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The metabolism of sennosides A and B by the intestinal microflora: in vitro and in vivo studies on the rat and the mouse

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The glycoside laxatives sennosides A and B should be considered as inactive precursors in which the sugar moiety acts as a transport group (Fairbairn & Moss 1970). They are hydrolysed in the organism into their aglycones at least in part by the action of bacterial enzymes (Lemmens 1979) and these aglycones by influencing the water and electrolyte transport in the colon are responsible for the laxative action (Lemmens 1974, 1976).

The present report deals with the in vitro and in vivo metabolism by the rat and mouse microflora of sennosides A and B. The role of the bacteria in the metabolism of sennoside A has been established by a comparative study on conventional (CVL) and germ free (GF) animals.

Materials and methods

Compounds. Sennosides A and B were commercially available (C. Roth, Karlsruhe, Germany) chrom. depur. Sennidins A and B were prepared by hydrolysing the sennosides with sulphuric acid (Lemmens 1977). Rhein was prepared from aloin by the method of Bellaart (1952). Rhein-9-anthrone was prepared by reduction of rhein in acid medium according to Auterhoff & Scherff (1960). Rhein-1-monoglucoside was prepared according to Bellaart (1952).

The compounds were checked chromatographically for purity and purified if necessary.

In vitro incubation with rat caecal contents. All test solutions were protected from light. The test substance (0·5–1·5 mg) was dissolved in 0·5% sodium bicarbonate solution and sterilized by filtration (Millipore 0·2 μm). One ml of this solution was added to a test tube containing 10 ml of sterile Bacto Tryptone Yeast extract broth (ISP medium 1—Difco Laboratories, Michigan) in 0·1 M phosphate buffer pH 7·00.

Caeca were obtained from CVL or GF female Fisher rats. Both CVL and GF animals were maintained on a commercial pelleted diet (Hope Farms, The Netherlands) enriched with vitamins to compensate for loss during sterilization (autoclaving at 121 °C for 25 min). Caecal extracts of CVL rats were prepared by mixing the entire caecal contents with 10 ml of sterile phosphate buffer and centrifuging for about 1 min at low speed to remove debris. Caeca of GF rats were surgically removed in the GF isolator (Trexler plastic isolators, H. G. Kleinfeld, Hannover, Germany) and the watery contents collected in a sterile test tube. The tube was centrifuged and 1 ml of the supernatant was aseptically added to the test solution before incubation. Two control samples were prepared similarly except that either the caecal extract or the test compound was omitted.

The tubes were incubated anaerobically at 37 °C. Test samples were removed at regular intervals and were immediately frozen at −20 °C.

Extraction of incubates. After the incubates had thawed, the samples were examined quantitatively and qualitatively as follows: the contents of a test tube were extracted with three 10 ml portions of acidified, peroxide-free diisopropylether, to avoid oxidation of labile metabolites such as rhein-9-anthrone. The ether extracts were divided in two equal portions and evaporated to dryness under reduced pressure without heating. One portion of the evaporated extract was dissolved in 0·5 ml of a 0·1% solution of *p*-nitrosodimethylaniline in pyridine to form the azomethine derivative of rhein-9-anthrone which gives a blue green colour. This derivative is chromatographically more easily separated from rhein than rhein-9-anthrone itself. The second portion of the evaporated extract was dissolved in 0·5 ml ethanol for chromatography of the other aglycones. The remaining aqueous solution was used for quantitative determination (Lemli 1965) of the glycosides as a function of time. The total amount of anthracene derivatives present after incubation was quantitatively determined by the same method, but without previous extraction of the aglycones.

Chromatography. The pyridine and methanol solution, together with appropriate standards, were examined by thin layer chromatography on 0·25 mm thick layers of precoated silicagel 60 GF_{254} (Merck, Darmstadt, Ger-

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Table 1. Metabolism of sennosides A and B and related compounds by the rat intestinal microflora. Test substances incubated anaerobically with rat caecal extracts in 0·1 M phosphate buffer (pH 7·00) ISP medium for 72 h.

Compounds	Metabolites		
	CVL rats	GF rats	Controls
Sennoside A	Sennidins A and B, Rhein-9-anthrone, Small amounts of rhein	None	None
Sennoside B	Sennidins A and B, Rhein-9-anthrone, Small amounts of rhein	None	None
Sennidins A and B	Rhein-9-anthrone, Small amounts of rhein	Rhein	Rhein
Rhein-9-anthrone	Unchanged rhein-9-anthrone Small amounts of sennidins A and B rhein	Rhein Small amounts of sennidins A and B	Rhein Small amounts of sennidins A and B
Rhein	None	None	None
Rhein-1-monoglucoside	Rhein	None	None

many) using mobile phases and detection systems already described (Lemmens 1979).

In vivo administration to mice. Mice were preferred to rats for the in vivo experiments because of ease of handling for oral administration. CVL and GF male and female C3H mice, ca 30 g, were used. The CVL mice were kept under conventional conditions at constant temperature with 15 h exposure to light a day. GF mice were kept in Trexler plastic isolators (Trexler 1959). The cages were provided with a wire mesh to facilitate the collection of faeces on blotting paper which was renewed after each collection of faeces.

The animals were divided into groups of male or female mice, at least 2 mice per group. Before the experiment, they were accustomed to the oral administration of solutions. During the experiment, both CVL and GF mice were fed the sterilized diet as for rats. Before and during the assay, food and water were freely available. At the beginning of the test 2 mg of sennoside A dissolved in 0·5 ml of 0·5% sodium bicarbonate solution and filter sterilized, was administered to each mouse by stomach tube. Control animals received 0·5 ml of the sodium bicarbonate solution.

Analysis of the faeces. Faeces of each group were collected at 3, 6, 12, 24 and 48 h after oral administration of sennoside A. The individual faecal samples were visually examined for normality; if a laxative action occurred, faeces were wet and stained the blotting paper. The faeces of each group were pooled and immediately frozen. After freeze-drying the powder was stored at −20 °C.

Qualitative determination of the metabolites was by thin layer chromatography. To determine the glycosidic compounds, the faecal powder was extracted by boiling with ethanol 50% v/v. The presence of anthrone derivatives was investigated after extraction with a 0·1% solution of *p*-nitrosodimethylaniline in pyridine. The aglycones were obtained by extraction with acidified, peroxide free diisopropylether. After extraction and centrifugation the supernatant was chromatographed as described.

Results

The results of the in vitro experiments in which the various test compounds were incubated anaerobically with CVL or GF rat caecal contents are summarized in Table 1.

Incubation of sennoside A or B with rat caecum contents shows that only CVL rats are capable of metabolizing the glycosides. After 72 h of incubation, no unchanged sennoside A or B could be recovered. In incubates without previous extraction of the aglycones formed by bacterial metabolism the total amount of anthracene derivatives present corresponded to about 95% of the added sennoside. No hydrolysis or other transformation occurs in the sennoside A or B samples incubated with caecal extracts from GF rats. About 90% of the administered quantity could be recovered from the remaining watery phase. The test compounds do not undergo any change when incubated without caecal extracts and recovery of sennosides was 100%.

When incubated with caecal extracts from CVL rats, sennidins A and B are reduced largely to rhein-9-anthrone, although some rhein can be detected. In the GF and control incubates they are slowly oxidized to rhein; no rhein-9-anthrone is present.

When incubated with caecal contents from CVL rats, rhein-9-anthrone remains practically unchanged. With GF rats or in control incubates, only traces of rhein-9-anthrone are still present after 72 h of incubation as oxidation occurs.

After incubation of rhein with or without caecum contents only unchanged rhein can be detected, no metabolism taking place.

Incubation of rhein-1-monoglucoside gives rise to the formation of rhein. After 72 h of incubation 20% unchanged glucoside is still present in the CVL rats incubates, as hydrolysis of the glucoside occurs. The other incubates show no transformation.

The results of the in vivo administration of sennoside A to mice are in keeping with those of the in vitro experiments. Starting from 3 h after oral administration of sennoside A, mice voided wet faeces and the laxative effect

lasted 12 h. In contrast GF animals showed no signs of diarrhoea, even after 48 h. There was no laxative action in control animals receiving the solutions without sennoside A.

On qualitative analysis, no metabolites could be demonstrated in the faeces of GF mice, only unchanged sennoside A (about 10% of the administered dose) was excreted. With CVL animals, we obtained the same metabolites as those from incubation of sennoides with caecal contents from the CVL rats in vitro (i.e. sennidins A and B, rhein and rhein-9-anthrone). Unchanged sennoside A was also recovered from CVL faeces. The total amount of excreted sennoside A and metabolites was also only about 10% of the administered dose.

Discussion

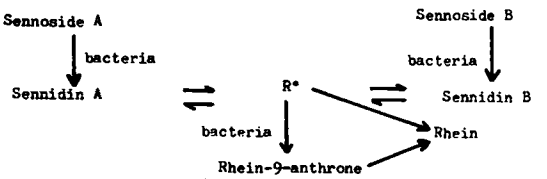
The results we have obtained on the metabolism of sennosides and their derivatives confirm earlier studies (Lemmens 1979; Lemmens & Dreessen 1979), but the major purpose of our experiments has been to establish the role of intestinal bacteria in the formation of the active compounds.

In GF animals no laxative action was observed and no metabolites were detected; these results demonstrate the importance of the microflora in producing active metabolites. The caecal flora extensively hydrolyses the sennosides into their corresponding sennidins; these dianthrone aglycones form free radicals when in solution (Lemli & Lemmens 1980) which can then be rearranged into dianthrones. This explains the equal amounts of sennidins A and B obtained after incubation of pure sennoside A or pure sennoside B respectively. Moreover the free radicals could be more easily hydrogenated than the dianthrones into rhein-9-anthrone by the intestinal microflora.

The role of the microorganisms in the oxidation of sennidin or rhein-9-anthrone to rhein is probably less important. Our experiments to find the relationship between the different steps in the metabolism of the sennosides point in that direction. The sennidins are readily reduced by intestinal bacteria to rhein-9-anthrone. These findings correspond with other results (Sasaki et al 1979). The amount of rhein formed in our incubation experiments is about the same in all incubates (CVL, GF or controls). Moreover rhein-9-anthrone remains practically unchanged in solutions with caecal extracts from CVL animals, but is oxidized to sennidin and rhein in solutions without bacteria. Therefore, the presence of rhein in the incubates is, at least in part, due to oxidation during manipulations of the test solutions or in the test solutions themselves.

The hydrolysis of the sennosides is complete after 72 h of incubation with bacteria. In vitro practically the total dose added is recovered from the incubates. Therefore, the microflora do not seem to be responsible for the formation of, as yet unknown, metabolites which would explain the loss of 90% of the sennosides after oral administration to GF and CVL rats. The same low recovery after oral administration of sennosides was observed by Lemmens (1979). Species or sexual variations do not seem to be limiting factors in the microbial transformations, as the same metabolites are detected in rats and mice without difference between male or female animals.

The various steps in the metabolism of sennoside A and B can be summarized as follows:



R* Free anthrone radicals.

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