Biochem. J. (1972) **129**, 881–890
Printed in Great Britain

The Role of the Gut Flora in the Metabolism of Cyclamate

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(*Received* 3 *May* 1972)

1. [^{14}C]Cyclamate was not metabolized when incubated with the liver, spleen, kidney or blood of rats or rabbits kept on a cyclamate-containing diet, and that had become converters of cyclamate into cyclohexylamine. 2. [^{14}C]Cyclamate was converted into cyclohexylamine when incubated under anaerobic conditions with the contents of the caecum, colon or rectum or with the faeces of cyclamate-pretreated rats. Similar results were obtained with cyclamate-pretreated rabbits. With cyclamate-pretreated guinea pigs, which did not readily convert cyclamate into cyclohexylamine, the colon contents showed only low activity in this respect. 3. The faeces of a human converter of [^{14}C]cyclamate into cyclohexylamine were also very active, but became less active when cyclamate was removed from his diet. 4. On subculturing the organisms from the contents of the colon and rectum of rats, the ability to convert cyclamate into cyclohexylamine was lost during three subcultures, but the loss of the activity was considerably decreased by subculturing in the presence of cyclamate. 5. Incubation of rat faeces in broths containing cyclamate increased their ability to metabolize cyclamate, but similar treatment of rabbit and human faeces suppressed this activity. 6. When rats are kept on a cyclamate diet the number of clostridia in the faeces increased considerably. In humans dietary cyclamate did not appear to alter the counts of various faecal micro-organisms. 7. The gut organisms that appear to develop the ability to convert cyclamate into cyclohexylamine are clostridia in rats, enterobacteria in rabbits and enterococci in man. 8. [^{14}C]Cyclohexylamine injected into the caecum or colon of rats is readily absorbed and excreted in the urine. 9. It appears that on continued intake of cyclamate the gut flora develop the ability to convert cyclamate into cyclohexylamine, which is then absorbed and excreted mainly in the urine, although a small proportion is metabolized to other compounds.

In the preceding paper (Renwick & Williams, 1972*b*) evidence was obtained which suggested that the conversion of cyclamate into cyclohexylamine in man, guinea pig, rabbit and rat was carried out in the gut and that the gut flora were involved. In the present paper further evidence for this view has been obtained and it would appear that the conversion of cyclamate into cyclohexylamine is carried out by certain gut micro-organisms but that any further metabolism of cyclohexylamine is performed by the tissues of the body. Part of this work has been briefly reported (Drasar *et al.*, 1971).

Materials and Methods

^{14}C-labelled cyclamate and cyclohexylamine and related compounds relevant to this work have been described in the preceding papers (Renwick & Williams, 1972*a*,*b*). Cyclohexylamine was determined by a combined g.l.c. and ^{14}C-counting method (Renwick & Williams, 1972*b*) and chromatographic separations were carried out as described by Renwick & Williams (1972*a*). The faeces used were stored when necessary at −20°C.

Micro-organisms

Bacteriological media. The media used were Todd–Hewitt broth, thioglycollate broth and tryptone–yeast-extract broth as described in the Oxoid Manual (1965).

Viable counts of faecal bacteria. These were carried out as described by Drasar (1967).

Isolation of individual strains of bacteria. Strains were isolated by using the method of Drasar (1967), with the exception of enterococci, which were isolated on a Methylene Blue–agar medium as described by Schaedler *et al.* (1965). The colonies were transferred from the isolation plates to tryptone–yeast-extract broth (1 ml).

Animals

These were Wistar albino rats, Duncan–Hartley strain albino guinea pigs and New Zealand White rabbits. The animals were pretreated with cyclamate as described in the preceding paper (Renwick & Williams, 1972*b*).

Incubation of tissue and gut homogenates. Immediately after the animals were killed the organs were removed and homogenized at 5°C in a Waring Blendor. The liver, kidneys and blood of rats and rabbits were made into 33% (w/v) homogenates and the spleen into 25% (w/v) homogenates in 1.15% (w/v) KCl. The hind-gut contents of rats (Table 1) were made into 25% (w/v) homogenates in 1.15% (w/v) KCl and the contents of other regions of the gut were prepared by suspending the whole contents in thioglycollate broth (10ml). The gut contents of rabbits (Table 6) were made up as 33% (w/v) homogenates in 0.1M-sodium phosphate buffer, pH7.4. For incubation the tissue homogenate or gut-contents preparation (1ml) was mixed with 0.1M-phosphate buffer, pH7.4 (1ml), and the [^{14}C]cyclamate or [^{14}C]cyclohexylamine solution (10–50μl) was then added. All solutions and apparatus were kept at 5°C before incubation. Controls were prepared by heating the homogenate and buffer at 100°C for 5min. The tubes containing the tissue homogenates were incubated at 37°C for 90min under air, after which they were analysed for [^{14}C]cyclohexylamine by combined g.l.c. and ^{14}C counting. Those containing homogenates of gut contents were similarly incubated but under N_2 or H_2 and are referred to later in the text.

Results

Rat tissues and gut flora

Tissue and gut contents. Table 1 shows the ability of the liver, kidney, spleen and blood and homogenates of gut contents from cyclamate-pretreated rats to metabolize [^{14}C]cyclamate. The rats used were able to convert ingested cyclamate into cyclohexylamine, but their liver, kidney, spleen and blood under the conditions used were unable to do so. Table 1 also shows that the ability to convert cyclamate into cyclohexylamine occurs in the contents of the hind-gut including the caecum, colon and rectum. The contents of the duodenum and ileum had little activity in this respect. Table 1 further suggests that the conversion in the active homogenates occurs better under anaerobic conditions (N_2 or H_2) than in the presence of air.

The experiments in Table 1 were also carried out by using [^{14}C]cyclohexylamine (0.1mg; 0.06μCi) instead of cyclamate and in each case the recovery of the added cyclohexylamine was 95–105%. The metabolism of the amine was negligible in these preparations from tissues and gut contents.

Faeces. Faecal homogenates (20% in Todd–Hewitt broth) were also prepared from the faeces of normal and cyclamate-pretreated rats. When

Table 1. *Metabolism of cyclamate by rat tissues and gut contents*

Rats nos. 22, 33 and 43 were kept on a cyclamate diet for 4.5 months. They were given orally 100mg of calcium cyclamate and the cyclohexylamine output in 24h was determined by g.l.c. After they were killed the activity of their tissues and hind-gut contents was determined with [^{14}C]cyclamate (0.1mg, 0.82μCi, per incubation mixture) as described in the text. Rats nos. 13, 14 and 16 were kept on a cyclamate diet for 4 months and the amount of [^{14}C]cyclamate used per incubation mixture was 0.2mg, 1.6μCi.

	Conversion into cyclohexylamine (% of administered or added cyclamate)						
Material tested — Rat no. ...	22	33	43	13	14	16	Normal animals
Whole animal	60	30	21	—	—	—	<0.01
Controls*	<0.05	<0.05	<0.05	—	—	—	<0.05
Liver, kidney, spleen, blood	<0.05	<0.05	<0.05	—	—	—	<0.05
Hind-gut contents							
Anaerobic (N_2)	3.25	1.94	0.78	—	—	—	<0.05
Aerobic (air)	2.40	0.62	0.43	—	—	—	<0.05
Contents of							
Duodenum–ileum (H_2)†	—	—	—	0.3	0.3	0.4	—
Caecum (H_2)†	—	—	—	33.6	61.5	54.0	—
Colon–rectum (H_2)†	—	—	—	34.0	69.6	14.9	—

* All controls; prepared by heating the homogenates (see the text).

† These results are expressed as the total activity in each region of the gut.

sodium [^{14}C]cyclamate (5mg; 3.2μCi) or [^{14}C]cyclohexylamine (3.7mg; 1.9μCi) was incubated with faecal homogenates (5ml) from normal rats for 48h at 37°C under H_2 neither was metabolized to any significant extent. However, when faecal homogenates from cyclamate-pretreated rats were used, the cyclohexylamine was recovered unchanged, whereas cyclamate was extensively metabolized to cyclohexylamine but no other product, as shown by radiochromatogram scanning of paper chromatograms prepared with four solvent systems (A, B, C and D; see Table 1 of Renwick & Williams, 1972*a*).

Effect of subculturing. Renwick & Williams (1972*b*) have shown that rats on a cyclamate diet that have acquired the ability to metabolize cyclamate lost this ability when cyclamate was removed from the diet. The organisms of the colon and rectum were subcultured in thioglycollate broth by using 1:10 dilutions and growing each for 48h, after which one-tenth was grown for the next subculture and nine-tenths were analysed for metabolizing ability. Table 2 shows that the ability to metabolize cyclamate is lost, since after one subculture about 10% of the cyclamate was converted into cyclohexylamine but after three subcultures only 0.1%. However, if the subculturing is done in the presence of calcium cyclamate (1mg/ml) the loss of this ability is less marked and in fact each subculture has more activity than expected from a straight 1:10 dilution, thus indicating that the system is self-replicating.

Effect of cyclamate on the bacterial population of the gut. The viable counts of various genera of

Table 2. *Cyclamate metabolism by subcultured micro-organisms of rat gut*

The contents of the colon and rectum from three cyclamate-pretreated rats were subcultured as described in the text. Suspensions of organisms were incubated at 37°C for 48h under H_2 with sodium [^{14}C]cyclamate (0.2mg, 1.6μCi, per incubation mixture) and the cyclohexylamine was determined by the combined g.l.c. and ^{14}C counting method.

No. of subcultures	Subculture conditions ...	Conversion into cyclohexylamine (% of added cyclamate)	
		Cyclamate absent	Cyclamate present
1		9.7	16.9
2		1.4	5.7
3		0.1	1.8

Table 3. *Effect of a cyclamate diet on the counts of various faecal micro-organisms*

Determinations were performed on freshly voided faeces (see the text). The normal values are the range found in individuals on an appropriate diet and the pretreated values are for four rats and three humans that had received cyclamate for 3 months and up to 1 month respectively (Renwick & Williams, 1972*b*). n.d., Values were not determined.

Organisms	$10^{-6}\times$No. of organisms/g			
	Rat		Man	
	Normal	Pretreated	Normal	Pretreated
Enterobacteria	0.5–6	0.4–20	0.001–100	0.003–0.07
Streptococci*	1–2	6–60	0–10	0.0002–0.0009
Enterococci	0.1–9	0.04–0.4	0.0001–1	0.004–0.02
Lactobacilli	1–1000	10–200	0.0001–10	0.03–0.06
Yeasts	0.008–1	0.005–1	0–0.01	0
Clostridia	0.00001–0.001	80–900	0–0.1	0.02–0.04
Bacteroides	10–200	3000–4000	10^4–10^5	10^4–2×10^4
Bifidobacteria	100–300	100–700	10^3–10^5	2×10^4–3×10^4
Veillonella	n.d.	n.d.	0–1	0.002–0.2
Total	n.d.	n.d.	10^4–10^5	3×10^4–5×10^4

* In rats total streptococci; in man *Streptococcus salivarius*.

bacteria in the faeces of normal and cyclamate-pretreated rats are shown in Table 3. The only significant change found was in the numbers of clostridia, which appear to have increased from the range 10^1–10^3 for controls to 8×10^7–9×10^8/g wet wt. of faeces in four rats that had been on a cyclamate diet for 3 months. Values for humans, which show little change, are also given in Table 3.

Metabolism of cyclamate by strains of bacteria isolated from faeces. The organisms were isolated from the faeces of cyclamate-pretreated rats. Table 4 shows that, in rat faeces, the organisms that were active in metabolizing cyclamate were clostridia. Table 4 also contains results for human faeces and rabbit caecal contents, which are discussed below.

Rabbit tissues and gut flora

Table 5 shows the ability of faecal homogenates (10% in Todd–Hewitt broth) from six rabbits, which had been on a cyclamate diet for 3 months and then for 5 months, to convert cyclamate into cyclohexylamine. The activity of the faeces in carrying out this conversion depends on the individual rabbit, no. 3 being a good converter and no. 5 a poor converter. After 5 months on cyclamate, the ability of faeces from rabbits nos. 1 and 4 to form cyclohexylamine had increased, whereas that from the others had diminished slightly. These results correlate well with the metabolism of cyclamate *in vivo* described in the preceding paper (Renwick & Williams, 1972*b*).

Table 6 shows the ability of the tissues and the gut contents of rabbits nos. 4, 5 and 6 after 5 months on a cyclamate diet to form cyclohexylamine from cyclamate. The tissues are inactive, as are also the intestinal contents of the three animals. Only the caecal and colon contents of rabbit no. 4 were significantly active, especially under anaerobic conditions. As Table 5 shows, the faeces of rabbit no. 4 were quite active in converting cyclamate into cyclohexylamine.

The organisms that appear to be able to convert cyclamate into cyclohexylamine in rabbit caecal contents are clostridia and enterobacteria (see Table 4). One colony out of five of enterobacteria and two colonies out of five of clostridia were able to form cyclohexylamine from cyclamate.

Guinea-pig tissues and gut contents

Table 7 shows the cyclamate-metabolizing activity of the gut contents of four guinea pigs that had been kept on a cyclamate diet for 3 months. Of these animals only nos. 1 and 4 were able to form significant amounts of cyclohexylamine from orally administered cyclamate. The contents of the duodenum and ileum of all four animals were inactive, but the contents of the caecum and colon showed some activity, especially the colon contents of guinea pig no. 4.

Table 4. *Metabolism of cyclamate by faecal or caecal bacteria from man, rat and rabbit*

The isolated colonies were incubated with sodium [^{14}C]cyclamate (0.05 mg; 0.3 μCi) for 48 h at 37°C under N_2. [^{14}C]Cyclohexylamine was determined by the combined g.l.c. and ^{14}C counting method.

Source of organisms ...	Rat faeces			Rabbit caecum			Human faeces		
	No. of colonies		Conversion of cyclamate (%)	No. of colonies		Conversion of cyclamate (%)	No. of colonies		Conversion of cyclamate (%)
Organisms	Tested	Active		Tested	Active		Tested	Active	
Enterobacteria	5	0	—	5	1	0.53	5	0	—
Enterococci	5	0	—	5	0	—	5	5	0.14–0.35
Clostridia	5	2	0.04, 0.68	5	2	0.21, 0.55	5	0	—
Bacteroides	5	0	—	5	0	—	5	0	—
Bifidobacteria	5	0	—	5	0	—	5	0	—

Table 5. *Conversion of cyclamate into cyclohexylamine by rabbit faecal homogenates*

The faecal homogenates (10ml) containing [^{14}C]cyclamate (0.1mg; 0.5μCi) were incubated at 37°C for 60h under N_2. Cyclohexylamine was determined by combined g.l.c. and ^{14}C counting. The rabbits used were on a cyclamate diet for 3 and then for 5 months.

Rabbit no.	Cyclohexylamine found after incubation (% of added cyclamate) Months on cyclamate ... 3	5
1	6.3	45.7
2	1.5	0.8
3	97.1	32.9
4	2.4	28.4
5	0.1	*
6	9.2	2.6

* No faeces passed.

Table 6. *Metabolism of cyclamate by tissues and gut contents of rabbits*

Rabbits nos. 4, 5 and 6 had been on a cyclamate diet for 5 months (see Table 5) and rabbits A and B had received no cyclamate. The activities of the tissues and gut contents were determined by incubation with sodium [^{14}C]cyclamate (0.05mg; 0.4μCi) as described in the text.

	Conversion into cyclohexylamine (% of added cyclamate)		
	Cyclamate-pretreated		Normal
Rabbit no. ...	4	5, 6	A, B
Controls	<0.1	<0.1	<0.1
Liver, kidney, spleen or blood	<0.1	<0.1	<0.1
Intestinal contents			
Anaerobic	<0.1	<0.1	<0.1
Aerobic	<0.1	<0.1	<0.1
Caecal contents			
Anaerobic	6.53	<0.1	<0.1
Aerobic	1.57	<0.1	<0.1
Colon contents			
Anaerobic	7.71	<0.1	<0.1
Aerobic	3.85	<0.1	<0.1

The correlation in this species between the metabolism *in vivo* and the metabolism *in vitro* by gut organisms was poor. This may be due to individual variations in the time taken for the oral dose of cyclamate to pass along the gastrointestinal tract (see preceding paper: Renwick & Williams, 1972*b*, Table 1).

Human faeces

The ability of faecal samples obtained from three human subjects before, during and after taking a course of 3g of calcium cyclamate daily to convert cyclamate into cyclohexylamine is shown in Table 8. In the preceding paper (Renwick & Williams, 1972*b*) it was shown that of the three subjects only B. S. D. became a fairly active converter of cyclamate into cyclohexylamine when on a cyclamate diet. Table 8 shows that only faeces from this subject were able to form cyclohexylamine in large amounts, and only after he had been on a cyclamate diet. When cyclamate was removed from his diet, his faeces lost a considerable amount of the activity; simultaneously the urinary excretion of cyclohexylamine was greatly decreased (Renwick & Williams, 1972*b*). Faeces from the other two subjects (A. G. R. and P. C. H.),

Table 7. *Metabolism of cyclamate by intestinal contents of guinea pigs*

Four guinea pigs were kept on a cyclamate diet for 3 months. Their gut contents (0.5g) in Todd–Hewitt broth (10ml) were incubated at 37°C for 60h under N_2 with 0.1mg of sodium [^{14}C]cyclamate (0.5μCi). [^{14}C]Cyclohexylamine was determined by combined g.l.c. and ^{14}C counting. With animals not on a cyclamate diet, the gut contents were inactive, the conversion into cyclohexylamine being <0.05% of the added cyclamate under the above conditions.

Guinea pig no.	Conversion into cyclohexylamine (% of added cyclamate)				% of oral dose of cyclamate*
	Duodenum	Ileum	Caecum	Colon	
1	<0.05	<0.05	0.07	<0.05	0.15
2	<0.05	<0.05	0.19	0.17	0.02
3	<0.05	<0.05	0.13	0.08	<0.01
4	<0.05	<0.05	0.08	2.12	0.39

* Results of tests carried out before the animals were killed. They were given orally calcium cyclamate (450mg each) and each 24h urine sample was analysed for cyclohexylamine by g.l.c. (Renwick & Williams, 1972*b*).

Table 8. *Metabolism of cyclamate by human faecal homogenates*

Faecal samples (2g) were suspended in Todd–Hewitt broth (10ml). To each mixture was added sodium [^{14}C]cyclamate (0.15mg; 0.8μCi) and then it was incubated under N_2 at 37°C for 60h. The cyclohexylamine content of each tube was determined by combined g.l.c. and ^{14}C counting. When the subjects were on a cyclamate diet they took 3g of calcium cyclamate daily.

Conditions	Subject ...	Conversion into cyclohexylamine (% of added cyclamate)		
		B.S.D.	A.G.R.	P.C.H.
Cyclamate-free diet		0.06	<0.05	<0.05
Cyclamate diet for 10 days		74.9	<0.05	<0.05
Cyclamate-free diet for 6 days after 18 days on cyclamate		0.14	—	—
Cyclamate diet for 28 days		—	0.06	<0.05

who did not become very active converters of cyclamate into cyclohexylamine on a cyclamate diet, showed little or no activity.

Samples (1g) of faeces from each of the three subjects when they were on the cyclamate diet were incubated in Todd–Hewitt broth (5ml) with sodium [^{14}C]cyclamate (0.4mg; 2.6μCi) at 37°C for 60h under N_2. The incubation mixtures were examined by paper chromatography and radiochromatogram scanning for metabolites, with solvents A, B, C and D (see Renwick & Williams, 1972*a*). Cyclohexylamine was found to be the only metabolite of cyclamate in the faecal homogenate from B. S. D. No cyclohexylamine or any other product apart from cyclamate was found in the faecal homogenates from A. G. R. and P. C. H.

The results with individual strains of organisms isolated from the faeces of B. S. D. on a cyclamate diet are shown in Table 4, which indicates that the organisms active in converting cyclamate into cyclohexylamine are enterococci.

Table 3 also contains data relevant to man and shows that the viable counts of organisms in human faeces are not significantly altered by incorporating calcium cyclamate (3g/day) into the diet.

Further evidence for bacterial activity. Active faeces (0.1g) from subject B. S. D. were suspended in tryptone–yeast-extract broth (5ml). This was incubated for 24h at 37°C under N_2 and then 0.1ml was removed and added to fresh broth (4.9ml). Both tubes were then incubated similarly for 24h and centrifuged for 15min at 3000rev./min. The bacteria were resuspended in fresh tryptone–yeast-extract broth (5ml), and sodium [^{14}C]cyclamate (0.05mg; 0.3μCi) was added. These suspensions were then incubated under N_2 at 37°C for 60h and the cyclohexylamine formed was determined by the combined g.l.c. and ^{14}C-counting method.

Table 9. *Effect of medium and various preincubation conditions on cyclamate metabolism by human faecal homogenates*

Media and conditions were as described in the text. Each incubation mixture contained sodium [^{14}C]cyclamate (0.05mg; 0.3μCi).

Conditions	Conversion into cyclohexylamine (% of added cyclamate)		
	In tryptone–yeast-extract broth	In Todd–Hewitt broth	In brain–heart infusion
No preincubation	86.7	72.0	—
After preincubation in the presence of			
No additive	41.8	0.20	—
0.2% Calcium cyclamate	13.8	0.13	—
0.5% Calcium cyclamate	5.8	0.10	—
1.0% Calcium cyclamate	2.9	0.11	—
2.0% Calcium cyclamate	2.7	0.08	—
No additive	69.7	—	58.0
0.5% Calcium cyclamate	0.18	—	0.15
0.5% Sodium cyclamate	0.68	—	0.41
No additive	81.0	—	—
0.01% Cyclohexylamine hydrochloride	88.8	—	—
0.1% Cyclohexylamine hydrochloride	88.1	—	—
0.5% Cyclohexylamine hydrochloride	82.7	—	—
No additive	32.5	—	—
0.1% Calcium cyclamate	1.5	—	—
0.1% Calcium cyclamate	0.01*	—	—
0.1% Calcium cyclamate	0.01†	—	—

* Broth diluted 10-fold.
† Broth diluted 100-fold.

The bacteria from the original suspension (4.9ml) metabolized, in duplicate results, 67 and 81% of the added cyclamate, whereas those from the subculture of 0.1ml metabolized 22 and 28%. Had there been a straight dilution of the activity the subculture should have metabolized only 1.5% of the cyclamate. This experiment demonstrates that the activity is bacterial and that the metabolizing system is self-replicating.

Factors affecting the bacterial activity in vitro

Human faecal samples. The effect of the medium and the presence of cyclamate and cyclohexylamine on the human faecal organisms converting cyclamate into the amine was investigated as follows. The faecal homogenate (1ml of a 10%, w/v, homogenate in meat-infusion broth) was added to the medium (5ml) and preincubated under N_2 at 37°C for 48h, alone or in the presence of various concentrations of cyclamate or cyclohexylamine hydrochloride. The bacteria were removed by centrifugation and suspended in 1ml of the same medium containing sodium [^{14}C]cyclamate (0.05mg; 0.3μCi). The suspension was then incubated under N_2 at 37°C for 60h. The [^{14}C]cyclohexylamine formed was determined by the combined g.l.c. and ^{14}C-counting method.

Table 9 shows that preincubation in Todd–Hewitt broth, which contains glucose, causes almost total loss of the bacterial activity, the value of 72% before incubation falling to 0.2% after incubation. In the tryptone–yeast-extract medium, which does not contain glucose, much of the activity is retained, i.e. the loss is from about 87 to 42% in one case, and other values in Table 9 for experiments in which no additive was used vary from 32.5 to 81%. Much activity (58%) is also retained from preincubation in brain–heart infusion, which does not contain glucose. However, if preincubation is carried out in the presence of cyclamate there is a loss of activity in all the media whether sodium or calcium cyclamate is used. Cyclohexylamine hydrochloride, however, has little effect on the activity of the organisms at a concentration of up to 0.5% in the medium.

When 10% homogenates of the active faeces of subject B. S. D., in sterile broths made from the faeces of subjects B. S. D. and A. G. R. when they were on a cyclamate-free diet, were preincubated with or without cyclamate the resultant activities were similar to those obtained with tryptone–yeast-extract broth. This indicates that no extrinsic factor in the faeces of the converter or non-converter is responsible

for the activation or inhibition of the bacterial metabolism of cyclamate to cyclohexylamine.

The tryptone–yeast-extract medium and brain–heart infusion, unlike Todd–Hewitt broth, contain no glucose and therefore no rich source of energy, but if the tryptone–yeast-extract medium is diluted the activity of the bacteria is abolished. It would appear that in the absence of a readily available energy-rich source in the medium, the organisms may derive a source of energy by splitting cyclamate, although dilution of the appropriate medium does not induce them to utilize cyclamate.

Rat and rabbit faecal samples. Faecal samples were prepared from two rats and from rabbit no. 4 (see Table 5), which were known converters of cyclamate into cyclohexylamine. These samples were prepared, preincubated under various conditions and then incubated with sodium [^{14}C]cyclamate as described above for the human material. The results are shown in Table 10, which indicates that the metabolic activity of the organisms from rat faeces was increased by preincubation with cyclamate and inhibited by cyclohexylamine, whereas that from rabbit faeces was suppressed by cyclamate, although the effect of cyclohexylamine was equivocal.

Absorption of [^{14}C]cyclohexylamine from the gut

For urinary cyclohexylamine to arise from the metabolism of cyclamate by gut bacteria it is essential that cyclohexylamine is absorbed from the gut. However, this absorption must occur at the site of formation, i.e. the caecum, colon and rectum, and not in the small intestine, which is probably the site of absorption of orally administered cyclohexylamine (Renwick & Williams, 1972*a*). Absorption from the gut could be measured by the appearance of [^{14}C]-cyclohexylamine in the blood after injection into the gut lumen. However, after intravenous administration of [^{14}C]cyclohexylamine the ^{14}C was rapidly removed from the blood and distributed to various tissues, mainly the liver, kidney, spleen and lung, as previously shown by Sonders *et al.* (1968).

The absorption of [^{14}C]cyclohexylamine from the gut was determined in rats anaesthetized with Nembutal (40mg/kg; intraperitoneally injected) and

Table 10. *Effect of cyclamate and cyclohexylamine on the activity of rat and rabbit faecal organisms*

Suspensions of bacteria from rat and rabbit faeces were prepared as described in the text. They were incubated with sodium [^{14}C]cyclamate and the cyclohexylamine formed was estimated as described in the text.

Conditions	Conversion into cyclohexylamine (% of added cyclamate)	
	Rat faeces	Rabbit faeces
No addition	0.4	2.8
0.1% Calcium cyclamate	0.54	1.2
0.5% Calcium cyclamate	1.22	0.6
1.0% Calcium cyclamate	2.22	0.3
0.5% Cyclohexylamine hydrochloride	0.04	0.5–4.0

Table 11. *Urinary excretion of ^{14}C in rats given [^{14}C]cyclohexylamine by various routes*

Anaesthetized animals were prepared as described in the text and given [^{14}C]cyclohexylamine (5mg/kg; 2.3μCi/kg). The results are the mean for three animals.

Time after injection (min)	Route of administration ...	Urinary excretion of ^{14}C (% of dose)		
		Intravenous	Intracolonic	Intracaecal
15		13	2	2
30		20	6	5
60		31	17	13
90		37	26	21
120		44	36	29
180		57	49	38
240		65	61	46
300		71	69	51

in which a cannula was inserted into the urinary bladder and the urethra ligated. [^{14}C]Cyclohexylamine was injected intravenously and into the lumen of various regions of the gut, and the ^{14}C in the bladder determined by collecting the urine and rinsing the bladder with 0.9% NaCl soln. (3× 0.3ml) at regular intervals (Table 11). Rapid elimination was found after intravenous administration (71% in 5h), whereas injection into the colon gave a slower initial excretion but a similar total (69% in 5h). Excretion, after injection into the caecum, was slower than in the other two cases (51% in 5h) but still appreciable.

Discussion

That cyclohexylamine is a metabolite of cyclamate in the urine was first observed by Kojima & Ichibagase (1966), but the site of its formation has been the subject of conflicting reports. Kojima & Ichibagase (1968) incubated cyclamate with rat liver homogenates and claimed to have detected by g.l.c. very small peaks due to cyclohexylamine, cyclohexanol and cyclohexanone. However, since the animals used had received seven daily doses of cyclamate before being killed, it is not clear whether these compounds were formed in the homogenate or were present in the liver as a result of pretreatment with cyclamate. Wallace *et al.* (1970), however, found no metabolism of [^{14}C]cyclamate when it was incubated with various tissues from normal rats and Prosky & O'Dell (1971) found no [^{14}C]cyclohexylamine to be formed when the livers of cyclamate-pretreated rats were perfused with [^{14}C]cyclamate. Our own results support the view that cyclamate is not metabolized by tissues of the rat or rabbit (see Tables 1 and 6).

That the gut plays a role in the conversion of cyclamate into cyclohexylamine is indicated by a number of authors. Sonders *et al.* (1969), in a brief report, stated that in a single cyclamate-pretreated rat 47% of an oral dose of [^{14}C]cyclamate was converted into [^{14}C]cyclohexylamine, whereas only 1% of an intravenously injected dose was converted. In another brief report, Golberg *et al.* (1969) stated that when [^{14}C]cyclamate was incubated anaerobically with the gut contents of dogs, up to 0.2% was converted into cyclohexylamine. More recently Dalderup *et al.* (1970) found that cyclamate was converted into cyclohexylamine when incubated anaerobically with the faeces of cyclamate-pretreated rats in broths containing no glucose. These workers also reported that the ability to metabolize cyclamate could be transferred from one rat to another, presumably through coprophagy. In contrast to these reports both Leahy *et al.* (1967) and Davis *et al.* (1969) were unable to show the formation of cyclohexylamine from cyclamate incubated with the faeces of human converters. Davis *et al.* (1969), however, prepared their faecal homogenates with normal saline and this may have killed many of the micro-organisms present (see Straka & Stokes, 1957).

Our own results show that the conversion of cyclamate into cyclohexylamine is a function of the gut flora, which, however, have to be trained to do this by the administration of cyclamate orally over a period of time to the whole animal. All of the Wistar albino rats, when kept on a cyclamate diet, become converters and their faeces are also able to do this (Table 1), particularly under anaerobic conditions. It would appear that the organisms capable of carrying out this reaction in rat faeces are clostridia (Table 4) and that the numbers of these organisms in the rat increase considerably (Table 3) when the animals are kept on a cyclamate diet for a prolonged period. The training of the organisms is illustrated in Table 2, for if the total organisms of the rat colon and rectum are subcultured they tend to lose the ability to convert cyclamate into cyclohexylamine, but if subcultured in the presence of cyclamate the loss of activity is retarded.

The faeces of the rabbit also acquire the ability to convert cyclamate into cyclohexylamine, although the activity acquired varies with each animal (Table 5); this variation corresponds to the urinary excretion of cyclohexylamine after oral administration of cyclamate (Renwick & Williams, 1972*b*). In the rabbit the activity is associated with the contents of the caecum and colon (Table 6) but not with the tissues. Furthermore, the activity in rabbit caecal contents seems also to be associated with clostridia, which are not numerous in the rabbit, and with enterobacteria, which are present in great numbers (Smith, 1965).

Our experience with guinea pigs was that they did not become very active converters of cyclamate into cyclohexylamine, and this is reflected by the poor ability of the caecum and colon contents to carry out the conversion (see Table 7) even though the animals were kept on a cyclamate diet for 3 months.

The variability in developing the ability to metabolize cyclamate is well illustrated by the three human subjects used in this study. Only one became a good converter on being put on a cyclamate diet, and the activity of his faeces reflected this ability (Table 8). Further, this activity was soon lost on a cyclamate-free diet. In this subject it appears that the organisms that acquire the converting ability are enterococci (Table 4), although there is no marked change in the counts of various faecal organisms in our subjects when put on a cyclamate diet (Table 3).

Our results suggest that the organisms that acquire the ability to split cyclamate are different in different animal species. In the rat, the organisms appear to be clostridia, and in the rabbit enterobacteria, since clostridia occur only in small numbers, and in man

enterococci. This view is supported by the observations that the activity of rat and rabbit faecal homogenates is affected in different ways by the presence of cyclamate or cyclohexylamine in the incubation mixture (Table 10). Cyclamate increases whereas cyclohexylamine abolishes the activity of rat faeces, but with rabbit faeces cyclamate diminishes the activity whereas the effect of cyclohexylamine is equivocal. Cyclamate also diminishes the activity of human faeces but cyclohexylamine has no effect (Table 9). The suggestion that the active organisms in human faeces are enterococci is of interest in relation to the ability of most Japanese to convert cyclamate into cyclohexylamine. According to Hill *et al.* (1971) the faeces of normal Japanese contain on average 10^8 enterococci/g, whereas the faeces of British and American subjects contain much fewer of these organisms (10^5/g).

Cyclohexylamine, once formed in the gut, is readily absorbed from the caecum and colon of rats as shown in Table 11. It has been shown that orally administered cyclohexylamine is mainly excreted in the urine (Renwick & Williams, 1972*a*), although in this case it could have been absorbed in the small intestine. If the cyclohexylamine formed from cyclamate were not absorbed in the rat it could inhibit the conversion of cyclamate into cyclohexylamine (see Table 10), although this would not necessarily be true for man and possibly the rabbit.

It appears therefore that cyclamate, being highly polar, is not readily absorbed and is therefore likely to pass along the intestine to areas containing high concentrations of micro-organisms. Some of these organisms then develop the ability to split cyclamate to cyclohexylamine, which is absorbed and mainly excreted in the urine, although a small proportion of the amine is further metabolized by deamination and ring hydroxylation. The mechanism of the splitting of cyclamate is unknown at the present time but it is clear that it occurs in the gut under anaerobic conditions. Two mechanisms are possible, namely hydrolysis and reductive fission:

$$C_6H_{11}NH\text{–}SO_3^- \xrightarrow{H_2O} C_6H_{11}NH_2 + HSO_4^-$$

$$C_6H_{11}NH\text{–}SO_3^- \xrightarrow{H_2} C_6H_{11}NH_2 + HSO_3^-$$

However, our results do not indicate which of these mechanisms is used; possibly both occur.

The present work suggests that prolonged continued intake of cyclamate could cause any person to become to some degree a converter of cyclamate into cyclohexylamine and its metabolites. Price *et al.* (1970) indicated that cyclohexylamine might be carcinogenic in the rat and it would appear that the production of cyclohexylamine from cyclamate by the gut flora could be of considerable importance in any toxic effect of the sweetener.

This work was in part supported by the International Sugar Research Foundation Inc., Bethesda, Md., U.S.A.

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