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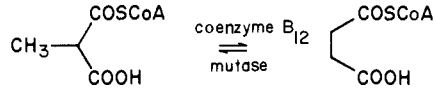
A Nonenzymic Model Intermediate for the Coenzyme B_{12} Dependent Isomerization of Methylmalonyl Coenzyme A to Succinyl Coenzyme A

Sir:

The coenzyme B_{12} dependent, enzyme catalyzed, reversible interconversion of methylmalonyl-SCoA (I) with succinyl-SCoA (II)[1] is crucial in human metabolism. It effects the return of propionic acid, resulting from amino acid and odd-chain fatty acid catabolism, to the tricarboxylic acid cycle.[2] Obstruction of the enzymic pathways necessary for the interconversion of methylmalonyl-SCoA (I) with succinyl-SCoA (II), as a consequence of any one of several possible genetic



I II

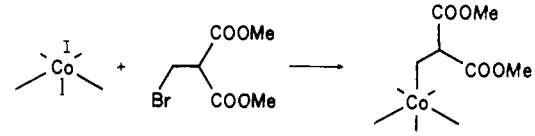
defects, leads to excesses of methylmalonic acid and propionic acid in the body and thence to some of the symptoms and malfunctions associated with the once fatal disorder pernicious anemia. In recent years it has become the practice, in appropriate instances, to monitor the levels of propionic and methylmalonic acids in the body fluids, in attempt to avert disastrous consequences from disorders related, at least in part, to pernicious anemia.[2]

In spite of the high level of interest in this important transformation (I ⇄ II), the mechanism of the reaction has remained chemically obscure. In particular, until recently,[3] there has been no nonenzymic, chemical model for such a carbon-skeleton rearrangemgnt.

A series of ingenious experiments on the enzyme system (I ⇄ II) has revealed that: the carbonyl-SCoA group is the migrating group[5] and that the migration is intramolecular.[6] The countervailing migration of hydrogen is an intermolecular reaction in which the substrate hydrogen is taken up by the 5′-methylene of the deoxyadenosine of the coenzyme, then later returned to the rearranged substrate.[7] This mechanism makes understandable the prior observation that isotopic hydrogen from deuterated or tritiated water is not incorporated into the substrate. Both the carbonyl-SCoA group and the hydrogen migrate in a way such that configuration is maintained at both termini of the rearranging system.[8]

It is difficult to explore the facets discussed above and impossible to delineate the respective contributions of the enzyme and coenzyme to the rearrangement reaction in the absence of an appropriate chemical model reaction. Thus, we have begun to explore the possibility of developing a nonenzymic, chemical model for this rearrangement. This series of experiments also serves as the first test of the general applicability of a recently proposed[3,4] model intermediate for the methylitaconate ⇄ α-methylene glutarate interconversion.

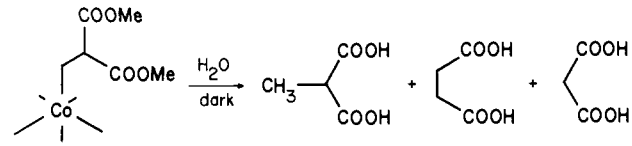
Accordingly, we have treated dimethyl bromomethylmalonate (III)[9] with vitamin B_{12s}[10,11] and obtained a metastable adduct IV. The adduct IV has all the ultraviolet and



III IV

visible spectral properties expected for an alkyl cobalamin[11,12] and it rapidly yields the ultraviolet and visible spectra of hydroxocobalamin upon exposure to light.

The alkyl cobalamin IV is, not unexpectedly, a very sensitive substance. It cannot be purified by extraction with phenol.[11] Even on attempted precipitation from the aqueous reaction mixture with acetone or tetrahydrofuran, the carbon-cobalt bond is cleaved to the extent of 50–70%, as judged by the changes in the visible spectrum. Thus, we have not been successful in isolating the solid adduct IV, and have been forced, as a consequence, to work with the total aqueous reaction mixture.[13] When the alkyl cobalamin IV was completely formed, the aqueous reaction mixture was extracted with ether in order to remove excess unreacted bromomethylmalonate III. The aqueous phase (pH 8–9) containing the alkylcobalamin IV was allowed to stand for 48 h at room temperature in the dark. At the end of this time, the visible spectrum showed complete conversion of IV to hydroxocobalamin (total cleavage of the carbon-cobalt bond). The reaction mixture was made acid with 10% aqueous hydrochloric acid and extracted continuously with ether for 24 h. The ether concentrate[14] was found to contain methylmalonic acid (V), 13.6%; succinic acid (VI), 3.7%; and malonic acid (VII), 18%.[16]



IV V VI VII

Isolation of pure, crystalline succinic acid (VI) from this model study of the methylmalonyl-SCoA (I) ⇄ succinyl-SCoA (II) interconversion demonstrates the fruitfulness and promise of this general approach to a chemical understanding of the mechanism of the coenzyme B_{12} dependent carbon-skeleton rearrangement reactions. It is worthwhile to summarize the attributes of this strategy. Since one has now observed *two* spontaneous rearrangements,[4] *in the dark*, at ambient temperature (20–25 °C), in aqueous solution, at or near physiological pH, of substances *identical in their carbon-skeletons* to the enzyme substrates, attached to the intact *cobalamin* nucleus, one is in position to suggest strongly that the carbon-cobalt substrate bond plays a crucial role in the rear-

rangement reactions which occur under enzyme control. It follows that conclusions drawn from model reactions lacking one or more of the above features[18] must be applied with circumspection to mechanistic questions surrounding the rearrangement reactions.

At the same time, it is proper to point out features of the model rearrangement reactions which have not yet been illuminated. Neither in this nor in our previous experiment[4] is the ionization state of either cobalt or carbon revealed.[19] We are just beginning to probe the possible salutory effects of the thioester grouping on the present rearrangement. The highly important question of stereochemistry remains completely open in the model series. These are examples and constitute only a few of the problems confronting us in attempting to understand the mechanism of the carbon-skeleton rearrangements from a chemical point of view. They will form the objectives of our continuing research effort in this area.

Acknowledgment. We are indebted to Dr. Kilmo Kang for early exploratory studies on this system and to Dr. Lalat Kumar for assistance in the course of the investigation. This work was generously supported by the National Institutes of Health through grant GM 19906 01.

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(3) We have recently presented a nonenzymic, chemical model reaction for part of an analogous transformation: the reversible, coenzyme B_{12} dependent, enzyme catalyzed interconversion of methylitaconic acid with α-methyleneglutaric acid.[4]

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(9) Prepared by treatment of dimethyl methoxymethylmalonate (from dimethyl malonate and chloromethylmethyl ether) with 3 mole equiv of 32% HBr in acetic acid. Excess HBr and acetic acid were removed at room temperature and 0.01 mmHg. The residual oil was then distilled, bulb-to-bulb, at room temperature and 10^{-5} mmHg. This is a modification of the procedure of J. L. Simonsen, *J. Chem. Soc.*, **93,** 1777 (1908). Our procedure attempts, to a greater extent than that of Simonsen, to take into account the sensitive nature of the bromo compound III. The NMR spectrum ($CDCl_3$) of the bromomethylmalonate III shows only a single line, somewhat broadened at the base, at δ 3.8. The mass spectrum is more informative, showing equal intensity doublets at *m/e* (rel int): 224, 226 (6, parent); 193, 195 (15, $M^+ - OMe$); 165, 167 (73, $M^+ - COOMe$) and a singlet at *m/e* 113 (100, base peak, $M^+ - HBr - OMe$). The mass spectrum is free of extraneous peaks in the higher mass range, which might arise from impurities.

(10) Vitamin B_{12s} was prepared by sodium borohydride reduction of hydroxocobalamin.[11]

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(12) λ_{max}^{water} 525 mμ(ϵ 6920), 500 (sh, ϵ 6450), 430 (ϵ 3590), 375 (ϵ 8000), 336 (ϵ 11 065), 315 (sh, ϵ 10 482), 285 (sh, ϵ 14 365), 282 (ϵ 15 530), and 260 (ϵ 17 280) with λ_{min}^{water} 410 (ϵ 3320). In acid solution (pH 3), the 525 mμ peak shifts to 274 mμ (ϵ 7740) and two peaks at 350 (ϵ 10 556) and 314 mμ (ϵ 11 494) become more apparent. This process is reversed upon the addition of base, but some decomposition can be observed, upon this relatively brief exposure to acid, in the rise of the peak at 352 mμ indicating the presence of increasing amounts of hydroxocobalamin. Since the alkyl cobalamin is a relatively unstable substance (see text), the ϵ values cited above could not be determined from a weighed sample. Instead, they were estimated by exposing a solution of the alkyl cobalamin to light, then calculating the ϵ values from those established prior[11] for hydroxocobalamin.

(13) In a typical reaction 1.346 g (1.0 mmol) of hydroxocobalamin *in* 85 ml of water was reduced under an atmosphere of nitrogen with 0.600 g (15.9 mmol) of sodium borohydride in 5 ml of water to the gray-green vitamin B_{12s}. This solution was treated in the dark with 0.700 g (3.1 mmol) of dimethyl bromomethylmalonate (III). After 6 min an ultraviolet spectrum showed the complete formation of the carbon–cobalt bond.

(14) The ether concentrate was a mixture of acids and esters. For this reason it was first stirred overnight with 1 ml of 10% sodium hydroxide, reacidified, and extracted again, continuously for 24 h, with ether. This ether concentrate was chromatographed on a 28 by 1.1 cm column of silicic acid, the products being eluted with a 55:45 mixture of ether–chloroform in 5-ml fractions.[15] Methylmalonic acid (V) was eluted first in fractions 7, 8, and 9; malonic acid (VII) was eluted next in fractions 10, 11 and 12; followed last by succinic acid (VI) in fractions 12, 13, and 14. Because of the overlap between malonic acid (VII) and succinic acid (VI), it was often necessary to combine fractions 12, 13, and 14 and to rechromatograph them on silicic acid. The yields cited in the text are those of recrystallized, sharp-melting solids, and are based on the limiting reagent, hydroxocobalamin. From 1.346 g (1.0 mmol) of hydroxocobalamin was isolated 16.1 mg (13.6%) of methylmalonic acid (V), 18.7 mg (18%) of malonic acid (VII), and 4.4 mg (3.7%) of succinic acid (VI). The spectral properties (ir, NMR, and MS) of the three crystalline solids were in excellent agreement with those of authentic samples.

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(16) Malonic acid may arise in this system from hydrolysis of the starting bromomethylmalonate III to hydroxymethylmalonate followed by loss of formaldehyde through a reverse aldol reaction. Alternatively, the presence of malonic acid (VII) may have significance for the carbon-skeleton rearrangements (IV $\rightarrow$ VI and I $\rightleftarrows$ II) in a way not yet fully apprehended. From the point of view of chemical reactivity, the bond between the methyl and methine carbons is an interesting one to consider being broken after the attachment to cobalt. However, the possible significance of malonic acid (VII) in the rearrangement reactions is lessened, although not ruled out, by the observation that malonic acid (VII) is also observed in the control reaction, whereas succinic acid (VI) is not seen.[17]

(17) A control reaction in which cobalt: (II) nitrate was substituted for hydroxocobalamin yielded no detectable succinic acid (VI) following the identical reaction conditions and chromatographic workup as those described above.

(18) G. Bidlingmaier, H. Flohr, U. M. Kempe, T. Krebs, and J. Retey, *Angew. Chem.*, **87,** 877 (1975), have recently reported a similar model reaction using cobaloximes. The rearrangement product was not isolated and the yield of rearrangement product was unstated. Cf. J. N. Lowe and L. L. Ingraham, *J. Am. Chem. Soc.*, **93,** 3801 (1971).

(19) H. A. O. Hill in "Inorganic Biochemistry", Vol. 2, G. L. Eichorn, Ed., Elsevier, Amsterdam, 1973, p 1118, has pointed out that the existence of three stable valence states of cobalt, Co(I), Co(II), and Co(III), gives rise to the possibility of wide mechanistic variation ranging from carbonium ion to free radical to carbanion, depending upon substrate demand. This suggestion was made prior to ours,[4] and should have been referred to in our earlier paper.[4]

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α-Aminoacrylate Schiff Base in Nonenzymatic Pyridoxal Catalysis

Sir:

This report describes a species absorbing at 467 nm, which we conclude to be the titled compound and a possible model for an intermediate in pyridoxal catalyzed α,β-elimination and β-replacement reactions of amino acids.

Many pyridoxal phosphate containing enzymes catalyze the elimination and/or replacement of a β-substituent of an amino acid. The generally accepted reaction sequence[1] involves two metastable intermediates: a quinoid (carbanion) intermediate, which is a Schiff base of the coenzyme and the amino acid deprotonated at the α-carbon atom, and a Schiff base of α-aminoacrylate formed from the quinoid intermediate with a loss of an electronegative group on the β-carbon atom.

Marked changes in spectra of enzymes during reactions with substrate or pseudosubstrate provided evidence for the reaction sequence. Intermediate species with an intense absorption in the 500-nm region have been studied in enzymatic[1,2] and in nonenzymatic systems[3,4] and identified as the quinoid intermediate.

On the other hand, there is less spectral evidence for the α-aminoacrylate Schiff base. Transient species absorbing at 455–470 nm have been reported in a few pyridoxal enzymes catalyzing β-elimination or β-replacement and have been suggested to be this intermediate.[5] A similar species has not been reported so far in nonenzymatic reactions.

Pyridoxal *N*-methochloride (1×10^{-4} M) and tryptophan