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The Properties and Assignment of Isomers of 2,4-Dinitrophenylhydrazones of Some Aliphatic α-Keto Acids

Hirohiko KATSUKI, Tsuya YOSHIDA,* Jun NAGAI, and Shozo TANAKA**
Department of Chemistry, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto

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Visible and infrared spectra and some other properties of *cis* and *trans* isomers of 2,4-dinitrophenylhydrazones of some aliphatic α-keto acids have been studied. It was observed that absorption maximum of the *cis* isomer in visible region occurred at a longer wavelength than that of the *trans* isomer in ethyl acetate as well as in bicarbonate solution and *vice versa* in sodium hydroxide solution. In infrared region, N–H and C=O stretching bands of the *cis* isomer were found at longer wavelengths than the corresponding bands of the *trans* isomer. It was concluded that a hydrogen bond is formed between C=O and N–H groups in the *cis* isomer. Further evidence which supports the above conclusion was given by the difference between both isomers in the nickel complex forming reaction, sodium carbonate reaction and in the properties of sodium salt. Instability of the *trans* isomer appeared to increase as the number of carbon atoms in the keto acid molecule increased. 2,4-Dinitrophenylhydrazones of some other α-keto acids were found to give polymorphs but not geometrical isomers.

2,4-Dinitrophenylhydrazine has been widely used as a reagent for the analysis of carbonyl compounds. A number of studies have been made on physical and chemical properties of the hydrazone derivatives. With α-keto acids, some of which are important metabolites in intermediary metabolism of organisms, investigations on the separation of geometrical isomers of the hydrazones were first carried out by us[1] and others[2-4] in 1955—6. We pointed out the importance of studies on both the isomerization between the *cis* and *trans* isomers of α-keto acid hydrazones and their properties for analysis, and a method for photometric determination of pyruvic acid irrespective of the existence of their isomers was reported.[5]

However, separation of the isomers of hydrazones of other α-keto acids other than those reported was not always possible because of lack of knowledge concerning isomerization between both isomers. The isomerization reaction was investigated and the first large scale separation became feasible by combining the salting-out method with isomerization treatment.[6]

In general, there should exist geometrical isomers in most condensation products of carbonyl compounds with carbonyl reagents including 2,4-dinitrophenylhydrazine. Spectroscopic studies in visible region have been reported since 1913 on the assignment of geometrical isomers of semicarbazones,[7] oximes,[8] and substituted[9,10] or non-substituted[11] phenylhydrazones of carbonyl compounds.

Studies on the infrared spectra were also made with the isomers of various hydrazone derivatives of α-keto acids.[1-4,12] The results indicated that a hydrogen bond was formed between C=O and N–H groups in the *cis* isomer but not in the *trans* isomer.

Proton nuclear magnetic resonance has also been applied successfully to the elucidation of structural isomerism involving restricted rotation. Karabatsos *et al.* studied NMR spectra of phenylhydrazones, semicarba-

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* Deceased May 4th, 1970.

** Present address; Kyoto Women's University, Higashiyama-ku, Kyoto.

[1] T. Moriwaki, H. Katsuki, and S. Tanaka, *Nippon Kagaku Zasshi*, **76**, 1367 (1955).

[2] F. A. Isherwood and R. M. Johnes, *Nature*, **175**, 419 (1955).

[3] A. Matsuyama, *J. Agr. Chem. Jap.*, **29**, 736, 977, 982 (1955).

[4] I. Hayashi, *Nature*, **178**, 40 (1956).

[5] H. Katsuki, C. Kawano, T. Yoshida, H. Kanayuki, and S. Tanaka, *Anal. Biochem.*, **2**, 433 (1961).

[6] H. Katsuki, C. Tanegashima, M. Tokushige, and S. Tanaka, This Bulletin, in press.

[7] F. J. Wilson and I. M. Heilbron, *J. Chem. Soc.*, **103**, 377 (1913).

[8] R. E. Raffauf, *J. Amer. Chem. Soc.*, **68**, 1765 (1946).

[9] H. Bredereck, *Ber.*, **65**, 1863 (1932).

[10] F. Ramirez and A. F. Kirby, *J. Amer. Chem. Soc.*, **75**, 6026 (1953).

[11] R. Kuhn and W. Munzing, *Chem. Ber.*, **85**, 29 (1952).

[12] M. Yamaguchi, "Infrared and Raman Spectra," Bunshibunkokenkyukai, Tokyo (1949), p.41.

zones, of aldehydes and ketones.[13–16)]

It was reported that 2,4-dinitrophenylhydrazones of some α-keto acids such as glyoxylic (I), pyruvic (II), α-ketobutyric (III), α-ketovaleric (IV), α-ketocaproic (V), and α-ketoisocaproic acid (VI) could be separated into two isomers by utilizing the solubility differences in 1 N sodium carbonate.[6)] These hydrazones were tentatively classified into group A. The hydrazones of α-ketoisovaleric (VII), α-keto-β-methylvaleric (VIII) and α-keto-β-dimethylbutyric acid (IX) could not be separated into two isomers by the above method and gave *x*- and *y*-forms by recrystallization from nonpolar and polar solvent, respectively. These hydrazones were classified into group B.

The present communication describes visible and infrared spectra and some properties of these isomers and forms, and deals with the assignment of the compounds. The study was successfully applied to the characterization,[17)] determination[18,19)] of α-keto acids which appear in biological materials by 2,4-dinitrophenylhydrazine method.

Experimental

Separation and Purification of Isomers and Forms of the Hydrazones. These were described previously.[6)]

Sodium Salts of the Hydrazones. The *cis* hydrazones of keto acids which belong to group A or the hydrazones of keto acids of group B were converted into their sodium salts by treating them with an excess amount of 1 N sodium carbonate. The sodium salts of the hydrazones, scarcely soluble in 1 N sodium carbonate, were filtered and washed twice with 1 N sodium carbonate and twice with a small amount of water. They were recrystallized from 50% aqueous acetone.

Some kinds of *trans* hydrazones of keto acids of group A, such as I and II, were dissolved in 1 N sodium carbonate, and the solution was saturated with sodium chloride. The precipitated sodium salts were filtered and washed twice with a small amount of 1 N sodium carbonate saturated with sodium chloride, and once with a small amount of water.

The *trans* hydrazones of III, IV, V, and VI were dissolved in a small amount of absolute ethanol. An excess amount of 1 N sodium carbonate was added to it. Sodium salts, thus precipitated, were washed with 1 N sodium carbonate and water, then recrystallized in the same way as for the *cis* isomers of the hydrazones.

Absorption Spectra in Visible Region. The hydrazones were dissolved in 0.1 N sodium bicarbonate or ethyl acetate. The spectra were taken with fresh solutions with the use of a Beckman Spectrophotometer, Model DU, and a Hitachi Recording Spectrophotometer, Model EPS. The spectra of the hydrazones in alkali were taken as follows: to the bicarbonate solution of the hydrazone, an equal volume of 2.0 N sodium hydroxide was added and the mixture was shaken thoroughly. After the mixture was left to stand for 10 min at 30°C, the spectra were taken within 10 min. The spectra showed no noticeable change during this period.

Infrared Spectra. Infrared spectra of the hydrazones were taken with a Nihonbunko Infrared Spectrophotometer, Model Koken DS 301 by the Nujol mull method, and also by the potassium chloride disc method in the case of the keto acid hydrazones which belong to group B.

Results and Discussion

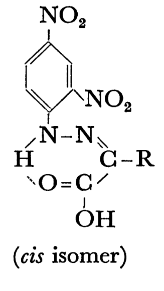
Absorption Spectra in Visible Region. In Table 1 are shown the wavelengths at which absorption maxima were observed and the molecular extinction coefficients of the hydrazones at those wavelengths in ethyl acetate, 0.1 N sodium bicarbonate and 1 N sodium hydroxide containing 0.05 N sodium bicarbonate.

TABLE 1. ABSORPTION SPECTRA OF THE HYDRAZONES IN VISIBLE REGION

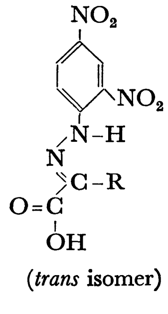
Hydrazones of keto acids		0.1 N Sodium bicarbonate		Ethyl acetate		1 N Sodium hydroxide[a)]	
		λ_{max}	ε_{max}	λ_{max}	ε_{max}	λ_{max}	ε_{max}
I	*trans*	365	2.45	350	2.48	453	2.63
	cis	373	2.57	360	2.54	380	1.75
II	*trans*	370	2.14	348	2.11	445	2.09
	cis	380	2.43	365	2.14	430	1.65
III	*trans*	373	2.32	348	2.13	440	1.97
	cis	383	2.45	368	2.33	435	1.70
IV	*trans*	373	2.33	348	2.10	440	1.93
	cis	383	2.48	363	2.34	430	1.75
V	*trans*[b)]	(373)	(2.35)	—	—	(435)	(1.81)
	cis	378	2.48	—	—	430	1.74
VI	*trans*	373	2.40	350	2.10	440	1.97
	cis	383	2.44	368	2.37	430	1.75
VII	*x*, *y*	380	2.38	355	2.55	430	1.79
VIII	*x*, *y*	380	2.32	363	2.78	430	1.76
IX	*x*, *y*	375	2.31	365	2.50	430	1.79

a) Containing 0.05 M sodium bicarbonate.
b) Contaminated with trace amounts of the *cis* isomer.

It is evident from the spectra of the free hydrazone and the sodium salt (taken in ethyl acetate and sodium bicarbonate solution, respectively) that absorption maximum of the *cis* isomer is at a longer wavelength than that of *trans* isomer, and that the molecular extinction coefficient of the *cis* isomer is greater than that of the *trans* isomer. This holds in all isomeric pairs of the hydrazons tested. These results would be expected from the stable six-membered ring structure of the *cis* isomer as follows:[1–4)]



(*cis* isomer)



(*trans* isomer)

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13) G. J. Karabatsos, J. D. Graham, and F. M. Vane, *J. Amer. Chem. Soc.*, **84**, 753 (1962).

14) G. J. Karabatsos, B. L. Shapiro, F. M. Vane, J. S. Fleming, and J. S. Ratka, *ibid.*, **85**, 2784 (1963).

15) G. J. Karabatsos, R. A. Taller, and F. M. Vane, *ibid.*, **85**, 2326 (1963).

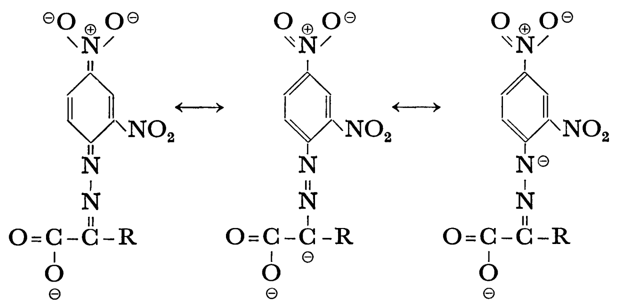
16) G. J. Karabatsos, F. M. Vane, R. A. Taller, and N. Hsi, *ibid.*, **86**, 3351 (1964).

17) H. Katsuki, T. Yoshida, C. Tanegashima, and S. Tanaka, *Anal. Biochem.*, **24**, 112 (1968).

18) C. Kawano, H. Katsuki, T. Yoshida, and S. Tanaka, *ibid.*, **3**, 361 (1962).

19) H. Katsuki, T. Yoshida, C. Tanegashima, and S. Tanaka, *ibid.*, (1971) in press.

It seems reasonable to assume that in sodium bicarbonate solution, only carboxyl group of the hydrazone of keto acid is dissociated, whereas both carboxyl and N–H groups are ionized in strong alkaline solution, giving red coloration. The fact that the addition of alkali to *N*-methyl-phenylhydrazones does not produce a red color[20] seems to support this assumption. The red coloration of alkaline solution is due to the formation of an unstable intermediate or intermediates. Some typical one are assumed to be as follows:



The *trans* isomer would be expected to give a deeper red than the *cis* isomer in alkaline solution since the hydrogen atom of N–H group of the latter is involved in a hydrogen bond formation and not easily ionizable. As a support of this hypothesis the results of spectral analyses of sodium hydroxide solution might be presented. The absorption maximum of the *trans* isomer was found to be at a longer wavelength than that of the *cis* isomer without exception and the molecular extinction coefficient of the *trans* isomer was greater than that of the *cis* isomer.

From the structure described above, a *cis-trans* interconversion would be expected to occur in alkaline solution. The observation that the red coloration produced in the *cis* isomer deepened as the concentration of sodium hydroxide was increased is in agreement with such a prediction.[21] Thus it is suggested that gradual breakage of the hydrogen bond in the *cis* isomer leads to conversion of the *cis* isomer into the *trans* isomer through the intermediate(s) described above.

In order to prove the interconversion of the two isomers in alkaline solution, as suggested by the development of red coloration under such conditions, the isomers were isolated as free hydrazone by the addition of hydrochloric acid to the alkaline solution. Further separation of the isomers was achieved by paper chromatography. Two spots corresponding to the *cis* and *trans* isomers were detected besides one spot presumably representing a decomposition product.

Two forms of the hydrazones of keto acids which belong to group B behaved differently in a solid state, but their solubilities, R_f values and partition coefficients were identical.[6] These two forms showed the same absorption spectra. Thus, they apparently behaved as the same substance in solution. From the spectral data in three different solvents, the form in solution seems to have the *cis* structure.[22]

Reaction with Sodium Carbonate. Although the spectra of the hydrazones in visible region in sodium carbonate solution and of those in sodium bicarbonate solution were nearly the same, the molecular extinction coefficient in the longer wave length region had slightly higher values in sodium carbonate than in sodium bicarbonate. It is difficult to differentiate the *cis* isomers from the corresponding *trans* isomers by visual inspection of the colored solution without comparing the spectra of isomeric pairs. However, a remarkable difference between the *cis* and *trans* isomers was found when the hydrazones reacted with sodium carbonate in ethanol. On the basis of this fact, a test for assigning the *cis* and *trans* isomers of α-keto acid hydrazones was devised as follows: A few milligrams of the hydrazone is dissolved in several drops of ethanol and to this solution is added one drop of 1 N sodium carbonate. The *trans* isomer produces red coloration, and the *cis* isomer none.

As shown in Table 2, the *trans* isomers gave a positive reaction and the *cis* isomers a negative reaction with one exception. Although all the hydrazones of the keto acids of group B were expected to give a negative reaction, those of IX gave a positive reaction.

Nickel Complex Formation. The nickel complex forming test[23] was applied to the series of isomers of

TABLE 2. SOME REACTIONS AND PROPERTIES OF THE HYDRAZONES

Hydrazones of keto acids		Sodium carbonate reaction	Nickel complex formation	Color of dehydrated sodium salt
I	*trans*	+	−	black brown (decomp.)
	cis	(−)[a]	+	orange
II	*trans*	+	−	dirty brown
	cis	−	+	red
III	*trans*	+	−	yellow
	cis	−	+	red
IV	*trans*	+	−	yellow
	cis	−	+	yellow
V	*trans*	+	−	yellow
	cis	−	+	red
VI	*trans*	+	−	yellow
	cis	−	+	orange red
VII	*x*, *y*	(−)[a]	+	orange red
VIII	*x*, *y*	(−)[a]	+	red brown
IX	*x*, *y*	+	+	dirty brown (decomp.)

a) Faint orange color

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[20] F. Bohlmann, *Chem. Ber.*, **84**, 490 (1951).

[21] H. B. Stewart, *Biochem. J.*, **55**, xxvi (1953).

[22] A shift of the absorption maximum of VII with increasing water content in ethanol solution had been attributed to isomerization. *Cf.* H. Katsuki, K. Sumizu. T. Moriwaki, and S. Tanaka, This Bulletin, **31**, 665 (1958). However, it appears that shift is due to a non-specific solvent effect, since such a phenomenon was also observed with the *cis*- as well as the *trans* hydrazone of II. The absorption maximum of the *cis* hydrazone of II shifted from 363 mμ to 378 mμ and that of the *trans* isomer from 348 mμ to 365 mμ under these conditions.

[23] H. Katsuki, K. Sumizu, T. Moriwaki, and S. Tanaka, *Nature*, **181**, 639 (1958).

hydrazones mentioned above. As shown in Table 2, the *cis* hydrazones of keto acids of group A and both forms of the hydrazones of keto acids of group B showed a positive reaction without exception, whereas the *trans* hydrazones of keto acids which belong to group A showed a negative reaction. The results of the nickel complex forming test were in line with the proposed structures of the isomers of keto acid hydrazones.

Properties of Sodium Salt. The characteristic properties of sodium salt of the *cis* hydrazone of II were reported.[1] The hydrated form of its sodium salt had a yellow color, but its color changed to red when dehydrated by standing *in vacuo* or by heating. The dehydrated form was extremely hygroscopic. It turned yellow as soon as it was exposed to air. The dehydrated sodium salt of the *trans* isomer had a dirty brown color and was not hygroscopic. These characteristic properties were also observed with sodium salt of the hydrazone of VII, which had a few molecules of water of crystallization and the dehydrated form showed an orange-red color. According to Hayashi,[24] sodium salt of *cis* isomer of phenylglyoxylic acid 2,4-dinitrophenylhydrazone also showed a similar behavior. In that case the hydrated form was orange, but the dehydrated form had a deep red color.

The characteristic color formation would be expected upon dehydration for the sodium salts of all of the *cis* isomers mentioned above. Inspection of Table 2 reveals that most keto acid hydrazones fulfilled this requirement. Exceptions were sodium salt of the *cis* hydrazone of IV, which was yellow, and that of IX, which decomposed when dehydrated.

Infrared Spectra. As N–H stretching band, each *trans* hydrazone of I, V, and VI showed a single absorption band and that of II, III, and IV showed two bands in the region 3500—3200 cm^{-1}, whereas each of all the *cis* hydrazones of these keto acids showed a single N–H stretching band which occurred at a longer wavelength than the corresponding band of the *trans* isomer. These results are shown in Figs. 1, 2, and 3.

We see that infrared spectra of most hydrazones showed a single stretching band for C=O group 1664—1750 cm^{-1}[1] except for the case of the *trans* hydrazones of III and IV, for which two and three bands were observed, respectively. The band for the *cis* isomer was seen at a longer wavelength than the corresponding band of the *trans* isomer. The difference in the positions of the stretching bands for C=O group between *cis* and *trans* isomers of the hydrazones of group A was the smallest for VI.

In the case of the hydrazones of keto acids which belong to group B, both *x* and *y* forms seemed to be polymorphic to each other because they showed identical spectra in Nujol mull (Fig. 4) as well as in potassium chloride disc except for those of VII. Both of the two forms of VIII hydrazones showed *cis* type N–H and C=O stretching bands. Absorption spectra in visible region and other properties of the two forms of VIII

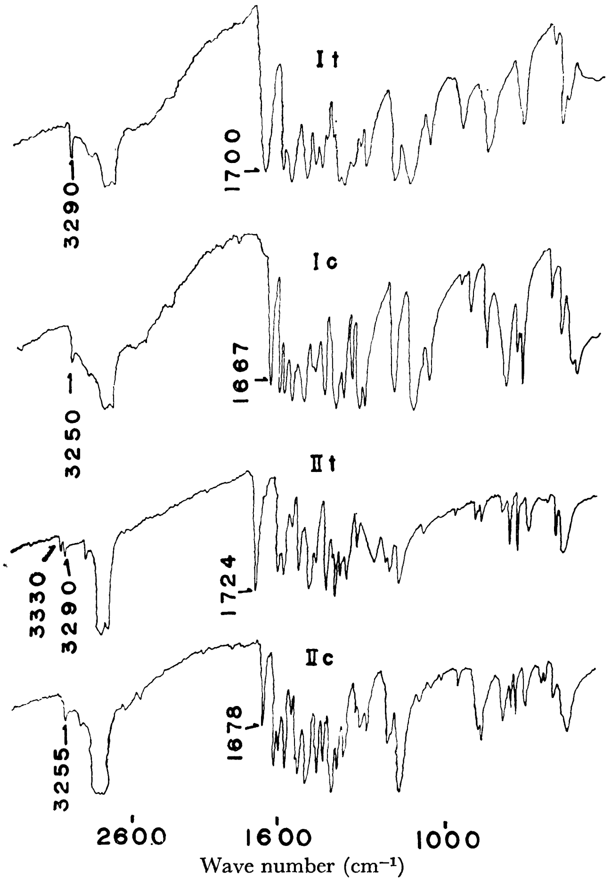

Fig. 1. Infrared spectra of the α-keto acid hydrazones (group A) (1).
by Nujol mull method.

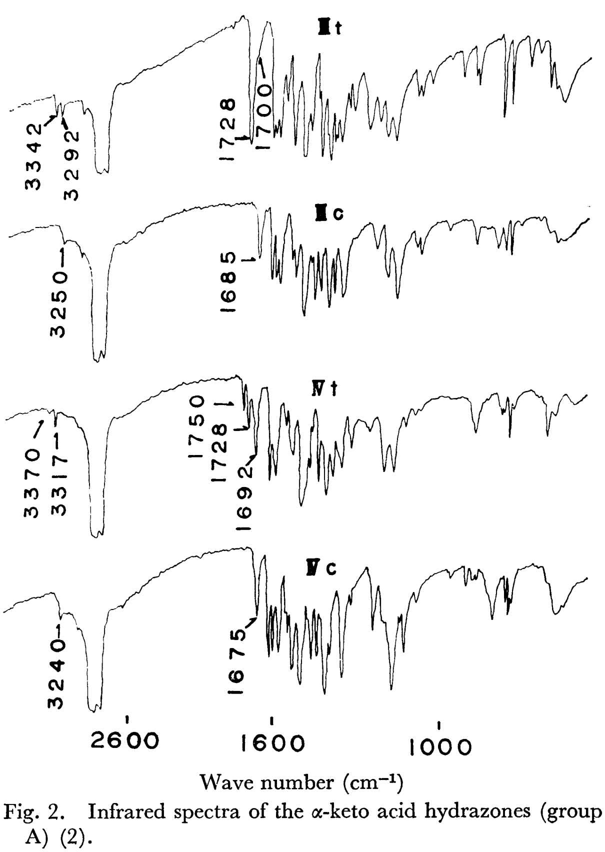

Fig. 2. Infrared spectra of the α-keto acid hydrazones (group A) (2).
by Nujol mull method.

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[24] I. Hayashi, private communication.

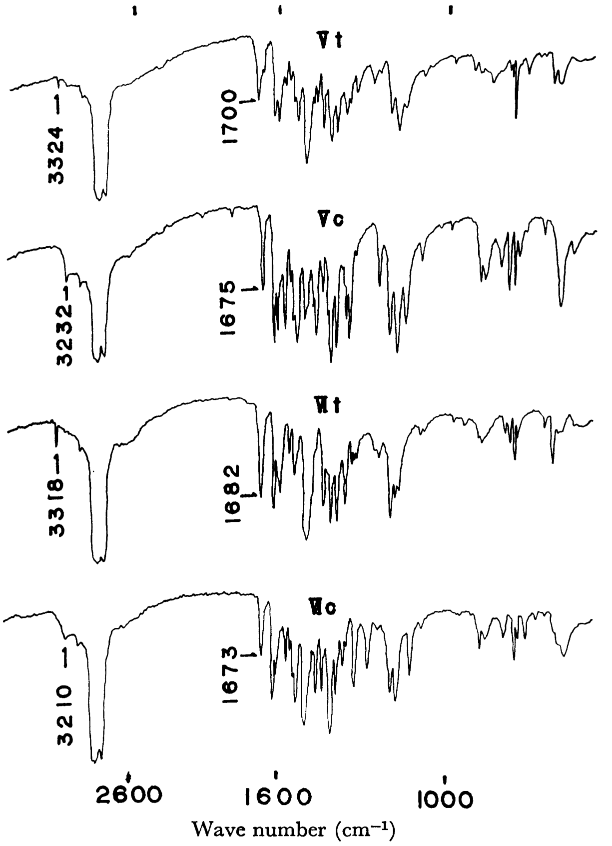

Fig. 3. Infrared spectra of the α-keto acid hydrazones (group A) (3).
by Nujol mull method.

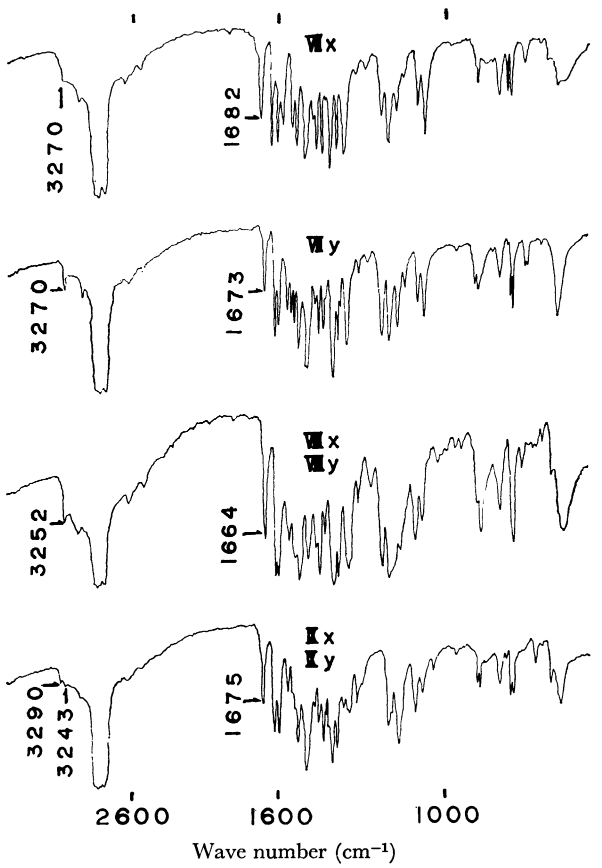

Fig. 4. Infrared spectra of the α-keto acid hydrazones (group B).
by Nujol mull method.

hydrazones supported their *cis* structure. Thus, it was concluded that the two hydrazones of VIII have *cis* structure in solid state as well as in solution.

In the case of IX, it was not possible to assign the hydrazone in solid state, since it showed an abnormal N–H band. Judging from the position of absorption maximum in visible region, the hydrazones of IX in solution seem to have *cis* structure but the result of the sodium carbonate reaction which is normally specific for the *trans* structure does not seem to be in line with it. No explanation could be given for this inconsistency.

The two forms of VII hydrazones, which showed quite different infrared spectra from each other, seem to be *trans* and *cis* isomers, respectively, but not polymorphs, because they showed similar stretching bands for C=O group to those of *cis* and *trans* hydrazones of VI, respectively. However, in solution, they behaved as the same substance to each other and they had the characteristics of *cis* type in all respects. If we assume that the *x* form (*trans* isomer) is stable only in solid state and isomerizes to the *y* form (*cis* isomer) as soon as it is dissolved in solution as reported by Abramovitch and Spenser for pyruvamide phenylhydrazone,[25] the behavior of VII hydrazones might be explained. Judging from its behavior in solid state, VII could be considered a member of group A, though it was classified as belonging to group B according to its behavior in solution.

It appears that the structures of the *cis* and *trans* isomers are stable with the hydrazones of lower keto acids. With the hydrazones of higher keto acids, however, the structure of the *trans* isomer seems to be less stable.

This seems to be supported also by the results of the isomerization of the hydrazones in solution as well as in melting state.[6]

The authors wish to thank Prof. Toru Takenaka, Institute for Chemical Research, Kyoto University, for his useful suggestion and Dr. Chizuko Tanegashima, Mukogawa Women's University, for her technical assistance.

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[25] R. A. Abramovitch and I. D. Spenser, *J. Chem. Soc.*, **1957**, 3767.