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THE MECHANISM OF ACTION OF VITAMIN B_{12}[1]

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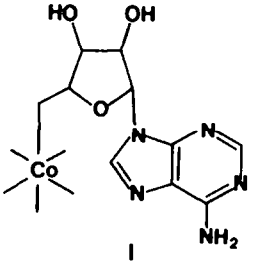
Abstract. A novel rearrangement reaction is introduced as a model for the rearrangement of methylitaconic acid (III) to α-methyleneglutaric acid (IV), one of three enzyme catalyzed, coenzyme B_{12}-dependent, carbon-skeleton rearrangements whose mechanism has been a source of puzzlement for many years. The key feature of the new model is the direct attachment of the substrate, methylitaconic acid, to the cobalt atom of vitamin B_{12}. This was accomplished by reacting butadiene-2,3-decarboxylic acid with hydrobromic acid generating bromomethylitaconic acid (VIII). Use of two moles of hydrobromic acid yielded **bis**-2,3-(bromomethyl)succinic acid (IX). Reaction of the monobromide VIII with vitamin B_{12s} did not yield the desired carbon-cobalt bonded adduct. Instead, the lactone α-methylene-γ-butyrolactone-β-carboxylic acid (X) was formed. Accordingly, the ester, dimethyl bromomethylitaconate (XIa), was reacted with vitamin B_{12s} and yielded the carbon-cobalt bonded adduct XIIa. **Bis**-trimethylsilyl bromomethylitaconate did not yield an adduct when reacted with vitamin B_{12s}, but **bis**-tetrahydropyranyl bromomethylitaconate (XIb) did yield the adduct XIIb. The ester cobalamin XIIb undergoes spontaneous decomposition at room temperature, in aqueous solution, at pH 8 and in the dark - biochemically ideal circumstances - yielding a mixture of: butadiene-2,3-decarboxylic acid (VII), methylitaconic acid (III) and α-methyleneglutaric acid (IV). The presence of the latter indicates that a skeletal change has taken place in a way which mimics the enzymatic reaction. This is the first non-enzymic model in this carbon-skeleton rearrangement series. The methyl ester cobalamin XIIa was stable in the dark but did decompose on irradiation with a sunlamp to butadiene-2,3-decarboxylic acid (VII) and methylitaconic acid (III). No α-methyleneglutaric acid IV was observed in the latter reaction.

Authentic methylitaconic acid (III) was prepared by alkylation of triethyl prop-2-ene-1,1,2-tricarboxylate (XIII) with methyl iodide followed by hydrolysis and decarboxylation. The lactone X and lactone α-methyl-γ-butyrolactone-β-carboxylic acid (XVI) were prepared by condensing the triester XIII with formaldehyde, hydrolyzing the lactone diester XV to the lactone X and hydrogenating to the saturated lactone XVI.

INTRODUCTION

The discovery of the coenzyme form (I) of vitamin B_{12} occurred in the course of Barker's



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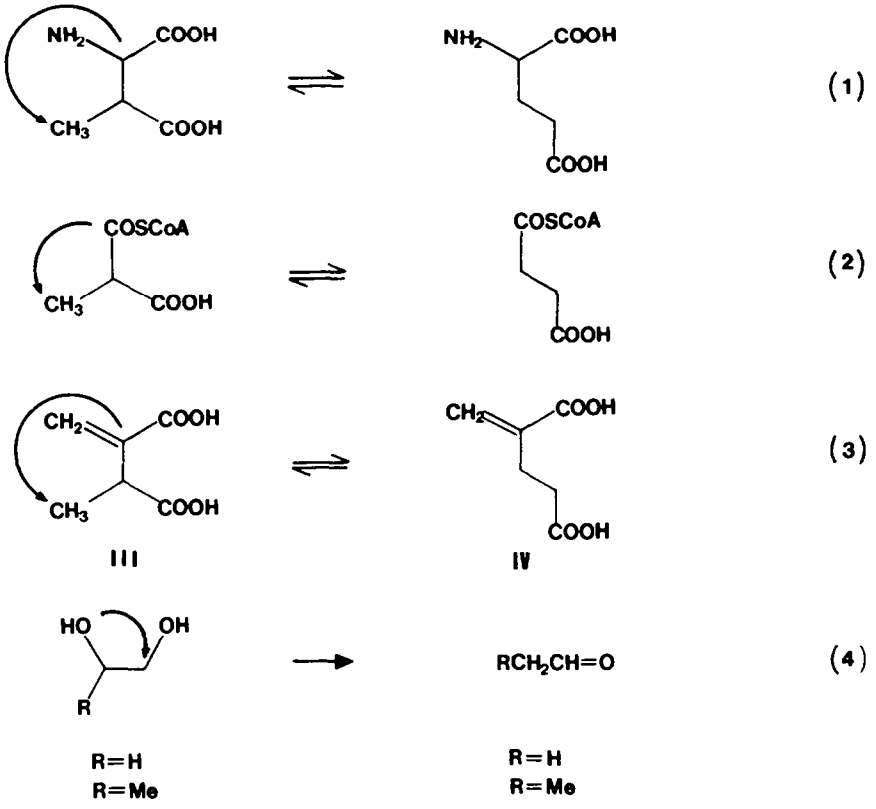
research into the metabolism of glutamic acid by the anaerobic bacterium **Clostridium tetanomorphum.**[2] From this beginning, eleven enzyme-catalyzed rearrangement reactions are now known to be dependent for their activity upon coenzyme B_{12}.[3,4,5] Of the eleven, three are the reversible carbon-skeleton rearrangements: β-methylaspartic acid ⇌ glutamic acid (equation 1),[6] methylmalonyl-SCoA ⇌ succinyl-SCoA (equation 2)[7] and β-methylitaconic acid (III) ⇌ α-methyleneglutaric acid (IV) (equation 3).[8] It is useful to include a fourth member of the series in this discussion, the intensively studied,[9,10] coenzyme B_{12}-dependent, dioldehydrase-catalyzed rearrangement of ethylene and propylene glycol to acetaldehyde and propionaldehyde (equation 4), irreversible reactions.



The rearrangement reactions involve the formal interchange of hydrogen with a migrating group (equations 1-4, arrows). Carbon-14 labelling studies have demonstrated that the glycyl fragment migrates in the β-methylaspartate ⇌ glutamate transformation[9] (equation 1, arrow), that the carbonyl-SCoA group migrates in the methylmalonyl-SCoA ⇌ succinyl-SCoA transformation[11] (equation 2, arrow), and that acrylate is the migratory group in the β-methylitaconate (III) ⇌ α-methyleneglutarate (IV) transformation (equation 3, arrow).[12] Oxygen-18 labelling studies[10] established that the hydroxyl group migrates as shown (arrow) in equation 4.

Early investigations[13] revealed that no hydrogen exchange occurs between substrate and deuterated or tritiated water.[14] For this reason, other mechanistic possibilities for the migration of hydrogen were explored. Thus, it was established that the migration of hydrogen is not intramolecular and concerted as is implied by the formalisms of equations 1-4 above; it is an intermolecular reaction in which the 5'-methylene of the deoxyadenosine of coenzyme B_{12} (I) is the agent of hydrogen transfer. First revealed by the tritium labelling studies of Abeles and his colleagues,[15] in the course of their research into the dioldehydrase-catalyzed rearrangements of ethylene and propylene glycol (equation 4), this mode of hydrogen transfer

has now been demonstrated to obtain for all three carbon-skeleton rearrangements (equations 1-3).[15] Similar hydrogen labelling experiments have been performed, with parallel results, in the ethanolamine deaminase,[16] L-β-lysine aminomutase[17] and D-lysine aminomutase[18] coenzyme B_{12}-dependent rearrangements.

THE MECHANISTIC PROBLEM

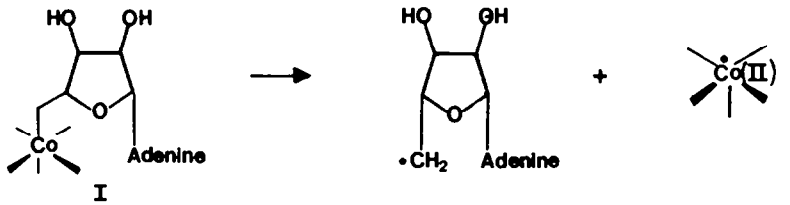
The coenzyme B_{12}-dependent carbon-skeleton rearrangements shown in equations 1-3 are unique. **At the time this work was initiated, there existed no analogous transformations in organic chemistry. There were no nonenzymic models for these reactions. Even now no reactive intermediates have been firmly established for any of the rearrangement reactions.**

The only acceptable substrates for the enzymes which govern the carbon-skeleton rearrangements are the substances shown in equations 1-3.[19] The nonexistence of chemical models made it difficult to define a role for coenzyme B_{12}, aside from hydrogen abstraction, distinct from that of the enzymes in the carbon-skeleton rearrangement reactions.

Although a number of organic ligands have been attached to the cobalt atom of vitamin B_{12}, none of the enzyme substrates of any of the eleven rearrangement reactions had been covalently linked to vitamin B_{12}. Thus, the question whether a coenzyme substrate covalent link might play a role in the enzyme-catalyzed rearrangement reactions of equations 1-3 had remained unresolved.

REACTIVE INTERMEDIATES

It is often assumed that the first step in the rearrangement sequence is enzyme induced cleavage of the coenzyme B_{12} deoxyadenosine carbon-cobalt bond followed by abstraction of hydrogen from substrate by the 5'-methylene group of the deoxyadenosine. The initial carbon-cobalt bond cleavage might occur homolytically to yield the deoxyadenosyl radical and vitamin B_{12r} (Co(II) oxidation level). The deoxyadenosyl radical can then abstract hydrogen from



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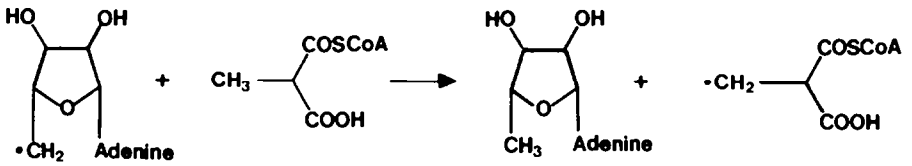
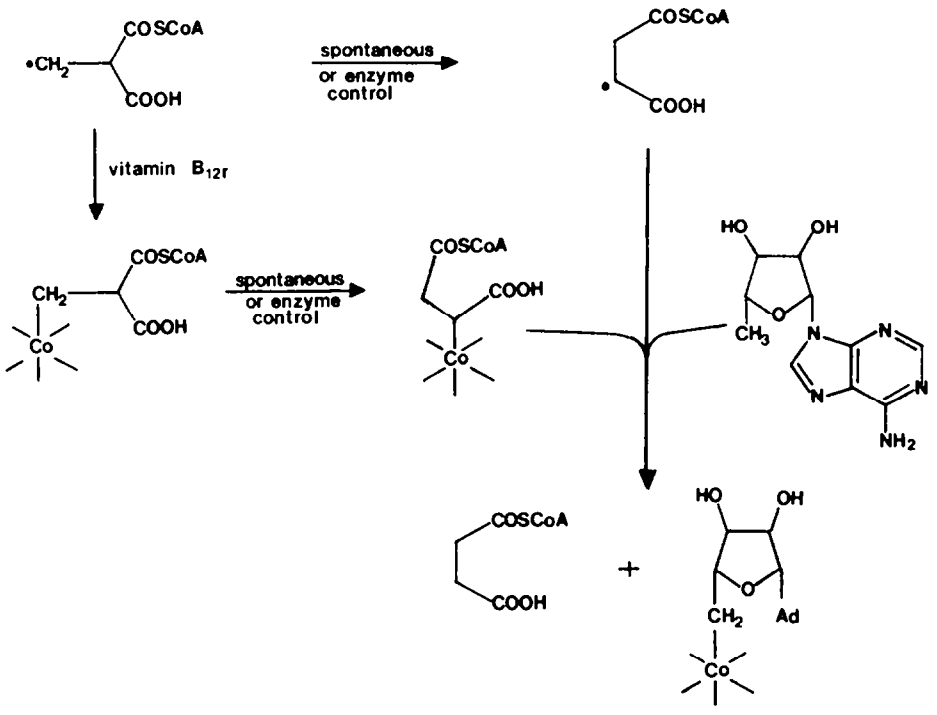
substrate, thereby satisfying the requirement of the labelling experiment[9] which demands transfer of isotopic hydrogen from substrate to the 5'-methylene group of the deoxyadenosine.

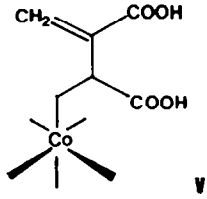


From this point, even this hypothetical picture becomes blurred. The substrate radical (or charged reactive intermediate - electron transfer between substrate and cobalt can occur, in principle, at any of a number of stages in the multi-step reaction sequence) might rearrange under enzyme control or rearrange spontaneously. Alternatively, the substrate radical can form a new carbon-cobalt bond to the B_{12} nucleus. This intermediate might then also rearrange spontaneously or under enzyme control. Confusion existed regarding the rearrangement reaction



because of the lack of experimental evidence for or against the various possibilities. Although the intervention of free radical intermediates has received support from the observation of electron-spin resonance (esr) signals during the dioldehydrase,[20] ethanolamine deaminase[21] and ribonucleotide reductase reactions;[22-24] in none of the three carbon-skeleton rearrangements have esr signals been detected. It has been suggested that free radicals are the most probable intermediates in the carbon-skeleton rearrangements (eq. 1-3), but there is, in fact, no experimental evidence to support this notion or, for that matter, to single out any other reactive intermediate in these three reactions. The case will be presented here for the intermediacy of the carbon-cobalt substrate bond at some stage in the reaction sequence.

To explore the possibile intermediacy of a direct link between carbon and cobalt required synthesis of an analogue of coenzyme B_{12} (I) with substrate attached to the apical position on cobalt. As noted above, a number of carbon-cobalt bonded derivatives of vitamin B_{12}, including coenzyme B_{12}, have been synthesized,[36] but no **bona fide** enzyme substrate had ever been attached to the B_{12} nucleus.

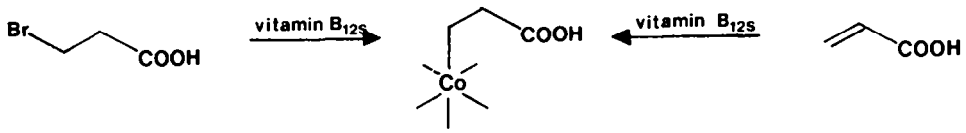


V

This inquiry was begun with the goal of preparing the vitamin B_{12} derivative V with methylitaconic acid (III) attached to cobalt through the methyl carbon. The choice of methylitaconic acid (III) as the subject of this series of experiments was deliberate. The linear substrates succinyl-CoA, glutamic acid and α-methyleneglutaric acid (IV) were set aside for the moment because it is known to be difficult to prepare stable adducts with secondary carbon attached to the cobalt atom of vitamin B_{12}. Of the remaining three branched substrates, methylitaconic acid (III) appeared to offer the fewest number of complicating features in terms of derivatization and formation of the carbon-cobalt bond to vitamin B_{12}.

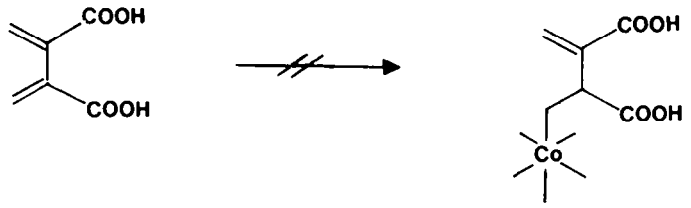
Results

Acrylic acid and β-bromopropionic acid react smoothly with vitamin B_{12s} yielding the β-substituted propionic acid adduct VI.[25] The extension to butadiene-2,3-dicarboxylic acid[26] VII



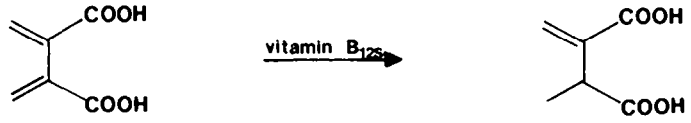
VI

appeared to provide a straightforward route to the desired adduct V. However, when this reaction was attempted, no carbon-cobalt bond formation took place. In the vitamin B_{12} series carbon-cobalt bonded adducts are characterized by moderately intense bands at 525 and 350 nm and the spectrum changes dramatically, to that of hydroxocobalamin with its strong band at 352 nm, upon exposure to light.[25]



VII V

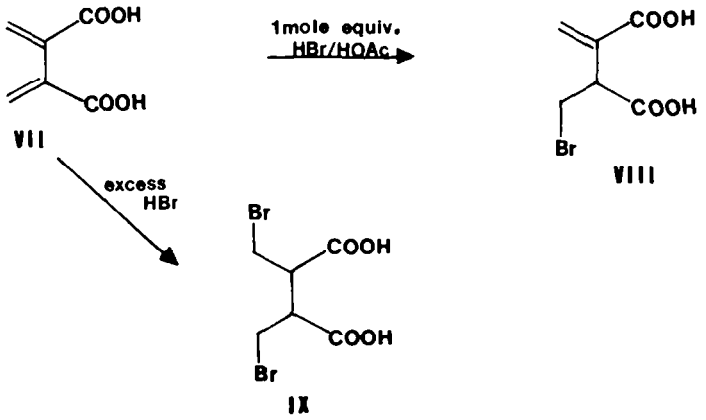
No such spectroscopic change was observed upon exposure of the butadiene-2,3-dicarboxylcic acid reaction mixture to light. No adducts were obtained when the dimethyl and bis-tetrahydropyranyl esters of butadiene-2,3-dicarboxylic acid (VI) were reacted with vitamin B_{12}. When the reaction between vitamin B_{12s} and butadiene-2,3-dicarboxylic acid (VII) was examined for products, methylitaconic acid III was isolated in 80% yield.



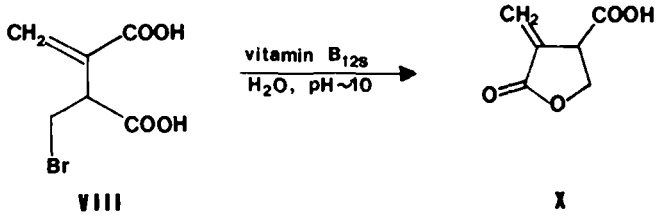
VII III

In the preliminary experiments with the model substances acrylic acid and β-bromopropionic acid it appeared, qualitatively, that the latter gave a somewhat faster reaction with vitamin B_{12s}. This observation, taken together with the failure of the butadiene diacid VII to form a carbon-cobalt bond and a later experiment in which methacrylic acid also failed to form a carbon-cobalt bond, leads to the conclusion that the successful reaction of the parent acrylic acid is exceptional. Moreover, the subsequent demonstration that the bromomethylitaconic esters do successfully react to form the desired carbon cobalt bond opens the door to the hypothesis that the trajectory of approach to the olefins may be the controlling factor and may be sensitive to steric interference. The two sp^2 centers comprising the double bond require the four groups attached to the double bond to lie in a plane. When the olefin approaches the hindered cobalt preparatory to bond formation, it encounters stiff resistance as a consequence of this rigidity. The corresponding bromo compounds are more flexible in having rotational freedom about the crucial carbon-carbon bond, and are thus better able to adapt to the crowded quarters in the space above the cobalt. An equally plausible explanation is that this is simply a manifestation of the generally lower reactivity of α-substituted acrylates in Michael-type addition reactions.

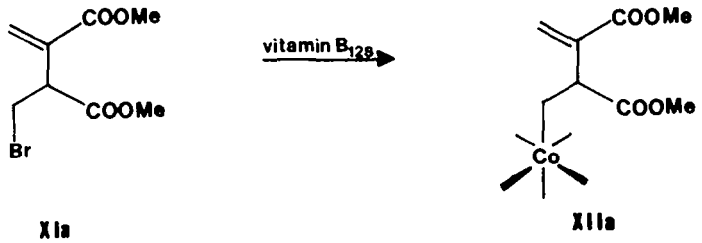
Accordingly, butadiene-2,3-dicarboxylic acid VII[26] was reacted in dioxane with slightly more than one equivalent of 32% hydrobromic acid in acetic acid, yielding cleanly (90%) the



monosubstituted bromomethylitaconic acid (VIII), mp 117-119°C. Use of an excess of hydrobromic acid yielded bis-2,3-bromomethylsuccinic acid (IX). The bromo acid (VIII) is stable in the crystalline state; in non-polar, non-hydroxylic solvents; and in strongly acidic medium. However, VIII is rapidly converted to the lactone X in neutral or basic hydroxylic solvents. Thus, the bromide VIII could not be used to alkylate the cobalt because of competing facile cyclization to the γ-lactone X.



To forestall lactone formation, the diester, dimethyl bromomethylitaconate (XIa), was prepared and reacted with vitamin B_{12s}. This reaction yielded the carbon-cobalt bonded adduct



XIIa demonstrating, for the first time, the feasibility of attaching one of the enzyme substrates to the cobalt atom of vitamin B_{12}. The adduct XIIa was purified by phenol extraction and crystallization from acetone-water to a state homogeneous judged by paper and thin-layer chromatographic analysis. The methyl ester adduct XIIa exhibited all the anticipated spectroscopic properties (Figure 1a),including the extraordinary sensitivity to light, associated with

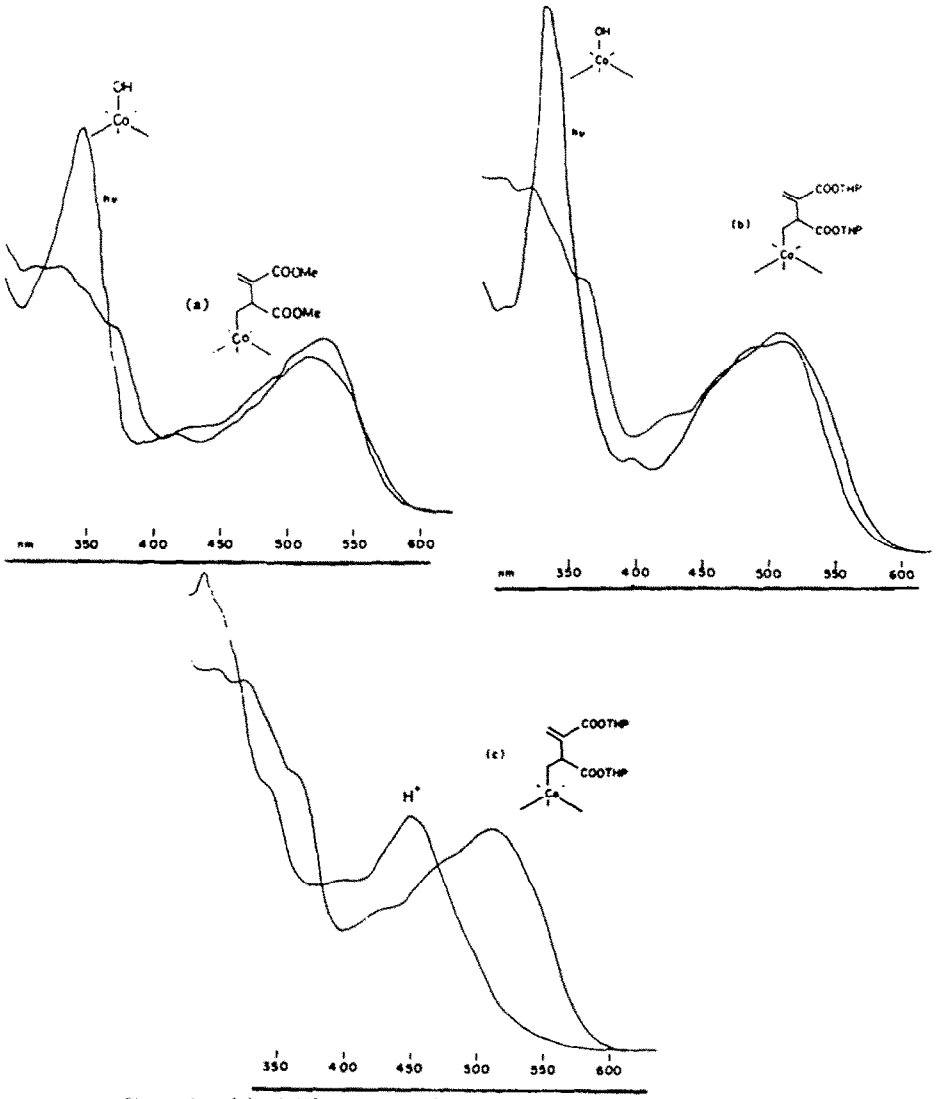


Figure 1. (a) Visible spectrum of the dimethyl methylitaconate - vitamin B_{12} adduct XIIa before and after exposure to light. (b) Bis-tetrahydropyranyl methylitaconate-vitamin B_{12} adduct XIIb before and after exposure to light. (c) Visible spectrum of adduct XIIb showing hypsochromic shift following acidification.

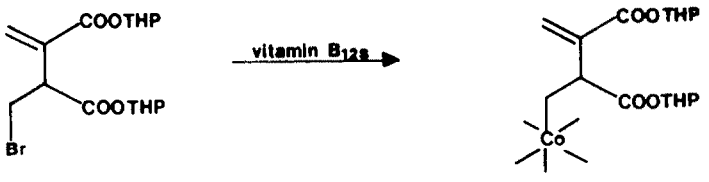
the alkylcobalamins. The structure assignment for XIIa is also supported by the observation of $M^{+}+H$ and $M^{-}-H$ peaks at m/e 1501 and 1499 in the positive and negative ion LD mass spectra.[36]

In the methylitaconate rearrangement (eq.3), the substrates are carboxylic acids. Accordingly, it was attractive to press on to construct the coenzyme B_{12}-methylitaconic acid adduct. Hydrolysis of the methyl ester adduct XIIa was not attractive; the potential for destruction of other sensitive functional groups in the B_{12} nucleus was too great.

The bromo diacid VIII not being a viable precursor, a labile ester was sought. Bis-trimethylsilyl bromomethylitaconate was prepared, but it hydrolyzed too rapidly under the aqueous conditions required for B_{12} adduct formation. Visible spectra characteristic of the carbon-cobalt bond were not observed. The lactone X was isolated from this attempt.

When the bromoacid VIII was stirred with dihydropyran in benzene at room temperature for 16 hours, the solid diacid VIII dissolved slowly as it reacted, yielding the bis-tetrahydropyranyl bromomethylitaconate XIb. The progress of the esterificatin was monitored using infrared spectroscopy, following the decrease in intensity of the broad carboxylic acid band between 3-4 μ.

The ester XIb reacted with vitamin B_{12s} yielding an adduct XIIb whose visible spectra (Figures 1b and 1c) demonstrated the formation of the desired carbon-cobalt bond. The bis-tetrahydropyranyl ester-vitamin B_{12} adduct XIIb was much more labile than the corresponding methyl ester adduct XIIa. It has not yet been found possible to effect purification of XIIb by phenol extraction or by column chromatography. In practice, the product XIIb was precipated from the aqueous reaction mixture by the addition of ice-cold acetone and was obtained, after drying, as a red powder.



XIIb XIIIb

Carbon-Skeleton Rearrangement

The tetrahydropyranyl ester alkyl cobalamin adduct XIIb was dissolved in water and allowed to stand in the dark for several hundred hours. Slow carbon-cobalt bond cleavage occurred leading to hydroxocobalamin with its prominent characteristic band in the visible spectrum at 352 nm. When there was no further change in the spectrum and the hydroxocobalamin band at 352 nm had reached its maximum, the aqueous reaction mixture was worked up by acidification and continuous extraction with ether. Examination of the total crude product using nmr (Figure 2a) revealed the presence of three products: methylitaconic acid (III), α-methyleneglutaric acid

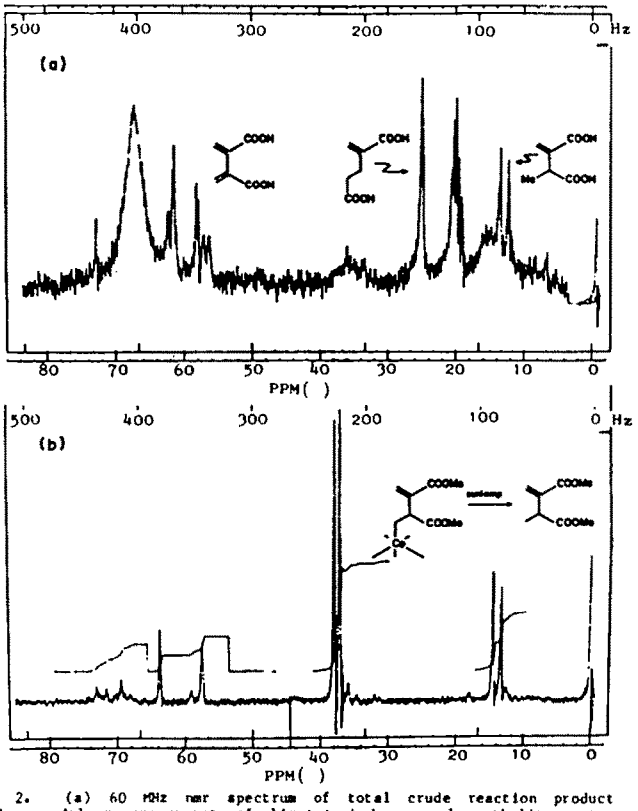
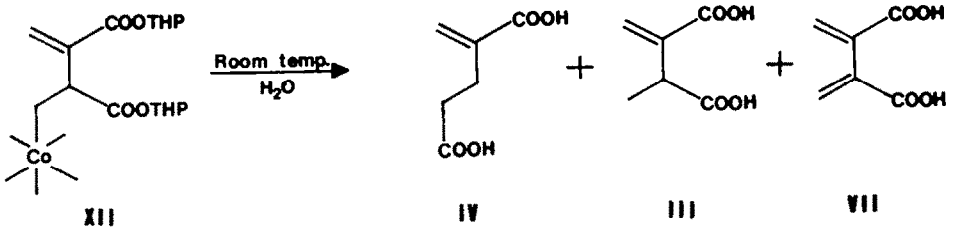


Figure 2. (a) 60 MHz nmr spectrum of total crude reaction product following model rearrangement of bis-tetrahydropyranyl methylitaconate-vitamin B_{12} adduct XIIb. (b) 60 MHz nmr spectrum of total crude reaction mixture from light promoted decompsotion of the dimethyl methylitaconate-vitamin B_{12} adduct XIIa.

(IV), and butadiene 2,3-dicarboxylic acid (VII). The mixture was resolved into its components by chromatography on silica gel. The yields of pure crystalline products were: α-methyleneglutaric acid (IV) 5-15%, methylitaconic acid (III) 3-7%, and butadiene-2,3-dicarboxylic acid (VI) 2-5%. These substances were identified by their spectroscopic properties and



XII IV III VII

by direct comparison with authentic samples.[27,28]

The enzyme-catalyzed reaction for which the above rearrangement is a model is not light dependent. However, because the cobalamin carbon-cobalt bond is light sensitive, it was of interest to learn whether the model rearrangement would occur under the influence of light. Accordingly, a pH 8-9 aqueous solution of the tetrahydropyranyl ester adduct XIIb was irradiated using a 250 watt sunlamp for approximately 60 hours at room temperature. The reaction was followed by monitoring the visible absorption spectra of aliquots from the reaction mixture. Acidification, continuous extraction and chromatography led to the following, isolated as sharp-melting crystalline products: α-methyleneglutaric acid (IV) 11%, methylitaconic acid (III) 6% and butadiene-2,3-dicarboxylic acid (VI) 3.5%. Thus, irradiation provided modest acceleration of the reaction while the products were not materially different from those found in the dark reaction.

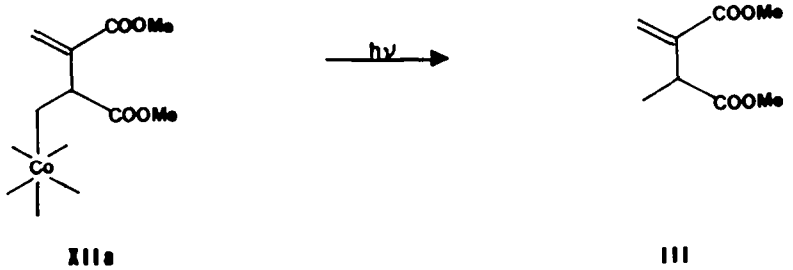
Control Reaction

When **bis**-tetrahydropyranyl bromomethylitaconate was treated with sodium borohydride in water, with cobalt nitrate in place of hydroxocobalamin, no rearrangement product IV was observed in the total crude reaction mixture or among the chromatographed products. The nmr spectrum of the rearrangement product, α-methyleneglutaric acid (IV), at 60 MHz (Figure 2a) shows a singlet at δ2.6 from the two chemically non-equivalent but magnetically coincident methylene groups. This relatively sharp peak makes the rearrangment product readily detected in the complex total crude reaction mixtures which characterize this series of experiments.

Non-Rearrangement of the Dimethyl Ester Adduct XIIa

In contrast to the **bis**-tetrahydropyranyl ester cobalamin adduct XIIb, the corresponding dimethyl ester adduct XIIa was well-behaved and readily purified. Extraction with phenol followed by chromatography on carboxymethyl cellulose (acid form) yielded carbon-cobalt bonded adduct XIIa homogeneous by thin-layer chromatography. In view of the apparent stability of the methyl ester XIIa as compared to the **bis**-tetrahydropyranyl ester XIIb, it was important to learn whether the dimethyl ester XIIa might also undergo carbon-skeleton rearrangement.

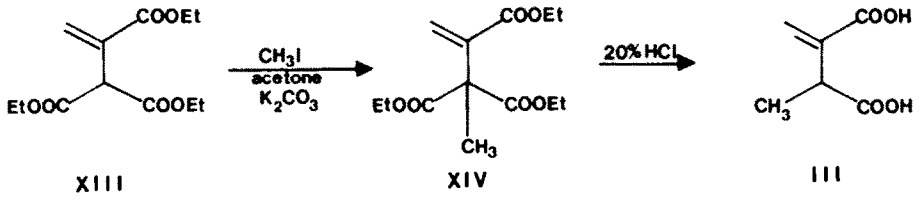
Upon standing in the dark for extended periods of time, in aqueous solution at room temperature, the dimethyl ester adduct XIIa was unchanged. Therefore XIIa was forced to decompose by irradiation of an aqueous solution with a sunlamp, destroying the carbon-cobalt bond. Acidification of the reaction mixture and continuous extraction yielded a total crude reaction mixture whose nmr spectrum is shown in Figure 2b. The spectrum shows that although the unrearranged methylitaconic acid III is present, the total crude reaction mixture contains, within the limits of detection of the nmr method, no α-methyleneglutaric acid IV, the carbon-skeleton rearrangement product.



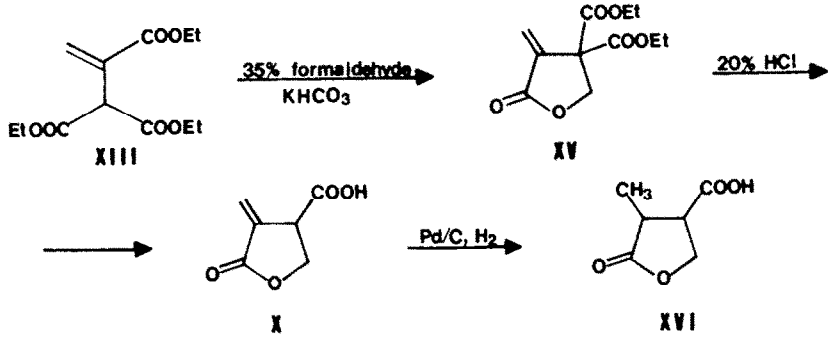
XIIa **III**

An authentic sample of α-methyleneglutaric acid IV was prepared according to Buchman, Reims and Schlatter[27] or by tri-n-butylphosphine catalyzed dimerization of acrylate.[34]

Authentic methylitaconic acid is available in the literature only by a short but awkward scheme in which dimethylmaleic anhydride is heated with KOH giving rise to an equilibrium mixture of maleate, fumarate and methylitaconate.[28] The latter is present to the extent of approximately 10% and is isolated by fractional crystallization. We have not used this antediluvian method. Instead, we developed the simple method shown below. It is described in detail in the experimental section.



Authentic lactones X and XVI are readily prepared as shown below:



Conclusion

The experiments described in this paper constitute the first non-enzymic rearrangement of a derivative of a **bona fide** substrate belonging to the coenzyme B_{12}-dependent carbon-skeleton rearrangements. Prior to these experiments, there existed no means of assessing the relative contributions of enzyme and coenzyme to the rearrangement reactions - apart from the well established hydrogen abstraction role of the 5'-deoxyadenosyl group. It had been concluded that the carbon-skeleton rearrangements of this series could only occur under enzymic influence. The experiments described here make possible a new and more explicit interpretation of the rearrangements: that the carbon-cobalt bond is formed and that the latter plays an integral part in the coenzyme B_{12}-dependent rearrangement reactions.

The model based on formation of the carbon-cobalt bond has been fruitful. Following the advent of the methylitaconate model above, there now have been developed working models for the methylmalonyl-SCoA rearrangement (eq.2)[29-31] and the ethylene glycol rearrangement (eq.4),[32] where none existed before.

A rival hypothesis holds that the rearrangements are free radical reactions. This is not incompatible with the the experimental models above. A conflict does arise with those who insist that the rearrangements are strictly free radical reactions and that the carbon-cobalt substrate bond plays no role in the reaction. There are two problems with the latter position at the present time. As noted above, no free radical esr signals have been observed in any of the carbon-skeleton rearrangement reactions. In addition, the paucity of free radical rearrangements has made it difficult to find suitable nonenzymic models. Indeed, so far, the free radical hypothesis has been a sterile one for the development, design and generation of chemical models for the carbon-skeleton rearrangement reactions. A nonenzymic model is a crucial adjunct to an understanding of the mechanism of action of coenzyme B_{12} if one believes with Abeles,[33] as we do, that, ".... an enzymatic reaction is not meaningfully defined until it can be related to known nonenzymatic reactions...".

EXPERIMENTAL SECTION

Melting points were taken with a Fisher-Johns hot stage melting point apparatus and are uncorrected. Elemental analyses were carried out by the Scandinavian Microanalytical Laboratories, Herlev, Denmark. Mass spectra were obtained using LKB-9000 and Varian MAT CH5 mass spectrometers for low and high resolution spectra, respectively. Infrared spectra were obtained using a Perkin-Elmer 247 grating infrared spectrophotometer with sodium chloride cells or potassium bromide pellets. Unless otherwise stated, nmr spectra were taken using a Varian T60 nmr spectrometer with tetramethylsilane as internal standard. Ultraviolet spectra were recorded on a Cary 14 instrument. In the thin-layer chromatographs R_{CN} indicates the R_f value relative to that of cyanocobalamin as reference in that solvent system.

β-Bromomethylitaconic Acid (VIII).

To a solution of 4.65 g (0.033 mole) of butadiene-2,3-dicarboxylic acid[26] (VII) in 140 ml of dioxane cooled in an ice-bath was added 9.97 g (0.04 mole) of Eastman 30-33% hydrobromic acid in acetic acid. The ice bath was removed after 5 min and the orange solution was stirred at room temperature for 15-30 h. The reaction was followed using nmr, observing the disappearance of the butadiene vinyl doublets at δ5.93 and 6.26 and the appearance of the bromomethylitaconate vinyl singlets at δ5.96 and 6.46. When the reaction was complete, the solvents were removed at room temperature by vacuum (10^{-2} mm) bulb-to-bulb distillation into a dry-ice trap. Residual solvent was removed by evaporation with four 30 ml portions of carbon tetrachloride. The resulting brown oil was dissolved in 50 ml of ether, treated with Norit and filtered through Celite. Concentration of the ether solution yielded 7.1 g of a pale yellow solid, mp 110-115°. Recrystallization was carried out at room temperature by dissolving the crude solid in a small amount of ether and precipitating the product by the addition of carbon tetrachloride; yield 6.45 g (90%) of white crystals, mp 117-120°, only slightly improved (mp 118-120°) on further crystallization.

Nmr (acetone-d_6): two one-proton vinyl singlets at δ6.46 and 5.96 and a three-proton aliphatic multiplet at δ3.85. At 250 MHz the aliphatic multiplet at δ3.85 is cleanly resolved in an ABX system showing: a triplet (J_{AX}= J_{AB}= 7 Hz) at ν_A = δ3.97, a quartet (J_{AB}= 7, J_{BX}= 10 Hz) at ν_B= δ3.87 and a quartet (J_{AX}= 7, J_{BX}= 10 Hz) at ν_X = δ3.72. Infrared (KBr): 1709 (s) and 3571-2326 (b) cm^{-1}. Mass spectrum (70 eV); m/e (relative intensity): 224, 222 (0.12, M^+); 223, 221 (0.2, M^+-H); 205, 203 (2, M^+-H-H_2O); 179, 177 (1.2, M^+-COOH); 143 (16.7, M-Br); 127 (100, M-CH_2Br); 125 (64, M^+-Br-H_2O). Exact mass: calc'd. for $C_6H_7{}^{79}BrO_4$, 221.9528; found, 221.9523.

Anal. Calc'd for $C_6H_7O_4Br$: C, 32.31; H, 3.16; Br, 35.83. Found: C, 31.93, 32.20; H, 3.06, 3.23; Br, 36.35, 36.39.

2,3-Bis-(bromomethyl)-succinic Acid (IX).

A solution of 1.015 g (0.007 mole) of butadiene-2,3-dicarboxylic acid (VII) in 25 ml of dioxane was cooled to 20° then treated with 3.65 g (0.015 mole) of Eastman 30-33% hydrobromic acid in acetic acid. The light brown mixture was stirred at room temperature for 22 hr. The reaction mixture was concentrated on the rotary evaporator to 2.215 g of a brown semisolid. The crude product was dissolved in 50 ml of ether, treated with Norit and filtered through Celite. The solution was then concentrated on the steam bath to ca. 10 ml, and the product was allowed to crystallize at room temperature. The resulting solid was triturated with a small amount of 50:50 ether-chloroform, to remove a light brown oil from the surface of the crystals; yield: 1.53 g (72%) of white crystals, mp 174-177°. Recrystallization from acetone yielded 1.182 g, mp 175-177°.

Nmr (acetone-d_6): two-proton multiplet at δ3.3 and four-proton multiplet at δ3.75. Infrared (KBr): 3571-2326 (b) and 1695 cm^1 (s). Mass spectrum (70 eV): m/e (reltive intensity): 224, 222 (1.2, M^+-HBr), 166, 164 (5.1, M^+-CH_2Br-CO_2H), 143 (15.3, M^+-HBr), 133, 135 (4.5, M^+-H_2O-CO_2-CO-Br), 125 (2, M^+-2HBr-OH).

Anal. Calc'd. for $C_6H_8Br_2O_4$: C, 23.71; H, 2.65; Br, 52.58. Found: C, 23.51; H, 2.76, Br, 52.71.

Dimethyl β-Bromomethylitaconate (XIa).

A solution of 2.62 g (0.012 mole) of bromomethylitaconic acid (VIII) in 40 ml of anhydrous methanol was cooled in an ice-bath, and two ml of acetyl chloride was added. The reaction was then allowed to stand at room temperature for 24 hr.

The solvent was removed on the rotary evaporator yielding an oil, which was dissolved in 50 ml of ether, washed with two 5 ml portions of 5% sodium bicarbonate solution, dried over anhydrous sodium sulfate then concentrated to 2.82 g (96%) of a light yellow oil.

Nmr (CCl_4): two one-proton vinyl singlets at δ6.3 and 5.75, two three-proton methyl ester singlets at δ3.76 and 3.68 and a three-proton aliphatic multiplet at δ3.7. Infrared (neat): 1724 cm^{-1}. Mass spectrum (70 eV): m/e (relative intensity) 219, 221 (4, M^+-OMe); 191, 193 (4.3, M^+-COOMe); 171 (1.5, M^+-Br) 157 (100, M^+-CH_2Br); 139 (61, M^+-Br-MeOH). Exact mass: calc'd. for $C_8H_{11}BrO_4$, 249.9841; found, 249.9841.

For analysis, a sample was distilled using a Hickman still, bp (bath temperature) 35°/0.0035 mm.

Anal. Calc'd. for $C_8H_{11}BrO_4$: C, 38.27; H, 4.42; Br, 31.83. Found: C, 39.00, 38.79; H, 4.44, 4.87; Br, 30.57, 30.36. The carbon analyses are high and the bromine analyses are low because of the tendancy of the molecule to lose hydrogen bromide on distillation.

Bis-(tetrahydropyranyl)Bromomethylitaconate (XIb).

A suspension of 5.5 g (0.02 mole) of bromomethylitaconic acid (VIII) in 12 ml of dry benzene was cooled in ice then treated with 10.4 g (0.121 mole) of dihydropyran. The ice-bath was removed and the reaction mixture was stirred for 12 h at room temperature. At this point, the solid had completely dissolved. The reaction was followed by observing the disappearance of the broad carboxylic acid band at 3330-2500 cm^1 in the infrared spectrum.

The completed reaction was evaporated on the rotary evaporator at 25° yielding 9.49 g of yellow oil. Nmr (CCL_4): two one-proton vinyl singlets at δ6.56 and 5.93, two-proton broad tetrahydropyranyl methine singlet at δ6.1, seven-proton broad multiplet at δ3.8 and twelve-proton multiplet at δ1.8. Infrared (neat): 1724 cm^{-1}. This is a relatively unstable substance and should be stored in the cold. It was reacted with vitamin B_{12s} directly as prepared with no further purification.

General Procedure for the Formation of Carbon-Cobalt Bonded Adducts with Vitamin B_{12}.

Adduct XIIa of Dimethyl Bromomethylitaconate (XIa) and Vitamin B_{12}.

A special double flask (Figure 3) was used in this and in all experiments leading to organo cobalamin adducts described here.

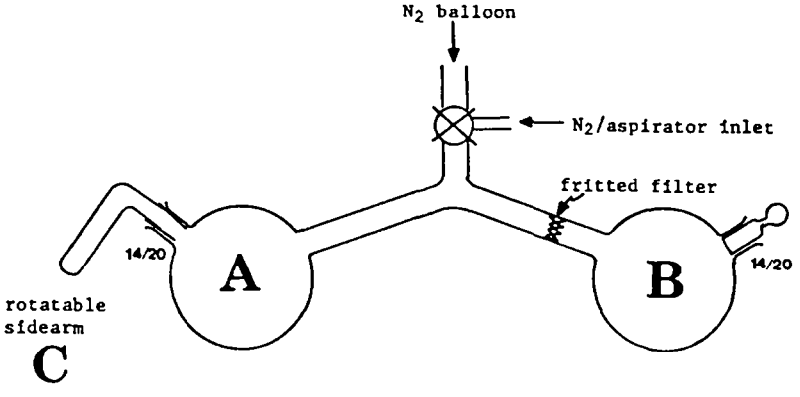


FIGURE 3

A solution of 4.0 g (0.003 mole) of hydroxocobalamin in 270 ml of water was placed in flask A (Figure 3). The right angle rotatable sidearm (C), attached to flask A by a 14/20 joint, contained a solution of 3.5 g (0.092 mole) of sodium borohydride in 30 ml of water. In flask B was placed 4.83 g (0.019 mole) of dimethyl bromomethylitaconate (XIa). A three-way stopcock with attached balloon was joined to the top of the curved tube connecting the two flasks. The apparatus was throughly purged of oxygen by evacuating (water aspirator) and flushing with nitrogen ten times. The balloon was then firmly inflated with nitrogen.

The closed side arm C containing the sodium borohydride solution was rotated 180° allowing the reducing agent to enter flask A and react with the hydroxocobalamin. The solution immediately became brown (vitamin B_{12r}). After 10 min, the grey-green color characteristic of vitamin B_{12s} was observed. The reaction was allowed to stir for an additional five minutes then removed to a dark room for the next stage of the reaction. Under dim red light, the grey-green solution of vitamin B_{12s} was filtered into flask B containing the dimethyl bromomethylitaconate (XIa) and allowed to react for 30 min. An ambient temperature water bath was used to prevent the exothermic reaction from becoming warm. A visible spectrum, taken after 30 min reaction time, showed absorption typical for an alkyl cobalamin (vide infra) with, in particular, the ratio of intensities at $\lambda_{337.5}:\lambda_{525}$= 1.65 indicating completion of the reaction.

The reaction mixture was treated with 30 ml of a 50:50 mixture of phenol and methylene chloride and extracted. The aqueous layer was washed with six 5 ml portions of the 50:50 phenol-methylene chloride solution. The combined phenol extracts were washed with two 10 ml portions of water and the water was backwashed with two 3 ml portions of the 50:50 methylene chloride-phenol mixture. The total combined phenol extracts were then diluted with 15 volumes of methylene chloride and extracted with fifteen 20 ml portions of water. The combined water extracts were then washed with three 200 ml portions of methylene chloride. The aqueous solution was concentrated at 0.1 mm. The residue from the evaportion was dissolved in 50 ml of water, precipitated by the addition of 1 L of acetone and allowed to crystallize overnight at 5°. Yield: 3.96 g of red solid adduct (XIIa). A portion of this material (0.315 g) was twice recrystallized at room temperature using 1.3 ml of water and 13 ml of acetone; yielding 0.296 g of product XIIa homogeneous on thin-layer chromatography (E. Merck, 0.1 mm, cellulose F plates); R_{CN} (solvent): 1.38 (2-butanol, water, 25% ammonium hydroxide; 50:36:14), 1.47 (1-butanol, 2-propanol, water; 37:26:37). Visible spectrum: λ_{max}^{water} (ε) 525 nm (6.510), 375 nm (7.960) and 333 nm (10,450) (Figure 1a). On brief (2 min) exposure to room light, the visible spectrum is transformed to that of hydroxocobalamin with its characteristic intense band at 352 nm. The mass spectra of the alkyl cobalamin XIIa showing M^++H and M^--H corresponding to the molecular ions at m/e 1501 and 1499 in the positive and negative ion mass spectra have been reported.[36]

Adduct XIIb of Bis-(tetrahydropyranyl) Bromomethylitaconate (XIb) with Vitamin B_{12}.

The double-flask apparatus, described above in Figure 3, for the preparation of the methyl ester XIa, was used. The experimental procedure was similar to that described above. A solution of 2.5 g (0.0018 mole) of hydroxocobalamin (Merck) in 160 ml of water was treated under an atmosphere of nitrogen with 1.25 g (0.032 mole) of sodium borohydride. After 10 minutes, the grey-green color indicative of the presence of vitamin B_{12s}, was produced. The reaction was removed to the dark room. Under dim red light, the solution of vitamin B_{12s} was added to 4.5 g (0.011 mole) of bis-(tetrahydropyranyl)-bromomethylitaconate (XIb).

The reaction mixture was stirred for 15 minutes at room temperature. The visible spectrum showed that the reaction was complete. The product alkyl cobalamin (XIIb) was precipitated from the aqueous reaction mixture by addition of 2.5 liters of ice-cold acetone. The resulting precipitate was removed by filtration, then twice suspended and dispersed in 100 ml portions of ice-cold acetone and refiltered, yielding 3.6 g of red solid. The product, a mixture of the desired alkyl cobalamin (XIIb) and inorganic salts from the reaction, resisted efforts to apply more satisfactory purification procedures. The alkyl cobalamin XIIb decomposes to hydroxocobalamin, shown by the rapid development of the band at 352 nm in the visible spectrum, on attempted extraction into phenol or on attempted chromatography.

The alkyl cobalamin XIIb was characterized by its visible absorption spectrum: λ_{max}^{water} (ε) 525 nm (7,280), 438 nm (4.480, sh), 372 nm (9,120, sh), 337 nm (12,150), 312 nm (12,660) and λ_{min}^{water} (ε) 410 nm (3,820) (Figure 1b). In acid solution the visible spectrum showed: $\lambda_{max}^{0.1N\ HCl}$ (ε) 460 nm (6,400), 375 nm (4,500, sh), 352 nm (3,200). When the acid solution was made basic with potassium hydroxide, the spectrum returned nearly to that of the original alkyl cobalamin with λ_{max} (ε): 510 mμ (5,400) and with, in addition, a new peak at 352 nm (9,400) signalling some decomposition into hydroxocobalamin. The intensity (ε) values cited above were obtained indirectly. The samples were exposed to light thereby converting the alkyl cobalamin to hydroxocobalamin. The 352 nm band of the latter substance, being of known intensity, was used as a standard with which the intensity of the band of the unknown XIIb could be compared.

Dark, Ambient Cleavage and Rearrangement of the Cobalamin Adduct XIIb.

A solution of 2.31 g of tetrahydropyranyl ester cobalamin XIIb, (from 1.639 g (0.0013 mole) of hydroxocobalamin), in 75 ml of water was prepared. The pH of the resulting solution was 10.4; it was lowered to 8.4 by the addition of acetic acid. The reaction was allowed to stand at ambient temperature (ca. 25°) under nitrogen, in the dark for 200-300 hrs. The reaction was monitored by daily withdrawal of a small sample for spectral analysis. A steady increase in intensity of the band at 352 nm, signaling the appearance of hydroxocobalamin, was observed. After 230 h when the increase in intensity of the 352 nm band had ceased, the reaction was made acidic by the addition of 10 ml of 10% hydrochloric acid. The reaction mixture was then extracted continuously overnight with 200 ml of ether. The ether extract was dried over anhydrous magnesium sulfate then concentrated to 0.107 g of an oily mixture containing the

organic ligand and its transformation products. The mixture was passed through a short (1.1 x 10 cm) column containing 3.6 g of silica gel and eluted with a 1:1 mixture of ethyl acetate and n-hexane yielding 0.073 g of an oil. The oil was then rechromatographed on a (1.1 x 33 cm) column of 12 g of silica gel. The column was developed by successive washing with 100 ml portions of 16, 16, 18 and 21% (vol/vol) of ethyl acetate in hexane. Further elution with 21% ethyl acetate in hexane, collecting 50 ml fractions, yielded 0.044 g of solid material largely α-methyleneglutaric acid (IV) in fractions 1-3. Fractions 4 and 5 contained 0.014 g of solid material which was large methylitaconic acid (III), contaminated with a small amount of α-methyleneglutaric acid (IV).

Fractions 1-3 were combined and recrystallized three times from ether-carbon tetrachloride; yield: 0.026 g (14%) of α-methyleneglutaric acid IV, mp 130.5-132.5°C, reported[27] mp 130-132°C. Fractions 4 and 5 were combined and recrystallized five times from ether-carbon tetrachloride; yield 0.012 g (7%) of methylitaconic acid III, mp 153-154°C, reported[28] mp 150-152°C.

Fraction 7 from this series of fractions contained a white solid which, after three recrystallizations from ether-chloroform, yielded 0.006 g (3%) of butadiene-2,3-dicarboxylic acid VII, mp 179-182°C reported[26] mp 185-187°C.

The three products III, IV, and VII from the chromatography exhibit spectral properties identical in all respects to those of authentic samples of III, IV and VII.

Photolysis of the Bis-(tetrahydropyranyl)methylitaconate Cobalamin Adduct XIIb.

The basic features and procedures of this experiment are similar to those of the dark reaction described above. A solution of 2.64 g of the tetrahydropyranyl ester cobalamin XIIb (from 1.78 g (1.32 mmol) of hydroxocobalamin), in 310 ml of water (pH 7-8) was placed in a two-neck, 500 ml round-bottom flask, equipped with a serum cap and an adapter carrying a nitrogen-filled balloon. The solution was evacuated and flushed with nitrogen five times, then the balloon was firmly inflated with nitrogen. The solution was irradiated with a 250 watt Westinghouse sunlamp placed 35 cm from the reaction vessel. Progress of the reaction was monitored by observing the development of the intense visible absorption band at 352 nm corresponding to the formation of hydroxocobalamin. The spectral change was complete after 56 hours of irradiation. The red solution was then extracted continuously with ether, for 12 hr. The ether solution was dried over sodium sulfate and evaporated to 0.058 g of a white semi-solid showing a nondescript proton nmr spectrum.

The aqueous solution was then made acidic by the addition of 2.8 ml of concentrated hydrochloric acid and extracted continuously overnight with ether. The resulting ether extract was dried over sodium sulfate and concentrated to 0.540 g of an oily semi-solid. Trituration with ether left 0.245 g of white substance, mp >300° which was not further investigated. The ethereal filtrate was concentrated to 0.295 g of a yellow semi-solid, shown by examination of the nmr spectrum to contain methylitaconic acid (III), α-methyleneglutaric acid (IV) and butadiene-2,3-dicarboxylic acid (VII) as components of the mixture.

Chromatogaphic separation of the reaction products, in the manner described for the dark reaction above, yielded 0.014 g (7%) of a α-methyleneglutaric acid (IV), 0.007 g (4%) of methylitaconic acid (III), and 0.005 g (3%) of butadiene-2,3-dicarboxylic acid (VII). The identities of the products were ascertained by spectral comparison with authentic samples.

Photolysis of the Dimethylmethylitaconate-Cobalamin Adduct XIIa.

In the dark, a solution of 3.96 g (0.0026 mole) of the dimethyl methylitaconate-cobalamin adduct XIIa in 280 ml of water was placed in a 500 ml, round-bottom flask equipped with three-way stopcock adapter carrying a nitrogen-filled balloon. The system was evacuated at the aspirator and flushed with nitrogen five times. The deep red solution was then irradiated with a Westinghouse 250 watt sunlamp placed 35 cm distant from the reaction vessel. The progress of the photolysis was monitored daily by following the development of the band at 352 nm in the visible spectrum, signalling the rise of the hydroxocoblamin and loss of the carbon-cobalt bond. After 120 hrs the reaction was complete, and it was worked up by continuous extraction for 24 hr with 300 ml of ether. The ether extract was dried over anhydrous sodium sulfate and concentrated to 0.064 g (14%) of a colorless liquid. This product was identical in its infrared and nmr spectra to those of an authentic sample of dimethyl methylitaconate. No α-methyleneglutarate was detected.

Control Reaction of Bis-(tetrahydropyranyl)bromoethylitaconate (XIb) with Sodium Borohydride and Cobalt Nitrate Under Rearrangement Reaction Conditions.

In this experiment, an attempt was made to follow the procedures of the above rearrangement reaction experiments. The purpose was to expose the bromomethylitaconate XIb to the reducing conditions of the rearrangement reaction. However, there are some unavoidable deviations. Since no vitamin B_{12} was present, the behavior and appearance of the control reaction mixtures differed from the actual rearrangement reaction experiments. In the step

requiring the addition of acetone, no precipitate formed in the absence of vitamin B_{12}. As noted below, the acetone was evaporated and the sequence was carried forward with the total aqueous reaction mixture.

Thus, a solution of 0.5 g (0.0017 mole) of cobalt nitrate hexahydrate in 100 ml of distilled water was placed under an atmosphere of nitrogen by evacuating and flushing with nitrogen. Under a positive pressure of nitrogen, 1.2 g (0.032 mole) of sodium borohydride was added. The resulting dark solution was then added to the bis-tetrahydropyranyl bromomethylitaconate (XIb) and stirred at room temperature in the dark for twenty minutes. The resulting dark brown solution was poured into 300 ml of acetone and allowed to stand for 30 min with occasional shaking. Acetone was evaporated at the aspirator. The resulting aqueous solution was diluted to a total volume of 120 ml by the addition of water. The solution was then irradiated with a 250 watt Westinghouse sunlamp placed 35 cm distant from the reaction vessel. After 84 hours of photolysis the reaction was stopped and work up by continuous overnight extraction with ether. The ether was dried with anhydrous sodium sulfate and concentrated to 0.412 g of a light-yellow oil which exhibited a nondescript nmr.

The aqueous solution was then made acidic (pH 2) by the addition of concentrated hydrochloric acid and extracted continuously overnight with ether. The ether solution was dried over anhydrous sodium sulfate and concentrated to 0.550 g of a somewhat green oil. The nmr spectrum of this substance showed the presence of butadiene-2,3-dicarboxylic acid (VII) and a product tentatively identified as the saturated lactone α-methyl-γ-butyrolactone-β-carboxylic acid by comparison with XVI. No absorption at δ2.6 characteristic of the rearrangement product α-methyleneglutaric acid (IV) was observed.

When the oil was dissolved in 1 ml of ether and allowed to crystallize overnight, 0.088 g (15%) of white powder, mp 185-188, appeared whose nmr spectrum was identical to that of butadiene-2,3-dicarboxylic acid (VII). Further crops of 0.080 g and 0.018 g were found by nmr to be a mixture of butadiene-2,3-dicarboxylic acid (VI) and the saturated lactone.

Reaction of Vitamin B_{12s} with Butadiene-2,3-dicarboxylic Acid (VI).

A solution of 0.236 g (0.175 mmole) of hydroxocobalamin in 5 ml of 50% aqueous ethanol was treated, under an atmosphere of nitrogen, with 0.235 g (0.006 mole) of sodium borohydride. After 10 min the grey-green color of vitamin B_{12s} appeared. The reaction was removed to the darkroom where the vitamin B_{12s} solution was added to 0.050 g (0.352 mmoles) of butadiene-2,3-dicarboxylic acid (VI). The course of the reaction was followed by visible spectroscopy. There was no evidence of the formation of any carbon-cobalt bonded product as judged both by the appearance of the visible spectrum and by the lack of sensitivity to light. The spectrum observed under normal conditions was that of vitamin B_{12r} and when taken under N_2 in a degassed cell, that of vitamin B_{12s}.

The reaction mixture was concentrated on the rotary evaporator at 30-35°(water bath). Trituration of the resulting red residue with ether-acetone yielded only 0.002 g of nondescript product. The red residue was then dissolved in 15 ml of water and made acid by the addition of concentrated hydrochloric acid. The acid solution was extracted with five 20 ml portions of ether. The resulting solution was dried over anhydrous sodium sulfate and concentrated to 0.045 g (90%) of a white powder mp 150-152°C. The infrared and nmr spectra of the product were identical to those of authentic methylitaconic acid (III).

Preparation of Authentic Samples.

α-Methyleneglutaric Acid (IV). α-Methyleneglutaric acid (IV), mp 130-132°C, was prepared according to Buchman Reims and Schlatter[27] or by the trialkyl phosphine promoted dimerization of ethyl or methyl acrylate[34] followed by ester hydrolysis. Nmr (acetone-d_6): four-proton aliphatic singlet at δ2.6 and two one-proton vinyl singlets (slightly split) at δ5.6 and 6.2.

Methylitaconic Acid (III).

Triethyl Prop-2-ene-1,1,2-tricarboxylate (XIII). Malachowski's 3:2 mixture of triethyl prop-2-ene-1,1,2-tricarboxylate and triethyl prop-1-ene-1,1,2-tricarboxylate[35] (73.5 g, 0.284 mole) in 200 ml of benzene was flushed with nitrogen and cooled in an ice-water bath. Sodium hydride (14.1 g, 0.312 mole, 53.4% in mineral oil) was added slowly over a period of approximately 30 min. The solution was allowed to warm to room temperature and stirred for 2.5 h.

The mixture was cooled in ice and quenched by the addition of 26 ml of cold concentrated hydrochloric acid in ice (~30 ml total volume). The mixture was stirred 5 min then poured into 200 ml of ether and extracted successively with 10 ml of 6% $NaHCO_3$ solution and two 10 ml portions of brine. The ether solution was dried over sodium sulfate, decolorized with Norit and evaporated to 83.8 g of an oil mixed with mineral oil. After removing the mineral oil, 74.3 g of product XIII was obtained. The product can be used directly or distilled, bp 105-110°/4mm. Nmr ($CDCl_3$): δ1.3 (t, J=7 Hz, 9H), 4.2 (q, J=7 Hz, 6H), 4.45 (s, 1H), 5.8 (s, 1H) and 6.4 (s, 1H). The vinyl methyl group at δ2.2 corresponding to the Δ^1-isomer was absent from the nmr spectrum.

Triethyl But-3-ene-2,2,3-tricarboxylate (XIV). The Δ^2-triester XIII (22.3 g, 0.086 mole) in 180 ml of acetone was treated with 16 g (0.11 mole) of methyl iodide. The solution was cooled in ice and 15.5 g of anhydrous potassium carbonate was added.

The orange-brown mixture was stirred for 48 h at room temperature. The mixture was filtered, the filter cake was washed with 200 ml of ether and the filtrate was concentrated. The residue was taken into 200 ml of ether and washed with saturated 10 ml of sodium thiosulfate solution and with two 10 ml portions of brine. The ether layer was dried over sodium sulfate and concentrated to 23.5 g of XIV as a light yellow oil. Nmr (CCl_4): δ1.28 (m, 9H), 1.8 (s, 3H), 4.2 (m, 6H), 5.6 (s, 1H), 6.2 (s, 1H).

Anal. Calc'd for $C_{13}H_{20}O_6$: C, 57.34; H, 7.40. Found: C, 57.35; H, 7.61.

Alternatively, Malachowski's mixture of Δ^1- and Δ^2-propenetricarboxylates can be converted to the common anion then alkylated with methyl iodide.

Methylitaconic Acid (III). Triethyl but-3-ene-2,2,3-carboxylate (XIV) (23.5 g, 0.087 mole) in 50 ml of water was cooled in an ice-water bath, treated with 46.4 g (0.15 mole) of $Ba(OH)_2 \cdot 8H_2O$ and stirred for one day at room temperature. The white suspension was transferred to a continuous extractor using 20 ml of water. The aqueous solution was continuously extracted for 3 h with ether then acidified by the addition of 25 ml of concentrated hydrochloric acid. After 24 h, 12.1 g of yellow-brown oil was obtained which partially crystallized on standing. The semisolid was taken up in 60 ml of 20% hydrochloric acid solution and heated at 40° for 48 h. Continous extraction then yielded, after two crystallizations from ether-$CHCl_3$, 10.035 g (82%) of III, mp 146-150°C, reported[39] mp 150-152°C. Nmr (acetone-d_6): δ1.35 (d, J=7 Hz, 3H), 3.36 (dq, J=7,1 Hz, 1H), 5.8 (t, J=1 Hz, 1H), 6.3 (d, J=1 Hz, 1H).

α-Methylene-γ-butyrolactone-β-carboxylic Acid (X).

Diethyl α-Methylene-γ-butyrolactone-β-dicarboxylate (XV). Triethyl prop-2-ene-1,1,2-tricarboxylate (XIII) (32.7 g, 0.13 mole) was treated with 13.4 g (0.16 mole) of 35% formaldehyde solution and 1.014 g (0.01 mole) of potassium bicarbonate. The mixture was stirred at 65°C for 25 min. Extraction with ether, followed by evaporation, yielded XV as a light-yellow oil weighing 30.5 g. This was used directly in the next step. Nmr (CCl_4): δ1.35 (t, J=7 Hz, 6H), 4.3 (q, J=7 Hz, 4H), 4.61 (s, 2H), 6.15 (s, 1H) and 6.5 (s, 1H).

α-Methylene-γ-butyrolactone-β-carboxylic Acid (X). The lactone diester XV (4.4 g, 0.018 mole) was heated at 38°C for 52 h in 125 ml of 20% hydrochloric acid. Removal of the solvent yielded 3 g of crude product which was dissolved in ether, filtered, then treated with hexane to the point of turbidity. This procedure yielded 1.4 g (54%) of crystalline X, mp 103-105°. Several further recrystallizations from ether-hexane yielded material with mp 105-107 from which point it was unchanged on further recrystallization. Nmr (acetone-d_6 and $CDCl_3$) δ 4.4 (m, 3H), 6.0 (d, J=1 Hz, 1H), 6.3 (d, J=1 Hz, 1H). Ir(KBr): 3400, 2950, 1770, 1710, 1670 cm^{-1} Mass spectrum (15 eV), m/e (relative intensity): 142 (M^+, 10), 113 (M^+-HCO, 100), 96 (M^+-46, 20), 84 (M^+-58, 15), 69 (M^+-83, 10). Exact mass: calc'd. for $C_6H_6O_4$: 142.0268. Found: 142.0266.

α-Methyl-γ-butyrolactone-β-carboxylic Acid (XVI). A solution of 0.045 g (0.3 mmole) of X in 4 ml of ethanol was hydrogenated for 2 hr using Pd/C as catalyst. After filtration and evaporation of the solvent, the product XVI was obtained as a solid, mp 115-118°C, weighing 0.032 g (70%). Nmr (acetone-d_6): δ1.25 (d, J=7 Hz, 3H), δ3.0 (p. J=7 Hz, 1H), δ3.4 (m, 1H), δ4.4 (m, 2H); ir(KBr): 3000, 1775 and 1740 cm^{-1}; mass spectrum (70 eV), m/e(rel. int.) 14 (M^+, 68), 113 (87), 100 (M^+-CO_2, 100), 85 (50).

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