Biochemical Pharmacology Vol 35, No 13, pp 2123–2128, 1986
Printed in Great Britain

0006-2952/86 $3 00 + 0 00
Pergamon Journals Ltd

INTESTINAL FIRST PASS METABOLISM OF AMYGDALIN IN THE RAT *IN VITRO**

GERHARD J. STRUGALA†, ADALBERT G RAUWS‡ and REMBERT ELBERS†

† Walther-Straub-Institut fur Pharmakologie und Toxikologie, Med Fakultat der Ludwig-Maximilians-Universitat Munchen, Nussbaumstr 26, D-8000 Munchen 2, Federal Republic of Germany, ‡ National Institute of Public Health and Environmental Hygiene, P O Box 1, 3720 BA Bilthoven, The Netherlands

(*Received* 7 *October* 1985, *accepted* 11 *December* 1985)

Abstract—The intestinal first pass metabolism of amygdalin has been investigated in rat small intestine *in vitro* The results show that amygdalin is hydrolyzed to prunasin, essentially in the wall of the proximal jejunum This specific β(1–6)hydrolytic cleavage of the terminal glucose residue is pH-dependent and can be inhibited by glucono-δ-lactone, a potent inhibitor of the lysosomal β-glucosidase of the rat intestine No substrate competition between phloridzin and lactose vs amygdalin was noted None of the more common soluble β- or α-enzymatic activities of mammalian intestine (α-glucosidase, α-amylase) or mammalian liver (β-galactosidase, β-glucuronidase) were capable of catalyzing the hydrolysis of the terminal glucose from amygdalin at pH's 5.0, 7 0 or 9.0. Furthermore, no metabolic activity of isolated rat livers toward amygdalin and prunasin was observed within two hours of recirculating perfusion However, cecal contents of conventional rats, exhibited both amygdalin- and prunasin-hydrolyzing activities The resulting mandelonitrile dissociates spontaneously into cyanide and benzaldehyde Therefore, our findings indicate that metabolism of amygdalin to prunasin occurring in the proximal part of jejunum is apparently mediated by enzymatic β(1–6)glucosidase activity of the gut wall In contrast, the toxicity of amygdalin due to the release of cyanide obviously requires microbiological activities of the gut flora

Amygdalin is a substance with a long history as a poison and a medicine [1]. During the later years of the "Laetrile" controversy [2] interest has grown in the fate of amygdalin in the mammalian organism [3]. The metabolism of amygdalin may clearly have implications for its alleged anti-cancer activity and its toxicity [1, 4–6]. Although the issue has subsided, amygdalin remains an interesting compound exhibiting both the character of a biochemical constituent and a xenobiotic substance

Amygdalin itself is not absorbed in the gastrointestinal tract to an appreciable extent. However, it is considered to be hydrolyzed to another cyanogenic glycoside, prunasin [1] Prunasin contains one molecule of glucose less than amygdalin [7]. In this respect amygdalin appears to be processed by the gut in a way, analogous to the disaccharides [8]. The pharmacokinetic inferences underlying the hypothesis of this intestinal 'first pass' effect [1] had to be substantiated by direct experiments. Moreover the relation, or lack of relation, of this pathway with the direct toxication of amygdalin by total hydrolysis [4, 9–11] had to be investigated. Therefore the metabolism of amygdalin in rat small intestine *in vitro* has been studied

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats (body weight 220–260 g) from Versuchstierzucht Jautz (Kisslegg, F.R.G.) were used Food was withdrawn about 20 hr before the experiments; the rats had free access to water.

Chemicals and solutions. Amygdalin (D-mandelonitrile-β-D-gentiobioside), mol wt. 457.44 from apricot kernels (approximately 99% pure), prunasin (D-mandelonitrile-β-D-glucoside) mol wt. 295 3 prepared by hydrolysis of amygdalin (approximately 98% pure), β-lactose (4-*O*-β-D-galactopyranosyl-β-D-glucose), phloridzin (Phloretin-2-β-D-glucoside), glucono-δ-lactone, bacitracin, neomycin sulfate, tetracycline, β-galactoside (EC 3.2.1.23) from bovine liver, β-glucuronidase (EC 3.2.1.31) from *Helix pomatia* and bovine liver, β-glucosidase (EC 3.2.1 21) from almonds, α-glucosidase (EC 3.2.1.20) from brewers yeast and α-amylase (EC 3 2.1.1) from human saliva were obtained from Sigma Chemical Company (Munchen, F.R G.) All other reagents were analytical grade chemicals from Merck (Darmstadt, F.R.G.).

Experiments on isolated Tyrode-perfused intestinal segments Isolated Tyrode-perfused segments of rat small intestine were used according to the method of Fisher and Parsons [12] as modified by Richter and Strugala [13]. Briefly, jejunal segments were prepared starting from the flexura duodenojejunalis and ileal segments backwards from the valvula ileocoecalis The length of the segments were approximately 10 cm The luminal side of the segments was perfused with recirculating Tyrode's solution (50 ml) Circulation rate: 55–65 ml/min. Composition of Tyrode's solution: Na^+ 137 8, K^+ 2.7, Ca^{2+} 1.4, Mg^{2+} 0.5, Cl^- 143 4, HCO_3 11 9, phosphate 0.4, glucose 15.1 mmol/l. The perfusion solution was oxygenated with a gas mixture of containing 95% O_2 and 5% CO_2. The pH value of the gassed perfusion medium

* These results were presented, in parts, at several meetings of the German Pharmacological Society, September 1982, March 1984, March 1985 and were published in abstract form [26–28]

† To whom correspondence and offprint requests should be addressed

was measured initially in either jejunal and ileal segments (pH 7 2–7.4) and remained constant throughout the duration of the experiment (2 hr). The incubation temperature was 37°. At the beginning of the experiments amygdalin was added to the test solution to final concentrations at 25, 50, 100 and 200 mg/l (=55, 110, 220, 440 μmol/l) At the end of the experiments the absorbate from the intestinal segments was collected and weighed. The adhering fluid of the segments was removed by careful blotting on filter paper and the length as well as the wet weight of the intestinal segments was determined.

In order to determine the microbiological contributions to amygdalin metabolism, rats were intubated five times at 12 hr intervals with either an antibiotic mixture in 1% aqueous carboxymethylcarboxycellulose as vehicle or an equal volume (0.75 ml) of the vehicle alone. The antibiotic mixture consisted of 100 mg of neomycin sulfate, 50 mg bacitracin and 50 mg tetracycline in 0 75 ml 1% aqueous carboxymethyl-cellulose [14]. After pre-treatment of the animals the small intestinal segments were prepared and perfused as described above and the hydrolysis of amygdalin was measured

The number of viable bacteria was determined in the perfusion medium, the homogenate of the jejunal and ileal segments as well as in the absorbate of the antibiotic-treated and untreated animals. Samples of 0 5 ml were diluted with sterile isotonic saline to 10 ml and 0.2 ml thereof were further diluted with sterile isotonic saline to 100 ml which were quantitatively filtered through membrane filters SM 13806, 0.45 μ of a Nutrient Pad Set SM 14005 (Sartorius, Gottingen, F.R.G) After flushing three times with 50 ml of sterile isotonic saline the filters were applied onto the nutrient pad and incubated at 37° for seven days in a Petri dish containing 3 ml of sterile isotonic saline.

Experiments with mucosa homogenate of the rat small intestine Mucosal epithelium from the upper small intestine was scraped off and centrifugated at 10,000 *g* for 5 min. The supernatant was removed, the mucosal epithelium was weighed and homogenized by cavitation (Ultraturrax, Janke & Kunkel, Munchen, F.R G.). Samples of this homogenate were added to 2.5 ml buffer (mucosa : buffer ratio = 1:20 (w/v)) (acetate pH 5), phosphate (pH 7) or pyrophosphate (pH 9) all buffers 100 mmol/l and incubated at 37° for 120 min. The homogenate contained amygdalin at a concentration of 2 mmol/l with or without one of the following substrates or inhibitors· phloridzin, lactose or glucono-δ-lactone.

Experiments with purified enzymes and rat cecal contents. Amygdalin or prunasin at a concentration of 2 mmol/l was incubated in 2 ml buffer (acetate, pH 5), (phosphate, pH 7) (pyrophosphate, pH 9) at 37° for 120 min with either β-glucosidase (0 84 U/ml), α-glucosidase (1.2 U/ml), β-galactosidase (0 33 U/ml), β-glucuronidase (0 22 U/ml, from bovine liver and 4 U/ml, from *Helix pomatia*) or α-amylase (0.28 U/ml)

Cecal contents (weighing approximately 1 g) of a conventional rat was removed and suspended by use of an Ultraturrax (Janke & Kunkel, Munchen, F.R.G) in 4 ml Krebs–Henseleit buffer (pH 7) containing glucose (15 mmol/l) and ammoniumchloride (20 mmol/l) Amygdalin or prunasin was added to this suspension at a final concentration of 2 mmol/l The reaction mixtures, and controls lacking the suspension of the cecal contents, were incubated at 37° in a shaking bath for 24 hr

Liver perfusion. Recirculating, hemoglobin-free liver perfusion was performed according to the method of Scholz *et al* [15] The perfusion medium used was Krebs–Henseleit bicarbonate buffer pH 7 4 at 37°, equilibrated with moistened O_2/CO_2 (95%/5%, v/v), supplemented with 10 mmol/l glucose and perfused at a flow rate of 4 ml/min/g liver (wet weight) Following a 10 min initial control period amygdalin or prunasin was added to the incubation medium (100 ml) at a final concentration of 0 5 or 2 mmol/l and perfusion was continued for 120 min The viability of the preparation was monitored by measuring the rate of glycolysis and oxygen consumption At 5, 10, 15, 30, 60, 75, 90, 105 and 120 min samples were withdrawn and subjected to HPLC-analysis.

Determination of amygdalin and prunasin Amygdalin and prunasin were determined in fluid samples by a HPLC technique according to the method of Rauws *et al* [16] Protein was removed either by ultrafiltration or precipitation by addition of 0 33 N perchloric acid and centrifugated The clear supernatant were neutralized with KOH and after additional centrifugation samples (20 μl, diluted 10 or 100 times) were injected onto a reversed-phase C18 column (Fa Gynkotek, Munchen, F R G) and eluted using a solvent system of water acetonitrile (85 15; v/v), detection 215 nm, flow rate 1 0 ml/min. The retention times of amygdalin and prunasin were 4.0 ± 0 2 and 6 2 ± 0 4, respectively With both analytical procedures the recoveries of amygdalin as well as prunasin added to intestinal tissue homogenate amounted to more than 90% Results were not corrected for recovery The variation coefficient of the standards was 2 5% The detection limit, twice the noise level, was 0 2 mg/l In experiments with the emphasis on the detection of benzaldehyde a detection wavelength of 254 nm was used Retention time for benzaldehyde was 12 min, detection limit, twice the noise level, was 0 05 mg/l

Statistics The results are expressed as mean values of n experiments ± the standard error of the mean (S E.M.). The significance of differences was estimated using Student's *t*-test for unpaired observations [17]

RESULTS

The results of the presystemic biotransformation of amygdalin into prunasin by rat jejunum is shown in Fig 1 An appreciable transformation of amygdalin into prunasin by cleavage of a glycosidic bond could be observed in the proximal jejunum With increasing concentration of amygdalin in the perfusion medium the relative amount of prunasin formed expressed as % of dose, decreased significantly from 10% at the lowest (25 mg/l) to about 5% at the highest (200 mg/l) amygdalin concentration Within an incubation period of 120 min about 65–80% of the total amount (TA) of prunasin formed was found in the 50 ml volume of the per-

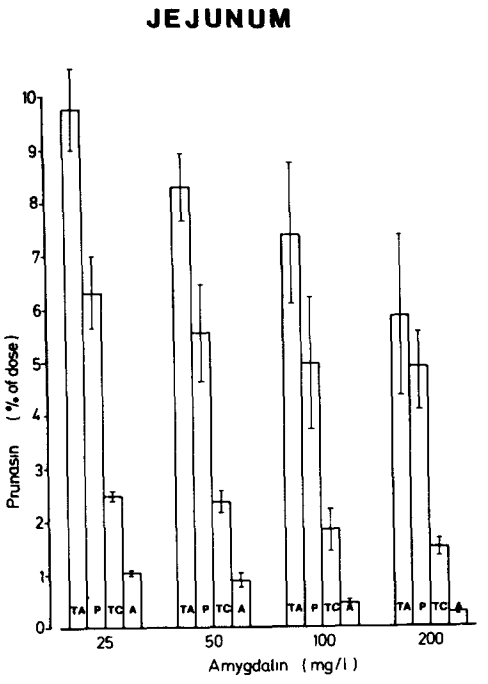

Fig 1 Hydrolysis of amygdalin into prunasin in isolated rat jejunum Amygdalin was initially present at 25–200 mg/l at the luminal side of isolated perfused segments of jejunum [13] Temperature, 37°, time, 120 min Ordinate formation of prunasin as expressed in % of amygdalin dose Each column represent the mean ± S E M of five to seven experiments TA, total amount, P, perfusate, TC, tissue content, A, absorbate

fusion medium (P), 25% in the intestinal tissue (TC), 5–10% in the absorbate (A). In the distal ileum only at higher amygdalin concentration (>100 mg/l) formation of prunasin could be observed accounting for approximately 1/10 that of the activity in the jejunum (not shown)

There were no indications, neither in the jejunum nor the ileum, for a further metabolism of prunasin, e.g. liberation of benzaldehyde, during the passage from the mucosa to the serosa.

In order to estimate the activity of the intestinal microbial flora in hydrolyzing the terminal glycosidic bond of amygdalin, the intestinal microflora was reduced by treating rats with an antibiotic mixture After pre-treatment of the animals isolated rat jejunum and ileum were reperfused for 2 hr with Tyrode's solution containing 100 mg/l amygdalin The results of these experiments are summarized in Fig. 2 It could be shown that the hydrolysis of amygdalin in the proximal jejunum treated with antibiotics was statistically significant reduced by about 50% as compared with the untreated controls. In contrast, in the distal ileum, where the microbial counts should be usually higher than in the proximal small intestine, no difference between antibiotic-treated and the control rats was found. Comparing the findings from experiments using the jejunum with those using the ileum no prunasin was detected in the perfusion fluid (P) of the ileum. The reason for this observation is unclear One plausible explanation, however, could be that the rate of hydrolytic cleavage of amygdalin is considerably lower than the rate of absorption for prunasin.

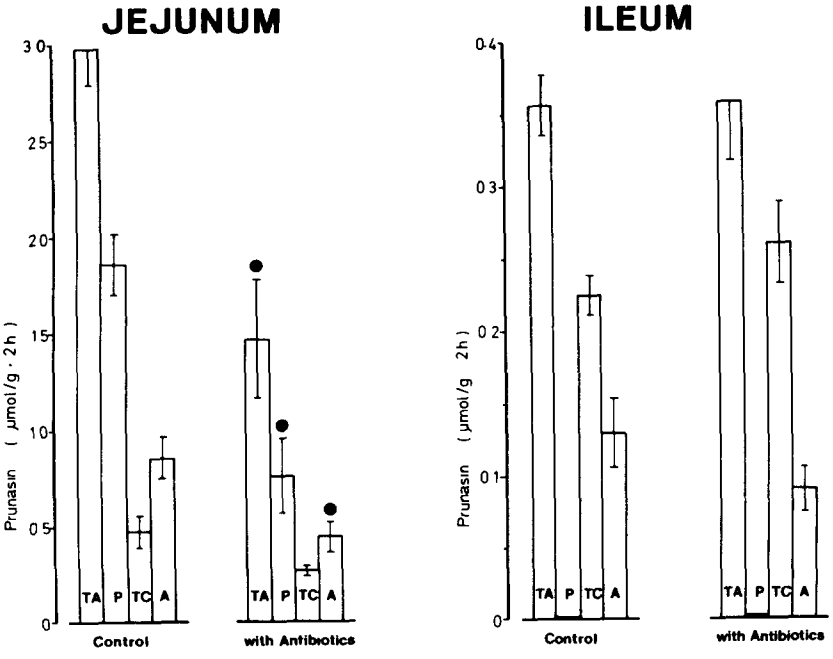

Fig 2 Effect of antibiotic-treatment on the hydrolysis of amygdalin in isolated rat jejunum and ileum Animals were intubated five times at 12-hr intervals with either an antibiotic mixture in 1% carboxymethylcellulose as vehicle or equal volume (0 75 ml) of the vehicle alone (control) After pre-treatment rats were prepared and jejunal and ileal segments were perfused with Tyrode's solution containing 100 mg/l amygdalin [13] Ordinates formation of prunasin within 2 hr in mmol/g tissue (wet weight) Columns represents mean ± S E M of five to six experiments TA, total amount, P, perfusate, TC, tissue content, A, absorbate ● $P \leq 0\ 05$ as compared with the control

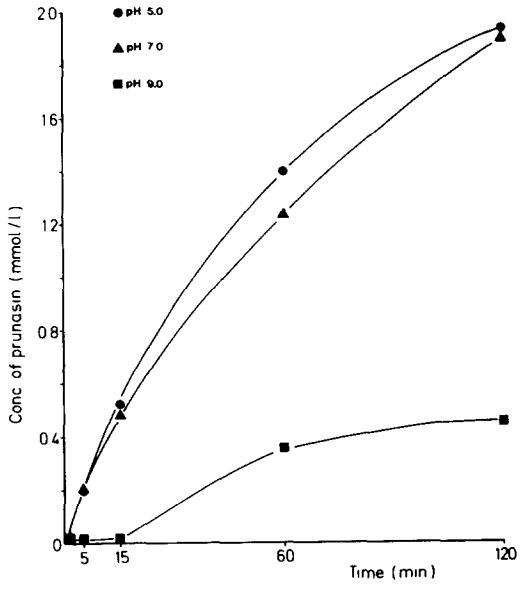

Fig 3 pH-Dependency of hydrolysis of amygdalin into prunasin Mucosal homogenates (mucosa-buffer-ratio 1 20, w/v) were incubated at pH 5, 7 and 9 for 120 min, temperature 37° Amygdalin concentration 2 mmol/l Ordinates concentration of prunasin formed (mmol/l), abscissa exposure time (min)

Interestingly, the number of intestinal bacteria in both jejunal and ileal segments was found to be very low (less than 100 counts/ml perfusion fluid) and did not differ from each other. Furthermore, the bacterial counts in treated and untreated animals were of the same order of magnitude

For a further elucidation of the hydrolysis of the terminal glucose moiety, experiments with mucosal homogenates from the upper small intestine were performed Since the pH-optimum for any putative enzyme of amygdalin or prunasin hydrolysis was unknown, experiments were carried out at pH 5, 7 and 9. When amygdalin (2 mmol/l) was added to the homogenate (mucosa.buffer-ratio 1 20, w/v) and incubated at 37° for 2 hr, formation of prunasin was found predominantly at acid and neutral pH (Fig 3). In the presence of glucono-δ-lactone (1 mmol/l), a potent inhibitor of the lysosomal β-glucosidase activity in the rat intestine [18], this hydrolysis was completely abolished under acidic conditions (pH 5) The inhibitory effect was less pronounced at pH 7 and was not present at pH 9, respectively (Fig 4) Under these conditions, the substrates, phloridzin and lactose at a 100-fold surplus did not affect amygdalin hydrolysis

In addition, experiments with purified β- or α-glucosidase activities of various origin were performed to elucidate whether one of the more common enzymes might be responsible for the specific β(1–6)hydrolysis of the terminal glucose moiety of amygdalin Table 1 shows that neither the examined intestinal (β-glucosidase, α-amylase) nor liver enzymes (β-galactosidase, β-glucuronidase) were capable of catalyzing the cleavage of the terminal glucose from amygdalin The data obtained with the cecal contents, however, exhibited always both amygdalin- and prunasin-hydrolyzing activities The resulting mandelonitrile dissociates spontaneously into cyanide and benzaldehyde

To investigate the ability of mammalian liver to further metabolize circulating prunasin or amygdalin, experiments with isolated perfused rat liver were performed Within an perfusion period of 2 hr neither the concentration of the two substrates examined (prunasin, amygdalin) decreased nor did any of the possible metabolites appear in the perfusate (not shown) As judged by the oxygen consumption, no sign of respiratory inhibition due to cyanide intoxication was observed

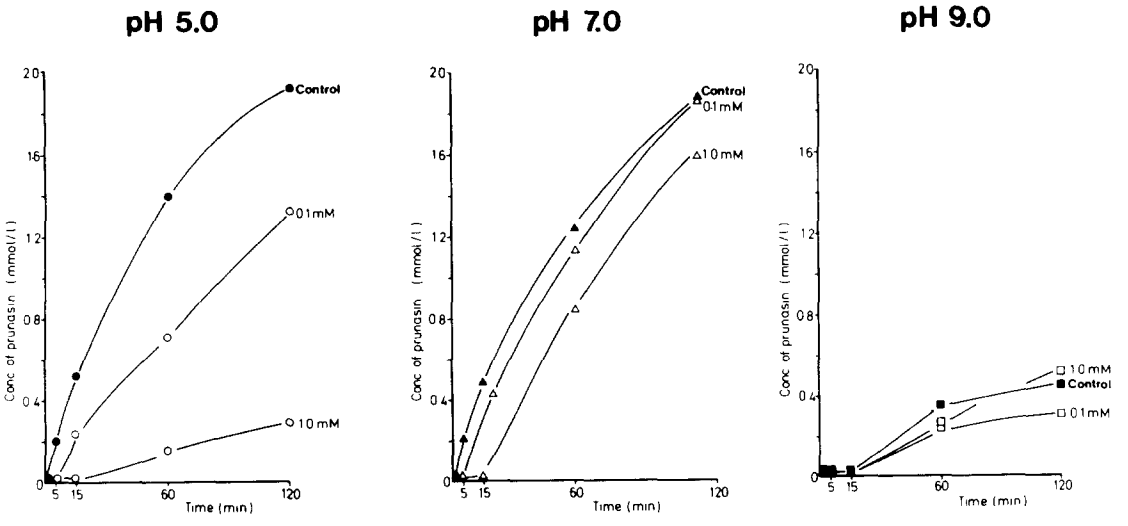

Fig 4 Effect of glucono-δ-lactone on the hydrolysis of amygdalin into prunasin Mucosal homogenates (mucosa–buffer ratio 1 20, w/v) were incubated at pH 5, 7 and 9 for 120 min with or without glucono-δ-lactone (0 1 or 1 mmol/l) as inhibitor Ordinates concentration of prunasin formed (mmol/l), abscissa exposure of time (min) Dark symbols denote formation of prunasin under control conditions at different pH s, open symbols denote the formation of prunasin in the presence of glucono-δ-lactone

Table 1 Incubation of amygdalin or prunasin with rat cecal contents as well as some common β- or α-enzymatic activities of various origin

Enzyme	Origin	Metabolites Prunasin	Benzaldehyde
β-glucosidase (EC 3 2 1 21)	almond	+	+ +
β-galactosidase (EC 3 2 1 23)	bovine liver	−	−
β-glucuronidase (EC 3 2 1 31)	bovine liver	−	−
β-glucuronidase (EC 3 2 1 31)	*Helix pomatia*	+	+ +
α-glucosidase (EC 3 2 1 20)	brewers yeast	−	−
α-amylase (EC 3 2 1 1)	human saliva	−	−
Cecal contents	rat	+	+ +

Amygdalin or prunasin was initially present at 2 mmol/l in 2 ml buffer (pH 5, 7 or 9) and incubated at 37° for 120 min with either one of the β- or α-activities Cecal contents were incubated at 37° for 24 hr in a Krebs–Henseleit buffer (pH 7 6) containing 15 mmol/l glucose and 20 mmol/l ammoniumchloride +, metabolite detected, −, no metabolite(s)

DISCUSSION

The intestinal 'first pass' metabolism of amygdalin into prunasin by cleavage of the terminal glucose residue, as inferred from pharmacokinetics results in the dog and excretion experiments in the rat [1], could be demonstrated mainly in the proximal rat jejunum This specific β(1–6)hydrolysis of amygdalin occurs predominantly at acidic and neutral pH and could be inhibited by glucono-δ-lactone, a potent inhibitor of the lysosomal β-glucosidases of rat intestine [18] In contrast, none of the more common β- or α-glucosidic activities of the mammalian intestine or liver were responsible for the amygdalin hydrolysis in the present study

Findings obtained with cecal contents, however, indicated both amygdalin- and prunasin-hydrolyzing activities The resulting mandelonitrile is unstable and will yield hydrocyanic acid and benzaldehyde [19]. Therefore, our results suggest that metabolism of amygdalin is obviously mediated by two different pathways (a) a 'first pass' metabolism of amygdalin to prunasin in the proximal part of the small intestine and (b) a total hydrolysis of amygdalin to glucose, benzaldehyde and cyanide by the microflora of the large intestine The latter pathway, microbial biodegradation, causes the well-known toxicity

These findings contrast, at least in part, with the observations of Carter *et al* [4] who concluded from experiments with conventional and germ-free rats receiving orally amygdalin that the gastrointestinal flora is obligatory for both metabolism and toxicity of amygdalin The reason for this discrepancy is that different analytical methods have been used in these two studies Carter *et al* [4] could not distinguish between amygdalin and its primary metabolite prunasin, as is done in the present study

Although pretreatment with antibiotics leads to a distinct reduction of amygdalin hydrolysis in rat jejunum (Fig 2), it appears unlikely that the gut flora contributes significantly to the biotransformation of amygdalin into prunasin. This is supported by the observation that in the distal ileum, where the microbial counts should be usually higher than in the upper part of the small intestine, no difference between antibiotic-treated and the untreated rats could be found (Fig. 2) Moreover, the number of intestinal bacteria in the whole small intestine was very low (less than 100 counts/ml perfusion solution) and was not different between treated and untreated animals. The inhibitory effect observed might be related to the properties of antibiotics (e g. neomycin) affecting the activities of intestinal enzymes as well as the absorption of naturally occurring compounds [20–22] The strongest argument against bacterial contribution to β(1–6)hydrolytic cleavage of the terminal glucose residue, however, is given by the fact that bacterial activities always produce a total hydrolysis of amygdalin to benzaldehyde and cyanide [4, 9–11] as is also shown in the present study

In this connection it should be mentioned that high levels of β-glucosidases have been demonstrated in rodent kidneys [23] as well as in the small intestinal mucosa of rat and human that should act on both amygdalin and prunasin [24]. Therefore, conversion of amygdalin into prunasin mediated by a β(1–6)glucosidase of the gut wall is more plausible

As indicated in this study no further metabolism of prunasin or amygdalin in isolated perfused rat liver was found. In particular, there was neither liberation of cyanide nor of benzaldehyde or its biotransformation products This finding agrees well with the observation that intravenous infusion of amygdalin produced neither cyanidemia nor signs of toxicity in humans and animals [1, 3, 5]

From our results and supported by other published results, the following picture emerges Orally administered amygdalin is hydrolyzed during absorption into prunasin by an intestinal 'first pass' effect The

prunasin produced is then excreted unchanged with the urine [1]. If glycosidic hydrolysis of amygdalin or absorption of prunasin is not complete in the jejunum, the substances will be totally hydrolyzed to cyanide and benzaldehyde in the colon. The absolute bioavailability of prunasin in dogs was found to be approximately 50% [6] which is much larger than that of amygdalin (2%) [1]. Moreover, there is evidence that prunasin is absorbed in rat jejunum not only by passive diffusion but also by a carrier-mediated transfer process [25]. That there is a difference between amygdalin and prunasin as far as concerns transport mechanisms through biological membranes is also suggested by the observation that prunasin is taken up by erythrocytes, whereas amygdalin is not (Rauws, unpublished results, 1985)

These findings may be less amazing if one realizes that amygdalin and prunasin are compounds which are glycosides of di- and monosaccharides, respectively It is well known that monosaccharides are readily absorbed by a specific transfer mechanism of the enterocytes, whereas disaccharides are only absorbed after hydrolysis by glucosidases at the brush border [8] Thus, our results show that the same mode of metabolism and subsequent absorption pertains to the cyanogenic glycoside amygdalin

Acknowledgement—The authors thank Mrs Margit Jourdan, Munich, for her skillful technical assistance

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