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Prefeeding-dependent anaerobic metabolization of xenobiotics by intestinal bacteria – methods for Acarbose metabolites in an artificial colon

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Summary: The biotransformation of Acarbose (Bay g 5421) by an artificial in vitro system with viable intestinal microorganisms was investigated. The bacteria were obtained from the colon of man or from the caecum and colon of rats and were incubated anaerobically with ^{14}C-Acarbose in a nutrient solution. The metabolites were separated and purified by chromatographic methods and identified by nuclear magnetic resonance (^{1}H; ^{13}C) spectrometry and by mass spectrometry.

Metabolites in man and rat are component 2 (minus the terminal glucose of Acarbose), a basic disaccharide consisting of rings B and C, and component 1. This latter substance is formed, after hydrolytic cleavage of the internal glucose of Acarbose, by spontaneous rearrangement of rings A and B (Acarviosine) into a tricyclic oxazolidine.

The metabolite pattern of Acarbose is changed profoundly after several weeks of pretreatment of man or rat with this compound. The microflora adapted in such a manner yields in addition methylated, hexosylated, and n-butyroylated derivatives of Acarbose and/or component 2.

Zusammenfassung: Acarbose wurde mit einem künstlichen In-vitro-System auf Biotransformation durch lebensfähige intestinale Mikroflora geprüft, die aus den unteren Darmabschnitten von Probanden und Ratten stammte. Dazu wurde ^{14}C-Acarbose mit Darmkeimen, Nährstoffen und Spurenelementen inkubiert.

Das Metabolitmuster von Acarbose setzt sich sowohl bei Menschen als auch bei der Ratte im wesentlichen aus Komponente 2, einem um Ring D ärmeren Homologen aus der Acarbose-Reihe, aus einem basischen Disaccharid, das aus den Ringen B und C des Ausgangsmoleküles besteht, und Komponente 1 zusammen. Letztere Verbindung entsteht durch spontane Umlagerung des nicht faßbaren Acarviosins (Ring A und B) in die trizyklische Verbindung mit Oxazolidin-Struktur. Acarviosin wird bei der Abspaltung von Ring C aus Komponente 2 gebildet.

Das Metabolitmuster von Acarbose verändert sich durch eine mehrwöchige Einnahme dieser Substanz im Vergleich zum nicht adaptierten Menschen oder zur nicht adaptierten Ratte gravierend. Adaptierte intestinale Mikroflora liefert sowohl aus Acarbose als auch aus der trizyklischen Komponente 1 reichhaltigere Metabolitmuster. Neben den oben beschriebenen Produkten konnten methylierte, hexosylierte und n-butyrylierte Derivate von Acarbose und/oder Komponente 2 nachgewiesen werden.

Key words: intestinal bacteria, colonic microflora, Acarbose, anaerobic metabolization, microorganisms

Introduction

Some substances remain unabsorbed after oral intake, e.g. the intense sweetener cyclamate (1), or sugars in β-glucosidic linkage like in cellobiose (2). Other substances may be designed for the gastrointestinal tract as their site of action, e.g. Acarbose (3, 4), an inhibitor of some carbohydrases, and are therefore essentially unabsorbed in the small intestine.

Whatever their source, be it the turnover of body constituents like intestinal mucosal cells (t/2 < 1.5 days) or food constituents like dietary fibre and polyols or drugs like the above-mentioned Acarbose, the highly versatile, essentially anaerobic microflora of the large intestine (5) degrades these materials, essentially in its strive for energy – albeit anaerobic conditions prevail. For review on mechanisms of such biotransformations see (5), and especially on carbohydrate degradation see (6).

Metabolic products of the caecal and colonic microflora may easily undergo absorption, e.g. as volatile fatty acids from carbohydrates (6) or as cyclohexylamine from cyclamate (1). Drug metabolites, if absorbed, may be metabolized further by tissues like liver or kidney before their excretion in the urine. The present report describes an experimental system which permits the anaerobic in vitro production of metabolites by the intestinal microflora without interference by host tissues. The main metabolites of Acarbose[1]) are presented.

Materials and Methods

Substances

^{14}C-Acarbose, ^{14}C-component 2 and ^{14}C-component 1 were obtained from Dr. Maul, Wuppertal, with a specific activity of 117 GBq/mol. The radioactivity was localized exclusively to the Acarviosine core (rings A and B) of Acarbose. In thin layer chromatography (see below), the purity of these substances was > 92 %. All other chemicals were of analytical grade or better.

Animals

Male Cara rats from the departmental colony were randomly assigned to control and preadaptation groups. Weaned animals of 70–90 g were kept in Makrolon® (Bayer AG) cages at 22° ± 2 °C, 38–54 % relative humidity, and a 6 p.m./6 a.m. dark/light cycle. They were fed a modified basal diet (ssniff, Soest) (7) containing 11 % sucrose for a couple of month. The preadaptation group received in addition 400 mg Acarbose/kg diet.

Collection of the caecal microflora

The rats were killed in CO_2 atmosphere and immediately transferred to an anaerobic chamber (Forma Scientific, Marietta, Ohio) in which all subsequent steps were carried out. The gas phase in the anaerobic chamber was 85 % N_2, 10 % H_2 and 5 % CO_2. After caecectomy 2 g of caecal contents were mixed with 0.5 ml ^{14}C-Acarbose, a mixture of sugars[2]) and "medium 10" (8) to an end volume of 2.5 ml and incubated anaerobically under stirring at 37 °C. Aliquots of the incubation mixture

[1]) Ph. D. Thesis, M. Pfeffer, Würzburg 1986

[2]) Joan Macy, Davis, CA; personal communication.

were withdrawn aseptically in intervals with 72 h as the longest incubation time, and kept at −30 °C until processed further.

Colonic flora from man

Two male healthy volunteers drank 2–8 l of an isotonic salt solution (9) until a spontaneous defecation set in whose middle portion was collected in a sterile plastic beaker and gassed with $N_2/H_2/CO_2$ immediately. After "anaerobic" centrifugation at 5000 rpm for 5 min a fluffy layer was collected. 3 g of this mainly bacterial material were incubated with 0.6 ml ^{14}C-Acarbose, a mixture of sugars, and 1.1 ml "medium 10" as described above. For adaptation to Acarbose up to 600 mg/day were taken orally for 4 weeks. For the isolation of mg quantities of metabolites, incubation conditions were scaled up 10–50 times.

Isolation and identification of metabolites

Aliquots of the incubations were lyophilized and then extracted with 60 % aqueous acetone (v/v) for rat material and with water for human material, giving yields of radioactivity from the incubate between 96 % and 99 %.

Separation of metabolites was done for small aliquots by thin-layer chromatography (TLC) on Kieselgel 100 (Merck, Darmstadt, F.R.G.) with the solvent mixtures A (acetic acid ethylester/methanol/H_2O/pyridine = 40/40/20/1 [v/v]) or B (chloroform/methanol/25 % NH_3 = 40/40/20 [v/v]). The hR_f values were 54, 65 and 87 in A and 13, 21 and 60 in B for Acarbose, compound 2, and compound 1, respectively. Autoradiography was performed with Cronex® 2/NIF 100 safety films (DuPont de Nemours) as contact autoradiography (exposition time: 1–100 days). Radioactive spots were scraped off, kept in H_2O at 45–50 °C for no less than 5 h, mixed with Ready Solv MP (Beckman Instruments, Frankfurt, F.R.G.) and counted in the liquid scintillation counter LS 7500 (Beckman Instruments, Frankfurt, F.R.G.).

Larger volumes of incubation mixtures were extracted as described above and chromatographed on CM-Sephadex® C-25 (Dt. Pharmacia) which was equilibrated with 0.2 M sodium acetate pH 5 and washed free of ions with degassed distilled water. Eluates with water and with 0.5 M NH_3 were collected in 10 ml fractions (Multirac 2111, LKB, Bromma) and the radioactive peaks were pooled and lyophilized. For a second chromatography on phosphocellulose (Serva, Heidelberg, F.R.G.), the adsorbent was kept for 4–5 h at pH 11 (25 % (v/v) aqueous NH_3) and then brought to pH 4.0 with formic acid. Eluates were obtained with 5 mM ammonium formate (pH 4) and radioactive peaks were lyophilized. TLC served as cross-check between column fractions and metabolite positions on Kieselgel 100.

For the identification of the metabolites, ^{1}H and ^{13}C NMR spectrometry was performed with a Bruker® AM 300 (Bruker Medizintechnik) instrument. Mass spectrometry was used with a Kratos® MS 80 RF instrument (Kratos Inc., U.S.A.) with DS-55 data system, with ionization either by fast atom bombardment with xenon, or by desorption chemical ionization by ammonia, or by a 70 eV electron impact. Controls consisted of sham eluates of the ion exchangers used, solvents, etc.

Results

The number of metabolites formed from Acarbose and component 1 increased with bacterial samples from adapted volunteers and rats (Table 1), when compared with patterns without preadaptation. However, preadaptation did not influence the number of metabolites formed with component 2 as the substrate.

Table 1. Metabolites formed from ^{14}C-Acarbose and its deglucosylated components 2 and 1 under different conditions.

Condition	Substrate	Number of metabolites formed
Rat, not adapted	Acarbose	5
	component 2	5
	component 1	0
Rat, adapted	Acarbose	7
	component 2	5
	component 1	13
Man, not adapted	Acarbose	5
Man, adapted	Acarbose	8

If however, the caecal and colonic microflora of rats was increased by adaptation to the D-glucosyl-alditol mixture Palatinit® (10) (Süddeutsche Zucker AG), the metabolite pattern of ^{14}C-Acarbose nevertheless remained the same as in non-adapted rats.

Those metabolites which comprised less than 1% of the Acarbose incubated, are listed in Table 1 but not isolated for structural identification. Thin-layer chromatographic techniques also served (detailed data not shown) for a study on how metabolites appeared or disappeared during the incubation period. From these data, and from the final identification of the major metabolites formed, a reaction scheme could be written which is given in Figure 1.

Acarbose (1) was hydrolyzed such that component 2 (4) was formed after removal of the terminal glucose (ring D); further cleavage of the internal glucose (ring C) resulted in the formation, after rearrangement, of component 1 (8). These hydrolytic steps had been known before (11) but the final identification of components 2 and 1 as metabolites of Acarbose was made on samples purified in the experiments of this paper. Another hydrolysis occurred on component 2 (4) by removal of ring A, a monounsaturated cyclohexitol which could not be recovered and could have been fermented totally. The resulting basic disaccharide (7) was unknown before from chemical experiments and does not carry a trivial name. This basic disaccharide is metabolized further in man only after preadaptation and disappears slowly in rat experiments.

Component 1 (8) was formed spontaneously from Acarviosine (12) and represents an oxazolidine derivative which after preadaptation apparently gave rise to quite some additional minor metabolites. Without adaptation in rats, and in man with or without preadaptation, component 1 (8) was not metabolized further. With preadaptation in the rat, only 2% of the total metabolites formed from Acarbose (1) were found to be component 1 (8) from which 13 minor metabolites were then observed (Table 1) whose biological relevance is most probably nil.

The major metabolite was component 2 (4) which was hydrolyzed further into component 1 (8) or the basic disaccharide (7) but also gave rise to conjugation reactions with n-butyric acid (6) onto ring A and with a

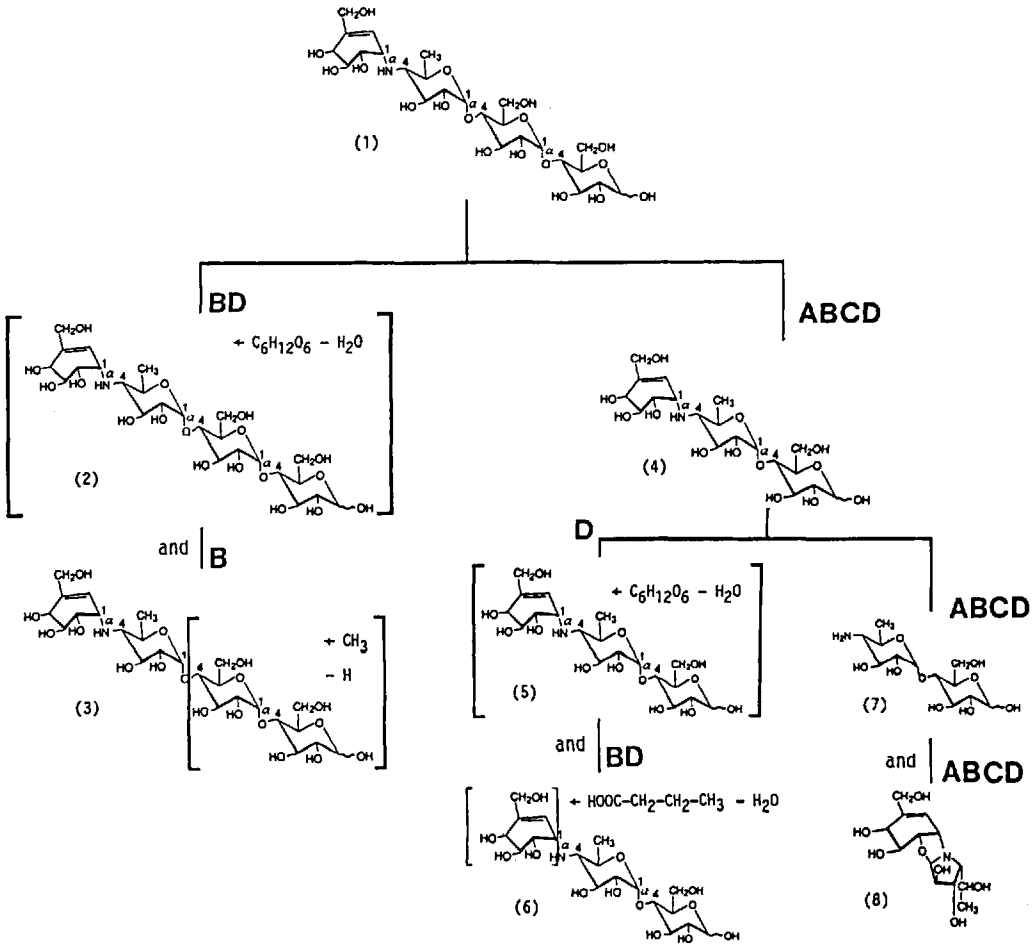

Fig. 1. Anaerobic biotransformation of Acarbose by intestinal bacteria.
In Acarbose (1), the monounsaturated cyclohexitol forms ring A, the dideoxy-aminosugar ring B, the internal glucose ring C and the terminal glucose ring D, e.g. as mentioned in the Summary and below.
Origin of metabolites: A = rats without preadaptation, B = rats after preadaptation, C = volunteer without preadaptation, D = volunteer after preadaptation.

Name and formation of metabolites: (1) Acarbose, (2) hexosylated Acarbose (hexosyl- on C-4 of ring A or on C-1 of ring D), (3) methylated Acarbose (on an oxygen of rings C or D; an artifactual methylation during the isolation is held to be highly improbable but could not be excluded with absolute certainty), (4) component 2 (by hydrolytic cleavage between rings C and D in Acarbose), (5) hexosylated component 2 (hexosyl on C-4 of ring A or on C-1 of ring C), (6) n-butyroylated component 2 (in ring A; n-butyrate), (7) basic disaccharide (by hydrolysis or hydrogenolysis of the N-glycosidic linkage between rings A and B of component 2), (8) component 1 (hydrolysis of the glycoside bond between rings B and C of component 2 and rearrangement of the Acarviosine formed).

hexosyl residue (molecular mass plus 162) on rings A or C. Hexosylation was also observed on Acarbose (1), leading to metabolite (2), however, neither the nature nor the position of the new hexosyl moiety are known.

Discussion

The quantity of metabolites that can be produced and isolated by the procedures described in this paper by far exceeds the amount which could be recovered from feces and urine of experimental animals. Although our system might be called an artificial colon it lacks an essential feature of in vivo systems, the dynamic state of apparent disequilibrium. Yet, when metabolites of ^{14}C-Acarbose containing extracts of rat urine and feces, kindly provided by Dr. Maul, Wuppertal, were compared with the metabolite pattern obtained in vitro without preadaptation, essential coincidence between in vivo and in vitro patterns could be noted. Thus, a wide applicability of the methods used in this study can be foreseen, including the variability of pretreatments of the animals and the possibility to perform microbiological studies in conjunction with the biochemical work as described above.

Some basic questions remain unanswered, e.g. whether metabolization of Acarbose requires intracellular events or not. Carbohydrases, catalyzing hydrolysis, might be expected as bacterial secretion products in the medium (13), and enzymes which catalyze hexosyl transfer reactions are also known to occur extracellularly with some species (14).

However, the idea of an n-butyroylation reaction occurring in the incubation medium is not so easily acceptable. But suppose that all metabolization of Acarbose did actually happen without participation of intracellular bacterial enzymes and intermediary metabolites: could this then explain the relative paucity of reactions which were actually observed, including the absence of redox reactions? Special efforts to check for ^{14}C-CO_2 and ^{14}C-carboxylic acids gave negligible amounts in the Acarbose studies described above.

The adaptation effect exerted by feeding Acarbose on the microflora of the lower gut has a rather specific nature, as is best seen from the increased number of metabolites formed of component 1 in the rat system (see Table 1). Although not investigated in man, it looks as if adaptation especially concerns component 1 and much less component 2 or Acarbose (see Fig. 1 and Table 1). In addition, a general increase of bacterial mass (and caecal weight) in the rat by feeding Palatinit® (Süddeutsche Zucker AG) did not affect the metabolization of Acarbose. A specific nature of the adaptation phenomena should be derived from these observations.

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