AN INTRAMOLECULAR REARRANGEMENT IN THE METHYLMALONYL ISOMERASE REACTION AS DEMONSTRATED BY POSITIVE AND NEGATIVE ION MASS ANALYSIS OF SUCCINIC ACID

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Marston and coworkers (1962) have proposed that the cobamide coenzyme-dependent isomerization of methylmalonyl CoA to succinyl CoA occurs by an intermolecular reaction involving a cyclic dimer, 2,5-dioxo-cyclohexane-1,4-dicarboxylic acid (succino-succinic acid). Bilateral cleavage of such a ring on alternate sides of the carbonyl group would lead to the formation of two molecules of either methylmalonic or succinic acid. These authors suggested that such a mechanism would also explain the glutamic-methyl aspartic isomerization, which likewise requires a cobamide coenzyme (Barker et al., 1958); however they were unable to label a pool of the proposed intermediate. The same scheme for methylmalonyl isomerase was suggested independently by Hegre et al. (1962). The dimer theory offers an elegant explanation for the two similar isomerization reactions. We have tested the possibility of this mechanism by the use of an equal mixture of C^{13}-carboxyl- and C^{13}-thioester-labeled methylmalonyl CoA as a substrate for the isomerase. An intermolecular transcarboxylation, as with the dimer mechanism, would label a predictable fraction of the product molecules with two C^{13} atoms, which could be detected by mass spectrometry. The mass spectrum of the enzymic succinate has been compared with calculated intermolecular and intramolecular spectra, as well as with that from a model chemical synthetic intermolecular reaction. The results reported here show that the transcarboxylation occurs by intramolecular rather than intermolecular rearrangement.

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Enzymic Isomerization: Methylmalonic acid-1-C^{13} was prepared by carbonation of t-butyl propionate with $C^{13}O_2$ containing 52 atom % excess C^{13} (Baumgarten and Hauser, 1944); conversion to the CoA ester (Trams and Brady, 1960) resulted in a mixture containing 26% each methylmalonyl-1-C^{13} CoA and methylmalonyl-3-C^{13} CoA, and 48% with no excess C^{13}. This mixture, 56 μmoles total, was incubated for 10 minutes at 30°C with 2 mg of a 10-fold purified methylmalonyl isomerase preparation from Propionibacterium shermanii (Stjernholm and Wood, 1961), in the presence of 10^{-4} M adenylcobamide coenzyme (from Micrococcus lactilyticus) and 0.1 M phosphate, pH 6.8. The final volume was 5 ml. Residual CoA esters were hydrolyzed with 0.01 M alkali, and the succinate product, 18 μmoles, was isolated from a Celite partition column by elution with chloroform-butanol.

Synthetic Succinic Acid-1,4-C^{13}: In order to test the mass analysis methods, succinic acid 1,4-C^{13} (C_2^{13}, i.e. a definite fraction of the molecules contained two C^{13} atoms) was synthesized by a chemical method which produced a labeling pattern similar to that of the proposed enzymic intermolecular reaction. Ethylene bromide was converted to succinonitrile using potassium cyanide containing about 20% C^{13}. After acid hydrolysis, the resulting labeled succinic acid was purified by partition chromatography and recrystallization from ether (M.P. 185°-186°C, uncorrected).

Mass Spectrometer Analysis of Succinic Acid: Analyses were made on a 6-inch radius spectrometer equipped to use solid samples for either positive or negative ion detection. Crystals of normal (C_0^{13}) succinic acid or succinic anhydride, 0.5-1 mg, were fused on a crimped tantalum filament, and spectra were obtained with no vaporizing heat other than the ionizing electron beam of 75 volts for positive ions and 50 volts for negative ions. Memory effects observed with liquid monocarboxylic acids are avoided by the direct introduction of solid crystalline samples. Predominant positive ion peaks were obtained for succinic acid at masses 74 and 100, with a small parent ion peak at mass 118. These peaks were accompanied by companions at the next higher mass number; these pairs appeared at

a constant ratio under constant operating conditions (McLafferty, 1962). Succinic anhydride gave a more pronounced peak at mass 100 and a negligible mass 118 peak. Negative ion analysis of succinic acid yielded an acid anion (mass 117) spectrum virtually identical with theoretical values for succinic acid (Table 1a). Ropp and Melton (1955) have shown that this clean parent anion is typical of negative ion mass analysis of the lower monocar-

Table 1. Relative Abundance of Ion Products from Succinic Acid

	Relative Abundance						
	Mass (Positive Ions)				Mass (Negative Ions)		
	100	101	102	103	117	118	119
a. Normal succinic acid							
Theory*	-	-	-	-	100	4.57	0.87
Found	100	39	2.5	0.4	100	4.52	0.98
b. Succinic acid-1,4-C^{13} (Chemically synthesized)							
Theory, 20% C^{13} KCN**	100	39	2.5	0.4	100	4.5	1.0
		50	19.5	1.25		50	2.27
			6.25	2.44			6.25
(Sum)**	100	89	28.3	4.1	100	54.5	9.5
Theory, 21% C^{13} KCN (Sum)**	100	92	30.2	4.5	100	57	10.7
Found	100	93.5	30.5	4.0	100	55.8	9.5
c. Succinic acid-C^{13} (Enzymic)							
Theory (Sums)**							
Intermolecular, 25% C^{13}	100	106	39.6	6.4			
26% C^{13}	100	109	42.1	7.0			
Intramolecular, 25% C^{13}	100	139	41.5	2.9***			
26% C^{13}	100	147	44.7	3.1			
Found	100	141.5	42.5	2.9 ± 0.14 (std. dev.)			

* Based on theoretical content of natural C^{13}, D, O^{17}, and O^{18}.

** In the theoretical calculations for the 20% C^{13} KCN, for example, the distribution of the parent masses, as calculated from the binomial product of the C^{12} and C^{13} isotope concentrations in the cyanide, are as follows: mass 100 ($C_0{}^{13}$), 0.8^2; mass 101 ($C_1{}^{13}$), $2 \times 0.8 \times 0.2$; mass 102 ($C_2{}^{13}$), 0.2^2; this yields a ratio of 100:50:6.25. Succeeding calculations are made from the ratios, 100:50:6.25 = 39:19.5:2.44, etc.

*** These last three values at mass 103 are not 0.0 due to the carry-over from the next lower mass, as noted immediately above.

boxylic acids. The only disadvantage of the negative ion technique is much lower sensitivity Nevertheless, samples of about one milligram gave excellent spectra under favorable operating conditions. Positive ion spectra at masses 100-103 and negative ion spectra at masses 117-119 were obtained from the chemically synthesized C_2^{13}-succinate. The labeling of the succinate product was calculated from the binomial product of the isotope concentrations of the reacting species. Table 1b lists values calculated for starting cyanide of 20 and of 21% C^{13} content, along with the experimental values. The experimental values agree with a calculated spectrum for approximately 21% (i.e. 21 atom %) C^{13}-CO_2. Carbon dioxide from total combustion of the succinate showed 11.8 atom % C^{13} or 21.4 atom % excess C^{13} for the two cyanide derived carbons. The negative ion data prove the reliability of the more complicated spectrum of the positive ion. The data correlate best at masses 103 and 119, where the C_2^{13}-succinate mass is least interferred with.

The amount of succinate from the isomerase reaction was limited, and a satisfactory negative ion spectrum was not obtained. This made necessary the use of the mass 100 positive ion spectrum. The predicted distribution for an intermolecular reaction was calculated by the same formula as for the model reaction. For the intramolecular mechanism only two parent mass numbers and their accompanying normal distribution are involved. The experimental results (Table 1c) compare closely with the calculated values for 25% average carboxyl C^{13}. They are distinctly different from the theoretical values calculated for an intermolecular reaction. Hence the cyclic dimer is not an intermediate in the isomerization reaction. Eggerer et al. (1960) proposed a rearrangement involving a free-radical intermediate. An additional intramolecular rearrangement, in which a carbanion intermediate is stabilized by cobalt, has been suggested (Ingraham, 1962); here the cobamide coenzyme is called a "biological Grignard reagent". No experimental evidence for these mechanisms is available.

The use of the simple negative ion mass spectra in conjunction with the multiple positive ion patterns for the study of C^{13} labeling of biologically derived carboxylic acids is

demonstrated. Spectrometry of the crystalline acids offers some distinct advantages over the method which involves conversion of the acid to the hydrocarbon by a two-step reduction (Wood, 1952).

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