Effect of Vitamin B_{12} Deprivation on the in Vivo Levels of Coenzyme A Intermediates Associated with Propionate Metabolism*

(Received for publication, May 1, 1974)

EUGENE P. FRENKEL, RICHARD L. KITCHENS, LOUIS B. HERSH, AND RENE FRENKEL

From the Evelyn L. Overton Hematology-Oncology Research Laboratory, Departments of Internal Medicine and Biochemistry, The University of Texas Southwestern Medical School, Dallas, Texas 75235, and Veterans Administration Hospital, Dallas, Texas 75216

SUMMARY

The in vivo coenzyme A intermediates involved in the metabolic pathway in which vitamin B_{12} serves as a coenzyme (conversion of methylmalonyl-CoA to succinyl-CoA) were measured in the livers of control and B_{12}-deprived animals. Succinyl-CoA was assayed by the succinate thiokinase arsenolysis of succinyl-CoA coupled with 5',5'-dithiobis(2-nitrobenzoic acid) measurement of the liberated coenzyme A. Methylmalonyl-CoA was measured by coupling the succinyl-CoA assay with methylmalonyl-CoA mutase. The assays were shown to be specific and reproducible and provided excellent recovery of added exogenous coenzyme A derivatives. Utilizing the in situ freeze-clamp technique, the mean liver propionyl coenzyme A levels were approximately 17-fold greater in the B_{12}-deprived animals than in the controls. As anticipated by the site of the known metabolic action of coenzyme B_{12}, methylmalonyl-CoA levels were also increased in the B_{12}-deprived animals, but were only 12-fold greater in the B_{12}-deprived than in the controls. Unexpectedly, succinyl-CoA levels were found to be on an average 4-fold greater in the livers of the B_{12}-deprived animals than in the controls. In individual liver samples the propionyl-CoA to methylmalonyl-CoA ratio was approximately 2:1. Acetyl-CoA levels were not reduced by the presence of increased endogenous propionyl-CoA, but were actually increased in livers from the B_{12}-deprived group. Study of selected Krebs' tricarboxylic acid cycle intermediates showed increased malate and normal citrate levels in the livers of the B_{12}-deprived group, yielding a 2-fold increase in the malate to citrate ratio. Thus, the present study provides a method of measurement of the CoA intermediates in the B_{12}-dependent pathway in the intact liver and demonstrates that B_{12} deficiency results in an increase in propionyl-CoA and methylmalonyl-CoA as well as in succinyl-CoA, an intermediate beyond the site of action of coenzyme B_{12}.

Shortly after it was demonstrated that vitamin B_{12} coenzyme A is required for the conversion of methylmalonyl coenzyme A (MM-CoA) to succinyl coenzyme A (Fig 1) by methylmalonyl-CoA mutase (methylmalonyl-CoA:CoA-carboxylmutase, EC 5.4.99.2) (1, 2), Cox and White provided the correlative clinical observation that methylmalonic aciduria occurred in B_{12} deficiency in man (3). Subsequent studies amply confirmed this latter observation (4-7) and demonstrated that both propionic and methylmalonic aciduria occurred in experimental and clinical B_{12} deprivation in animals and man (8).

Vitamin B_{12} deficiency has been shown to reduce the activity of methylmalonyl-CoA mutase; and this has been assumed to result in the accumulation of MM-CoA in the tissues (9-11). The subsequent conversion of MM-CoA to methylmalonate has been reported to be catalyzed by liver, brain, and kidney homogenates of both normal and deficient rats (9), but the mechanism has not been studied. Unlike methylmalonyl-CoA mutase, the tissue level of propionyl-CoA carboxylase is not altered by vitamin B_{12} deficiency (10). It is noteworthy that the actual levels of MM-CoA in tissues have not been measured (12).

Recent evidence in man has documented that vitamin B_{12} deficiency results in altered fatty acid synthesis and the accumulation of odd chain fatty acids in the myelin of peripheral nerves (13). Increased fatty acid synthesis and an increase in the content, activity, and synthesis of the enzyme of fatty acid synthesis have now been demonstrated in the liver of B_{12}-deprived animals (14, 15). In addition, these studies have shown that in B_{12} deprivation, propionyl-CoA served as an effective substrate for both enzymes of fatty acid synthesis (fatty acid synthetase and acetyl-CoA carboxylase), thus providing a reasonable mechanism for odd chain fatty acid synthesis (14). The mechanism for the increased fatty acid synthesis in vitamin B_{12} deprivation is unknown. Since succinyl coenzyme A, the product of the mutase reaction, has been proposed as a competitive inhibitor for acetyl-CoA in the citrate synthase reaction by Williamson and co-workers (16, 17), this potential product provided speculation as an effector of synthesis.

1 E. P. Frenkel and R. L. Kitchens, submitted for publication.
2 The abbreviations used are: MM-CoA, methylmalonyl coenzyme A; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); STK, succinate thiokinase.

* This work was supported in part by the Heddens-Good Foundation and Robert A. Welch Foundation Grant I-988.
In an attempt to clarify these interrelationships the present study was designed to determine the levels of CoA derivatives in the livers of vitamin B12-deprived animals and to investigate possible correlations between the levels of these intermediates and the citric acid cycle.

### METHODS

**Animals and Their Maintenance**—Animal care and maintenance and the manner of achieving vitamin B12 deficiency have been previously described (14, 18). Animals were maintained on one of three dietary programs: (a) routine rat chow (obtained from Feeders Supply Co., Dallas, Texas), hereafter referred to as “normal diet”; (b) vitamin B12-deficient diet (obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio), subsequently referred to as “B12-deprived diet”); or (c) vitamin B12-deficient diet that was supplemented with intramuscular injections of 100 μg of vitamin B12 (cyanocobalamin obtained from Calbiochem, San Diego, Calif.) given three times per week, hereafter referred to as the “B12-supplemented diet.” The development of the B12-deprived state required approximately 8 months beyond the post-weaning period (18). Animals in each dietary group were serially evaluated for propionyl-CoA and methylmalonyl-CoA and serum vitamin B12 levels before death (19, 20).

**Preparation of Tissue Extracts**—Animals were stunned by a blow on the head and a portion of the liver was rapidly freeze-clamped in situ with modified Wollenberger brass plate clamps precooled in liquid nitrogen (21). The time elapsed to complete the freezing procedure ranged between 14 and 25 s. The freeze-clamped tissue was then transferred into liquid nitrogen until further processing. The remaining liver tissue was then weighed and assayed for vitamin B12 content (22). Tissue B12 assays were also performed on spleen and other selected tissues. The freeze-clamped liver aliquot was weighed, pulverized in a mortar precooled in liquid nitrogen, and homogenized in 2 volumes of ice-cold 10% (νν) perchloric acid. The homogenate was centrifuged for 10 min at 18,000 × g, and the supernatant fluid recovered, and the volume measured. Aliquots were then separated for assay of intermediates. Recovery determinations were performed for each intermediate by adding a measured amount of a standard solution of the respective CoA derivative to a duplicate aliquot. Endogenous free thiols were removed from extracts used for subsequent assay of acetyl-CoA, MM-CoA, and succinyl-CoA. This pretreatment consisted of adding 10 μl of 30% H2O2 to 2.0 ml of extract, and the mixture was allowed to stand at room temperature for 5 min to oxidize the thiols. The samples were then placed on ice, 50 μl of 2 M Tris (Cl-), pH 7, were added, and the pH was adjusted to 7.0 by the slow addition of 2 M KOH with vigorous agitation on a Vortex mixer. The precipitate was removed by brief centrifugation (2,000 × g for 5 min), and excess H2O2 was removed from the supernatant fraction by the addition of 1 ml of a 10-mg solution of catalase per ml (Sigma Chemical Co., St. Louis, Mo.). After 10 min incubation at room temperature, the samples were placed on ice and assayed.

Extracts for the propionyl-CoA assay were prepared by a modification of the method of Suling and Volkmann (23). 2 M Tris-Cl* (pH 7), 200 μl were added per ml of extract, and the mixture was neutralized to pH 7 by the slow addition of 2 N KOH (freshly prepared with boiled glass-distilled water kept free of atmospheric CO2 by infusion of nitrogen gas) while stirring on a Vortex mixer. Standard curves of measured amounts of propionyl-CoA were prepared in parallel with the tissue extracts using equivalent amounts of 10% perchloric acid, since the slope of the curve varied with the concentration of contaminating bicarbonate.

**Assay of Propionyl-CoA**—Assay of the neutralized extracts (200 μl) was performed by the method of Suling and Volkmann (23), using 12.5 μCi of 14CO2 (specific activity 50 μCi per pmole; New England Nuclear) with no added carrier and 25 millimoles of propionyl-CoA carboxylase in a final volume of 1.0 ml. Incubations were terminated by the addition of 50 μl of 50% trichloroacetic acid. Determination of 14CO2 fixation was performed as described by Gregolin et al. (24). Aliquots (250-μl) of the reaction fluid were placed into counting vials and heated to dryness in an 85° oven for 45 min to remove free 14CO2. Samples were counted in a Beckman LS-150 liquid scintillation system as previously described (13).

**Assay of Succinyl-CoA**—Succinyl-CoA was measured spectrophotometrically with succinate thiokinase (STK) obtained from Escherichia coli by an arsenolysis method coupled with DTNB. This reaction can be depicted as follows:

\[
\text{Succinyl-S-CoA} + \text{Mg}^{++}, \text{AsO}_4^{3-} \rightarrow \text{succinate} + \text{CoA-SH.}
\]

The sulfhydryl group released was determined by its reaction with DTNB. The total volume of reaction mixture was 1.0 ml (pH 7.4) and contained final concentrations of 100 μM Tris-Cl, 100 μM KC1, 25 mM arsenate (Na+), 10 mM MgCl2, 0.5 mM DTNB, and 100 to 400 μl of tissue extract. Arsenolysis was initiated by the addition of 0.15 unit of STK, and the amount of free sulfhydryl released was determined at 412 nm in a Gilford recording spectrophotometer. Pig heart succinate thiokinase (Sigma) proved unsuitable because of instability and too rapid inactivation by DTNB. E. coli STK proved stable when stored frozen, and yielded a sharp end point when DTNB was used as described above.

**Assay of Methylmalonyl-CoA**—MM-CoA was determined by coupling the method described above for the succinyl-CoA assay

\[
\text{Methylmalonyl-CoA} + \text{AsO}_4^{3-} \rightarrow \text{aconitate} + \text{AsO}_4^{2-}
\]

\[
\text{aconitate} + \text{CoA-SH} \rightarrow \text{fumarate} + \text{CoA-SH}
\]

\[
\text{fumarate} \rightarrow \text{malate} + \text{CO}_2
\]

\[
\text{malate} \rightarrow \text{pyruvate} + \text{CO}_2
\]

\[
\text{pyruvate} \rightarrow \text{acetyl-CoA}
\]

\[
\text{acetyl-CoA} \rightarrow \text{acetate} + \text{CoA-SH}
\]

\[
\text{acetate} \rightarrow \text{propionate}
\]

\[
\text{propionate} \rightarrow \text{methylmalonyl-CoA}
\]

\[
\text{methylmalonyl-CoA} \rightarrow \text{succinyl-CoA}
\]

\[
\text{succinyl-CoA} \rightarrow \text{fumarate}
\]

\[
\text{fumarate} \rightarrow \text{malate}
\]

\[
\text{malate} \rightarrow \text{citrate}
\]

\[
\text{citrate} \rightarrow \text{oxaloacetate}
\]

\[
\text{oxaloacetate} \rightarrow \text{glutamate}
\]

\[
\text{glutamate} \rightarrow \text{aspartate}
\]

\[
\text{aspartate} \rightarrow \text{citrate}
\]

\[
\text{citrate} \rightarrow \text{oxaloacetate}
\]

\[
\text{oxaloacetate} \rightarrow \text{pyruvate}
\]

\[
\text{pyruvate} \rightarrow \text{acetyl-CoA}
\]

\[
\text{acetyl-CoA} \rightarrow \text{acetate} + \text{CoA-SH}
\]

\[
\text{acetate} \rightarrow \text{propionate}
\]

\[
\text{propionate} \rightarrow \text{methylmalonyl-CoA}
\]

\[
\text{methylmalonyl-CoA} \rightarrow \text{succinyl-CoA}
\]

\[
\text{succinyl-CoA} \rightarrow \text{fumarate}
\]

\[
\text{fumarate} \rightarrow \text{malate}
\]

\[
\text{malate} \rightarrow \text{citrate}
\]

\[
\text{citrate} \rightarrow \text{oxaloacetate}
\]

\[
\text{oxaloacetate} \rightarrow \text{glutamate}
\]

\[
\text{glutamate} \rightarrow \text{aspartate}
\]

\[
\text{aspartate} \rightarrow \text{citrate}
\]

\[
\text{citrate} \rightarrow \text{oxaloacetate}
\]

\[
\text{oxaloacetate} \rightarrow \text{pyruvate}
\]

\[
\text{pyruvate} \rightarrow \text{acetyl-CoA}
\]

\[
\text{acetyl-CoA} \rightarrow \text{acetate} + \text{CoA-SH}
\]

\[
\text{acetate} \rightarrow \text{propionate}
\]

\[
\text{propionate} \rightarrow \text{methylmalonyl-CoA}
\]

\[
\text{methylmalonyl-CoA} \rightarrow \text{succinyl-CoA}
\]

\[
\text{succinyl-CoA} \rightarrow \text{fumarate}
\]

\[
\text{fumarate} \rightarrow \text{malate}
\]

\[
\text{malate} \rightarrow \text{citrate}
\]

\[
\text{citrate} \rightarrow \text{oxaloacetate}
\]

\[
\text{oxaloacetate} \rightarrow \text{glutamate}
\]

\[
\text{glutamate} \rightarrow \text{aspartate}
\]

\[
\text{aspartate} \rightarrow \text{citrate}
\]
with methylmalonyl-CoA mutase. The assay was performed by the addition of 0.4 unit of the mutase to the succinyl-CoA assay mixture, the reaction was then begun with STK and carried out as previously described. Since the mutase was partially contaminated with methylmalonyl-CoA racemase and coenzyme B12, these were not required additions. The mutase was shown to be highly resistant to inactivation by the amount of MM-CoA was determined by subtracting the value obtained for succinyl-CoA alone from the total amount determined in the presence of the mutase.

**Assay of Acetyl-CoA**—Acetyl-CoA was determined by a modification of the method of Serre (25, 26) utilizing citrate synthase (pig heart citrate synthase, crystalline suspension in ammonium sulfate; Sigma) coupled to DTNB in the following manner:

\[
\text{Acetyl-CoA + oxaloacetate } \rightarrow \text{citrate synthase } \rightarrow \text{citrate + CoA-SH.}
\]

The sulfhydryl released was then determined by its reaction with DTNB. The reaction mixture, in a total volume of 1 ml, contained (final concentrations) 100 mM Tris-Cl− (pH 8.0), 0.1 mM DTNB, 0.25 mM oxaloacetic acid, and 100 to 200 μl of tissue extract. The reaction was begun by the addition of 0.5 unit of citrate synthase and followed spectrophotometrically to the end point as described above.

**Assays for Citrate and Malate**—Citrate was determined in tissue extracts (100 μl) using citrate lyase and malate dehydrogenase (Boehringer Mannheim Corp., New York, N. Y.) as described by Moellering and Gruber (27). The amount of malate present in the extracts was determined with malic enzyme isolated from rat liver by the method of Isohashi et al. (28). The reaction mixture (total volume 1.0 ml) contained final concentrations of 100 mM MOPS buffer (morpholinopropanesulfonic acid, obtained from Sigma) adjusted to pH 7.5 with KOH, 10 mM MgCl2, 0.15 mg per ml of TPN+ (P-L Biochemicals, Milwaukee, Wis.), and 100 μl of tissue extract. The reaction was begun by the addition of 0.1 unit of malic enzyme and followed to completion spectrophotometrically at 340 nm.

**Purification of Enzymes**—Propionyl CoA carboxylase was purified from bovine liver by the method of Halen et al. (29) as modified by Giorgio and Pflaut (30) and Söling and Vollkann (23). Enzymatic activity was assayed spectrophotometrically (23) and the protein content determined by the method of Lowry et al. (31). The final specific activity was 2.4 μmol units per min per mg of protein at 25°. Equivalent substrate concentrations of acetyl-CoA and n-butyryl-CoA produced rates of 1 and 6%, respectively, of that of propionyl-CoA.

The partially purified methylmalonyl-CoA mutase6 from Propionibacteria was obtained as the eluate from the cellulose phosphate column separation (32, 33). This was then carried through the remaining steps of purification according to the methods of Koshui et al. (32, 33) omitting only the TEAE-cellulose gradient separation. Transcarboxylase6 was utilized in the assay for enzymatic activity (32, 33) and the protein determined by the method of Lowry et al. (31). The final specific activity was 2.5 μmol units per min per mg of protein at 25°. The mutase was shown to be contaminated with methylmalonyl-CoA racemase and active, enzyme-bound coenzyme B12, but free of transcarboxylase. The final enzyme preparation was dialyzed overnight against 4 liters of 100 mM Tris-Cl−, pH 7.0, in order to remove inorganic phosphate, an inhibitor of ammonolysis catalyzed by STK.7

**Standardization of CoA Derivatives**—Standard solutions of 10 mM concentration were prepared in distilled water for acetyl-CoA, MM-CoA, and succinyl-CoA (P-L Biochemicals) and their concentrations determined by the respective assays. Propionyl-CoA (P-L Biochemicals) was standardized with carnitine acetyltransferase (34), according to the following reaction:

\[
\text{Propionyl-CoA + carnitine } \rightarrow \text{transferase } \rightarrow \text{propionyl carnitine + CoA-SH}
\]

The sulfhydryl release was then assayed with DTNB. The reaction mixture (final volume 1.0 ml) contained final concentrations of 100 mM Tris-Cl−, pH 8.0, 1 mM dl-carnitine, 0.1 mM DTNB, and the propionyl-CoA standard. The reaction was started with 1 unit of carnitine acetyltransferase (Sigma) and followed spectrophotometrically at 412 nm to the end point. Assays in the absence of propionyl-CoA were used to correct the small optical density changes which result from the reaction of the sulfhydryl group of the enzyme with the DTNB.

Standard solutions of CoA derivatives were stored frozen and re­såayed every 2 weeks; each solution was found to be stable for up to 1 month.

**RESULTS**

**Characterization of CoA Assays**— Pretreatment of the tissue extracts with hydrogen peroxide was shown to be virtually 100% effective in removing endogenous free thiols. It was not possible to use the molar extinction coefficient of DTNB (13.6 X 106) to calculate directly the amount of substrate in the tissue extracts because of a quenching phenomenon. The correct relationship of substrate concentration to changes in optical density was determined by use of standard curves prepared with tissue extract and increasing concentrations of exogenously added CoA derivatives. Fig. 2 demonstrates this phenomenon in tissue extracts in the succinyl-CoA assay. These effects were seen in the acetyl-CoA, MM-CoA, and succinyl-CoA assays. Results were calculated from the standard curves.

Recovery of standards added to tissue extracts was in excess of 80% for acetyl-CoA, 90% for propionyl-CoA, 90% for MM-CoA, and 80% for succinyl-CoA. The results were corrected by parallel recovery determinations.

**Specificity of Assays**—The specificity of the assay for propionyl-CoA was documented with standard preparations of acetyl-CoA and n-butyryl-CoA. Fig. 3 compares the standard curve obtained with different concentrations of propionyl-CoA with those obtained with acetyl-CoA and butyryl-CoA. Equivalent concentrations of acetyl-CoA and butyryl-CoA resulted in 2 and 10%, respectively, the amount of 14CO2 fixation produced by propionyl-CoA. Further evidence for the specificity of the assay was provided by thin layer chromatographic separation and identification of the labeled products of the reaction after hydrolysis. These analyses were performed on tissue extracts prepared from livers removed from animals on the normal, supplemented, and β2-depleted diets as previously described.

![Fig. 2. Standard curve for the assay of succinyl-CoA. The abscissa indicates the amount of succinyl-CoA added in nanomoles, and the ordinate the absorbance change (Δ O.D.) at 412 nanometers. The upper line was the assay in the absence of tissue extract; the lower line was the assay in the tissue extract to which the succinyl-CoA was added.](http://www.jbc.org/)

---

6 The methylmalonyl-CoA mutase and transcarboxylase were graciously provided by Dr. Harland G. Wood, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio.

7 L. B. Hersh, submitted for publication.
Malonic, methylmalonic, and ethylmalonic acids, the respective carboxylation products resulting from the action of propionyl-CoA carboxylase on acetyl-CoA, propionyl-CoA, and n-butyryl-CoA (35, 36), were used as carriers for the radiolabeled hydrolyzed products. More than 90% of the radioactivity was found in the methylmalonic acid spot. The remaining activity was found diffusely along the plate without specific preferential localization.

Specificity of the arsenolysis reaction for succinyl-CoA utilizing the E. coli STK was demonstrated by the absence of reaction with acetyl-CoA, propionyl-CoA, n-butyryl-CoA, and malonyl-CoA over a wide range of concentrations. The reaction with succinyl-CoA was linear (Fig. 2) and reached a sharp end point within 5 min.

The methylmalonyl-CoA assay was coupled to the succinyl-CoA reaction for specificity. Initial studies demonstrated that the methylmalonyl-CoA mutase was contaminated with an enzyme which catalyzed a very slow decarboxylation of propionyl-CoA and acetyl-CoA, and corrections could easily be made for the slight spectrophotometric "drift" that these produced (see Fig. 2). Reproducibility was improved by starting the reaction with STK, rather than with the mutase, since the mutase preparation absorbed significantly at 412 nm.

The specificity of the acetyl-CoA assay has been firmly documented by Sere et al. (25, 26) and Tubbs and Garland (34). In studies not shown, no reaction was detected with propionyl-CoA, over a wide range of concentrations.

Characteristics of Study Animals — The status of the animals in each dietary group is shown in Table I. The vitamin B12-deprived group had low liver and spleen vitamin B12 values. Since the study animals were killed by cervical dislocation in order to freeze-clamp the liver, serum samples could not be easily obtained. Serum samples, however, were obtained from paired mates for assay and the values are shown in Table I. Propionic and methylmalonic aciduria were also demonstrated in the B12-deprived group before death. As previously shown (18), another characteristic of the B12-deprived animals was increased liver size and a resultant high liver weight to body weight ratio based in part on the liver weight and the failure of the animals to gain the same amount of weight as their paired normal controls. The B12-supplemented control animals had specifically been given supplemental B12 for only 2 weeks before death. It is noteworthy that these animals still retained the altered weight ratios in spite of their otherwise normal characteristics.

Liver CoA Intermediates — The values for propionyl-methylmalonyl, succinyl, and acetyl-CoA intermediates in the liver extracts are shown in Table II. Several observations were unexpected in light of the evidence that coenzyme B12 deprivation produces a defect in the conversion of MM-CoA to succinyl-CoA (1, 2), which could then reasonably cause the major increment in MM-CoA. Actually, mean propionyl-CoA levels were 17-fold greater (ranging to an extreme of 34-fold) in the B12-deprived group than in the controls. In addition, although MM-CoA was increased approximately 12-fold (with a range to an extreme of 34-fold) in the B12-deprived group than in the controls. In addition, although MM-CoA was increased approximately 12-fold (with a range to an extreme of 19-fold) in the livers of the B12-deprived animals as compared to the controls, propionyl-CoA levels were greater than MM-CoA in all but one animal studied. As shown in Table II, this relationship provided a propionyl-CoA to MM-CoA ratio of nearly 2:1. Although it was expected that suc-

![Diagram](http://www.jbc.org/)

**FIG. 3.** Standard curve and specificity of the propionyl-CoA assay. The curve obtained with different amounts of propionyl-CoA is compared to those obtained with n-butyryl-CoA or acetyl-CoA.

**TABLE I**

<table>
<thead>
<tr>
<th>Study group*</th>
<th>Body weight</th>
<th>Liver weight</th>
<th>Ratio liver weight to body weight</th>
<th>Liver vitamin B12 content</th>
<th>Spleen vitamin B12 content</th>
<th>Serum vitamin B12 level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (8)</td>
<td>260 ± 10</td>
<td>7.9 ± 0.4</td>
<td>3.1 ± 0.11</td>
<td>120.6 ± 0.9</td>
<td>55.8 ± 7.6</td>
<td>922 ± 79</td>
</tr>
<tr>
<td>B12-supplemented (4)</td>
<td>175 ± 8</td>
<td>10.0 ± 0.6</td>
<td>5.7 ± 0.3</td>
<td>130 ± 27</td>
<td>62.6 ± 4.5</td>
<td>1047 ± 111</td>
</tr>
<tr>
<td>B12-deprived (12)</td>
<td>198 ± 8</td>
<td>10.1 ± 0.5</td>
<td>5.1 ± 0.12</td>
<td>20.2 ± 1.0</td>
<td>9.4 ± 0.6</td>
<td>140 ± 10</td>
</tr>
</tbody>
</table>

* Numbers in parentheses refer to the number of animals in each group.
TABLE II

Values of liver CoA intermediates

Data obtained from in situ freeze-clamp of liver. Conditions for each assay are as delineated under "Methods." For each assay replicate tissue extracts with added standards were prepared and run simultaneously to validate the assay and determine the recovery. Numbers in parentheses are the number of animals studied. Results are ± S.E.

<table>
<thead>
<tr>
<th>Study animals</th>
<th>Propionyl-CoA</th>
<th>Methylmalonyl-CoA</th>
<th>Succinyl-CoA</th>
<th>Acetyl-CoA</th>
<th>Ratio of propionyl-CoA to methylmalonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.8 (8) ± 0.47</td>
<td>&lt;5* (4)</td>
<td>&lt;5* (4)</td>
<td>43.5 (3) ± 1.8</td>
<td>1.0*</td>
</tr>
<tr>
<td>B₃₃-supplemented</td>
<td>2.7 (4) ± 0.51</td>
<td>&lt;5* (3)</td>
<td>6.7 (3) ± 0.72</td>
<td>31 (3) ± 5.2</td>
<td>-</td>
</tr>
<tr>
<td>B₃₃-deprived</td>
<td>81.5 (10) ± 15.6</td>
<td>57.0 (7) ± 11.9</td>
<td>23.7 (7) ± 3.3</td>
<td>78.6 (6) ± 8.9</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

- The lower limits of resolution of the method limited more specific definition of the amount of respective intermediate.
- Because of the limits of resolution of the amount of methylmalonyl-CoA, this is an estimated value.

Fig. 4. Relationship of propionyl-CoA concentration in the liver extracts (nanomoles per g of liver) to liver vitamin B₃₃ levels (nanograms per g of liver).

Cinyl-CoA levels would be reduced, the concentration of this intermediate was in fact nearly 4-fold greater in the livers of the B₃₃-deprived animals than in those of the controls. Since the in situ freeze-clamping technique caused a time lapse of several seconds between the stunning blow and the freeze-clamping, the effects of delaying the freezing of the tissue on the levels of intermediates was also studied. In studies not shown rapid and delayed freeze-clamping (ranging from 15 to 60 s after the stunning blow) demonstrated that the absolute levels of intermediates decreased as a result of increasing time delay, but the relative ratios were not significantly altered from those shown in Table II. Levels of hepatic propionyl-CoA, MM-CoA, and succinyl-CoA were approximately 5, 40, and 30% lower when freeze-clamp delay of 60 s (from time of the stunning blow) was compared to the usual 15-s interval. Thus, the increased values for succinyl-CoA could not be ascribed to an artifact of the tissue handling.

An interesting relationship was seen between the liver vitamin B₃₃ value and propionyl-CoA and MM-CoA levels. As shown in Fig. 4, propionyl-CoA was increased above the normal range, when the tissue vitamin B₃₃ levels were less than 40 ng per g of liver (approximately 30% of the normal tissue B₃₃ level). A similar relationship (Fig. 5) was seen for MM-CoA, which was measurably elevated when the tissue B₃₃ level was less than 30 ng per g of liver.

Finally, as shown in Table II, acetyl-CoA levels were approximately 2-fold greater in B₃₃-deprived animals than in the control animals. Although the mean values in the B₃₃-supplemented group were slightly lower than in the animals on a normal diet, the differences were not statistically significant. The values in the liver extracts obtained from the normal animals agreed with those reported by Allred and Guy (37) where a different assay method was used.

Levels of Krebs' Cycle Intermediates—The finding of increased levels of succinyl-CoA in the livers from the B₃₃-deprived ani-
TABLE III

Values of liver Krebs' cycle intermediates

<table>
<thead>
<tr>
<th>Study animals</th>
<th>Malate (nmol/g liver)</th>
<th>Citrate (nmol/g liver)</th>
<th>Ratio of malate to citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (4)</td>
<td>437 ± 67</td>
<td>137 ± 13</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>B12-supplemented (4)</td>
<td>522 ± 68</td>
<td>212 ± 38</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>B12-deprived (7)</td>
<td>600 ± 84</td>
<td>151 ± 26</td>
<td>6.9 ± 1.4</td>
</tr>
</tbody>
</table>

Data obtained from the liver extracts from the animals for the CoA intermediates analysis. Conditions of the assays were as outlined under "Methods." Figures in parentheses indicate the number of animals studied. Results are ± S.E.

DISCUSSION

The evidence that coenzyme B12 was required in the conversion of methylmalonyl-CoA to succinyl-CoA (1, 2) combined with the observation of propionic and methylmalonic aciduria in experimental and clinical circumstances of B12 deficiency (2-8) has led to the general conclusion that such deprived states result in increased tissue levels of the CoA intermediates (propionyl-CoA and methylmalonyl-CoA). To date, actual measurements of these intermediates have not been reported.

Inferential evidence from the measurements of total methylmalonate have supported the expected increase in the concentration of CoA derivatives (11, 39, 40). However, the lack of knowledge concerning the deacylation of these derivatives (9, 10), combined with the evidence that the rates of disappearance of MM-CoA in liver and brain were no different in B12-deprived animals than in the normal (9), suggest that such inferential data must be cautiously interpreted. The present study provides the first direct measurements of these CoA derivatives in B12-deprived animals coupled with the previous evidence from this laboratory of a 2-fold increase in citrate synthase activity in this state (38), led to the study of selected Krebs' cycle intermediates in this tissue. As shown in Table III, measurements of malate and citrate were performed. The concentration of malate was approximately 2-fold greater in the liver extracts obtained from the B12-deprived than in those obtained from control animals. By contrast, the mean for citrate in the livers of the B12-deprived animals was not significantly different than that from the normal animals. The resultant malate to citrate ratio was over 2-fold greater in the B12-deprived group.

The increased levels of succinyl-CoA in vitamin B12 deprivation (Table II) were quite unexpected (10). It was anticipated that succinyl-CoA levels would be reduced because of the decreased contribution from MM-CoA. The basis for the increased succinyl-CoA in the livers of B12-deprived animals is not clear. That these levels were not the result of artifacts in the assay technique is supported by several points. First, the arsenolysis method, using E. coli succinate thiolkinase, was shown to be highly specific for succinyl-CoA. In addition, recovery in excess of 90% was demonstrated when standards were added to tissue extracts and then assayed in parallel with the unknowns. The data obtained from the freeze-clamp study performed at increasing intervals following the cranial injury failed to demonstrate either a relative or absolute increase in succinyl-CoA levels; in fact, such delay resulted in a decrease in succinyl-CoA levels.

Selected Krebs' cycle intermediates, malate and citrate were also measured. As shown in Table III, malate was 2-fold greater in the livers from B12-deprived animals when compared to the controls, whereas there was little difference in the concentration of citrate in the different groups, resulting in an increase in the malate to citrate ratio. Since propionyl-CoA has been shown to inhibit citrate synthase (17), one possible explanation is that the high propionyl-CoA level inhibits citrate synthase causing a decrease in cycle activity. This decrease could result from the high propionyl-CoA level, which inhibits citrate synthase, causing a decrease in cycle activity.
in an increase in succinyl-CoA which may be derived from glutamate via α-ketoglutarate. The high succinyl-CoA levels could further inhibit citrate synthase activity. Studies are now in progress to clarify these relationships.

Finally, the level of acetyl-CoA in the livers of B12-deprived animals was somewhat higher than in the controls. Although our control values were slightly lower than those described by Williams et al. (45), the differences may be the result of their use of starved animals, which has been shown by Guynn et al. (46) to increase acetyl-CoA levels. Of primary interest is that the relationship between the values in the controls and those in the B12-deprived tissues follow the pattern they reported (45). Their proposal that increased concentrations of propionyl-CoA would result in decreased levels of acetyl-CoA (because of substrate function in thiolase related reactions) is not consistent with the present observations; no evident relationship existed between propionyl-CoA values in any given liver and acetyl-CoA levels.

The importance of intracellular compartmentation, between the cytoplasm and mitochondria, in terms of the measured levels of CoA derivatives cannot be answered with the present study. The freeze-clamp technique was shown to be critical for accurate measurements of the CoA derivatives involved. Thus, until a rapid separation technique for subcellular units becomes available, it will not be possible to critically examine the issue of compartmentation in a direct manner. At least one factor known to affect the compartmentation for propionyl-CoA is transport by L-carnitine (42). In vivo levels of L-carnitine and propionyl-L-carnitine have been measured and shown to be normal in B12 deprivation.8 Certain inferences concerning the compartmentation of the CoA intermediates can be made from the localization of the enzymes involved in their interconversion. Thus, the mitochondria is the known site of propionyl-CoA carboxylase (44) as well as the site of activation of propionate synthetase (14). Since acetyl-CoA carboxylase, another cytoplasmic enzyme, readily carboxylates propionyl-CoA to methylmalonyl-CoA (14,24) a mechanism for the formation of MM-CoA exists in the cytoplasm. To date, no evident basis exists for the occurrence of succinyl-CoA outside of the mitochondrial compartment.

Acknowledgment.—The authors wish to express their appreciation to Dr. Paul Sreer for his advice and suggestions.

REFERENCES


8 E. P. Frenkel and R. L. Kitchens, unpublished observations.
Effect of Vitamin B$_{12}$ Deprivation on the in Vivo Levels of Coenzyme A Intermediates Associated with Propionate Metabolism
Eugene P. Frenkel, Richard L. Kitchens, Louis B. Hersh and Rene Frenkel


Access the most updated version of this article at [http://www.jbc.org/content/249/21/6984](http://www.jbc.org/content/249/21/6984)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/21/6984.full.html#ref-list-1](http://www.jbc.org/content/249/21/6984.full.html#ref-list-1)