Accumulation of methylmalonic acid caused by vitamin B₁₂-deficiency disrupts normal cellular metabolism in rat liver

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To clarify the relationship between intracellular concentrations of methylmalonic acid and metabolic and growth inhibition in vitamin B₁₂-deficient rats, hepatic methylmalonic acid levels were assayed and inhibition of glucose and glutamic acid metabolism by methylmalonic acid was studied in isolated hepatocytes. Vitamin B₁₂-deficient rats (14 weeks old) excreted more urinary methylmalonic acid and had lower body weights than the control rats. Hepatic methylmalonic acid levels (3-6 (SD 1.30)-5.3 (SD 0.51) μmol/g tissue; 7.9 (SD 2.90)-11.8 (SD 1.14) mM) were increased and correlated with the extent of the growth retardation during vitamin B₁₂-deficiency. Isolated hepatocytes and mitochondria from normally fed rats were labelled with [¹⁴C(U)]glucose and [¹⁴C(U)]glutamic acid respectively, in the presence or absence of 5 mM-methylmalonic acid. Although methylmalonic acid did not affect the incorporation of [¹⁴C] into protein and organic acid fractions in the hepatocytes, it inhibited [¹⁴C]O₂ formation (an index of glucose oxidation by the Krebs cycle) by 25% and incorporation of [¹⁴C] into the amino acid fraction by 30%. In the mitochondria, methylmalonic acid inhibited [¹⁴C]O₂ formation (indicating glutamic acid oxidation by the Krebs cycle) by 70%, but not the incorporation of [¹⁴C] into the protein fraction. The incorporation of [¹⁴C] into the organic acid fraction was significantly stimulated by the addition of methylmalonic acid. These results indicate that the unusual accumulation of methylmalonic acid caused by vitamin B₁₂-deficiency disrupts normal glucose and glutamic acid metabolism in rat liver, probably by inhibiting the Krebs cycle.

Vitamin B₁₂: Methylmalonic acid: Liver

Vitamin B₁₂ (or cobalamin; Cbl)-deficiency causes a severe growth retardation (Dryden & Hartman, 1966; Fehling et al. 1978; Watanabe et al. 1991; Kennedy et al. 1992) and various metabolic disorders (Weidemann et al. 1970; Williams & Spray, 1971; Fehling et al. 1978; Brass & Stabler, 1988; McCully, 1992; Metz, 1992) in animals. The activities of certain hepatic enzymes involved in fatty acid synthesis (e.g. fatty acid synthase, EC 2.3.1.85, and acetyl-CoA carboxylase, EC 6.4.1.2) (Frenkel et al. 1973), in the Krebs cycle (e.g. citrate synthase, EC 4.1.3.7) (Frenkel et al. 1976; Mukherjee et al. 1976) and in Cbl coenzyme synthesis (e.g. aquacobalamin reductase, EC 1.6.99.8) (Watanabe et al. 1991) are increased severalfold during Cbl-deficiency, whereas that of 5'-deoxyadenosyl-cobalamin-dependent methylmalonyl-CoA mutase (EC 5.4.99.2) is decreased significantly.

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(Cardinale et al. 1969; Watanabe et al. 1994). Consequently, hepatic levels of propionyl- and methylmalonyl-CoA are significantly increased (Frenkel et al. 1974; Brass et al. 1990), resulting in inhibition of hepatic pyruvate oxidation, gluconeogenesis, ureagenesis and fatty acid oxidation (Weidemann et al. 1970; Martin-Requero et al. 1973; Arinze et al. 1979; Brass et al. 1986).

Hepatic methylmalonyl-CoA, when it accumulates, is hydrolysed to form methylmalonic acid (MMA), which is excreted in significant amounts in urine in Cbl-deficient animals (Watanabe et al. 1991). The elevated level of urinary and/or serum MMA, which have been used as an index of Cbl-deficiency (Moelby et al. 1990), suggest that MMA accumulates in tissues of Cbl-deficient animals and that the MMA accumulation may be toxic to the cell. There is, however, little information concerning the extent of MMA accumulation in mammalian livers during Cbl-deficiency or the mechanism by which the MMA accumulation interferes with normal cellular metabolism.

To clarify whether increased intracellular concentrations of MMA impair hepatic metabolism (glucose and amino acids) and retard growth in Cbl-deficient rats, we measured hepatic MMA levels in Cbl-deficient rats and demonstrated that the MMA accumulation inhibits normal cellular metabolism in isolated hepatocytes.

**METHODS**

We first investigated the relationship between hepatic MMA concentrations and growth in Cbl-deficient rats. An inhibition study was then undertaken in which the effect of increased hepatic MMA concentrations on the metabolism of glucose and glutamic acid was investigated.

Forty male weanling Wistar rats (3 weeks old, 40 (sd 5) g), born to 14-week-old parents fed on a Cbl-deficient diet for 8 weeks, were used. Parent rats aged 6 weeks were obtained from Clea Inc., Tokyo, Japan. The Cbl-deficient diet fed to the parents was prepared as described previously (Watanabe et al. 1991). The control diet was identical to the Cbl-deficient diet, except that 5 μg of cyanocobalamin (CN-Cbl) (Sigma Chemical, St Louis, MO, USA) per kg diet was included. The 3-week-old weanling rats were housed in individual metabolism cages at 23° in a room with a 12 h light–dark cycle. They were given free access to the control or Cbl-deficient diet and to tap water for 5 weeks. The animals used in these studies were maintained in accordance with the guidelines of the National Research Council (NRC, 1985).

Effects of feeding MMA-precursor amino acids on growth and tissue-MMA levels in the Cbl-deficient rats were studied by the use of the diets shown in Table 1. The Cbl-deficient 8-week-old rats were given free access to the four experimental diets, which were either sufficient or deficient in Cbl and supplemented with either four MMA-precursor amino acids (L-methionine, 5·8 g; L-isoleucine, 5·1 g; L-threonine, 4·6 g; and L-valine, 4·5 g per kg diet) (MITV) or L-glutamic acid (20 g/kg diet) (GLU), instead of the original Cbl-deficient diet. Feed consumption by each rat given the Cbl-deficient GLU and MITV diets was recorded three times weekly. On the basis of this feed consumption, the control rats were pair-fed along with each rat in the Cbl-deficient groups. The control diets were identical to the experimental diets, except that 5 μg CN-Cbl/kg diet was included. All rats had free access to water. Rats were fed for 6 weeks and used in the experiments for MMA assay.

Rat hepatocytes were isolated by the method of Berry & Friend (1969). All buffers were purged with O₂-CO₂ gas (95:5, v/v) for 30 min before using. Male Wistar rats (body weight, 150–200 g) fed on a non-purified commercial rat diet (CE-2, Clea Inc.) were anaesthetized with pentobarbital (50 mg/kg body weight). The liver was perfused through the portal vein with a perfusion buffer at a flow rate of 50 ml/min for 15 min, placed in a recirculating chamber, and perfused with a collagenase (EC 3.4.24.3) buffer at a flow rate...
of 50 ml/min for 30 min at 37°. Hepatocytes were obtained from the collagenase-treated liver and dispersed in a washing buffer. The hepatocytes were filtered through two nylon filters (50 and 150 meshes, Masuda Chemicals Co. Ltd, Osaka, Japan) and washed three times with the washing buffer. The hepatocytes obtained showed high viability (90 (SD 3.0) % n 10, as estimated by trypan-blue exclusion) and were used in radiolabelling experiments. The recovery of hepatocytes from liver was 4-6 × 10⁷ cells/g tissue.

Rat liver mitochondria were isolated as follows: livers were obtained from male Wistar rats (body weight, 150-200 g) fed on a non-purified commercial rat diet (CE-2, Clea Inc.) and washed with chilled saline (9 g NaCl/l). The livers were cut into small pieces with a razor blade and homogenized in about nine volumes of 5 mM-Tris-HCl buffer, pH 7.4, containing 0.25 M-sucrose by using a glass homogenizer with a Teflon pestle. The homogenate was filtered through a double layer of gauze to remove unbroken tissues and then centrifuged at 700 g for 10 min to remove unbroken cells and nuclei. The supernatant fraction was centrifuged at 7000 g for 10 min. The precipitate, containing mitochondria, was washed twice with the same buffer, suspended in a small amount of the same buffer and used in radiolabelling experiments. All procedures were carried out at 4°.

Radiolabelling experiments were conducted using the following methods. [¹⁴C(U)]glucose (11 000 MBq/mmol, American Radiolabeled Chemicals Inc., St Louis, MO, USA) labelling experiments with isolated hepatocytes were done at 25° in a reaction mixture (20 ml) containing 25 mM-Krebs-Ringer bicarbonate buffer, pH 7.4, and 1 mM-glucose (containing 0.0185 MBq [¹⁴C(U)]glucose). The gas phase in a rubber-capped vessel (25 ml volume) containing the reaction mixture without radioactive glucose solution was exchanged with O₂-CO₂ (95:5, v/v). The reaction was started by the addition of radioactive glucose, allowed to proceed for the indicated times and stopped by the addition of 100 μl 7M-HClO₄. The CO₂ formed in the vessel was trapped for 60 min by a filter paper soaked in 990 g/l monoethanolamine (the filter paper was fixed inside the rubber cap). In the case
of $[^{14}\text{C}](\text{U})$glutamic acid (9.25 MBq/mmol, American Radiolabeled Chemicals Inc.) labeling experiments in isolated rat liver mitochondria, the reaction mixture (2.0 ml) containing 25 mM-Krebs–Ringer bicarbonate buffer, pH 7-4, 1 mM-glutamic acid (containing 0.0185 MBq $[^{14}\text{C}](\text{U})$glutamic acid) was used.

Labelled hepatocytes and mitochondria were centrifuged at 1000 $g$ for 10 min. The cell pellet was extracted twice with 1.0 ml 0.3 M-HClO$_4$ and used as a protein fraction. The supernatant fraction was extracted twice with 4.0 ml diethyl ether. The combined ether fractions were evaporated to dryness and used as a lipid fraction. The remaining fraction was neutralized by the addition of 2 M-KOH to pH 6–7 and centrifuged at 1000 $g$ for 10 min. The supernatant fraction was applied to a column (10.0 x 5.0 mm) of Dowex 50 (H$^+$ form) (Muromachi Kagaku Kogyo Kaisha Ltd, Tokyo, Japan), washed with 80 ml distilled water and eluted with 12.0 ml 1 M-NH$_4$OH solution. The NH$_4$OH eluate was used as an amino acid fraction. The void and washing fractions were combined and applied to a column (10.0 x 5.0 mm) of Dowex 1 (HCOO$^-$ form) (Muromachi Kagaku Kogyo Kaisha Ltd). The column was washed with 80 ml distilled water and eluted with 12.0 ml 1 M-HCl solution. The HCl eluate was used as an organic acid fraction. Radioactivity in each fraction was counted by an Aloka LSC-903 liquid scintillation counter (Aloka Co. Ltd, Tokyo, Japan).

Tissue MMA levels were assayed by HPLC as described previously (Toyoshima et al. 1994). Urine of rats was sampled for 24 h with the rats in individual metabolism cages. Livers, kidneys and blood were obtained from the rats after killing by decapitation under diethyl ether anaesthesia. Plasma was obtained from the blood by centrifuging at 1000 $g$ for 10 min at 4$^\circ$. The plasma and tissues were immediately frozen in liquid N$_2$ and stored at $-85^\circ$ until analysis. To urine or plasma, an equal volume of 0.7 M-HClO$_4$ (Nacalai Tesque, Kyoto, Japan) was added, mixed vigorously and centrifuged at 1000 $g$ for 10 min at 4$^\circ$. The supernatant fraction was used as a HClO$_4$ extract. The tissues were cut into small pieces with a razor blade, homogenized in an equal volume of 0.7 M-HClO$_4$ by use of a mortar and pestle, and centrifuged at 1000 $g$ for 10 min at 4$^\circ$. The supernatant fraction was used as a HClO$_4$ extract. In samples containing a large amount of MMA, the extracts were diluted with distilled water. The HClO$_4$ extract was applied to a column (10.0 x 50 mm) of Dowex 50 x 4 (H$^+$ form) (Muromachi Kagaku Kogyo Kaisha Ltd). The void fraction was collected and subjected to HPLC analysis. More than 85% of the MMA was recovered in the void fraction when a known amount of authentic MMA was added to urine, plasma and tissues, and extracted by the above method.

The samples obtained were analysed on an ion-exchange HPLC column (Shodex Ionpak C-811, 8 x 500 mm; Showa-denko Co. Ltd, Tokyo, Japan) with a JASCO (Japan Spectroscopic Co. Ltd, Tokyo, Japan) HPLC apparatus (880-PU pump, 870-UV spectrophotometer and 807-IT Chromato-data processor) and a column oven (CS-600, Chromato Science Co. Ltd, Tokyo, Japan). Hepatic concentration of MMA was calculated on the basis of water space (0.448 ml/g fresh liver) of rat liver cytosol and mitochondria (Siess et al. 1982).

Other assays were conducted using the following methods. Protein was assayed by the method of Bradford (1976) with bovine serum albumin as a standard. Extraction of total Cbl from liver was done using the KCN method (Frenkel et al. 1980) and total Cbl content was assayed by a radiodilution method using Euglena pellicle fragments (Watanabe et al. 1993). Serum homocysteine was assayed by the methods of Jacobsen et al. (1989, 1994).

Statistical significance was determined using Student’s $t$ test, $P < 0.05$ was considered significant. All values are presented as means and standard deviations.
RESULTS AND DISCUSSION

Plasma MMA (196.8 (SD 53.40) μM) and homocysteine (92.4 (SD 5.98) μM) levels as indices of Cbl deficiency were about 95- and 2.5-fold greater in the 8-week-old rats fed on the Cbl-deficient diet than in the control rats fed on the Cbl-sufficient diet, indicating that the rats fed on the Cbl-deficient diet developed a slight Cbl deficiency. The 8-week-old Cbl-deficient rats were used in the following experiments.

To clarify the relationship between hepatic MMA accumulation and growth retardation in rats during Cbl deficiency, feeding experiments were conducted using diets supplemented with MMA-precursor amino acids (methionine, isoleucine, threonine, and valine) (MITV) which are considered to increase tissue MMA levels in Cbl-deficient rats. The Cbl-deficient rats were pair-fed with the MITV- and GLU-supplemented control diets. The MITV and control rats showed identical growth curves in the presence of CN-Cbl (5 μg/kg diet). The rats fed on each of the experimental diets weighed significantly less in the absence of Cbl than in the presence of Cbl on all occasions (Fig. 1); body weights of 14-week-old rats fed on Cbl-deficient MITV and control diets were about 46 and 66% respectively, of those of the rats fed on the Cbl-sufficient experimental diets. These results indicate that the lower body weights of the Cbl-deficient MITV rats are due to factors other than a decrease in feed consumption during Cbl-deficiency.

Both the 14-week-old Cbl-deficient MITV and control rats had hepatic Cbl contents that were about 96% lower (30.2 (SD 0.54) ng/total tissue) than those of the corresponding Cbl-sufficient rats (894.3 (SD 57.65) ng/total tissue). There were no differences in the hepatic Cbl contents between the Cbl-deficient MITV and control rats. These results indicate that both groups of 14-week-old Cbl-deficient rats developed severe Cbl deficiency.

Fig. 2 shows urinary MMA excretion per g body weight of the rats fed on the experimental diets. Urinary MMA excretion in the 8-week-old Cbl-deficient rats became undetectable 1 week after feeding the Cbl-sufficient experimental diets. The Cbl-deficient MITV rats excreted higher levels of MMA in urine relative to the control rats during the experiment.

Amounts of MMA in livers, kidneys and plasma of the 14-week-old rats fed on the experimental diets were assayed (Table 2). The levels of MMA in livers of the Cbl-deficient MITV and control rats were 5.30 (SD 0.51) μmol/g tissue (1.18 (SD 1.14) mM as calculated on the basis of water space (0.448 ml/g tissue) of rat liver cytosol and mitochondria) (Siess et al. 1982) and 3.6 (SD 1.30) μmol/g tissue (7.9 (SD 2.90) mM) respectively. MMA also accumulated in the kidneys (2.1 (SD 0.30)-3.0 (SD 0.33) μmol/g tissue) of Cbl-deficient rats, while concentrations of plasma MMA (0.75 (SD 0.18)-0.92 (SD 0.06) mM) were (about 90%) lower than those of the hepatic MMA. No MMA could be found in the tissues and plasma of the Cbl-sufficient rats. The results indicate that the amounts of urinary excretion and hepatic accumulation of MMA are increased by feeding of MMA-precursor amino acids and are correlated to the extent of the growth retardation during Cbl deficiency.

Kennedy et al. (1990) have reported that MMA levels of liver (333 nmol/g tissue) and kidney (185 nmol/g tissue) increase considerably in Cbl-deficient sheep. Although normal levels of plasma MMA in man have been reported to be less than 0.34 μM, the levels increase significantly in severely Cbl-deficient patients with methylmalonic aciduria (Moelby et al. 1990), suggesting that substantial amounts of MMA accumulate in the liver. Differences in the hepatic MMA levels would be due to different Cbl status and foods of the Cbl-deficient patients or animals. These observations suggest the possibility that the unusual MMA accumulation caused by Cbl deficiency may interfere with hepatic metabolism.

To study the effect of MMA on cellular metabolism, isolated rat hepatocytes from normally fed animals were labelled with [14C(U)]glucose in the presence or absence of 5 mm-
Fig. 1. Effect of vitamin B₁₂ deficiency on body-weight gain in rats. Cobalamin (Cbl)-deficient rats (8 weeks old) were fed for 6 weeks on Cbl-sufficient diets supplemented with methionine, isoleucine, threonine and valine (MITV, ●) or glutamic acid (GLU, △), or on Cbl-deficient diets with MITV (○) or GLU (△). For details of diets and procedures, see Table 1 and p. 930. Values are means for four rats per dietary group, with standard deviations represented by vertical bars. *Mean values were significantly different from those of the corresponding Cbl-sufficient groups (P < 0.05). †Mean value was significantly different from that of the Cbl-deficient GLU group (P < 0.05).

Fig. 2. Effect of vitamin B₁₂ deficiency on methylmalonic acid excretion in rats. Cobalamin (Cbl)-deficient rats (8 weeks old) were fed for 6 weeks on Cbl-sufficient diets supplemented with methionine, isoleucine, threonine and valine (MITV, ●) or glutamic acid (GLU, △), or on Cbl-deficient diets with MITV (○) or GLU (△). For details of diets and procedures, see Table 1 and pp. 930–932. Values are means for four rats, with standard deviations represented by vertical bars. *Mean values were significantly different from those for the Cbl-deficient GLU group (P < 0.05).

MMA. No radioactivity was found in the lipid fraction. The \(^{14}\text{CO}_2\) formation (as an index of glucose oxidation via the mitochondrial Krebs cycle) from the \([^{14}\text{C}(U)]\)glucose taken up by the hepatocytes without MMA increased linearly up to 40 min, and decreased slightly thereafter. Addition of MMA inhibited \(^{14}\text{CO}_2\) formation by 25% (Fig. 3(a)), suggesting that the decrease in \(^{14}\text{CO}_2\) formation is mainly due to metabolic inhibition of the Krebs cycle by MMA, and not to that of the glycolytic pathway. The incorporation of \(^{14}\text{C}\) into the protein fraction increased linearly with the length of reaction time and MMA did not affect the incorporation of radioactivity (Fig. 3(b)). The incorporation of \(^{14}\text{C}\) into the amino acid fraction was inhibited 30% by MMA after 60 min reaction (Fig. 3(c)). Although
Table 2. Methylmalonic acid concentrations in the liver, kidney and plasma of rats fed on diets sufficient or deficient in cobalamin (Cbl) and containing supplements of glutamic acid (GLU) or methionine, isoleucine, threonine and valine (MITV)†

(Mean values and standard deviations for four rats per dietary group)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Amount of methylmalonic acid</th>
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<tr>
<td></td>
<td>Liver (µmol/g)</td>
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<tr>
<td></td>
<td>Mean</td>
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<tr>
<td>Cbl-deprived MITV</td>
<td>5-30*</td>
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<tr>
<td>Cbl-deprived GLU (control)</td>
<td>3-55</td>
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<tr>
<td>Cbl-sufficient MITV</td>
<td>ND</td>
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<tr>
<td>Cbl-sufficient GLU (control)</td>
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ND, not detectable.

* Mean values were significantly different from those of the Cbl-deprived GLU rats, \( P < 0.05 \).

† For details of diets and procedures, see Table 1 and pp. 930–932.

Fig. 3. Effect of methylmalonic acid (MMA) accumulation on various aspects of hepatic metabolism in rats. (a) \( \text{CO}_2 \) formation from radioactive glucose taken up by hepatocytes with (●) or without (○) 5 mM-MMA; (b) incorporation of radioactivity into the protein fraction with (●) or without (○) MMA; (c) incorporation of radioactivity into amino acids with (●) or without (○) MMA; (d) incorporation of radioactivity into the organic acid fraction with (●) or without (○) MMA. For details of procedures, see pp. 930–932. Values are means for six assays, with standard deviations represented by vertical bars. * Mean values were significantly different from controls without MMA (\( P < 0.05 \)).
Fig. 4. Effects of methylmalonic acid (MMA) on glutamic acid metabolism in isolated rat liver mitochondria. (a) CO₂ formation from radioactive glutamic acid taken up by the mitochondria with (●) or without (○) 5 mM-MMA; (b) incorporation of radioactivity into the protein fraction with (●) or without (○) MMA; (c) incorporation of radioactivity into the organic acid fraction with (●) or without (○) MMA. Values are means for six assays, with standard deviations represented by vertical bars. *Mean values were significantly different from controls without MMA (P < 0.05).

The incorporation of ¹⁴C into the organic acid fraction showed slightly higher levels in the presence of MMA than in the absence of the acid during the experiment (Fig. 3(d)), there were no significant differences between the two assays. These results suggest that the decrease in radioactivity of the amino acid fraction is due to the metabolic inhibition of the Krebs cycle by MMA since some amino acids (alanine, glutamic acid and aspartic acid) are derived from glucose via the Krebs cycle intermediates, α-ketoglutarate and oxaloacetate respectively.

To study the effect of MMA on mitochondrial amino acid metabolism, rat liver mitochondria from normally fed animals were labelled with [¹⁴C(U)]glutamic acid in the presence or absence of 5 mM-MMA. No radioactivity was found in the lipid fraction. The
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$^{14}$CO$_2$ formation (as an index of glutamic acid oxidation by the Krebs cycle) from the radioactive glutamic acid taken up by the mitochondria without MMA increased linearly up to 40 min, and decreased slightly thereafter (Fig. 4(a)). Addition of MMA inhibited $^{14}$CO$_2$ formation by 70%, indicating that the decrease in $^{14}$CO$_2$ formation is mainly due to metabolic inhibition of the Krebs cycle by MMA. MMA did not affect the incorporation of $^{14}$C into the protein fraction (Fig. 4(b)). The incorporation of $^{14}$C into the organic acid fraction was significantly stimulated up to 20 min and then maintained at a constant level (Fig. 4(c)). These results indicate that accumulation of MMA disrupts normal glutamic acid metabolism in rat liver mitochondria, probably due to inhibition of the Krebs cycle.

Halperin et al. (1971) have reported that MMA is a potent inhibitor of the dicarboxylic acid carrier system of the inner mitochondrial membrane of rat liver. The inhibition of the Krebs cycle by MMA may be due to the impairment of the mitochondrial dicarboxylate carrier system. Arinze et al. (1979) have reported that 5–20 mM-MMA inhibits gluconeogenesis in mammalian hepatocytes. These observations and the results presented here indicate that the unusual accumulation of MMA caused by Cbl deficiency disrupts normal hepatic metabolism; it would thus become one of the causes of the severe growth retardation seen in Cbl-deficient animals. To clarify the mechanism of the inhibition of the Krebs cycle by MMA, further biochemical studies are required.

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REFERENCES


