y*			46.23		4.61		18.17

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washed and dried as described for the *trans* isomer in order to prevent isomerization. Recrystallization was carried out in exactly the same way as described for the *trans* isomer.

In the case of the hydrazone of keto acid in group **B**, the two forms were obtained by the following method: crystals of *x* form were obtained by recrystallization of the crude hydrazone from benzene and those of *y* form from 50% aqueous ethanol.

The results of elementary analyses of the hydrazones obtained are summarized in Table 1.

Each isomer as well as each form thus obtained contained no impurities detectable by paper chromatographic analysis.[8] The *cis* isomers gave higher R_f values than the corresponding *trans* isomers in normal phase systems and *vice versa* in reversed phase systems.

Isomerization of the Hydrazones. Determination of the proportions of the two isomers of the hydrazones during their formation was carried out as follows: One m*l* of 2,4-dinitrophenylhydrazine reagent (2.5 μ mol/m*l* in 1.2 N hydrochloric acid) was added to 2 m*l* of keto acid solution containing 0.2 to 0.7 μ mole of keto acid at 30°C. At varying time intervals up to 1 hr, 7 ml of 1.6 N sodium hydroxide solution was added to an aliquot of the mixture to stop the reaction. The red color produced upon addition of alkali was measured at two different wave lengths (W_1 and W_2) after allowing the solution to stand for 10 min at room temperature. W_1 is the wavelength at which two curves representing the absorption spectra of the hydrazone isomers in alkali intersect each other. Thus both isomers show the same absorption coefficient. W_2 is the wavelength at which the difference of the two curves is the largest. If the measured absorbances at W_1 and W_2 are represented as A_1 and A_2, respectively, A_1 or A_2 is equal to the sum of the absorbance due to each isomer of hydrazones formed and the one due to the remaining hydrazine reagent. Thus the following two equations are derived:

$$A_1 = k_1(m+n) + l_1(2.5-m-n)$$

$$A_2 = k_{2c}\cdot m + k_{2t}\cdot n + l_2(2.5-m-n)$$

where m and n represent the amounts of *cis* and *trans* hydrazones (μ moles), respectively, and k_1 and l_1 represent the absorbances of 1 μ mole of *cis* and *trans* hydrazones and of the hydrazine reagent at W_1 under these conditions, respectively. Similarly, k_{2c}, k_{2t}, and l_2 represent the absorbances of 1 μ mole of the *cis* and *trans* hydrazones and of the hydrazine reagent at W_2, respectively. The wavelengths, W_1 and W_2, and the specific absorption coefficients of the hydrazones in group **A** and of the hydrazine, *viz.*, k_1, l_1, k_{2c}, k_{2t}, and l_2, are summarized in Table 2.

Experiments on the isomerization of hydrazones in organic solvent containing hydrochloric acid were carried out as follows: A definite amount of hydrazone (0.3 to 0.6 μ mole) was dissolved in 1 m*l* of organic solvent and to this solution was added 4 m*l* of the same organic solvent saturated with 6 N hydrochloric acid. The solution was kept at 30°C for various periods of time. A few drops of concd. aqueous ammonia were then added to neutralize the solution and stop the isomerization reaction. The organic solvent was evaporated *in vacuo* and the residual hydrazone was dissolved in 5 m*l* of 0.1 N sodium hydrogen carbonate. Five m*l* of 2.0 N sodium hydroxide was added and the absorbances at W_1 and W_2 were determined as described above. In this case, l_1 and l_2 in the equations were negligible because no excess reagent was present. The interference due to the decomposition products from the hydrazone was also negligible under these conditions.

Table 2. Absorption coefficient of the hydrazones and the hydrazine

Hydrazones of keto aicds	W_1	k_1	l_1	W_2	k_{2c}	k_{2t}	l_2
	mμ			mμ			
I	406	1.12	0.088	460	0.40	2.60	0.017
II	417	1.54	0.068	470	1.00	1.68	0.013
III	416	1.59	0.070	470	1.22	1.63	0.013
IV	422	1.68	0.059	470	1.26	1.63	0.013
V	420	1.65	0.060	470	1.25	1.65	0.013
VI	420	1.67	0.060	470	1.25	1.68	0.013

The method for determination of the hydrazone isomers of I and II in ethyl acetate, ethyl ether and isopropyl ether was modified in the following way: After a few drops of aqueous ammonia were added to stop the reaction, 5 m*l* of petroleum ether and 2 m*l* of 0.1 N sodium hydrogen carbonate were added. By shaking the mixture in a separatory funnel, the hydrazones were transferred completely from the upper to the lower layer, and were determined spectrophotometrically by the method described above.

Solubility in Aqueous Sodium Carbonate. An excess amount of the hydrazone was shaken thoroughly with 1 m*l* of 1 N sodium carbonate for 1 hr at 25°C or 0°C, to let the hydrazone remain undissolved in the solution. The concentration of the hydrazone in the saturated solution was determined spectrophotometrically.

Partition between ethyl acetate and 0.1 N sodium carbonate. The hydrazone was dissolved in 2 m*l* of 0.1 N sodium carbonate containing 0.01 N sodium hydrogen carbonate. The hydrazone in the solution was then extracted with 10 m*l* of ethyl acetate and the amount of residual hydrazone in the lower layer was determined spectrophotometrically.

Rusults and Discussion

Isomerization during Hydrazone Formation. The proportion of *cis* to *trans* isomer during the course of hydrazone formation in dilute hydrochloric acid upon addition of the hydrazine reagent was determined. In the case of II, completion of hydrazone formation required 5 min at 30°C. The proportion of *cis* to *trans*

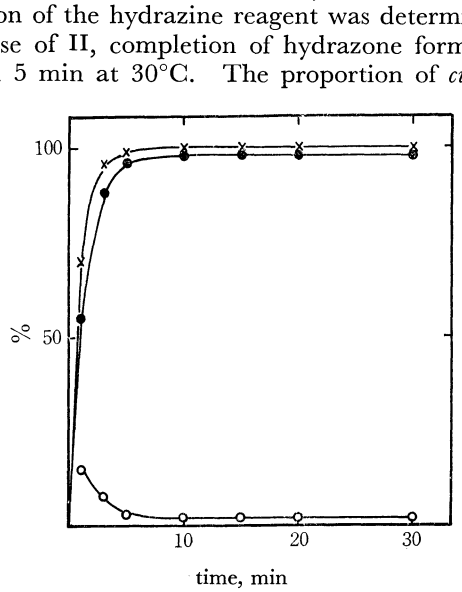

Fig. 1. Proportions of the *cis* and *trans* isomers during the formation of the hydrazone of II.
○, *cis* isomer; ●, *trans* isomer; ×, total hydrazone

isomer was found to vary depending upon experimental conditions. After the reaction for 1 min, about 23% of the hydrazone was present as the *cis* isomer. However, the *cis* isomer once produced was gradually converted into the *trans* isomer during further reaction by the action of hydrogen ions. The fraction of the *cis* isomer at equilibrium was negligible as shown in Fig. 1.

The reaction of I with hydrazine was rapid as in the case of II, but the other keto acids (III–VI) required 10—20 min for the completion of the reaction. The fraction of *cis* isomer in isomeric mixture after attainment of equilibrium in the hydrazone formation was as follows: **I**, negligibly small; III, 62%; IV, 50%; V, 81%; VI, less than 5%. Unlike the case of II, the fraction of *cis* isomer in V after 1-min reaction was 71% and increased to 81% at equilibrium state.

Although isomerization of the hydrazone in dilute hydrochloric acid in the absence of the hydrazine reagent occurred in a similar manner to that in the presence of the hydrazine reagent, hydrolysis of the hydrazone took place to a considerable extent. In the case of II, decomposition by hydrolysis after 30 min was more than 28%. The proportions of the two isomers at equilibrium were similar to those in the presence of the reagent.

The fact that one isomer is separated from the hydrazones of keto acids in group **B**, together with the above results, indicate various differences in the relative stabilities of both isomers of the hydrazones of keto acids. It might be said that, in general, the *trans* isomer is more stable than the *cis* isomer for lower keto acids, and *vice versa* for higher keto acids.

Isomerization in Organic Solvents. The hydrazones are stable in pure organic solvents unless exposed to strong light. However, in organic solvents containing hydrogen ions isomerization took place. Table 3 shows the isomerization of the hydrazones in ethyl acetate containing hydrogen ions. The fractions of *cis* isomers in an equilibrium mixture showed high values except for VI. The values in ethyl acetate were larger than the corresponding values in aqueous solution without exception.

The proportions of the two isomers at equilibrium also varied depending upon the nature of the organic solvent. The isomerization of the hydrazones of II at 10°C in several organic solvents containing hydrogen ions was investigated. The fractions of *cis* isomers in isomeric mixture at equilibrium were as follows: 14% in isopropyl ether, 23% in ethyl ether, 58% in acetone, 44% in ethanol and 56% in 50% aqueous ethanol. The decomposition of the hydrazone of II was much less (less than 7.5%) in isopropyl ether, ethyl ether and ethyl acetate than in acetone, ethanol, and aqueous ethanol. In the latter, it reached 25—55%.

Preparation of the Isomers. For the preparation of *trans* isomer, hydrazones of the keto acids in group **A** were conveniently prepared by the usual method. The hydrazine reagent dissolved in dilute hydrochloric acid was added to the aqueous solution of keto acid and the mixture was left standing for 1 hr to convert the *cis* hydrazone into the *trans* hydrazone as completely as possible. The crude hydrazones thus obtained contained more *trans* isomer than in those obtained from organic solvent as is shown in Table 3. The separation of the *trans* isomer from the *cis* isomer was carried out based on the fact that the *cis* isomer is scarcely soluble in a concentrated sodium carbonate solution.

The difference in solubilities of the two isomers in

Table 3. Isomerization of the hydrazones in ethyl acetate containing hydrogen ions

Hydrazones of keto acids	Time (min)	Hydrazone decomposed (%)	*Cis* isomer in isomeric mixture (%)
I	20	—	71
	60	—	72
II	20	8.2	64
	60	8.6	63
III	20	—	82
	60	—	86
IV	20	2.4	86
	60	2.5	88
VI	20	1.2	<5
	60	1.2	31

Table 4. Solubilities of the hydrazones in 1N sodium carbonate

Hydrazones of keto acids	Solubilities (mmol/ml)	
	0°C	25°C
I*t*[a)]	434	
I*c*[a)]	0.12	
II*t*[a)]	78	
II*c*[a)]	0.03	
III*t*	3.92	6.83
III*c*	0.01	0.07
IV*t*		1.50
IV*c*		0.18
V*t*		5.11
V*c*		0.28
VI*t*[a)]	60.9	
VI*c*[a)]	0.18	
VII*x,y*		0.28
VIII*x,y*		0.53
IX*x,y*		0.27

a) Solubilities of these hydrazones were measured at 0°C instead of 25°C for economical reasons.

Table 5. Partition of the hydrazones between ethyl acetate and 0.1N sodium carbonate

Hydrazones of keto acids	Amounts of hydrazones in 0.1N Na_2CO_3	
	trans isomer (%)	*cis* isomer (%)
I	100	66
II	98	59
III	82	26
IV	69	18
V	49	8
VI	67	68
	x form (%)	*y* form (%)
VII	22	22
VIII	13	13
IX	22	22

1N sodium carbonate varied considerably depending upon the kinds of keto acids as can be seen in Table 4. The difference was the largest in the case of I and gradually decreased as the number of carbon atoms of the keto acid molecule increased, though VI showed an unexpectedly large difference. Separation of the isomers from each other was thus more difficult in the case of the hydrazones of higher keto acids as compared to those of I and II. The hydrazones of keto acids in group **B** were found to be scarcely soluble in sodium carbonate solution in a similar way to the *cis* hydrazones in group **A**.

The difference in behavior of the two isomers in the salting-out is also seen in the partition of each isomer from ethyl acetate and sodium carbonate solution. Table 5 shows the amount of the hydrazone remaining in sodium carbonate solution when the hydrazone was extracted with ethyl acetate from sodium carbonate solution. Thus we see that the *cis* isomer is more easily extracted into ethyl acetate than the *trans* isomer, with the exception of VI, where the two isomers showed nearly the same value. A considerable portion of the hydrazones in group **B** showed the values of the same order of magnitude as those of *cis* isomers in group **A**. Since each hydrazone shows a characteristic value varying greatly in magnitude, measurement of the value is useful for the characterization of the hydrazone.

For the purpose of isolation of the *cis* isomer, isomerization treatment was carried out before the separation procedure by salting-out in order to increase the fraction of the *cis* isomer in the mixture. (For the details of the separation method of both isomers (group **A**), see Experimental).

Although, in general, the existence of *cis* and *trans* isomers is feasible in 2,4-dinitrophenylhydrazones of most unsymmetrical carbonyl compounds, α-keto acids can form highly stable hydrazone derivatives of *cis* form.[3,16] Curious behavior of the *cis* isomers in salting-out, solubility in sodium carbonate solution and in partition between ethyl acetate and 0.1 N sodium carbonate is due to their characteristic structures. Thus, the separation method described above is applicable only to the isomers of α-keto acid hydrazones.

Failure of isolation of the two isomers from keto acid hydrazones in group **B** indicates the instability of one isomer under the usual conditions. So far the behavior of the hydrazones seems to be concerned with keto acids with such structure as $\begin{matrix}R\\R'\end{matrix}>CHCOCOOH$ or $\begin{matrix}R\\R'\\R''\end{matrix}>CCOCOOH$.

Isomerization during Melting Point Determination. When a bath with melting point apparatus was heated gradually in the usual manner, some hydrazones did not show sharp melting points. Some pairs of the isomers showed no appreciable difference in their melting points when heated by the usual technique. However, when a capillary containing a sample was steeped into a bath preheated up to a temperature a few degrees below melting point by the usual procedure, sudden melting was often observed in one of the isomers. In such a case, the procedure was repeated in the above mentioned manner, lowering the temperature of the bath by a few degrees each time until the hydrazone did not melt any more. The melting point thus obtained was regarded as the true melting point of the hydrazone. On the other hand, no depression of the melting point was observed on the other isomer even by this method. In Table 6 shown are the melting pontis of the isomers of the hydrazones obtained by this method. The values by the conventional technique are also given when a difference was observed between the two measurements. *Trans* isomers of I and II showed higher melting points than their corresponding *cis* isomers, whereas *cis* isomers higher melting points than their corresponding *trans* isomers. Such a remarkable difference between the melting points of the isomers measured by the two methods suggests the possibility of isomerization of one isomer to the other during heating process. In fact, occurrence of isomerization could be demonstrated by subjecting the hydrazone to paper chromatography after it was kept for some time at certain temperature between the two melting points.

It is noticeable that the relative stabilities of both isomers, when their crystals are being heated, seem to accord with those in solution.

Although the two forms of hydrazones of keto acids in group **B** showed different melting points, they were not affected by the manner in which measurement was made. No isomerization was observed during the heating process.

Table 6. Melting points of the hydrazones

Hydrazones of keto acids	Mp (Corrected) *cis* isomer	Mp (Corrected) *trans* isomer	Mp given in literature
I	182.5—183.5	205.5	203[17]
II	210—211	217—218.5	216[18]
III	202—202.5	170—171 (202—202.5)[a]	198[19]
IV	166.5—167.5	129—130 (166—168)[a]	167[19]
V	156—157	120	153[19]
VI	177—178	145.5—146.5	162[19]
	x form	*y* form	
VII	187.5—188	196—196.5	196[19]
VIII	169—170	170—171	169[20]
IX	172.5—173.5	177—178	180[15]

a) The values in parentheses show melting points measured by the usual method.

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