Studies in Detoxication

79. THE METABOLISM OF *cyclo*[^{14}C]HEXANE AND ITS DERIVATIVES*

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Little is known about the metabolic transformations of simple alicyclic hydrocarbons such as *cyclo*hexane. In contrast, there is a considerable amount of information about the more complex alicyclic compounds such as the steroids. A knowledge of the fate in the body of *cyclo*hexane and some of its derivatives, such as *cyclo*hexanol and *cyclo*hexanone, could be of value in the study of the metabolism of the more complex compounds. Their widespread use in industry has prompted many studies of their toxic effects on animals (see Browning, 1953), but previous investigations of their metabolic fate have produced little definite information. According to Filippi (1914) *cyclo*hexane is converted into *cyclo*hexanone in rabbits; Torboli (1937) reported that it formed adipic acid. Bernhard (1937) was unable to find any urinary metabolites of *cyclo*hexane in the dog except small amounts of oxalic acid, and presumed that it was totally oxidized in this animal. Treon, Crutchfield & Kitzmiller (1943), however, reported that, in rabbits, conjugated glucuronic acid corresponding to 5% of the dose was excreted after dosage with *cyclo*hexane. Incubation of *cyclo*hexane with ox or sheep kidney or liver is believed to produce small amounts of a substance giving reactions for ketones (Fabre, Truhaut & Peron, 1952); *cyclo*hexane is oxidized by *Pseudomonas aeruginosa* to adipic acid, valeric acid and formic acid, *cyclo*hexyl hydroperoxide being considered as an intermediate (Imelik, 1948).

Bernhard (1937) and Weitzel (1950) found no urinary metabolites of *cyclo*hexanol in dogs, but Treon *et al.* (1943) found that in rabbits this alcohol caused an increased excretion of conjugated glucuronic acid corresponding to 50% of the dose.

Filippi (1914) reported that *cyclo*hexanone yielded small amounts of adipic acid in rabbits, whereas Treon *et al.* (1943) found that it gave rise to the excretion of substantial amounts of conjugated glucuronic acid.

One of the difficulties in investigating the metabolism of *cyclo*hexane is the lack of specific tests for the compound and its metabolites. By using ^{14}C-labelled *cyclo*hexane many of these difficulties can be overcome, and we shall show that, as far as the rabbit is concerned, the main metabolite of *cyclo*hexane, *cyclo*hexanol and *cyclo*hexanone is *cyclo*hexyl glucuronide, which was isolated, and that *cyclo*hexane and *cyclo*hexanol are also hydroxylated to *trans-cyclo*hexane-1:2-diol.

* Part 78: Baldwin, Robinson & Williams (1959).

MATERIALS AND METHODS

Materials. The following compounds were prepared or purchased, and purified (melting points are corrected): *cyclo*hexane (British Drug Houses Ltd.), b.p. 81°, n_D^{20} 1·4262; *cyclo*hexanol (British Drug Houses Ltd.), b.p. 161–162°, n_D^{20} 1·4650; *cyclo*hexanone (Harrington Bros. Ltd.), b.p. 155·5°, n_D^{20} 1·450; *cis-cyclo*hexane-1:2-diol, m.p. 98° (Vogel, 1956); (±)-*trans-cyclo*hexane-1:2-diol, m.p. 103° (Horning, 1955); *cis-* and *trans-cyclo*hexane-1:3-diol were separated from a commercial mixture (L. Light and Co.) according to Clarke & Owen (1950), *cis-*, m.p. 86°, and *trans-*, m.p. 116°; *cis-* and *trans-cyclo*hexane-1:4-diol, m.p. 108–110° and 141° respectively, were the gift of Dr L. N. Owen; *cyclo*hex-1-enyl acetate, b.p. 181° (Machinskaya, 1952); *cyclo*hexane-1:2-dione, m.p. 36° (Arnold, 1952); *trans*-2-amino*cyclo*hexanol, m.p. 65° (Wilson & Read, 1935); *cyclo*pentanealdehyde, b.p. 136° (McCasland, 1951); *cyclo*hexene oxide, b.p. 130° (Gilman & Blatt, 1941); *cyclo*hex-2-enol, b.p. 68°/15 mm. Hg, n_D^{25} 1·4832, and its phenylurethane, m.p. 105·5° (Bartlett & Woods, 1940). The derivatives of the above-mentioned compounds needed for identification and isotopic dilution were prepared and purified by standard methods. All derivatives used have been described in the literature.

cyclo[^{14}C]Hexane was purchased from The Radiochemical Centre, Amersham, Bucks. After a number of biological experiments had been carried out, this material was found to contain a small percentage of [^{14}C]benzene. By conversion into *m*-dinitrobenzene after adding excess of carrier benzene, the [^{14}C]benzene content of the *cyclo*hexane was found to be 1·9%. By conversion into phenol toluene-*p*-sulphonate through sulphonation and alkali-treatment after adding excess of carrier benzene, the [^{14}C]benzene content was found to be 1·5%. One small sample of *cyclo*hexane freed from traces of benzene by gas chromatography was later supplied by The Radiochemical Centre and used in one experiment (see Table 4).

Analytical methods. Glucuronic acid and ethereal sulphate in urine were determined by methods previously described in this series (e.g. Mead, Smith & Williams, 1958).

Paper chromatography. R_F values for the *cyclo*hexane-1:2-diols are shown in Table 1, and agree with those given by Henbest & Wilson (1957). The glucuronides of *cyclo*hexanol and of the *cyclo*hexane-1:2-diols could be separated on paper, but the glucuronides of the *cis-* and *trans*-diol could not.

Table 1. *R_F values of* cyclo*hexanediols and related glucuronides*

R_F values for descending chromatography on Whatman no. 4 paper: A, in butan-2-one–xylene–water (1:1:1, by vol.); B, in propanol–aq. NH_3 soln. (sp.gr. 0·88) (7:3, v/v); C, in butanol–acetic acid–water (4:1:2, by vol.). The *cyclo*hexanediols were detected on paper with ammoniacal silver nitrate solution according to Henbest & Wilson (1957). The glucuronides used were obtained biosynthetically as gums (see text). An aqueous 1% solution (50 μl.) of the glucuronide gums was placed on the paper. They were detected by spraying the paper with a fresh solution of naphtharesorcinol (2% in aqueous 33% trichloroacetic acid), drying at room temperature and then heating at 135° for about 5 min. The glucuronides showed as deep-blue spots, the glucuronide of *cyclo*hexanol giving a more intense colour than the glucuronides of the diols.

	R_F		
Solvent	A	B	C
*trans-cyclo*Hexane-1:2-diol	0·25	—	—
*cis-cyclo*Hexane-1:2-diol	0·38	—	—
*cyclo*Hexyl glucuronide	—	0·55	0·59
*trans-cyclo*Hexane-1:2-diol glucuronide	—	0·46	0·42
*cis-cyclo*Hexane-1:2-diol glucuronide	—	0·43	0·40

Animals and collection of expired air. Adult chinchilla doe rabbits were used and were kept on a constant diet (60 g. of diet 41, Associated Flour Millers Ltd., plus 100 ml. of water daily). Compounds were administered with water by stomach tube. For collection of expired air an animal was housed in a sealed Perspex chamber through which a current of air could be drawn (cf. Parke & Williams, 1950, 1953) and which had an arrangement for collecting urine periodically without breaking the air current. The air after passing through the chamber was led through various Drechsel bottles for trapping CO_2 (in 4N-NaOH), ketones (1% of 2:4-dinitrophenylhydrazine in N-HCl) and hydrocarbons (ethanol at −70°), as described by Parke & Williams (1953). The recovery of *cyclo*hexane from the apparatus was checked with *cyclo*[^{14}C]hexane. Thus in 10 hr. 96% of 0·844 g. of *cyclo*hexane placed on a watch-glass in the chamber could be recovered by aeration into ethanol, the material being estimated from its radioactivity.

Measurement of radioactivity. All measurements were carried out on solid or liquid samples of 'infinite thickness' in an end-window counter. The specific activities were determined by comparison with a stable reference polymer (The Radiochemical Centre) and sufficient counts to give a standard error of ±2% were taken.

Radioactivity in urine was measured by counting the residues obtained by evaporation of the urines on planchets under an infrared lamp or by direct counting of liquid samples. The results by the two procedures did not differ significantly. With tissues and faeces, radioactivity was determined after conversion into $BaCO_3$ by wet oxidation (Van Slyke, Plazin & Weisiger, 1951) and in some cases by direct measurement on tissue homogenates after drying. Radioactive carbon dioxide in expired air was determined in the usual way, as $BaCO_3$, and total hydrocarbons trapped in the ethanol absorption bottles were estimated by direct counting of liquid samples.

cyclo*Hexane in expired air.* Each absorption bottle used to trap *cyclo*hexane contained 20 ml. of ethanol. Carrier *cyclo*hexane (0·8 g.) was added to half of the contents of each bottle. Then an equal volume of water was added, the *cyclo*hexane separated by centrifuging and allowed to stand over sodium for 24 hr. It was then distilled in the Craig microdistillation apparatus, the fraction of b.p. 81° being collected. This was converted into $BaCO_3$ by the wet-oxidation method and counted. *cyclo*Hexanol was determined by adding 1 ml. of carrier to 5 ml. of the ethanolic absorption fluid, the mixture was fractionally distilled and the fraction, b.p. 161–162°, treated with 3:5-dinitrobenzoyl chloride. The *cyclo*hexanol was counted as the dinitrobenzoate. *cyclo*Hexanone was determined by adding 0·5 ml. of carrier to 5 ml. of ethanolic fluid, then isolating the ketone as the 2:4-dinitrophenylhydrazone. Neither *cyclo*hexanol nor *cyclo*hexanone was found in the expired air and therefore further details of their determination are omitted.

Determination of urinary metabolites

cyclo*Hexanol.* Carrier *cyclo*hexanol (1 g.) was added to a volume of urine (usually 3–5 ml.) sufficient to contain about 1 μc of radioactivity. Half a volume of 10N-HCl was added and the mixture refluxed for 3 hr. The cooled mixture was continuously extracted with ether for 3 hr. After washing the extract with saturated $NaHCO_3$ soln., the ether was evaporated and the residual *cyclo*hexanol dried with anhydrous Na_2SO_4. The 3:5-dinitrobenzoate was prepared and washed successively with 2N-HCl, saturated Na_2CO_3 soln. and water until it was neutral. It was recrystallized from ethanol (charcoal) to constant activity (m.p. 110°). Free *cyclo*hexanol in the urine was determined in a similar manner but the hydrolysis with HCl was omitted.

cyclo*Hexanone.* This ketone (0·3 g.) was substituted for the *cyclo*hexanol described in the preceding paragraph and the hydrolysis was carried out similarly. The hydrolysate was steam-distilled and the *cyclo*hexanone in the distillate was extracted with ether. The dried (anhydrous Na_2SO_4) extract was evaporated and the recovered ketone dissolved in ethanol and added to a boiling solution of 2:4-dinitrophenylhydrazine [10%, w/v, in H_2SO_4–ethanol (1:5, v/v)]. After boiling for 1 min., the solution was cooled and the 2:4-dinitrophenylhydrazone of *cyclo*hexanone which separated was recrystallized to constant activity (m.p. 157–158°). Free *cyclo*hexanone was determined by the same procedure but the acid hydrolysis was omitted. No free *cyclo*hexanone was found and the ketone found after hydrolysis is probably an artifact (see below).

cis- *and* trans-cyclo*Hexane-1:2-diol.* Carrier amounts (0·25 g.) of each diol were added to urine and the hydrolysis was carried out as for *cyclo*hexanol. Extraction of the diols was made when the hydrolysate had been adjusted to pH 8 with NaOH. The ether was evaporated and the residue was then washed with ether to remove *cyclo*hexanol. The residue, dissolved in pyridine, was then treated with *p*-nitrobenzoyl chloride at 100° for 10 min. The solid residue was stirred with water and extracted successively with 2N-HCl, saturated Na_2CO_3 soln. and finally with water until neutral and free from pyridine. The di-*p*-nitrobenzoates of the diols (m.p. *trans*-, 130°; *cis*, 149°; see Elliott, 1958) were then

repeatedly recrystallized from ethanol to constant activity. The free diols were determined in the same way but the hydrolysis was omitted.

Some determinations of *cyclo*hexanol, *cyclo*hexanone, *cyclo*hexane-1:2-diols and *cyclo*pentanealdehyde were carried out after hydrolysis of the urine with β-glucuronidase. Generally, the urine (8–10 ml.) was adjusted to pH 4·6 and then incubated at 30° for 24 hr. with 10 mg. of an ox-liver β-glucuronidase preparation (3000 Fishman units: L. Light and Co.). The necessary carrier was then added to the mixture, which was then incubated for a further 24 hr., and the material to be determined was then isolated and counted as described above.

trans-cyclo*Hexane*-1:3-*diol*. This was determined similarly after the addition of 250 mg. of carrier, except that it was isolated finally as the dibenzoate [m.p. 124° after crystallization from methanol, light petroleum (b.p. 60°), acetic acid and finally ethanol].

trans-cyclo*Hexane*-1:4-*diol*. The above described procedure could not be successfully applied to the 1:4-diol owing to its low solubility in ether. The 1:4-diol (150 mg.) was added to 3–4 ml. of urine and the mixture (adjusted to pH 4·5) incubated for 24 hr. at 37° with a β-glucuronidase extract from *Helix pomatia*. The incubation mixture was evaporated to dryness at not more than 40° and the residue extracted repeatedly with ethanol. The ethanol was removed and the residue dissolved in pyridine and benzoylated with benzoyl chloride. The dibenzoate of *trans-cyclo*hexane-1:4-diol was recrystallized from ethanol until it had no activity (m.p. 150°).

cyclo*Hexane*-1:2-*dione*. The ketone (0·25 g.) was dissolved in 1·7 ml. of urine and hydroxylamine hydrochloride (0·44 g.) added, the solution being kept at 0°. A 50% (w/v) solution of KOH (0·7 ml.) was added drop by drop at 0°, and after keeping at 0° for 1 hr. the *cyclo*hexane-dione dioxime was collected and recrystallized from water (m.p. 187–188°). It had no activity.

cyclo*Hexene oxide*. The oxide (0·4 ml.) was added to urine (8 ml.) adjusted to pH 7. After 24 hr. the mixture was centrifuged. The supernatant oxide was separated and converted into *trans-cyclo*hexane-1:2-diol with 2N-H_2SO_4 (8 ml.). The diol was extracted with ethyl acetate, the extract dried with anhydrous Na_2SO_4 and the solvent evaporated. The diol was then counted as its di-*p*-nitrobenzoate as already described. No activity was found.

cyclo*Pentanealdehyde*. This highly volatile compound was lost during acid hydrolysis. A mixture of urine (8 ml.) and concentrated HCl (4 ml.) was heated in a sealed tube at 115° for 3 hr. After cooling, the tube was opened and its contents were neutralized with solid $NaHCO_3$. An excess of acetic acid (about 1 ml.) was added, followed by *trans*-2-amino*cyclo*hexanol (230 mg.). The mixture was cooled to 0° and sodium nitrite (690 mg. in 2 ml. of water) added to convert the amino compound into *cyclo*pentanealdehyde (McCasland, 1951). Excess of nitrous acid was removed with aq. 1% ammonium sulphamate soln. and the mixture steam-distilled. The distillate (10 ml.) was treated with an excess of a saturated solution of 2:4-dinitrophenylhydrazine in ethanol. The *cyclo*pentanealdehyde 2:4-dinitrophenylhydrazone which separated was recrystallized from ethanol to constant specific activity (m.p. and mixed m.p. 158°). For enzymic hydrolysis, the urine (8 ml.) was brought to pH 4·6 and incubated in a sealed tube with 1 ml. of a β-glucuronidase preparation from the giant African snail (*Achatina fulica*) at 30° for 24 hr. The carrier *cyclo*pentanealdehyde was prepared in the mixture from *trans*-2-amino-*cyclo*hexanol and counted as described above.

Succinic acid. This was determined as described by Parke & Williams (1953) except that the benzhydrol derivative of succinic acid was not prepared.

Oxalic acid. This was also determined according to Parke & Williams, but acetic acid was used instead of HCl for acidifying the urine.

Adipic acid. A solution of adipic acid (0·25 g.) in 2 ml. of 2N-Na_2CO_3 was added to urine (3–5 ml.) which had been previously acidified with HNO_3 and titrated with $AgNO_3$ soln. to remove chlorides. The mixture was treated with 3 ml. of aq. 10% (w/v) $AgNO_3$ soln. and the silver adipate was precipitated by dropwise addition of aq. 2N-NH_3 soln. The precipitate was filtered off and twice reprecipitated from solution in 2N-HNO_3 by addition of ammonia. The adipic acid was then removed from an acid solution by extraction with ethyl acetate and was recrystallized from ethanol until its activity disappeared (m.p. 152°).

Maleic acid. The acid (250 mg.) was added to 8 ml. of urine previously incubated for 24 hr. at 30° with 10 mg. of an ox-liver β-glucuronidase preparation (3000 Fishman units). After 3 hr. an excess of aq. 10% (w/v) $BaCl_2$ soln. was added to the solution and the barium maleate precipitated by dropwise addition of aq. 2N-NH_3 soln. The precipitate was dissolved in 2N-HCl and the maleic acid extracted from the solution with ethyl acetate. Removal of the ethyl acetate gave maleic acid, m.p. 135°, which showed no radioactivity.

Malonic acid. The acid (0·25 g.) dissolved in 2 ml. of 2N-NaOH was added to a volume of urine containing approximately 1 μc of radioactivity and the mixture adjusted to pH 2 by the addition of 2N-HCl. The urine was then continuously extracted with ether for 16 hr. The crystalline precipitate obtained was dissolved in ethanol, and the solution, after decolorizing with charcoal, was evaporated to dryness. Barium hydroxide solution (0·25N) was added to the residue, and the precipitated barium salt was dissolved in 2N-HCl, reprecipitated by the addition of barium hydroxide (0·25N) and digested with ethanol. Malonic acid (m.p. 133°) was extracted with ether in a continuous extraction from the barium salt after acidification with HCl, and it was finally recrystallized from ethanol until its activity disappeared.

Isolation of metabolites

From cyclo*hexane*. The 24 hr. urine of four rabbits, which had received collectively 4·8 g. of *cyclo*hexane, was neutral, did not reduce Benedict's reagent and gave an intense naphtharesorcinol reaction. Chromatography of the urine in solvents B and C (see Table 1) showed the presence of two glucuronides (one of R_F 0·55 in B, and 0·59 in C; the other, R_F 0·45 in B and 0·42 in C). The first corresponded to *cyclo*hexyl glucuronide and the second to *cyclo*hexane-1:2-diol glucuronide. One of the glucuronides was isolated through the basic lead acetate fraction of the urine (Kamil, Smith & Williams, 1951) as a gum, which on methylating with diazomethane and acetylating with pyridine and acetic anhydride yielded 2·2 g. of a crude solid. On repeated recrystallization from aq. 70% ethanol and finally ethanol, *methyl* (cyclo*hexyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* (0·4 g.) was obtained as white needles, m.p. 139° and $[\alpha]_D^{20}$ − 35·7° in

$CHCl_3$ (*c*, 1) (Found: C, 54·7; H, 6·9; $C_{19}H_{28}O_{10}$ requires C, 54·8; H, 6·8%). (Optical rotations are given to ±1°.)

From cyclo*hexanol*. Paper chromatography of the urine showed the same glucuronide spots as in *cyclo*hexane urine (see above). The same methyl (*cyclo*hexyl tri-*O*-acetyl-β-D-glucosid)uronate was isolated in a similar manner (see above) from the urine of six rabbits which had received 1 g. each of *cyclo*hexanol. The yield of crude ester was 6·5 g., which on repeated recrystallization had m.p. and mixed m.p. 139° and $[\alpha]_D^{20}$ – 35·6° in $CHCl_3$ (Found: C, 55·0; H, 7·0; CH_3CO, 31·2. $C_{19}H_{28}O_{10}$ requires C, 54·8; H, 6·8; CH_3CO, 31%). The ester (0·5 g.) was refluxed for 3 hr. with N-HCl (5 ml.). After cooling, the solution was extracted with ether. The extract was dried (anhydr. Na_2SO_4) and evaporated. The residue was esterified with 3:5-dinitrobenzoyl chloride and the resulting *cyclo*hexyl 3:5-dinitrobenzoate had m.p. and mixed m.p. 110° after repeated recrystallization from ethanol.

From cyclo*hexanone*. Four rabbits were each given 1·5 g. of *cyclo*hexanone. The urine, which was collected for 18 hr., was non-reducing and was free from ketones, but the naphtharesorcinol reaction was strongly positive. The glucuronide was isolated as above (yield 6·1 g. crude) and from this pure methyl (*cyclo*hexyl tri-*O*-acetylglucosid)-uronate was obtained, m.p. and mixed m.p. 139° and $[\alpha]_D^{26}$ – 36·0° in $CHCl_3$.

From cyclo*hex*-1-*en*-1-*yl acetate*. Free *cyclo*hex-1-en-1-ol is unstable and readily isomerizes to *cyclo*hexanone. The acetate (6 g. to four rabbits) was therefore fed. The urine (after 18 hr.) was non-reducing and contained a glucuronide. This proved to be that of *cyclo*hexanol, for 4·3 g. of methyl (*cyclo*hexyl tri-*O*-acetylglucosid)uronate (m.p. 139° after crystallization and $[\alpha]_D^{26}$ – 35·2° in $CHCl_3$) was isolated.

From (±)-trans-cyclo*hexan*-1:2-*diol*. A total of 6 g. of the diol was fed to four rabbits. The 18 hr. urine was non-reducing, contained no ketones and gave a strong naphtharesorcinol reaction. Paper chromatography of the urine with solvents B and C (Table 1) revealed one spot reacting with naphtharesorcinol, with R_F 0·45 in B and 0·42 in C. The glucuronide gum (5·5 g.) was prepared through basic lead acetate precipitation and part of it slowly crystallized to give a glucuronide, m.p. 135–140°, which could not be purified readily. The main bulk was methylated and acetylated to give 7·9 g. of crude ester. Repeated recrystallizations from ethanol yielded white needles of *methyl* (2-*acetoxy*cyclo*hexyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate*, m.p. 165·5–166°; $[\alpha]_D^{20}$ – 31·9° in $CHCl_3$ (*c*, 1) (Found: C, 53·9; H, 6·3; CH_3CO, 36·9. $C_{21}H_{30}O_{12}$ requires C, 53·2; H, 6·4; CH_3CO, 36·3%). Hydrolysis of this ester (0·35 g.) with N-HCl, extraction with ether and esterification of the residue with *p*-nitrobenzoyl chloride as described above for the *cyclo*hexyl glucuronide yielded the di-*p*-nitrobenzoate of (±)-*trans*-*cyclo*hexane-1:2-diol, m.p. and mixed m.p. 148·5° after recrystallization.

cis-*cyclo*Hexane-1:2-diol was also fed to rabbits (1 g. each), but although a glucuronide was formed, it was isolated as a gum which was not successfully crystallized, nor could a crystalline derivative be prepared from it.

RESULTS AND DISCUSSION

Glucuronide formation. Quantitative results for the glucuronide output of rabbits receiving oral doses of *cyclo*hexane and its derivatives are shown in Table 2. At a dose of 460 mg./kg., about 30% of *cyclo*hexane is excreted as glucuronides, of which there are two. The main one is *cyclo*hexyl glucuronide, which was isolated, and the minor one is *trans*-*cyclo*hexane-1:2-diol glucuronide. The latter was shown to be present by paper chromatography, and by the detection of *trans*-*cyclo*hexane-1:2-diol by isotope dilution after hydrolysis with β-glucuronidase of the urine obtained after dosing with *cyclo*[^{14}C]hexane.

*cyclo*Hexanol is highly conjugated with glucuronic acid (Table 2) and gives rise to the same two glucuronides as *cyclo*hexane. *cyclo*Hexanone also gives rise to considerable amounts of conjugated glucuronic acid which was proved, by isolation, to be mainly *cyclo*hexylglucuronide. *cyclo*Hexanone is therefore reduced to *cyclo*hexanol *in vivo*. *cyclo*Hex-1-enyl acetate is metabolized also to *cyclo*hexyl glucuronide. This suggests that it is hydrolysed to *cyclo*hex-1-enol, which is known to rearrange rapidly to *cyclo*hexanone; the latter is then reduced and conjugated as shown in Fig. 1.

Glucuronide formation is also the main fate of the *cis*- and *trans*-*cyclo*hexane-1:2-diol. The *cis* isomer seems to be more highly conjugated than the *trans*, but only in the latter was a crystalline derivative of the glucuronide, which proved to be a monoglucuronide, isolated.

Metabolism of cyclo*hexane*. The quantitative aspects of the metabolism of *cyclo*hexane are shown in Tables 3 and 4. Most of the experiments were carried out at a dose of 300–400 mg./kg. In one experiment, however, the dose was a thousand times smaller, namely 0·3 mg./kg. Table 3 shows the results for metabolites eliminated in the ex-

Table 2. *Conjugation of* cyclo*hexane and its derivatives by the rabbit*

Figures for conjugation are the means for three animals, the ranges being in parentheses, except for *cyclo*hexanol, where two animals were used.

Compound administered	Dose† (mg./kg.)	Percentage of dose excreted as glucuronide
*cyclo*Hexane*	460	30 (27–37)
*cyclo*Hexanol‡	260	65 (61, 68)[2]
*cyclo*Hexanone	248	66 (51–86)
*cyclo*Hex-1-enyl acetate	350	39 (37–42)
(±)-*trans*-*cyclo*Hexane-1:2-diol	280	49 (42–56)
cis-*cyclo*Hexane-1:2-diol	280	75 (63–89)

* Ethereal sulphate and mercapturic acid outputs were measured for *cyclo*hexane, but they were not significantly different from normal.

† About 5·5 m-mole/kg. for *cyclo*hexane, and 2·5 m-mole/kg. for the other compounds.

‡ *cyclo*[^{14}C]Hexanol of low activity, and prepared biosynthetically from *cyclo*[^{14}C]hexane. In the two animals used the total radioactivity of the urine was 68 and 75% of the dose; these figures are slightly higher than the glucuronide figures of 61 and 68% respectively.

pired air. When the dose is 300–400 mg./kg., about 35–45 % of the dose is eliminated in the expired air, some 10 % of the dose as carbon dioxide and 25–35 % as unchanged *cyclo*hexane (b.p. 81°). When the dose is very small (Expt. 4) no unchanged *cyclo*hexane is eliminated, but about 5 % is converted into carbon dioxide, the rest being mainly excreted in the urine as metabolites. In Expts. 2–4, quoted in Table 3, the radioactivity accounted for was almost quantitative and amounted to about 95 % of the dose.

Table 4 summarizes our isotope-dilution experiments on the identification of the metabolites of *cyclo*hexane. According to this table, the main metabolite is *cyclo*hexanol, which accounts for 30–40 % of the high dose and 60 % of the low dose. The next main metabolite is (±)*trans-cyclo*hexane-1:2-diol, which accounts for 5–8 % of the high dose and nearly 17 % of the low dose. These two compounds, conjugated with glucuronic acid, appear to be the only urinary metabolites of *cyclo*hexane in the rabbit. However, there are quoted in this table small positive values for *cis-cyclo*hexane-1:2-diol, *cyclo*hexanone and phenol. Furthermore, in Table 5 a small positive value is also recorded for *cyclo*pentanealdehyde. We have evidence, however,

Table 3. *Metabolites of* cyclo*hexane in the expired air*

[^{14}C]*cyclo*Hexane was administered orally to rabbits and the expired air was collected for 2 days.

Expt. no.	2	3	4
Dose of *cyclo*hexane (mg./kg.)	390	360	0·3†
Dose of ^{14}C (μc/animal)	108	185	50
Metabolites (% of dose)			
Carbon dioxide	10·1	8·5	5·5
*cyclo*Hexane	24·5	38·0	0
*cyclo*Hexanol	0	0	0
*cyclo*Hexanone	0	0	0
Total in expired air	34·6	46·5	5·5
(Urine, faeces and tissues*	58·7	49·8	89·2)
(Total accounted for	93·3	96·3	94·7)

* See Table 4.

† This small dose consisted of 0·7 mg. of *cyclo*hexane dissolved in 0·8 ml. of arachis oil.

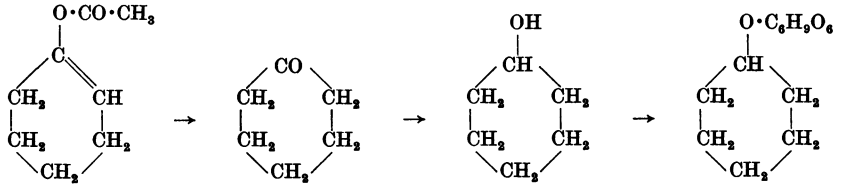


Fig. 1. Reduction and conjugation of *cyclo*hexanone.

Table 4. *Urinary metabolites of* cyclo*hexane*

Metabolites found are expressed in % of dose.

Expt. no.	1	2	3	4	5
Dose of *cyclo*hexane (mg./kg.)	330	390	360	0·3	100‡
Dose of ^{14}C (μc/animal)	18·5	108	185	50	14
Duration of experiment (days)	5	6	3	4	2
Metabolites examined‖					
*cyclo*Hexanol	—	41 (1·0)*	34·4	61·0	48·0
(±)-*trans-cyclo*Hexane-1:2-diol	4·5	8·5 (0·07)*	4·7	16·7	—
*cis-cyclo*Hexane-1:2-diol§	0·4	0·9	1·1	3·5	—
*cyclo*Hexanone§	1·5	3·6 (0)*	5·4 (0)*	4·5 (0)*	—
*cyclo*Hexane-1:2-dione	—	0	0	0	—
*trans-cyclo*Hexane-1:3-diol	—	0	0	0	—
*trans-cyclo*Hexane-1:4-diol	—	0	0	0	—
*cyclo*Hexene oxide	—	—	—	—	0
Adipic acid	0	0	0	0	—
Succinic acid	—	0	0	0	—
Malonic acid	—	0	0	0	—
Oxalic acid	0	0	0	0	—
(Phenol)	—	(0·6)†	(0·5)†	(1·1)†	(<0·02)
Sum of urinary metabolites	—	54·6	46·2	86·8	48·0
Radioactivity of urine	33·0	56·0	41·0	98·0	59
Radioactivity of faeces	—	0·2	0·1	—	—
Radioactivity of tissues	—	2·6	2·4	—	—

* These figures in parentheses are for the free compound, that is before acid hydrolysis.

† This phenol is derived from the [^{14}C]benzene present in the *cyclo*hexane.

‡ Benzene-free *cyclo*hexane. § Artifacts: see Table 5 and text. ‖ Determined by isotope-dilution.

which suggests that three of these compounds are artifacts of the acid-hydrolysis procedures used in the experiments. The appearance of phenol in amounts of 0·5–1·1% of the dose in Expts. 2–4 and 7 is due to the presence of small amounts of benzene in the *cyclo*[^{14}C]hexane supplied to us. When a specially purified sample of *cyclo*[^{14}C]-hexane which had been freed of [^{14}C]benzene by gas chromatography was fed to rabbits, no phenol was found in the urine (see Expt. 5, Table 4). Thus it was proved that *cyclo*hexane was not aromatized and converted into phenol *in vivo*.

*cis-cyclo*Hexane-1:2-diol, *cyclo*hexanone and *cyclo*-pentanealdehyde appear to be artifacts. When the urine is hydrolysed with β-glucuronidase instead of hydrochloric acid, these compounds were not found in the hydrolysate (see Table 5). However, the origin of these artifacts is not entirely clear, but they could arise from *trans-cyclo*hexane-1:2-diol glucuronide. It was found that when either (±)-*trans-cyclo*hexane-1:2-diol or its non-crystalline glucuronide was heated with 5N-hydrochloric acid, small amounts of *cyclo*pentanealdehyde could be isolated (the 2:4-dinitrophenylhydrazone, m.p. 155–156°, not depressing the m.p. of the authentic compound, m.p. 158–159°, but depressing the m.p. of *cyclo*hexanone 2:4-dinitrophenylhydrazone, m.p. 157–158°). On heating *cis-cyclo*hexane-1:2-diol in the same way, small amounts of *cyclo*-hexanone were isolated (2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 158°, but depressing the m.p. of *cyclo*pentanealdehyde 2:4-dinitrophenylhydrazone). When the non-crystalline *cis-cyclo*-hexane-1:2-diol glucuronide was heated with hydrochloric acid, the 2:4-dinitrophenylhydrazone (m.p. 125–127°) of an aldehyde or a ketone was isolated but it depressed the m.p. of both the 2:4-dinitrophenylhydrazone of *cyclo*pentanealdehyde and of *cyclo*hexanone; we suspect that this compound was a mixture of the two dinitrophenylhydrazones, but we were unable to separate them. Under certain conditions derivatives of the *trans*-diol can be converted into the *cis*-diol, and there is a considerable literature on this point (see, for example, Clarke & Owen, 1949; Winstein, Hess & Buckles, 1942; Fujita & Nomura, 1942). We suggest, tentatively, that the small amount of *cis-cyclo*hexane-1:2-diol, *cyclo*hexanone and *cyclo*-pentanealdehyde found in the urine after acid hydrolysis are derived from *trans-cyclo*hexane-1:2-diol glucuronide thus:

trans-diol glucuronide → *cis*-diol → *cyclo*hexanone
trans-diol glucuronide → *cyclo*pentanealdehyde

Other *cyclo*hexane derivatives tested for were *cyclo*hexane-1:2-dione, *trans-cyclo*hexane-1:3- and -1:4-diol, and *cyclo*hexene oxide, but they were not found.

Since *cyclo*hexane is converted into carbon dioxide, possible intermediates are the dicarboxylic acids adipic, succinic, maleic, malonic and oxalic, but these were not detected in the urine by isotope dilution.

Table 6 shows the ratio of *cyclo*hexanol to *trans-cyclo*hexane-1:2-diol in the urine of rabbits at

Table 5. *Urinary metabolites of* cyclo*hexane determined after acid and enzymic hydrolysis of urine*

Expt. no.	6	7
Dose of *cyclo*hexane (mg./kg.)	272	221
Dose of ^{14}C (μc/animal)	20·1	91·5
Duration of experiment (hr.)	48	48
	Acid hydrolysis	Enzymic hydrolysis
Metabolites (% of dose)*		
*cyclo*Hexanol	39·5	41·1
(±)-*trans-cyclo*Hexane-1:2-diol	2·95	3·1
*cis-cyclo*Hexane-1:2-diol	1·8	0
*cyclo*Hexanone	1·4	0
*cyclo*Hex-2-enol	—	0
*cyclo*Pentanealdehyde	1·3	0
Adipic acid	—	0
Maleic acid	—	0
(Phenol)	—	(0·6)
Sum of metabolites	47·0	44·8
Radioactivity of urine	49·3	51·0

* Determined by isotope-dilution.

Table 6. *Relative excretion of* cyclo*hexanol and* trans-cyclo*hexane-1:2-diol at various times after dosing with* cyclo*hexane*

Expt. 3
(dose of *cyclo*hexane, 360 mg./kg.)

Time of collection of urine after dosing (hr.)	Ratio mono-ol/diol*
0–18	18·0
18–42	7·0
42–48	6·8

Ratio for total excretion is about 7.

Expt. 4
(dose of *cyclo*hexane, 0·3 mg./kg.)

Time of collection of urine after dosing (hr.)	Ratio mono-ol/diol*
0–1·5	9·0
1·5–6	5·3
6–24	4·6
24–48	3·0

Ratio for total excretion is about 4.

* This is the ratio of the amount of *cyclo*hexanol to *trans-cyclo*hexane-1:2-diol excreted in that time.

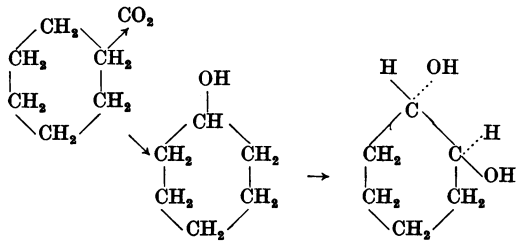
Table 7. *Some metabolites of* [^{14}C]cyclo*hexanol*

Expt. no.	1	2
Dose (mg./kg.)	270	260
Dose of radioactivity (μc/animal)	1	1
	Percentage of dose*	
*cyclo*Hexanol	58·0	64·5
*trans-cyclo*Hexane-1:2-diol	7·3	5·5
*cyclo*Hexanone†	5·3	6·1
Sum of metabolites	65·3‡	70·0‡
Total radioactivity of urine	—§	75·0

* These figures were obtained after acid hydrolysis of the urine, which had been collected for 2 days after dosing.
† *cyclo*Hexanone is probably an artifact, see text.
‡ Excluding *cyclo*hexanone.
§ Urine lost.

various times after dosing. It is to be noted that, in both experiments, the ratio falls with time. This suggests that the primary metabolite is *cyclo*hexanol and that the diol is being formed from the mono-ol. The diol is, in fact, a metabolite of *cyclo*hexanol, as shown in Table 7. In the two experiments quoted, 58 and 64 % of the dose was excreted as conjugated *cyclo*hexanol, and 7·3 and 5·5 % as conjugated *trans-cyclo*hexane-1:2-diol. The sum of these two metabolites, 65 and 70 %, is in agreement with the corresponding glucuronide figures, 61 and 68 % quoted in Table 2.

Our results suggest that the gross outlines of the metabolism of *cyclo*hexane are as follows:



At present there is no evidence to show how carbon dioxide is formed or in which metabolite ring-opening occurs.

SUMMARY

1. When *cyclo*[^{14}C]hexane is administered to rabbits in doses of about 350–400 mg./kg., about 40 % of the ^{14}C appears in the expired air and about 50 % in the urine in 2 days after dosing. With a dose of 0·3 mg./kg., only about 5 % of the dose of ^{14}C is found in the expired air and 90 % in the urine.

2. With the large dose, the ^{14}C-labelled compounds found in the expired air were carbon dioxide (about 10 % of the dose) and unchanged *cyclo*hexane (about 30 %), whereas with the small dose only labelled carbon dioxide (5·5 %) was found.

3. The urinary metabolites of *cyclo*hexane were *cyclo*hexanol and *trans-cyclo*hexane-1:2-diol, excreted as glucuronides. *cyclo*Hexyl glucuronide was isolated and characterized. With the larger doses the *cyclo*hexanol accounted for 35–50 % and the diol for 3–8 % of the dose, whereas the values for the small dose were *cyclo*hexanol, 61 %, and the diol, 16·7 %. The ratio, *cyclo*hexanol/*cyclo*hexane-diol, was about the same for the large and small doses.

4. *cis-cyclo*Hexane-1:2-diol, *cyclo*hexanone, *cyclo*hexane-1:2-dione, *trans-cyclo*hexane-1:3- and -1:4-diol, *cyclo*hexene oxide, *cyclo*hex-2-en-1-ol, and adipic acid, succinic acid, maleic acid, malonic acid and oxalic acid, and phenol, do not appear to be urinary metabolites of *cyclo*hexane.

5. *cyclo*Hexanol is highly conjugated (65 % of the dose) with glucuronic acid in the rabbit. The main metabolite is *cyclo*hexyl glucuronide. About 6 % of the dose is excreted as conjugated *trans-cyclo*hexane-1:2-diol.

6. *cyclo*Hexanone is reduced in rabbits and excreted mainly as *cyclo*hexyl glucuronide. This glucuronide is also the main metabolite of *cyclo*hex-1-en-1-ol, which was fed as its acetate.

7. Both *cis-* and (±)*trans-cyclo*hexane-1:2-diol are excreted by rabbits, highly conjugated with glucuronic acid. The monoglucuronide of the *trans*-diol was isolated and characterized.

One of us (T.H.E.) participated in this work during sabbatical leave from the University of Malaya, Singapore.

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Haemorrhage and Tissue Electrolytes

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A survey of the literature reveals considerable variability in the concentration of chloride in skeletal muscle. The variability has been both from one investigator to another and from one animal to another. Darrow, Harrison & Taffel (1939), for example, analysed the muscle of four normal dogs and found the chloride concentration to vary from 18·9 to 22·8 m-equiv./kg., and Nichols, Nichols, Weil & Wallace (1953) obtained similar figures. Kerpel-Fronius (1937), however, found 12·7 m-equiv./kg. Most of the published figures for the skeletal muscle of rats, rabbits and cats have been of the order of 12–14 m-equiv./kg. (Kerpel-Fronius, 1937; Darrow *et al.* 1939; Hines & Knowlton, 1939; Manery & Hastings, 1939; Lowry, Hastings, Hull & Brown, 1942). The concentration of chloride in human muscle has generally been found to be as high as or higher than those given by Darrow *et al.* (1939) for dogs. Kerpel-Fronius (1937) found 16·9 and 18·6 m-equiv./kg. in samples from two men, Talso, Spafford & Blaw (1953) 19·1, Wilson (1955) an average of 25·6, with a range from 19·3 to 38·3, Mudge & Vislocky (1949) 27·4, 28·1 and 29·7 and Barnes, Gordon & Cope (1957) an average of 23·1 with a range from 13·9 to 39·1 m-equiv./kg. There has not been the same variability in the concentration of chloride in other tissues, but since chloride is generally considered to be outside the cells, and to give a fair measure of the extracellular-fluid volume of an adult muscle we began to consider why the chloride in muscle was so variable. Even taking into consideration the facts that some authors express their results on a fat-free basis, whereas others do not, and that some but not others rinse their samples and blot them to remove superficial blood, the variation still remained very great, and it was clear that there was some other cause of the variability. The fact that bleeding the animal might have something to do with it was brought home to us when we compared the chloride concentrations in the muscles of pigs which had been killed and bled at the slaughter-house with those from pigs which had not been bled. The average for the former group was 13 m-equiv./kg., that for the latter 22 m-equiv./kg. It was decided to look into this matter further and to investigate the effect of bleeding on the chemical composition of skeletal muscle of the rat, the fowl, the pig and man. Since, moreover, we wished to know whether chloride could be used as a measure of the extracellular fluid in young animals it was decided to try the effect of bleeding and at the same time to compare the chloride and inulin spaces in the tissues of the newborn pig.

EXPERIMENTAL

Animals and experiments

Rats. Five adult black-and-white hooded rats weighing about 300 g. were anaesthetized with ether and as much as possible of the adductor and quadriceps muscles were taken from the right thigh. Then each animal was killed by bleeding from the abdominal aorta, and when it was dead the same muscles were taken from the other thigh. The amount of blood that could be removed before the animal died was not more than 5–8 ml.

A muscle taken from a living body is quite different in appearance from one taken from the body after it is dead. It is bright pink, with blood oozing from the small capillaries, whereas there is no visible blood in an autopsy specimen. It was thought that this in itself might affect the composition quite apart from any differences due to the removal of blood from the animal. In order to control this point, five other similar rats were anaesthetized as before, muscle was removed from the right thighs, the animals were killed with an overdose of ether and muscle was taken from the other side.

Cockerels. A healthy Rhode Island cockerel aged 26 weeks, weighing 3·5 kg., was killed by decapitation and the bird was exsanguinated by hanging it head downwards.