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ROLE OF THE GASTROINTESTINAL MICROFLORA IN AMYGDALIN (LAETRILE)-INDUCED CYANIDE TOXICITY*

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Abstract—When conventional rats were given single oral doses of amygdalin (600 mg/kg), they sometimes experienced lethargy and convulsions, and usually died within 2 to 5 hr. Rats affected in this way had high concentrations of cyanide in their blood (2.6–4.5 μg/ml). Germfree rats receiving the same dose of amygdalin did not exhibit these symptoms and had blood cyanide concentrations (< 0.4 μg/ml) indistinguishable from those of conventional rats which did not receive amygdalin. After a non-toxic oral dose (50 mg), the recovery of amygdalin was higher in germfree than in conventional rats and only germfree rats had amygdalin in their feces. Furthermore, the intestinal contents of conventional rats, but not of germfree rats, catalyzed the release of benzaldehyde from amygdalin. It is concluded that the gastriointestinal flora are obligatory for the reactions which lead to the release of toxic amounts of cyanide from amygdalin (laetrile).

Amygdalin (mandelonitrile-β-D-glucosido-6-β-D-glucoside) is the major cyanogenic constituent of laetrile preparations [1,2], and is presumably the source of cyanide which is responsible for its toxicity [1,3,4]. In plant tissues, β-glucosidase-mediated hydrolysis of amygdalin yields glucose and mandelonitrile; the latter forms benzaldehyde and hydrocyanic acid either spontaneously or enzymically [5]. The fate of amygdalin and, hence, how cyanide is released has not been described in mammalian tissues. Cycasin (methylazoxymethanol-β-D-glucoside), another β-glucoside of plant origin, depends on the presence of the gastrointestinal flora for its metabolism and toxicity [6], and thus it seems possible that the release of cyanide from amygdalin *in vivo* may also require enzymes that are of bacterial rather than mammalian origin. If the release of cyanide from amygdalin were dependent on the flora, it would explain why mice are killed by relatively low oral doses of amygdalin and are resistant to much higher doses administered intraperitoneally [7].

This study compares the toxicity and metabolism of amygdalin when administered to germfree and conventional rats, and shows that neither cyanide release nor toxicity occurs in germfree rats.

MATERIALS AND METHODS

Materials. Amygdalin from apricot kernels (Lot 97C-74I0; approximately 99 per cent pure), β-glucosidase (EC 2.2.1.21) from almonds and mandelonitrile benzaldehyde-lyase (EC 4.1.2.10) from almonds were obtained from the Sigma Chemical Co. (St. Louis, MO).

Animals. Rats were of the Sprague–Dawley strain and weighed between 450 and 550 g. Conventional rats (Charles River Breeding Laboratories, Wilmington, MA) and germfree rats (Charles River Breeding Laboratories or the Smith and Phillips Germfree Supply Co., Salem, NH) were housed individually in metabolism cages (Acme Research Products, Cleveland, OH) which permitted separate collection of urine and feces. Germfree rats were maintained within a sterile isolator (Standard Safety Equipment, Palantine, IL) as described previously [8]. Animals were allowed chow (sterilizable 7-RF diet, Agway Inc., Syracuse, NY) and water *ad lib.*, and were maintained on a schedule of 12 hr of darkness and 12 hr of light.

Animal experiments. Freshly prepared aqueous solutions of amygdalin were administered to rats by gastric intubation. For metabolism studies, feces and urine were collected continuously for 72 hr and stored at −20° until analyzed. Fecal extracts were prepared by homogenizing the feces collected during 24 hr with 50 ml of water in a Sorvall Omni-Mixer (Sorvall Inc., Newtown, CT). The mixture was centrifuged at 31,000 *g* for 10 min and the supernatant solution retained for analysis.

In vitro *studies*. Cecal and stomach contents (weighing approximately 1 g) were removed from either a conventional rat or asceptically from a germfree rat and suspended by use of a Vortex Genie mixer (Fisher Scientific, Boston, MA) in 9 ml of sterile saline. Samples (0.1 ml) of this suspension were added to 10 ml of either Phenol Red Broth (BBL, Cockeysville, MD) or anaerobically sterilized Basal Medium–Peptone Yeast Extract [9], each containing amygdalin at a final concentration of 8 mg/ml. The reaction mixtures, and controls lacking the suspension of intestinal contents, were incubated at 37° either aerobically or anaerobically in an atmosphere

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of 10 per cent hydrogen: 5 per cent carbon dioxide: 85 per cent argon (Med-Tech Gases, Boston, MA) [8].

Measurement of amygdalin. Amygdalin was assayed in urine and fecal extracts by enzymic release of benzaldehyde which was determined by high pressure liquid chromatography (h.p.l.c.). Samples of urine (0.05–0.5 ml) or fecal extracts (0.5 ml) were incubated with β-glucosidase (7 units) and mandelonitrile lyase (5 units) in a total volume of 1.0 ml which contained 0.1 M sodium acetate buffer at pH 5.2. The reaction mixture was incubated at room temperature for 15 min and was then extracted with 1.0 ml of ether by shaking the mixture for 30 sec. A sample of the ether extract (10 μl) was injected onto a reverse-phase μBondapak C_{18} column (Waters Associates, Milford, MA) and eluted using a solvent system of water–acetonitrile (70:30). Benzaldehyde was quantified by its absorption at 254 nm. These assay conditions permitted stoichiometric recovery of benzaldehyde from solutions with concentrations of amygdalin up to 2 mg/ml. The addition of as little as 2 mg amygdalin to a 24 hr sample of urine or feces was assayed quantitatively by this method which is similar to that of Flora *et al.* [10]. It should be noted that this assay does not distinguish between amygdalin and mandelonitrile-β-glucoside. Mandelonitrile was quantified by the release of benzaldehyde when incubated as above except that β-glucosidase was omitted.

To determine the loss of amygdalin from bacterial incubations, samples (1.0 ml) of the reaction mixtures were filtered through a 0.22 μm filter (Millipore Corp., Bedford, MA), and 20 μl of the clear filtrate were injected onto a μBondapak/Carbohydrate column (Waters Associates) for h.p.l.c. Chromatography was effected by a linear gradient in a solvent system of 10 mM phosphate buffer at pH 7.0 and tetrahydrofuran which extended from 5 to 20 per cent (v/v) buffer during 7 min at a flow rate of 2.0 ml/min.

Measurement of hippuric acid. Samples of urine (5.0 ml) were acidified with 0.2 ml of 10 N HCl and extracted three times with 5.0 ml of ether. The combined ether extracts were concentrated to dryness under a stream of nitrogen. The residue was dissolved in 1.0 ml of anhydrous acetonitrile and again concentrated to dryness after which it was redissolved in 1.0 ml of diazomethane in ether [11] and allowed to stand for 15 min at 0°. Samples (10 μl) were analyzed by gas–liquid chromatography on an OV-17 column (Applied Science Laboratories Inc., State College, PA) maintained at 215°. Salicyluric acid (2.0 mg), added to each urine sample prior to acidification, served as an internal standard for the quantification of hippuric acid.

Measurement of cyanide and thiocyanate. Cyanide and thiocyanate concentrations were determined in whole blood and in urine according to the method of Bruce *et al.* [12] using a Gilford spectrophotometer (model 240).

RESULTS

When conventional rats were given a single oral dose of amygdalin (600 mg/kg), they became increasingly lethargic and experienced respiratory difficulty and convulsions. Death usually occurred within 2 to 5 hr. Germfree rats, on the other hand, did not exhibit any visible signs of toxicity after receiving the same dose of amygdalin. The condition of the rats at various times after administration of amygdalin was noted and the rats were killed to determine the concentration of cyanide in the blood. The results (Fig. 1) show that rats exhibiting signs of toxicity had high blood cyanide levels (between 2.6 and 4.5 μg/ml), while rats which were healthy even 4 hr after the administration of amygdalin had low or normal blood cyanide levels. Of particular interest is the observation that cyanide concentrations in germfree rats were less than 0.4 μg/ml and thus were indistinguishable from those of rats which had not received amygdalin.

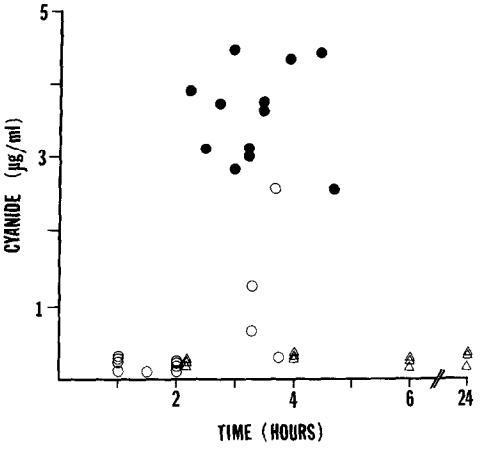

Fig. 1. Relationship between health status of rats and concentration of cyanide (CN^-) in blood at various times after the administration of amygdalin. Germfree (triangles) and conventional (circles) rats were given a single dose of amygdalin (600 mg/kg) by gavage. At the times indicated, their health status was noted. (Open circles denote healthy conventional rats while closed circles denote sick conventional rats.) No germ free rats were sick. The rats were then killed, and the concentration of cyanide in the blood was determined.

The higher blood cyanide in conventional rats after the ingestion of amygdalin might be explained by an obligatory role of the flora in the metabolism of amygdalin. Thus, the recovery of amygdalin in the urine and feces of germfree and conventional rats was compared. A 50 mg dose was used in order to avoid toxicity. As shown in Table 1, amygdalin was recovered in the feces of germfree but not of conventional rats. This observation, together with the significantly higher recovery of amygdalin from germfree rats, is compatible with a role for the flora in the metabolism of amygdalin.

If the metabolites of amygdalin, in addition to cyanide, could be recovered in conventional but not in germfree rats, it would be additional evidence in favor of the role of the flora in amygdalin metabolism. The data of Table 1 indicate that no more than an average of about 20 mg of the 50 mg dose of amygdalin is metabolized in conventional rats. This would be expected to yield approximately 16 mg of glucose, 4.5 mg of benzaldehyde and 1.1 mg of cyanide [1]. The release of glucose and cyanide

Table 1. Recovery of amygdalin after oral administration to conventional and germfree rats*

Type of rat	Amygdalin recovered (mg)		
	Urine	Feces	Total
Conventional	29.2 ± 8.9†	0.0	29.2 ± 8.9
Germfree	38.8 ± 7.4	2.6 ± 1.0	41.5 ± 7.0‡

* Six rats of each kind were given 50 mg amygdalin by gavage, and urines and feces were collected during 48 hr.
† Mean value ± S.D.
‡ Significantly different from the conventional group ($P < 0.05$).

Table 2. Metabolism of amygdalin *in vitro* by intestinal contents from conventional rats*

Intestinal contents	Recovery (%)	
	Amygdalin	Benzaldehyde
Cecum (aerobic)	62	10
(anaerobic)	100	0
Stomach (aerobic)	80	6
(anaerobic)	100	0

* Reaction mixtures containing either cecal or stomach contents were incubated together with amygdalin (8 mg/ml) for 24 hr under either aerobic or anaerobic conditions, as described in Materials and Methods.

(measured as thiocyanate) in these amounts cannot be detected above normal background measurements. Benzaldehyde is converted to benzoic acid which is excreted as hippuric acid in the rat [13]. A single oral dose of at least 5 mg of benzaldehyde must be administered to see a significant elevation of urinary hippuric acid above the levels found in rats on the chow diet.

Table 2 shows that amygdalin is lost and benzaldehyde is released in aerobic but not in anaerobic incubations with either cecal or stomach contents of the conventional rat. With stomach or intestinal contents of germfree rats, the recovery of amygdalin is complete and no benzaldehyde is formed. The poor recovery of benzaldehyde in terms of the amygdalin metabolized is explained by the observation that only 30 per cent of benzaldehyde added to cecal contents is recovered under these conditions. Cyanide release is not detected in the experiments of Table 2 because the method of Bruce *et al.* cannot detect cyanide when it is bound to cysteine and iron salts which are present in the bacterial medium [14].

The capacity of the flora of the stomach and the cecum to metabolize amygdalin, together with the smaller recovery of amygdalin in conventional rats, supports the hypothesis of an obligatory role for the flora in the metabolism of amygdalin. It is puzzling, however, that amygdalin is not metabolized under anaerobic conditions, since this is the physiological environment under which most reactions attributed to the flora occur [15].

An alternative explanation for the high concentrations of cyanide observed only in conventional rats is that the conventional rat may have a decreased ability to detoxify cyanide by converting it to thiocyanate. This possibility is excluded, however, by measurements of blood thiocyanate concentrations (Table 3). Thiocyanate concentrations, like those of cyanide, remain normal in germfree rats exposed to amygdalin. In conventional rats, however, the concentration of thiocyanate is elevated and tends to correlate with that of cyanide. There is no evidence that pathways known to detoxify cyanide are more active in germfree rats.

Mandelonitrile is unstable and is converted spontaneously to benzaldehyde [5]. We were unable to demonstrate an enhancement of the rate of this reaction *in vitro* with rat cecal contents.

DISCUSSION

The absence of either significant toxicity or cyanide release when amygdalin is administered to germfree rats in doses which are lethal to the conventional rat suggests that cyanide release is dependent on the presence of the gastrointestinal flora. Most likely the flora is obligatory for cleavage of the β-glycosidic bonds which release the aglycone, mandelonitrile. β-Glucosidase activity is present in cecal contents of the conventional rat as well as in several strains of bacteria which are indigenous to the gastrointestinal tract. Indeed, hydrolysis of amygdalin forms the basis of a biochemical diagnostic test for the identification of several such strains [9].

Table 3. Concentrations of cyanide (CN^-) and thiocyanate (SCN^-) in blood of conventional and germfree rats given amygdalin*

Rat	Health status	N†	Cyanide (μg/ml blood)	Thiocyanate (μg/ml blood)
Germfree controls	Healthy	6	0.13 ± 0.07‡	5.10 ± 1.24
Germfree	Healthy	12	0.27 ± 0.08	6.54 ± 2.18
Conventional	Healthy	13	0.50 ± 0.70	4.69 ± 1.29
Conventional	Sick	12	3.59 ± 0.66	15.85 ± 8.94

* All rats except those referred to as controls were given a single dose of amygdalin (600 mg/kg) by gavage. Rats were killed at various times between 1 and 24 hr. After recording their health status, the concentrations of cyanide and thiocyanate in the blood were determined.
† Number of rats in the group.
‡ Mean value ± S.D.

Although the intestine of the rat also contains β-glucosidase activity [16], it is unlikely that this activity contributes significantly to the *in vivo* metabolism of amygdalin. This is not unexpected, however, since methylazoxymethanol, the carcinogenic aglycone of the related β-D-glucoside, cycasin, is not released in the germfree rat. Thus, cycasin is metabolized and is carcinogenic for the conventional rat whereas it is neither metabolized nor is it carcinogenic for the germfree rat [6,17].

When administered parenterally to man, amygdalin is excreted largely unchanged in the urine [18]. It is of interest, therefore, that cases of laetrile-induced cyanide toxicity have occurred after enteral administration of laetrile [1,19], even though much higher doses are routinely administered intravenously. A recent report, for example, cites a case of acute cyanide toxicity which occurred when the customary dose of laetrile was given in the form of an enema rather than intravenously [20]. Thus, routes of administration which provide the most direct contact of the cyanide-containing glycoside with the gastrointestinal flora appear to maximize the possibility of cyanide release and subsequent toxicity.

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