GLYCOGEN METABOLISM
IN
MEAL-FED PYRIDOXINE-DEFICIENT RATS

by

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We accept this thesis as conforming to the
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THE UNIVERSITY OF BRITISH COLUMBIA
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Date April 30, 1973
ABSTRACT

Pyridoxine-deficient rats are known to exhibit little, if any, weight gain; they also have decreased fat stores in comparison with their pair-fed controls. The defect in energy metabolism responsible for this phenomenon is not well understood at present. This study was undertaken to investigate some aspects of glycogenesis and glycogenolysis in order to add to the present information on energy metabolism in the pyridoxine deficiency state. Meal-fed animals were used, in order to eliminate differences due to the mode of feeding between the experimental and the pair-fed control animals.

Male weanling rats were fed a pyridoxine-deficient diet in one 2-hour daily meal, while the controls were pair-fed. This eliminated differences due to feeding frequency when these groups were compared with each other.

Aspartate amino-transferase and alanine amino-transferase activities were assayed in liver and erythrocytes in order to verify the presence of a pyridoxine deficiency state under the conditions used in this laboratory.

The activities of glycogen phosphorylase, the rate-limiting enzyme in glycogenolysis, and glycogen UDP-glucosyltransferase were assayed in liver and muscle. Glycogen storage in these tissues was also measured.
Finally, the incorporation of labelled carbon atoms into blood glucose and liver glycogen following intraperitoneal injection of L-alanine-$^{14}$C was assayed.

Glycogen phosphorylase activity was reduced in pyridoxine-deficient animals. This defect was not accompanied by a concomitant increase in the deposition of glycogen. There was, therefore, the possibility of a decreased ability to form glycogen.

Glycogen UDP-glucosyltransferase activity was normal in muscle and elevated in liver indicating, if anything, an unimpaired ability to synthesize glycogen from UDPG.

A trend towards a lesser incorporation of labelled carbon atoms into the blood glucose by the pyridoxine-deficient group appeared when the results were expressed as a percent of administered dose per ml. This became statistically significant when the data was expressed in terms of the circulating glucose pool. Although not at a statistically significant level, there was a greater incorporation of labelled carbon atoms into the liver glycogen of the pyridoxine-deficient group.

It appeared from these findings that the defect in energy metabolism in pyridoxine deficiency may be the result of a reduced availability of carbon skeletons and occurred prior to the formation of glycogen. Further study in this area is necessary to reveal the exact point at which energy loss occurred.
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INTRODUCTION

Pyridoxine-deficient rats gain less weight and store less fat than their pair-fed controls, but the cause of this defect has not been clarified to date. The excess energy loss in the pyridoxine deficiency state could not be accounted for by increases in heat production, physical exercise or loss through excreta.

An impairment in fat synthesis could account for lowered body fat stores. However, it has been observed that the liver and the adipose tissue have a normal capacity to synthesize fat.

The other major dietary source of energy is carbohydrate. With the inclusion of pyridoxal phosphate in the structure of glycogen phosphorylase, the impact of pyridoxine deficiency on this enzyme could manifest itself as an overall change in energy metabolism.

In studies of energy utilization by animals subject to different nutritional treatments, it is important to standardize not only the amounts eaten by the experimental and control groups, but also the mode of feeding.

The present study was undertaken to look at the changes, if any, that occur during the synthesis, storage and subsequent breakdown of glycogen in pyridoxine deficient rats under a meal-feeding regime utilized to eliminate differences due to mode of feeding rather than the deficiency itself.
2 REVIEW OF LITERATURE

2.1 The Role of Pyridoxine in Metabolism

In 1934, Gyorgy discovered a B-vitamin that cured acrodynia in rats (Gyorgy, 1934). This vitamin was isolated and identified as pyridoxol in 1938 (Gyorgy, 1938; Kereztesy and Stevens, 1938). Much of the early work on the role of this factor in nutrition and metabolism was reviewed by Snell (Snell, 1958; Snell, 1961).

Initially, the term "vitamin B₆" was employed to designate pyridoxol (Fig. 1a). However, Snell (1944) found that amination or oxidation produced pyridoxamine (Fig. 1b) or pyridoxal (Fig. 1c), respectively.¹ Either of these derivatives possessed a greater growth-promoting activity for lactic acid bacteria than pyridoxol itself (Snell, 1944).

2.1.1 Role of Pyridoxine in Metabolic Pathways

Much of the information on the mechanism of action of pyridoxal phosphate in enzymes related to amino acid metabolism has been based on studies with nonenzymatic model systems. It was suggested that the nonenzymatic reactions involve the formation of Schiff-base-metal

¹The terminology for vitamin B₆ is as follows: collective name is pyridoxine; vitamin B₆ alcohol is pyridoxol; vitamin B₆ aldehyde is pyridoxal; vitamin B₆ amine is pyridoxamine.
Figure 1. Composition of pyridoxol, pyridoxamine and pyridoxal.

Pyridoxol
Fig. 1a

Pyridoxamine
Fig. 1b

Pyridoxal
Fig. 1c
Figure 2. Schiff base formed between pyridoxal-5-phosphate and amino acid.
chelate complexes of pyridoxal (Fig. 2), in which the aldehyde group is combined with the amino group of an amino acid (Sebrell and Harris, 1968; Snell, 1958; Snell, 1961). Although a bi- or trivalent metal ion participates in Schiff-base formation in model systems, this requirement appears to be fulfilled in the enzymatic reaction by interactions between pyridoxal phosphate and the apoenzyme itself (Sebrell and Harris, 1968).

The phosphorylated form of pyridoxal, pyridoxal-5'-phosphate, was shown to be a highly specific coenzyme while pyridoxamine-5-phosphate has limited coenzyme activity (Roberts and Frankel, 1951). Pyridoxal phosphate-dependent enzymes are involved in a wide variety of reactions, almost entirely related to amino acid metabolism. These include: transaminations, decarboxylations, amine oxidations, aldol condensations, cleavage reactions, and deaminating dehydrations (Sebrell and Harris, 1968). Some 50 pyridoxal phosphate-dependent enzymes have been listed by Sauberlich (Sebrell and Harris, 1968).

A role for pyridoxal-5-phosphate in carbohydrate metabolism is suggested by its involvement with polysaccharide phosphorylases. The polysaccharide phosphorylases differ from most of the pyridoxal phosphate enzymes in that they do not require a free aldehyde group for their activity. This is borne out of the fact that reduction of glycogen phosphorylase with NaBH$_4$ has no influence on the activity
of the enzyme (Fischer et al., 1958). It was shown that the role of pyridoxal phosphate in the activation of this enzyme is structural: in maintaining its tetrameric form intact (Fischer and Krebs, 1966).

Pyridoxine has also been implicated in lipid metabolism, but its role has not been fully characterized. Thus, several reports have suggested its involvement with fatty acid elongation, cholesterol metabolism, body and liver fat storage and unsaturated fatty acid interconversions (Sebrell and Harris, 1968). However, no pyridoxal phosphate-dependent enzyme systems involved in lipid metabolism have been isolated.

2.2 Energy Utilization in Pyridoxine Deficiency

Pyridoxine deficiency has long been known to result in alterations in carbohydrate and fat metabolism, but the exact nature and specificity of this relationship has not yet been adequately clarified. The main alteration is a reduction in the ability of animals to store energy as body fat in the deficiency state (Mueller, 1964; Sakuragi, 1959; Sherman, 1950). In addition, several interferences with carbohydrate metabolism have been reported (Krebs and Fischer, 1964).

One of the earliest responses to nutritional deficiency is inanition. Accordingly, it is important in studies concerned with the effects of deficiency on energy storage
to equalize the caloric intakes of the experimental and the control animals. Since in most studies reported in the literature comparison has been made between pyridoxine-deficient and pair-fed control animals, the discrepancy in energy storage in vitamin B₆ deficiency must be related to energy utilization.

The amount of energy stored by animals under any condition is the balance between intake and output. Output represents the fraction of energy spent for maintaining basal metabolism, for heat production, and for physical activity. Any excess would be stored.

2.2.1 Basal Metabolic Rate

Orsini et al. (1942) observed a lowered metabolic rate in pyridoxine-deprived rats as compared to ad libitum-fed controls. J.R. Beaton et al. (1956) found no difference in the basal metabolic rate between pyridoxine-deprived and pair-fed rats. Paul (1968) confirmed that the basal metabolic rates of pyridoxine-deficient rats and their pair-fed controls were similar. However, the level of oxygen consumption of both groups was greater than that attained by the ad libitum-fed group.

2.2.2 Heat Production

Direct quantitative measurements on heat production in pyridoxine deficiency are lacking. However, Yeh and Weiss (1963) found that when pyridoxine-deprived rats were
exposed to a cold environment, their temperature decreased more rapidly than that of pair-fed controls. When trained to press a lever to obtain heat, the pyridoxine-deficient rats did so more often than the pair-fed group. The authors concluded that this reflected either increased heat loss or inability to maintain body temperature under cold stress.

2.2.3 Physical Activity

There are no data in the literature on the physical activity levels of pyridoxine-deficient animals. Therefore, it is not possible to evaluate this aspect of energy expenditure.

2.2.4 Energy Loss in Excreta

Carter and Phizackerley (1951) found that the absorption of carbohydrate, fat and the products of protein digestion was not impaired in pyridoxine deficiency. It was mentioned in the studies of Huber et al. (1964) that unreported data on urinary excretion in pyridoxine deficiency showed no increased loss of calories.

Paul (1968) confirmed that the loss of energy in the faeces of pyridoxine-deficient rats was unaltered. However, the animals showed energy losses in the urine that would account for approximately half the calculated discrepancy in energy storage between the experimental and the pair-fed control rats.
2.2.5 Body Fat Stores

As early as 1949, Sure and Easterling (1949) demonstrated that pyridoxine-deprived rats store less carcass fat than their pair-fed controls. This was confirmed subsequently by Beare et al. (1953).

While the lowering of body fat levels in pyridoxine deficiency has been demonstrated repeatedly, the observations on liver lipids have not been consistent. Guggenheim and Diamant (1957) reported increased deposition of fat in the liver of pyridoxine-deprived rats. This was in direct opposition to the findings of Carter and Phizackerly (1951) who found no alteration in liver fat in pyridoxine-deprived rats, even when a diet containing 20% fat was used.

An impairment in fat synthesis could account for lowering body fat stores. Desikachar and McHenry (1954) demonstrated decreased fat deposition in pyridoxine-deficient rats as compared to pair-fed controls. When $^{14}$C-labelled glucose was given orally, the rates of radioactivity incorporation into carcass fatty acids were similar in both groups precluding any impairment in fat synthesis under those conditions. The authors suggested that increased fat oxidation is a plausible mechanism of lowering body fat levels. This was in disagreement with the suggestion of Carter and Phizackerly (1951), that pyridoxine was required for the conversion of carbohydrate to fat.
Sabo et al. (1971) using glucose-$1^{14}$C and acetate-$1^{14}$C, studied fat synthesis by liver slices from animals deprived of pyridoxine for 3-4 weeks. There was no difference in lipid synthesis from acetate or glucose in fasted or fed deficient and pair-fed control rats. These authors concluded that the liver and the adipose tissue of the deprived rats had a normal capacity to synthesize fat when glucose was metabolically available.

2.3 Carbohydrate Metabolism in Pyridoxine Deficiency

The only known pyridoxal phosphate-dependent enzyme that could possibly affect energy metabolism directly is glycogen phosphorylase. Information on this area of carbohydrate metabolism should be useful in the study of energy metabolism in pyridoxine deficiency.

Pyridoxine has long been known to affect carbohydrate metabolism, but the exact mechanism is not clear. J.R. Beaton and Goodwin (1954) found that there was a significant decrease in the levels of blood sugar and liver glycogen in pyridoxine-deficient rats as compared to pair-fed controls after only 5 days of deprivation. After 21 days, there was also a decrease in fasting levels of pyruvic and lactic acids suggesting that carbohydrate stores were either rapidly depleted or not formed in normal amounts. Other work by J.R. Beaton (1955) revealed a decrease in a pyruvic acid synthesizing enzyme (i.e. aldolase) in the deficiency.
Huber et al. (41) reported hypoglycemia in pyridoxine-deprived rats. This was in agreement with the results of Lyon and Porter (1962). Guggenheim and Diamant (1957) showed no differences in liver glycogen synthesis of pyridoxine-deficient rats as compared to ad libitum-fed rats; however, the pair-fed controls had elevated blood glucose and liver glycogen levels. This explained previous findings from experiments in which only pair-fed controls were used. The elevated levels of liver glycogen and blood sugar in pair-fed animals possibly reflected the meal-eating pattern of food consumption. This effect has been well-documented by many workers (Fuller and Diller, 1970; Leveille, 1966; Leveille and Chakrabarty, 1967).

Muscle glycogen levels have also been studied. Illingworth et al. (1960) found no significant difference in muscle glycogen levels between pyridoxine-deprived rats and ad libitum-fed controls. Lyon and Porter (1962) reported decreased levels of muscle glycogen in two strains of pyridoxine-deprived mice compared to ad libitum-fed controls.

Angel (1968) found that meal-fed pyridoxine-deficient rats deposited less glycogen following the meal than their unrestricted but meal-fed controls. The subcutaneous administration of 300 μg pyridoxol HCl/rat/day for 2 days increased glycogen deposition over that of the controls as long as
the repleted animals had unrestricted access to food during
the daily meal. Thus, it appears that the effect of pyri-
doxine on glycogen deposition may be mediated through its
effect on food intake.

As shown in Fig. 3 (Fischer and Krebs, 1966),
glycogen-UDP glucosyltransferase (Glycogen synthetase)
and glycogen phosphorylase are the two enzymes involved
directly in glycogen synthesis and breakdown, respectively.
These enzymes probably act to control the rate of glycogen
deposition and mobilization. Glinsman et al. (1970) noted
that hyperglycemia caused the inactivation of phosphorylase
i.e. liver phosphorylase $\rightarrow$ dephospho phosphorylase
and the activation of glycogen synthetase i.e. glucose-6-
phosphate dependent (D) $\rightarrow$ glucose-6-phosphate independ-
ent (I) in perfused rat liver. Bishop and Larner (1967)
and Larner (1967-68) found that glucagon had an effect on
these enzymes similar to that of glucose and insulin reversed
this effect.

2.3.1 Glycogen Phosphorylase

Pyridoxal-5-phosphate has been isolated from phos-
phorylase preparations of lobster muscle, cat muscle, human
striated and heart muscle, pig liver and potatoes (Baranowski
et al., 1957; Fischer et al. 1963). Phosphorylase from
muscle tissue has been thoroughly studied and extensive
reviews are available (Fischer et al., 1963; Krebs and
Figure 3. Role of glycogen-UDP glucosyltransferase and glycogen phosphorylase in glycogen synthesis and glycogenolysis, respectively.
The glycogen phosphorylase of muscle exists in two forms: 'a' and 'b', the former being the active one. The relationship between the two forms is as follows:

\[
2 \text{phosphorylase } 'b' + 4 \text{ ATP} \xrightarrow{\text{Cyclic-AMP}} \text{phosphorylase } 'a' + 4 \text{ ADP}
\]

\[
\text{phosphorylase } 'a' + 4 \text{ H}_2\text{O} \rightarrow 2 \text{phosphorylase } 'b' + 4 \text{ Pi}
\]

The 'b' form is inactive without AMP while the 'a' form is stimulated by AMP but does not require it for activity. Each molecule of phosphorylase 'b' consists of two subunits of a molecular weight of 125,000 each, while phosphorylase 'a' is composed of four of these subunits.

Liver phosphorylase is a different type of protein than that of muscle. Unlike muscle phosphorylase, liver phosphorylase has the same molecular weight i.e. 237,000 in both the active and inactive forms called liver phosphorylase and dephospho phosphorylase respectively and both consist of two subunits. A problem exists in assaying liver phosphorylase in that, unlike that of muscle, the inactive or dephospho phosphorylase form is only slightly activated by AMP and therefore usually only the active form is included in the assay for total phosphorylase (Krebs and Fischer, 1964).

Pyridoxal phosphate is bound to phosphorylase as a substituted aldamine derivative involving an amine group and another unidentified group X from the protein (Fischer et al., 1964).
1963). In acid or base, form I (Fig. 4) is converted to yellow Schiff base (II) which can hydrolyze to give free pyridoxal phosphate and apophosphorylase (III). The amino group to which pyridoxal phosphate is bound was shown to be the epsilon-amino group of a lysine residue in phosphorylase.

There have been varying reports on the effect of pyridoxine deficiency on phosphorylase activity. Eisenstein (1962) and Lyon and Porter (1962) found a decrease in muscle phosphorylase 'a' while Illingworth et al. (1960) found no change. Total muscle phosphorylase (Eisenstein, 1962; Illingworth et al., 1960; Lyon and Porter, 1962) and total liver phosphorylase (Eisenstein, 1962; Lyon and Porter, 1962) activities were decreased in pyridoxine deficiency. Illingworth postulated that increased 'a' to 'b' conversion may occur as a protective measure to maintain the enzyme activity levels needed to supply energy for muscle contractions.

Takami et al. (1968) found that pyridoxal phosphate level in the liver was decreased by 30% after sixty days on a pyridoxine-free diet, and then it remained constant. It was suggested that the source of pyridoxal phosphate to maintain this level was muscle phosphorylase. Krebs and Fischer (1964) have stated the belief that there is a large reservoir of pyridoxal phosphate in muscle
Figure 4: Binding of pyridoxal phosphate to phosphoprotein.
phosphorylase (perhaps one-half of all pyridoxine in the body). About 60% of all pyridoxine in muscle is associated with phosphorylase; in liver, much of the pyridoxine is not associated with phosphorylase. The importance of this is unknown. Pyridoxine in muscle exists in a pyridoxal rather than pyridoxamine form (Krebs and Fischer, 1964) and thus does not need further conversion before use.

2.3.2 Glycogen-UDP glucosyltransferase (Glycogen Synthetase)

Glycogen synthetase does not contain pyridoxal phosphate in its structure; nevertheless, it may be important as a possible regulator of the rate of glycogen synthesis. The radioactive glycogen formed from UDPG-\(^{14}\)C indicates that the glucose residue becomes attached to an \(\alpha(1\rightarrow4)\) linkage, the same link hydrolyzed by glycogen phosphorylase (Leloir et al., 1959). Leloir et al. (1959) found that the in vitro reaction required the presence of a polysaccharide primer and that it is activated by hexose-6-phosphates.

Just as glycogen phosphorylase exists in two forms, so does glycogen synthetase. These are known as glucose-6-phosphate dependent (D) and glucose-6-phosphate independent (I) forms (Glinsmann et al., 1970). Gold (1968) disagreed with the implications of this terminology, stating that the EDTA used in the assay medium masked glucose-6-phosphate activation of the so-called independent form. Gold found that in vitro activation results in the appearance of a
form with increased sensitivity to glucose-6-phosphate and an increased affinity to Mg$^{++}$.

The only published study on the effect of pyridoxine deficiency on glycogen synthetase activity is that of Illingworth et al. (1960). The activity of the muscle enzyme remained unaltered after seven to eight weeks on a pyridoxine-free diet, as comparison with the controls revealed.

Another means by which pyridoxine may play a role in carbohydrate metabolism was suggested by the results of Glazer and Weber (1971). These authors found that pyridoxal phosphate inhibited glucose conversion to lactate in rat liver homogenate, but had little effect on the conversion of glucose-6-phosphate to lactate. Therefore, it was suggested that pyridoxal phosphate inhibits glucose-6-phosphate formation, possibly by depressing hexokinase activity. On the basis of this, it would follow that the inhibition would be reduced in pyridoxine deficiency, thus increasing glucose-6-phosphate availability for utilization.

2.4 Effect of Mode of Feeding

To obtain results directly caused by pyridoxine deficiency, the effects of the accompanying inanition must be eliminated. This is best accomplished using pair-fed controls. G.H. Beaton et al. (1956) tried to overcome
inanition by administering insulin to pyridoxine-deprived rats and found that fat stores were increased to the level of the controls. The authors concluded that inanition could, therefore, be the cause of decreased fat stores. Huber et al. (1964) also found that insulin reversed many of the effects of pyridoxine deficiency, but concluded that more than just inanition was involved, as insulin sensitivity of adipose tissue segments was also increased.

In pair-feeding trials, the deficient rats are allowed food *ad libitum* while the pair-fed animals are forced to consume less than they would eat if fed *ad libitum*. Under these conditions the pair-fed controls consume their food quickly, and become 'meal-eaters' rather than 'nibblers'. Thus, they undergo long periods of fasting, the effects of which include hyperplasia of the gastrointestinal tract and hyperlipogenesis (Cohn and Joseph, 1959; Cohn and Joseph, 1960; Cohn and Joseph, 1968; Cohn and Joseph, 1970; Fabry and Brown, 1967; Hollifield and Parson, 1962; Leveille and Hanson, 1965; Tepperman and Tepperman, 1964). Leveille and O'Hea (1967) reported a decreased activity level of meal-fed animals such that the animals were able to maintain normal weight gain on 70% of the food consumption of the *ad libitum*-fed animals.

Therefore it does not seem reasonable to compare the *ad libitum*-fed i.e. nibbling, deprived rat to either a
pair-fed or a pair-weighed control. A logical alternative would be to adapt the group to be deprived of pyridoxine to a meal-feeding schedule. This would eliminate not only differences due to caloric intake, but also differences due to feeding frequency.

Evidence in support of the value of meal-feeding as suggested can be obtained from the work of Emerson et al. (1959) and Cockburn and Van Bruggen (1959). They demonstrated that the length of time after eating must be known in work measuring lipid-labelling because of the rapid increase and subsequent drop that occurs after the ingestion of food.

Fuller and Diller (1970) point out the danger of comparing results of liver glycogen deposition in meal-fed and ad libitum-fed rats, in that one may conclude meal-fed had greater, lesser or the same degree of deposition depending on whether the measurement was taken twelve hours after the start of the meal, just before the meal, or halfway in between, respectively. This was suggested bearing in mind that ad libitum-fed rats usually eat mostly at night and meal-fed animals are usually fed in the morning. Before sacrifice both groups are commonly fasted, a 'normal' condition for the rat adapted to meal-feeding but not for the nibbling rat. Thus the two groups must be at a similar point after food ingestion.
Using pyridoxine-deprived rats adapted to a two-hour daily feeding schedule, Paul (1968) showed that, after 60 days of deprivation, the deficient rats weighed significantly less than either the *ad libitum*-fed or pair-fed controls. Of interest is the fact that although body fat levels were significantly less in the pyridoxine-deficient than in the *ad libitum*-fed controls, they were only slightly less than those of the pair-fed group. Thus, it appears that the effect of pyridoxine deficiency on body fat (cf. Section 2.2.5) may be attributed to differences in the mode of feeding between the experimental and the control rats.

2.5 Summary

Pyridoxine deficiency leads to alterations in overall energy production, utilization and storage. As the pyridoxine-deprived animal stores less energy as fat, it must be expending more energy. This increased energy expenditure of the animal should be reflected in its basal metabolic rate, its level of physical activity and heat production and/or the balance of energy between intake and excreta. However, the reason for the excess energy loss in pyridoxine deficiency cannot be explained in these terms.

Studies on fat metabolism revealed that adipose
and liver tissues from pyridoxine-deprived rats have, if anything, a normal ability to form lipids from labelled acetate or glucose. This indicates that lack of fat precursors may be responsible for the observed reduction in body fat stores.

The most immediate source of energy is carbohydrate. With the inclusion of pyridoxal phosphate in the structure of glycogen phosphorylase, the impact of pyridoxine deficiency on energy metabolism in general is important. A large gap in information exists at present between the expected decrease in carbon skeletons available because of faulty transamimation and the well-documented effects of pyridoxine deficiency on glycogen phosphorylase in ad libitum-fed deprived rats.

The literature contains many conflicting reports as to the effect of pyridoxine deficiency on the various aspects of carbohydrate metabolism. One possible reason for this is the difference in the modes of feeding between the control and the deprived rats.

In order to obtain a clearer picture of energy metabolism during pyridoxine deficiency it seemed necessary to observe some aspects of carbohydrate metabolism using comparable conditions, by adapting the deficient group to meal-feeding.
3 MATERIALS AND METHODS

3.1 Materials

Male weanling Wistar rats (40-50 g initial body weight) purchased from Woodlyn Laboratories, Guelph, Ontario, were used throughout this investigation.

The composition of the diet is shown in Table I. The vitamin mixture (Table II) and the mineral mixture (Table III) were prepared by General Biochemicals, Inc., Chagrin Falls, Ohio, U.S.A. Corn oil (Mazola brand) and sucrose were purchased locally.

Chemicals were Fisher-brand reagent grade and were obtained from Fisher Scientific Ltd., Vancouver, B.C. Amberlite MB-3 ion-exchange resin was purchased from Rohm and Haas, Philadelphia, Pennsylvania, U.S.A. Biochemicals were supplied by Sigma Chemical Co., St. Louis, Missouri, U.S.A. Radiochemicals were obtained from Amersham/Searle Corporation, Don Mills, Ontario. Rat liver glycogen, used in the glycogen phosphorylase assay mixture, was prepared by the method of Cowgill and Pardee (1957).

3.2 Methods

3.2.1 Handling of Animals

The animals were housed individually in screen-bottomed cages in an air-conditioned room maintained at
TABLE I. Composition of the diet.\textsuperscript{1}

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<tbody>
<tr>
<td>Sucrose</td>
<td>667 gm/kg diet</td>
</tr>
<tr>
<td>Casein (92% protein)</td>
<td>170</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
</tr>
<tr>
<td>Vitamin mix\textsuperscript{2}</td>
<td>25</td>
</tr>
<tr>
<td>Mineral mix\textsuperscript{3}</td>
<td>38</td>
</tr>
</tbody>
</table>

\textsuperscript{1}The diet was mixed in 4 kg lots and stored at 4°.

\textsuperscript{2}The composition of the vitamin mix is shown in Table II.

\textsuperscript{3}The composition of the mineral mix is shown in Table III.
TABLE II. Composition of vitamin mix for the pyridoxine-deficient test diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>gm/kg diet</td>
<td></td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>0.005</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.01</td>
</tr>
<tr>
<td>d-Calcium pantothenate</td>
<td>0.02</td>
</tr>
<tr>
<td>Niacinamide (nicotinamide)</td>
<td>0.05</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0003</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin B12 with mannitol (0.1%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.0002</td>
</tr>
<tr>
<td>Choline dihydrogen citrate</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>110.0 IU/kg</td>
</tr>
<tr>
<td>Vitamin A palmitate</td>
<td>10,000.0 IU/kg</td>
</tr>
<tr>
<td>Vitamin D-2</td>
<td>1,000.0 IU/kg</td>
</tr>
<tr>
<td>Non-nutritive fibre (cellulose)(^1)</td>
<td>19.4009</td>
</tr>
</tbody>
</table>

\(^1\)The same formulation was used for pyridoxine control diet except pyridoxol HCl was added at 0.01 gm/kg diet at expense of non-nutritive fibre.
TABLE III. Composition of mineral mix for all diets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium carbonate (CaCO₃)</td>
<td>7.260698</td>
</tr>
<tr>
<td>Calcium phosphate dibasic (CaHPO₄)</td>
<td>14.31973</td>
</tr>
<tr>
<td>Sodium phosphate dibasic (Na₂HPO₄)</td>
<td>6.00875</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>7.310174</td>
</tr>
<tr>
<td>Magnesium sulphate (MgSO₄)</td>
<td>2.302268</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.589836</td>
</tr>
<tr>
<td>Manganese sulphate (MnSO₄·H₂O)</td>
<td>0.152216</td>
</tr>
<tr>
<td>Zinc carbonate (ZnCO₃)</td>
<td>0.0532756</td>
</tr>
<tr>
<td>Potassium Iodate (KIO₃)</td>
<td>0.0030443</td>
</tr>
</tbody>
</table>
24° subjected to a light/dark cycle (light: 6:00 am - 6:00 pm). Water was provided ad libitum. The pyridoxine-deprived rats were given access to food for 2 hours daily, between 0900 and 1100 hours. The pair-fed controls were offered, each day, an amount of the pyridoxol-supplemented diet isocaloric to that just consumed by the deprived group during the meal. Pair-feeding was performed at approximately 1130 hours. Food consumption records were kept and were corrected for spillage. The animals were handled frequently and were weighed upon arrival and at weekly intervals thereafter.

The rats were fed the experimental and the control diets for 45 days. Subsequently they were killed by decapitation 4 hours after the initiation of the last feeding.

In experiment III, the rats were given an injection of L-alanine-U-\textsuperscript{14}C, 45 minutes before sacrifice. It was diluted to a specific activity of 2.3 \textsuperscript{\textmu}Ci/m mole. Radioactivity was administered interperitoneally at the rate of m mole/100 g body weight in 1.0 ml saline.

3.2.2 Enzyme Assays

3.2.2.1 Glycogen Phosphorylase (\textsuperscript{\alpha}-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) This assay was carried out on both liver and white leg muscle (gastrocnemius), using the method of Niemeyer et al. (1961).
Approximately 1 g of liver was weighed and a 1:10 homogenate in 0.15 M KF was prepared in 30 sec using a glass homogenizer equipped with a Teflon pestle. The assay medium contained 0.0125 μmoles glucose-1-phosphate, 0.0335 μmoles citrate buffer, pH 6.0, 0.0375 μmoles KF, 0.0125 μmoles AMP, and 5.0 mg glycogen in a volume of 0.5 ml. Incubations were carried out at 37°C. Inorganic phosphate was determined on a sample from the final assay mixture by the method of Taussky and Shorr (1953).

The muscle enzyme was assayed in the same manner as that of the liver, except that a 1:20 homogenate was prepared and strained through cheesecloth prior to use.

3.2.2.2 Glycogen-UDP glucosyltransferase (uridine diphosphate glucose: glycogen α-1-glucosyltransferase, EC 2.4.1.11) Glycogen-UDP glucosyltransferase activity was determined by measuring the amount of radioactivity incorporated into glycogen from 14C-labelled UDP-glucose following the method of Gold and Segal (1967) as modified by Wiley and Leveille (1970). Activity in both liver and white leg muscle (gastrocnemius) was assayed.

Approximately 1 g of each tissue was homogenized in 0.1 M glycylglycine buffer, pH 7.4. The assay mixture contained 2.5 mg glycogen, 2.5 μmoles UDP-glucose-U-14C (0.1 μCi/μmole), 3.5 μmoles glucose-6-phosphate, and
3.3 μmoles glycylglycine, pH 7.4, in a total volume of 0.5 ml.

The purified glycogen pellets were hydrolyzed by heating with 2 ml 1 N H₂SO₄. One ml of hydrolyzed sample was added to 10 ml scintillation solvent (Patterson and Greene, 1965) and counted in a Picker Nuclear Liquimat 220.

### 3.2.2.3 Aspartate amino-transferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1)

This method employs the reaction between aspartic acid and α-ketoglutarate, and measures the dinitrophenylhydrazone of pyruvate formed from the chemical decarboxylation of oxalacetate. Both liver and erythrocytes were used.

A 1:10 liver homogenate in 0.25 M sucrose was prepared. The blood was collected in heparinized tubes. After the buffy coat and the plasma were discarded, the cells were washed with saline and then hemolyzed in 2 volumes of distilled water. The assay was carried out as described by Umbreit et al. (1957).

### 3.2.2.4 Alanine amino-transferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2)

Both liver and erythrocytes were prepared as described above. The assay was performed according to Wroblewski and Cabaud (1957).
3.2.3 Other Methods

3.2.3.1 Glycogen Glycogen deposition was determined on liver and white leg muscle (gastrocnemius) by the method of Hassid and Abraham (1957).

As the glycogen content of muscle was low, it was co-precipitated with Na$_2$SO$_4$ and had to be purified for assay purposes in order to avoid interference. The modified method described by Hassid and Abraham (1957) was used. However, rather than hydrolyzing the extracted glycogen pellet, it was dissolved in 5 ml of water for assay by the anthrone reagent, as for liver.

The amount of radioactivity incorporated into liver glycogen was determined as follows: the glycogen was extracted as described for muscle (Hassid and Abraham, 1957) except that more washings were applied. To eliminate any labelled protein that may have remained, 2 ml of 10\% trichloroacetic acid (TCA) were added to 2 ml glycogen solution and the clear supernatant after centrifugation was used to form the final pellet. One ml of hydrolyzed glycogen and 10 ml scintillation solvent (Patterson and Greene, 1970) were then counted.

3.2.3.2 Blood Glucose Blood was collected in heparinized vials containing KF and was deproteinized by the method of Somogyi (1945).
For the determination of radioactivity incorporated into circulating glucose, 2 ml of the protein-free filtrate were passed through a 0.8x20 cm Amberlite MB-3 column (Friedmann et al., 1965). The column was washed with H$_2$O at a flow rate of 1 ml/min until 40 ml eluate were collected (Deodhar and Mistry, 1969). Preliminary tests with labelled L-alanine-U-$^{14}$C or glucose-U-$^{14}$C established that a negligible amount of alanine was passed through the column ($>$0.2%), while the glucose passed through with approximately 92% efficiency. A 1 ml sample of the eluted labelled glucose and 10 ml scintillation solvent (Patterson and Greene, 1965) were mixed and counted at an efficiency of 78%.

Glucose determination was performed on the protein-free filtrate by the glucose oxidase method recommended by Sigma Bulletin No. 510.

3.2.3.3 Protein Protein content of enzyme solutions was determined using the Folin-Ciocalteu phenol reagent, following the method of Lowry et al. (1951).

3.2.4 Statistics
The animals were randomly assigned to groups using a table of random numbers (Huntzberger, 1967). The selection for sacrifice on specific days was done in a similar manner. Student's t-tests were carried out to determine differences between two group means.
4 EXPERIMENTAL AND RESULTS

4.1 Experiment I

This study was conducted to evaluate the effects of pyridoxine deprivation in meal-fed rats on the activities of liver and muscle glycogen phosphorylases and on glycogen storage in these tissues. One group of weanling rats was meal-fed the pyridoxine-free diet in the manner described in Section 3.2.1, while the other group was given the pyridoxine-supplemented diet in quantities equal to those consumed by the deprived group daily. The experimental period was 45 days, at which time the animals were sacrificed. The terminal procedures were arranged so that the animals in each group were killed 4 hours after their last meal.

The food consumption and weight gain data are presented in Figs. 5 and 6, respectively. Although the deprived and the pair-fed controls consumed equal amounts of food, the latter animals exhibited a greater weight gain over the 45-day experimental period (P < 0.005).

In order to verify the state of deficiency in the deprived rats, the activities of liver and erythrocyte aspartate and alanine aminotransferases were assayed. As shown in Table IV, pyridoxine deprivation in meal-fed rats was associated with decreases in the activities of both of the liver enzymes, as comparison with the pair-fed
Figure 5  Food Consumption of Meal-fed Pyridoxine-deprived Rats
Figure 6 Weight Gain of Meal-fed Pyridoxine-deprived Rats

- • pair-fed controls
- ○ pyridoxine-deprived
Table IV. Liver and erythrocyte aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) in meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Erythrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GOT (µmoles keto acid formed/g/minute)</td>
<td>GPT (µmoles keto acid formed/ml haemolyzate/minute)</td>
</tr>
<tr>
<td>Pyridoxine-deficient&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>51 ± 5.7&lt;sup&gt;5&lt;/sup&gt;</td>
<td>14 ± 1.7&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pair-fed control&lt;sup&gt;4&lt;/sup&gt;</td>
<td>96 ± 10.2</td>
<td>50 ± 6.3</td>
</tr>
</tbody>
</table>

1. Mean ± standard error of the mean for 12 rats.
2. Fed the pyridoxine-free diet for 45 days after weaning.
3. Had access to food from 9:00 to 11:00 a.m. daily.
4. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.
5. Significantly different from the control group at P < 0.001.
controls revealed \( (P < 0.001 \text{ in both cases}) \). In erythrocytes, only aspartate aminotransferase was decreased in the deficiency state \( (P < 0.001) \), while alanine aminotransferase activity remained unaltered.

The activities of liver and muscle glycogen phosphorylases are summarized in Table V. Pyridoxine deficiency was associated with significant decreases in the activities of both the liver \( (P < 0.05) \) and the muscle \( (P < 0.001) \) enzymes, as shown by comparison with the pair-fed controls. This was true whether the activities were expressed in terms of fresh tissue weight or in terms of tissue protein content. It is noteworthy that the deficiency did not affect the liver and the muscle phosphorylases to the same degree i.e. whereas the activity of the liver enzyme (per mg protein) was 68% of that observed in the control group, that of the muscle enzyme was 40% as much.

The effects of pyridoxine deficiency on glycogen deposition are presented in Table VI. As comparison with the pair-fed controls revealed, the accumulation of glycogen in both the liver and the skeletal muscle during the 4-hour period after the initiation of feeding was clearly impaired \( (P < 0.05) \). It appears that at least in the liver, differences in glycogen deposition were not due to differences in tissue size, as both the deprived and the control groups had comparable liver weights.
Table V. Liver and gastrocnemius muscle glycogen phosphorylase activities of meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver 1</th>
<th>Muscle 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α moles P/g/min</td>
<td>α moles P/mg protein/min</td>
</tr>
<tr>
<td>Pyridoxine-deficient 2,3</td>
<td>70 ± 8.35</td>
<td>0.62 ± 0.0745</td>
</tr>
<tr>
<td>Pair-fed controls 4</td>
<td>101 ± 12.3</td>
<td>0.95 ± 0.122</td>
</tr>
</tbody>
</table>

1. Mean ± standard error of the mean for 12 rats.
2. Fed the pyridoxine-deficient diet for 45 days after weaning.
3. Access to food from 9:00 - 11:00 a.m. daily.
4. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.
5. Significantly different from the control group at P < 0.05.
6. Significantly different from the control group at P < 0.001.
Table VI. Liver weights and liver and gastrocnemius muscle glycogen levels of meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver weight</th>
<th>Liver glycogen</th>
<th>Muscle glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100g body wt</td>
<td>mg/g tissue</td>
<td>mg/g tissue</td>
</tr>
<tr>
<td>Pyridoxine-deficient&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>3.6 ± .33</td>
<td>26.7 ± 1.69&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.17 ± 0.013&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pair-fed controls&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.4 ± .20</td>
<td>38.5 ± 4.79</td>
<td>0.21 ± 0.011</td>
</tr>
</tbody>
</table>

1. Mean ± standard error of the mean for 12 rats.
2. Fed the pyridoxine-deficient diet for 45 days after weaning.
3. Had access to food from 9:00 - 11:00 a.m. daily.
4. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.
5. Significantly different from the control group at P  0.05.
4.2 Experiment II

Glycogen-UDP glucosyltransferase (glycogen synthetase) is the other enzyme beside phosphorylase which could affect glycogen levels in mammalian tissues. Therefore, the present experiment was conducted in order to correlate the effects of pyridoxine deficiency on glycogen phosphorylase with the activities of liver and muscle glycogen synthetases. The general experimental protocol was similar to that described previously (cf. Section 3.2.1 and Subheading 4.1).

As shown in Table VII, the responses of glycogen phosphorylase to pyridoxine deprivation were similar to those observed in Experiment I (Table V). This was true for both the liver and the muscle enzymes.

The activities of liver and muscle glycogen synthetases are shown in Table VIII. In the former tissue, pyridoxine deprivation led to a rise in the activity of glycogen synthetase to levels significantly greater than those attained by the pair-fed control group, both when activity was expressed in terms of fresh tissue weight ($P < 0.001$) and tissue protein ($P < 0.01$). In contrast, the activity of muscle glycogen synthetase remained unaltered in relation to the control group, regardless of the manner in which results were expressed.
Table VII. Liver and gastrocnemius muscle glycogen phosphorylase activities of meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moles P/g/min</td>
<td>moles P/mg protein/min</td>
</tr>
<tr>
<td>Pyridoxine-deficient$^2,^3$</td>
<td>130 ± 10.0$^5$</td>
<td>1.23 ± 0.0747$^5$</td>
</tr>
<tr>
<td>Pair-fed controls$^4$</td>
<td>170 ± 9.1</td>
<td>1.56 ± 0.0795</td>
</tr>
</tbody>
</table>

1. Mean ± standard error of the mean for 12 rats.
2. Fed the pyridoxine-deficient diet for 45 days after weaning.
3. Had access to food from 9:00 - 11:00 a.m. daily.
4. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol·HCl/kg diet.
5. Significantly different from the control group at P < 0.01.
6. Significantly different from the control group at P < 0.001.
Table VIII. Liver and gastrocnemius muscle glycogen-UDP glucosyltransferase activities of meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver 1 (dpm/g/min) x 10^{-3}</th>
<th>Liver 1 (dpm/mg protein/min) x 10^{-3}</th>
<th>Muscle 1 (dpm/g/min) x 10^{-3}</th>
<th>Muscle 1 (dpm/mg protein/min) x 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine-deficient^2,^3</td>
<td>616 ± 24.8</td>
<td>5.01 ± 0.20</td>
<td>828 ± 75.8</td>
<td>15.64 ± 1.546</td>
</tr>
<tr>
<td>Pair-fed controls^4</td>
<td>482 ± 22.6</td>
<td>4.20 ± 0.20</td>
<td>986 ± 26.8</td>
<td>18.46 ± 1.033</td>
</tr>
</tbody>
</table>

1. Mean ± standard error of the mean for 12 rats.
2. Fed the pyridoxine-deficient diet for 45 days after weaning.
3. Had access to food from 9:00 - 11:00 a.m. daily.
4. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.
5. Significantly different from the control group at P< 0.01.
6. Significantly different from the control group at P< 0.001.
4.3 Experiment III

The possibility exists that the effects of pyridoxine deficiency on tissue glycogen deposition (Experiment I) may reflect a decrease in the ability of the animal to produce glycogen from sources other than carbohydrate, viz., gluconeogenesis from amino acids. Therefore, Experiment III was conducted to test this hypothesis, using alanine-$^{14}$C as a tracer.

The animals were fed the pyridoxine-free and the control diets in the same manner described previously (cf. Section 3.2.1). At the end of depletion, the rats were given 1 mmole alanine-$^{14}$C/100 g body weight intraperitoneally, 4 hours after the initiation of their last meal. All animals were killed 45 minutes after injection and the appearance of radioactivity in liver glycogen and blood glucose was determined.

As shown in Table IX, there were no differences in blood glucose levels between the deficient and the pair-fed control groups at the time of sacrifice. This was also true with regard to the incorporation of $^{14}$C from alanine-$^{14}$C into blood glucose, when the data were expressed as a percentage of administered radioactivity/100 ml blood. However, the amount of radioactivity present in the circulating glucose space (assumed to be 30% of body weight) (Friedmann et al., 1965) was considerably smaller in the
Table IX. Incorporation of radioactivity from alanine-U$^{14}$C into blood glucose and liver glycogen in meal-fed pyridoxine-deficient rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose $^2$</th>
<th>Liver glycogen $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml</td>
<td>Radioactivity $^3$ (dpm/ml) x 10$^{-3}$</td>
</tr>
<tr>
<td>Pyridoxine-deficient$^4,5$</td>
<td>114 ± 5.2</td>
<td>4.17 ± 0.524</td>
</tr>
<tr>
<td>Pair-fed control$^6$</td>
<td>114 ± 5.3</td>
<td>5.16 ± 0.348</td>
</tr>
</tbody>
</table>

1. L-alanine-U$^{14}$C (2.3 μCi/mmole) was administered intraperitoneally at the rate of 1 mmole/100 g rat in 1 ml physiological saline 45 minutes before sacrifice.

2. Mean ± standard error of the mean for 10 rats.

3. This value represents the radioactivity present in the circulating glucose pool which is assumed to be 30% of body weight (Friedmann et al., 1965).

4. Fed the pyridoxine-free diet for 45 days after weaning.

5. Had access to food from 9:00 - 11:00 a.m. daily.

6. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.

7. Significantly different from the control group at P< 0.001.
deficient group than in the controls ($P < 0.001$).

The amount of radioactivity incorporated into liver glycogen, expressed/\(g\) tissue, was somewhat greater in the pyridoxine-deficient rats than in the controls, but the difference between the two groups did not reach the level of statistical significance. As live weight in both groups was comparable (cf. Appendix), the same statements may be made regarding total glycogen in this tissue. In any case, this amount of radioactivity was small compared to that present in circulating glucose.
A decrease in voluntary food consumption results from many treatments in nutritional studies. The difficulty encountered in investigating energy utilization under these conditions is the necessity of distinguishing the consequences of nutritional alteration itself from those of the accompanying inanition. The usual approach is to equalize the caloric intakes of the experimental and the control animals, by employing pair-feeding techniques. However, when appetite depression is severe, the daily allotment of food offered to the pair-fed animal will be considerably less than that required to satisfy its appetite and will be eaten within a relatively short period of time. Thus, the pair-fed animal becomes an intermittent eater analogous to the meal-fed rat. Meal-feeding, the restriction of food availability to a short daily period, leads to several adaptations related to energy storage and utilization by the rat (Leveille, 1970). These responses include increases in the activities of enzymes involved with fatty acid synthesis (Chakrabarty and Leveille, 1969; Leveille and Hanson, 1966), increased lipogenic potentials of the liver and the adipose tissue (Braun and Fabry, 1969; Cohn and Joseph, 1960; Fabry and Braun, 1967; Hollifield and Parson, 1962 a,b; Leveille, 1966, 1967 a-d; Leveille and Hanson, 1965; Tepperman and
Tepperman, 1958), as well as enhanced glycogen deposition in liver, muscle and adipose tissue (Leveille and Chakrabarty, 1967).

Because of the foregoing considerations, it is important in studies of energy utilization in nutritional deficiency to standardize not only the level of food intake, but also the mode of feeding of the experimental and the control groups. In the studies reported herein, the pyridoxine-deprived rats were adapted to meal-feeding, while the controls were pair-fed. In this manner, differences in both caloric intake and feeding frequency between the two groups of rats were minimized.

It appears that pyridoxine deficiency in meal-fed rats is associated with a decrease in feed efficiency (growth in relation to food intake), since the deprived rats showed lower weight gain than the pair-fed controls. This was in agreement with the findings of Paul (1968) under the same experimental conditions. Paul (1968) also showed that the magnitude of the difference in weight gain between the meal-fed deficient and pair-fed control groups was considerably smaller than that observed when ad libitum-fed (nibbling) deficient rats were compared with their respective controls. Thus, the effects of pyridoxine deficiency on growth as reported in the literature (Sure and Easterling, 1949) may be attributed largely to differ-
ences in the mode of feeding between the experimental and control animals.

It has been suggested that the activities of aspartate and alanine aminotransferases in the liver (Radhakrishnamurty et al., 1968) and erythrocytes (Brin et al., 1960; Cheney et al. 1967; Radhakrishnamurty et al., 1968) reflect the pyridoxine status of animals. In the present studies, the activities of liver and erythrocyte aspartate aminotransferases and liver alanine aminotransferase were significantly reduced to levels below those attained by the pair-fed controls. However, the activity of erythrocyte alanine aminotransferase remained apparently unaltered. This may have been a reflection of food restriction in the control group, which is known to elevate the activity of this enzyme in red blood cells to a point that could mask the effects of deficiency on the experimental group (Cheney et al., 1965).

The results of Experiments I and II show that the meal-fed pyridoxine-deficient rats possess less glycogen phosphorylase activity in the liver, and particularly in the muscle, than their pair-fed controls. These responses were consistent with those observed by Eisenstein (1962), using nibbling rats with pair-weighed controls. Mice deprived of pyridoxine have been also reported to have less
phosphorylase activity in their tissues than animals fed a complete diet ad libitum (Lyon and Porter, 1962). It should be noted that only 'total' phosphorylase activity was measured in the studies reported herein. This represents the enzyme active in the presence of AMP in the assay medium (Krebs and Fischer, 1962).

Glycogen phosphorylase catalyzes the rate-limiting step in glycogen utilization (Krebs and Fischer, 1964). Accordingly, a reduction in the activity of this enzyme might be expected to impair glycogenolysis and thereby increase tissue glycogen levels. However, the results of Experiment I showed that liver and muscle glycogen stores in the deficient rats were decreased below those observed in the controls. That tissue glycogen levels are decreased in pyridoxine deficiency has been also demonstrated in nibbling rats (Beaton and Goodwin, 1954; Beaton, 1955; Guggenheim and Diamant, 1957; Eisenstein, 1962), and mice (Lyon and Porter, 1962). Accordingly, it appears that the phosphorylase activity remaining in the tissues of the deficient animal may be sufficient to maintain a 'normal' ability to mobilize glycogen.

The results of Experiment II demonstrated that the total activities of liver and muscle glycogen synthetase were not impaired in meal-fed pyridoxine-deficient rats.
Illingworth et al. (1960) reported similar findings in nibbling rats with ad libitum-fed controls. However, the data of these authors are open to question, because the deprived rats were given a semi-synthetic diet while the controls were maintained on "laboratory chow". In either case, the reduction of tissue glycogen levels in pyridoxine deficiency does not appear to be a reflection of impaired glucose transfer from UDPG to glycogen. This does not rule out the possibility that the activities of other enzymes related to glycogen synthesis may be decreased. Unfortunately, this cannot be ascertained at the present time because of the lack of published data.

The glucose utilized in glycogenesis is supplied by the diet and by gluconeogenesis from non-carbohydrate sources such as lactate and amino acids. Since pyridoxine deficiency decreases the activities of liver aminotransferases (Experiment I), it would be reasonable to expect that the availability of amino acid carbon for glucose synthesis is impaired under these conditions. The results of Experiment III support this contention, since the conversion of labelled alanine to glucose was lower in the meal-fed deficient rats than in the pair-fed controls, particularly when the radioactivity of blood glucose was calculated in terms of the circulating pool. However, it was not possible to demonstrate this response in liver glycogen, probably
because of the relatively small amount of radioactivity incorporated and the high variability of the values obtained.

Judged by the results obtained in Experiment I, the reduction in tissue glycogen stores in pyridoxine-deficient animals appears to be 20-30% of those attained by the pair-fed controls. Although the proportion of total body glycogen derived from amino acid carbon is not known, the overall findings of the present investigation suggest that decreased tissue glycogen levels may be attributed, at least partly, to the limited supply of substrates for gluconeogenesis resulting from impaired transamination.


<table>
<thead>
<tr>
<th>Experiment</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>116 ± 7.2(^1)</td>
<td>3.95 ± 0.0371(^1)</td>
</tr>
<tr>
<td>Pyridoxine-deficient(^3,4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed controls(^5)</td>
<td>124 ± 1.3</td>
<td>4.38 ± 0.320</td>
</tr>
<tr>
<td>Experiment II</td>
<td>105 ± 4.9(^1,6)</td>
<td>4.19 ± 0.00495(^1)</td>
</tr>
<tr>
<td>Pyridoxine-deficient(^3,4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed controls(^5)</td>
<td>112 ± 1.1</td>
<td>4.21 ± 0.0660</td>
</tr>
<tr>
<td>Experiment III</td>
<td>110 ± 6.7(^2,6)</td>
<td>3.75 ± 0.130(^2,7)</td>
</tr>
<tr>
<td>Pyridoxine-deficient(^3,4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed controls(^5)</td>
<td>122 ± 1.4</td>
<td>3.33 ± 0.0650</td>
</tr>
</tbody>
</table>

1. Mean ± standard error for 12 rats.
2. Mean ± standard error for 10 rats.
3. Fed the pyridoxine-deficient diet for 45 days after weaning.
4. Had access to food from 9:00 - 11:00 a.m. daily.
5. Fed the pyridoxine-deficient diet plus 10 mg pyridoxol.HCl/kg diet.
6. Significantly different from the control group at \( P < 0.01 \).
7. Significantly different from the control group at \( P < 0.001 \).