Total Synthesis of Acetate from Carbon Dioxide. Retention of Deuterium during Carboxylation of Trideuteriomethyltetrahydrofolate or Trideuteriomethylcobalamin†

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ABSTRACT: A mass analysis of acetate formed from trideuteriomethylcobalamin or 5-trideuteriomethyltetrahydrofolate in fermentations of α-ketobutyrate by an enzyme preparation from *Clostridium thermoaceticum* has shown that mono-, di-, and trideuterioacetate are formed. More than 50% was trideuterioacetate whether the precursor was trideuteriomethylcobalamin, 5-trideuteriomethyltetrahydrofolate, or a 1:1 mixture of the deuterio and hydrogen methyl precursors. No deuterium isotope effect was observed in the formation of acetate. Three mechanisms have been considered: (1) a Grignard-type reaction involving addition of CO_2 to a methyl carbanion, (2) carboxylation of the methyl group with displacement of hydrogen to form a carboxymethyl group and then return of the displaced hydrogen to form the acetate by an intramolecular reaction, and (3) as in two but the reaction to form acetate is by an intermolecular reaction. Mechanism three seems to be excluded since the proportion of tri-, di-, and monodeuterioacetate was the same whether or not only a trideuterio precursor or a 1:1 mixture of deuterio and hydrogen precursors was used. Mechanism two, the intramolecular hydrogen transfer, would account for this unaltered proportion of the types of deuterioacetates but an isotope effect would be expected because of the cleavage and re-formation of the hydrogen bond. The isotope effect would only be observed, however, if the step was or became a rate-limiting step. Thus the results favor the carbanion mechanism but are not completely conclusive. The intramolecular mechanism or the carbanion mechanism does not account for the formation of di- and monodeuterioacetate from the trideuteriomethyl precursors; these forms may result from secondary exchange reactions.

The bacterium *Clostridium thermoaceticum* performs a total synthesis of acetate from CO_2. The methyl group of the acetate is formed by reduction of CO_2 to formate which is converted to 5,10-formyl-THF[1] and by further reduction to H_3C-THF. The methyl group is then transferred to a cobalt of a corrinoid enzyme yielding a cobalt–methylcorrinoid enzyme complex which is carboxylated to yield acetate (Ljungdahl and Wood, 1969).

We have earlier proposed two mechanisms for the carboxylation step (Ljungdahl *et al.*, 1966) and these are outlined in Figure 1 using deuterated precursors. The first mechanism involving a carboxymethylcorrinoid as an intermediate was proposed by Ljungdahl *et al.* (1965). They isolated a highly radioactive compound from *C. thermoaceticum* after exposure to $^{14}CO_2$ for 15 sec which on photolysis under aerobic conditions yielded radioactive products similar to those obtained by photolysis of synthetically prepared *Co*-carboxymethylcobalamin (Ljungdahl and Irion, 1966). In addition the cleavage of *Co*-carboxymethylcobalamin to acetate was catalyzed in the presence of TPNH by a protein isolated from *C. thermoaceticum* (Ljungdahl *et al.*, 1965, 1967). The second mechanism is based on a suggestion by Ingraham (1964) that H_3C-B_{12} may act as a biological Grignard reagent. A third mechanism has recently been proposed by Schrauzer and Sibert (1970). They suggest that a dithiol facilitates the reductive cleavage of H_3C-B_{12} with formation of a methyl carbanion, which reacts with CO_2 to form acetate. Evidence for the participation of a thiol in the formation of acetate in extracts of *C. thermoaceticum* has been obtained by Poston *et al.* (1966) who found that the formation of acetate is inhibited by sulfhydryl reagents.

Ghambeer *et al.* (1971) have prepared extracts of *C. thermoaceticum* which effectively convert the methyl group of $H_3{}^{14}C$-B_{12} or $H_3{}^{14}C$-THF to the methyl group of acetate during fermentations of pyruvate or α-ketobutyrate. To further elucidate the mechanism of carboxylation such extracts have been used to convert D_3C-B_{12} and D_3C-THF to acetate. The resulting deuterioacetate has been analyzed in the mass spectrometer. The rate of formation of acetate from D_3C-B_{12} compared to H_3C-B_{12} has been determined as a measure of the deuterium isotope effect.

Methods and Materials

Chemicals. D_3C-B_{12} was prepared from cyanocobalamin using trideuteriomethyl iodide (Hogenkamp and Rush, 1969; Mervyn and Smith, 1969). D_3C-THF was synthesized by

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[1] Abbreviations used are: THF, tetrahydrofolate; H_3C-THF, 5-methyltetrahydrofolate; D_3C-THF, 5-trideuteriomethyltetrahydrofolate; B_{12}, cobalamin; H_3C-B_{12}, *Co*-methylcobalamin; D_3C-B_{12}, *Co*-trideuteriomethylcobalamin; CoE, corrinoid enzyme; H_3C-CoE, cobalt–methylcorrinoid enzyme complex.

catalytic hydrogenation of folic acid with Pt_2O followed by condensation of the resulting tetrahydrofolate with D_2-formaldehyde and reduction to D_3C-THF by $NaBD_4$ as described by Sakami (1963). The D_3C-THF was purified by column chromatography on QAE-Sephadex (Parker *et al.*, 1971) and stored in sealed evacuated vials. All other chemicals were commercial products.

The isotopic purity of the D_3C-B_{12} was not determined directly with the mass spectrometer; instead the D_3CI from which it was synthesized was examined. The results were D_3CI, 95.3%; HD_2CI, 3%; H_2DCI, 1%; and H_3CI, 0.7%. The deuterium content of D_3C-THF was estimated by nuclear magnetic resonance (nmr) spectrometry in D_2O. Methyltetrahydrofolate has a peak at τ 7.5, that is characteristic of the hydrogen of the 5-*N*-methyl group. When the D_3C-THF was assayed the peak at τ 7.5 was absent, indicating that the 5-methyl group was composed of greater than 90% of D_3C groups.

Procedure. Enzyme preparations from *C. thermoaceticum* were precipitated with ammonium sulfate and dialyzed as described by Ghambeer *et al.* (1971). α-Ketobutyrate was chosen as the substrate since it is converted to propionate and thus does not dilute the deuterioacetate formed from D_3C-B_{12} or D_3C-THF. The α-ketobutyrate was determined before and after fermentation in deproteinized portions by use of lactate dehydrogenase and the acetate by the spectrophotometric method described by Schulman and Wood (1971). Deproteinization was done by addition of H_2SO_4 to give pH 1. The amount of methyltetrahydrofolate utilized during the fermentation was estimated by determination of the THF liberated as a consequence of methyl transfer. The assay involved the conversion of the THF to the methenyl derivative by addition of formic acid and heating for 5 min at 100° (Rosenthal *et al.*, 1965). The resulting methenyltetrahydrofolate was measured spectrophotometrically at 350 nm. Protein was assayed according to Gornall *et al.* (1949).

It was found that the amount of methyl-B_{12} converted to B_{12} could not be determined by spectral change since the B_{12} was absorbed on the protein during the deproteinization and could not be recovered quantitatively. By use of a tracer amount of $H_3{}^{14}C$-B_{12} the conversion of the methyl of H_3C-B_{12} to the methyl of acetate was determined by measuring the radioactivity in the acetate as described by Ghambeer *et al.* (1971). The ^{14}C was determined by the liquid scintillation technique.

The acetate was separated from the deproteinized solution by continuous extraction with ether for 48 hr. The acid in the ether extract was titrated with 0.2 N sodium hydroxide every 8 hr to determine that the extraction was complete. The resulting extract was evaporated to dryness, dissolved in 2 N H_2SO_4, and applied to a column of 10 g of Celite 535 (Swim and Kramptiz, 1954). The propionic acid which emerged first from the Celite column was determined by titration. Those fractions which contained acetic acid also contained α-ketobutyric acid. These fractions from expt I–IV were titrated with 0.2 N NaOH and were pooled, taken to dryness, redissolved in 1.0 ml of water, and treated with 10 ml of saturated ceric sulfate (in 10 N H_2SO_4) followed by 5 ml of 5 N dichromate. The α-ketobutyric acid is oxidized to propionic acid by ceric sulfate (Krebs, 1937) and the dichromate oxidizes some other compounds which might be present. The oxidized solution was steam distilled, and the acetic acid was separated from the propionic acid resulting from the oxidation of α-ketobutyric acid by chromatography on a second Celite column. The neutralized acetic acid fractions were combined and dried, and the amount of deuterioacetate was determined by mass analysis. In expt V and VI the acetic, α-ketobutyric acid fraction of the first Celite column was oxidized with 25 ml of the ceric sulfate solution exclusively instead of the combination of ceric sulfate and dichromate.

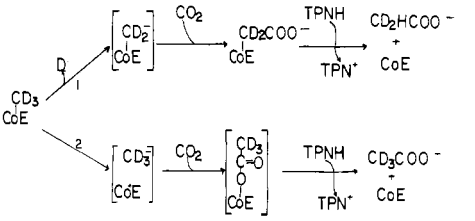

FIGURE 1: The carboxymethyl mechanism (1) and the Grignard mechanism (2) for formation of acetate from methyl-B_{12} and CO_2 and proposed use of trideuteriomethyl precursors to differentiate between the mechanisms.

Mass Analysis of Deuterioacetate. The acetate was dissolved in H_2O and approximately 100 μmoles in no more than 0.2 ml of solution was placed in a clean dry 10-ml test tube with a ground-glass stopper. Concentrated HCl (10–15 μl) was added so that the resulting pH was approximately 1. Anhydrous diethyl ether (1 ml; distilled and stored over sodium wire in a dark bottle) was added; the tube was stoppered and shaken for about 2 min to equilibrate the phases. The aqueous phase was removed with a Pasteur pipet and the ether phase which contained about 60% of the original acetic acid was shaken with two portions of P_2O_5 (about 10 mg). The ether solution was placed in a 0.5 × 9 cm test tube fitted with a high-vacuum stopcock and a 10/30 ground-glass joint for attachment to the mass spectrometer. The main portion of the ether was removed at −78° (Dry Ice–propanol) on a vacuum system and the rest at −78° by the pumping system of the mass spectrometer which was a cyclical type (Model 21–130). After being evacuated to a pressure of 1×10^{-7} mm, the frozen acetic acid mixture was allowed to warm. Under these conditions the entire sample was vaporized and introduced into the sample inlet system to give pressures of 30–80 μ measured by a flexible membrane micromanometer. The sample was introduced into the mass spectrometer through a gold foil leak and ionized at 70 eV and 20 μA of filament current. Voltage measurements to five decimals of *m/e* 60, 61, 62, and 63 corresponding to H_3CCOOH, $H_2DCCOOH$, HD_2CCOOH, and D_3CCOOH were determined using a John Fluke differential voltmeter connected across the output of the amplifier. The data are presented as the per cent composition of the types of deuterated acetate.

Conditions for the Enzyme Reaction. The experiments of Table I were carried out in 125-ml conical flasks. The enzyme preparation, α-ketobutyrate, ferrous ammonium sulfate, thiamine pyrophosphate, and sufficient water to give the desired volume were added at 4°. The flask was stoppered with a serum cap, evacuated through a syringe needle, and then flushed with argon. The process was repeated five times at 4°. The $KHCO_3$ and the methyl substrates were then injected with a syringe, the contents were mixed, and incubation was at 57°. Small aliquot samples were withdrawn with a syringe and were analyzed for utilization of $H_3{}^{14}C$-B_{12} and H_3C-THF by methods described above. At the conclusion of the incubation the mixture was acidified with sulfuric acid to pH 1. The suspension was centrifuged at 3000*g* for 10 min, and the supernatant solution was used for isolation of the acetate.

TABLE I: Formation of Acetate from Deuteriomethyl-B_{12} and from Deuteriomethyl-THF by an Enzyme Preparation from *C. thermoaceticum* Fermenting α-Ketobutyrate.[a]

Experiment		I D_3C-B_{12} (μmoles)	II D_3C-B_{12} H_3C-B_{12} (μmoles)	III D_3C-THF (μmoles)	IV D_3C-THF H_3C-THF (μmoles)	V D_3C-B_{12} (μmoles)	VI D_3C-THF (μmoles)
Methyl compound utilized			85	128	134		
Acetate by spectroassay	Final	365	371	368	336		
	Initial	16	16	34	35		
	Net	349	355	334	301		
Acetate isolated		575	566	880	824	290	264
α-Ketobutyrate utilized		2410	2370	2310	2180	2900	2195
Propionate formed		787	845	670	776	1100	710

[a] The results are given in μmoles/100 ml of fermentation mixture. The volume in expt I, II, and V was 120 ml with 1950 mg of protein in expt I and II and 1620 mg in expt V and contained 0.1 M potassium phosphate (pH 7.0), 4×10^{-3} M cysteine, 0.06 M α-ketobutyrate, 0.003 M ferrous ammonium sulfate, 0.001 M thiamine pyrophosphate, and 0.03 M $KHCO_3$, and in expt I 200 μmoles of D_3C-B_{12}, expt II 200 μmoles of methylcobalamin containing 50% D_3C-B_{12} and 50% $H_3{}^{14}C$-B_{12}, and in expt V 220 μmoles of D_3C-B_{12}. Incubations were at 57° and for 80 min (expt I and II) and 135 min (expt V). The volume in expt III, IV, and VI was 100 ml with 1840 mg of protein in expt III and IV and 1500 mg in expt VI. The concentrations of other components were the same as in experiments I, II, and V except the methyl-B_{12} was replaced by 400 μmoles of DL-D_3C-THF in expt III and VI and by 400 μmoles of DL-methyl-THF containing 50% D_3C-THF and 50% H_3C-THF in expt IV. Incubations were at 57° and for 110 min (expt III and IV) and for 90 min (expt VI).

The experiments of Table III were done similarly but in a volume of 3 ml in test tubes. In these experiments the rates of the formation of acetate from D_3C-B_{12} or H_3C-B_{12} were measured to determine if there was an isotope effect.

Results

Six experiments for mass analysis of the acetate were done with the deuteriomethyl compounds, two with D_3C-B_{12}, one with an equal mixture of D_3C-B_{12} and $H_3{}^{14}C$-B_{12}, two with D_3C-THF, and one with an equal mixture of D_3C-THF and H_3C-THF. The purpose of using mixtures of deuterated and nondeuterated methyl compounds was for the detection of intermolecular hydrogen transfer as will be considered in the Discussion.

Products of Fermentation. The progress of the conversion of the $H_3{}^{14}C$-B_{12} to acetate in expt II was determined by removal of samples at 30, 50, and 80 min. The radioactivity in the isolated acetate was 2800, 7740, and 7640 cpm, respectively, from 1 ml of the fermentation mixture. Apparently the utilization of $H_3{}^{14}C$-B_{12} stopped after 50 min. About 50% of the methyl-B_{12} had been utilized at this time. The yield of THF from the methyl-THF was 0.4, 0.77, and 1.28 μmoles per ml in expt III at 30, 50, and 110 min. It was 0.66, 0.96, and 1.34 in expt IV at the same times. These amounts are equivalent to 64 and 67%, respectively, of the available methyl-THF if it is assumed that only one isomer is utilized.

The yields of acetate, amounts of methyl compounds and α-ketobutyrate utilized and the propionate formed are shown in Table I. Although α-ketobutyrate was used as a substrate to avoid formation of acetate from this source it is evident from the results of Table I that there was a very substantial yield of acetate which did not originate from the methyl precursors. The amounts of methyl precursors utilized were 85, 128, and 134 μmoles, respectively, in expt II, III, and IV but the net acetate formed as determined by spectrophotometric assay varied from 301 to 355 μmoles for expt I–IV. Furthermore, during the isolation of the acetate from these experiments there was additional formation of acetate since the isolated acetate varied from 566 to 880 μmoles. Thus in expt II 85% of the acetate [(566–85/566)100] is estimated to have been formed from nonmethyl precursors, and in expt III and IV 86 and 84%, respectively. The source of the acetate which was formed during the isolation *per se* is unknown. The fractions from the first Celite column which contained the acetate and α-ketobutyrate were oxidized with ceric sulfate and dichromate to remove the α-ketobutyrate. There may have been unknown compounds present in these fractions which were oxidized to acetate. Possibly the ether used in the extractions contained contaminants. During the isolation of acetate from expt V and VI, the oxidation was performed with ceric sulfate only and this was done carefully to avoid excess of oxidant. In these experiments much less acetate was isolated, which seems to indicate that at least some of the acetate in expt I–IV was formed during the oxidation. Unfortunately the acetate formed during the incubations in expt V and VI was not determined.

The amount of α-ketobutyrate utilized during the fermentation was substantial, 2180 to 2900 μmoles. The fermentation is considered to occur in whole cells as follows

$$4CH_3CH_2COCOOH + 4H_2O \longrightarrow 4CH_3CH_2COOH + 4CO_2 + 8H$$

$$8H + 2CO_2 \longrightarrow CH_3COOH + 2H_2O$$

sum:

$$4CH_3CH_2COCOOH + 2H_2O \longrightarrow 4CH_3CH_2COOH + 2CO_2 + CH_3COOH$$

Accordingly unlabeled acetate would be formed during the fermentation by net synthesis of acetate from CO_2 and a substantial amount of acetate which was formed during the fer-

TABLE II: Types of Deuterioacetate Formed from D_3C-B_{12} or D_3C-THF, during Fermentation of α-Ketobutyrate by an Enzyme Preparation from *C. thermoaceticum*.

Expt No.	Methyl Compound	Non-deuterated Acetate (%)	Deuterated Acetate[a] (%)	Composition of Deuterated Acetate[a]		
				$H_2DCCOOH$ (%)	HD_2CCOOH (%)	D_3CCOOH (%)
I	D_3C-B_{12}	79.2	20.8	11.1	20.7	68.2
II	D_3C-B_{12} + H_3C-B_{12}	80.6	19.4	17.4	23.1	59.5
III	D_3C-THF	82.1	17.9	25.4	25.4	49.2
IV	D_3C-THF + H_3C-THF	88.7	11.3	26.5	23.0	50.5
V	D_3C-B_{12}	77.3	22.7	1.3	4.4	94.3
VI	D_2C-THF	65.9	34.1	16.7	30.8	52.5

[a] These values were determined from the peak heights of *m/e* 60, 61, 62, and 63 and represent the average of seven measurements. There was no measurable ion beam at *m/e* 59, 58, 57, or 56. A sample calculation from the results of the CD_3-B_{12} (expt V) is given. The voltage measurements from the respective *m/e* values were: 60, 0.5444; 61, 0.1498; 62, 0.01002; 63, 0.1507. The contribution of CH_3COOH to *m/e* 61 and 62 is 2.4 and 0.44% of *m/e* 60 due to the normal abundance of ^{13}C and ^{18}O, ^{17}O. The correction is 0.5444 × 0.024 = 0.01307 and 0.5444 × 0.0044 = 0.00239. These values were subtracted from *m/e* 61 and 62 to give *m/e* 61 = 0.01498 − 0.01307 = 0.00191 and *m/e* 62 = 0.01002 − 0.00239 = 0.00763. The total corrected voltage from *m/e* 60, 61, 62, and 63 thus was: 0.5444 + 0.00191 + 0.00763 + 0.1507 = 0.7046. The per cent composition of the isolated acetate was calculated as follows: H_3CCOOH = (0.5444/0.7046)100 = 77.3, $H_2DCCOOH$ = (0.00191/0.7046)100 = 0.3, HD_2CCOOH = (0.00763/0.7046)100 = 1.0, D_3CCOOH = (0.1507/0.7046)100 = 21.4. The per cent composition of the deuterated acids was calculated for expt V as follows: 0.3 + 1.0 + 21.4 = 22.7 and $H_2DCCOOH$ = (0.3/22.7)100 = 1.3, HD_2C-$COOH$ = (1/22.7)100 = 4.4, D_3CCOOH = (21.4/22.7)100 = 94.3.

mentation as determined by spectrophotometric assay could be accounted for from this source. However, tests with $^{14}CO_2$ have shown that enzyme preparations like those used in these experiments (Ghambeer *et al.*, 1971) do not catalyze a significant total synthesis of acetate from CO_2; CO_2 is fixed in acetate only when the methyl precursor is added and almost exclusively in the carboxyl group.

The source of the acetate appears to have been from endogenous compound(s) present in the crude extract. In the absence of added substrates (α-ketobutyrate and the methyl precursor) about 0.12 μmole of acetate was formed per mg of protein in 90 min at 57°. Thus about 220 μmoles might be formed from this source. Attempts to reduce the endogenous formation of acetate by extensive dialysis of the enzyme preparation were not successful and led to inactivation of the enzyme.

It is noted that the propionate formed from the α-ketobutyrate was much less than a mole for mole yield. The additional products which are formed were not identified but under similar conditions, in addition to acetate, acetoin has been observed as a product from pyruvate.

Mass Analysis of the Acetate. The data from the mass analysis of the acetate from experiments with D_3C-B_{12} and D_3C-THF are shown in Table II. The data of columns five to seven show the composition of the acetate based on only those molecules which contained deuterium. These data are presumed to reflect the results which would have been obtained if endogenous acetate had not been produced and diluted the acetate produced from the methyl precursors.

The results from only those molecules which contained deuterium show that from 49 to 94% of the deuterioacetates contained three deuterium with a much higher value for D_3CCOOH in expt V than in any of the other experiments. The latter conversion is almost quantitative since the methyl iodide was 95.3% trideuterio. There were substantial amounts of both mono- and dideuterioacetates formed in expt I–IV and VI.

Test for Exchange of the Methyl Hydrogen of Acetate during Incubation with Enzyme. An enzyme preparation, similar to those used in the deuterium experiment, was tested for capacity of catalyzing an exchange of methyl protons of acetate. Such an exchange would give rise to mono- or dideuterioacetate. For this purpose an enzyme preparation containing 128 mg of protein was incubated for 2 hr at 55° under N_2 with 0.5 μmole of tritiated acetate containing 416,500 cpm in a total volume of 6 ml. The incubation contained 600 μmoles of potassium phosphate (pH 7.0), 24 μmoles of cysteine, 150 μmoles of sodium α-ketobutyrate, and 15 μmoles of ferrous ammonium sulfate and similar incubations were done in absence of enzyme or without the α-ketobutyrate or ferrous ammonium sulfate. After the incubation an aliquot part of the mixture was passed through a small Dowex 2 (50–100 mesh) column which retained the tritiated acetate, while tritium in the water passed through the column. The total amount of tritium found in the water from the incubations in the presence of enzyme varied from 3000 to 6600 cpm, while that from the control without enzyme was 4200 cpm. The acetate was recovered from the incubations by steam distillation and chromatography on Celite and contained from the nonenzyme incubation 416,000 cpm, while from the enzyme incubations 406,000–421,000 cpm. It is concluded that acetate once formed was stable and that the enzyme preparation did not catalyze an exchange of the methyl hydrogens of acetate and water, nor cause loss of hydrogen by side reaction involving the methyl hydrogens of acetate.

Test for an Isotope Effect on the Conversion of Methyl-B_{12} to Acetate. If the conversion of methyl-B_{12} to acetate involved the transfer or replacement of a hydrogen atom from the methyl group as indicated in Figures 2 or 3, there might be an isotope effect and the acetate would be formed at a lower rate

TABLE III: Test for an Isotope Effect on the Conversion of Methyl-B_{12} to Acetate.[a]

Time (min)	I Endogenous (μmoles/ml)	II α-Keto-butyrate (μmoles/ml)	III α-Keto-butyrate + CH_3-B_{12} (μmoles/ml)	IV α-Keto-butyrate + CD_3-B_{12} (μmoles/ml)
30	0.69	0.23	0.67	0.62
60	1.29	0.76	1.52	1.65
90	1.62	1.12	2.37	2.25

[a] The acetate at zero time in micromoles per milliliter was in I, 0.052; II, 0.056; III, 0.074; IV, 0.106. These values have been subtracted from the values shown in the table. The reaction mixture contained in 3 ml 60 mg of protein, 0.03 M $Fe(NH_4)_2SO_4$, 0.001 M thiamine pyrophosphate, 0.03 M $KHCO_3$, and where indicated 0.1 M α-ketobutyrate, 1.1 mM CH_3-B_{12}, and 1.5 mM CD_3-B_{12}.

from D_3C-B_{12} than from H_3C-B_{12}. A comparison of the rates of acetate formation from these substrates is shown in Table III. In addition the rates of formation of acetate are given when α-ketobutyrate but no methyl-B_{12} was added to the enzyme preparation and also without substrates (endogenous formation of acetate). There was a substantial formation of endogenous acetate (expt I) and when α-ketobutyrate was added (expt II) the production of acetate was somewhat lower than the endogenous rate. This inhibition by α-ketobutyrate has been observed repeatedly. Addition of methyl-B_{12} increased the rate of formation of acetate and the increase was the same with H_3C-B_{12} (expt III) as with D_3C-B_{12} (expt IV). Thus there was no observed isotope effect in the formation of acetate from D_3C-B_{12}.

Discussion

The use of D_3C-B_{12} and D_3C-THF appeared to be an attractive tool to distinguish between the carboxymethyl and the Grignard type of mechanism (or any other mechanisms involving a methyl carbanion) for the formation of acetate (Figure 1). If the reaction occurred according to the carboxymethyl mechanism one would expect the formation of dideuterated acetate, while according to the Grignard mechanism trideuterated acetate would be the product. However, the results of these experiments could only have been conclusive

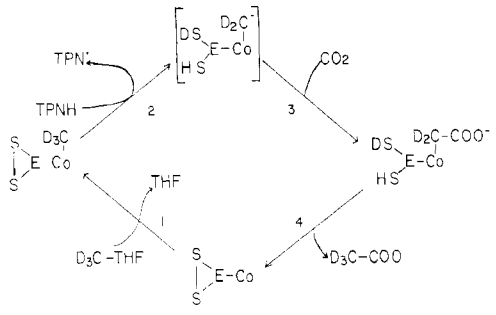

FIGURE 2: Formation of trideuterioacetate *via* a carboxymethylcorrinoid involving intramolecular hydrogen transfer through a disulfide of the enzyme.

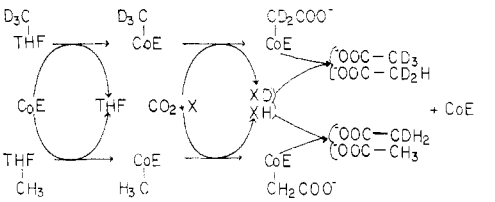

FIGURE 3: Formation of tri-, di-, and monodeuterioacetate *via* a carboxymethylcorrinoid involving intermolecular hydrogen transfer mediated by a hydrogen carrier X.

if dideuterioacetate was the product, since it is possible to form trideuterioacetate by either mechanism. For example, the hydrogen atom (or deuterium) in reaction 1 (Figure 1) may not leave as a proton freely exchangeable with the solvent; instead it may combine with a carrier, *e.g.*, a disulfide as shown in Figure 2. The hydrogen atom may then be added back to the same carbon from which it was abstracted by an intramolecular reaction. The disulfide mechanism in Figure 2 was chosen because Poston *et al.* (1966) found that the formation of acetate from CH_3-B_{12} is inhibited by arsenite and cadmium which react with vicinal sulfhydryl groups. Normally hydrogens on sulfhydryl groups exchange with water rapidly but if the reaction occurred in a hydrophobic region of the enzyme the exchange might be prevented or be slow and there would be retention of deuterium during the reaction.

A second hypothesis, involving a carboxymethyl intermediate, is outlined in Figure 3 which involves an intermolecular reaction. The hydrogen is abstracted from the *Co*-methyl group by a carrier X, which is not tightly bound to the enzyme. Thus when the reaction is with a mixture of D_3C-THF and H_3C-THF a pool would be formed of XD and XH. The "hydrogen" added back in the formation of acetate would be either a D or an H. Consequently a mixture of tri- and dideuterioacetate would be formed from D_3C precursors, while H_3C precursors would yield mono- and nondeuterioacetate *via* the intermolecular reaction. The purpose of presenting these schemes is only to illustrate the effect of mechanisms involving (Figure 1) loss of D as a deuteron and (Figures 2 and 3) retention of D by an intramolecular and intermolecular transfer. Other mechanisms illustrating these points may be just as plausible as those chosen.

The result of our experiments was that the predominant acetate formed from the trideuterated methyl donors was trideuterioacetate. Thus there was retention of all three hydrogens of the methyl group during the carboxylation reaction. From Table II it can be seen that more trideuterioacetate was formed from the D_3C-B_{12} than from D_3C-THF and this was especially evident in expt V. These results seem to favor the Grignard-like mechanism involving a methyl carbanion intermediate, or the intramolecular carboxymethyl mechanism (Figure 2). However, the relatively high amounts of mono- and dideuterioacetate could indicate a mechanism involving an intermolecular transfer of hydrogen as illustrated in Figure 3. The experiments with 1:1 mixture of trideuterated and nondeuterated methyl precursors were designed to detect the latter mechanism. It would be expected that the ratios of the different acetate species would differ with a 1:1 mixture from those formed from 100% D_3C-B_{12} or D_3C-THF if the reaction was by the intermolecular mechanism. The data presented in Table II show that the addition of H_3C-B_{12} and H_3C-THF to the D_3C-B_{12} and D_3C-THF did not result in a change of ratios of the mono-, di-, and trideuterioacetate. The large amount of endogenous acetate synthesis may indicate a large pool of

nondeuteriomethyl precursors and therefore the effect of added H_3C-B_{12} or H_3C-THF would be masked. However, this is not likely since extracts used in these experiments do not catalyze fixation of a substantial amount of $^{14}CO_2$ when incubated alone or in the presence of α-ketobutyrate or pyruvate (Ghambeer *et al.* 1971). Thus the endogenous acetate appears to be formed by a pathway which does not involve CO_2 fixation and H_3C-THF or H_3C-B_{12}. Apparently the endogenous acetate is formed *via* pyruvate formed from substrates present in the enzyme preparation. The fact that α-ketobutyrate inhibits the endogenous acetate formation (Table III) is in agreement with this assumption. It is concluded from the results of the experiments with mixed deuterio and hydrogen precursors that a carboxymethyl mechanism is excluded which involves an intermolecular hydrogen transfer. The fact that D_3CCOO^- was the predominant product in all experiments also makes the occurrence of an intermolecular hydrogen transfer unlikely.

Retention of all three hydrogens of methyl-B_{12} and methyl-THF during the carboxylation step suggests the mechanism is *via* the carbanion or a reaction involving an intramolecular hydrogen transfer. In the latter mechanism a hydrogen–carbon bond is broken twice, first in the transfer from the methyl group to the enzyme-bound carrier and second when the hydrogen atom is transferred back to the methyl group of the acetate (Figure 2). It is well known that mechanisms involving hydrogen–carbon bond cleavage generally exhibit an isotope effect. For example, in the reaction catalyzed by propanediol dehydrase, deuterated propanediol is converted to deuterated propionaldehyde at one-twelfth the rate of the nondeuterated substrate (Frey *et al.*, 1965). Similarly the deuterium isotope effect in the ethanolamine ammonia-lyase reaction is 7.4 (Weisblat and Babior, 1971). Both these reactions involve two hydrogen–carbon bond cleavages. We did not observe an effect on the rate of acetate formation from D_3C-B_{12} compared to the nondeuterated substrate. However, the isotope effect may not be evident because the cleavage of the hydrogen–carbon bond may not be the rate-limiting step; such a step might well be the carboxylation reaction.

If the acetate is formed by a carbanion mechanism, the formation of di- and monodeuterioacetate must occur by secondary reactions, since only trideuterioacetate is formed by this mechanism. A possible explanation of the formation of mono- and dideuterioacetate is that the D is lost by exchange reactions. Such exchange might occur by reversible oxidation of 5-methyltetrahydrofolate to 5,10-methylenetetrahydrofolate and also to 5,10-methenyltetrahydrofolate. The result would be formation of mono- and dideuterio-5-methyltetrahydrofolate, which would be converted to mono- and dideuterioacetate. The enzymes 5,10-methylenetetrahydrofolate reductase and dehydrogenase, which catalyze these reactions, are both present in *C. thermoaceticum* as well as *Clostridium formicoaceticum* (O'Brien *et al.*, 1971, and unpublished results). The latter organisms also perform the synthesis of acetate from CO_2 by a mechanism similar to that of *C. thermoaceticum* (O'Brien and Ljungdahl, 1972; Schulman *et al.*, 1972). Since there was formation of mono- and dideuterioacetate with trideuterated methyl-B_{12}, it would be necessary in this case to assume that the methyl group of D_3C-B_{12} is converted to D_3C-THF which then is subject to the above reversible reactions. The fact that more di- and monodeuterioacetate were formed from D_3C-THF than from D_3C-B_{12} may support the above suggestion. The formation of 94% D_3C-COOH in expt V may have resulted because for unknown reasons the methyl group of D_3C-B_{12} was not transferred to THF to form D_3C-THF in this experiment. Another possibility is that there is an exchange reaction which occurs at the site of the acetate formation. An exchange at the corrinoid site is proposed by Penley *et al.* (1970) to account for the formation of methane from difluoromethylcobalamin by methane bacteria.

It is of interest that formation of methane from acetate by *Methanosarcina barkeri* occurs exclusively from the methyl carbon and that all hydrogens are retained (Pine and Barker, 1956). It has been suggested that the methane formation is *via* a methylcorrinoid and that it occurs in a manner which is the reverse of acetate formation (Stadtman, 1967). Our results showing retention of the hydrogens during formation of acetate seem to support this idea. Furthermore, Kisliuk (1963) has studied the formation of methionine in *Escherichia coli* and demonstrated with tritium labeling that the methyl group of H_3C-THF is transferred intact to homocysteine. This reaction also involves a methyl corrinoid as intermediate.

In conclusion it might appear, since D_3CCOO^- is the predominant product, that the Grignard or a similar reaction is the most probable mechanism. However, it is well known from studies of other reactions catalyzed by corrinoid enzymes that it is characteristic of these enzymes that there is hydrogen transfer without exchange with the protons of the water. One exception is the ribonucleotide triphosphate reductase reaction. These studies have been reviewed extensively (Barker, 1967; Hogenkamp, 1968; Stadtman, 1971). Our results do not exclude a carboxymethylcorrinoid as intermediate, but any mechanism proposed must account for the formation of trideuterioacetate.

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Arene Oxides as Intermediates in the Oxidative Metabolism of Aromatic Compounds. Isomerization of Methyl-Substituted Arene Oxides†

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ABSTRACT: Arene oxides are strongly implicated as key intermediates in monoxygenase-catalyzed metabolism of aromatic compounds. To provide further information on their metabolic significance, the arene oxides of toluene, of the three xylenes, of mesitylene, and of 2-methyl- and 1,2-dimethylnaphthalene 1,2-oxide were synthesized, and their phenolic rearrangement products were compared to the phenols obtained by hepatic metabolism of the parent hydrocarbons. The range of phenolic products obtained from microsomal metabolism of the alkyl-substituted aromatic substrates was compatible with the intermediacy of certain extremely labile arene oxides. Formation of arene oxides with alkyl substituents on the oxirane ring did not appear to be a significant pathway for hepatic metabolism except with mesitylene. Migrations of methyl groups as well as apparent migrations of oxygen occurred during rearrangement of methyl-substituted arene oxides. Formation of 2,4-dimethylphenol from 1,4-dimethylbenzene oxide provides a chemical model for the conversion of 4-methylphenylalanine to 3-methyltyrosine by phenylalanine hydroxylase. Phenolic isomers formed on rearrangement of the arene oxides were qualitatively predictable by a simple carbonium ion theory. The *ratios and nature* of isomerization products, however, varied with reaction conditions, indicating that multiple mechanistic pathways are operative in these rearrangements.

The arene oxide, naphthalene 1,2-oxide, has been established as the obligatory intermediate in the metabolic formation of naphthol, a dihydrodiol and a glutathione conjugate from the bicyclic hydrocarbon, naphthalene (Jerina *et al.*, 1968c, 1970a). In addition, an arene oxide was recently reported as a metabolite of the polycyclic hydrocarbon, dibenzanthracene (Selkirk *et al.*, 1971). Arene oxides derived from monocyclic hydrocarbons have yet to be isolated from a biological system (*cf.* Jerina *et al.*, 1968b). However, a variety of evidence does *suggest* arene oxides as key intermediates in the hepatic metabolism of monocyclic hydrocarbons. For example, benzene and a variety of other monocyclic aromatic compounds are converted to dihydrodiols and premercapturic acids (Sato *et al.*, 1963; Jerina *et al.*, 1967; Smith *et al.*, 1950; Chang *et al.*, 1970, etc.), presumably *via* intermediate arene oxides. In addition, formation of phenols from both monocyclic (Daly *et al.*, 1968b) and bicyclic (Boyd *et al.*, 1972) aromatic hydrocarbons occurs with varying degrees of retention of the ring substituent originally present at the position to which the hydroxyl group is introduced. This migration and retention of substituent, known as the NIH shift (Guroff *et al.*, 1967; Daly *et al.*, 1968a; Jerina *et al.*, 1971b), is *incompatible* with the direct formation on phenols by an insertion reaction, but is compatible with the migration of substituents which occur during isomerization of deuterated arene oxides to phenols (Jerina *et al.*, 1968a; Boyd *et al.*, 1972). The present investigation has attempted to determine whether the intermediacy of arene oxides is compatible with the observed metabolism of various alkylated aromatic hydrocarbons and whether such arene oxides would have the requisite stability for isolation from the metabolic system.

Experimental Section

General. All compounds synthesized were routinely checked for structure and purity by proton magnetic resonance (Varian HA-100) and mass spectrometry (Hitachi RMU-7). In many instances, combustion data were also obtained. The general synthetic schemes are shown in Table I and the proton magnetic resonance spectra of the olefins are presented in Table II.

Preparation of Dihydrobenzenes and Dihydronaphthalenes. Birch reductions of methyl-substituted benzoic acids and

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