Contribution of the digestive tract microflora to amylomaize starch degradation in the rat

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To study in vivo the contribution of the bacterial flora to amylomaize starch degradation in the rat, germ-free and conventional rats were fed on a diet containing either a normal maize starch or an amylomaize starch. In germ-free rats maize starch was almost totally digested in the small intestine, whereas 40% of the ingested amylomaize starch reached the caecum and 30% was excreted, despite the very high endogenous amylase activity. Study by transmission electron microscopy of germ-free caecal contents showed an endocorrosion of the starch granule. In conventional rats, as in germ-free rats, digestibility of maize starch reached 98% in the small intestine, whereas that of amylomaize starch was only 60%. In the caecum of these rats amylomaize starch was fermented, and this led to a decrease in caecal pH and to formation of short-chain fatty acids (SCFA), especially propionate. Comparison between conventional rats fed on maize starch or amylomaize starch showed that caecal SCFA concentrations during a circadian cycle varied in the same way whereas total SCFA and lactic acid concentrations were much higher in rats fed on amylomaize starch. Amylase (*EC* 3.2.1.1) activity was similar in the caecal contents of conventional rats whatever the ingested starch. It was lower in conventional than in germ-free rats, but no starch granule remained in the caecum of conventional rats. These results showed that bacterial amylase was more efficient at degrading resistant amylomaize starch than endogenous amylase.

Starch digestion: Amylomaize starch degradation: Gut microflora: Rats

In man and in animals, appreciable proportions of starch may escape digestion in the small intestine (Stephen *et al.* 1983; Englyst & Cummings, 1985; Asp *et al.* 1986; Berry, 1986; Flourié *et al.* 1986; Cummings & Englyst, 1987). In fact, some starches such as amylose-rich starch or uncooked potato starch are naturally resistant to hydrolysis by salivary and pancreatic amylases, whereas others become resistant to digestion after cooking and cooling (Englyst & Cummings, 1985; Englyst & Macfarlane, 1986; Ring *et al.* 1987, 1988).

From a nutritional point of view, the new interest devoted to these resistant starches is related to the fact that the fraction resistant to small intestinal digestion can be compared with some dietary fibres in that they possess some of the same physiological properties (Bjorck *et al.* 1986; Behall *et al.* 1989). Fermentation of poorly digestible starches, as with that of fibres, reduces the activity of bacterial enzymes such as β-glucuronidases and nitroreductases (Mallet *et al.* 1988) which are involved in the genesis of recto-colic cancers and in the bacterial transformations of bile acids (Andrieux *et al.* 1989).

The amylomaize starch studied here is a native starch containing 700 g amylose/kg. In a former study, Andrieux & Sacquet (1986) have demonstrated that it is not totally digested by the germ-free rat, whereas it is totally degraded by the conventional rat. However, although there is a good understanding of amylomaize starch digestibility in germ-free rats,

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the structure of resistant starch in the digestive contents is unknown. In addition, since Reddy *et al.* (1969) have shown large differences between germ-free and conventional rats in the amylase (*EC* 3.2.1.1) and maltase (*EC* 3.2.1.20) activities in the small intestine, the proportion of starch fermented by the microbial flora in conventional rats might be different from the fraction of starch excreted by germ-free rats.

The purposes of the present study were: (1) to estimate the respective role of digestive and bacterial enzymes in the digestion of maize and amylomaize starches in the small and large intestine of germ-free and conventional rats; (2) to compare the amylolytic activities and other glycolytic activities in germ-free and conventional rats fed on either maize starch or amylomaize starch; (3) to study the ultrastructure of amylomaize starch granules and the carbohydrate hydrolysis products in the caecum of germ-free and conventional rats.

MATERIALS AND METHODS

Animals and diets

Male Fisher 344 germ-free and conventional rats (3 months old, body-weight 285 (SE 5) g) were fed for 4 weeks on a semi-synthetic diet containing either 580 g/kg of a normal maize starch (including 300 g amylose and 700 g amylopectin/kg) or an amylomaize starch (including 700 g amylose and 300 g amylopectin/kg) (Roquette frères, Lestrem, France). The other dietary ingredients were (g/kg): casein 205, maize oil 90, cellulose 50, minerals and vitamins 75 (Andrieux & Sacquet, 1986).

Diets were sterilized by gamma-irradiation at 40 kGy in vacuum-sealed plastic bags and offered *ad lib.* to animals as a paste containing 600 g demineralized water/kg. This paste was prepared daily at 16.00 hours.

Two groups of sixteen germ-free rats were fed on maize or amylomaize starch diets and two groups of sixteen conventional rats were fed on irradiated maize or amylomaize starch diets.

During the third week of feeding, a transit marker (^{14}C-labelled polyethylene glycol (PEG), relative molecular mass 4000) was incorporated into the diets (0·21 μCi/g). At the end of this period daily marker ingestion and excretion were similar and a 5 d balance study was conducted to measure food intake and total starch digestibility. Fresh faecal samples were collected to measure bacterial glycolytic activities. Then two rats from each group were killed every 3 h for 24 h to measure starch digestibility in the small intestine and in the hind-gut, and variations in caecal weight and caecal contents (pH and short-chain fatty acid (SCFA) concentrations) during the 24 h period.

Preparation of intestinal contents and of faeces samples

The caecal pH was determined under diethyl ether anaesthesia using a microelectrode. Then intestinal contents were removed immediately and portions of caecal contents were mixed with mercuric chloride for SCFA determinations or with triethanolamine buffer (pH 7·0) for lactic acid determinations. The faeces and intestinal samples were frozen in liquid nitrogen and maintained at $-20°$ until analysed.

For ultrastructural analysis of amylomaize starch, portions of caecal contents of rats killed at 10.00 hours were immediately treated for electron microscopy analysis as described previously (Gallant *et al.* 1973).

Analysis

Starch digestibility at different sites in the digestive tract. The content of the last 50 mm of the ileum, the last 50 mm of the colon and the caecum of rats fed on the transit marker during the week and killed every 3 h, were collected, washed out and the relative concentrations of starch and transit marker (^{14}C-labelled PEG) were determined.

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The ratio (R), starch concentration:transit marker concentration, was measured in the intestinal contents, in faeces and in the diet. Since rats eat several meals daily and transit time is different in germ-free and conventional rats (Riottot, 1987), and because transit rate of marker and starch may be different, we computed the arithmetic mean of starch digestibility at each level of the digestive tract. The mean value of R was used to calculate the mean digestibility of starch (D_S) at different levels (S) of the digestive tract (ileum, caecum, colon and faeces) for 24 h. D_S at site S of the digestive tract was expressed as a percentage of intake:

$$D_S = (1 - R \text{ site } S / R \text{ in diet}) \times 100.$$

Starch analysis. In diets, faeces or intestinal contents, starches were dispersed in dimethylsulphoxide (800 ml/l) by autoclaving for 20 min. Samples of this suspension containing at most 5 g starch/l were then hydrolysed at 55° for 3 h in a 10 mM-phosphate buffer (pH 4·5) by amyloglucosidase (*EC* 3.2.1.3; Optidex 1200; Roquette fréres, Lestrem, France).

Glucose formed was determined enzymically with glucose oxidase (*EC* 1.1.3.4; Biotrol, Paris).

Amylase activity. This was determined using an enzymic colorimetric test with a chromophore blue as substrate (Phadebas, Pharmacia, France). Activity was expressed as U/g fresh weight. One unit of amylase was equivalent to 1 μmol maltose produced/min at 37°.

SCFA. SCFA were determined using gas–liquid chromatography after water extraction of acidified samples. The chromatograph used was a GC 121 Del (Delsi) apparatus with a flame-ionization detector and a semi-capillary column (15 m × 0·53 mm) impregnated with SP 100. Carrier gas (N_2) flow-rate was 30 ml/min, column temperature 125°, detector temperature 155°, hydrogen flow-rate 40 ml/min, compressed air flow-rate 40 ml/min. Isobutyric acid was used as an internal standard. The integrator was a Delsi Enica 21 apparatus.

L- and D-Lactic acids. These were determined enzymically using lactate dehydrogenase (*EC* 1.1.1.27; Boehringer-Mannheim).

Identification of products of starch hydrolysis in caecal contents. Carbohydrates were determined after water extraction (1:10, w/v) and protein precipitation. Total reducing sugars were determined by the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1945). Carbohydrates were separated by thin-layer chromatography on silica gel plates as described by Weber *et al.* (1985).

Chromatograms were developed with the following system: propanol–ethyl acetate–ethanol–acetic acid–pyridine–water (7:3:3:2:2:4, by vol). Spots were revealed by heating plates sprayed with a solution of sulphuric acid in acetone (50 ml/l). Glucose was determined enzymically with glucose oxidase (Biotrol, Paris).

Glycolytic activities were measured by the rate of release of *p*-nitrophenol from their *p*-nitrophenylglucosides. The reaction mixture contained 0·3 ml substrate solution (5 mM) and 0·2 ml of a dilution 1:2 of caecal content in phosphate buffer (0·1 M) at the caecal pH. Incubation was at 37° and *p*-nitrophenol concentration was measured as the optical absorbance at 400 nm after addition of 2·5 ml 0·25 M-sodium carbonate.

Statistical analysis

Results are expressed as means with their standard errors. Values were compared by analysis of variance and the Newman–Keuls test using STAT ITCF software.

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Table 1. *Nutritional variables and digestive utilization of maize and amylomaize starches in germ-free and conventional rats**

(Mean values with their standard errors for eighteen rats per group)

Group...	Germ-free				Conventional			
Starch in diet...	Maize		Amylomaize		Maize		Amylomaize	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Live wt (g)	316[b]	7	300[a]	5	322[b]	6	297[a]	8
Food intake (g)	14[a]	0·6	17·2[b]	0·4	14[a]	1	14·5[a]	1·4
Digestive utilization (%) at:								
Terminal ileum	97·5[e]	1·5	61·9[c]	2·7	97·5[e]	1·4	61·1[c]	2·5
Caecum	98·5[ef]	0·4	70·4[d]	2·5	99·7[f]	0·4	96·1[e]	0·5
Distal colon	98·5[ef]	0·2	71·8[d]	1·5	99·8[f]	0·5	96·5[e]	0·6
Faeces	98·6[ef]	0·2	70·0[d]	0·4	99·6[f]	0·1	97·1[e]	0·3

[a, b] For each nutritional variable, values with different superscript letters were significantly different (analysis of variance and Newman–Keuls test), $P < 0·05$.

[e–f] Values with different superscript letters were significantly different (analysis of variance and Newman–Keuls test), $P < 0·05$.

* For details of diets and procedures, see pp. 490–491.

RESULTS

Nutritional variables and digestive utilization of maize and amylomaize starches

The live weight of germ-free and conventional rats fed on the amylomaize starch diet was slightly lower than that of rats fed on maize starch (Table 1). Feed intake was similar in conventional rats, but in germ-free rats it was higher with the amylomaize starch diet than with the maize starch diet.

Maize starch digestion was close to 97% in the terminal ileum of germ-free and conventional rats. It was not enhanced in the caecum of germ-free rats but it was total in the caecum of conventional rats.

Amylomaize starch digestion was only 60% in the terminal ileum of germ-free and conventional rats. In the caecum of germ-free rats amylomaize starch degradation was improved, but the total digestibility remained 30% lower than that of maize starch. In the caecum of conventional rats amylomaize starch degradation was much higher than that in germ-free rats, but it remained slightly lower than maize degradation.

Glycolytic activities in caecum and faeces

Amylolytic activity (Table 2) was fourfold higher in the caecum and the faeces of germ-free rats fed on amylomaize starch than in those fed on maize starch. After centrifugation of a tenfold dilution of caecal and faecal contents, this activity was mainly fixed to insoluble particles. By contrast, in conventional rats the amylolytic activity did not vary significantly with the nature of the diet in the caecum, whereas in the faeces it was very low when rats received maize and it remained at the same level as that in the caecum of rats fed on amylomaize starch. After centrifugation of a tenfold dilution of caecal or faecal contents, 90–95% of amylolytic activity was found in the supernatant fluid.

Mucolytic activities (*N*-acetyl-β-D-glucosaminidase (*EC* 3.2.1.30), *N*-acetyl-β-D-galactosaminidase (*EC.* 3.2.1.49), α-L-fucosidase (*EC* 3.2.1.51)) were negligible in germ-free rats; β-D-galactosidase (*EC* 3.2.1.23) and β-D-glucuronidase (*EC* 3.2.1.31) were very low (0·015 (SE 0·007) and 0·006 (SE 0·0004) U/g respectively). They were lower in

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Table 2. *Glycolytic activities (U/g) in caecum and faeces of germ-free and conventional rats fed on maize- or amylomaize-starch diets**

(Mean values with their standard errors for eighteen rats per group)

Starch in diet...	Caecum				Faeces			
	Maize		Amylomaize		Maize		Amylomaize	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
				Germ-free				
Amylase (*EC* 3.2.1.1)	60[b]	5	280[a]	4	58[b]	2	210[a]	8
				Conventional				
Amylase	51[b]	6	65[b]	6	5[c]	3	55[b]	5
β-D-glucuronidase (*EC* 3.2.1.31)	0·88[c]	0·14	0·28[a]	0·01	1·53[d]	0·12	0·38[b]	0·06
β-D-galactosidase (*EC* 3.2.1.23)	1·25[c]	0·07	0·63[a]	0·02	1·45[d]	0·02	0·88[b]	0·05
N-acetyl-β-D-glucosaminidase (*EC* 3.2.1.30)	0·70[b]	0·05	0·28[a]	0·02	1·2[c]	0·1	0·56[b]	0·1
N-acetyl-β-D-galactosaminidase (*EC* 3.2.1.49)	0·30[b]	0·04	0·16[a]	0·01	0·40[c]	0·02	0·16[a]	0·01
β-D-glucosidase (*EC* 3.2.1.21)	0·27[a]	0·01	0·29[a]	0·02	0·47[b]	0·09	0·47[b]	0·06
α-L-fucosidase (*EC* 3.2.1.51)	0·12[a]	0·01	0·11[a]	0·01	0·22[b]	0·03	0·09[a]	0·02

[a–d] Values with different superscript letters were significantly different (Newman–Keuls test), $P < 0·05$.

* For details of diets and procedures, see pp. 490–491.

caecal contents than in faeces of conventional rats (Table 2). The activity of β-D-glucuronidase was three to fourfold lower in rats fed on amylomaize starch than in rats fed on maize starch, while *N*-acetyl-β-D-glucosaminidase, *N*-acetyl-β-D-galactosaminidase and β-D-galactosidase were also lower with the amylomaize-starch diet, but β-D-glucosidase (*EC* 3.2.1.21) and α-L-fucosidase were similar with the two diets.

Amylomaize starch degradation in the hind-gut of germ-free and conventional rats

Ultrastructure of amylomaize starch granules. Observation of the caecal contents of germ-free rats by transmission electron microscopy showed that amylomaize starch granules were partly hydrolysed, as shown by endocorrosion of the granule (Plate 1). In the conventional rat, examination of the caecal contents revealed no trace of starch granules (Plate 2).

Hydrolysis products. The caeca of germ-free rats contained 46·0 (SE 0·5) μmol reducing sugars/g. These carbohydrates seem to be mostly composed of glucose (45·0 (SE 0·2) μmol/g). In fact, thin-layer chromatographic analysis showed that glucose was the major hydrolysis product, followed by maltose and maltotriose, without any intermediary between long-chain malto-oligosaccharides and maltotriose. Traces of maltose and maltotriose had disappeared from the faeces of rats. The caeca of conventional rats contained 10 μmol reducing sugars/g, of which 7·8 (SE 0·2) μmol was glucose. Maltose, maltotriose and short-chain malto-oligosaccharides could also be observed. The faeces of these rats contained only a small amount of maltose as shown by thin-layer chromatographic analysis.

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Table 3. *Caecal variables and caecal concentrations of short-chain fatty acids (SCFA) in conventional rats fed on maize- and amylomaize-starch diets**

(Mean values with their standard errors for eighteen rats per group)

Group...	Germ-free				Conventional			
Starch in diet...	Maize		Amylomaize		Maize		Amylomaize	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Caecal wt (g/kg body-wt)	54[c]	1	62[d]	2	13[a]	1	34[b]	2
pH	7·0[c]	0·1	7·1[c]	0·1	6·75[b]	0·02	5·8[a]	0·1
SCFA (μmol/g)	0·2[a]	0·1	0·2[a]	0·1	48[b]	5	77[c]	4
Total SCFA (μmol)	1·1[a]	0·1	1·2[a]	0·1	200[b]	12	700[c]	33

[a-d] Values with different superscript letters were significantly different (Newman–Keuls test), $P < 0.05$.

* For details of diets and procedures, see pp. 490–491.

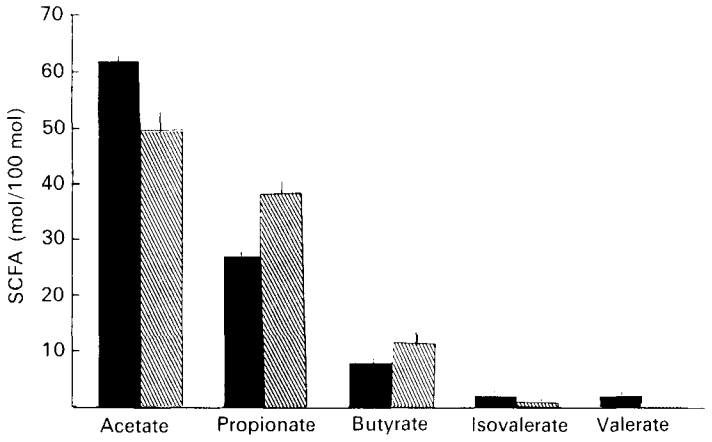

Fig. 1. Relative concentrations (mol/100 mol) of the different short-chain fatty acids (SCFA) in caecal contents of conventional rats fed on a maize-starch diet (■) or an amylomaize-starch diet (▨). Values are means with their standard errors represented by vertical lines for eighteen rats per group. For details of diets and procedures, see pp. 490–491.

Fermentation of starches in conventional rats

Caecal variables and caecal SCFA concentrations (Table 3). In germ-free rats caeca were enlarged: 5·4 (SE 0·1)% of live weight when feeding was based on maize starch and 6·2 (SE 0·2) % when it was based on amylomaize starch, which corresponds to a weight increase of 25%. The caecal pH of these rats was neutral and the caecum contained only traces of acetate. In conventional rats, compared with maize, amylomaize starch caused a 300% increase in caecal weight and a significant reduction in intestinal pH. The caecal SCFA concentration increased significantly and the total amount of SCFA was increased 3·5-fold. The relative concentrations of the different SCFA were considerably modified by amylomaize ingestion (Fig. 1). The mean concentration of acetate was reduced by one-third with corresponding increases in propionate and to a less extent in butyrate; that of valerate, already very low, decreased still further or disappeared.

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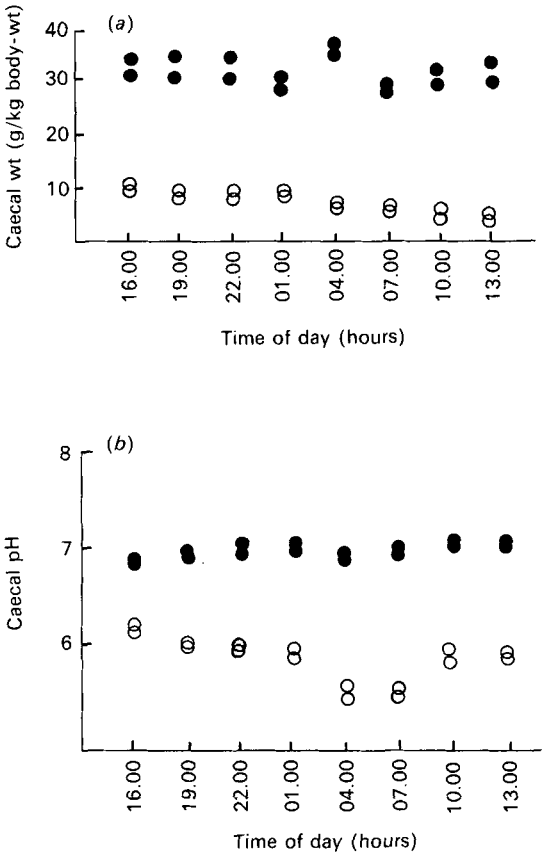

Fig. 2. Circadian variation in (*a*) the caecal weight and (*b*) caecal pH in conventional rats fed on a maize-starch diet (○) or an amylomaize-starch diet (●); each value corresponds to one rat. For details of diets and procedures, see pp. 490–491.

Circadian variation in the caecal weight, pH, SCFA and lactic acid concentrations. In conventional rats individual values for caecal variables showed that caecal weight was always higher in rats fed on amylomaize starch than in those fed on normal starch (Fig. 2(*a*)). The caecal pH was always lower in rats fed on amylomaize starch, with a more marked decrease between 04.00 and 13.00 hours (Fig. 2(*b*)). Total lactic acid concentration (L- and D-lactic acid) was very low in rats fed on maize starch; it was higher at every time-point in rats fed on amylomaize starch with a maximum at 04.00 hours (Fig. 3(*a*)) without change of the L-:D-lactic acid ratio (1·8 (SE 0·1)). Variations in caecal SCFA concentrations were similar in rats fed on the two types of starches (Fig. 3(*b*)). At 16.00 hours, before the diets were given to the rats, this concentration was at a minimum. During the feeding period (16.00–07.00 hours) the caecal SCFA concentration increased progressively to reach a maximum value almost twice as high as the initial value. The circadian variation in the total amount of SCFA in the caecum exhibited much more marked differences between the two types of starch. With maize the variation was small, the maximum values being observed between 04.00 and 10.00 hours. With amylomaize it increased considerably (Fig. 3(*c*)).

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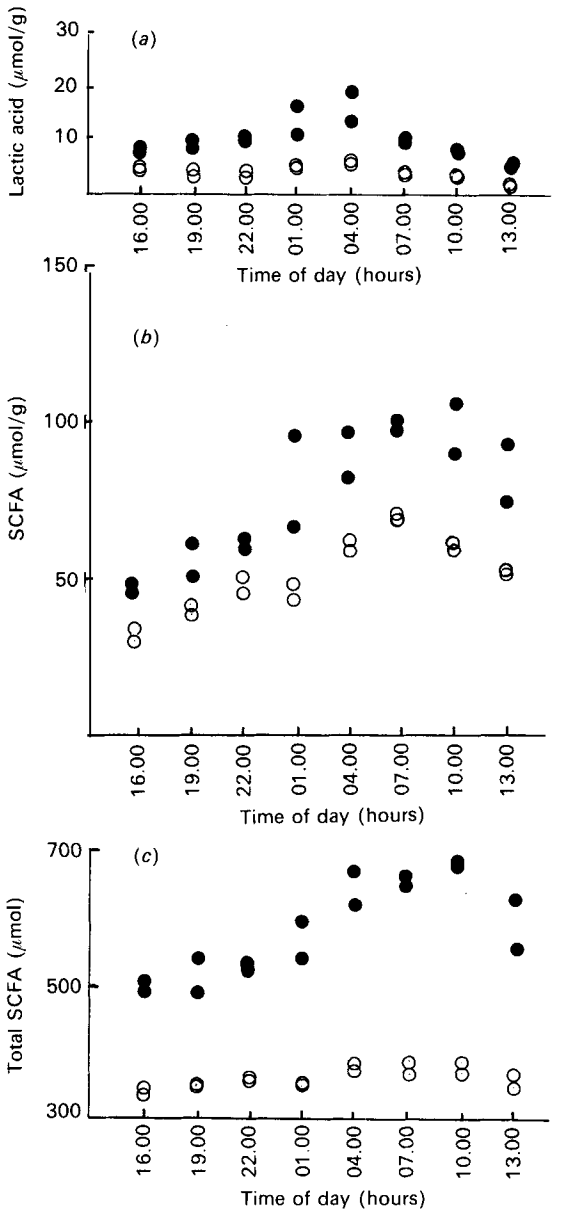

Fig. 3. Circadian variation in (*a*) lactic acid and (*b*) short-chain fatty acid (SCFA) caecal concentrations, and (*c*) total amount of SCFA in the caecum of conventional rats fed on a maize-starch diet (○) or an amylomaize-starch diet (●); each value corresponds to one rat. For details of diets and procedures, see pp. 490–491.

DISCUSSION

These results show that ingestion of amylomaize starch modifies the digestive physiology of rats. Differences between germ-free and conventional rats show the contribution of the microflora to amylomaize degradation in the hind-gut. They confirm some published data such as the slightly depressive effect of amylomaize on growth (Ayano *et al.* 1977; Sacquet *et al.* 1983), increased feed intake in the germ-free rat (Andrieux & Sacquet, 1986) or the resistance of amylose-rich starches to hydrolysis by salivary and pancreatic amylases

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observed in animals (Szylit *et al.* 1974; Ayano *et al.* 1977) and in man (Stephen *et al.* 1983; Englyst & Cummings, 1985; Asp *et al.* 1986; Berry, 1986; Cummings & Englyst, 1987).

The crystalline structure of these starches, and particularly that of amylose, may be responsible for this resistance to hydrolysis by endogenous amylases (Ring *et al.* 1988; Sievert & Pomeranz, 1989), resulting in the small intestine in a slower hydrolysis of amylose into glucose and in a delayed postprandial insulin response (Thorne *et al.* 1983; Goddard *et al.* 1984; Behall *et al.* 1989).

We have described starch digestion in different parts of the digestive tract and shown that the microbial flora does not affect starch digestion in the small intestine. However, Reddy *et al.* (1969) observed a halving of the amylase concentration in the intestinal lumen and the maltase content of the enterocyte in the conventional rat compared with the germ-free rat.

Our results show that amylomaize starch granules are partly hydrolysed when entering the caecum. They present an endocorrosion similar to that observed by Gallant *et al.* (1973) after enzymic attack by pig pancreatic enzyme.

In germ-free rats, feeding on amylomaize starch compared with maize starch led to a higher amylase activity in the hind-gut. However, in these rats the degradation of amylomaize starch granules by the digestive enzymes is probably very slow, since even after a transit time of several days in the large intestine (Riottot, 1987), 30% of starch is not digested and is excreted. In germ-free rats, amylase activity was similar in the faeces and in the caecum, whatever the ingested starch, and it was mainly fixed to starch granules. By contrast, in conventional rats this activity is mainly free in the supernatant fraction, as observed by Macfarlane & Englyst (1986) in human faeces. However, these authors showed that bacteria-bound activity was considerably more efficient in breaking down starch, and that amylolytic bacteria belonging to the genera *Bifidobacterium*, *Bacteroides*, *Fusibacterium* and *Butyrivibrio* can play a major role in hydrolysis of starch in the colon.

There was no difference between the amylase activity in the caeca of conventional rats fed on maize or amylomaize starch. If this activity remained unchanged in the faeces of rats fed on amylomaize starch, it was almost zero in the faeces of rats fed on normal maize starch. These results are in agreement with those of Reddy *et al.* (1969), who showed that the intestinal flora inactivates endogenous enzymes. The persistence of a higher amylase activity in conventional rats fed on amylomaize starch suggests that the activity of bacterial proteases may be inhibited or reduced by this starch, either because these amylases are protected by their substrate (Rosenblum *et al.* 1988), or because the production of proteolytic enzymes is lower in these rats. The decreased proportion of isovalerate, a metabolite produced by bacterial proteolysis (Prins, 1977), supports this hypothesis. Mallet *et al.* (1988) have also demonstrated a reduced activity of azo- and nitro-reductases in conventional rats fed on partly resistant starches such as amylomaize.

Our results confirm that amylomaize starch fermentation in the large intestine of the conventional rat considerably modifies bacterial activity and the bacterial ecosystem (Mallet *et al.* 1988; Andrieux *et al.* 1989). The pH decrease and the quantitative and qualitative variations in the SCFA may indicate a change in the equilibrium of bacterial populations. Indeed, conventional rats fed on amylomaize for 3 months then isolated from any bacterial contamination except their own microbial flora and again fed on normal maize starch do not recover their initial bacterial activity (Andrieux *et al.* 1989).

The kinetic study of variations in pH and SCFA caecal concentrations showed that intestinal fermentation induces changes in the intestinal environment between the fasting and digestion periods. In rats fed on maize starch these changes only affected the SCFA levels, but the pH remained stable. In rats fed on amylomaize starch both the pH and the SCFA varied. In these rats the increase in the caecal volume probably led to a better

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absorption of SCFA by enhancing the absorption surface of the caecum and by lengthening the duration of intestinal transit (Riottot, 1987). However, circadian variations in the caecal environment may lead to the disappearance of some bacterial populations sensitive to variations in the environment, such as the pH decrease, to the benefit of amylolytic bacteria capable of degrading the resistant starch. It may be interesting to isolate these bacteria and to study their metabolism in gnotoxenic rats.

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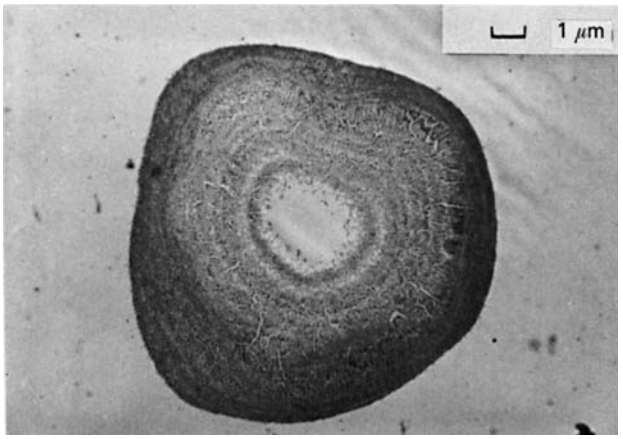

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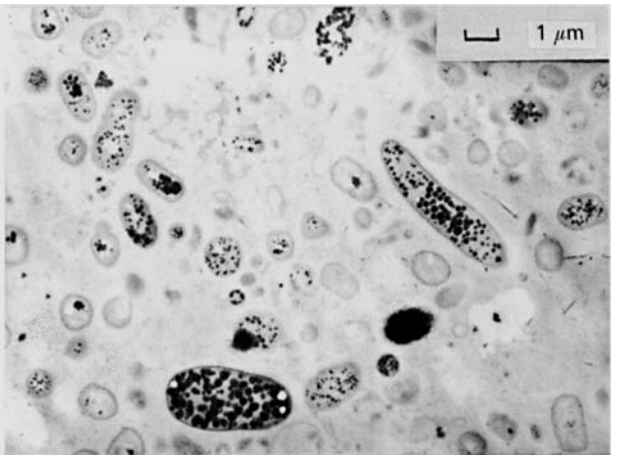

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EXPLANATION OF PLATES

Plate 1. Amylomaize starch granule in the caecum of germ-free rats fed on an amylomaize-starch diet. × 5000.
Plate 2. Caecal content of conventional rats fed on an amylomaize-starch diet. × 5000.

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