PATHOLOGICAL CHANGES IN RATS FED THE CRAMBE MEAL-GLUCOSINOLATE HYDROLYTIC PRODUCTS, 2S-1-CYANO-2-HYDROXY-3,4-EPITHIOBUTANES (ERYTHRO AND THREO) FOR 90 DAYS

D. H. GOULD* and M. R. GUMMBANN

US Department of Agriculture, Science and Education Administration, Western Regional Research Center, Berkeley, CA 94710

and

M. E. DAXENBICHLER

US Department of Agriculture, Science and Education Administration, Northern Regional Research Center, Peoria, IL 61604, USA

(Received 20 February 1980)

Abstract—2S-1-Cyano-2-hydroxy-3,4-epithiobutanies (erythro and threo) isolated from the seed of Crambe abyssinica, were fed to groups of six weanling rats at levels of 0.75, 150 or 300 ppm in the diet for 90 days. The higher dose groups showed poor growth and increased serum levels of alkaline phosphatase, total bilirubin, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and ornithine carbamyl transferase. The severity and occurrence of renal and hepatic lesions were dose-dependent. Renal alterations consisted of hypertrophy of proximal tubular epithelial cells with prominent karyomegaly. Hepatic lesions consisted primarily of megalocytosis of the hepatocytes and bile-duct hyperplasia with disruption of the normal hepatic architecture. Half of the rats in the high-dose group had karyomegaly of pancreatic acinar cells.

INTRODUCTION

Cruciferous plants, which are commonly cultivated for use as vegetables, condiments, oilseeds and forage, contain glucosinolates (GS) that are enzymatically hydrolysed to a number of biologically active nitriles or isothiocyanates. It has been shown that nitrile formation is more likely to occur during autolysis of the crushed, wet, natural plant material (VanEtten & Daxenbichler, 1971; VanEtten, Daxenbichler, Peters & Tookey, 1966; VanEtten & Tookey, 1979). Epiprogoitrin (5S-2-hydroxy-3-butenyl-GS), the major GS of crambe seed, may be hydrolysed in defatted meal by endogenous enzymes to yield goitrin (R-5-vinyloxazolidine-2-thione) which suppresses thyroidal iodine uptake and causes thyroid hyperplasia and hypertrophy (Greer, 1962). Alternatively, during autolysis of crushed, recently harvested seed below 45°C, the hydrolysis of epiprogoitrin preferentially follows a pathway that results in the formation of three nitriles, S-1-cyano-2-hydroxy-3-butene and two diastereomic 2S-1-cyano-2-hydroxy-3,4-epithiobutanies (CHEB; Daxenbichler, VanEtten & Wolff, 1968). The conditions for the formation of these substances have been defined and the biological effects of the autolysed meal and of the nitrile mixture isolated from autolysed meal have been briefly described (VanEtten, Gagne, Robbins, Booth, Daxenbichler & Wolff, 1969). This study was undertaken to determine the pathological effects of feeding two of these purified nitriles, erythro- and threo-CHEB at various concentrations to young rats for 90 days.

EXPERIMENTAL

Seed of Crambe abyssinica Hochst ex R. E. Fries was stored at 5°C until it was used. The seed was dehulled, flaked, and defatted by percolation with pentane-hexane (b.p. 33-57°C) at room temperature. The preparation of a typical batch of CHEB was as follows. A total volume of 400 ml water was added, in 50-100-ml aliquots, to 250 g defatted seed meal. After each addition, the mixture was quickly blended with a large spatula. After all of the water had been added, the wet meal was allowed to autolyse for a further 20 min. Sodium chloride (200 g) was blended into the slurry, followed by 3 litres dichloromethane. The resultant mixture was intermittently stirred by hand for 15 min and then the solids were removed by filtration through paper with the aid of Celite filter-aid. The solids were then extracted twice with 2 litres dichloromethane. The combined dichloromethane extracts were concentrated to 250 ml under vacuum at 45°C. A 0.5-ml aliquot was analysed by gas-liquid chromatography (GLC) as previously described (Daxenbichler & VanEtten, 1977) and it was found that the total dichloromethane extract of 250 g seed meal typically contained 1.4 g threo-CHEB, 1.6 g erythro-CHEB, 0.08 g 1-cyano-2-hydroxy-3-butene and 0.0 g 5-vinyloxazolidine-2-thione.

*Present address: College of Veterinary Medicine and Biomedical Sciences, Department of Pathology, Colorado State University, Fort Collins, Colorado 80523, USA.
These combined extracts were again reduced to a syrup and dissolved in 40 ml water. After centrifugation at 1500 g for 15 min to remove traces of solid material, the aqueous solution was applied to a 5 × 100 cm column of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) using water as the eluent. The sample was allowed to enter the Sephadex bed by gravity flow. Fractions were collected at 30-min intervals (flow rate 53 ml/hr). The fractions were qualitatively analysed by thin-layer chromatography (TLC) on Precast Silica Gel 60 F-254 (EM Laboratories, Inc., Elmsford, NY) plates. Ether–hexane (3:1, v/v) was used as the developing solvent and charring with H_2SO_4-dichromate was used for detection. Fractions 1–23 made up the column void volume (610 ml). Fractions 51–59 contained l-cyano-2-hydroxy-3-butene, fractions 63–70 contained erythro-CHEB. The appropriate fractions were combined and extracted four times with twice their volume of dichloromethane. After evaporation of the solvent, 44 parts three-CHEB were combined with 56 parts erythro-CHEB for use in the rat feeding studies. The only significant impurity that could be detected in the CHEB preparation by TLC and GLC analysis was a minor amount (about 1%) of 1-cyano-2-hydroxy-3-butene. Because the CHEB mixture was unstable and tended to polymerize when neat, it was stored in acetic solution at 0°C until it was incorporated into the feed.

Weanling male Sprague–Dawley rats, obtained from Simonsen Laboratories, Gilroy, CA, were fed a basal diet with the following composition: corn meal, 50%; soybean meal, 30%; casein, 7%; corn oil (Mazola), 5%; Bernhart and Tomarelli salt mixture (United States Biochemical Corp., Cleveland, OH), 4%. Vitamin diet fortification mixture (ICN, Cleveland, OH), 2.2%; corn starch, 1.65%; dt-methionine (ICN), 0.15%. The rats were housed three to a cage and were given feed and water ad lib. The animal room was maintained at 73°F and 50% relative humidity with equal periods of light and darkness.

Groups of six rats were fed a diet containing 0 (control), 75, 150 or 300 mg CHEB/kg. To prepare the test diets, the CHEB, dissolved in reagent-grade acetone, was thoroughly mixed into the basal diet to give a premix containing 900 mg/kg. After drying, the premix was appropriately diluted with additional basal diet. The diets were prepared at approximately 1-month intervals, placed in feed containers with screw caps and stored at 10°C until needed. Feed containers in the animal cages were replaced weekly. A check of the stability of the CHEB in the diet showed 100% recovery for at least 1 month when the diet was stored at −10°F. At room temperature, the recovery was 65% after 1 wk. Thereafter, the rate of loss decreased and recovery was 59% after 2 wk.

The rats were observed daily. Weight and feed intake were determined at approximately 1-wk intervals. After 90 days the rats were killed under ether anaesthesia by exsanguination via the brachial artery, and using potassium oxalate-treated syringes blood samples were obtained from the axillary space. Determinations on blood included total protein, leukocyte and platelet counts (Coulter Counter Model ZBI, Coulter Electronics, Hialeah, FL), packed cell volume, haemoglobin concentration, and differential leucocyte counts. Plasma levels of glucose, albumin, total protein, alkaline phosphatase, urea nitrogen, glutamic-oxaloacetic transaminase, glutamic–pyruvic transaminase, ornithine carbamyl transferase and total bilirubin were determined using a Technicon Auto-Analyser-II (Technicon Corp., Tarrytown, NY). Plasma sodium and potassium were determined using a Klina Flame Spectrophotometer (Beckman Instruments, Fullerton, CA). Analysis was carried out on samples of urine collected in the last week of the test. Occult blood, ketones, glucose, protein, bilirubin, urobilinogen and pH were estimated using Multistix (Ames Company, Elkhart, IN). Colour, specific gravity and the presence of urinary sediments were also recorded.

Complete autopsies were carried out and tissues were fixed in buffered neutral formalin (BNF) and 4% formaldehyde–1% glutaraldehyde (GF) solution (McDowell & Trump, 1977). In both the control group and the 300-ppm group two animals were fixed by intracardiac perfusion with GF. The bladders and lungs of all of the animals were inflated with BNF. The adrenal glands, thyroid glands, heart, liver, kidney, spleen, testes and brain were blotted and weighed before immersion in the fixative. The following tissues were embedded in paraffin, sectioned at 6 μm, stained with haematoxylin and eosin, and examined microscopically: adrenal glands, aorta, auditory sebaceous gland, sphenoid bone marrow (decalcified with RDO) obtained from DuPage Kinetic Labs, Inc., Downers Grove, IL, brain and thoracic spinal cord, colon, duodenum, epididymis, oesophagus, eyes, heart, ileum, jejunum, kidney, liver, lung, lymph node (mesenteric, renal, anterior mediastinal and retropharyngeal), mammary gland, nerve (sciatric, pancreas, parathyroid, pituitary, prostate, salivary gland, seminal vesicles, skeletal muscle (semimembranosus), skin, spleen, stomach, testes, thyroid, thymus, tongue, trachea, and urinary bladder. Perl’s, Van Gieson and Wilder stains (Luna, 1968) were also used on some of the paraffin-embedded tissue sections. GF-fixed liver and kidney tissue from animals treated with 0 or 300 ppm CHEB was embedded in Epon 812 (Shell Chemical Co., New York), cut at 1 μm with glass knives, and stained with toluidine blue. Impression smears were prepared from femoral bone marrow and enlarged lymph nodes and were stained with Giemsa stain.

Measurements on blood and urine, body weights and organ weights were analysed by one-way analysis of variance. Means were compared by Duncan’s multiple range test (Duncan, 1955). Log transformed data were used whenever the within-group variation was proportional to the arithmetic mean and if the transformation resulted in greater homogeneity of variance among groups. Where appropriate, other data were analysed by Fisher’s Exact Test for 2 × 2 tables (Bliss, 1967).

RESULTS

Mean body weights were inversely related to the concluded erythro-CHEB in the diet throughout the course of the study. In rats fed 150 or 300 ppm CHEB, mean body weights were always significantly
Lesions in rats fed glucosinolate products

less than those of the controls. In the 75-ppm group, mean body weights were significantly less than those of the controls only between days 44 and 64. Feed consumption generally reflected growth, with significantly decreased values occurring in the 300-ppm group throughout the study.

Despite the decreased feed intake, the overall mean daily dose of CHEB ingested per kg body weight was proportional to the concentration of CHEB in the diet and was 5.4, 10.6 and 21.9 mg/kg body weight/day for the 75-, 150- and 300-ppm test groups, respectively, while the mean total amount of CHEB ingested per rat was 124, 227 and 269 mg, respectively. One rat out of the six given 300 ppm CHEB in the diet gradually lost weight after 44 days of treatment. became moribund and was killed and autopsied on day 73.

Studies of blood samples taken at the end of the test showed a statistically significant decrease in circulating erythrocytes in the 150- and 300-ppm groups, along with small, significant increases in erythrocyte haemoglobin content. Packed cell volume was inversely related to CHEB concentration and was significantly decreased in the 300-ppm group. Platelet counts appeared to increase with increasing CHEB in the diet; however, differences among group means were not statistically significant. A slight elevation (not significant) in the leucocyte count for the 300-ppm group was accompanied by a statistically significant shift ($P < 0.01$) in the relative numbers of lymphocytes to neutrophils. Compared to control values, lymphocytes were decreased while neutrophils were increased. In addition, the absolute number of eosinophils was significantly elevated ($P < 0.01$) in this group. There were numerous significant differences in plasma- and serum-constituent concentrations between the control rats and those fed 300 ppm CHEB. In the latter group, glucose, albumin, albumin:globulin ratio and urea nitrogen were all decreased, whereas alkaline phosphatase, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, ornithine carbamyl transferase and total bilirubin were increased. There were no significant differences in the concentrations of plasma and serum constituents between the rats fed the two lower CHEB levels and the controls.

Urine taken during the last week of the study from rats fed 300 ppm CHEB contained small amounts of blood. The incidence of blood in the urine of this group was significantly increased ($P < 0.005$. Fisher's exact test) compared with the controls. Similarly, glucose was also present in significant ($P < 0.005$) amounts in the urine of the 300-ppm group (at levels estimated to be 1-1.5 g/100 ml). Other urinary factors were not noteworthy.

In rats given 300 ppm CHEB, there were statistically significant decreases in all absolute organ weights. In the other two groups of rats fed CHEB, only heart weight in the 150-ppm group and adrenals weight in the 75-ppm group were significantly lower than control values. These differences in absolute organ weights were considered to be largely related to changes in body weight rather than to any specific effects of CHEB treatment. Similarly, relative organ weights (expressed as a simple ratio to body weight) did not indicate any specific effects of CHEB; they generally increased with increasing concentration of CHEB in the diet and were all (including relative brain weight) significantly elevated in the 300-ppm group. An allometric adjustment of organ weights to a common body weight using growth constants (Trieb, Pappritz & Lützen, 1976) helped to remove the influence of body weight. With this approach, the weights of kidneys, heart, thyroid, and brain were essentially uniform throughout all of the treated groups and were not significantly different from control values. However, allometrically adjusted weights of the liver and the spleen, and particularly of the testes, showed statistically significant increases apparently in response to dietary CHEB. Adrenals weight in the low-dose group (75 ppm) was less than that of the control and high-dose (300-ppm) groups.

Apart from the alterations in body condition associated with poor growth, gross lesions were limited to the livers of the CHEB-fed animals and the testes of animals in two of the groups. The affected livers were firmer and had irregular capsular surfaces. One animal in the 300-ppm group had bilateral diminution of testicular size with softening, while one control animal had unilateral testicular discoloration and softening. Histological lesions associated with CHEB ingestion occurred in the liver, kidney and pancreas (Table 1). Livers of rats in the 300-ppm group were severely affected and were characterized by varying combinations of bile-duct hyperplasia, fibrosis, megaocytosis, disruption of lobular architecture and individual hepatocyte necrosis. The bile-duct hyperplasia usually involved portal areas, although the hyperplasia in well developed lesions involved regions as large as several hepatic lobules. The ducts, usually lined by simple cuboidal epithelium, were often surrounded by dense fibrous connective tissue lightly

<table>
<thead>
<tr>
<th>Dose of CHEB (ppm)</th>
<th>No. of rats with lesions of the</th>
<th>Liver</th>
<th>Kidney (nephrocytomegaly)</th>
<th>Pancreas (acinar cell karyomegaly)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>75</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>150</td>
<td>5</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

CHEB = erythro- and three-2S,1-cyano-2-hydroxy-3,4-epithiobutane

*There were six rats in each treatment group. One rat in the 300-ppm group became moribund and was killed on day 73.
infiltrated with lymphocytes and macrophages, the latter occasionally bearing haemosiderin. In the large areas of bile-duct proliferation, the lumina tended to be larger and the epithelial lining flattened, presenting a pseudovascular pattern at low magnifications (Fig. 1a). These larger areas of biliary hyperplasia were frequently present in subcapsular locations. Tracts of proliferating biliary tissue, sometimes appearing as less differentiated structures without lumina, were composed of cells with oval, vesicular nuclei and often extended from portal to central areas resulting in the formation of pseudolobules and further architectural disruption (Fig. 1b).

Megalocytic hepatocytes occurred primarily in periportal areas, although in severely affected livers the distribution was more diffuse. The megalocytes, some of which were up to 30–40 μm in diameter, had homogeneous, basophilic cytoplasm, and enlarged, vesicular nuclei up to 20 μm in diameter with multiple, large nucleoli (Fig. 1c). Border zones between adjacent megalocytes were frequently quite distinct due to a reduced stain affinity of peripheral cytoplasm. Individually necrotic hepatocytes, which were never numerous, tended to occur in portal areas and occasionally mitotic figures were also seen.

Bile-duct hyperplasia, limited to portal areas, was the major alteration in the less severely affected livers. In a few areas of the less affected livers, megalocytosis seemed to predominate over the bile-duct lesion.

The renal lesion was characterized by well developed cytomegaly, consisting of both nuclear and cytoplasmic enlargement of proximal tubular epithelial cells throughout the cortex and outer medullary stripe (Fig. 2a). The most striking aspect of the alteration was karyomegaly, with some nuclear diameters as large as 20 μm. In the control group, proximal tubular epithelial nuclei were approximately 7–9 μm in diameter (Fig. 2b), whereas in affected animals nuclear diameters in excess of 9 μm were common, with most falling in the range of 9–11 μm. Some of the large nuclei had eosinophilic 'inclusion bodies' distinctly characteristic of cytoplasmic invaginations. Other morphological characteristics of the kidney appeared normal.

Pancreatic alterations were seen in half of the high-dose rats and consisted of karyomegaly of acinar cells (Table 1). While unaffected acinar cell nuclei were approximately 6–9 μm in diameter, the diameters of affected nuclei were consistently 9–11 μm and occasionally 14–15 μm.

Histologically, the abnormal tests detected grossly were characterized by degeneration of seminiferous epithelium and tubular atrophy. Because one animal bearing this testicular lesion was in the control group and the other was in the high-dose group, these lesions were considered incidental findings. The tests of all of the other animals were histologically normal.

**DISCUSSION**

Feeding young male rats CHEB for 90 days resulted in a number of dose-dependent pathological alterations. Liver and kidney lesions were present even at the 75-ppm dose level, and increased in severity and incidence at the 150- and 300-ppm levels. The kidney appeared to be more sensitive to the effects of CHEB since the fully developed lesion was present in both the 150- and 300-ppm groups. Associated with the presence of well developed liver lesions were elevations of serum alkaline phosphatase, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, ornithine carbamyl transferase and total bilirubin, indicating both hepatocellular damage and cholestasis. The reduced levels of blood glucose, albumin, and urea nitrogen, the altered albumin:globulin ratio, and the decreased erythrocyte and packed-cell-volume values in the high dose group were probably related to the low nutritional status of the animals. The reason for the increased corpuscular haemoglobin content is not evident. The haematuria and glucosuria observed in the high-dose group were probably not related to the nephrocytomegaly, since animals in the 75- and 150-ppm groups had nephrocytomegaly but no urinary alterations.

The renal and hepatic lesions present in these rats are remarkably similar to those seen in both aflatoxin and pyrrolizidine alkaloid toxicosis. Subacute aflatoxicosis in rats is characterized by biliary and oval cell proliferation, distortion of lobular architecture, and hepatocytic karyomegaly (Newberne & Butler, 1969). Renal tubular karyomegaly is produced by aflatoxin B1 (Butler, 1964) and to a greater extent by aflatoxin G1 (Butler & Lijinsky, 1970). Both agents produce liver tumours and aflatoxin G1 is a potent renal carcinogen than aflatoxin B1. Hepatocellular basophilic foci seen early in livers of aflatoxin-treated rats (Newberne, 1976), and thought by some to be significant with respect to liver tumour development (Newberne, 1976; Squire & Levitt, 1975), were not observed in rats fed CHEB. Chronic ingestion of pyrrolizidine alkaloids results in hepatic megalocytosis, bile-duct proliferation and fibrosis (Bull, Culvenors & Dick, 1968), and renal karyomegaly in intoxicated swine and rats. The pattern of the karyomegaly in rats varies with the specific alkaloid; some primarily affect proximal tubules while others affect distal tubules and glomeruli (McLean, 1970).

Other agents that cause renal tubular karyomegalic alterations include alkali-treated soya protein and lysinoalanine (Woodard & Short, 1977), S-dichlorovinyl-L-cysteine (Terracini & Parker, 1965), 4-fluoro-L-α-amino diphenyl (Mathews & Walpole, 1958), dimethylxanthine (Zak, Holzner, Singer & Popper, 1960), lead (Mathews & Walpole, 1958), certain gold compounds (Payne & Saunders, 1978), and the flame retardant, tris-(2,3-dibromopropyl) phosphate (Reznik, Ward, Hardisty & Russfield, 1979). The last five substances are renal carcinogens.

Karyomegaly of pancreatic acinar cells occurred in only half of the rats in the high-dose group and appeared to be an effect of CHEB. The nature of the lesion was unclear, but it was similar to the liver and kidney changes in that there appeared to be an alteration in nuclear regulatory processes. That the pancreatic acinar cell karyomegaly is related to the reduced nutritional status of the high-dose animals, and not directly to CHEB, is possible, but unlikely, since the usual alteration associated with nutritional deprivation is acinar cell atrophy (Jubb & Kennedy, 1970).

The glucosinolate hydrolysis product, goitrin, was not present in this diet, and thyroid lesions were not
Fig. 1. Liver sections from rats given 300 ppm erythro- and threo-2S-1-cyano-2-hydroxy-3,4-epithiobutanes showing: (a) a large area of bile-duct proliferation with flattened epithelium and dilated lumina; (b) the proliferation of small bile ducts from the portal area (top) into the lobule parenchyma; (c) megalocytic hepatocytes, fibroplasia and light mononuclear cell infiltration adjacent to the portal area—the centrallobular area (top) shows normal hepatocytes. Each section was stained with haematoxylin and eosin and the length of each bar represents 30 μm.
Fig. 2. Sections of the kidney of (a) a rat given 300 ppm erythro- and three-2S-cyano-2-hydroxy-3,4-epithiobutanes and (b) a control rat. Note the marked enlargement of the nuclei and cytoplasm in the kidneys of the treated rat compared with the size and uniformity of those of the control. Each section was stained with haematoxylin and eosin and the length of each bar represents 20 μm.
detected. It is not clear what relationship there is between the hepatic lesions described in this study and those that are found in poultry fed rapeseed products and which are presumed to be caused by hydrolysis products of glucosinolates. The lesions induced in the poultry were characterized by centrilobular fibrosis and hepatocellular degenerative changes (Umemura, Yamashiro, Bhatnagar, Moody & Slinger, 1977).

It should be noted that the lesions present in rats fed CHEB were similar in many respects to those that preceded tumour development in animals after exposure to a number of carcinogenic agents. However, the significance of the CHEB-induced lesions is unclear and knowledge of the consequences of these potentially widespread, naturally occurring substances awaits additional study.

Acknowledgements—We wish to thank Lu Rossi, Glenda Dugan, Claire Lash, MacDonald Calhoun, Dorothy Robbins and William Schroeder for their skillful technical assistance.

REFERENCES