(4.1 mmol.) of silver nitrate in 25 ml. of ethanol and 10 ml. of water was allowed to stand at room temperature in the dark for 27 hr. Silver chloride (322 mg., 59%) was removed by filtration, an additional 0.7 g. of silver nitrate and 20 ml. of water was added to the filtrate, and the reaction was allowed to continue for 5 more days to give an additional 62 mg. of silver chloride, which brought the total to 384 mg. (71%). After removal of the silver chloride, the ethanol was evaporated from the filtrate under reduced pressure and the aqueous residue extracted with ether. The ether was removed and the residue treated with carbon tetrachloride to give a yellow solution and an insoluble white solid. This solid was recrystallized three times from water to give 20 mg. (6%) of the hydroxylactone XVI, m.p. 152–158°; the melting point was not depressed on admixture with XVI obtained by the hydrolysis of the methoxylactone III.

Synthesis of 3-Phenyl-4-hydroxy-4-methoxy-2-butenoic Acid Lactone (III).—A solution of 0.6 g. (0.0034 mole) of the hydroxylactone XVI and 1.5 ml. of concentrated sulfuric acid in 35 ml. of absolute methanol was allowed to stand at room temperature for 24 hr. and then concentrated to 20 ml. on a steam-bath over a period of 2 hr. The solution was cooled to 0°, diluted with ether, and washed with water and 5% aqueous sodium bicarbonate. The ethereal solution was dried over anhydrous magnesium sulfate, filtered, and the ether evaporated. The residue was distilled to give 0.4 g. (62%) of the methoxylactone III, b.p. 131° (1 mm.), identified by its infrared and n.m.r. spectra.

[CONTRIBUTION FROM THE ENZYME SECTION OF THE NATIONAL HEART INSTITUTE, BETHESDA, MARYLAND]

Methylmalonyl Coenzyme A Isomerase: Investigation of a Carbon Monoxide Transfer Mechanism

BY MARTIN FLAVIN[1] AND CLARENCE SLAUGHTER

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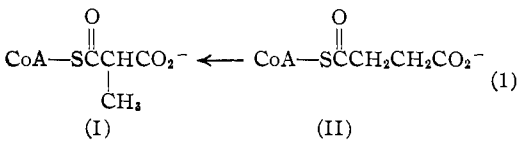
Investigation of the mechanism of the isomerization of methylmalonyl coenzyme A to succinyl coenzyme A, catalyzed by an enzyme from kidney cortex, has been hampered by the absence of any analogous model non-enzymatic "transcarboxylation" reaction. This deficiency has recently been supplied by the discovery that the cinenic acid rearrangement involves a carboxyl rather than an alkyl migration. Evidence that the non-enzymatic reaction takes place by a carbon monoxide and carbonium ion mechanism prompted an investigation of the possible intermediary formation of carbon monoxide in the reversible isomerization of methylmalonyl coenzyme A. Preliminary results do not support this mechanism for the enzymatic reaction.

Introduction

The reversible rearrangement of methylmalonyl coenzyme A (I) to succinyl coenzyme A (II)

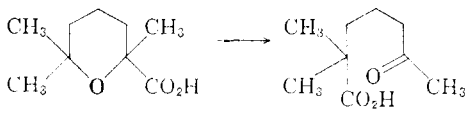


(1)

(I) (II)

is catalyzed by an enzyme discovered 5 years ago in extracts of animal tissues, where it plays a role in the metabolism of propionic acid.[2] Interest in this reaction has recently been stimulated by the discovery that a coenzyme form of vitamin B_{12}[3] is an essential cofactor.[4] The rearrangement, which is effectively a carboxyl transfer, has been without apparent analogy in organic or biological chemistry, and the mechanism has remained obscure.

An interesting analogy has now come to light through the demonstration that the rearrangement, in concentrated sulfuric acid, of α-cinenic acid (III) to geronic acid (IV)



(2)

III IV

(1) Established Investigator of the American Heart Association.

(2) (a) M. Flavin, *Federation Proc.*, **14**, 211 (1955); (b) M. Flavin P. J. Ortiz and S. Ochoa, *Nature*, **176**, 823 (1955); (c) M. Flavin and S. Ochoa, *J. Biol. Chem.*, **229**, 965 (1957); (d) W. S. Beck, M. Flavin and S. Ochoa, *J. Biol. Chem.*, **229**, 997 (1957).

(3) H. Weissbach, J. Toohey and H. A. Barker, *Proc. Natl. Acad. Sci., U. S.*, **45**, 521 (1959).

(4) (a) R. M. Smith and K. J. Monty, *Biochem. Biophys. Res. Comm.*, **1**, 105 (1959); (b) E. R. Stadtman, P. Overath, H. Eggerer and F. Lynen, *ibid.*, **2**, 1 (1960); (c) J. R. Stern and D. L. Friedman, *ibid.*, **2**, 82 (1960); (d) S. Gurnani, S. P. Mistry and B. C. Johnson, *Biochim. Biophys. Acta*, **38**, 187 (1960).

involves a carboxyl-group transfer, rather than the previously assumed long range methyl migration.[5a,b] Evidence that the mechanism is a decarbonylation, followed by isomerization of the resultant carbonium ion, and recapture of carbon monoxide,[5c] has prompted the reinvestigation of the mechanism of the methylmalonyl coenzyme A (CoA) isomerase reaction reported here.

While the analogy suffers from lack of obvious potentiality for resonance stabilization of intermediate carbonium ions in the enzymatic reaction (equation 1),[6] this shortcoming was weighed against the unknown factors represented by the intervention of enzyme and coenzyme and the potentiality of the coenzyme to serve as a carbon monoxide carrier through its cobalt moiety.[7]

Experimental

Methylmalonyl CoA was prepared by a procedure yielding the monothioester unequivocally and in high yield.[8] Labeled succinyl CoA could be prepared in good yield from succinic-1,4-C^{14} acid (28 μcuries/mg.) on a 4-mg. scale, by the method previously described for methylmalonyl CoA,[2d] with the aid of microglassware. Tetrahydrofuran, which inhibits the isomerase reaction, was removed from the thioester solution by ether extraction at *p*H 6. The $C^{14}O$ (500 μcuries in 250 μmoles) was obtained in a break-seal bulb and freed of traces of $C^{14}O_2$ by alkali extraction before use.

(5) (a) J. Meinwald, THIS JOURNAL, **77**, 1617 (1955); (b) J. Meinwald and J. T. Ouderkirk, *ibid.*, **82**, 480 (1960); (c) J. Meinwald, H. C. Hwang, D. Christman and A. P. Wolf, *ibid.*, **82**, 483 (1960).

(6) Since, unlike the case of cinenic acid, hydrogen atoms are bonded to the relevant carbons in the enzymatic rearrangement, the latter could be alternately formulated as being mediated by a hydride-shift or an unsaturated intermediate (acrylyl CoA); M. Flavin and C. Slaughter, *J. Biol. Chem.*, **235**, 1112 (1960).

(7) Spectral evidence for a carbon monoxide derivative of vitamin B_{12} has been reported by J. G. Heathcote; quoted in R. T. Williams (editor), "Biochemistry of Vitamin B_{12}," Biochemical Society Symposium, No. 13, Cambridge University Press, 1955, p. 15.

(8) E. G. Trams and R. O. Brady, THIS JOURNAL, **82**, 2972 (1960).

The isomerase used in all experiments was the step 2 fraction from lamb kidney cortex acetone powder.[2d] Activity was measured as previously described,[2d] after alkaline hydrolysis of thioesters. Table I illustrates the effect on the reaction rate, with this dialyzed enzyme fraction,

TABLE I

B_{12} COENZYME REQUIREMENT FOR KIDNEY METHYLMALONYL CoA ISOMERASE

Coenzyme added	Concentration, M	Succinate formed, μmole/hour × mg. protein
None		0.11
DBC	5×10^{-6}	.49
DBC	5×10^{-7}	.51
DBC	5×10^{-8}	.38
DBC	5×10^{-9}	.09
BC	5×10^{-6}	.23
BC	5×10^{-7}	.45
BC	5×10^{-8}	.32
BC	5×10^{-9}	.13
Vitamin B_{12}	5×10^{-7}	.13
Vitamin B_{12}	5×10^{-8}	.13

of added 5,6-dimethylbenzimidazole-B_{12} coenzyme (DBC), benzimidazole-B_{12} coenzyme (BC)[3] and vitamin B_{12}. Isomerase reaction mixtures contained, besides coenzyme as indicated and 0.2 mg. of enzyme in a final volume of 0.9 ml.: 50 μmoles of potassium phosphate, pH 7.3, and 0.3 μmole of methylmalonyl CoA. Incubated 30′ at 30° in the dark.

Results

Two experiments were performed to test for intermediary formation of CO, possibly in a bound form, as to the cobalt of the coenzyme. Both were contingent on the assumption of a measurable degree of isotopic equilibration between the latter and the rather small amount of free CO which can be dissolved in water. The occurrence of measurable exchange between free CO and the postulated bound intermediary CO seems likely to the authors, in a reversible reaction. In the first experiment, carboxyl-labeled succinyl CoA was incubated with enzyme and unlabeled CO, and C^{14} in CO was determined after its oxidation by Pd^{++} to CO_2. In the second, unlabeled methylmalonyl CoA was incubated with enzyme under a gas phase of pure $C^{14}O$, and C^{14} was determined in the newly formed succinate.

The incubation with labeled succinyl CoA was carried out in a Dixon–Keilin flask with mercury-filled manometer, permitting periodic replacement of the alkali in the center-well without exposure of gas phase to the atmosphere.[9] To a final volume of 2.75 ml. in the main compartment were added 10 units of isomerase, 20 μmoles of succinyl-1,4-C^{14} CoA (2×10^7 c.p.m.), 70 μmoles of $KHCO_3$, 0.3 of DBC and 150 of potassium phosphate, pH 7.3. Gas phase contained 130 μmoles of CO and 900 of N_2. A concentration of coenzyme greater than that optimal for the net reaction (Table I) was chosen to facilitate exchange between free CO and the postulated coenzyme-bound $C^{14}O$. With the center-well closed to the main compartment, the vessel was incubated 3 hr. at 30°. Succinyl CoA deacylase assays in a control vessel indicated 50% thioester remaining after 30 minutes, 10% after 120 minutes. From a sidearm, 0.25 ml. of 5 N H_2SO_4 was tipped in, and CO_2 was collected in 3 successive portions of 0.2 ml. of 3 N NaOH (Table II). Carrier $KHCO_3$ was added to fractions 2 and 3 before precipitation as $BaCO_3$ for counting. Slow evolution of minute amounts of $C^{14}O_2$ continues due to decomposition of some reaction product which is catalyzed by acid alone (Table II). After 240 minutes, 0.3 ml. of 0.3 N HCl, containing 90 μmoles of $PdCl_2$ and 15 of $ZnSO_4$,[9] was added through the vent to a second sidearm. During the final 2 hr. CO_2 produced by oxidation of about half the CO present was collected in a fourth portion of alkali. No significant amount of C^{14} was found in the CO (Table II).

TABLE II

RADIOACTIVITY IN CO_2 AND CO AFTER METHYLMALONYL CoA ISOMERASE INCUBATION WITH SUCCINYL-1,4-C^{14} CoA

Fraction collected	Collection time, min.	Amount of $BaCO_3$, μmoles	Total C^{14} present, c.p.m.
CO_2 #1	60	65	1900
CO_2 #2	30	0	150
CO_2 #3	150	0	240
CO	120	65	150

The second experiment was carried out with the aid of a Toepler pump attached to a large evacuated bulb through which $C^{14}O$ could be quantitatively cycled between a storage bulb and a reaction vessel (11 ml. volume). The latter contained, in a volume of 3 ml., 8 units of isomerase, 10 μmoles of methylmalonyl CoA, 0.015 of DBC and 300 of potassium phosphate, pH 7.3. The reaction vessel was connected to the system, placed in liquid N_2 and evacuated. About 120 μmoles (240 μcuries) of $C^{14}O$ were admitted, and the vessel was rocked vigorously at 25° for 210 minutes. Succinic acid was isolated chromatographically,[2c] and an aliquot of 1.2 μmoles was found to contain no radioactivity. No residual, unreacted methyl malonate could be detected.

The interconversion of I and II was originally defined, on the basis of evidence that neither CO_2 nor propionyl CoA were intermediates,[2b,10] as an intramolecular isomerization which did not involve decarboxylation followed by recarboxylation.[2a,b] The present negative results provide somewhat comparable, though not entirely conclusive, evidence against a decarbonylation–recarbonylation mechanism.

A free-radical mechanism recently has been postulated by Eggerer, *et al.*[11]; their evidence that the one-carbon fragment in the rearrangement is derived from the thioesterified rather than the free carboxyl would also be difficult though not impossible to reconcile with transcarbonylation.

Acknowledgments.—The authors are indebted to Dr. Lin Tsai for calling their attention to the

(9) F. J. Simpson, G. Talbot and D. W. S. Westlake, *Biochem. Biophys. Res. Comm.*, **2**, 15 (1960).

(10) Similar evidence has been presented against the intermediary formation of formate or formaldehyde; E. F. Phares, E. A. Delwiche and S. F. Carson, *J. Bact.*, **71**, 604 (1956).

(11) H. Eggerer, P. Overath, F. Lynen and E. R. Stadtman, THIS JOURNAL, **82**, 2643 (1960).

cinenic acid rearrangement; to Dr. Herbert Weissbach for samples of DBC and BC; to Dr. Arnold Pratt for advice concerning gas manipulation; and to Dr. Roscoe Brady for making available before publication the method for preparation of methylmalonyl CoA.

[CONTRIBUTION FROM AVERY LABORATORY, UNIVERSITY OF NEBRASKA, LINCOLN 8, NEBR.]

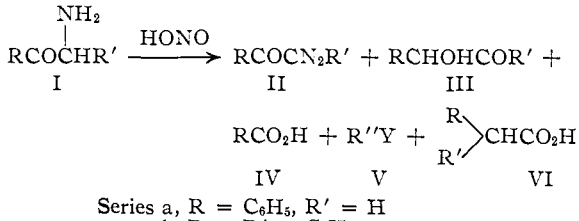
Reactions of Amines. VII. The Reactions of α-Amino Ketones with Nitrous Acid[1,2]

BY HENRY E. BAUMGARTEN AND CHARLES H. ANDERSON[3]

RECEIVED JUNE 21, 1960

The reactions of several α-amino ketones, $RCOCH(NH_2)R'$, with nitrous acid yielded, in addition to α-hydroxy ketones and α-diazo ketones, acidic products, RCO_2H and $RR'CHCO_2H$. When R′ = H only *cleavage* product RCO_2H was formed but when R′ ≠ H both the cleavage acid and the *rearrangement* product $RR'CHCO_2H$ were formed. The remaining cleavage fragments appear to be the alcohols or olefins to be expected from the acid-catalyzed decomposition of the diazo hydrocarbon intermediate, $R'CHN_2$. Mechanisms to explain these observations are given.

Based on the work of previous investigators and on theoretical considerations one would predict that α-amino ketones should react with nitrous acid to give any or all of four feasible products (or sets of products), as illustrated in the sequence I → VI.



Series a, R = C_6H_5, R′ = H
b, R = R′ = C_6H_5
c, R = C_6H_5, R′ = $CH_3CH_2CH_2$
d, R + R′ = $-CH_2CH_2CH_2CH_2-$

The reaction of I with nitrous acid to give a diazoketone (II) was first described by Schiff and Maissen[4] and later was used with modest success by Angeli.[5] The formation of benzoin from desylamine is an example of the formation of a product of type III.[6] Apparently the only report of the formation of an acid of type IV is that of Angeli,[5c] who reported the formation of benzoic acid (IVa) when phenacylamine (Ia) was treated with nitrous acid. Angeli suggested two possible sources of the benzoic acid, oxidation of the diazoketone IIa to the acid plus an unspecified fragment V and hydrolytic cleavage of the diazoketone to the acid plus diazomethane (V, R″ = CH_2, Y = N_2). Very recently Edwards and Lesage[7] reported the first example of the formation of a product of type VI, cyclopentanecarboxylic acid (VId), from the reaction of α-aminocyclohexanone with nitrous acid. The latter communication prompts us to report the results of an examination of the reaction of a number of α-amino ketones with nitrous acid, which has been and is being carried out in this Laboratory.

Our completed studies have been restricted largely to investigations of the *cleavage reaction* (I → IV + V) and of the *rearrangement reaction* (I → VI); therefore, we have little to contribute at this time relative to the formation of products like II and III other than that their presence was noted in almost every experiment (by infrared techniques) and that yields of II seem to be better when R′ ≠ H. Reactions leading to these products will be ignored in the following discussion and comparisons will be made between only the cleavage and rearrangement reactions.

α-Amino ketones (I) for which R = aryl and R′ = H appear to give only cleavage products (IV + V). Rigorous examination of this generalization has been made only for phenacylamine (Ia), but in the examination of a number of substituted phenacylamines no evidence has been obtained for the presence of other acidic products (Table I). In the case of phenacylamine, the expected acidic products would be benzoic (IVa) and phenylacetic acids (VIa). In model experiments mixtures of these acids could be converted to the methyl esters and the latter separated by gas chromatography in such a manner as to permit the facile determination of less than 1% of phenylacetic acid in the presence of benzoic acid. When the crude acid obtained from the reaction of phenacylamine with nitrous acid was converted into the methyl ester and the ester examined by gas chromatography, no peak corresponding to methyl phenylacetate was present. This result indicates that either the rearrangement reaction does not occur with phenacylamine under the conditions used or that it occurs to the extent of well below 1% of the total acid product. Examination of a number of crude acid fractions with infrared techniques tended to confirm this observation.

A relatively large number of experiments were carried out with phenacylamine to determine the optimum conditions for the cleavage reaction. These appeared to involve the slow addition of acid (sulfuric, hydrochloric or acetic acid) to an aqueous solution of phenacylamine hydrochloride and three equivalents of sodium nitrite at room temperature.[8] Under these optimum conditions the yield of benzoic acid was as high as 68%. A number of substituted phenacylamines were examined under the same conditions and gave yields in the range 70–78% (Table I).

(1) Paper VI, THIS JOURNAL, **82**, 4422 (1960).

(2) The work was supported in part by grant G-3689 of the National Science Foundation.

(3) Abstracted from the Ph.D. thesis of C. H. A., June, 1959.

(4) R. Schiff and P. Maissen, *Gazz. chim. ital.*, **11**, 471 (1881).

(5) (a) A. Angeli, *Ber.*, **26**, 1715 (1893); (b) A. Angeli, *Gazz. chim. ital.*, **23II**, 345 (1893); (c) A. Angeli and G. Malagnini, *ibid.*, **24II**, 318 (1894); (d) A. Angeli, *ibid.*, **25II**, 394 (1895).

(6) P. W. Neber and G. Huh, *Ann.*, **515**, 283 (1935).

(7) O. E. Edwards and M. Lesage, *J. Org. Chem.*, **24**, 2071 (1959).

(8) *Cf.* D. W. Adamson and J. Kenner, *J. Chem. Soc.*, 838 (1934).