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Vitamin B_{12} deficiency results in the abnormal regulation of serine dehydratase and tyrosine aminotransferase activities correlated with impairment of the adenylyl cyclase system in rat liver

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The aim of the present study was to elucidate the mechanism of the vitamin B_{12} deficiency-induced changes of the serine dehydratase (SDH) and tyrosine aminotransferase (TAT) activities in the rat liver. When rats were maintained on a vitamin B_{12}-deficient diet, the activities of these two enzymes in the liver were significantly reduced compared with those in the B_{12}-sufficient control rats (SDH 2·8 (SD 0·56) *v.* 17·5 (SD 6·22) nmol/mg protein per min (*n* 5); $P<0·05$) (TAT 25·2 (SD 5·22) *v.* 41·3 (SD 8·11) nmol/mg protein per min (*n* 5); $P<0·05$). In the B_{12}-deficient rats, the level of SDH induction in response to the administration of glucagon and dexamethasone was significantly lower than in the B_{12}-sufficient controls. Dexamethasone induced a significant increase in TAT activity in the primary culture of the hepatocytes prepared from the deficient rats, as well as in the cells from the control rats. However, a further increase in TAT activity was not observed in the hepatocytes from the deficient rats, in contrast to the cells from the controls, when glucagon was added simultaneously with dexamethasone. The glucagon-stimulated production of cAMP was significantly reduced in the hepatocytes from the deficient rats relative to the cells from the control rats. Furthermore, the glucagon-stimulated adenylyl cyclase activity in the liver was significantly lower in the deficient rats than in the controls. These results suggest that vitamin B_{12} deficiency results in decreases in SDH and TAT activities correlated with the impairment of the glucagon signal transduction through the activation of the adenylyl cyclase system in the liver.

Vitamin B_{12} deficiency: Serine dehydratase: Tyrosine aminotransferase: Glucagon

Vitamin B_{12} is taken-up into mammalian cells and converted to its coenzyme forms, methylcobalamin (MeCbl) and 5′-deoxyadenosylcobalamin (AdoCbl)[1,2]. MeCbl functions in methionine synthase, which catalyses the synthesis of methionine from homocysteine with 5-methyltetrahydrofolate as a methyl donor, and participates in one-carbon and folate metabolism[3,4]. In contrast, AdoCbl is required by methylmalonyl-CoA mutase, which converts L-methylmalonyl-CoA to succinyl-CoA, an intermediate of the TCA cycle, during the final stage in the degradation pathways of some amino acids, odd-numbered fatty acids and cholesterols[5]. It is known that B_{12} deficiency causes haematological and neurological abnormalities, hepatic injury and growth retardation in mammals[6–8].

Serine dehydratase (SDH) is the enzyme that catalyses the pyridoxal 5′-phosphate-dependent deamination of serine and threonine to produce pyruvate and 2-oxobutyrate, respectively, in the liver. The expression level of SDH in the liver is dramatically increased under conditions of gluconeogenesis, such as the feeding of a high-protein diet, starvation and diabetic mellitus[9–11]. Glucagon, glucocorticoids and insulin play pivotal roles in the regulation of SDH expression[12,13]. Tyrosine aminotransferase (TAT) catalyses the rate-limiting step of tyrosine degradation, and the expression of TAT is also regulated by glucagon, glucocorticoids and insulin[14,15].

It has been reported that amino acid metabolism is disordered by B_{12} deficiency, and certain amino acids, in particular serine and threonine, are abnormally increased in the plasma and excreted into the urine of animals with B_{12} deficiency[16–18]. Previously, we found that SDH activity in the liver was significantly reduced due to B_{12} deficiency in rats[16], although neither MeCbl nor AdoCbl is required for the enzyme as a cofactor. Thus, it is possible that a decrease in hepatic SDH activity results in the abnormal increase in the plasma and urinary levels of serine and threonine in the B_{12}-deficient rats. However, the mechanisms by which SDH

Abbreviations: AC, adenylyl cyclase; AdoCbl, 5′-deoxyadenosylcobalamin; CN-Cbl, cyanocobalamin; Gs, GTP-binding protein; MeCbl, methylcobalamin; SDH, serine dehydratase; TAT, tyrosine aminotransferase; Tris, tri(hydroxymethyl)-aminomethane.

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activity in the liver is affected by B_{12} deficiency have not yet been determined.

In the present study, we examined the effects of vitamin B_{12} deficiency on the hormonal regulation of SDH and TAT activities in the rat liver. We report here that glucagon signal transduction through activation of the adenylyl cyclase (AC) system is impaired by B_{12} deficiency, and consequently the activities of SDH and TAT are abnormally regulated in the rat liver under B_{12}-deficient conditions.

Materials and methods

Diets

In the present study, standard and non-protein diets with or without vitamin B_{12} were used (Table 1). In the non-protein diet, defatted soyabean in the standard diet was substituted by glucose. When vitamin B_{12} was fed to the rats, cyanocobalamin (CN-Cbl) was added at 25 or 100 μg/kg diet. The concentration of folic acid in the experimental diet was 2 mg/kg diet on the basis of AIN-93VX.

Animal treatments

All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University. Male weanling Wistar rats (3 weeks old), born to 14-week-old parent rats, which had been fed the standard diet without vitamin B_{12} for 8 weeks, were used. The parent rats were purchased from Kiwa Laboratory Animals (Wakayama, Japan). The weanling rats were randomly allocated to the B_{12}-deficient and control (B_{12}-sufficient) groups, and individually housed under controlled temperature (22 ± 2°C), humidity (55 ± 10 %) and lighting (from 08.00 to 20.00 hours) conditions. The vitamin B_{12}-deficient and control rats were allowed free access to the standard diet without or with CN-Cbl (25 μg/kg), respectively, and water. After being maintained for 17 weeks, at 20 weeks of age these rats were anaesthetised with diethyl ether, and their livers excised.

In the vitamin B_{12}-supplementation experiment, the B_{12}-deficient rats at 20 weeks old were fed the standard diet supplemented with CN-Cbl at 100 μg/kg for 2 weeks. The livers were obtained from the B_{12}-supplemented rats (22 weeks old) under diethyl ether anaesthesia. All experimental rats were killed at 10.00 hours.

Table 1. Composition of the experimental diets (g/kg)*

	Diet (g/kg diet)	
	Standard	Non-protein
Defatted soyabeans†	400	–
Glucose	453	853
Soyabean oil	100	100
Mineral mixture‡	35	35
Vitamin B_{12}-free vitamin mixture‡	10	10
Choline chloride	2	2

* When vitamin B_{12} was fed to the rats, cyanocobalamin was added at 25 or 100 μg/kg diet.

† Defatted soyabeans contained about 50 % crude protein and 50 % carbohydrate.

‡ Mineral mixture (AIN-93G-MX) and vitamin B_{12}-free vitamin mixture (AIN-93G-VX without vitamin B_{12}) were purchased from Clea (Tokyo, Japan).

Enzyme assays

The livers were homogenised using a Teflon homogeniser in tri(-hydroxymethyl)-aminomethane (Tris)-HCl buffer (0·1 mol/l; pH 7·4) containing KCl (0·15 mol/l) and EDTA (1 mmol/l) for the SDH assay, or in potassium phosphate buffer (50 mmol/l; pH 7·6) containing KCl (0·15 mol/l), EDTA (1 mmol/l) and pyridoxal 5′-phosphate (0·2 mmol/l) for the TAT assay. The homogenate was centrifuged at 100 000 ***g*** for 15 min, and the supernatant fraction obtained was used as a crude enzyme solution.

SDH activity was determined with serine as a substrate[19]. Briefly, the reaction mixture (1 ml) contained potassium phosphate buffer (0·1 mol/l; pH 8·0), KCl (0·15 mol/l), serine (0·2 mol/l), pyridoxal 5′-phosphate (0·4 mmol/l), NADH (0·15 mmol/l), five units of lactate dehydrogenase (from rabbit muscle) and the crude enzyme solution. The reaction was monitored at 37°C by following the decrease in absorbance at 340 nm due to the consumption of NADH.

TAT activity was determined on the basis of previous reports[20]. The assay depends on the alkali-catalysed conversion of the reaction product, *p*-hydroxyphenylpyruvate, to *p*-hydroxybenzaldehyde and oxalate. The reaction mixture contained potassium phosphate buffer (0·1 mol/l; pH 7·6), tyrosine (5·6 mmol/l), α-ketoglutarate (10 mmol/l), pyridoxal phosphate (50 μmol/l), and the crude enzyme solution in a total volume of 0·93 ml, at pH 7·6. The reaction was started by the addition of enzyme and allowed to proceed for 15 min at 37°C, at which time it was stopped by the addition of 0·07 ml of 10 M-KOH. The reaction was read at 331 nm by using a value of 19 900/M per cm for the molar absorbance of *p*-hydroxybenzaldehyde.

The K_m values of SDH for serine and TAT for tyrosine were determined from Lineweaver–Burk plots. The protein concentration was measured according to Bradford[21] with bovine serum albumin as a standard.

Measurement of hepatic vitamin B_{12} content

The liver (about 1 g) was homogenised in four volumes of 10 mM-sodium acetate buffer (pH 4·8) containing 0·2 % (w/v) potassium cyanide, and the homogenate was boiled for 30 min. The boiled suspension was centrifuged at 3000 ***g*** for 10 min. The supernatant fraction was used for the vitamin B_{12} assay by a microbiological method using *Lactobacillus leichmannii* ATCC 7830 and B_{12} assay medium (Nissui, Tokyo, Japan). The liver extract was diluted with distilled water to a B_{12} concentration range of 0·01–0·1 μg/l and used as the assay sample. The turbidity (%T) of a test culture of *L. leichmannii* ATCC 7830 grown at 37°C for 16–21 h was measured at 660 nm with a spectrophotometer.

Administration of glucagon and dexamethasone in rats fed the non-protein diet

The vitamin B_{12}-deficient or control rats at 20 weeks of age, which had been maintained on the standard diet, were fed the non-protein diet without or with supplementation with

CN-Cbl (25 μg/kg), respectively, for 5 d; then the food was removed 12 h before the hormonal administration. Glucagon (Sigma-Aldrich, St Louis, MO, USA) and dexamethasone (Sigma-Aldrich) were suspended in tricaprylin and emulsified with an equal volume of PBS, and the emulsion (0·5 ml), containing 1 mg glucagon and 5 mg dexamethasone, was injected intraperitoneally[22]. At 1 d after the administration, the livers were excised for the determination of SDH activity.

Primary culture of hepatocytes

Parenchymal hepatocytes were isolated from rats (age 20 weeks) maintained on the standard diet without (vitamin B_{12}-deficient group) or with (control group) supplementation with CN-Cbl, by perfusion of the liver with collagenase according to a previous paper[23]. The cells were suspended in Dulbecco's modified Eagle's medium supplemented with 1 mM-insulin, seeded in a collagen-coated 60 mm dish at a density of 7×10^4 cells/cm^2, and cultured at 37°C in 5 % CO_2–95 % air. After 6 h, the medium was replaced by Dulbecco's modified Eagle's medium containing 0·8 trypsin inhibitor units/ml trypsin inhibitor and aprotinin (0·12 μg/ml). These cells were then cultured for an additional 18 h, and used as the primary cultured hepatocytes for the following experiments.

To study the induction of SDH and TAT, the primary cultured hepatocytes were stimulated with glucagon (0·5 μmol/l) and/or dexamethasone (10 μmol/l) in Dulbecco's modified Eagle's medium containing 0·8 trypsin inhibitor units/ml trypsin inhibitor and aprotinin (0·12 μg/ml) for 24 h. These cells were then harvested and disrupted by repeated freeze-thawing in Tris-HCl buffer (0·1 mol/l; pH 7·4) containing phenylmethylsulfonyl fluoride (1 mmol/l), leupeptin (0·5 μg/ml) and pepstatin (1 μg/ml) for the SDH assay, or in potassium phosphate buffer (20 mmol/l; pH 7·0) containing pyridoxal 5′-phosphate (0·2 mmol/l) for the TAT assay. After centrifugation at 40 000 ***g*** for 20 min, the supernatant fraction obtained was used as a crude enzyme solution for the determination of SDH or TAT activity.

To examine the glucagon-induced production of cAMP, the primary cultured hepatocytes were stimulated with glucagon (0·5 μmol/l) in Hank's salt solution at 37°C. After 0, 1, 3 or 6 min incubation, HCl was added to the culture medium at the final concentration of 0·1 mol/l. The amount of cAMP produced during the incubation was determined by measuring the concentration of cAMP in the culture medium using a commercially available kit (cAMP enzyme immunoassay system; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) according to the instructions of the manufacturer.

Measurement of adenylyl cyclase activity

The plasma membrane fraction was prepared from the livers of the B_{12}-deficient or control rats (20 weeks old) according to a previously described method[24]. The AC activity was assayed as described by Salomon *et al.*[25] with some modifications. Briefly, the AC reaction was performed at 30°C for 10 min in a mixture (0·1 ml) which contained Tris-HCl buffer (25 mmol/l; pH 7·6), [α-^{32}P]ATP (1 mmol/l; 180 MBq), [^{3}H]cAMP (1 mmol/l; 2 MBq), GTP (0·1 mmol/l), $MgCl_2$ (5 mmol/l), dithiothreitol (1 mmol/l), creatine phosphate (20 mmol/l), creatine phosphokinase (100 units/ml), 3-isobutyl-1-methylxanthine (0·5 mmol/l) and the plasma membrane preparation. The reaction was stopped by the addition of an equal volume of 10 % (w/w) SDS solution containing cAMP (2·5 mmol/l) and ATP (10 mmol/l), and heated at 100°C for 10 min. cAMP was isolated by sequential chromatography of the reaction mixture on Dowex 50-X4 and neutral alumina, and its radioactivity was counted for determination of AC activity.

Statistical analyses

The B_{12} content, and SDH and TAT activities, in the liver were compared among the vitamin B_{12}-deficient, B_{12}-supplemented and control rats by one-way ANOVA, followed by the Scheffé *post hoc* test. The other data were evaluated by two-way ANOVA, and *post hoc* analyses were done by the Scheffé *post hoc* test. These analyses were performed using GB-Stat 5.4 (Dynamic Microsystems, Silver Spring, MD, USA). All data are presented as mean values and standard deviations, and statistical significance was defined as $P < 0·05$.

Results

Hepatic serine dehydratase and tyrosine aminotransferase activities in vitamin B_{12}-deficient rats

To induce vitamin B_{12} deficiency, weanling rats (3 weeks old) were fed a standard diet (containing about 20 % protein) without B_{12} for 17 weeks. At 20 weeks of age, the body weight of the B_{12}-deficient group (305 (SD 24·4) g) was about 50 % of that of the B_{12}-sufficient control groups (613 (SD 36·4) g) ($P < 0·05$). The hepatic B_{12} content in the B_{12}-deficient rats was reduced to less than 10 % of that in the control rats ($P < 0·05$) (Table 2). The B_{12} deficiency resulted in a significant decrease in SDH activity in the liver, with the activity in the deficient rats being reduced to less than 20 % of that in the control rats. When the B_{12}-deficient rats were fed a B_{12}-supplemented diet (in which CN-Cbl was included at 100 μg/kg) for 2 weeks, SDH activity was increased to a level comparable with that in the control rats, in response to a significant increase in the hepatic B_{12} content. TAT activity in the liver was also significantly lower in the deficient group

Table 2. Effects of vitamin B_{12} deficiency on serine dehydratase (SDH) and tyrosine aminotransferase (TAT) activities and vitamin B_{12} content in liver of the B_{12}-deficient, B_{12}-supplemented and B_{12}-sufficient control groups

(Mean values and standard deviations for five rats)

	SDH (nmol/mg protein per min)		TAT (nmol/mg protein per min)		Vitamin B_{12} content (pmol/g liver)	
Diet group	Mean	SD	Mean	SD	Mean	SD
Control	17·5^{a}	6·22	41·3^{a}	8·11	245^{a}	67·9
B_{12}-deficient	2·8^{b}	0·56	25·2^{b}	5·22	14^{c}	5·0
B_{12}-supplemented	20·7^{a}	1·16	37·1^{a}	10·36	89^{b}	14·7

^{a,b,c}Values within a column with unlike superscript letters are significantly different ($P < 0·05$).

relative to the control. Furthermore, the decreased TAT activity in the deficient rats was restored to a normal level by supplementation with B_{12} for 2 weeks. The K_m values of SDH for serine and TAT for tyrosine were not significantly different among the experimental groups (SDH, 115·8 (SD 34·2), 99·0 (SD 24·7) and 126·6 (SD 26·0) mmol/l; TAT, 2·3 (SD 0·33), 2·8 (SD 0·49) and 2·6 (SD 0·30) mmol/l in the control, vitamin B_{12}-deficient and B_{12}-supplemented rats, respectively).

Abnormalities in the dietary and hormonal regulations of the serine dehydratase activity in the liver under the vitamin B_{12}-deficient conditions

It is well known that SDH activity in the liver is dramatically changed depending on the content of dietary protein[11]. When the vitamin B_{12}-sufficient control rats, which had been maintained on the standard diet (containing about 20 % protein) for 2 weeks, were fed the non-protein diet for 5 d, a significant decrease in SDH activity was induced in the liver, as expected (Fig. 1). However, in the vitamin B_{12}-deficient rats, feeding of the non-protein diet did not cause any significant effect on the hepatic SDH activity. Thus, the significant difference in SDH activity between the B_{12}-deficient and control rats was lost when these rats received a non-protein diet. When glucagon and dexamethasone were administered by intraperitoneal injection into the B_{12}-sufficient control rats that had been maintained on the non-protein diet for 5 d, a significant increase (over 10-fold) in SDH activity was induced in the liver 24 h after the injection. In response to the injection of glucagon and dexamethasone, an increase in the hepatic SDH activity was also observed in the B_{12}-deficient rats; however, the extent of the increase in the deficient group was significantly lower than that in the control. These results suggest that the dietary and hormonal regulations of SDH activity are disordered in the rat liver under vitamin B_{12}-deficient conditions.

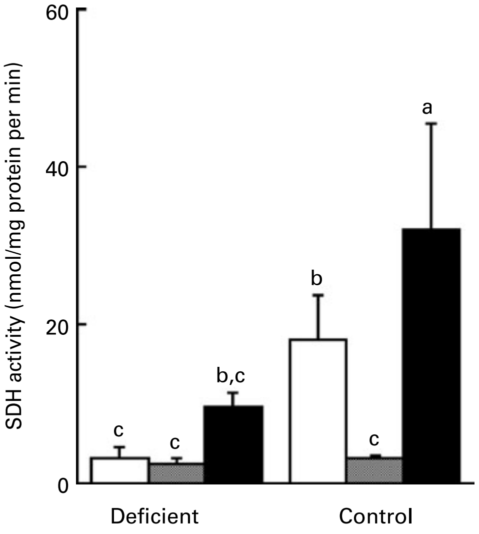

Fig. 1. Induction of serine dehydratase (SDH) in the liver by the administration of glucagon and dexamethasone under vitamin B_{12}-deficient conditions. Hepatic SDH activity was determined in the deficient or control rats just before the feeding of the non-protein diet (□), at 5 d after the feeding of the non-protein diet (▨) or at 24 h after the administration of glucagon and dexamethasone (■). Values are the means of five rats, with their standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters are significantly different ($P<0·05$).

Effects of vitamin B_{12} deficiency on the induction of serine dehydratase and tyrosine aminotransferase by glucagon and dexamethasone in primary culture of hepatocytes

Hepatocytes were isolated from the vitamin B_{12}-deficient or control (B_{12}-sufficient) rats (20 weeks old), and cultured in serum-free medium without vitamin B_{12}. To the primary culture of the hepatocytes, glucagon and/or dexamethasone was added, and SDH activity in the cells was determined 24 h after the stimulation (Fig. 2). A significant increase (about 6-fold) in SDH activity was observed in the hepatocytes prepared from the control rats when they were stimulated simultaneously with glucagon and dexamethasone, although neither glucagon nor dexamethasone alone caused any significant effect. These results are consistent with a previous report showing that both glucagon and dexamethasone are required to induce SDH[26]. However, in the hepatocytes from vitamin B_{12}-deficient rats, even when glucagon and dexamethasone were added at the same time, SDH activity was not significantly enhanced.

TAT activity was significantly increased in the primary cultures of the hepatocytes prepared from either the vitamin B_{12}-deficient or control rats when stimulated with dexamethasone (Fig. 3). In the hepatocytes from the control rats, a further increase in TAT activity was induced when glucagon was simultaneously added with dexamethasone, although TAT activity was not increased by the addition of glucagon

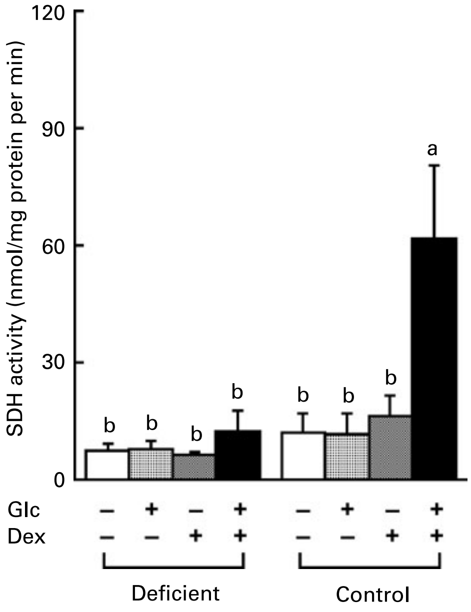

Fig. 2. Induction of serine dehydratase (SDH) by glucagon (Glc) and dexamethasone (Dex) in the primary culture of hepatocytes prepared from vitamin B_{12}-deficient rats. The primary cultures of hepatocytes were incubated in the presence or absence of Glc and/or Dex for 24 h, and SDH activity was then determined. Values are the means of five rats, with their standard deviations represented by vertical bars. [a,b] Mean values with unlike letters are significantly different ($P<0·05$).

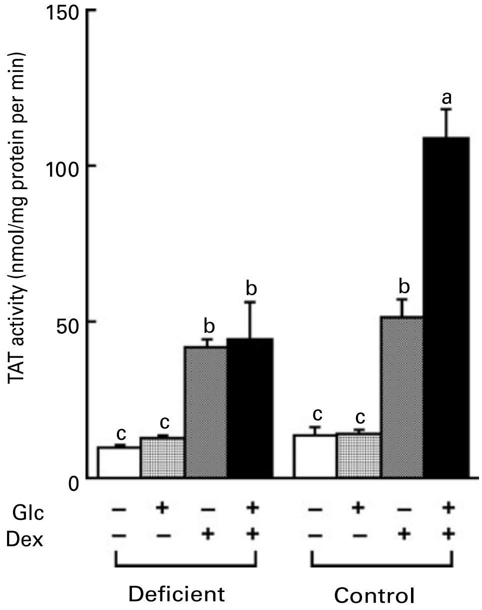

Fig. 3. Induction of tyrosine aminotransferase (TAT) by glucagon (Glc) and dexamethasone (Dex) in the primary culture of hepatocytes prepared from vitamin B_{12}-deficient rats. The primary cultures of hepatocytes were incubated in the presence or absence of Glc and/or Dex for 24 h, and TAT activity was then determined. Values are the means of four rats, with their standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters are significantly different ($P < 0{\cdot}05$).

alone. In contrast, in the cells from the B_{12}-deficient rats, glucagon did not cause any effect on TAT activity even in the presence of dexamethasone.

Glucagon-induced cAMP production in the hepatocytes of the vitamin B_{12}-deficient rats

A primary culture of the hepatocytes was prepared from the vitamin B_{12}-deficient rats and incubated with glucagon, and cAMP production was followed during the incubation. As shown in Fig. 4, cAMP was produced in response to the stimulation with glucagon in the hepatocytes from the B_{12}-deficient rats, as well as the hepatocytes from the B_{12}-sufficient control rats. However, the level of cAMP production was significantly lower in the hepatocytes from the deficient rats after 3 and 6 min of the stimulation.

Adenylyl cyclase activity in the liver of the vitamin B_{12}-deficient rats

The plasma membrane fraction was prepared from the liver of the vitamin B_{12}-deficient or control (B_{12}-sufficient) rats, and AC activity in the membrane fraction was compared between the two groups. As shown in Fig. 5, the basal AC activity without any stimulation was slightly lower in the B_{12}-deficient rats compared with the controls, but no significant difference was observed. When the plasma membrane fraction from the control rats was stimulated with glucagon, AC activity was increased about 3-fold compared with that without such stimulation ($P < 0{\cdot}05$). However, the stimulation with glucagon did not induce a significant increase in AC activity in the B_{12}-deficient rats. In addition, AC activity obtained after stimulation with forskolin was significantly lower in the B_{12}-deficient rats than in the control rats.

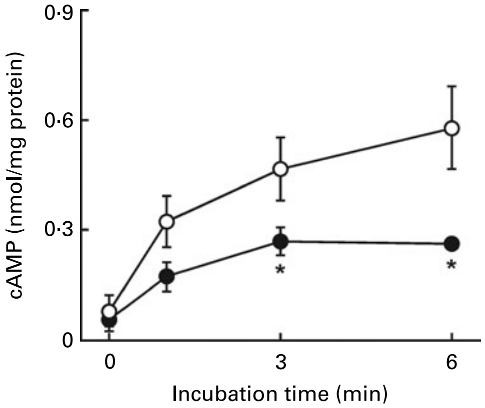

Fig. 4. Glucagon-stimulated cAMP production in the primary culture of hepatocytes from vitamin B_{12}-deficient rats. The primary cultures of hepatocytes prepared from B_{12}-deficient (●) or control (○) rats were incubated with glucagon for 6 min. Values are the means of five rats, with their standard deviations represented by vertical bars. * Mean value is significantly different from that of the primary cultures of hepatocytes prepared from the control rats at the same time point ($P < 0{\cdot}05$).

Discussion

In mammalian cells, MeCbl and AdoCbl are required for methionine synthase and methylmalonyl-CoA mutase, respectively, as coenzymes[1,5]. It is well known that in mammals with a vitamin B_{12} deficiency these enzyme activities are significantly lower and the plasma and urinary levels of homocysteine and methylmalonic acid are abnormally elevated[5,27,28]. We have previously reported that SDH activity in the liver is significantly reduced in rats with dietary vitamin B_{12}

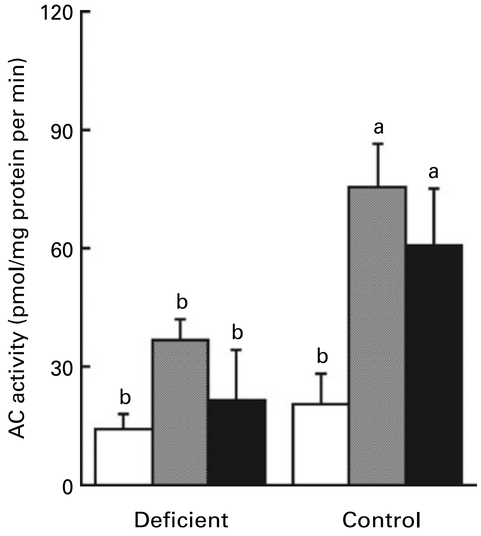

Fig. 5. Effects of vitamin B_{12} deficiency on adenylyl cyclase (AC) activity in the liver. AC activity in the plasma membrane was determined in the absence (□) or presence of forskolin (100 μmol/l) (▨) or glucagon (1 μmol/l) (■). Values are the means of four rats, with their standard deviations represented by vertical bars. [a,b] Mean values with unlike letters are significantly different ($P < 0{\cdot}05$).

deficiency[16], although neither MeCbl nor AdoCbl participates in the SDH enzyme reaction. Furthermore, we also found that serine and threonine are abnormally elevated in the plasma and excreted into the urine in the B_{12}-deficient rats. Impairment of folic acid metabolism due to a decrease in the activity of methionine synthase might cause the abnormalities of the conversion of serine and tetrahydrofolate to glycine and 5,10-methylene-tetrahydrofolate, which is catalysed by serine hydroxymethyltransferase. However, it is conceivable that the decrease of SDH activity is largely responsible for the accumulation of serine in plasma.

SDH, which catalyses the conversion of serine and threonine to pyruvate and 2-oxobutyrate, respectively, is thought to be one of the key enzymes involved in gluconeogenesis, and the nutritional and hormonal regulations of the enzyme expression in the liver have been well studied. It is reported that rats fed a high-protein diet show a 100-fold increase of hepatic SDH activity compared with that in rats maintained on a protein-free diet[11,29]. In addition, it is also known that glucagon and glucocorticoids play pivotal roles in the induction of SDH, whereas insulin suppresses the enzyme induction[12,13].

SDH activity in the liver was significantly lower in the vitamin B_{12}-deficient rats than in the B_{12}-sufficient controls when the rats were maintained on the standard diet, which contained about 20 % protein (Table 2). In the B_{12}-deficient rats, no significant change in SDH activity occurred even if a non-protein diet was fed, in contrast to the effect in the B_{12}-sufficient control rats, in which the enzyme activity was markedly decreased by the absence of dietary protein (Fig. 1). These results suggest that vitamin B_{12} deficiency results in the impairment of the nutritional regulation of SDH activity, and that the activity is maintained at a low level in the liver of B_{12}-deficient rats even if the dietary protein level is increased.

The level of SDH induction in response to the administration of glucagon and dexamethasone was significantly lower in the vitamin B_{12}-deficient rats than in the B_{12}-sufficient controls (Fig. 1). Furthermore, in the primary culture of hepatocytes prepared from B_{12}-deficient rats, in contrast to the cells from the control rats, SDH was not induced even when glucagon and dexamethasone were simultaneously added to the medium (Fig. 2). This indicates that the B_{12} deficiency results in impairment of the hormonal regulation of SDH activity in rat liver.

The activity of TAT, as well as SDH, in the liver was significantly lower in the vitamin B_{12}-deficient rats than in the controls when the rats were maintained on a standard diet that contained about 20 % protein (Table 2). Since TAT is rate limiting for tyrosine degradation[14], it is reasonable to postulate that the decrease in TAT activity due to B_{12} deficiency would result in abnormal tyrosine metabolism. Indeed, in our previous study[16], it was observed that tyrosine, together with serine and threonine, increased in the plasma due to vitamin B_{12} deficiency in rats.

In contrast to SDH, whose induction requires both glucagon and glucocorticoids[26], TAT can be induced in primary cultures of hepatocytes by the action of glucocorticoids alone without any need for glucagon, although glucagon is required in addition to glucocorticoids for the maximal induction of TAT[23]. TAT was induced by dexamethasone in the primary culture of hepatocytes prepared from the vitamin B_{12}-deficient rats at a level comparable with that observed in the cells from the B_{12}-sufficient control rats (Fig. 3), suggesting that B_{12} deficiency does not have any effect on the function of glucocorticoids in the liver. However, in the hepatocytes from the deficient rats, in contrast to the cells from the control rats, glucagon did not potentiate the induction of TAT by dexamethasone, suggesting that the glucagon signal transduction is impaired under the B_{12}-deficient conditions. It is well known that glucagon regulates the expression of many kinds of enzymes involved in glycolysis, glyconeogenesis and the urea cycle, in addition to SDH and TAT; therefore, energy and amino acid metabolism is thought to be broadly affected by the impairment of glucagon signal transduction in mammals with B_{12} deficiency. Actually, it has been reported that B_{12} deficiency results in abnormal increases in many kinds of amino acids, in addition to serine, threonine and tyrosine, in the plasma and urine[16,18], and induces growth retardation[16,28] in mammals; however, the details of the mechanisms underlying these effects remain to be elucidated.

Glucagon stimulates AC in the plasma membrane, increasing the intracellular concentration of cAMP. After activation of cAMP-dependent protein kinase A, this enzyme catalyses the phosphorylation of cAMP response element-binding protein. The binding of cAMP response element-binding protein to the cAMP response element, located upstream of the transcriptional start sites, modulates the expression of SDH and TAT[30,31]. The glucagon-stimulated AC consists of three integral proteins in the plasma membrane: the glucagon receptor, the catalytic unit of AC, and the GTP-binding protein (Gs) which links the receptor and the catalytic unit[32,33]. The level of glucagon-stimulated cAMP production was significantly reduced in the primary culture of hepatocytes from the vitamin B_{12}-deficient rats compared with the cells from the B_{12}-sufficient controls (Fig. 4), indicating that the glucagon-stimulated AC system is impaired in the liver under B_{12}-deficient conditions. The forskolin-stimulated AC activity in the liver was significantly lower in the B_{12}-deficient rats than in the controls, although no significant difference between the two groups was observed in the basal level of AC activity (Fig. 5). It is known that forskolin, when added at higher concentrations (over 1 μM), directly interacts with the catalytic unit of AC without the participation of activated Gs and stimulates its catalytic activity[34]. Thus, the reduced responsiveness of AC to forskolin at 100 μM observed in the B_{12}-deficient rats suggests that B_{12} deficiency results in dysfunction of the catalytic unit of AC in the liver. In addition, in the B_{12}-deficient rats, in contrast to the B_{12}-sufficient controls, a significant increase in AC activity was not induced by treatment with glucagon. This result may indicate that the functional coupling between Gs and the catalytic unit of AC is also disordered in the liver under B_{12}-deficient conditions. The reduction of cAMP production due to the dysfunction of the AC system would cause a decrease in the level of cAMP response element-binding protein phosphorylated by cAMP-dependent protein kinase A, leading to attenuation of the effect mediated through the cAMP response element located upstream of the transcriptional start site of SDH or TAT, and thereby decrease the expression of their SDH and TAT proteins in the B_{12}-deficient rats.

The AC system functionally depends on the maintenance of a suitable membrane environment, and changes in the plasma membrane lipid composition result in alteration of the basal and Gs-mediated stimulation of AC activity[32,33,35]. Phosphatidylcholine has been shown to act as a dominant factor that regulates the function of the catalytic unit of AC and contributes to the functional coupling between Gs and the catalytic unit[36–38]. Since MeCbl acts as a cofactor for methionine synthase in mammalian cells, the synthesis of *S*-adenosyl-methionine, which is utilised as a methyl donor in the conversion of phosphatidylethanolamine to phosphatidylcholine, would be affected under B_{12}-deficient conditions[39]. The synthesis of methionine from betaine, which is derived from choline, by betaine-homocysteine methyl transferase is also linked to the synthesis of *S*-adenosyl-methionine. Indeed, Åkesson *et al.*[40] reported that in rats fed a B_{12}-deficient diet, a significant decrease in the proportion of phosphatidylcholine in the phospholipids, with a concomitant increase in that of phosphatidylethanolamine, occurred in the liver, even though the diet was supplemented with choline. Therefore, as a hypothesis, a decrease in the proportion of phosphatidylcholine in the plasma membrane might account, at least in part, for the impairment of the glucagon-stimulated AC system in the liver of B_{12}-deficient rats. Previously, Hatta *et al.*[41] reported that the β-adrenoceptor–Gs–AC system was impaired in rats fed a vitamin B_{12}-deficient diet.

In summary, the data obtained in the present study show that vitamin B_{12} deficiency results in impairment of the glucagon-stimulated AC system in the rat liver. As a result of the dysfunction of glucagon signalling, the regulation of the activity of SDH and TAT in the liver would be disordered in vitamin B_{12}-deficient rats.

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References

1. Riedel B, Fiskerstrand T, Refsum H & Ueland PM (1999) Co-ordinate variations in methylmalonyl-CoA mutase and methionine synthase, and the cobalamin cofactors in human glioma cells during nitrous oxide exposure and the subsequent recovery phase. *Biochem J* **341**, 133–138.
2. Suormala T, Baumgartner MR, Coelho D, *et al.* (2004) The cblD defect causes either isolated or combined deficiency of methylcobalamin and adenosylcobalamin synthesis. *J Biol Chem* **279**, 42742–42749.
3. Oltean S & Banerjee R (2003) Nutritional modulation of gene expression and homocysteine utilization by vitamin B_{12}. *J Biol Chem* **278**, 20778–20784.
4. Yamada K, Gravel RA, Toraya T & Matthews RG (2006) Human methionine synthase reductase is a molecular chaperone for human methionine synthase. *Proc Natl Acad Sci U S A* **103**, 9476–9481.
5. Stabler SP (1999) B_{12} and nutrition. In *Chemistry and Biochemistry of B_{12}*, pp. 343–365 [R Banerjee, editor]. New York: John Wiley & Sons, Inc..
6. Ebara S, Adachi S, Takenaka S, Enomoto T, Watanabe F, Yamaji R, Inui H & Nakano Y (2003) Hypoxia-induced megaloblastosis in vitamin B_{12}-deficient rats. *Br J Nutr* **89**, 441–444.
7. Nakao M, Kono N, Adachi S, Ebara S, Adachi T, Miura T, Yamaji R, Inui H & Nakano Y (2006) Abnormal increase in the expression level of proliferating cell nuclear antigen (PCNA) in the liver and hepatic injury in rats with dietary cobalamin deficiency. *J Nutr Sci Vitaminol* **52**, 168–173.
8. Scalabrino G & Peracchi M (2006) New insights into the pathophysiology of cobalamin deficiency. *Trends Mol Med* **12**, 247–254.
9. Ishikawa E, Ninagawa T & Suda M (1965) Hormonal and dietary control of serine dehydratase in rat liver. *J Biochem (Tokyo)* **57**, 506–513.
10. Snell K (1984) Enzymes of serine metabolism in normal, developing and neoplastic rat tissues. *Adv Enzyme Regul* **22**, 325–400.
11. Ogawa H, Fujioka M, Su Y, Kanamoto R & Pitot HC (1991) Nutritional regulation and tissue-specific expression of the serine dehydratase gene in rat. *J Biol Chem* **266**, 20412–20417.
12. Noda C, Yakiyama M, Nakamura T & Ichihara A (1988) Requirements of both glucocorticoids and glucagon as co-inducers for activation of transcription of the serine dehydratase gene in cultured rat hepatocytes. *J Biol Chem* **263**, 14764–14768.
13. Kanamoto R, Su Y & Pitot HC (1991) Effects of glucose, insulin, and cAMP on transcription of the serine dehydratase gene in rat liver. *Arch Biochem Biophys* **288**, 562–566.
14. Granner DK, Lee A & Thompson EB (1977) Interaction of glucocorticoid hormones and cyclic nucleotides in induction of tyrosine aminotransferase in cultured hepatoma cells. *J Biol Chem* **252**, 3891–3897.
15. Nitsch D, Boshart M & Schutz G (1993) Activation of the tyrosine aminotransferase gene is dependent on synergy between liver-specific and hormone-responsive elements. *Proc Natl Acad Sci U S A* **90**, 5479–5483.
16. Ebara S, Toyoshima S, Matsumura T, Adachi S, Takenaka S, Yamaji R, Watanabe F, Miyatake K, Inui H & Nakano Y (2001) Cobalamin deficiency results in severe metabolic disorder of serine and threonine in rats. *Biochim Biophys Acta* **1568**, 111–117.
17. Stabler SP, Sampson DA, Wang L-P & Allen RH (1997) Elevations of serum cystathionine and total homocysteine in pyridoxine-, folate-, and cobalamin-deficient rats. *J Nutr Biochem* **8**, 279–289.
18. van der Westhuyzen J, van Tonder SV, Gibson JE, Kilroe-Smith TA & Metz J (1985) Plasma amino acids and tissue methionine levels in fruit bats (*Rousettus aegyptiacus*) with nitrous oxide-induced vitamin B_{12} deficiency. *Br J Nutr* **53**, 657–662.
19. Peraino C (1967) Interactions of diet and cortisone in the regulation of adaptive enzymes in rat liver. *J Biol Chem* **242**, 3860–3867.
20. Granner DK, Hayashi S, Thompson EB & Tomkins GM (1968) Stimulation of tyrosine aminotransferase synthesis by dexamethasone phosphate in cell culture. *J Mol Biol* **35**, 291–301.
21. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
22. Su Y, Kanamoto R, Miller DA, Ogawa H & Pitot HC (1990) Regulation of the expression of the serine dehydratase gene in the kidney and liver of the rat. *Biochem Biophys Res Commun* **170**, 892–899.
23. Noda C, Nakamura T & Ichihara A (1983) α-Adrenergic regulation of enzymes of amino acid metabolism in primary cultures of adult rat hepatocytes. *J Biol Chem* **258**, 1520–1525.
24. Emmelot P & Bos CJ (1969) Studies on plasma membranes. IX. A survey of enzyme activities displayed by plasma membranes isolated from normal and preneoplastic livers and primary and transplanted hepatomas of the rat. *Int J Cancer* **4**, 705–722.
25. Salomon Y, Londos C & Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Anal Biochem* **58**, 541–548.

26. Noda C, Tomomura M, Nakamura T & Ichihara A (1984) Hormonal control of serine dehydratase mRNA in primary cultures of adult rat hepatocytes. *J Biochem (Tokyo)* **95**, 37–45.
27. Stabler SP, Lindenbaum J & Allen RH (1996) The use of homocysteine and other metabolites in the specific diagnosis of vitamin B-12 deficiency. *J Nutr* **126**, 1266S–1272S.
28. Toyoshima S, Watanabe F, Saido H, Pezacka EH, Jacobsen DW, Miyatake K & Nakano Y (1996) Accumulation of methylmalonic acid caused by vitamin B_{12}-deficiency disrupts normal cellular metabolism in rat liver. *Br J Nutr* **75**, 929–938.
29. Pitot HC & Peraino C (1963) Carbohydrate repression of enzyme induction in rat liver. *J Biol Chem* **238**, 1910–1912.
30. Haas MJ & Pitot HC (1999) Glucocorticoids stimulate CREB binding to a cyclic-AMP response element in the rat serine dehydratase gene. *Arch Biochem Biophys* **362**, 317–324.
31. Nichols M, Weih F, Schmid W, DeVack C, Kowenz-Leutz E, Luckow B, Boshart M & Schutz G (1992) Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. *EMBO J* **11**, 3337–3346.
32. Neelands PJ & Clandinin MT (1983) Diet fat influences liver plasma-membrane lipid composition and glucagon-stimulated adenylate cyclase activity. *Biochem J* **212**, 573–583.
33. Needham L, Finnegan I & Houslay MD (1985) Adenylate cyclase and a fatty acid spin probe detect changes in plasma membrane lipid phase separations induced by dietary manipulation of the cholesterol:phospholipid ratio. *FEBS Lett* **183**, 81–86.
34. Seamon KB & Daly JW (1986) Forskolin: its biological and chemical properties. *Adv Cyclic Nucleotide Protein Phosphorylation Res* **20**, 1–150.
35. Stubbs CD & Smith AD (1984) The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim Biophys Acta* **779**, 89–137.
36. Calorini L, Mugnai G, Mannini A & Ruggieri S (1993) Effect of phosphatidylcholine structure on the adenylate cyclase activity of a murine fibroblast cell line. *Lipids* **28**, 727–730.
37. Hebdon GM, LeVine HIII, Sahyoun NE, Schmitges CJ & Cuatrecasas P (1981) Specific phospholipids are required to reconstitute adenylate cyclase solubilized from rat brain. *Proc Natl Acad Sci U S A* **78**, 120–123.
38. Ross EM (1982) Phosphatidylcholine-promoted interaction of the catalytic and regulatory proteins of adenylate cyclase. *J Biol Chem* **257**, 10751–10758.
39. Metz J (1992) Cobalamin deficiency and the pathogenesis of nervous system disease. *Annu Rev Nutr* **12**, 59–79.
40. Åkesson B, Fehling C & Jagerstad M (1978) Effect of vitamin B_{12} deficiency on phosphatidylethanolamine methylation in rat liver. *Br J Nutr* **40**, 521–527.
41. Hatta S, Watanabe M, Ikeda H, Kamada H, Saito T & Ohshika H (1995) Impairment of adenylyl cyclase signal transduction in mecobalamin-deficient rats. *Eur J Pharmacol* **291**, 351–358.