Applicability of an Enzymatic Quantitation of Methylmalonic, Propionic, and Acetic Acids in Normal and Megaloblastic States

By Eugene P. Frenkel and Richard L. Kitchens

A rapid sensitive spectrophotometric assay for the measurement of methylmalonic and propionic acids in urine is described. The assay is based upon the quantitation of propionic acid using acetyl coenzyme A synthetase isolated from baker's yeast. This enzyme is highly specific for acetate and propionate, and acetate interference is eliminated by conversion to citrate. Methylmalonic acid was assayed by converting it to propionate by heat decarboxylation and then measuring the propionate increment over the endogenous amount in the noncarboxylated sample. Studies of urine obtained from normal subjects (by isolation, partial purification, and then assay by the isotope dilution technique) demonstrated urinary excretion of less than 1 mg of propionic acid and 1–5 mg of methylmalonic acid per day. In 22 consecutive patients with documented vitamin B₁₂ deficiency, methylmalonic acid excretion in excess of 30 mg/24 hr was found. In four other patients, with only neurologic involvement methylmalonic aciduria aided in identifying B₁₂ deficiency as an etiologic factor. Methylmalonic acid excretion was measured by direct assay of an aliquot of urine, requiring neither a valine load nor special extraction procedures. Propionic aciduria was variably increased in B₁₂ deficiency and did not correlate either with the severity of the deficit or degree of methylmalonic aciduria. The assay was performed on urine, but it is potentially applicable to tissue extracts. In addition, this assay method can be utilized for the quantification of urine acetate levels as well.

CONGENITAL DEFECTS and ACQUIRED DEFICIENCIES in the coenzyme B₁₂-dependent oxidation of propionate (Fig. 1) are known to result in propionic (PA) and methylmalonic (MMA) aciduria. The potential for rapid identification and quantification of these acidurias provides a valuable approach to the difficult differential diagnosis of ketoacidotic states in childhood, as well as an aid in the diagnosis of acquired B₁₂ deficient states.

The applicability of the currently available techniques has been limited largely to investigative programs, since the one method with reasonable resolution and reproducibility is that of gas chromatography. However, the gas chromatographic analytic approach requires either the isolation of the free acids or the making of an appropriate derivative or special extraction of the
Propionic acid $\rightarrow$ Propionyl coenzyme A $\rightarrow$ D-methylmalonyl coenzyme A $\downarrow$

Succinyl coenzyme A $\xrightarrow{\text{Coenzyme B12 mutase}}$ L-methylmalonyl coenzyme A

Fig. 1. B12-dependent oxidation of propionate.

urine, combined with, of course, the absolute requirement of the chromatographic apparatus and technical expertise in its use.

The purpose of the present communication is to describe a highly specific, sensitive, and rapid spectrophotometric assay applicable to the identification and quantification of propionic and methylmalonic acids in urine. In addition, the method provides the potential for the measurement of acetate in urine as well. The rationale for the assay is based upon the highly specific reactivity of the enzyme acetyl coenzyme A synthetase with acetate and propionate:27,28

\[
\text{Acetate or propionate} + \text{ATP} + \text{coenzyme A} \xrightarrow{\text{acetyl CoA synthetase}} \text{acetyl CoA or propionyl CoA} + \text{AMP} + \text{PPi}. 
\]

In the assay described, the simultaneous removal of acetyl CoA permits the spectrophotometric determination of propionate (PA) in the specimen. Subsequent decarboxylation of endogenous methylmalonic acid (MMA) to propionic acid then provides a potential increment in total propionate equivalent to the amount of methylmalonic acid in that specimen. The present study further documents, by corroborative isotope dilution assay, that urine from normal human subjects contains small but measurable amounts of both propionic and methylmalonic acid.

MATERIALS AND METHODS

Principles of the Assay Method

The enzymatic assay quantitates propionic acid in the sample. In order to measure methylmalonic acid, this moiety is first decarboxylated by heat and acid to propionic acid and the latter is then determined.

Preparation of the urine sample. Urine samples from patients were collected into bottles and capped to prevent volatilization of the acids. When the assay was used for rat urine, the specimens were collected from animals in single unit metabolic cages into bottles containing 0.5 ml of 1 N NaOH to prevent volatilization of the acids; rat urine was neutralized and diluted 1:4 prior to assay.

Propionic acid assay. For assay of specimens, 100 µl of urine was utilized; no special treatment or extraction was required.

Methylmalonic acid assay. In order to determine the MMA in the specimen, the MMA was initially decarboxylated by acid and heat to its decarboxylation product, propionic acid.29 A 2.0-ml aliquot of urine was placed in a heavy duty, screw-cap glass centrifuge tube and 0.2 ml of concentrated HCl was added. The tightly capped tube was then incubated for 4 hr in a routine “hospital-type” autoclave at 250°F and 18 lb pressure. After cooling, the pH was determined. Adequacy of this decarboxylation procedure was evaluated using triplicate samples with added MMA standards. After 2 hr incubation, approximately 65% of the MMA was identified as propionate and 4 hr incubation yielded in excess of 90% conversion of MMA to propionic acid.
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When the postincubation pH was 3 or greater, incomplete decarboxylation was frequently noted. Although this occurred uncommonly, when the pH was noted to be above 3, HCl was again added and the specimen reautoclaved. Following decarboxylation, the specimen was neutralized (pH 7-8) with 100-200 μl of saturated KOH, and any precipitate was removed by centrifugation. For the final assay, 100 μl of this aliquot was utilized.

Methods of Assay

Reagents and enzymes. Acetyl CoA synthetase was isolated from baker’s yeast as described. The specific activity of the purified preparation was 44 l.U./mg protein at 25°C. For routine assay, use of this highly purified preparation was not required, and enzyme with specific activity in the range 20-40 l.U./mg (obtained by purification through the Bio-Gel fractionation step) was used. Excellent results were obtained with preparations with specific activities as low as 6 l.U./mg.* The enzyme was stored at 0°-4°C in suspension with 2.4 M ammonium sulfate (buffered with 0.1 M potassium phosphate, 5 mM of 2-mercaptoethanol, and 0.5 mM EDTA, pH 7.0-7.5), under which conditions it was stable for at least 2 mo.* Citrate synthase (pig heart: 80 units/mg), malate dehydrogenase (pig heart: 1000 units/mg), catalase (beef liver: 3000 units/mg), carnitine acetyl transferase (pigeon breast muscle: 80 units/mg), ATP (disodium salt, Grade I, neutralized with KOH to pH 7), L-malic acid (neutralized with KOH to pH 7), and DL-carnitine-HCl (similarly neutralized) were all obtained from Sigma Chemical Company, St. Louis, Mo. Coenzyme A (lithium salt, P&L Biochemicals, Inc., Milwaukee, Wis.) aliquots were dissolved in distilled water and were frozen for storage periods of up to 1 mo in a 20 M solution with 100 μM 2-mercaptoethanol. Nicotinamide-adenine dinucleotide, oxidized (NAD⁺), in 100-μmol aliquots, was dissolved in 1 ml 0.05 M Tris HCl, pH 7.5-8.0, and stored at 0.4°C for up to 2 wk. DTNB [5,5′dithiobis-(2-nitrobenzoic acid)] was obtained from Aldrich Chemical Co., Milwaukee, Wisc., and made up daily by dissolving it in 0.2 M Tris-HCl buffer, pH 7.6. Methylmalonic acid (A grade) and propionic acid were obtained from Calbiochem, LaJolla, Calif., and radiolabeled methylmalonic acid (2-[methyl-14C]; sp act 7 mCi/mmol) was obtained from New England Nuclear, Boston, Mass.

Preparation of the Assay Reaction Mixture

The reaction mixture for each assay contained 100 μmol Tris-HCl buffer, pH 7.6; 10 μmol MgCl₂; 6 μmol ATP; 5 μmol malate; 2 μmol NAD⁺; 25 μg citrate synthase; 25 μg malate dehydrogenase; 1 μmol of 2-mercaptoethanol or dithiothreitol; and 0.2 μmol coenzyme A in 0.8 ml aliquots.

For expediency, it was demonstrated that a “stock” reaction cocktail could be prepared for 20 assays and that this cocktail was stable when stored 0°-4°C for 2-3 days. This stock reaction mixture was made up in 13.8 ml distilled water and contained 1.0 ml 2 M Tris-HCl buffer, pH 7.6; 0.2 ml 1 M MgCl₂; 0.4 ml 300 mM ATP; 0.1 ml 1 M malate; 0.4 ml 100 mM NAD⁺; 0.02 ml citrate synthase (25 mg/ml); 0.02 ml malate dehydrogenase (25 mg/ml); 0.2 ml of 20 M coenzyme A; and 100 μmol 2-mercaptoethanol. In the individual assay, an aliquot of 0.8 ml was used. For frequent assay use, aliquots of the above reaction mixture were prepared (omitting the distilled water and the enzymes) and stored at –70°C.

Assay procedure. The assay sequence is delineated in Table 1. For assay, 100 μl of the specimen were added to 0.8 ml of the above reaction mixture (in a 12- × 75-mm glass tube), and the reaction was begun by the addition of 10 μl (2 units) of acetyl CoA synthetase. After 1 hr incubation at room temperature, the tubes were placed on ice and 50 μl of 10% (1 N) perchloric acid added. After 2-3 min of reaction time, the specimen was neutralized by the addition of 50 μl 2 M Tris base (which consistently provided a pH range of 7.5-8, eliminating the need for pH monitoring). Fifty microliters of 0.36 M H₂O₂ (freshly prepared by diluting 30% H₂O₂ [9 M] 1:25 with distilled water) were added, and the specimen was incubated for 15 min at room temperature. Then, 25 μl (2 mg/ml) of catalase were added, and the incubation continued for 5 min more. (Fresh catalase solutions should be utilized, since residual H₂O₂ decreases development and duration of the sub-

*A partially purified preparation of the enzyme (2 l.U./mg) is commercially available from Boehringer-Mannheim Co., New York, N.Y.
Table 1. Assay for Methylmalonic Acid (MMA)

<table>
<thead>
<tr>
<th>Approach</th>
<th>Reaction Involved</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Decarboxylation of MMA</td>
<td>MMA $\xrightarrow{\text{heat}}$ propionic acid + CO$_2$</td>
<td>Chemical reaction activated by temp $&gt; 250^\circ F$</td>
</tr>
<tr>
<td>II. Assay for propionic acid</td>
<td></td>
<td>Acetyl CoA synthetase is specific for acetate and propionate</td>
</tr>
<tr>
<td>A. Conversion of propionic acid to propionyl CoA</td>
<td>Acetate + ATP + CoA $\xrightarrow{\text{Acetyl CoA synthetase}}$ Acetyl CoA + AMP + PPi</td>
<td>Malate serves as source for OAA</td>
</tr>
<tr>
<td></td>
<td>Malate + NAD$^+$ $\xrightarrow{\text{Malate dehydrogenase}}$ OAA + NADH</td>
<td>Although reaction equilibrium favors production of malate and NAD$^+$, the OAA produced adequately provides for citrate synthase reaction</td>
</tr>
<tr>
<td></td>
<td>$\xrightarrow{\text{Citrate synthase}}$ Acetyl CoA + OAA + Citrate + CoA</td>
<td>By converting acetyl CoA $\rightarrow$ citrate and free CoA it no longer affects subsequent reaction steps</td>
</tr>
<tr>
<td></td>
<td>Acidification (by perchloric acid, pH 2)</td>
<td>Removal of extant enzymes</td>
</tr>
<tr>
<td>C. Enzyme inactivation</td>
<td>Subsequent neutralization</td>
<td>Eliminate possible reversibility of acetyl CoA synthetase with resultant production of propionate</td>
</tr>
<tr>
<td>D. Oxidation of free thiols</td>
<td>H$_2$O$_2$</td>
<td>Eliminates interference of thiols in subsequent DTNB reaction</td>
</tr>
<tr>
<td>E. Determination of propionyl CoA by carnitine acyl transferase reaction and simultaneous assay of resultant CoA by DTNB</td>
<td>Propionyl CoA + carnitine $\xrightarrow{\text{Carnitine acyl transferase}}$ Propionyl carnitine + CoA SH</td>
<td>H$_2$O$_2$ excess removed</td>
</tr>
</tbody>
</table>
CONCENTRATIONS OF PROPIONATE

The level of MMA in urine specimens obtained from normal human donors was separately determined by diethyl ether extraction of the urine, thin-layer chromatographic purification, and assay by isotope dilution. 14C-methylmalonic acid was added to a 2-mI acidified aliquot of a measured 24-hr urine volume and the specimen extracted with diethyl ether as previously described.17 The solution was acidified with 4 N HCl to pH 2 and saturated with 1.8 g NaCl. The solution was then extracted three times with 10-ml volumes of diethyl ether (previously treated with NaOH). The ether phases were pooled and washed with 0.6 ml 2 M Tris base (pH 9). The ether phase was discarded. One aliquot of the residual was used to measure radioactivity to determine the recovery of the separation,17 and one was assayed for propionate. Recovery of added radioactivity was in excess of 96% on each specimen studied.

**Assay for acetate in urine.** The present assay method is applicable for the determination of urine acetate. During the evaluation of this method, a similar assay, employing beef heart acetyl CoA synthetase, was reported by Guynn and Veech.30 The assay for acetate was carried out with the reaction mixture described above, with the addition of (final concentration) 0.1 mM NADH. The reaction was begun with 0.5 units of acetyl CoA synthetase and followed to end point (occurring within 5 min) spectrophotometrically at 340 nm. Acetate was calculated directly, using the extinction coefficient for NADH (6.22 × 105 sq cm/mol). Acetate contamination was noted in some of the reaction reagents (primarily malate). L-malic acid was therefore purified by extraction five times with equal volumes of a 1:1 mixture of diethyl ether and petroleum ether (previously washed with 10% KOH to remove organic acids). The ether phases were discarded, and the aqueous phase was lyophilized to provide aliquots for subsequent acetate assays.

**RESULTS**

**Effect of incubation time and enzyme activity.** The effect of incubation time on the enzymatic conversion of added propionate to propionyl CoA by acetyl CoA synthetase (2 I.U.) was determined. As shown in Fig. 2A, complete enzymatic conversion was evident by 45 min under the assay conditions described.

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Fig. 2. Effect of incubation time and enzyme (acetyl CoA synthetase) on the conversion of propionate in the assay. (A) Exogenous propionate (50 nmol/assay) was added under conditions of the assay and the completeness of enzymatic conversion and recovery of the added propionate measured at different intervals of incubation. (B) With a 1-hr incubation time, under conditions described in Materials and Methods, the effect of varying amounts of acetyl CoA synthetase on the conversion of added propionate was determined.

above. Based upon these observations, a 1-hr incubation time has been utilized. Figure 2B demonstrates the effect of varying amounts of acetyl CoA synthetase in the assay employing the 1-hr incubation time. Under the conditions of the assay, levels beyond 1.5 U of enzyme provided complete enzymatic conversion.

Recovery and quantification of MMA and PA in urine. A typical standard curve obtained by assay of urine containing measured quantities of exogenous propionic acid is shown in Fig. 3. Similar studies were performed by the addition of varying amounts of MMA to the urine; the recovery was approximately 90%.

Proportional dilution studies were performed by the addition of a measured amount of propionate to the urine. Assay of this specimen and serial dilution of aliquots, with assay of these dilutions, were performed. As shown in Fig. 4, excellent proportionality was noted on serial dilution of the native urine, providing evidence against the existence of substances in urine that interfere with the assay.

The minimal limit of accurate resolution for propionate is approximately
5 nmol//assay or 50 µmol/liter of urine (approximately 4 mg propionic acid/liter of urine). For methylmalonic acid, the method will detect approximately 60-70 µmol of MMA/liter (or 7-8 mg of MMA/liter urine).

Propionic and methylmalonic acids were shown to be unaffected by storage at room temperature for at least 48 hr. Urine was assayed on freshly collected specimens from patients with propionic and methylmalonic aciduria, as well as from normal subjects to which propionic and methylmalonic standards were added. The specimens were then left at room temperature and reassayed at 24 and 48 hr. No significant volatilization of the acids occurred, as evidenced by less than a 5% difference in values over this period of storage.

Specificity of the assay. Specificity of the enzymatic reaction catalyzed by acetyl CoA synthetase for acetate, propionate, and acrylate has been documented.\textsuperscript{27,28} Under the conditions of the assay, acetate was removed by conversion to citrate by citrate synthase and malate dehydrogenase (Table I) in the presence of NAD and malate. Citrate synthase has been shown to be highly specific for acetyl CoA under the conditions described.\textsuperscript{31} The effect of acetate on the reliability of the measurement of propionic acid was assessed by assay of a fixed quantity of the former in the presence of varying amounts of acetate. Acetate did not interfere with the assay except at very high concentrations. Only at levels above 1.2 µmol/assay (i.e., approximately 15 mmol/liter or 900 mg/liter acetic acid) was some interference noted. Although we have not encountered such levels in human urine, its evidence would be easily observed spectrophotometrically. Additions of up to 1000 nmol per assay of formate, acrylate, n-butyrate, or isobutyrate were not detectable by the assay method.

Clinical Application of the Assay

Levels of propionic and methylmalonic acid in urine from normal subjects. Urine obtained from ten normal subjects was assayed as described above and then reassayed by isotope dilution before isolation and partial purification. As shown in Table 2, MMA excretion was below the level of detection (less than 5 mg/24 hr) by the spectrophotometric assay. By isotope dilution, MMA was consistently identified in the urines from normal subjects with values in the range of 1.0-4.9 mg/24 hr. Since this latter assay involved the partial purification of MMA, the study provided further support that the identified propionic acid had its source in MMA, rather than from some unidentified breakdown.
Table 2. Urinary Excretion of Methylmalonic, Propionic, and Acetic Acids in Normal Subjects

<table>
<thead>
<tr>
<th>Acid Excreted</th>
<th>mg/24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylmalonic</td>
<td>2.2 ± 0.3*</td>
</tr>
<tr>
<td>Propionic</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Acetic</td>
<td>3.8 ± 0.6</td>
</tr>
</tbody>
</table>

Methylmalonic and propionic acids were measured by the isotope dilution technique after an aliquot of a freshly collected 24-hr urine specimen was appropriately extracted as previously described.17 Acetate was measured directly.

*MSEM.

product resulting from the autoclave procedure. Similar studies of propionate excretion in normal subjects (Table 2) identified propionic acid only by the isotope dilution technique, and the normal subjects excreted less than 1 mg/24 hr.

Methylmalonic and propionic aciduria in disease states. Measurement of MMA and PA was performed on aliquots of 24-hr urine collections obtained from 22 consecutive patients with documented evidence of acquired vitamin B12 deficiency. These patients had megaloblastic anemia, low serum vitamin B12 levels,32 absence of gastric intrinsic factor activity, and all demonstrated a complete repair of their hematologic abnormalities with vitamin B12 repletion. As shown in Table 3, all of these patients had increased MMA excretion, ranging between 30 and 2850 mg/24 hr. Propionic acid excretion was normal in 15 of these patients; in the other 7 patients the values ranged between 10 and 145 mg/24 hr. No correlation was seen between the degree of propionic aciduria and methylmalonic aciduria. For instance, a patient with 2850 mg of MMA in his 24-hr urine collection had normal propionate excretion. One patient with a urinary MMA of 230 mg/24 hr had a urinary propionate of 145 mg in that specimen. Neither the severity of the anemia nor the duration of clinical symptoms correlated with the degree of propionic or methylmalonic aciduria. In addition, four patients have been studied with no hematologic abnormalities (absence of anemia, normal red cell mean corpuscular volume, normal mean lobe average of polymorphonuclear leukocytes, absence of hypersegmentation of the polymorphonuclear leukocytes, and a normal bone marrow examina-

Table 3. Propionic and Methylmalonic Aciduria in Man

<table>
<thead>
<tr>
<th></th>
<th>Propionic Aciduria (mg/24 hr)</th>
<th>Methylmalonic Aciduria (mg/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects (26)</td>
<td>&lt;1*</td>
<td>&lt;5*</td>
</tr>
<tr>
<td>Hepatic necrosis (4)</td>
<td>&lt;1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Megaloblastic dyspoiesis</td>
<td>Secondary to folate deficiency (12)</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>Long-term anticonvulsant</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>therapy with peripheral macrocytosis (3)</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>DiGuglielmo syndrome (2)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Vitramin B12 deficiency</td>
<td>With megaloblastic anemia (22)</td>
<td>0–145</td>
</tr>
<tr>
<td></td>
<td>With neurologic deficit (4)</td>
<td>0–16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>230–705</td>
</tr>
</tbody>
</table>

Assays were performed by the described spectrophotometric assay on aliquots of 24-hr urine specimens.

*Below the level of resolution of the spectrophotometric assay method.
tion), but with neurologic changes compatible with those seen in vitamin B₁₂ deficiency. These patients all had a peripheral neuropathy with variable involvement of their posterolateral spinal cord tracts. Each had serum vitamin B₁₂ values below 125 pg/ml (normal 200–1000 pg/ml), and all demonstrated improvement in their peripheral neuropathy with vitamin B₁₂ repletion. Increased methylmalonic aciduria (Table 3) was present in all four ranging between 230 and 705 mg/24 hr. Only one demonstrated increased propionic aciduria (16 mg/24 hr). No correlation between the severity of the neurologic deficit and the degree of methylmalonic aciduria was noted in these patients.

Propionic and methylmalonic aciduria were also evaluated in other circumstances where the clinical findings may be confused with the B₁₂-deficient state. As noted in Table 3, megaloblastic anemia secondary to folate deficiency, peripheral macrocytosis associated with long-standing anticonvulsant therapy, and patients with that form of autonomous megaloblastic dyspoiesis termed DiGuglielmo syndrome all had normal urinary excretion of PA and MMA. This result was also true of patients with hepatic necrosis (Table 3). Similarly, patients with renal failure (with metabolic acidosis and proteinuria) and adult ketoacidosis had normal urinary PA and MMA.

**Urinary Acetate Quantitation**

Utilizing the assay modification noted above, urine acetate levels were measured in freshly collected specimens from ten normal subjects. Their values were all below 4.5 mg/24 hr (Table 2). Recovery studies, employing acetate standards added to urine, demonstrated that greater than 95% of the added acetate was measured. Similarly, proportional dilution studies documented proportionality in both native and autoclaved urine, suggesting that other substances in the urine did not interfere with the assay. Normal urine permitted to stand at room temperature had elevated acetate levels, probably due to breakdown of acetate-containing compounds, making quantitation of 24-hr specimens unreliable. Addition of alkali or heating enhanced this breakdown.

**DISCUSSION**

In 1962, Cox and White⁸ provided the initial documentation that methylmalonic aciduria was a sensitive index of the acquired vitamin B₁₂-deficient state. Subsequently they demonstrated that, as one might expect from the site of the metabolic defect, propionic aciduria also was seen in B₁₂ deficiency.¹⁵ The identification and measurement of these acidurias gained further potential clinical importance when congenital defects in the B₁₂-dependent metabolic pathway were described as one cause of childhood ketoacidosis,¹⁰⁻¹⁸ since the classical criteria of B₁₂ deficiency were usually absent. The present spectrophotometric assay provides a sensitive, reproducible method that can be performed with standard clinical laboratory instruments. In addition, the assay provides quantification of both propionic acid and methylmalonic acid, which is of value in the recently identified congenital abnormalities of the propionate oxidative pathway,¹⁸⁻²⁰ where methylmalonic aciduria is not seen.

As can be noted from a typical standard curve (Fig. 3), the quantitation of propionate (or the decarboxylated methylmalonic acid) does not correspond to
the usual extinction coefficient for DTNB (13.6), when corrected for the dilution utilized. This result is due to the quenching effect of the unextracted urine on the DTNB color development, a phenomenon noted also in studies employing tissue extracts, and a small but consistent thiol ester loss when the measurement is made with the reported extinction coefficient of DTNB. In experiments not shown, it has clearly been established that exogenous propionyl CoA, under the conditions described, has an apparent thiol ester loss when the assay values are expressed simply by the extinction coefficient of DTNB. Thus the loss is not due to incomplete enzymic conversion of propionate to propionyl CoA, but, rather, due to these described factors. However, it has further been established that reliable and reproducible circumvention of this effect is easily achieved by the use of appropriate standards and a standard curve.

Utilizing an extensive extraction and chromatographic isolation sequence, Thomas and Stalder in 1957 demonstrated the presence of “trace” amounts of methylmalonic acid in normal subjects. The limits of detection by virtually all of the methods subsequently applied for the identification and determination of methylmalonic acid have been so poor that often a valine load to stress the pathway has been required in order to identify methylmalonic acid in the urine. In order to answer the question of whether methylmalonic acid was a normal urinary product, we extracted urine obtained from normal subjects, separated the MMA chromatographically, and quantitatively assayed for MMA by the isotope dilution technique. Methylmalonic acid was identified in all of the normal subjects. In addition to this identification, this procedure, which depended upon partial purification, helped to establish that the material assayed as propionate had MMA as its exclusive origin and that it was not derived from some unknown compound broken down to propionate during the procedure. Such specificity of the measured moiety is a serious problem in most of the colorimetric procedures. Loss as well as specificity also applies to the gas chromatographic techniques, since thermal lability of methylmalonic acid, even in the ester form, may affect the results.

That the spectrophotometric assay for MMA is sensitive is corroborated by the evidence of abnormal values identifiable in 22 consecutive patients with clinical evidence of a megaloblastic anemia secondary to vitamin B12 deficiency (Table 3). Additional clinical interest in this method in the screening of patients with neurologic deficit in the absence of megaloblastic anemia is provided by the identification of four such patients, whose subsequent clinical improvement with B12 repletion has suggested that B12 deficiency was etiologic in their clinical presentation. Chanarin and co-workers have described a group of patients with vitamin B12 deficiency, and in 7 of 23 cases MMA excretion has been normal. He has suggested that this finding relates to the severity of the B12 deficiency, since MMA excretion is identified only in those patients with relatively low serum vitamin B12 levels. It is of some interest that two patients included in the present series had been studied at other centers prior to being evaluated in our laboratory. Those studies employed a gas chromatographic procedure utilized by Chanarin et al. and were reported as “normal (below the limit of resolution).” Spectrophotometric assay revealed MMA values of 40 and 65 mg/24 hr. This finding suggests that the failure to identify MMA in
clinically evident B₁₂-deficient states may have been methodologic. In addition, we, unlike Chanarin et al.,¹⁴ have not been able to correlate the degree of methylmalonic aciduria with the serum vitamin B₁₂ level. This failure is not too surprising, in light of the only modest correlation of the absolute serum B₁₂ level with measured tissue stores of vitamin B₁₂.³⁸

The present study demonstrated that propionic aciduria was a variable finding in patients with established vitamin B₁₂ deficiency (Table 3). In addition, no correlation could be made to the degree of methylmalonic aciduria in these patients. In each case studied, the degree of methylmalonic aciduria was much greater than that of propionic aciduria. Little attention has been paid to propionic aciduria in the B₁₂-deprived state since the original studies of Cox et al.¹⁵ This dichotomy between the degree of propionic and methylmalonic aciduria is of considerable interest. A simplistic explanation could relate these differences to the fact that the B₁₂ coenzyme defect is, in fact, specific for the conversion of methylmalonyl coenzyme A to succinyl CoA; furthermore, B₁₂ deficiency is known to have no effect on propionyl CoA carboxylase. Nevertheless, recent measurements of the actual hepatic CoA intermediates in the B₁₂ deprived state demonstrated that such deficiency resulted in higher levels of propionyl CoA than methylmalonyl CoA.³³ This observation suggests that a specific (for methylmalonyl CoA) intracellular deacylase is responsible for this effect. Studies are now in progress to identify and characterize such a deacylase.

In addition to the above unresolved problem, there has only been limited characterization of the pathophysiologic interrelationships of propionic and methylmalonic aciduria in the B₁₂-deficient state. One explanation for variable methylmalonic aciduria in B₁₂ deprivation could be correlated with the level of the specific B₁₂ coenzyme involved (5'-deoxyadenosylcobalamin [AdCo]) in the propionate catabolic pathway. Since Linnell et al.³⁹,⁴⁰ have shown that the decline in specific cobalamin forms was not homogeneous in the deficient state, one might anticipate that the MMA excretion would correlate not with the total B₁₂ level, but rather with the specific amount of AdCo. In the only study to date, that of seven patients by Cooper and co-workers,⁴¹ the level of AdCo did not correlate with the degree of methylmalonic aciduria. The simplicity and availability of the present spectrophotometric assay should help provide data to resolve some of these questions.

Previous studies by Cox et al.¹⁵ had demonstrated increased urine acetate excretion in vitamin B₁₂-deficient patients and had demonstrated a correlation between the level of acetate excretion and associated neurologic deficit related to the B₁₂ deficiency. A noteworthy application of the present assay was its ability to be utilized for the assay of urine acetate content. Serial studies demonstrated that the amount of free acetate in normal urine was less than 1 mg/24 hr. However, considerably higher levels were demonstrated when the breakdown of (labile) acetate-containing compounds was permitted to occur. Such breakdown was identified when specimens were permitted to stand at room temperature after collection (thereby invalidating routine 24-hr urine collections), or when the specimen was made alkaline or heated. Under such circumstances, the measured level of acetate was high and very variable. All of the above conditions were involved in the assay reported by Cox et al.¹⁵ We have
not demonstrated increased free acetate excretion in carefully collected specimens from patients with B12 deficiency, and no evidence of a relationship of urine acetate levels to the neurologic sequelae of B12 deficiency was seen.

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Applicability of an enzymatic quantitation of methylmalonic, propionic, and acetic acids in normal and megaloblastic states

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