Nephrocalcinosis and Diets Containing Alkali- and Non-Alkali-Treated Soy Protein

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Diets containing alkali-treated soy protein have been shown to cause nephrocalcinosis in rats. In order to determine if alkali-treated soy protein is the dietary component that induces nephrocalcinosis, the effects of a purified diet containing 20% α-protein (an alkali-treated soy protein) were compared with the effects of the same diet containing 20% promine-D (a non-alkali-treated soy protein) on renal morphology and renal calcium and phosphorus metabolism. After a 9-week feeding trial, light and transmission electron microscopy revealed that the animals fed either the α-protein or promine-D diet developed nephrocalcinosis. In fact, the type of nephrocalcinosis was the same in both groups of animals. Moreover, quantitative determinations of total renal calcium and phosphorus showed that the severity of nephrocalcinosis was also the same in the two groups. No signs of nephrocalcinosis were detected in rats fed a standard commercial laboratory diet. Since nephrocalcinosis was present in the animals fed the promine-D diet, and that it was identical to that found in the animals fed the α-protein diet, it appears that alkali-treated soy protein is not the factor responsible for nephrocalcinosis in rats fed a diet containing the protein.

INTRODUCTION

Alkali treatment of soybean protein has become popular in the food industry. The treatment is used in the isolation of protein from soy flour and in the preparation of protein solutions suitable for the production of spun soy proteins. The textured proteins produced by spinning have the consistency of meat fiber, and therefore are used in the manufacture of processed meat products, as well as other edible goods.

Some concern has arisen about the safety of ingesting foods containing alkali-treated soy protein, since it was found that rats fed diets containing alkali-treated soy protein developed renal lesions (Woodard and Alvarez, 1967; DeGroot and Slump, 1969; Woodard, 1969). In several early studies (Woodard and Alvarez, 1967; Woodard, 1969) it was observed that rats fed a diet containing α-protein (an alkali-treated soy protein) developed nephrocalcinosis and a condition termed nephrocytomegaly, which was characterized by hypertrophy of epithelial cells in the pars recta segments of proximal tubules. It has been determined that alkali treatment of soy protein produces an unusual amino acid, Nε-(DL-2-amino-2-carboxyethyl)-L-lysine (DeGroot and Slump, 1969), which has been given the common name lysinoalanine. Woodard and Short (1973) found that there was a correlation between the ingestion of lysinoalanine and the development of nephrocytomegaly, and suggested that this amino acid was the toxic agent in α-protein that was responsible for the induction of nephrocytomegaly. However, no relationship was shown between lysinoalanine and the development of nephrocalcinosis.
Woodard (1969) has reported that when a non-alkali-treated soy protein (promine-D) was substituted for α-protein in the diet, nephrocalcinosis, similar to that seen with α-protein, occurred. Based on this finding he concluded that the ingestion of α-protein did not cause nephrocalcinosis. This conclusion is somewhat questionable, since very little information was given about the development and characteristics of the nephrocalcinosis induced by either the α-protein or promine-D diets. In later reports, Woodard (1971a, b) did characterize in some detail nephrocalcinosis induced by various diets containing promine-D. However, many of the characteristics that Woodward (1971a) described of nephrocalcinosis induced by the promine-D diets were very similar to those seen in phosphate-induced nephrocalcinosis (MacKay and Oliver, 1935; Zalups et al., 1983; Zalups and Haase, 1983). Recently, Zalups et al. (1983) have described in detail the development of nephrocalcinosis induced by an α-protein diet (similar to that used by Woodard), and found that the nephrocalcinosis was not a phosphate-induced type of renal calcification. In addition, they found that the severity of nephrocalcinosis was considerably less than that which Woodard (1971a) reported with the promine-D diets. Since the nephrocalcinosis Woodard observed in rats fed diets containing promine-D or α-protein may have been caused by an imbalance of minerals (particularly phosphate) in the diet, there is some uncertainty about Woodard’s conclusion that alkali-treated soy protein does not cause nephrocalcinosis.

The aim of the present study is to compare the effects of a purified diet containing an alkali-treated soy protein (α-protein) with the effects of the same diet containing a non-alkali-treated soy protein (promine-D) on serum, urinary, and renal calcium and phosphorus as well as on renal morphology. These comparisons will aid in determining whether alkali-treated soy protein is the principal dietary factor responsible for nephrocalcinosis induced by a purified diet containing the protein.

MATERIALS AND METHODS

Animals and Diets

Thirty young female Sprague–Dawley rats weighing 42–57 g were used in the present study. The animals were divided equally into three groups. One group of 10 rats were fed a purified diet containing 20% α-protein (an alkali-treated soy protein), while another group of 10 rats was fed the same diet, with only promine-D (a non-alkali-treated soy protein) being substituted for the α-protein. The ingredients of the basal diet (modeled after Woodard and Short, 1973) containing either 20% α-protein or 20% promine-D are listed in Table 1. The third group of 10 rats was fed a standard commercial laboratory diet (Ralston Purina Co., St. Louis, Mo.). All the animals were fed their respective diets for a period of 9 weeks.

During the feeding trial all animals were housed individually in stainless-steel metabolic cages and were allowed food and water ad libitum. Once a week each animal was weighed and a 24-h food consumption measurement was made.

Quantitative Determinations

From four randomly selected rats of each group, 24-hr urine samples were collected after 6 and 9 weeks of feeding and blood was drawn from the orbital
TABLE I
Composition of α-Protein and Promine-D Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Content (%)</th>
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<tbody>
<tr>
<td>Alpha protein or Promine-D</td>
<td>20.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>5.0</td>
</tr>
<tr>
<td>Alphacel</td>
<td>2.5</td>
</tr>
<tr>
<td>Dextrin</td>
<td>57.0</td>
</tr>
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</table>

\( ^a \) I.C.N. Nutritional Biochemicals, Montreal, Quebec, Canada.

\( ^b \) Central Soya Company, Chicago, Ill.

\( ^c \) Salt mix composition (g/100 g mix): CaHPO\(_4\) \( \cdot \) 2H\(_2\)O, 43.33; CaCO\(_3\), 7.59; NaCl, 3.81; KCl, 7.86; MgO, 1.99; Fe Citrate, 2.99; ZnCO\(_3\), 0.43; MnSO\(_4\) \( \cdot \) H\(_2\)O, 0.32; CuSO\(_4\) \( \cdot \) 5H\(_2\)O, 0.24; KIO\(_3\), 0.0055.

\( ^d \) Vitamin mix composition (g/100 g mix): thiamine HCl, 0.32; pyridoxine HCl, 0.32; riboflavin, 0.32; d-calcium pantothenate, 0.8; nicotinamide, 1.0; folic acid, 0.1; 1-inositol, 2.0; α-DL-tocopherol acetate, 0.7; menadione, 0.004; vitamin B\(_{12}\), 0.02; biotin, 0.002, vitamin A acetate, (500,000 IU/g) 0.001; vitamin D\(_3\) (500,000 IU/g) 0.024.

sinus under ether anesthesia after 9 weeks. Once the blood was taken, the right kidneys from these animals were removed and dehydrated in an incubator at 80°C for 2 weeks. After dehydration, the kidneys were immersed in petroleum ether for 4–6 hr for the removal of fat. The fat-free dry kidneys were ashed in a Sybron Thermolyne 1500 muffle furnace at 600°C for 48 hr. The resulting residue from each kidney was dissolved in three 1-ml aliquots of 1 N HCl for determination of total renal calcium and phosphorus.

Serum, urinary, and renal phosphorus determinations were made using the Phosphorus Auto/Stat Kit (Pierce Chemical Co., Rockford, Ill.) spectrophotometric method, and all calcium measurements were made with a Varian atomic absorption spectrophotometer.

**Light Microscopy**

At the end of the 9-week feeding period, the left kidney from each of the 10 animals from each group was removed and cut transversely into slices. One slice was fixed in 80% ethanol for 24 hr at 5°C and then for a further 24 hr at room temperature. Another slice was fixed in neutral-buffered Formalin for 48 hr at room temperature. The fixed tissue was subsequently dehydrated in a series of gradedethanols, cleared in toluene, and embedded in paraffin wax. The ethanol-fixed tissue was sectioned at 10 μm and stained with alizarin red S (Dahl, 1952) or by the combined von Kossa–PAS method (Moffat, 1958) for the demonstration of calcium salts. Sections of the Formalin-fixed tissue (5 μm) were stained with hematoxylin and eosin (H and E) for general histology.

**Transmission Electron Microscopy**

An additional slice from some of the left kidneys was taken for transmission electron microscopy. The tissue was rapidly immersed in a solution containing 2.5% glutaraldehyde and 2.0% paraformaldehyde buffered with 0.1 M s-collidine (pH 7.4). The tissue slices were diced into small cubes (1 mm\(^3\)) while in the fixative. The cubes of tissue were fixed for 4–6 hr. After fixation, the samples
were postfixed for 1 hr in 1% osmium tetroxide, buffered with 0.1 M s-collidine. The samples were then stained en bloc for 1 hr with 2% uranyl acetate. Subsequently, the specimens were dehydrated in a series of graded ethanols and washed in propylene oxide. The blocks were then infiltrated with a half-and-half mixture of propylene oxide and Araldite CY212 for 4 hr or overnight, followed by pure Araldite for 4–6 hr. The specimens were embedded in Araldite at 60°C for 24 hr.

Initially, 0.5-μm sections were taken from the embedded tissue using glass knives and a Sorvall "Porter–Blum" MT-1 ultramicrotome. The sections were stained with 1% toluidine blue in 1% borax and viewed with the light microscope. When suitable areas were located for electron microscopy, the tissue was sectioned at 70–90 nm and then, without grid staining, examined with an A.E.I. 801 transmission electron microscope.

Statistical Analysis of Quantitative Data

Statistical evaluation of differences between means of each set of data was accomplished by means of one-way analysis of variance followed by multiple comparison testing using Tukey's method.

RESULTS

Throughout the feeding trial no differences in weight gain or food consumption were observed among the three groups of animals.

Quantitative Data

After 9 weeks of feeding, the levels of serum calcium and phosphorus in the rats fed either the α-protein diet or the promine-D diet were similar to those in the rats fed the commercial laboratory diet (Table II). In addition, all three groups of rats excreted similar amounts of calicum and phosphorus after both 6 and 9 weeks.

Total renal calcium and phosphorus, after 9 weeks, were significantly greater in the animals fed the α-protein and promine-D diets than in the animals fed the commercial laboratory diet. There was no difference, however, in the amount of renal calcium and phosphorus between the group fed the α-protein diet and the group fed the promine-D diet.

Structural Data

α-Protein diet. In all 10 animals fed the α-protein diet, a mild to moderate form of nephrocalcinosis was detected. The nephrocalcinosis was characterized mainly by intraluminal calciferous deposits localized along the junction of the outer and inner stripes of the outer medulla (Fig. 1). Most of the deposits were situated in the terminal segments of proximal tubules. Some were, however, found in the descending thin limbs of Henle. As a note, all the deposits stained with both alizarin red S and von Kossa methods, suggesting that they contained both calcium and phosphorus. Tubular necrosis was also observed in the terminal segments of proximal tubules, but only in the proximity of the calciferous deposits. Many of the calciferous deposits appeared to be intimately associated with necrotic or degenerated epithelial cells of the proximal tubule. In many instances, the apical portion of these cells appeared to be missing, while the remaining portion of the cells seemed to be in direct contact with a deposit. Occasionally, nuclei of epithelial cells were seen within the matrix of the deposits. These find-
<table>
<thead>
<tr>
<th></th>
<th>Serum calcium (mg/dl)</th>
<th>Serum phosphorus (mg/dl)</th>
<th>Urinary calcium (mg/kg body wt/24 hr)</th>
<th>Urinary phosphorus (mg/kg body wt/24 hr)</th>
<th>Renal calcium (mg/g tissue)(^{a})</th>
<th>Renal phosphorus (mg/g tissue)(^{a})</th>
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<tr>
<td><strong>α-Protein diet</strong></td>
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<tr>
<td>6 weeks</td>
<td>—</td>
<td>—</td>
<td>4.9 ± 0.8</td>
<td>23.2 ± 10.2</td>
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<tr>
<td>9 weeks</td>
<td>10.8 ± 0.3</td>
<td>5.9 ± 0.5</td>
<td>5.4 ± 1.0</td>
<td>26.9 ± 12.3</td>
<td>1.4 ± 0.6(^{b})</td>
<td>10.7 ± 1.0(^{b})</td>
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<tr>
<td><strong>Promine-D diet</strong></td>
<td></td>
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<tr>
<td>6 weeks</td>
<td>—</td>
<td>—</td>
<td>5.0 ± 1.0</td>
<td>21.1 ± 8.5</td>
<td>—</td>
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<tr>
<td>9 weeks</td>
<td>11.0 ± 0.1</td>
<td>5.7 ± 0.4</td>
<td>5.3 ± 1.1</td>
<td>27.3 ± 1.8</td>
<td>1.7 ± 0.7(^{b})</td>
<td>10.3 ± 0.5(^{b})</td>
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<tr>
<td><strong>Commercial laboratory diet</strong></td>
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<tr>
<td>6 weeks</td>
<td>—</td>
<td>—</td>
<td>5.0 ± 1.2</td>
<td>19.5 ± 8.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9 weeks</td>
<td>10.9 ± 0.1</td>
<td>5.6 ± 0.6</td>
<td>5.4 ± 2.8</td>
<td>32.2 ± 7.7</td>
<td>0.3 ± 0.1</td>
<td>8.6 ± 0.1</td>
</tr>
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**Note.** Values are means ± SD.

\(^{a}\) Based on fat-free dry kidney weight.

\(^{b}\) Statistically different \((P < 0.05)\) from commercial laboratory diet values.
FIG. 1. Calciferous deposits in the kidney of a rat fed a diet containing 20% α-protein for 9 weeks. Most of the deposits (black) are localized along the junction of the outer and inner stripes of the outer medulla (OS/IS). Some are also present in the inner stripe of the outer medulla (IS). Alizarin red S. ×50.

...ings suggest that some of the deposits may have been formed in part by or from degenerating and/or necrotic epithelial cells of the proximal tubule. Besides the localized changes seen in the area of the calciferous deposits, no other pathological changes were observed in the kidneys.

The ultrastructural features of nephrocalcinosis in the rats fed the α-protein diet for 9 weeks were identical to those described previously by Zalups et al. (1983). As a review, the most frequently encountered features were intraluminal deposits and necrotic epithelial cells in the terminal segments of proximal tubules. The majority of deposits were composed of concentric lamellae wrapped around a central core, and varied in size from small lamellar bodies to deposits that completely filled the tubular lumen. As observed in the light microscope, the lamellar deposits appeared to be formed at least in part from degenerated apical portions of the epithelial cells in the proximal tubules. The cytoplasmic contents of the apical regions of some of the epithelial cells seemed to have been transformed and organized into a series of lamellae, which subsequently became incorporated into the deposits. Another feature, observed less frequently, in the terminal segments of proximal tubules, was the presence of apatite-like crystals in the tubular epithelial cells. Lamellar deposits were also observed in the lumina of some descending thin limbs of Henle. In general, though, very few changes were seen in the epithelial cells (of the descending thin limbs) that surrounded the deposits, suggesting that the structures originated from more proximal segments of the nephron, presumably the pars recta segments of the proximal tubules. No other pathological changes were found.

Promine-D diet. All the rats fed the promine-D diet also developed a mild to moderate form of nephrocalcinosis. Just as in the rats fed the α-protein diet, most of the calciferous deposits were localized along the junction of the outer and inner...
FIG. 2. Calcification in the kidney of a rat fed a diet containing 20% promine-D for 9 weeks. As in Fig. 1, most of the deposits are situated at the OS/IS, but some are also present in the IS. Alizarin red S. ×50.

stripes of the outer medulla (Fig. 2). In fact, the histologic and ultrastructural features of nephrocalcinosis in the rats fed the promine-D diet for 9 weeks were identical to those found in the rats fed the α-protein diet for the same period of time.

Commercial laboratory diet. At both the light and electron microscopic level, no signs of nephrocalcinosis or any other pathological condition were detected in the rats fed the commercial laboratory diet.

DISCUSSION

In the present investigation, young female rats fed a diet containing 20% α-protein for 9 weeks developed nephrocalcinosis. The calciferous deposits were found primarily at the junction of the outer and inner stripes of the outer medulla (also referred to as the cortico-medullary junction by some investigators), which is consistent with the findings of Woodard and Alvarez (1967), who apparently were the first to report the incidence of nephrocalcinosis in rats fed diets containing α-protein.

Woodard and Alvarez (1967) and Woodard (1969) found that, in addition to nephrocalcinosis, enlarged epithelial cells with large nuclei were present in the terminal segments of proximal tubules in the kidneys of rats fed α-protein diets, and termed this type of renal lesion nephrocytomegaly. It has been demonstrated that alkali-treated soy protein contains an unusual amino acid called lysinoalanine (DeGroot and Slump, 1969), and it has been suggested that it is the lysinoalanine that is responsible for the development of nephrocytomegaly (Woodard and Short, 1973). More recently, it has been suggested that the ingestion of lysinoalanine as a free amino acid, rather than in a protein-bound form, is directly related to the induction of nephrocytomegalic changes (Struthers et al., 1977). There was, however, no evidence of nephrocytomegaly in the rats fed the α-protein diet in this
study. Nephrocalcinosis in the absence of nephrocytomegaly in rats fed diets containing alkali-treated soy protein has also been observed in other studies (DeGroot and Slump, 1969; Van Beek et al., 1974; Zalups et al., 1983). A possible explanation for the absence of nephrocytomegaly in the present study, as well as in previous studies, is that the level of nonprotein-bound lysinoalanine in the diet was not sufficient enough to bring about the nephrocytomegalic changes. It is also possible, that some component, other than lysinoalanine, in the diets used by Woodard (1969) and Struthers et al., (1977) may have been responsible for the renal lesions.

Woodard (1969) also observed that rats fed a diet containing 20% promine-D, a non-alkali-treated soy protein, developed nephrocalcinosis. He claimed that the type of renal calcification was the same as that found in rats fed an α-protein diet, although no detailed description of the characteristics of nephrocalcinosis were given. Woodard concluded from this finding that alkali-treated soy-protein was not responsible for the induction of nephrocalcinosis. In a later study, Woodard (1971a) did trace out, in some detail, the development of nephrocalcinosis induced by a promine-D diet. Many of his findings, however, were considerably different from those found in this study. First, Woodard found that nephrocalcinosis was quite severe after feeding rats the promine-D diet for 8 weeks, while in the present study the severity of nephrocalcinosis in rats fed the promine-D diet was at worst moderate after 9 weeks. Woodard also observed thickened basement membranes, dilated tubules, and signs of epithelial hyperplasia, and a chronic inflammatory response in the kidneys of the experimental animals, none of which were found by this investigator. Moreover, Woodard observed that the urinary excretion of calcium was decreased and phosphorus increased in the experimental animals. Such changes were not found in this study. In fact, there were no changes in either serum or urinary calcium and phosphorus in the rats fed the purified diets. In general, the structural and physiological changes found by Woodard appeared to be very similar to those found in nephrocalcinosis induced by excess dietary phosphate (MacKay and Oliver, 1935; Zalups et al., 1983). Since the type of renal calcification Woodard was observing may have been caused by an imbalance of phosphate or other mineral(s) in the diet, it is virtually impossible to deduce from his findings whether alkali-treated soy protein induces nephrocalcinosis or not.

Although different from the Woodard studies, the characteristics of nephrocalcinosis in the rats fed either the α-protein or promine-D diet were identical to those reported by Zalups et al. (1983), who fed rats an α-protein diet, identical to the one used in this study, for up to 12 weeks. Zalups et al. (1983) and Zalups and Haase (1983) investigated the etiology of the nephrocalcinosis induced by the α-protein diet and found that it was not a phosphate-induced type of renal calcification, which suggests that the nephrocalcinosis found in the present study was also not phosphate induced.

Since the morphological and kidney ash results of the present study show that the type and severity of nephrocalcinosis induced by the α-protein diet was the same as that induced by the prominc-D diet, it appears that α-protein is not the factor in the diet that induces the nephrocalcinosis. Therefore, in the final analysis the conclusion made by Woodard (1969) was correct, although it was based on misleading data.

The question as to what factor is responsible for the nephrocalcinosis induced
by either the α-protein or promine-D diet is still without an answer. What is perhaps most perplexing is the fact that the amounts of nutrients present in the purified diets fall within the range recommended by the American Institute of Nutrition (1977) and the National Research Council (1978) for the normal growth and development of the rat. Further research is required in order to better understand the effects of purified diets on renal mineral metabolism in the rat as well as other laboratory animals.

In summary, the results of the present study indicate that alkali-treated soy protein is not responsible for the nephrocalcinosis found in rats fed a purified diet containing the protein.

ACKNOWLEDGMENTS

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