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The Fate of Cyclamate in Man and Other Species

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1. ^{14}C-labelled cyclamate has been administered to guinea pigs, rabbits, rats and humans. When given orally to these species on a cyclamate-free diet, cyclamate is excreted unchanged. In guinea pigs some 65% of a single dose is excreted in the urine and 30% in the faeces, the corresponding values for rats being 40 and 50%, for man, 30–50% and 40–60%, and for rabbits, 90 and 5%, the excretion being over a period of 2–3 days. 2. Cyclamate appears to be readily absorbed by rabbits but less readily by guinea pigs, rats and humans. 3. If these animals, including man, are placed on a diet containing cyclamate they develop the ability to convert orally administered cyclamate into cyclohexylamine and consequently into the metabolites of the latter. The extent to which this ability develops is variable, the development occurring more readily in rats than in rabbits or guinea pigs. In three human subjects, one developed the ability quite markedly in 10 days whereas two others did not in 30 days. Removal of the cyclamate from the diet caused a diminution in the ability to convert cyclamate into the amine. 4. In rats that had developed the ability to metabolize orally administered cyclamate, intraperitoneally injected cyclamate was not metabolized and was excreted unchanged in the urine. The biliary excretion of injected cyclamate in rats was very small, i.e. about 0.3% of the dose. 5. The ability of animals to convert cyclamate into cyclohexylamine appears to depend upon a continuous intake of cyclamate and on some factor in the gastrointestinal tract, probably the gut flora.

Cyclamic acid (cyclohexylsulphamic acid) as its sodium or calcium salt has been widely used as an artificial sweetening agent since 1959, when it was pronounced to be 'generally regarded as safe' (GRAS) by the Food and Drugs Administration (U.S.A.). It was, however, banned at the end of 1969 because it was suspected of being a bladder carcinogen in rats (Price *et al.*, 1970). It is a strong acid of pK_a 1.9 (Kojima *et al.*, 1966) and therefore would be expected to be excreted mainly unchanged when administered to animals (see Williams, 1959). The earlier work on the metabolism of cyclamate (e.g. Taylor *et al.*, 1951; Miller *et al.*, 1966) seemed to confirm this view, but Kojima & Ichibagase (1966) found cyclohexylamine in the urine of dogs and humans, but not in urine of rabbits, after oral doses of sodium cyclamate. This was supported by Leahy *et al.* (1967), who found cyclohexylamine in the urine of a small proportion of a number of human volunteers who had taken sodium cyclamate orally. Subsequently several authors have reported the excretion of cyclohexylamine in the urine of man and animals receiving cyclamate orally.

The excretion of cyclohexylamine in the urine of cyclamate-pretreated rats has been reported, in some cases briefly, by Kojima & Ichibagase (1968), Oser *et al.* (1968), Sonders *et al.* (1969), Dalderup *et al.* (1970), Wallace *et al.* (1970) and Prosky & O'Dell (1971) and in the urine of humans on a cyclamate diet by Wills *et al.* (1968), Davis *et al.* (1969), Kojima & Ichibagase (1969), Asahina *et al.* (1971) and Litchfield & Swan (1971). Kojima & Ichibagase (1968) have also shown that rabbits on daily oral doses of cyclamate excreted undefined amounts of cyclohexylamine, cyclohexanol and cyclohexanone.

The present work, which has in part been briefly reported (Renwick & Williams, 1969, 1970), describes the metabolism of [^{14}C]cyclamate in normal and cyclamate-pretreated rats, rabbits, guinea pigs and humans in more quantitative detail than earlier work. It will be shown that in guinea pigs, rats and rabbits, orally administered cyclamate is almost entirely excreted unchanged in the urine and faeces, but if these animals are kept on a diet containing cyclamate for several months they can develop the ability to convert cyclamate into cyclohexylamine and its metabolites. Some experiments have also been carried out on humans with similar results. Evidence will also be presented to show that the conversion of cyclamate into cyclohexylamine is probably carried out by the gut flora, an aspect which is further investigated in the next paper (Drasar *et al.*, 1972).

Materials and Methods

Chemicals

Calcium cyclamate dihydrate was purchased from Abbott Laboratories Ltd., Queenborough, Kent, U.K. As measured by g.l.c. its cyclohexylamine

content was less than 0.0015% and did not increase over 2 years. Cyclohexylsulphamic acid, m.p. 182°C, was purchased from R. N. Emanuel Ltd., Wembley, Middx., U.K. [U-^{14}C]Cyclohexylsulphamic acid (9.42μCi/mg) was supplied by Mallinckrodt Nuclear, St. Louis, Mo., U.S.A. and was a gift of the International Sugar Research Foundation, Bethesda, Md., U.S.A. Paper chromatography (see Renwick & Williams, 1972; preceding paper) showed it to contain 0.6% cyclohexylamine. The [^{14}C]cyclamic acid (96mg; 910μCi) in water (2ml) was passed through an ion-exchange column (10ml; Zeo-Karb 225 SRC 13; Na^+ form) and eluted with water. The first 50ml of eluate contained 99% of the ^{14}C and paper chromatography showed it to contain no cyclohexylamine, whereas solvent extraction indicated no more than 0.02–0.03% of cyclohexylamine. The solution of purified [^{14}C]cyclamic acid was neutralized (pH7) with $NaHCO_3$ and stored at −15°C. Freeze-drying of the aqueous solution of free [^{14}C]cyclamic acid resulted in the loss of 90% of the ^{14}C, which was recovered in the 'cold finger' (at −70°C) of the apparatus. Freeze-drying of aqueous solutions of cyclamic acid was therefore carried out only after their adjustment to pH7. Other compounds were prepared as described in the preceding paper (Renwick & Williams, 1972).

Animals

Female Wistar albino rats (Oxford Laboratories Animal Centre), Duncan–Hartley strain albino guinea pigs and New Zealand White rabbits were used. Sodium cyclamate was administered in aqueous solution by intragastric intubation or intraperitoneal injection. Animals were housed in appropriate metabolism cages so that urine and faeces could be collected separately; when necessary, the expired air could also be collected. During experiments the animals had free access to suitable cyclamate-free food and water.

Pretreatment with cyclamate. The animals were allowed free access to food and water but their water contained 0.5% (w/v) of calcium cyclamate. During pretreatment, which lasted several months, six or seven rats were housed to a cage and three or four guinea pigs to a cage. Rabbits were housed singly.

Collection of expired air. For CO_2 collection the expired air was drawn through two wash-bottles fitted with sintered-glass inlets and each containing 300ml of 4M-NaOH. For other volatile metabolites, the expired air was drawn through two tubes packed with anhydrone [anhydrous $(MgClO_4)_2$] and then through two tubes each containing ethanol (50ml) kept at −70°C. For simultaneous collection of CO_2 and volatile metabolites these tubes were used in series: 1, NaOH, 2, anhydrone and 3, ethanol.

Determination of cyclamate, cyclohexylamine and metabolites

Cyclamate and its metabolites. [^{14}C]Cyclamate and its metabolites were normally determined by paper chromatography and liquid-scintillation counting as described by Renwick & Williams (1972).

Cyclohexylamine. (*a*) By g.l.c. Sodium chloride (0.2g/ml of the 24h urine, or a sample not exceeding 1 litre) was dissolved in the urine and the solution was adjusted to pH>11 with 10M-NaOH. The solution was extracted four times with 0.1vol. of dichloromethane. Each extract was centrifuged for 15min at 2000rev./min and the aqueous phases, together with any emulsion which formed, were pooled for the next extraction. The extracts were passed through cotton-wool and then extracted twice with 0.2M-HCl (0.05vol. of original urine). The acid extract was cooled, adjusted to pH>11 with 10M-NaOH, saturated with NaCl and extracted with dichloromethane (2×5ml). This extract was passed through a column (3cm×3mm diam.) of anhydrous Na_2SO_4 and then diluted to 10ml with dichloromethane. A sample (2μl) of this solution was analysed for cyclohexylamine by g.l.c. The instrument used was a Hewlett Packard Chromatograph (F. and M. Scientific 402 High Efficiency Gas Chromatograph) with a flame ionization detector and fitted with a 5ft (1.54m) glass column (3mm internal diam.) packed with polyethylene glycol 6000 (10%) and KOH (2%) on Chromosorb G (80–100 mesh). Cyclohexylamine had a retention time of 4.5min under the conditions used, i.e.: column temp. 115°C; detector temp. 160°C; flash heater temp. 150°C; hydrogen pressure 20lb/in^2 (138kN·m^{-2}); air pressure 25lb/in^2 (172kN·m^{-2}), nitrogen pressure 40lb/in^2 (276kN·m^{-2}); flow rate 38ml/min. The detector response was linear between 0.5 and 25μg of cyclohexylamine, and 0.1–0.5μg could be detected but not accurately measured. If no cyclohexylamine was detected the whole urine extract was concentrated to 1ml at 30°C under N_2 and again subjected to g.l.c. The recovery of cyclohexylamine at 5mg/litre of urine was 80–90% before the evaporation step and 60–70% after evaporation.

(*b*) By ^{14}C counting. When [^{14}C]cyclamate was administered cyclohexylamine was also determined by reverse isotope dilution as described in the preceding paper (Renwick & Williams, 1972).

(*c*) By combined g.l.c. and ^{14}C counting. This was a rapid method for determining [^{14}C]cyclohexylamine and was used particularly for human urine and for faecal homogenates incubated with cyclamate [see the next paper (Drasar *et al.*, 1972)].

To the neutral sample of urine or homogenate expected to contain [^{14}C]cyclohexylamine was added an aq. 13.6% (w/v) non-radioactive cyclohexylamine hydrochloride soln. (0.5ml, i.e. 50mg of cyclohexylamine). The mixture was saturated with NaCl,

treated with 10M-NaOH (1ml) and then extracted by shaking for 30min with dichloromethane (10ml). The mixture was centrifuged to break any emulsion and the dichloromethane phase was separated and passed through cotton-wool. The extract was then shaken for 30min with 2M-HCl (5ml) and the aqueous phase separated, brought to pH12 with 10M-NaOH, saturated with NaCl and then extracted by shaking for 30min with dichloromethane (10ml). The organic phase was separated and filtered through cotton-wool. ^{14}C in a sample (1ml) was determined by liquid-scintillation counting and the extent of extraction of cyclohexylamine was measured by g.l.c. With the internal standard of cyclohexylamine, this method allowed a rapid and accurate determination of [^{14}C]-cyclohexylamine, the recoveries being in the range

Table 1. *Excretion of ^{14}C by normal and cyclamate-pretreated guinea pigs after dosing with [^{14}C]cyclamate*

Two groups of three guinea pigs were used. One group was normal and the other had been pretreated with cyclamate for 6 months. Each animal was given an oral dose of sodium [^{14}C]cyclamate (56mg/kg) containing 14μCi of ^{14}C. Urine and faeces were analysed daily for ^{14}C by liquid-scintillation counting. Results for days 1, 2 and 6 or 7 only are quoted. Ranges are quoted only for the first and last days.

	^{14}C excreted (cumulative % of dose)					
	Normal animals			Pretreated animals‡		
Day	Urine	Faeces	Total	Urine	Faeces	Total
1	54 (26–90)	13 (0.4–36)	67 (46–93)	23 (13–34)	16 (6–26)	39 (37–40)
2	62	26	88	33	23	56
6 or 7*	65	29	94 (88–97) (c.w. 1%)†	39	26	65 (55–71) (c.w. 20%)†

* 6 days for pretreated animals and 7 days for normal animals.

† c.w., Cage wash. The value for the pretreated animals is high, but it appeared to be derived mainly from urine. The recovery of ^{14}C, however, is 85% including c.w.

‡ Expired air in day 1 was trapped and analysed for $^{14}CO_2$ and ethanol-soluble volatile metabolites, both of which were negligible (0.03–0.07% and <0.01% respectively).

Table 2. *Metabolites of cyclamate in normal and pretreated guinea pigs*

Animals, doses and pretreatment were as described in Table 1. The urine and faeces were analysed by paper chromatography.

		^{14}C in the first 24h excreta (% of dose)			
Animal no.	Material analysed	^{14}C in sample	Cyclamate	Cyclohexylamine	Other metabolites
Normal animals					
1	Urine	45.0	45.0	0.0	0.0
2		26.4	26.4	0.0	0.0
3		89.6	89.6	0.0	0.0
1	Faeces	24.3*	24.3	0.0	0.0
2		35.7	35.7	0.0	0.0
3		3.4	—	—	—
Pretreated animals					
5	Urine	33.9	33.0	0.3	0.6
6		21.9	18.8	2.5	0.6
7		13.4	12.8	0.4	0.2
5	Faeces	5.6	5.6	0.0	0.0
6		15.4	15.0	0.4	0.0
7		25.9	25.9	0.0	0.0

* Sample for second 24h after dosing, since this animal passed very little faeces during the first 24h.

95–104% (0.06μCi; 0.1mg). The method is non-specific and was used when cyclohexylamine was the only ^{14}C-labelled amine likely to be present.

Results

Guinea-pig urine

Table 1 shows the recovery of ^{14}C in normal and cyclamate-pretreated guinea pigs. The recovery of ^{14}C was satisfactory but it would appear that the elimination was slower in the pretreated guinea pigs than in normal animals. About one-third of the ^{14}C was excreted in the faeces and the rest in the urine in 6–7 days. Table 2 shows the nature of the ^{14}C in the urine excreted in 24h after dosing. In the normal animals the radioactivity was entirely in the form of cyclamate and no other product was detected. In the pretreated animals, although the main metabolite in the urine was cyclamate, the urine contained cyclohexylamine (0.3–2.5% of the dose) and small amounts of other compounds. When chromatographed on paper these other compounds separated with R_F 0.06 in solvent D (see the preceding paper: Renwick & Williams, 1972) and appeared to be similar to the metabolites found after administration of [^{14}C]-cyclohexylamine, i.e. mainly conjugates of *trans*-cyclohexane-1,2-diol (Renwick & Williams, 1972). The ^{14}C in the faeces of the pretreated animals was entirely in the form of cyclamate, except in one animal in which cyclohexylamine was found, but

Table 3. *Excretion of ^{14}C by normal and cyclamate-pretreated rabbits receiving [^{14}C]cyclamate*

Two groups of three rabbits were used, one normal and the other having received a cyclamate-containing diet for 5 months. Each animal was given an oral dose of sodium [^{14}C]cyclamate (56mg/kg), 15μCi to normal animals and 20μCi to pretreated animals. The urine and faeces were analysed daily for ^{14}C. c.w., Cage wash.

	^{14}C excreted (cumulative % of dose)					
	Normal animals			Pretreated animals		
Time after dosing (days)	Urine	Faeces	Total	Urine	Faeces	Total
1	82 (71–92)	5 (2–8)	87 (79–94)	70 (65–75)	5 (3–7)	75 (68–82)
2	89	6	95	75	6	81
3	90	6	96	76	6	82
6	91	6	97 (93–97) (c.w. 1%)	—	—	—
8	—	—	—	77	7	84 (77–89) (c.w. 5%)

Table 4. *Metabolites of [^{14}C]cyclamate in the urine of normal and cyclamate-pretreated rabbits*

Two groups of three rabbits were used, one normal and the other having received a cyclamate diet for 5 months. The oral dose of sodium [^{14}C]cyclamate was as described in Table 3. The urine excreted in the first 24h after the oral dose of [^{14}C]cyclamate was analysed by paper chromatography.

	^{14}C (% of dose)				
	In sample	Cyclamate	Cyclohexylamine	Cyclohexyl-glucuronide	Other metabolites
Normal animals					
A	70.9	70.2	0.04*	0.0	0.7
B	92.1	91.3	0.01*	0.0	0.9
C	81.7	80.9	0.07*	0.0	0.8
Pretreated animals					
1	69.5	40.8	6.9 (6.6)*	8.2	13.5
2	74.7	73.4	0.0 (0.03)*	0.0	0.0
3	65.1	45.0	4.2 (4.5)*	6.2	9.3

* Determined by isotope dilution; the concentration in the cyclamate administered was 0.03–0.04%.

some contamination of the faeces by urine cannot be excluded.

Rabbit urine

Table 3 shows the recovery of ^{14}C in normal and cyclamate-pretreated rabbits. In both the ^{14}C was excreted mainly in the urine, 91% in 6 days in normal rabbits and 77% of the dose in 8 days in the pretreated animals. The faecal excretion in both was about 6–7%. The recovery of ^{14}C was better in the normal animals (98%) than in the pretreated (89%) and again it would appear that the elimination was a little slower in pretreated than in normal animals.

In Table 4 the nature of the ^{14}C excreted in the urine in 24h after dosing is shown. In normal animals there may have been a slight metabolism of cyclamate, since 0.01–0.07% of the dose of ^{14}C appeared as cyclohexylamine and 0.7–0.9% as other compounds. This unknown material had a lower R_F value than cyclamate in all four solvents and was possibly an artifact similar to that reported previously by Miller *et al.* (1966). In two of the pretreated animals there was an appreciable metabolism of cyclamate, but the third animal excreted the cyclamate almost entirely unchanged. Both cyclohexylamine (6.9 and 4.2% of the dose) and cyclohexyl glucuronide (8.2 and 6.2%) were detected in the urine of these two animals together with other metabolites (13.5 and 9.3% of the dose), which were separated from cyclamate, cyclohexylamine and cyclohexyl glucuronide in solvent D with R_F 0.59. Paper chromatography in all four solvents indicated that this material contained compounds similar to those excreted after the administration of [^{14}C]cyclohexylamine (Renwick & Williams, 1972). The unknown material found in the urine of normal rabbits given [^{14}C]cyclamate was also detected in the urine of cyclamate-pretreated animals [0.8% (0.4–1.3%) of the dose in three rabbits].

The variability in the metabolism of cyclamate in individual pretreated rabbits is shown in Table 5. After 3 months' pretreatment five out of six rabbits were producing detectable amounts of cyclohexylamine in quantities from 0.03 to 7.4% of the dose of cyclamate, but one rabbit (no. 5), produced no detectable cyclohexylamine. On continuing the pretreatment for 5 months, the output of cyclohexylamine showed a tendency to increase.

Human urine

The recovery of ^{14}C in three human subjects receiving [^{14}C]cyclamate orally is shown in Table 6. On a cyclamate-free diet the recovery was 87–90% in 4–5 days after dosing. In two of the subjects (P.C.H. and A.G.R.) more ^{14}C was recovered in the faeces (50 and 60%) than in the urine (37 and 30%), but the other subject (B.S.D.) excreted rather more in the urine (49%) than in the faeces (38%). The three subjects were next given orally 3g of calcium cyclamate daily. After B.S.D. had been 17 days and the other subjects 30 days on this regime they were given orally 1g of calcium cyclamate containing 8.8μCi of ^{14}C. In this experiment (see Table 6) the recovery of ^{14}C in 4–5 days was 96–99% of the dose. In each case, the recovery of ^{14}C in the urine was rather higher than when the subjects were on a cyclamate-free diet, but the faecal excretion was the same as before the cyclamate diet. Table 7 shows the analysis of the urine samples from these three subjects for cyclohexylamine. One subject, B.S.D., excreted cyclohexylamine to the extent of 5% of the dose of ^{14}C before being put on a cyclamate diet, but after 17 days on a cyclamate diet the output of cyclohexylamine increased to nearly 8% of the dose. The

Table 5. *Cyclohexylamine excretion by rabbits pretreated with cyclamate*

Six rabbits were kept on a cyclamate-containing diet for 5 months. Each was then given an oral dose of calcium cyclamate (500mg/kg) and the cyclohexylamine in the next 24h urine sample was determined by g.l.c. None of the animals excreted cyclohexylamine (i.e. <0.01%) when given a similar dose before pretreatment. The cyclohexylamine content of the calcium cyclamate administered was <0.0015%.

Animal no.	Period of pretreatment (months) ...	Conversion into cyclohexylamine (% of dose)	
		3	5
1		0.21	4.3
2		0.05	0.06
3		7.4	4.8
4		0.03	7.2
5		<0.01	0.01
6		0.04	0.02

Table 6. *Excretion of ^{14}C by human subjects receiving [^{14}C]cyclamate*

Three male human subjects were given orally, in water, calcium [^{14}C]cyclamate (1 g containing 8–9 μCi of ^{14}C). Their urine and faeces were analysed for ^{14}C by scintillation counting. Subjects were pretreated with cyclamate as described in the text.

		^{14}C excreted (cumulative % of dose)					
		Normal subject			Pretreated subject		
Subject	Time after dosing (days)	Urine	Faeces	Total	Urine	Faeces	Total
B.S.D.	0.25–0.5	7	6	13	14	2	16
	1	21	—	27	50	30	80
	2–3	40	16	56	59	—	89
	3	45	34	79	63	33	96
	4–5	49	38	87	63	33	96
P.C.H.	0.25	10	—	10	8	—	8
	1	20	7	27	24	—	24
	2–3	33	30	63	40	46	86
	3	35	—	65	43	47	90
	4–5	37	50	87	45	54	99
A.G.R.	0.25	9	—	9	18	—	18
	1	17	28	45	27	—	27
	2–3	25	38	63	40	46	86
	3	29	—	67	41	51	92
	4–5	30	60	90	42	54	96

Table 7 *Excretion of cyclohexylamine by human subjects receiving [^{14}C]cyclamate*

The human subjects were given, before and after being kept on a cyclamate diet, 1 g of calcium [^{14}C]cyclamate containing 9.5 μCi (normal subjects) or 8.8 μCi (pretreated subjects) of ^{14}C. Their urine was analysed daily by solvent extraction (see the text).

		^{14}C excreted (cumulative % of dose)			
		Normal subjects		Pretreated subjects	
Subject	Day no.	^{14}C in sample	Cyclohexylamine	^{14}C in sample	Cyclohexylamine
B.S.D.*	1	20.7	0.2	44.5	1.9
	2	35.5	1.7	59.3	5.4
	3	44.6	4.3	63.0	7.7
	4	48.8	5.2	—	—
P.C.H.†	1	20.0	0.02	24.1	0.01
	2	31.2	0.02	40.1	0.01
	3	34.6	0.02	43.4	0.01
	4	36.9	0.02	44.6	0.01
A.G.R.†	1	17.2	0.02	26.8	0.01
	2	23.7	0.02	39.5	0.01
	3	28.7	0.02	41.4	0.01
	4	29.7	0.02	—	—

* Pretreated for 17 days.
† Pretreated for 30 days.

other two subjects excreted little or no cyclohexylamine before or after being put on a cyclamate diet. Other metabolites of cyclohexylamine were not detected in human urine by using paper chromatography.

The three subjects were examined in more detail

Table 8. *Excretion of cyclohexylamine in human subjects receiving daily doses of cyclamate*

Three human subjects took 3g of calcium cyclamate each day, day 1 being the day on which the first dose was taken. Each day's urine was analysed for cyclohexylamine by g.l.c.

Time on cyclamate (days)	Subject ...	Cyclohexylamine in urine (% of dose) B.S.D.	P.C.H.	A.G.R.
0†		<0.002	<0.002	<0.002
1		0.07	<0.002	0.003
5		5.8	0.005	0.013
10		17.3	0.015	0.014
12		13.5	0.005	0.007
15		16.4	0.034	0.071
20		—	0.053	0.160
25		—	0.052	0.140
26		(0.25*)	—	—
27		(1.6*)	—	—
28		—	0.014	0.050

* B.S.D. stopped taking daily cyclamate on day 18 and remained on a cyclamate-free diet. On day 26 he was given 3g of calcium cyclamate and his urine analysed for cyclohexylamine on days 26 and 27.

† No cyclamate taken.

during the period of cyclamate ingestion, as shown in Table 8. Their urine samples were examined for cyclohexylamine by g.l.c. on various days during a regime in which they took orally 3g of calcium cyclamate each day. On the first day of the cyclamate regime two of the subjects excreted detectable cyclohexylamine, but it can be seen that B.S.D. developed the ability to convert cyclamate into cyclohexylamine, the conversion being as much as 17% on the tenth day. The other two subjects did not develop this ability during 30 days on cyclamate, but there was a slight increase in the conversion, which reached a maximum of 0.05% in P.C.H. and 0.16% in A.G.R. B.S.D. ceased taking daily cyclamate on the eighteenth day, and after 8 days on a cyclamate-free diet he was given 3g of calcium cyclamate and his urine analysed for cyclohexylamine by g.l.c. Table 8 shows that by then his ability to convert cyclamate into the amine had diminished to about the same value as found before pretreatment (see Table 7).

A sample of the urine of B.S.D., shown by g.l.c. to contain 256mg of cyclohexylamine, was extracted with dichloromethane. The extract was treated with ether saturated with HCl gas and cooled to 0°C. The cyclohexylamine hydrochloride which separated (266mg; yield 77%) was filtered off and was identified by m.p. and mixed m.p. (203°C) and i.r. spectrum and then converted into its *N*-benzoyl derivative, m.p. and mixed m.p. 149°C from aq. ethanol, and checked by its i.r. spectrum.

Rat urine

The excretion of ^{14}C by normal and cyclamate-pretreated rats after an oral dose of [^{14}C]cyclamate is shown in Table 9. In the three groups of rats the recovery of ^{14}C was satisfactory, being over 90%. In the normal rats more ^{14}C was recovered in the faeces (52%) than in the urine (39%), but the reverse was true for both groups of pretreated rats. The nature of the ^{14}C-containing compounds in the urine and faeces of the first 24h is shown in Table 10. In the normal rats cyclamate was excreted entirely unchanged in the urine and faeces, but in the pretreated rats there was a considerable excretion of cyclohexylamine in both the urine and faeces, the urine containing much more than the faeces. There was a considerable individual variation in the amount of amine excreted. Thus rat no. 11 excreted 3.8% of the dose (urine, 3.2%; faeces, 0.6%) as cyclohexylamine, whereas rat no. 45 excreted 47.8% (urine, 37.7%; faeces, 10.1%). Table 10 also shows that in the pretreated rats other minor metabolites (up to 5% of dose) were excreted in the urine but not in the faeces. The amounts of the other minor metabolites were proportional to the output of cyclohexylamine, being larger with the higher outputs of the amine. They were separated from cyclamate and cyclohexylamine in solvents A and D, in which they had R_F values of 0.57 and 0.58 respectively. Since the R_F values were similar to those for the metabolites of cyclohexylamine in rat urine, these minor metabolites are probably conjugates of 3- and 4-aminocyclohexanol (Renwick & Williams, 1972).

The variability of the conversion of cyclamate into cyclohexylamine in 22 pretreated rats is illustrated in Table 11. Four groups of five or six rats were pretreated with cyclamate for 3 months and the cyclohexylamine output of each of the rats was determined

Table 9. *Excretion of ^{14}C after a dose of [^{14}C]cyclamate by normal rats and rats pretreated with cyclamate*

Groups of three rats were used. One group was not pretreated; the other two groups were pretreated with cyclamate for 3 and 5 months. Each rat was given orally 56mg of sodium [^{14}C]cyclamate/kg (8μCi/animal) and the urine and faeces were analysed daily for ^{14}C. Average values are quoted. Ranges are given only for the first day to indicate the spread of the results, and for the final totals. c.w., Cage wash.

		^{14}C excreted (% of dose)		
Day no.	Pretreatment period (months)	Urine	Faeces	Total
1	None*	37 (34–40)	46 (41–49)	83 (78–89)
2		39	52	91
3		39	52	91 (87–96) (c.w. 5%)
1	3*	58 (50–73)	22 (8–42)	80 (58–92)
2		63	26	89
3		63	27	90 (84–94) (c.w. 2%)
1	5†	46 (29–63)	35 (21–52)	81 (80–83)
2		47	38	85
3		47	38	85 (82–89) (c.w. 4%)

* Day 1 expired CO_2 was trapped and analysed for ^{14}C content. The amounts found were negligible, i.e. <0.1%.

† Day 1 expired air was trapped for the collection of ethanol-soluble volatile metabolites. None was detected, i.e. <0.01%.

Table 10. *Excretion of metabolites of cyclamate in rats*

Rats, dosing and pretreatment were as described in Table 9. The urine and faeces for the first 24h after dosing were analysed by paper chromatography.

			^{14}C excreted (% of dose)			
Rat no.	Pretreatment period (months)	Material analysed	^{14}C in sample	Cyclamate	Cyclohexyl-amine	Other metabolites
Three un-numbered rats	None	Urine	37 (34–40)	37	0	0
		Faeces	46 (41–49)	46	0	0
34	3	Urine	50.3	24.6	24.1	1.9
42			73.1	27.8	39.5	4.6
24			50.2	43.6	5.0	0.7
34		Faeces	41.7	30.5	10.0	0
42			15.6	6.2	9.4	0
11	5	Urine	28.7	21.6	3.2	0.7
25			47.9	19.8	22.6	3.9
45			63.0	16.2	37.7	5.1
11		Faeces	51.8	51.5	0.6	0
25			32.5	28.2	4.7	0
45			20.7	9.5	10.1	0

by g.l.c. after an oral dose of 200 mg of calcium cyclamate. It is clear that the rats in group 1 were poor converters whereas those in group 4 were good converters, but in each group there was a wide range of ability to convert cyclamate into the amine.

Table 12 shows the effect on the production of cyclohexylamine in cyclamate-pretreated rats of removing cyclamate from the diet. These rats were given daily a small dose (0.8mg) of [^{14}C]cyclamate and the output of ^{14}C and [^{14}C]cyclohexylamine was determined daily. Table 12 shows that on the removal of cyclamate from the diet the ability to produce the

Table 11. *Cyclohexylamine excretion in rats pretreated with cyclamate*

Groups of rats pretreated with cyclamate for 3 months were each given orally a dose of 200mg of calcium cyclamate. The 24h urine sample of each rat was analysed for cyclohexylamine by g.l.c. Ranges are given in parentheses. None of the animals excreted cyclohexylamine when given a similar dose before pretreatment.

Group no.	No. of rats in group	Cyclohexylamine in urine (% of dose)
1	6	2.6 (0.7–6.1)
2	5	5.0 (0.5–9.6)
3	5	13.2 (1.5–28.2)
4	6	16.6 (6.1–34.6)

amine diminished with time. In 5 days the extent of conversion in rat no. 15 fell from 2.5 to 0.1%, in rat no. 21 from 11.4 to 0.5%, and in rat no. 32 from 25.0 to 0.7% of the dose.

Table 13 shows what happened when three rats, cyclamate-pretreated for 4 months, were given simultaneously [^{14}C]cyclamate intraperitoneally and non-radioactive cyclamate orally. The ^{14}C was almost entirely excreted in the urine (83%; range 80–90% in 24h) and there was little in the faeces (1.4%; range 0.6–2.0%), possibly owing to contamination with urine. Rats nos. 23 and 30 were good converters of cyclamate into the amine and, although their urine contained large amounts of the non-radioactive amine (64% and 49% of the dose) as determined by g.l.c., there was very little (1.3% and 0.9%) [^{14}C]cyclohexylamine. Rat no. 12 was a poor converter and this animal produced negligible amounts of [^{14}C]-cyclohexylamine (0.05%), but significant amounts (1.4%) of the non-radioactive amine. This experiment showed that, in cyclamate-pretreated rats, cyclamate was metabolized only if it was given by mouth. When injected the cyclamate was excreted in the urine unchanged.

Rat bile

The small amount of ^{14}C in the faeces of rats injected with [^{14}C]cyclamate could arise partly by biliary excretion or from contamination by urine or both. The extent of the biliary excretion of cyclamate was examined in two bile-duct-cannulated female rats. They were prepared as described by Abou-El-Makarem *et al.* (1967) and injected intraperitoneally with sodium [^{14}C]cyclamate (10mg/kg; 1.4μCi/animal) and their urine, bile and faeces were collected for 24h. The urinary output of ^{14}C in the two animals was 51 and 93% and the biliary output 0.3% of the

Table 12. *Effect of stopping cyclamate pretreatment on cyclohexylamine production*

Three rats (nos. 15, 21 and 32) had been pretreated with cyclamate for 4 months. The cyclamate was removed from the diet. Each rat was then given orally 0.8mg of sodium [^{14}C]cyclamate/kg (1.7μCi/rat) each day for 5 successive days. The urine and faeces were collected daily and analysed for ^{14}C, and urine also for cyclohexylamine, by solvent extraction.

Time after end of cyclamate pretreatment (days)	^{14}C or cyclohexylamine excretion (% of dose)									
	Rat no. 15			Rat no. 21			Rat no. 32			
	^{14}C		Cyclohexylamine in urine	^{14}C		Cyclohexylamine in urine	^{14}C		Cyclohexylamine in urine
	Urine	Faeces		Urine	Faeces		Urine	Faeces	
1	28	53	2.5	45	54	11.4	58	11	25.0
2	45	41	0.8	44	43	2.6	38	31	3.3
3	52	42	0.1	45	42	0.5	38	39	0.9
4	36	59	0.2	44	45	0.4	42	34	0.6
5	41	43	0.1	34	59	0.5	48	40	0.7
Total ^{14}C recovered from the five doses	91			94			87		

Table 13. *Excretion of cyclohexylamine in rats receiving cyclamate orally and intraperitoneally*

Three rats pretreated with cyclamate for 4 months were given sodium cyclamate orally at a dosage of 320mg/kg, and sodium [^{14}C]cyclamate intraperitoneally in water at a dosage of 11mg/kg, each rat receiving 1.7μCi of ^{14}C. ^{14}C in the urine and faeces collected for 24h after dosing was determined by liquid-scintillation counting and cyclohexylamine in the urine, after extraction with dichloromethane, by g.l.c. and scintillation counting. Values for cyclohexylamine in parentheses are expressed as mg of cyclohexylamine/rat.

	^{14}C excreted in 24h		Cyclohexylamine in urine in 24h	
Rat no.	Urine	Faeces	By ^{14}C counting (% of intraperitoneal dose)	By g.l.c. (% of oral dose)
12	88.9	2.0	0.05 (0.001)	1.4 (0.7)
23	83.0	1.5	1.3 (0.018)	64.1 (29.4)
30	79.5	0.6	0.9 (0.013)	48.8 (22.4)

dose in each rat. Little injected cyclamate was therefore excreted in the bile.

Discussion

Absorption of cyclamate

Cyclamic acid is a fairly strong acid (pK_a 1.9) and therefore it may not be readily absorbed. In the normal guinea pig, man and rat a significant amount of orally administered cyclamate is excreted unchanged in the faeces, that is about one-third in the guinea pig (Table 1) and about one-half in man (Table 6) and the rat (Table 9). In the rabbit, however, cyclamate seems to be readily absorbed, since some 90% of an oral dose is excreted in the urine (Table 3). When these species are given a cyclamate-containing diet, it appears that those animals that become relatively good converters of cyclamate into cyclohexylamine also tend to excrete more of the ^{14}C radioactivity in the urine. This is particularly true for the rat (Table 9), which after pretreatment excretes 30–40% in the faeces, compared with over 50% before pretreatment. The guinea pig, which is a poor converter after pretreatment, excretes about the same amount in the faeces before and after pretreatment (Table 1). The one human subject (B.S.D.) who became a good converter excreted more ^{14}C in the urine after pretreatment (63%) than before (49%), but the two subjects who did not become converters showed less-marked differences in the route of excretion before or after the cyclamate diet (Table 6). In the rabbit cyclamate pretreatment made little difference (Table 3) to the route of excretion and cyclamate was mainly excreted in the urine, whether it was metabolized or not. However, apart from the rabbit the observations in general suggest that if cyclamate undergoes metabolism then this metabolism occurs in the gastrointestinal tract, because the metabolite, cyclohexylamine, when given orally, is excreted mainly in the urine (see the preceding paper: Renwick & Williams, 1972).

Since cyclamate is not readily absorbed from the gastrointestinal tract in the guinea pig, rat and man it can therefore reach the rectum, where, particularly in man, the main concentration of gut bacteria occurs.

Fate of cyclamate in normal animals

In normal guinea pigs (Table 2), rabbits (Table 4) and rats (Table 10), orally administered cyclamate is excreted entirely unchanged in the urine and faeces. This is its expected fate since it occurs as a highly polar anion at physiological pH values. In humans (Tables 7 and 8), one individual (B.S.D.) excreted cyclohexylamine in the urine with very small amounts in the first day (0.2%) but larger amounts in the second and third days after dosing (1.7 and 2.5%). This delay is probably due to the time taken for the dose to reach the gut bacteria (Table 6) and to induce their cyclamate metabolism (see the next paper: Drasar *et al.*, 1972).

Fate of cyclamate in cyclamate-pretreated animals

Some animals maintained on a diet containing cyclamate develop the ability to convert cyclamate into cyclohexylamine, but the extent of this development appears to depend on the individual animal and to some extent on the species. Table 2 shows that three guinea pigs kept for 6 months on a cyclamate diet developed the ability to convert cyclamate into cyclohexylamine, but to a small extent. All six rabbits kept for 5 months on cyclamate (Table 5) developed the ability, but to varying degrees (0.01–7.2%). In three human males, one became a good converter in a few days whereas the other two became only very poor converters even after 30 days on a cyclamate diet (see Tables 7 and 8). The animals most readily trained to convert cyclamate into cyclohexylamine were rats, and Table 11 shows that 22 rats kept on a cyclamate diet for 3 months all became converters but to varying degrees. In this connexion it is important to remember that, of the four species

studied, the rat has organisms all along the gastrointestinal tract from stomach to rectum whereas in man they are largely confined to the rectum and large intestine (see Drasar *et al.*, 1970).

Effect of stopping cyclamate pretreatment

When cyclamate was removed from the diet of rats that had become converters of cyclamate into cyclohexylamine while on the cyclamate diet, the ability of these animals to carry out the reaction diminished (Table 12). This was also shown to be the case with the one human converter, B.S.D. (Table 8), who when on a cyclamate diet converted some 17% of a dose of cyclamate into cyclohexylamine but when tested 8 days after being taken off the cyclamate diet had lost the ability to a considerable extent.

Cyclohexylamine formation after intraperitoneal injection of cyclamate

In cyclamate-pretreated rats, the conversion of cyclamate into cyclohexylamine occurred only when the sweetener was given orally. This was shown by giving simultaneously [^{14}C]cyclamate by injection and non-radioactive cyclamate orally and determining cyclohexylamine by g.l.c. and by liquid-scintillation counting (Table 13). When [^{14}C]cyclamate was injected into rats, it was almost entirely excreted unchanged in the urine. This experiment shows quite clearly that in cyclamate-pretreated rats the ability to form cyclohexylamine is associated with the gastrointestinal tract.

Our present results suggest that normal animals are unlikely to be able to metabolize cyclamate, but that they can be trained to do so by repeated ingestion of cyclamate. The main metabolite is then cyclohexylamine, which is itself further metabolized to a minor extent to the metabolites described in the preceding paper (Renwick & Williams, 1972). The metabolism of cyclamate occurs in the gastrointestinal tract and, as is shown in the next paper (Drasar *et al.*, 1972), the gut flora are responsible for the conversion of cyclamate into cyclohexylamine but not for the further metabolism of this amine.

The main metabolite of cyclamate is cyclohexylamine, but once this is formed it is possible that small amounts of cyclohexanol, cyclohexanone, *trans*-cyclohexane-1,2-diol, the *cis* and *trans* isomers of 3- and 4-aminocyclohexanol and possibly *N*-cyclohexylhydroxylamine could be produced. The relationship of these metabolites to the suspected carcinogenicity of cyclamate is unknown at the present time, but it would appear that the continued ingestion of cyclamate could result in the production of increasing amounts of cyclohexylamine and consequently of the minor metabolites of cyclohexylamine.

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References

Abou-El-Makarem, M. M., Millburn, P., Smith, R. L. & Williams, R. T. (1967) *Biochem. J.* **105**, 1269–1274

Asahina, M., Yamaha, T., Watanabe, K. & Sarrazin, G. (1971) *Chem. Pharm. Bull.* **19**, 628–632

Dalderup, L. M., Keller, G. H. M. & Schouten, F. (1970) *Lancet* **i**, 845

Davis, T. R. A., Adler, N. & Opsahl, J. C. (1969) *Toxicol. Appl. Pharmacol.* **15**, 106–116

Drasar, B. S., Hill, M. & Williams, R. E. O. (1970) in *Metabolic Aspects of Food Safety* (Roe, F. J. C., ed.), pp. 245–255, Blackwell Scientific Publications, Oxford and Edinburgh

Drasar, B. S., Renwick, A. G. & Williams, R. T. (1972) *Biochem. J.* **129**, 881–890

Kojima, S. & Ichibagase, H. (1966) *Chem. Pharm. Bull.* **14**, 971–974

Kojima, S. & Ichibagase, H. (1968) *Chem. Pharm. Bull.* **16**, 1851–1854

Kojima, S. & Ichibagase, H. (1969) *Chem. Pharm. Bull.* **17**, 2620–2625

Kojima, S., Ichibagase, H. & Iguchi, S. (1966) *Chem. Pharm. Bull.* **14**, 965–971

Leahy, J. S., Taylor, T. & Rudd, C. J. (1967) *Food Cosmet. Toxicol.* **5**, 595–596

Litchfield, M. H. & Swan, A. A. B. (1971) *Toxicol. Appl. Pharmacol.* **18**, 535–541

Miller, J. P., Crawford, L. E. M., Sonders, R. C. & Cardinal, E. V. (1966) *Biochem. Biophys. Res. Commun.* **25**, 153–157

Oser, B. L., Carson, S., Vogin, E. E. & Sonders, R. C. (1968) *Nature (London)* **220**, 178–179

Price, J. M., Biava, C. G., Oser, B. L., Vogin, E. E., Steinfeld, J. & Ley, H. L. (1970) *Science* **167**, 1131–1132

Prosky, L. & O'Dell, R. G. (1971) *J. Pharm. Sci.* **60**, 1341–1343

Renwick, A. G. & Williams, R. T. (1969) *Biochem. J.* **114**, 78P

Renwick, A. G. & Williams, R. T. (1970) *Proc. Int. Sugar. Res. Conf.*, 1st, pp. 3–14, International Sugar Research Foundation Inc., Bethesda

Renwick, A. G. & Williams, R. T. (1972) *Biochem. J.* **129**, 857–867

Sonders, R. C., Netwal, J. C. & Wiegand, R. G. (1969) *Pharmacologist* **11**, 241

Taylor, J. D., Richards, R. K. & Davin, J. C. (1951) *Proc. Soc. Exp. Biol. Med.* **78**, 530–533

Wallace, W. C., Lethco, E. J. & Brouwer, E. A. (1970) *J. Pharmacol. Exp. Ther.* **175**, 325–330

Williams, R. T. (1959) *Detoxication Mechanisms* 2nd. edn., pp. 730–732, Chapman and Hall Ltd., London

Wills, J. H., Jameson, E., Stoewsand, G. & Coulston, F. (1968) *Toxicol. Appl. Pharmacol.* **12**, 292