ALANINE METABOLISM IN PYRIDOXINE-DEPLETED RAT LIVER

Mitsuko OKADA and Midori ABE

Department of Nutrition, School of Medicine, Tokushima University, Tokushima, Japan

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Summary Alanine metabolism in normal and pyridoxine-deficient rats was studied in vivo and in vitro. Incorporation of 14C-alanine into various liver components was determined and no difference was shown between normal and deficient animals in the incorporation into liver homogenates, lipid, protein and plasma glucose. Using the liver slice system, gluconeogenic activity from alanine or pyruvate was 40% lower in deficient rats compared with the activity of normal rats. However, inhibition was completely removed by the addition of 2-oxoglutarate to alanine. Penicillamine did not affect glucose formation from alanine in the liver slice.

We have reported that a severe pyridoxine deficiency is observed in rats fed a high protein diet without pyridoxine as judged from decreases in the activities of some hepatic B6-enzymes and pyridoxal content of tissues (1,2). The activity of alanine aminotransferase [EC 2.6.1.2], in particular, suffers a marked decrease in tissues of pyridoxine-deficient rats (1). In the deficient animals, many kinds of amino acids were accumulated in the liver and plasma and excreted in the urine (3). The hepatic alanine content was, however, found to be lower in the deficient rat (3). The present study deals with alanine metabolism in vivo and in vitro in the pyridoxine-deficient rats, and the importance of alanine as a source of plasma glucose will be discussed.

EXPERIMENTAL

Male rats of the Wistar strain, weighing about 50 g, were fed a diet containing 70% casein with and without supplemented pyridoxine. The dietary composition used was the same as described previously (3). Unless otherwise specified, animals the pyridoxine-supplemented diet received an amount of food equal to that eaten by the deficient group for 3 weeks, and all animals were fasted over-
night before being sacrificed. The body weight of rats at the time of sacrifice was in the range of 80 to 100 g. $^{14}$C-Alanine(U) was obtained from the Radiochemical Center, Amersham and 1-$^{14}$C-sodium pyruvate came from Daiichi Pure Chemicals Co., Ltd.

Incorporation of $^{14}$C-alanine into body components in vivo. Rats fed experimental diets for 3 weeks were injected intraperitoneally with 5 μCi of $^{14}$C-alanine (173 mCi/mmole). At the various intervals after the injection, rats were killed, and blood and livers were pooled for analyses. Plasma was separated by centrifugation and aliquots were chromatographed on a silicic acid-coated plate (4). The solvent system used was n-butanol-95% ethanol-5.4% acetic acid (500:316:184), and the glucose spots were analysed for both glucose and radioactivity. A portion of the liver homogenate was precipitated by the addition of 10% trichloracetic acid and the precipitate was used for analyses of protein and radioactivity after repeated washings with 70% ethanol containing 5% trichloracetic acid at 80°C. Liver lipid was prepared essentially by the method of Folch and Less as described previously (1).

Experiments with the slice system. In slice experiments, rats fed the experimental diet for 3 weeks or fed laboratory chow for 1 week were used. Liver slices were prepared using a slicer devised by STADIE and RIGGS (5). Warburg or Erlenmeyer flasks were used for incubation and the incubation mixture contained appropriate substrates in 3 ml of Krebs-Ringer bicarbonate buffer in which Na$^+$ was replaced by K$^+$ (6). Prior to incubation, a gas mixture of O$_2$-CO$_2$ (95:5) was introduced to the flasks. Warburg flasks, used for the determination of liberated CO$_2$, contained 0.3 ml hyamine solution in the center well, and the reaction was carried out for 30 min at 37°C with shaking. After the reaction was stopped by addition of 0.5 ml 15% perchloric acid, shaking was further continued for 30 min to complete the trapping of CO$_2$. 0.1 ml of hyamine solution was used for the determination of radioactivity. For the assay of glucose formation, the reaction was usually carried out for 60 min and was terminated by chilling in ice water. The reaction mixture was then homogenized, 2 volumes of ethanol were added, heated at 70°C for 2 min and the supernatant was obtained by centrifugation. When alanine was used as a substrate, the supernatant was separated by a thin-layer chromatography described above and the fraction corresponding to glucose was used for analyses of glucose and radioactivity. When pyruvate was a substrate, the supernatant was applied onto a Dowex-1 (Cl$^-$) column to remove pyruvate by adsorption and the washing solution was used for the analyses of glucose and its radioactivity.

Determination of radioactivity. An aliquot of lipid fraction dissolved in chloroform-methanol (2:1) was mixed with the toluene scintillator containing 0.4% PPO and 0.04% POPOP and radioactivity was determined with a Packard-Tri-Carb liquid scintillation counter. A scintillator of dioxane system (7) was used for the determination of the radioactivities of water soluble components,
i.e., glucose as a aqueous solution, and protein and CO₂ as a hyamine solution.

**Analyses.** Glucose was analysed by the method of glucose oxidase using Glucostat obtained from Worthington Biochemical Corp. Protein was determined by the biuret reaction (8), and alanine was enzymatically measured as described before (1). Alanine aminotransferase activity was determined by measuring the pyruvate produced from L-alanine and 2-oxoglutarate as phenylhydrazone (9).

**RESULTS**

**Alanine metabolism in vivo**

To compare alanine metabolism between normal and deficient rats, the incorporation of ¹⁴C-alanine into some liver components was determined during the period of 2 hr after the injection. The total radioactivity in liver tissues and incorporation of ¹⁴C-alanine into lipid fractions showed a very similar pattern in both normal and deficient animals as indicated in Figs. 1 and 2. Incorporation of ¹⁴C-alanine into liver protein resulted in a higher specific activity in deficient rats than normal rats (Fig. 3). This seems to be due to the difference of alanine pool sizes in the livers of the normal and deficient rats; the alanine content in the deficient rat liver (1.93±0.08 μmoles/g) was found to be much lower than that in the normal rat liver (2.94±0.20 μmoles/g).

Since alanine is known to be a preferable substrate for the hepatic gluconeogenesis (10), incorporation of ¹⁴C-alanine into plasma glucose was studied. A higher radioactivity was detected in deficient rats during the first 15 min after the injection, but after the initial period, the activities fell in a similar manner in both

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**Fig. 1.** Total radioactivity in liver tissues. ○―○, Normal; ×―×, deficient. Each point represents the average of data from 3 rats. 0.2 ml of 20% homogenate was mixed with 0.2 ml 60% KOH, dissolved by heating and 0.1 ml 3% H₂O₂ was added. 0.1 ml of sample was taken, dissolved in 10 ml scintillator and radioactivity was counted.
Fig. 2. Incorporation of $^{14}$C-alanine into liver lipid. O—O, normal; ×—×, deficient. Liver lipid extracted was dissolved in C:M (2:1), an aliquot was dissolved in a toluene scintillator and the radioactivity was determined.

Fig. 3. Incorporation of $^{14}$C-alanine into liver protein. O—O, normal; ×—×, deficient. Liver protein was prepared described in the text.

Fig. 4. Incorporation of $^{14}$C-alanine into plasma glucose. O—O, normal; ×—×, deficient. Plasma glucose was separated as described in the text.
groups (Fig. 4). The plasma glucose concentrations were not different in the two groups (the normal; 95.4±4.2 mg/dl, the deficient; 99.4±9.4 mg/dl).

**Alanine metabolism in vitro**

The activity of gluconeogenesis in the liver of rats fed the normal or the deficient diet was studied using the slice system (Table 1). When 

| Substrate                  | Normal        | Deficient    |
|----------------------------|---------------|--------------|
|                            | Glucose production |            |
|                            | cpm/mg protein/hr | μmoles/mg protein/hr | cpm/mg protein/hr | μmoles/mg protein/hr |
| 14C-Alanine                | 537±22 (3,803) | 0.1412±0.0170 (4,711) | 327±56* (7,237) | 0.0694±0.0150* (22,504) |
| 14C-Alanine+2-oxoglutarate | 870±84 (2,437) | 0.1202±0.0097 (27,284) | 1,276±156* (7,237) | 0.0567±0.0048* (22,504) |
| 14C-Pyruvate               | 2,581±82 (3,803) | 0.1095±0.0042 (4,711) | 1,610±86* (7,237) | 0.0462±0.0064* (22,504) |

* Statistically significant between normal and deficient groups, p < 0.05.

14C-pyruvate was used as the substrate, about 40% inhibition of gluconeogenesis was observed in slices from the deficient animals as compared with the control animals. The addition of 2-oxoglutarate to the deficient slices greatly increased the glucose formation from 14C-alanine. 2-Oxoglutarate exhibited only a slight effect on the incorporation of 14C-alanine in the normal slices. No difference in 14CO₂ liberation was observed between the normal and deficient slices from 14C-alanine, with or without 2-oxoglutarate, and from 14C-pyruvate.

**Effect of penicillamine on hepatic gluconeogenesis**

The role of alanine aminotransferase isozymes on hepatic gluconeogenesis was studied. The gluconeogenesis from 14C-alanine in the slice system was essentially unaffected by penicillamine at the concentration where alanine aminotransferase activity in the solubilized system was completely inhibited (Fig. 5). Since penicillamine, a powerful inhibitor of alanine aminotransferase, is known to be impermeable to the mitochondrial membrane, this result indicates that the mito-
Fig. 5. Effect of penicillamine on alanine aminotransferase activity and gluconeogenic activity from alanine. —— gluconeogenesis from $^{14}$C-alanine in the slice system. —— alanine aminotransferase activity in the solubilized system.

chondrial enzyme is solely responsible for the conversion of alanine to glucose in the hepatic cells.

**DISCUSSION**

In a previous paper, we reported that the alanine content in the liver of rats fed a high-protein, pyridoxine-deficient diet was lower than that of the control rats (3). Since the alanine levels in portal blood were the same in both groups, we suspected that alanine is more efficiently utilized in the deficient animals. Since the kinetics of incorporation of the total radioactivity from $^{14}$C-alanine into various constituents was similar in both the normal and the deficient animals (Fig. 1), the permeability of alanine into liver cells should not be affected by pyridoxine deficiency.

The incorporation of alanine into liver lipids was studied, because an induction of fatty liver in the deficient animals is known (1,11). As shown in Fig. 2, alanine was incorporated into the lipid fractions at the same extent in the livers of both groups. The decrease in alanine content in deficient rat liver is, therefore, not due to an increased utilization of alanine for the synthesis of lipids.

The incorporation of $^{14}$C-alanine into liver protein was significantly higher in deficient rats than control rats in terms of specific activity (Fig. 3). Considering the difference of pool sizes of alanine in the livers of both animals, however, the amount of alanine utilized for protein synthesis in the liver of the deficient rat under the present experimental condition seems to be maintained at the normal level and this observation confirms the data presented in the previous paper where $^{14}$C-leucine was used as a tracer (3).

Alanine is known to be efficiently used for hepatic gluconeogenesis in rats (10). We have observed a severe decrease in cytosolic and a moderate decrease in
mitochondrial alanine aminotransferases in the liver of deficient rat fed a high-protein diet (1). The incorporation of alanine into plasma glucose was very similar in both the normal and the deficient groups except for an initial higher incorporation in the deficient group. The difference in the rate of the initial incorporation might be due to a difference of pool size of alanine and other intermediates for gluconeogenesis.

To estimate gluconeogenic activities in the livers of normal and deficient rats, the slice system was used (Table 1). Net formation of glucose in normal rats was twice that in deficient rats in all cases. A part of this difference would be due to glycogenolysis of stored glycogen in normal rats. When alanine was the sole substrate, relatively small amount of the substrate was converted to glucose in both groups. This must have been resulted from a shortage of endogeneous amino acceptor of alanine in these slices. When the substrate was alanine or pyruvate, about 40% inhibition of glucose production was observed in deficient rat. This observation suggests that some steps involved in the gluconeogenic process from pyruvate are impaired in the deficient liver. Upon the addition of 2-oxoglutarate, a higher incorporation of 14C-alanine into glucose was observed in the deficient slices. This observation suggests that greater amount of 2-oxoglutarate is used for glucose formation in normal slices than deficient slices.

On pyridoxine-deficiency, the activities of most of B6-enzymes are decreased and the metabolic conversion of many amino acids are impaired, resulting in the accumulation in the liver and excretion in the urine of those amino acids (3). Alanine is the only exception to this general rule. The probable reason for this is that the decreased activity of alanine aminotransferase is still sufficient to convert alanine to glucose via pyruvate in the deficient animal. As a consequence, alanine is used for glucose production in preference to other amino acids.

Recently a stimulation of renal gluconeogenesis by alanine was reported by FRIEDRICHS (12), while alanine is established as a negative effector of pyruvate kinases from liver (13-17) and kidney (18,19). CORNELL et al. (20) reported that various amino acids influence gluconeogenic activity in various ways, i.e., some amino acids accelerate gluconeogenesis and some others inhibit. Thus amino acids that accumulate in the livers of deficient rats may influence hepatic gluconeogenesis in various ways.

It is interesting to determine which isozyme of alanine aminotransferase plays an important role for hepatic gluconeogenesis from alanine because this enzyme exists in both cytosolic and mitochondrial fractions. Penicillamine was used as an inhibitor of alanine aminotransferase in cytosol fraction. Glucose production from alanine in slice system was not affected by penicillamine at the concentration where the solubilized alanine aminotransferase activity was almost completely inhibited (Fig. 5). This observation suggests that the mitochondrial alanine aminotransferase is mainly responsible for gluconeogenesis from alanine. This finding should be contrasted with the work of KATSUNUMA (21), who found
that glucose formation from aspartate was exclusively carried out by cytosolic aspartate aminotransferase. In his experiment, addition of 10 mM DL-penicil-lamine inhibited glucose formation from aspartate completely. This difference may come from the compartmentation of enzymes involved in gluconeogenesis, i.e., pyruvate carboxylase, which is located in mitochondria, must function in the glucose formation from alanine, whereas no mitochondrial enzyme is involved in the overall process on gluconeogenesis from aspartate. Processes of gluconeogenesis from both alanine and aspartate are depicted in Scheme I.

![Scheme 1. Supposed mechanism of hepatic gluconeogenesis from aspartate or alanine by transaminase isozymes.](image)

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