β -Aspartylglycine, a Substance Unique to Caecal Contents of Germ-Free and Antibiotic-Treated Mice

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The caecal supernatants from germ-free, antibiotic-treated and control mice were compared with respect to their content of low-molecular-weight substances (less than 3500 mol.wt.). The supernatants contained about the same amount of free amino acids. After acid hydrolysis, the caecal supernatants of germ-free and antibiotic-treated mice showed a 2.9-fold increase in free amino acids, whereas a similar treatment of the supernatant from control mice resulted in a 2.6-fold increase. By gel filtration on Sephadex G-25, and high-voltage paper electrophoresis at pH 3.5 of the fractions eluted after the void volume, it was found that the caecal supernatants of germ-free and antibiotic-treated mice contained a substance more acidic than aspartic acid. Preparative high-voltage electrophoresis, dansylation, amino acid analysis and a specific colour reaction showed the substance to be β -aspartylglycine. After a minimal 36 h of treatment with neomycin and bacitracin, a high concentration of β -aspartylglycine was found, and no enterococci and aerobic Gram-negative rods could be cultured from the caecal contents. The possibility that in one mouse the appearance of β -aspartylglycine was related to a decrease in Gram-negative rods was ruled out by selective elimination of aerobic Gram-negative rods by using polymyxin B. This suggests that other bacteria concomitantly eliminated with the enterococci and aerobic Gram-negative rods, directly or indirectly, could play a role in the accumulation of β -aspartylglycine.

The supernatant of caecal contents from germ-free mice has been found to contain substances affecting smooth muscle tissue *in vitro* (Gordon, 1965, 1975). Caecal contents from conventional controls showed these activities to a much lower degree. Increased amounts of free amino acids observed in an 80% ethanol extract of germ-free caecal contents (Combe *et al.*, 1965) could be implicated in this phenomenon (Gordon & Bruckner, 1974; Kokas & Valentine, 1974). This observation has prompted us to investigate further the nature of the water-soluble low-molecular-weight substances in the caecal contents from germ-free mice, antibiotic-treated mice and conventional controls.

Experimental

Materials

α -L-Aspartylglycine and β -L-aspartylglycine were from Serva (Heidelberg, Germany). Dansyl chloride was from Fluka A.G. (Buchs, Switzerland). Xylene Cyanol FF was from Merck A.G. (Darmstadt, Germany). HCl (Aristar) and ϵ -Dnp-lysine were from BDH (Poole, Dorset, U.K.). Neomycin

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Dnp, dinitrophenyl.

sulphate and bacitracin [61 units/mg; 1 unit of bacitracin is contained in 0.01351 mg of the Second International Standard Preparation (1964) of Bacitracin Zinc, which contains 74 units/mg] were from Centrafarm (Rotterdam, The Netherlands). Polymyxin B sulphate was from Pfizer (Rotterdam, The Netherlands). Brain heart infusion broth and agar products were from Difco Laboratories (Detroit, MI, U.S.A.). Sephadex G-25 was a product of Pharmacia (Uppsala, Sweden). Whatman 3MM chromatography paper was from W. and R. Balston (Maidstone, Kent, U.K.). Spectrapor-3 membrane tubing was from Spectrum Medical Industries (Los Angeles, CA, U.S.A.), and polyamide layers were from Schleicher und Schüll (Dassel, Germany). All other reagents were analytical-grade products from Merck A.G.

Animals

Germ-free mice (ND2-strain) were kindly provided by REPGO-Institutes TNO, Rijswijk, The Netherlands. They were reared in plastic isolators under standard germ-free conditions and fed on AM pelleted diet (Hope Farms, Woerden, The Netherlands). Control and antibiotic-treated mice were non-inbred Swiss mice supplied by TNO (Zeist, The Netherlands).

Caecal supernatants

Batches of caecal contents were prepared by pooling the material from five to eight germ-free mice, 15–20 antibiotic-treated mice and 40–50 control animals. After homogenizing (Sorvall, Omnimixer), dry weight was determined at 90°C in a vacuum oven. Subsequently the batches were diluted to 15% dry weight and centrifuged at 27000g for 30min in a Sorvall RC-5 centrifuge. These batches were used for preparation of diffusates and subsequent gel filtration. Supernatants of caecal and faecal contents of individual mice were prepared by thoroughly mixing a sample of the caecal or faecal contents with water (670mg/ml of water) and subsequent centrifugation at about 2000g for 15min. These batches were used for high-voltage paper electrophoresis. The supernatants were stored at –25°C.

Isolation and analysis of low-molecular-weight substances

Dialysis was performed at 0–2°C with Spectrapor-3 dialysis tubing with a molecular-weight cut-off of 3500. Routinely 0.5ml of caecal supernatant was dialysed overnight against 50ml of demineralized water. The diffusate was freeze-dried and dissolved in 1.5ml of 0.1M-acetic acid; 1.0ml of this solution was freeze-dried and dissolved in 1.5ml of 0.1M-acetic acid; 1.0ml of this solution was applied to a column (0.9cm × 160cm) of Sephadex G-25 (fine) and eluted at a flow rate of 10ml/h with 0.1M-acetic acid; 2.3ml fractions were collected. A_{280} was measured in a Zeiss PMQ-II spectrophotometer. Samples of alternate fractions eluted after the void volume (V_0) were spotted on Whatman 3MM chromatography paper and subjected to high-voltage paper electrophoresis at pH3.5 (pyridine/acetic acid/water, 1:10:89, by vol.) for 60–90min at 3000V. A reference mixture was used containing all the common amino acids and the marker dyes, ϵ -Dnp-lysine and Xylene Cyanol FF. After drying at 70–80°C, the paper was sprayed with 0.2% ninhydrin in ethanol and heated at 70–80°C for 10–15min.

Preparative high-voltage paper electrophoresis at pH3.5 was carried out as described by Ambler (1963). For additional purification high-voltage paper electrophoresis at pH6.5 in pyridine/acetic acid/water (25:1:225, by vol.) was used. After detecting the amino acids and peptide-like material by treatment with ninhydrin, the paper was heated for 10min at 150°C. This treatment results in a clear blue colour for all β -aspartyl peptides (Buchanan *et al.*, 1962a). Determination of N-terminal amino acids was carried out as described by Gros & Labouesse (1969) by reaction with dansyl chloride in a mixture of equal volumes of acetone and 0.05M-NaHCO₃, pH10.0 (with 0.1M-NaOH), for

30min at 37°C. After drying under vacuum the dansylated derivatives were hydrolysed in 6M-HCl at about 110°C for 4–16h. After drying, identification of dansyl-amino acids was achieved by t.l.c. on polyamide layers (5cm × 5cm) (Woods & Wang, 1967) by using a standard mixture of dansyl-amino acids (Hartley, 1970). Amino acid analyses were performed with a Kontron Liquimat III amino acid analyser with norleucine as an internal standard. Samples were hydrolysed in 0.4ml of 6M-HCl at about 110°C in evacuated sealed glass tubes for 18–22h.

Antibiotic treatment

Female mice (non-inbred Swiss mice), 7 weeks of age, were treated with antibiotics. These were supplied *ad libitum* with drinking water containing 4mg of neomycin sulphate and 2mg of bacitracin/ml (28 mice were used), or 1mg of polymyxin B/ml (four mice were used).

Sampling programme

Supernatants obtained from the pooled caecal contents of five to eight germ-free mice, 15–20 antibiotic treated mice and 40–50 control mice were used for: (i) determination of the amino acid composition before and after acid hydrolysis; (ii) preparation of diffusates; (iii) gel filtration of diffusates and subsequent high-voltage paper electrophoresis; (iv) in some cases, preparative high-voltage paper electrophoresis, dansylation and amino acid analysis. In separate experiments during treatment with antibiotics, caecal and faecal contents of individual mice were investigated with respect to the presence of aerobic Gram-negative rods, enterococci and β -aspartylglycine. Some 20 mice were treated with neomycin and bacitracin and the caecal contents of four individual mice were analysed every 12h up to 48h. Faecal samples of eight mice that received the same antibiotic treatment and of eight control mice were investigated in groups of four in the same manner as the caecal content of each individual mouse. Four mice were treated with polymyxin and the bacterial concentration in their faecal contents was investigated every 24h. After 48h the caecal contents of each individual mouse were analysed as described above. In another experiment two control female mice, 7 weeks of age, received drinking water *ad libitum* containing 1mm- or 10mm- β -aspartylglycine. Faeces were sampled daily for 3 days and supernatants were subjected to high-voltage paper electrophoresis. Occasionally urine samples (10–15 μ l) were also subjected to high-voltage paper electrophoresis.

Culturing of bacteria

A 33.5mg sample of caecal contents or faeces was suspended in 0.5ml of brain/heart infusion broth. Subsequently serial dilutions were made in plastic trays with 64 cups of 1 ml capacity each. The trays were filled with 0.5ml of brain/heart infusion broth per cup. Eight dilution steps (1:10) were routinely carried out for each sample. After overnight incubation at 37°C, the cultures were subcultured on Endo-agar and aesculin-azide agar prepared as described by Sneath (1956) for identification of aerobic Gram-negative rods and enterococci respectively.

Results

Caecal supernatants were dialysed against demineralized water and the diffusate was freeze-dried. The water-soluble low-molecular-weight substances present in the diffusate were fractionated on Sephadex G-25 (fine) and the A_{280} elution patterns obtained with the diffusates from control and germ-free mice are shown in Figs. 1(a) and 1(b) respectively. Gel filtration of the diffusate from antibiotic-treated mice showed an intermediate pattern with similarities to both (not shown). Figs. 2(a) and 2(b) show the results of the electrophoresis of particular fractions eluted after the void volume (see corresponding arrows). One very acidic spot was present in the germ-free diffusate (see arrow in Fig. 2b) that was totally absent from the diffusate from control mice. The diffusate from antibiotic-treated mice also showed this extra spot.

This acidic material was purified and isolated by preparative paper electrophoresis at pH 3.5 and 6.5. After hydrolysis and subsequent amino acid analysis, equal amounts of aspartic acid and glycine were observed. Dansylation and subsequent hydrolysis showed aspartic acid to be the *N*-terminal amino acid. Without hydrolysis the substance was eluted ahead of aspartic acid from the column of the amino acid analyser with a molar absorbance after reaction with ninhydrin of about 50% of that of the constituent amino acids. When electrophoretograms of the substance were heated to 150°C after spraying with ninhydrin a clear blue spot resulted. A spot of the same colour was observed with this treatment, and migrating to the same position, after electrophoresis of commercial β -aspartylglycine. Also a weak yellowish brown spot was observed in the latter case, which is probably the cyclized form of β -aspartylglycine (Naughton *et al.*, 1960). Commercial α -aspartylglycine showed a purple spot less acidic than aspartic acid. These results clearly show the substance to be β -aspartylglycine.

When the 15% dry weight caecal supernatant from germ-free or antibiotic-treated mice was directly

Fig. 1. Gel filtration of the supernatant of caecal contents from (a) control and (b) germ-free mice on a Sephadex G-25 (fine) column (0.9 cm $\times$ 160 cm)

Elution was carried out with 0.1 M-acetic acid at a flow rate of 10 ml/h, 2.3 ml fractions being collected. $\circ$, A_{280} ; V_0 , void volume; V_1 , total volume.

investigated on the amino acid analyser the material running in the β -aspartylglycine position was not homogeneous. Two overlapping peaks were observed about equal in height. One of these was β -aspartylglycine, representing a concentration of about 4 μ mol/ml. When the caecal supernatant of control mice was analysed in the same manner, a small peak ran in the β -aspartylglycine position, containing one-twentieth the amount of ninhydrin-reacting material found with germ-free and antibiotic-treated mice. However, semi-quantitative data from high-voltage paper electrophoresis show that this peak was not β -aspartylglycine.

The amino acid compositions before and after acid hydrolysis of caecal supernatants from germ-free, antibiotic-treated and control mice are shown in Table 1.

Caecal contents of control mice showed a normal concentration of bacteria. After 12h of antibiotic treatment (neomycin/bacitracin), the number of enterococci was decreasing, while in one case there

Fig. 2. Analysis by paper electrophoresis at pH 3.5 of the fractions indicated between the arrows in Figs. 1(a) and 1(b). For details see the text. →, β -Aspartylglycine. The drawing shows the result of an electrophoresis of a reference mixture containing all amino acids and two marker dyes. ●, Yellow; ●, blue; a, Xylene Cyanol FF; b, aspartic acid; c, glutamic acid; d, ϵ -Dnp-lysine; e, neutral amino acids; f, basic amino acids.

Table 1. Amino acid composition before and after acid hydrolysis of caecal supernatants from control, germ-free and antibiotic-treated mice

Caecal contents were diluted to 15% dry weight and centrifuged. Supernatant amino acid concentrations were determined with an amino acid analyser before and after acid hydrolysis. See the Experimental section for details.

	Amino acid content ($\mu\text{mol/ml}$)					
	Control		Germ-free		Antibiotic-treated	
	Before	After	Before	After	Before	After
Asp	6.7	30.2	4.7	38.8	5.1	38.1
Thr	5.4	14.9	6.5	14.8	3.9	11.3
Ser	8.6	16.3	11.8	18.1	6.7	15.7
Glu	12.2	28.3	8.9	26.6	9.7	34.2
Pro	10.1	16.3	7.5	23.7	15.8	14.2
Gly	6.4	47.3	12.8	67.6	13.1	61.9
Ala	15.3	37.8	10.7	27.9	9.1	25.1
Val	7.0	15.7	6.5	11.0	5.1	8.7
Met	2.7	4.3	1.4	1.8	.8	1.7
Ile	5.6	10.1	4.0	7.5	2.6	7.1
Leu	8.9	18.1	6.6	14.0	4.1	13.6
Tyr	2.7	5.2	2.6	4.3	1.5	3.4
Phe	4.4	7.1	3.1	4.6	1.9	4.7
Lys	8.8	18.8	7.7	19.0	7.8	15.7
His	1.5	4.5	2.7	6.7	1.5	5.7
Arg	3.3	6.2	4.2	6.2	2.2	4.3

were no enterococci. The number of aerobic Gram-negative rods was not decreasing except for this one case. The caecal contents of this particular mouse

only contained a small amount of β -aspartylglycine. After 24 h of antibiotic treatment, the concentration of enterococci had not decreased further, except in one mouse in which no enterococci and aerobic Gram-negative rods were present. A rather intense spot of β -aspartylglycine was observed after paper electrophoresis in this case. The number of Gram-negative rods in the other caecal supernatants had not changed significantly and no β -aspartylglycine was found. After 36 and 48 h of antibiotic treatment no enterococci and aerobic Gram-negative rods could be found and now β -aspartylglycine was present in all supernatants. Similar results were obtained with the faecal samples. In urine samples β -aspartylglycine was always present. No β -aspartylglycine could be found in faecal samples of mice treated with 1 mM- or 10 mM- β -aspartylglycine supplied in drinking water.

The polymyxin treatment resulted in a total elimination within 24 h of aerobic Gram-negative rods in faecal samples. The caecal contents obtained after 48 h of polymyxin treatment did not contain β -aspartylglycine.

Discussion

Gel filtration

The elution patterns of control and germ-free caecal supernatants show a high degree of similarity with respect to the elution position of most peaks (Figs. 1a and 1b). The first peak (fraction 40/41), however, is higher in the pattern obtained after gel filtration of the diffusate from a germ-free

caecal supernatant. The second and third peaks (fractions 49 and 53/54 respectively) are about equal in height. The fourth and fifth peaks of the pattern obtained after gel filtration of the control diffusate (fractions 60 and 70 respectively) are not present in the elution diagram of diffusate from germ-free mice. In the latter, however, a new peak appears at fraction 66. The peak at fraction 78 in both elution patterns, which is twice as high in the pattern obtained from germ-free mice, contains tryptophan.

In mice treated with neomycin and bacitracin the diffusate of a caecal supernatant fractionated on Sephadex G-25 (fine) apparently contains some substances comparable with those in a germ-free diffusate (peak at fraction 40) and others comparable with those in a control diffusate (peaks at fractions 60 and 70), and thus appears to occupy some intermediate position.

A similar analysis of the diet resulted in quite a different pattern, except for the peaks at fractions 65 (also present in the germ-free diffusate) and 78 (tryptophan).

High-voltage paper electrophoresis

Our analysis shows that the difference in the electrophoretic pattern at the anode (Figs. 2*a* and 2*b*) between the water-soluble low-molecular-weight components present in the supernatants of caecal contents from germ-free and control mice is due to the presence of β -aspartylglycine in the caecum of germ-free mice. Even after overloading the chromatography paper, no β -aspartyl peptides were found in the caecal supernatant from control mice. From semi-quantitative data of high-voltage paper electrophoresis it is estimated that the amount of β -aspartylglycine in the caecal contents of germ-free and antibiotic-treated mice is at least 200 times that in the caecal contents of control mice.

In human urine, β -aspartyl peptides are commonly present (Buchanan *et al.*, 1962*a,b*). Buchanan *et al.* (1962*b*) and Dorer *et al.* (1966) provided evidence that β -aspartylglycine in urine is largely endogenous and results from protein catabolism. The remaining part is derived more directly from protein in the diet.

Our results show that β -aspartylglycine was always present in urine samples of germ-free, antibiotic-treated and control mice. Administration of β -aspartylglycine to control mice did not result in its accumulation in the faeces, in contrast with the situation in normally fed germ-free and antibiotic-treated animals.

Amino acid analysis

Combe *et al.* (1965) investigated the presence of free amino acids in the caecal contents of germ-free and control rats by extraction with 80% ethanol

and subsequent analysis of the amino acids by paper chromatography. The caecum of germ-free rats contained twice as much soluble nitrogen, and 50–100 times the quantity of free amino acids as in control animals.

In the present study using the supernatant obtained after homogenization and centrifugation of the caecal contents, strikingly different results were observed. The amounts of free amino acids in the caecal contents of germ-free, antibiotic-treated and control mice were found to be rather similar (see Table 1).

Combe & Pion (1966) reported little change in the amino acid composition of the caecal contents from germ-free and control rats after acid hydrolysis. In our studies a 2.6–2.9-fold increase in the concentration of amino acids was observed on acid hydrolysis of a caecal supernatant from germ-free, antibiotic-treated or control mice. After hydrolysis, a relatively high content of aspartic acid and glycine was observed in all supernatants. However, it is evident that the increases in aspartate and glycine on acid hydrolysis of the caecal supernatants of germ-free and antibiotic-treated mice exceed what is observed for control mice. The difference in amino acid concentration before and after hydrolysis of a supernatant from germ-free mice minus the difference in amino acid concentration before and after hydrolysis of a supernatant from control mice is 10.6 $\mu\text{mol/ml}$ for aspartic acid and 13.9 $\mu\text{mol/ml}$ for glycine. A similar comparison for antibiotic-treated mice shows an increase of 9.5 $\mu\text{mol/ml}$ in aspartic acid concentration and of 7.9 $\mu\text{mol/ml}$ in glycine concentration. Part of this increase could be ascribed to hydrolysis of β -aspartylglycine. Other aspartyl peptides and peptides containing glycine may be responsible for the remainder.

Antibiotic treatment

After at least 36 h of treatment with neomycin and bacitracin, no enterococci and aerobic Gram-negative rods could be found in the caecal contents. However, in one case after 12 h of treatment, high-voltage paper electrophoresis showed that the caecum contained a low concentration of β -aspartylglycine. No enterococci could be found in this case and the concentration of Gram-negative rods was decreased. This suggested that the decrease in concentration of Gram-negative rods could have an indirect or direct effect on the presence of β -aspartylglycine in the caecum. This possibility was ruled out by decontamination of four mice with polymyxin B. After 48 h of treatment with polymyxin, Gram-negative rods could no longer be cultured from the caecal contents and no β -aspartylglycine was present. This suggests that the elimination of other bacteria, not tested for, but also removed by the treatment with neomycin and bacitracin, could

directly or indirectly cause the accumulation of β -aspartylglycine in the intestinal tract.

Experiments by Dorer *et al.* (1968) revealed the presence of a β -aspartyl peptidase in tissue homogenates of rats, and *Escherichia coli* B has been found to contain a β -aspartyl peptidase (Haley, 1968). The fact that β -aspartylglycine already accumulates in the caecum after 12–24 h of antibiotic treatment (neomycin/bacitracin) points to a possible absence or malfunctioning of β -aspartyl peptidases, or to difficulties with absorption of β -aspartylglycine in germ-free and antibiotic-treated mice. Whether this has any relation to other anomalies inherent to the germ-free state deserves investigation.

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