Intracellular Localization of Hepatic Propionyl-CoA Carboxylase and Methylmalonyl-CoA Mutase in Humans and Normal and Vitamin B₁₂ Deficient Rats

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SUMMARY. The intracellular localization of the enzymes in the vitamin B₁₂ dependent pathway which involves the oxidation of propionate was studied in rat liver obtained from normal and vitamin B₁₂ deficient rats as well as from man. The subcellular site of propionyl CoA carboxylase and the vitamin B₁₂ dependent-methylmalonyl CoA mutase were determined. All of the activity of these two enzymes was demonstrated to be in the mitochondria and those enzymes were shown to be loosely bound to the inner membrane-matrix portion of the mitochondria. Vitamin B₁₂ deficiency did not alter the subcellular localization. Finally, a rapid enzymatic assay for methylmalonyl-CoA mutase was described.

Propionyl-CoA carboxylase (E.C. 6.4.1.3) and methylmalonyl-CoA mutase (E.C. 5.4.99.2) are critical enzymes in the oxidation of propionate (Kaziro & Ochoa, 1964) and the branched amino acids valine and isoleucine; threonine and methionine degradation have also been demonstrated via this pathway (Marston et al, 1961). Interest in this pathway relates to the coenzyme B₁₂ requirement of methylmalonyl-CoA mutase. Since the primary site for fatty acid oxidation in the cell is the mitochondria (Tubbs & Garland, 1968), and since the end product of propionate oxidation, succinyl CoA, is an intermediate in the Krebs' tricarboxylic acid cycle located in the mitochondria, it has generally been assumed that these two enzymes exist within the mitochondria. However, recently Morrow et al (1973) provided data that led them to propose an intracellular distribution in both mitochondria and cytoplasm for methylmalonyl CoA mutase in liver. An earlier study by Scholte (1969) had raised a similar question for the localization of propionyl CoA carboxylase in rat liver. Although he demonstrated that propionyl CoA carboxylase was primarily a mitochondrial matrix enzyme (Scholte, 1969), 25% of the enzyme activity was found in the cytosol. Scholte (1969) was unable to differentiate between a physiologic 'bimodal' distribution (i.e. in cytosol and mitochondria) of the enzyme or a leak of the enzyme due to mitochondrial injury during preparation.

The mitochondrial marker enzyme utilized in the above studies (Morrow et al, 1973; Scholte, 1969) was cytochrome oxidase, an enzyme so tightly bound to the inner mitochondrial membrane (Seligman et al, 1968) that it is a relatively insensitive indicator of leakage of
soluble matrix contents during mitochondrial injury. The present study sought the intra-
cellular localization of these two enzymes, employing sensitive markers of leakage due to
membrane injury, in normal rat and human liver. In addition, we compared the findings in
normal rat liver with those in vitamin B_{12} deficient animals, since these enzymes are in the
metabolic pathway in which vitamin B_{12} is required as a coenzyme. The intramitochondrial
distributions of these two enzymes were also studied. Finally, we have provided a new spec-
trophotometric assay method for methylmalonyl CoA mutase.

MATERIALS AND METHODS

Animals and their Maintenance
The care and maintenance of the rats as well as the dietary programmes for the normal and
the vitamin B_{12} deprived groups were as previously described (Frenkel et al., 1973a, b, 1974).
The development of vitamin B_{12} deficiency generally required approximately 6 months. The
animals were sacrificed in the fed state.

Human Liver
Specimens were obtained at autopsy from two adults; 4 h after the death of one subject
from a gunshot wound and the second 12 h after the death of a patient due to pulmonary
embolism. Subjects were otherwise normal and histologic evaluation corroborated the ab-
sence of underlying hematologic or hepatic disease.

The Preparation of Rat Liver Subcellular Fractions
The rats were killed by decapitation and the livers quickly removed and placed in cold
saline. An aliquot of spleen and liver were removed for tissue vitamin B_{12} assay (Frenkel,
1972). The liver was blotted and a 5 g portion weighed and suspended in 6 vol of 0.25 M
sucrose and 0.005 M MOPS, pH 7.4 (morpholinopropane sulphonic acid, Sigma Chemical
Corp., St Louis, Missouri, U.S.A.); the MOPS previously adjusted to pH 7.4 by the addition
of KOH. Brief and gentle homogenization was then carried out in a glass vessel with a
mitochondrial injury. An aliquot of the homogenate was removed for enzyme and protein
assay, and the remainder then fractionated by a modification of a method of De Duve et al
(1955). Sequential fractions separated by centrifugation were: first, the nuclear fraction
separated as the 600 g pellet after 10 min of centrifugation, then the mitochondrial fraction
obtained as the 8000 g pellet from a 10 min centrifugation, the light mitochondrial fraction
obtained as the 20 000 g pellet obtained after 10 min of centrifugation, and the microsomal
and cytosol fractions as the pellet and supernatant portions, respectively, from the 100 000 g
centrifugation for 60 min. The mitochondrial fraction was then washed by resuspending in
the homogenization medium and centrifuging again. All of the separated pellet fractions
were resuspended in a measured volume of homogenization medium for the enzyme assays.

Preparation of Human Liver Subcellular Fractions
The human liver specimens were immediately placed in cold, buffered, sterile saline and a
5 g portion weighed and homogenized into 5 vol of homogenization medium. The fractions
were subsequently separated as above, except that the nuclear pellet was washed in 5 vol of media, re-homogenized and re-centrifuged, and the supernatant fractions were combined for the subsequent separations.

**Preparation of Rat Liver Mitochondrial Subfractions**

Mitochondria were isolated from the liver by the method of Johnson & Lardy (1967). The mitochondria were washed twice, an aliquot removed for enzyme assay and the remainder was fractionated with digitonin (Calbiochem, San Diego, California, U.S.A.) by the method of Schnaitman et al (1967) and Schnaitman & Greenawalt (1968) as modified by Scholte (1969). The separate fractions obtained by this method (Scholte, 1969) were labelled Fraction 1, the pellet from centrifugation at 9500 g; Fraction 2, the pellet obtained at 35 000 g; and, Fraction 3, the pellet from the 230 000 g centrifugation. Fraction 4 was the residual supernatant fraction.

In order to further differentiate between the inner membrane and matrix compartments, mitochondrial subfractions were prepared in a separate series of studies using the nonionic detergent Lubrol WX as described by Chan et al (1970).

**Enzyme Assays**

*Methylmalonyl CoA mutase* was assayed by a method that exploited a spectrophotometric quantitation of the amount of succinyl CoA formed. An aliquot of the tissue fractions was added to the assay reaction mixture which contained final concentrations of 100 mM MOPS buffer (K+, pH 7.4), 20 μg per ml of antimycin A, 0.1% Triton X-100, 0.1 mM coenzyme B₁₂ (Calbiochem, San Diego, California, U.S.A.), and 0.5 mM methylmalonyl CoA (P & L Biochemicals, Milwaukee, Wisconsin, U.S.A.) in a total vol of 0.5 ml. The racemic (1) form of methylmalonyl CoA was produced by heating for 1.5 min in a 65°C water bath (Mazumder et al, 1961). All steps which involved the presence of coenzyme B₁₂ were carried out in the dark. Mixtures were preincubated for 5 min at 37°C in the absence of methylmalonyl CoA, and the reaction was begun by the addition of methylmalonyl CoA. After 5 min of incubation the reaction was terminated with 50 μl of 10% perchloric acid, the specimen was immediately placed on ice and 25 μl of 2 M tris (Cl), pH 8, added. The specimen was then neutralized to the point where the evident vitamin B₁₂ tinctorial quality was identified by a colour change from yellow to pink (pH range of 5.5–6) by addition of microlitre amounts of 3 M potassium carbonate while agitating vigorously on a vortex mixer. Brief centrifugation was carried out and 100 μl was removed for the assay of succinyl CoA formed as previously described (Frenkel et al, 1974). This spectrophotometric measurement used an arselenalysis reaction with succinate thiokinase (prepared from *E. coli* and kindly supplied by Dr Jonathan Nishimura of the University of Texas at San Antonio). The arselenalysis product of succinyl CoA was coupled with DTNB (5′,5′-dithiobis-[2-nitrobenzoic acid]), and the release of free CoA was measured at 412 nm (Frenkel et al, 1974). Each unit of mutase activity is defined as that capable of forming 1 μmol of succinyl CoA per min at 37°C. Duplicate assays were performed at varying levels of enzyme activity to ensure linearity. In studies not shown, the presence of Triton X-100 was documented to have no effect on the activity of solubilized enzyme.

Validation of this assay method for methylmalonyl-CoA mutase was supported by several
observations. As shown in Fig 1(a), the assay was linear from 0 to 50 mU of enzyme when an incubation time of 5 min was used. Variation in the incubation times (Fig 1b), also demonstrated linearity from 0 to 10 min. Furthermore, this assay method was compared to the radioassay technique of Whitaker & Giorgio (1973). Isotopically labelled methylmalonyl-CoA was obtained in two forms (DL-[3-14C]methylmalonyl-CoA through the kind gift of Dr Theodore Whitaker, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A., and as DL-[2-14C]methylmalonyl-CoA by special order from New England Nuclear, Boston, Mass.). Thin layer chromatographic isolation on Whatman Cellulose Powder CC41 (W. R. Balston, England) demonstrated 95% radiopurity. The labelled methylmalonyl-CoA was diluted with methylmalonyl-CoA to a specific activity of 0.88 μCi/μmol.

Fig 1. Characteristics of the methylmalonyl-CoA mutase assay. The conditions of the assay were as described in Methods. (a) With increasing amounts of enzyme protein the assay was linear between 0 and 50 nmol of succinyl-CoA formed during a 5 min incubation time at 37°C. A mU of enzyme activity is defined as that activity which forms 1 nmol of succinyl-CoA at 37°C per min. Results of comparative radioassay are depicted (□) when DL-[2-14C]methylmalonyl-CoA was used as substrate and the product hydrolysed, and the 14C-succinate isolated and counted as described (Whitaker & Giorgio, 1973). (b) Variation in the incubation time (horizontal axis) was shown to yield a linear conversion to succinyl-CoA from 0 to 10 min where the amount of enzyme protein was constant.

The radioassay employed the [14C]methylmalonyl-CoA as substrate and the product was hydrolysed with KOH, the [14C]succinate product isolated and counted as described by Whitaker & Giorgio (1973). Assay results were corrected for radiopurity. As shown in Fig 1(a), the spectrophotometric and radioisotopic assays gave essentially the same results. The requirement for and specificity of coenzyme B12 in the mutase reaction were demonstrated. Essentially no mutase activity was seen in the absence of coenzyme B12 and coenzyme methyl-B12 failed to restore activity. Activity was restored by the coenzyme deoxyadenosyl-B12.

Propionyl-CoA carboxylase was assayed by the 14CO2-fixation technique described by
Halenz & Lane (1960) and Gregolin et al (1968), modified by addition of Triton X-100 (0.1%) to release latent activity in the mitochondria. The presence of the Triton X-100 was demonstrated not to affect the solubilized enzyme activity.

Glutamate dehydrogenase was assayed spectrophotometrically by the NADH oxidation method of King & Frieden (1970). The reaction mixture contained final concentrations of 100 mM tris Cl, pH 8.0, 50 mM ammonium chloride, 0.1 mM EDTA, 0.1 mM NADH, 5 μM rotenone, 0.1% Triton X-100 and 10 mM α-ketoglutarate (K+) in a 1 ml vol. The reaction was begun by the addition of α-ketoglutarate and was determined spectrophotometrically at 340 nm.

Citrate synthase was assayed by the method of Srere (1969). The reaction mixture contained final concentrations of 100 mM tris (Cl), pH 8.0, 0.1 mM DTNB, 0.1% Triton X-100, 0.05 mM acetyl-CoA and 0.25 mM oxaloacetate (K+) in a final volume of 1 ml. The reaction was begun with oxaloacetate, and the release of free thiol groups was determined spectrophotometrically at 412 nm. In separate studies it was shown that Triton X-100, used to release latent activity from mitochondria, did not affect citrate synthase activity.

Succinate dehydrogenase was assayed spectrophotometrically using oxidized cytochrome c coupled with phenazine methosulphate (King, 1967). The assay mixture contained final concentrations of 100 mM potassium phosphate buffer, pH 7.5, 0.05 mM cytochrome c (type 4, Sigma Chemical Co., St Louis, Missouri, U.S.A.), 0.05 mM phenazine methosulphate, 0.3 mM KCl, 0.05 mM rotenone, and 5 mM succinate (K+) made up to a final volume of 1 ml. The reaction was begun by the addition of succinate and cytochrome c reduction was measured spectrophotometrically at 550 nm.

Glucose-6-phosphate dehydrogenase (G6PD) was assayed spectrophotometrically by NADPH formation (Lohr & Waller, 1965). The reaction mixture consisted of 100 mM MOPS buffer, pH 7.4, 10 mM MgCl₂, 0.2 mM NADP⁺ and 1 mM glucose-6-phosphate.

Adenylate kinase was assayed spectrophotometrically by the method of Sottocasa et al (1967a). The hexokinase and G6PD for the assay were obtained from Behringer Corporation, New York, U.S.A.

Rotenone insensitive NADH-cytochrome c reductase was assayed by the method of Sottocasa et al (1967b).

Protein was determined by the method of Lowry et al (1951).

RESULTS

The Intracellular Enzyme Distribution in Rat Liver

Schematic representation of the distribution of methylmalonyl-CoA mutase and propionyl-CoA carboxylase in cellular fractions from normal rat liver is demonstrated in Fig 1, and the quantitative delineation of the data is expressed in Table I. Two marker enzymes are displayed: one, glutamate dehydrogenase, a soluble mitochondrial matrix enzyme used as a marker for mitochondria (King & Frieden, 1970), and G6PD as the marker enzyme for the cytosol. A degree of mitochondrial contamination of the nuclear fraction cannot be avoided, and, as noted in Fig 2, those enzymes associated with the mitochondria are more evident in the nuclear separation than in the cytosol fraction (Scholte, 1969; De Duve et al, 1955). The amount of activity of the methylmalonyl-CoA mutase and propionyl-CoA carboxylase in
TABLE I. Intracellular distribution of methylmalonyl CoA and propionyl CoA in rat liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mitochondrial</th>
<th>'Light mitochondrial'</th>
<th>Microsomal</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>93.3</td>
<td>1.8</td>
<td>3.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Methylmalonyl CoA mutase</td>
<td>97.5</td>
<td>0.5</td>
<td>0.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Propionyl CoA carboxylase</td>
<td>92.8</td>
<td>1.0</td>
<td>0.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>4</td>
<td>1.8</td>
<td>1.0</td>
<td>93.2</td>
</tr>
</tbody>
</table>

Representative fractionation study from normal rat liver. Mitochondrial and nuclear fractions are combined.

the nuclear fraction was virtually identical to that of glutamate dehydrogenase, a known mitochondrial marker enzyme, further suggesting that the degree of mitochondrial contamination of the nuclear fraction could be used as a correlative expression of the localization of the two enzymes in question. In addition, the amount of methylmalonyl-CoA mutase in the cytosol fraction was very low and corresponded to the amounts of glutamate dehydrogenase in that fraction. Only a slightly greater amount of propionyl-CoA carboxylase was found in the cytosol, and when contrasted to classical cytosol situated enzymes (i.e. G6PD), its quantitative presence was clearly trivial. Utilizing the marker enzymes, corrections can be made for the amount of cross contamination in each of the fractions. Intracellular localization studies were done on four separate occasions on liver from normal and vitamin B12 deficient

**Fig 2.** Pattern of intracellular enzyme distribution in normal rat liver. The per cent of total enzyme activity per percentage of total protein (vertical axis) is expressed for each enzyme relative to the per cent of total enzyme protein (horizontal axis) for glutamate dehydrogenase, methylmalonyl-CoA mutase, propionyl-CoA carboxylase and G6PD.
Enzymes of a Vitamin B₁₂ Dependent Pathway

rats. In these separate experiments the cytosol fraction was shown to contain as low as 1% and as high as 4% of the total methylmalonyl-CoA mutase activity and 5-7% of the total propionyl-CoA carboxylase activity. The identification of the separate light mitochondrial and microsomal fractions were shown to contain less than 1% of the total activity of each of the enzymes. The intracellular distribution of the enzymes in the vitamin B₁₂ deficient rats was exactly the same as that identified in the liver from normal animals. Thus, methyl-malonyl-CoA mutase and propionyl-CoA carboxylase are located exclusively in the mitochondrial compartment and the distribution of the enzymes is not affected by the presence of vitamin B₁₂ deficiency.

![Diagram](image)

Fig 3. Schematic representation of selected enzymes from the subcellular fractionation of human liver obtained 4 h and 12 h after death. Nuclear and mitochondrial fractions were combined.

Intracellular Enzyme Distribution in Human Liver

The amount of tissue needed for an adequate determination of the subcellular (and mitochondrial) distribution of the enzymes made it necessary to use post-mortem tissue for the studies in human liver. In order to characterize the degree of autolysis three mitochondrial marker enzymes were used. Succinate dehydrogenase, a mitochondrial marker which, like cytochrome oxidase, is known to be tightly bound to the inner mitochondrial membrane (De Duve et al, 1962). Two other enzymes less well bound to the mitochondrial substructures were also utilized: glutamate dehydrogenase, a loosely bound mitochondrial matrix enzyme, and citrate synthase, an even more loosely bound mitochondrial matrix enzyme, were also used. Because of their known binding characteristics, these reference markers could be used to quantitate the leakage from damaged mitochondria (Scholte, 1969; Sottocasa et al, 1967a, b; Srere, 1972).

The intracellular distribution of the methylmalonyl-CoA mutase and propionyl-CoA carboxylase in the human liver obtained 4 and 12 h after death is contrasted to two mitochondrial marker enzymes in Fig 3. As shown, succinate dehydrogenase is very tightly
### Table II. Intracellular distribution of methylmalonyl-CoA mutase and propionyl CoA carboxylase in human liver

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Nuclear 4h</th>
<th>Mitochondrial 4h</th>
<th>Light mitochondrial 4h</th>
<th>Microsomal 4h</th>
<th>Cytosol 4h</th>
<th>Recovery 4h</th>
<th>Nuclear 12h</th>
<th>Mitochondrial 12h</th>
<th>Light mitochondrial 12h</th>
<th>Microsomal 12h</th>
<th>Cytosol 12h</th>
<th>Recovery 12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase</td>
<td>39</td>
<td>42</td>
<td>37</td>
<td>13</td>
<td>3</td>
<td>4</td>
<td>31</td>
<td>44</td>
<td>93</td>
<td>103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>23</td>
<td>44</td>
<td>43</td>
<td>14</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>20</td>
<td>99</td>
<td>92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylmalonyl-CoA mutase</td>
<td>21</td>
<td>50</td>
<td>40</td>
<td>13</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>22</td>
<td>91</td>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase</td>
<td>18</td>
<td>33</td>
<td>35</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>33</td>
<td>93</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>13</td>
<td>39</td>
<td>43</td>
<td>18</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>66</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>99</td>
<td>106</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>21</td>
<td>34</td>
<td>17</td>
<td>8</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>35</td>
<td>37</td>
<td>89</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

Studies performed on subcellular fractions of human liver obtained 4 and 12 h after death. Values recorded are the percentage of that total activity for the enzyme identified in the whole homogenate. The recovery from that original whole preparation is recorded on the right. (—) indicates that no pellet was formed and those specimens (from the light mitochondrial fraction) were combined with the microsomal fraction.
bound, so that even 12 h post mortem the 'leak' into the cytosol was virtually zero. Under the same conditions, citrate synthase appears to have significant cytosolic activity which further increases at 12 h after death. The pattern for glutamate dehydrogenase was very similar (see Table II). The patterns for methylmalonyl-CoA mutase and propionyl-CoA carboxylase were strikingly similar to those of known matrix enzymes citrate synthase and glutamate dehydrogenase. It is evident from the marker enzymes that the presence of these enzymes in the cytosol represented contamination from mitochondria. Table II demonstrates that data in a quantitative fashion. When the mitochondrial marker enzymes were utilized to correct for leakage from the mitochondrial compartments, less than 2% of the methylmalonyl-CoA mutase and the propionyl-CoA carboxylase were found in the cytosolic fraction. These matrix markers strongly suggest that the methylmalonyl-CoA mutase and propionyl-CoA carboxylase are in fact located exclusively in the mitochondrial compartment in human liver just as was demonstrated in the rat liver.

Intramitochondrial Distribution of Methylmalonyl-CoA Mutase and Propionyl-CoA Carboxylase

Four separate mitochondrial marker enzymes (Fig 4) were utilized in the digitonin fractionation for submitochondrial localization (Scholte, 1969). These included succinate dehydrogenase, a marker for the inner membrane; glutamate dehydrogenase, a mitochondrial matrix marker; rotenone-insensitive NADH-cytochrome c reductase for the outer membrane; and adenylate kinase for the intermembrane space. As shown in Fig 4, the enzymes on the mitochondrial membrane and matrix (succinate dehydrogenase and glutamate dehydrogenase) were found almost entirely in Fractions 1 and 2 as previously described by Scholte (1969). Although inner mitochondrial membrane is absent from Fraction 4, a small portion of the matrix material (5–10%) was found in that fraction, due to the leakage from the inner
membrane-matrix unit. The distribution of adenylate kinase indicated that the contents of the intermembrane space occur almost entirely in Fraction 4. The outer membrane was partially separated from the inner membrane matrix unit, and the outer membrane marker rotenone-insensitive NADH-cytochrome c reductase was seen primarily in Fraction 3 (Fig 4). Utilizing these marker enzymes, methylmalonyl-CoA mutase and propionyl-CoA carboxylase (Fig 4) were clearly localized to the inner membrane-matrix fraction. Table III provides the quantitative data, corrected for the marker enzymes, in studies of liver from normal and vitamin B\textsubscript{12} deprived animals. Subcellular localization was further corroborated by whole mitochondrial fractionation by the lubrol technique described by Chan et al (1970). In studies not shown, such fractionation released propionyl-CoA carboxylase and methylmalonyl-CoA mutase into the supernatant fraction along with known matrix markers, glutamate dehydrogenase, citrate synthase and malate dehydrogenase (Srere, 1972). These enzymes were clearly separated from succinate dehydrogenase, an inner-membrane marker, which appeared in the pellet fraction. Thus, both separation methods demonstrated that virtually all of the methylmalonyl-CoA mutase and propionyl-CoA carboxylase could be accounted for on the inner membrane-matrix portion of the mitochondria, and both enzymes appeared to be loosely bound.

### Table III. Intramitochondrial membrane distribution of enzymes in liver

<table>
<thead>
<tr>
<th></th>
<th>Inner membrane-matrix fraction</th>
<th>Vitamin B\textsubscript{12} deprived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylmalonyl CoA mutase</td>
<td>99%</td>
<td>100%</td>
</tr>
<tr>
<td>Propionyl CoA carboxylase</td>
<td>89%</td>
<td>92%</td>
</tr>
</tbody>
</table>

### DISCUSSION

Interest in the coenzyme B\textsubscript{12} dependent-methylmalonyl coenzyme A mutase reaction (Fig 5) has been stimulated by the identification of congenital forms of methylmalonicaciduria (Oberholzer et al, 1967; Barness, 1973) as well as its implication in altered fatty acid synthesis in neural tissue in acquired forms of vitamin B\textsubscript{12} deficiency (Frenkel, 1973c; Barley et al, 1972). Methodological evaluation of assays for the mutase by Goodey & Gompertz (1972) revealed the lack of applicability of a classic spectrophotometric assay (Wood et al, 1964) to tissue extracts and problems in resolution and sensitivity with the usual isotopic methods which require separation of the radioactive methylmalonic and succinic acids (Oberholzer et al, 1967; Morrow et al, 1969; Cardimale et al, 1969; Cannata et al, 1965; Morrow & Barness, 1969). The present assay exploited the spectrophotometric quantitation of the amount of succinyl-CoA formed by the arsenolysis reaction, previously shown to be highly specific and sensitive (Frenkel et al, 1974). We propose this simple spectrophotometric assay as an alternative to the gas-liquid radiochromatographic analysis described by Goodey & Gompertz (1972).
The present study evaluated the intracellular site of the controlling enzymes in this metabolic pathway that involves the oxidation of propionate and aliphatic amino acids (Fig 3), which requires the vitamin B₁₂ dependent mutase reaction. In attempting to dissect some of the biochemical sequelae of vitamin B₁₂ deprivation, knowledge of the sites of enzyme activity are helpful clues in the pursuit of interrelated reactions. Thus, Morrow et al (1973) described methylmalonyl-CoA mutase activity in both mitochondrial and soluble portions of fractionated cells. They correlated this observation with earlier evidence of low urinary succinate excretion in their patient with congenital methylmalonic aciduria (Morrow & Barness, 1969) and proposed a cytoplasmic requirement for succinyl-CoA (Morrow et al, 1973). By the use of multiple enzyme markers, the present study demonstrated that virtually all of the activity of methylmalonyl-CoA mutase, as well as that of propionyl-CoA carboxylase, was present only in the mitochondrial fraction. Utilizing enzyme markers that were both loosely and tightly bound to the mitochondria, it was evident that the previous identification of the mutase in the cytoplasmic fraction related in large part to its loose binding in the mitochondria and, in addition, post-mortem autolysis. Fractionation studies of the mitochondria further substantiated this loose binding and localized them to the inner membranes portion of the mitochondria. As one would anticipate, vitamin B₁₂ deficiency did not affect the site of localization of these enzymes.

Previous studies from this laboratory (Frenkel et al, 1974) have demonstrated that the tissue levels of the CoA derivatives (propionyl, methylmalonyl and succinyl) showed poor correlation with urinary levels of their respective hydrolysed acids (propionic, methylmalonic and succinic). These observations suggest that the low urinary succinate excretion described in the patient with methylmalonic aciduria by Morrow & Barness (1969) must be interpreted with caution since that value may have no relationship to actual tissue levels of the related CoA derivations or the subcellular distribution of the related enzymes. Available methodology for accurate tissue quantification of the actual levels of CoA derivatives (Frenkel et al, 1974) will make it possible to specifically characterize the changes in the patients with congenital methylmalonic aciduria in the future.

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