Effect of Methylmalonyl Coenzyme A, a Metabolite Which Accumulates in Vitamin B₁₂ Deficiency, on Fatty Acid Synthesis*

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SUMMARY

We have proposed that the accumulation of methylmalonyl-CoA, which occurs when a deficiency of B₁₂ coenzyme exists, may lead to its incorporation into fatty acids in place of malonyl-CoA. This leads to the production of fatty acids, which are not usually produced and which may be physiologically undesirable. As a first step in the test of this hypothesis, we have examined the effect of methylmalonyl-CoA on fatty acid synthesis in vitro, in rat liver supernatant fractions. It was shown that methylmalonyl-CoA inhibits fatty acid synthesis and is incorporated into fatty acids.

In mammals, the conversion of methylmalonyl-CoA to succinyl-CoA is catalyzed by methylmalonyl-CoA isomerase (EC 5.4.99.2) an enzyme which utilizes vitamin B₁₂ coenzyme (1). This conversion fails to occur in certain pathological conditions and consequently relatively large amounts of methylmalonic acid are excreted in the urine (2, 3). The best known of these conditions is pernicious anemia in which the ability to absorb vitamin B₁₂ from the intestinal tract is impaired and a generalized vitamin B₁₂ deficiency occurs (4). Recently, a disease has been described in children, methylmalonic aciduria, in which large amounts of methylmalonic acid are excreted in the urine (5). In this case, the excretion of methylmalonic acid is due either to the inability to convert vitamin B₁₂ to vitamin B₁₂ coenzyme (6–8) or to absence of or a defect in methylmalonyl-CoA isomerase (7). The question arose whether the accumulation of methylmalonic acid under these conditions may be partially responsible for some of the pathological symptoms associated with these diseases. Possible detrimental effects of methylmalonyl-CoA are suggested by toxicity of valine, a precursor of methylmalonyl-CoA, in B₁₂-deficient pigs. Doses of valine which have no effect on normal pigs can be fatal to B₁₂-deficient pigs.¹ A mode of action of methylmalonyl-CoA is suggested by structural similarities to malonyl-CoA. As a result of this structural similarity, methylmalonyl-CoA could interfere with fatty acid synthesis or be incorporated into fatty acids and thus give rise to the production of branched chain fatty acids which might not be synthesized when methylmalonyl-CoA does not accumulate. Recently, evidence has become available which suggests that the production of or the incorporation of abnormal fatty acids can lead to biological malfunctions. It has, for instance, been shown that the properties of membranes can be significantly affected by substitution of fatty acids in the membrane (9) and it is conceivable that total malfunction could occur. Also, a disease has been described (10, 11), Refsum’s disease, in which a large percentage of the body’s fatty acids are replaced by tetramethylhexadecenoic acid. This disease leads to neurological defects and eventually to death. In connection with this, it should be noted that, in the later stages of pernicious anemia, neurological symptoms also occur.

It, therefore, appeared of interest to us to investigate the effects of methylmalonyl-CoA on fatty acid synthesis in mammals. So far, this effect has not been extensively investigated. The effect of methylmalonic acid on the biosynthesis of lipids in rat adipose tissue and rat liver has been investigated (12). The data suggest that methylmalonic acid may be incorporated into lipids in adipose tissue, although the possibility that it was incorporated after prior conversion to propionate is not eliminated. No significant incorporation into the lipids of liver was observed. We have now undertaken a study of the effect of methylmalonyl-CoA on fatty acid synthesis in a soluble system derived from rat liver. The use of methylmalonyl-CoA seemed more appropriate than methylmalonic acid, since it is not known whether an activating system for methylmalonic acid exists and since methylmalonyl-CoA is a metabolic product derived from a number of precursors such as valine, isoleucine, and propionate.

The results of our studies are reported here.

MATERIALS AND METHODS

Substrates—n-Glucose 6-phosphate was purchased from Sigma; coenzyme A and acetyl-CoA were from P. L. Biochemicals; boron fluoride-methanol and labeled and unlabeled fatty acid methyl ester standards were from Applied Science Laboratories; and 2-methyl-¹⁴C-malonic acid was from New England Nuclear. ¹⁴C-Acetyl-CoA and 1, 3-¹⁴C-methylmalonyl-CoA were gifts of Dr. Konrad Bloch and Dr. J. M. Lowenstein, respectively.

Unlabeled methylmalonyl-CoA was prepared by the method

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of Overath et al. (18), and unlabeled malonyl-CoA by a similar procedure (14). Propionyl-CoA was prepared according to Stadtman (15). Methylmalonyl-CoA labeled with tritium in the methyl group was synthesized from 2-methyl-3-H-malonic acid through its mixed anhydride with ethylchloroformic acid (16). Paper chromatography, first in isobutyric acid-HCl-NaOH (66:33:1) and then in 0.1 M potassium phosphate, pH 4.5-ethanol (1:1) at 4°C (17), yielded a radiochemically pure product. The concentration of acyl-CoA esters was determined with the hydroxamate procedure of Lipmann and Tuttle (18).

Rat Liver Extract—Rats were fasted for 2 days and then refed for 3 days with a high fructose diet (19). After decapitation, a high speed supernatant liver fraction was prepared according to Fang and Lowenstein (20). This high speed supernatant preparation was used in all of the succeeding experiments without further purification.

Fatty Acid Synthesis Assay—The assay is based on the methods of Brady (21) and Fang and Lowenstein (20). At the end of the reaction time, 2.0 ml of 2 N NaOH were added to a 1-ml reaction mixture and the reaction was heated at 90°C for 2 hours. The cooled reaction mixture was first extracted three times with 4 ml of low boiling petroleum ether. The aqueous phase was acidified with 2.0 ml of 2.5 N H2SO4 and the fatty acids were isolated by extracting five times with 4 ml of low boiling petroleum ether. The petroleum ether extract was then backwashed with an equal volume of 0.05 N HCl and transferred to a counting vial. The solvent was evaporated by gentle warming, toluene scintillation counting fluid was added, and the sample was shaken and counted. Within a range of 0.05 to 0.20 mg of protein, the incorporation of radioactivity from 1,3-14C-malonyl-CoA was shown to be linear.

Identification of Fatty Acids—The fatty acids formed in the above assay were analyzed by gas-liquid chromatography. The petroleum ether extracts were dried over Na2SO4. After filtration and removal of the solvent, the fatty acids were esterified with boron fluoride-methanol (22). A small amount of saturated even chain fatty acid methyl ester standards (C₆-C₂₀) was added and the combined esters were streaked on thin layer chromatography plates coated with Silica Gel G. The plates were developed in benzene and the purified methyl esters were recovered by scraping off the appropriate band and eluting with diethyl ether. After concentration, these solutions were analyzed on a F and M gas chromatograph, model 402, equipped with a stream splitter leading to a Nuclear-Chicago gas radiochromatography counting system, model 4998. The column was 6 feet × 0.25 inch outside diameter packed with 6% H3EII-F-14P on 100 to 120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.) and was operated from 100° to 180° with a temperature program of 3° per min.

Catalytic hydrogenations of isolated fatty acid methyl esters were carried out according to Kishimoto and Radin (23). Unsaturated standards were added to the samples to show that reduction was complete.

Isolation of Pig Liver Fatty Acids—Livers from normal and R12-deficient pigs (24) were examined to ascertain the composition of their lipid fatty acids. Portions of the liver were homogenized in a Waring Blendor at 0°C in 20 volumes of CHCl₃-methanol (2:1). After filtration, the CHCl₃-methanol extract was concentrated under reduced pressure to a viscous liquid. The lipids were then saponified by refluxing for 3 hours in 10% methanolic KOH. The methanol was removed, H₂O was added, and nonacidic moieties were removed by extraction with low boiling petroleum ether. The fatty acids were extracted with low boiling petroleum ether after acidifying with 5 N H₂SO₄. The petroleum ether solution of the fatty acids was dried over Na₂SO₄ and the petroleum ether was removed under reduced pressure. The residue was taken up in a small volume of diethyl ether and the fatty acids were esterified with diazomethane. The fatty acid methyl esters were streaked on thin layer chromatography plates coated with Silica Gel G. After development in 95% hexane-5% diethyl ether, the appropriate band was scraped off and eluted with ether. The concentrated solutions were then analyzed on the gas-liquid chromatography system described above without the stream splitter attachment.

RESULTS AND DISCUSSION

Experiments were carried out to investigate the effects of methylmalonyl-CoA upon the incorporation of 14C-acetyl-CoA and 1,3 14C malonyl-CoA into fatty acids and to determine whether methylmalonyl-CoA is itself incorporated into fatty acids when added. Reactions were carried out at 37°C for 30 min. Reaction was stopped, and fatty acids were determined as described under "Materials and Methods." Protein concentrations are in the range in which product formation is linear with protein concentration.

### TABLE I

**Inhibition by methylmalonyl-CoA**

<table>
<thead>
<tr>
<th>Total radioactivity in reaction products</th>
<th>Minus methylmalonyl-CoA</th>
<th>Plus methylmalonyl-CoA</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>epm</td>
<td>epm</td>
<td>%</td>
</tr>
<tr>
<td>Omit protein, replace malonyl-CoA with 1,3-14C-malonyl-CoA (3.0 × 10⁶ epm/μmole)</td>
<td>2</td>
<td>321</td>
<td>47</td>
</tr>
<tr>
<td>Omit acetyl-CoA, replace malonyl-CoA with 1,3-14C-malonyl-CoA (3.0 × 10⁶ epm/μmole)</td>
<td>2.77 × 10⁴</td>
<td>1.46 × 10⁴</td>
<td>38</td>
</tr>
<tr>
<td>Replace acetyl-CoA with 14C-acetyl-CoA (4.68 × 10⁶ epm/μmole)</td>
<td>4.58 × 10⁴</td>
<td>2.86 × 10⁴</td>
<td>68</td>
</tr>
<tr>
<td>Replace malonyl-CoA with 1,3-14C-malonyl-CoA (3.0 × 10⁶ epm/μmole)</td>
<td>4.58 × 10⁴</td>
<td>2.86 × 10⁴</td>
<td>68</td>
</tr>
</tbody>
</table>

* Since one carboxyl group is lost as CO₂ during the condensation reaction, the effective specific activity is 1.5 × 10⁶ epm per μmole.

* With 0.20 μmole of methylmalonyl-CoA added.
acids. The results of these experiments are summarized in Tables I and II. The data in Table I show that the addition of methylmalonyl-CoA to fatty acid-synthesizing systems reduces the amount of U-acyt-CoA and 1,3-14C-malonyl-CoA.

**Table II**

**Incorporation of 3H-methylmalonyl-CoA into fatty acids**

The 1-ml reaction mixture contained 50 μmoles of potassium phosphate buffer (pH 7.0), 0.68 μmole of TPNH, 5.0 μmoles of glucose 6-phosphate, 4.8 μmoles of Cleland's reagent, 0.14 mg of high speed supernatant protein as well as 0.35 μmole of acetyl-CoA, 0.36 μmole of malonyl-CoA, and 0.41 μmole of 3H-methylmalonyl-CoA (5.1 × 10^6 cpm per μmole). Reactions were carried out at 37° for 30 min. Reactions were stopped and fatty acids were determined as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Changes in reaction mixture</th>
<th>Total radioactivity in reaction products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omit protein</td>
<td>1</td>
</tr>
<tr>
<td>Omit acetyl-CoA</td>
<td>241</td>
</tr>
<tr>
<td>Omit malonyl-CoA</td>
<td>66</td>
</tr>
<tr>
<td>Complete system</td>
<td>2.09 × 10^6</td>
</tr>
<tr>
<td></td>
<td>6.83 × 10^6</td>
</tr>
</tbody>
</table>

*With 0.43 mg of protein used in assay.*

The fatty acids synthesized in the presence of methylmalonyl-CoA were examined by gas chromatography in order to gain additional information concerning their structure. Separate experiments were carried out in which a specific component of the reaction system was labeled. Fig. 1b represents the radioactive acids detected when 14C-acetyl-CoA and unlabeled malonyl-CoA and methylmalonyl-CoA were used. This is to be compared with Fig. 1a which shows the fatty acids obtained when methylmalonyl-CoA is omitted. Two new peaks (indicated by arrows) are detected in Fig. 1b, which are not seen in

**Fig. 1.** Gas-liquid chromatography of labeled fatty acid methyl esters. The basic 1-ml reaction mixture consisted of 50 μmoles of potassium phosphate buffer (pH 7.0), 0.68 μmole of TPNH, 5.0 μmoles of glucose 6-phosphate, 4.8 μmoles of Cleland's reagent, 1.3 mg of protein, 0.35 μmole of acetyl-CoA, 0.36 μmole of malonyl-CoA, and 0.40 μmole of methylmalonyl-CoA when added. Reactions were carried out for 30 min at 37°. Fatty acids were isolated, esterified, and purified as described under "Materials and Methods." Saturated, even chain, fatty acid methyl esters (C16:0) were added as internal standards. Details of the gas-liquid chromatographic procedure are given in the text. Traces represent mass peaks and radioactive peaks.
Fig. 1a. Fig. 1, c and d, represents a similar experiment in which 1,3-3C-malonyl-CoA was used. Again, two new peaks appear in the presence of methylmalonyl-CoA, which are not found in its absence. These peaks have the same retention time as those in Fig. 1b, and therefore probably represent structurally identical acids, most likely acids derived from acetyl-CoA, malonyl-CoA, and methylmalonyl-CoA. Fig. 1d represents the chromatographic patterns of the fatty acids obtained when 1H-methylmalonyl-CoA and unlabeled acetyl-CoA and malonyl-CoA are used. In this chromatograph, radioactivity is more diffuse and individual peaks cannot be easily distinguished. We attribute this diffuseness to the presence of many peaks which cannot be resolved under these conditions. The chromatographic pattern shows that all fatty acids derived from 1H-methylmalonyl-CoA are structurally different from those obtained in its absence; i.e., no peaks corresponding to those in Fig. 1, a and c, are seen. It should also be noted that radioactivity is present in the area corresponding to the elution point of the acids, attributed to the presence of methylmalonyl-CoA in the experiment represented by Fig. 1, b and d. This is consistent with the conclusions that fatty acids are derived from all three substrates, methylmalonyl-CoA, malonyl-CoA, and acetyl-CoA.

The presence of peaks when 1,3-3C-malonyl-CoA and methylmalonyl-CoA are used, or 1H-methylmalonyl-CoA (Fig. 1e), which are not apparent when 3C-acetyl-CoA and methylmalonyl-CoA are used (Fig. 1b), does not contradict this conclusion. A given fatty acid molecule will have a higher specific activity when derived from 1,3-3C-malonyl-CoA or 1H-methylmalonyl-CoA than when derived from 3C-acetyl-CoA; consequently, fatty acids present in low concentrations will not be detected when the label is derived from 3C-acetyl-CoA.

To determine whether methylmalonyl-CoA is converted to propionyl-CoA prior to incorporation into fatty acids, an experiment was carried out in which propionyl-CoA was added to the fatty acid-synthesizing system containing 1,3-3C-malonyl-CoA. The gas chromatographic pattern obtained is shown in Fig. 1f. It is different from that obtained in the presence of methylmalonyl-CoA (Fig. 1d). Therefore, the major fraction of methylmalonyl-CoA is not converted to propionyl-CoA prior to incorporation into fatty acids.

The fatty acids obtained in the presence of methylmalonyl-CoA were subjected to catalytic reduction (23) prior to gas chromatography. This treatment did not produce a change in the chromatographic pattern. It was concluded that the fatty acids derived from methylmalonyl-CoA were not unsaturated, since the procedure used is known to reduce unsaturated fatty acids.

The result obtained from a gas chromatographic examination of the fatty acids derived from methylmalonyl-CoA can be summarized as follows. (a) The retention time on gas liquid chromatography is intermediate between 14:0 and 16:0, and 16:0 and 18:0. (b) The major fraction of the gas-liquid chromatography peaks does not coincide with 15:0 and 17:0 standards. (c) They are saturated. These facts taken together suggest that the majority of fatty acids derived from methylmalonyl-CoA are branched, and that therefore methylmalonyl-CoA is incorporated in nonterminal positions. This conclusion is also consistent with data summarized in Tables I and II.

We next attempted to see whether fatty acids similar to those formed in the system in vitro could be detected in vitro. The only suitable material available was lipids from pigs which had been maintained on a vitamin B12-deficient diet. The characteristics of the pigs maintained on such a diet have been described elsewhere (24). For our purposes, B12-deficient pigs are not the ideal source of material since the deficiency in these animals does not lead to symptoms either resembling human pernicious anemia or methylmalonic aciduria. In spite of this, we thought it of interest to examine the lipid composition of these animals. It has previously been reported that vitamin B12 deficiency leads to the accumulation of lipids in the liver (25). We have qualitatively confirmed this finding in the case of all B12-deficient pigs which we have examined. The liver lipids of pigs were hydrolyzed and the gas chromatographic pattern of the liver fatty acids was examined. A total of three deficient pigs and two control pigs were examined. In no case did we find significant amounts of fatty acids corresponding to those obtained in the system in vitro containing methylmalonyl-CoA. We did, however, find what we believe to be a significant change in fatty acid pattern. In all cases B12-deficient animals had a higher percentage of the 18:1 monoenoic fatty acids. The ratio of 18:0 to 18:1 for a normal pig (0.059 μg of B12 coenzyme per g of liver tissue) was 1.8, while for two deficient pigs it was 1.2 (0.006 μg of B12 coenzyme per g of liver tissue) and 0.3 (0.002 μg of B12 coenzyme per g of liver tissue). In the animal in which the ratio of 0.3 was obtained, the most severe deficiency was present. In view of the small number of animals used, these results can at best be considered indicative. Whether this accumulation of unsaturated fatty acids, if significant, can be related to the accumulation of methylmalonyl-CoA can, of course, not be stated at this time. Our experiments in vitro would not have led to this prediction. The failure to find branched chain fatty acids in vitamin B12 deficiency does not disprove our initial hypothesis, i.e., that such fatty acids are extensively incorporated as a result of the deficiency in humans since, as was previously mentioned, the deficiency symptoms in pigs differ greatly from those found in humans. A more rigorous test of our hypothesis will depend upon the availability of more suitable models or the availability of autopsy material from human patients.

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