Biochem. J. (1965) **95**, 70

The Metabolism of Methylcyclohexane

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(*Received* 20 *July* 1964)

1. When [U-^{14}C]methylcyclohexane is fed to rabbits (dose 2–2·5m-moles/kg. body wt.), 65% of the radioactivity is excreted in the urine as metabolites, 0·5% appears in the faeces and about 15% in the expired air, some 4–5% remaining in the body in about 60hr. after dosing. The 15% of the dose appearing in the expired air consists of unchanged methylcyclohexane (10%) and $^{14}CO_2$ (5%). The low output of $^{14}CO_2$ shows that reactions leading to complete oxidation of methylcyclohexane are of minor importance. 2. The main metabolite found in the urine was the glucuronide of *trans*-4-methylcyclohexanol which was isolated. Seven methylcyclohexanols were found in the urine as conjugated glucuronides. The amounts of these were determined by isotope dilution to be as follows: *cis*-2-, 0·6%; *trans*-2-, 1·2%; *cis*-3-, 11·5%; *trans*-3-, 10·5%; *cis*-4-, 2·4%; *trans*-4-methylcyclohexanol, 14·7%, cyclohexylmethanol, 0·3%. No 1-methylcyclohexanol was found. There was evidence also that a small amount (approx. 1%) of the hydrocarbon aromatized to benzoic acid, probably via cyclohexylmethanol and cyclohexanecarboxylic acid. 3. The pattern of hydroxylation and the various amounts of the isomers found suggest that the hydroxylation *in vivo* of methylcyclohexane is dependent on steric factors in the molecule, hydroxylation occurring to the greatest extent at the carbon atom furthest away from the methyl group.

Methylcyclohexane, a component of certain crude petroleums, is used as a solvent and as a starting material and intermediate in synthesis in the chemical industry. It is not highly toxic (Gerarde, 1962). Little is known about the details of its metabolism in the animal body. Bernhard (1937) reported that it was probably completely oxidized in the dog, for he was unable to detect any of its metabolites in the urine, but Treon, Crutchfield & Kitzmiller (1943*a*,*b*) found that it caused a small increase (4·5% of dose) in glucuronide output in the rabbit. However, it is shown in the present paper that methylcyclohexane undergoes metabolism in the rabbit to yield several metabolites, the main one being the glucuronide of *trans*-4-methylcyclohexanol. The methylcyclohexane molecule is of considerable interest from a metabolic point of view because it contains primary, secondary and tertiary carbon atoms, any of which may be preferentially attacked. Further, if secondary alcohols are formed *in vivo*, these can occur as *cis–trans* isomers that differ in their thermodynamic stability. There are also the possibilities that the molecule may be aromatized and that the ring may be split to yield, eventually, carbon dioxide. By using [U-^{14}C]methylcyclohexane, it was shown that almost all these possibilities occur. Several reference compounds used in the present work were prepared and described by Elliott, Tao & Williams (1965).

MATERIALS

All melting points are uncorrected.

Methylcyclohexane (British Drug Houses Ltd., Poole, Dorset) was redistilled twice, b.p. 100–102°, $n_D^{21\cdot5}$ 1·4222, and shown to be pure by gas–liquid chromatography and infrared spectroscopy.

[U-^{14}C]Methylcyclohexane (The Radiochemical Centre, Amersham, Bucks.) was stated to contain not more than 5% of [^{14}C]cyclohexane and had a specific activity of 238μc/g. The activity was checked by conversion into $BaCO_3$ (Van Slyke, Plazin & Weisiger, 1951) and determining the activity with an end-window counter. The purity was assessed by gas–liquid chromatography. A sample on a column of Halamide at 68° gave three peaks with retention times corresponding to those of cyclohexane, methylcyclohexane and toluene. By comparison with authentic mixtures, the amount of toluene was found to be less than 1% (v/v).

Cyclohexanol (Hopkin and Williams Ltd., Chadwell Heath, Essex) was redistilled and had b.p. 159–161°, n_D^{24} 1·4650; 3,5-dinitrobenzoate, m.p. 111·5–112·5°. Cyclohexanecarboxylic acid (white label; Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) had m.p. 31°; amide, m.p. 184–185°. Other compounds used were: benzoic acid (May and Baker Ltd., Dagenham, Essex), m.p. 122°;

hippuric acid (British Drug Houses Ltd.), m.p. 188°; 1-methylcyclohexanol, (±)-*cis*-2-methylcyclohexanol, (±)-*trans*-2-methylcyclohexanol, (±)-*cis*-3-methylcyclohexanol, (±)-*trans*-3-methylcyclohexanol, *cis*-4-methylcyclohexanol, *trans*-4-methylcyclohexanol, cyclohexylmethanol, (±)-2-methylcyclohexanone, (±)-3-methylcyclohexanone and 4-methylcyclohexanone (see Elliott *et al.* 1965).

METHODS

Animals and diet. Adult chinchilla doe rabbits were used and fed as described by Elliott *et al.* (1965). Methylcyclohexane was administered with water by stomach tube. After receiving the dose the animals showed an increased respiration rate and tended to be lethargic, but no narcosis was observed.

Analytical methods. Glucuronic acid was determined by the method of Hanson, Mills & Williams (1944) as modified by Paul (1951); ethereal sulphate was determined by the turbidimetric method of Sperber (1948); mercapturic acid was determined by the iodine-titration method of Stekol (1936).

Isolation of metabolites and hydrolysis experiments. The method of Kamil, Smith & Williams (1951) was used for the isolation of glucuronides. Methylcyclohexane (3·5ml.) was fed to two rabbits and from the 22hr. urine, by using this method, the glucuronide was isolated. The aqueous solution containing the glucuronide was evaporated to dryness *in vacuo* at 45°, giving 1·8g. of crude hygroscopic gum, which deposited, on cooling, a small amount of needles that turned pink on standing. Attempts to crystallize the gum were unsuccessful. It was therefore methylated with ethereal diazomethane to give a gummy methyl ester, which was then treated with acetic anhydride and pyridine (equal parts) overnight. When the mixture was poured into water, a product was obtained that solidified on repeated treatment in order with $NaHCO_3$, dilute HCl and water to give 3·5g. of crude triacetyl methyl ester. Five recrystallizations from ethanol yielded 30mg. of pure ester, m.p. 142° not depressed when mixed with methyl [*trans*-4-methylcyclohexyl tri-*O*-acetyl-β-D-glucosid]uronate, $[\alpha]_D^{24}$ $-32·8\pm1°$ (*c* 3 in $CHCl_3$) (Found: C, 56·2; H, 7·0; O, 37·1. $C_{20}H_{30}O_{10}$ requires C, 55·8; H, 7·0; O, 37·2%). In two similar experiments simple recrystallization of the triacetyl methyl ester from ethanol failed to raise the melting point to that of the pure compound; accordingly, in one of these experiments a systematic three-stage recrystallization (Tipson, 1950) was attempted and in all three stages the pure ester was obtained.

Hydrolysis of the triacetyl methyl ester. The small yield of the glucuronide triacetyl methyl ester was insufficient for hydrolysis. Therefore the crude evaporated mother liquors left after the three-stage recrystallization were combined and hydrolysed with 20ml. of N-HCl for 3hr. in a glycerol bath maintained at 130°. The alcohol was then steam-distilled from the mixture and the steam-distillate extracted with three portions of ether. The combined extract was dried over $MgSO_4$ overnight. The solvent was evaporated and the residual alcohol was esterified with 3,5-dinitrobenzoyl chloride in pyridine. After five recrystallizations from ethanol, pure *trans*-4-methylcyclohexyl 3,5-dinitrobenzoate was obtained, m.p. and mixed m.p. 141°.

Hydrolysis of the glucuronide. Methylcyclohexane (12ml.) was fed to six rabbits and from the 24hr. urine there was isolated 6g. of crude glucuronide gum, which was hydrolysed with 12ml. of N-HCl for 3hr. in a glycerol bath maintained at 130°; the liberated alcohol was recovered by steam-distillation, yielding 1·5ml. of an oil, 1ml. of which was esterified with 3,5-dinitrobenzoyl chloride in pyridine, and the ester, after three recrystallizations from ethanol, yielded 0·2g. of pure *trans*-4-methylcyclohexyl 3,5-dinitrobenzoate, m.p. and mixed m.p. 141–141·5°.

Paper chromatography. Urine (30μl.) from a rabbit receiving 4·4m-moles of methylcyclohexane/kg. body wt. was chromatographed on Whatman no. 1 paper with urines obtained after feeding the isomeric methylcyclohexanols and methylcyclohexanones. The solvent systems used were: *A*, butan-1-ol–pyridine–water (4:1:5, by vol.); B, butan-1-ol–acetic acid–water (4:1:5, by vol.). The glucuronides were detected by spraying with 0·2% naphtharesorcinol in ethanol (5vol.) and aq. 85% (w/v) H_3PO_4 (1vol.) freshly mixed before spraying. The chromatograms were then heated at 90–100° for 5–10min. in an oven in which a beaker of hot water had stood for some time. Glucuronides gave a well-defined blue spot on a pinkish background.

Radioautography. [U-^{14}C]Methylcyclohexane (0·3482g.) containing 83μc of radioactivity was fed to a rabbit weighing 1·57kg. The first fraction of urine collected, with approx. 0·54μc of activity/ml., was used for radioautography. The urine (40μl.) was placed on a Whatman no. 2MM paper and allowed to run in two solvent systems: *I*, butan-1-ol–butyric acid–water (2:2:1, by vol.); *II*, butan-1-ol–acetic acid–water (4:1:5, by vol.)

After being dried, the chromatographs were placed in contact with Kodirex No-Screen X-ray films for a fortnight. The chromatographs were sprayed with: (i) naphtharesorcinol for glucuronides; (ii) bromocresol green spray [0·04% (w/v) bromocresol green in 60% (v/v) ethanol, and adjusting the colour to just blue with ammonia] for organic acids; (iii) ninhydrin spray (0·2% ninhydrin in butan-1-ol previously saturated with water) for amino acids.

Distribution of radioactivity, mode and rate of excretion. All measurements of radioactivity 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 were taken to give a standard error of ±2%.

The animal, after being dosed with [U-^{14}C]methylcyclohexane, was kept in a sealed Perspex chamber so designed that collection of urine could be made separate from the faeces, and expired air could be collected in convenient fractions (Parke & Williams, 1950, 1953).

Collection of expired air. The method adopted was similar to that described by Elliott, Parke & Williams (1959). In our first experiment, the recovery of radioactivity was low, and hence quantitative recovery of methylcyclohexane from the metabolic chamber was checked with [U-^{14}C]methylcyclohexane. It was found that, by increasing the air-flow rate through the absorption train, in 20hr. 95% of 10μc of [U-^{14}C]methylcyclohexane placed on a watch-glass in the chamber could be recovered in the ethanol absorbers. The $^{14}CO_2$ in each NaOH fraction was determined by direct counting of the liquid sample and as $BaCO_3$ (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The activities of ethanol fractions were estimated by counting liquid samples, and the actual substance present

was estimated by isotopic dilution carried out on portions of suitably combined fractions.

Methylcyclohexane in the expired air. Unlabelled methylcyclohexane (1 ml.) was added to 10 ml. of each ethanol fraction shown to contain activity. To this was added an equal volume of distilled water. The non-aqueous layer was separated and dried over sodium overnight to remove traces of water and ethanol, and the liquid was distilled in a micro-distillation apparatus, the material boiling at 100° and showing n_D^{20} 1·4226 being collected. The distillate was converted into $BaCO_3$ by the wet-oxidation procedure (Van Slyke *et al.* 1951) and counted. A similar method was used to detect *trans*-4-methylcyclohexanol in the ethanol fractions, but it was found to be absent. Since the radioactivity of the unchanged [U-^{14}C]methylcyclohexane in the breath accounted for virtually all the radioactivity occurring in the ethanol fractions, these were not examined further.

Excretion of radioactivity in the faeces. Faeces were combined and homogenized with water, and the activity was determined by counting portions of the homogenate dried on planchets.

Distribution of radioactivity in tissues. When the collection of urine was discontinued, the animal was killed and the tissues were examined for radioactivity by conversion into $BaCO_3$ (Van Slyke *et al.* 1951).

Urinary excretion. Urine was collected as excreted, and the radioactivity determined by counting both liquid samples and solid residues (Parke, 1956). Collection of urine was discontinued when it was found to contain negligible activity.

Determination of metabolites in the urine by isotopic dilutions. Fractions of urine containing significant radioactivity were combined and portions of the combined urine were used for isotopic dilution.

2-, 3- *and* 4-*Methylcyclohexanols and cyclohexylmethanol.* The carrier alcohol (usually 0·5 ml.) was added to a volume of urine (4–6 ml.) containing approx. 1 μc of activity. The solution was made up to 20 ml. with N-HCl and then heated for 15 or 20 min. (see Table 1) and the alcohol recovered by steam-distillation. The distillate was extracted with ether, and the material recovered from the ethereal extract was converted into a suitable derivative (see Table 1), which was then recrystallized to constant activity. These derivatives had m.p. and mixed m.p. identical with those of the corresponding authentic compounds.

1-*Methylcyclohexanol.* Attempts to recover this alcohol after acid, alkaline or neutral hydrolysis were unsuccessful, insoluble decomposition products being formed. Partial hydrolysis of the glucuronide of 1-methylcyclohexanol could be effected by a β-glucuronidase preparation. At present, a satisfactory method for complete enzyme hydrolysis has not been obtained, and therefore it is not possible to estimate quantitatively the amount of 1-methylcyclohexanol by isotopic dilution. However, both paper-chromatographic and radioautographic evidence suggest that 1-methylcyclohexanol cannot be present in more than minute traces, if at all.

Cyclohexanol. Cyclohexanol (0·481 g.) was added to 4 ml. of urine, the mixture was hydrolysed and the alcohol recovered according to the method of Elliott *et al.* (1959). The 3,5-dinitrobenzoate was prepared and purified to constant activity (m.p. and mixed m.p. 111·5–112·5°).

(±)-2-*Methylcyclohexanone,* (±)-3-*methylcyclohexanone and* 4-*methylcyclohexanone.* The appropriate ketone (0·5 ml.) was mixed with 4 ml. of urine and 2 ml. of water in a separating funnel and the mixture kept for 1 hr. with occasional shaking to establish equilibrium. The ketone was then extracted with ether, the combined extracts were dried and the ether was evaporated. A suitable derivative [2,4-dinitrophenylhydrazone of (±)-2-methylcyclohexanone, m.p. 138°; semicarbazone of (±)-3-methylcyclohexanone, m.p. 179°; 2,4-dinitrophenylhydrazone of 4-methylcyclohexanone, m.p. 134°] was prepared and purified by recrystallization. In all cases the derivative entrained no radioactivity.

Cyclohexanecarboxylic acid. The acid (1 g.) was added to 4 ml. of urine, to which 4·5 ml. of 5N-NaOH was then added. The solution was made up to 15 ml. with water and then maintained in a glycerol bath at 130° for 0·5 hr. The acid was liberated with HCl and extracted with ether. It was then converted into its silver salt with ammoniacal $AgNO_3$, and the salt was washed successively with water, ethanol and ether, and then counted. The free acid was liberated from the silver salt by acidification with dilute HNO_3, and then reconverted into its silver salt, the activity of which was redetermined. This process was repeated seven times, after which the activity became very low. It was then converted into its amide by refluxing the free acid with thionyl chloride for 0·5 hr. and then adding aq. ammonia to the cooled mixture. The amide was extracted with ether, from which it was crystallized by concentration. It was recrystallized once from hot water with charcoal, and the material (m.p. and mixed m.p. 184°) contained no significant activity.

Total benzoic acid. This acid (0·5 g.) was added to 4 ml. of

Table 1. *Hydrolytic procedures in isotopic-dilution experiments*

Experimental details are given in the text.

Compound	Time of heating at 130° with N-HCl (min.)	Derivative prepared	m.p. of pure derivative
(±)-*cis*-2-Methylcyclohexanol	15	3,5-Dinitrobenzoate	99–100°
(±)-*trans*-2-Methylcyclohexanol	30	3,5-Dinitrobenzoate	117–117·5
(±)-*cis*-3-Methylcyclohexanol	30	3,5-Dinitrobenzoate	98–99
(±)-*trans*-3-Methylcyclohexanol	15	*p*-Nitrobenzoate	61·5–62
cis-4-Methylcyclohexanol	20	3,5-Dinitrobenzoate	107
trans-4-Methylcyclohexanol	30	3,5-Dinitrobenzoate	141
Cyclohexylmethanol	30	3,5-Dinitrobenzoate	94·5–95

urine and the mixture hydrolysed as described by Kingsbury & Swanson (1921). By this method any combined form of benzoic acid present would be converted quantitatively into the free acid (Quick, 1926). After hydrolysis the benzoic acid was extracted with ether, and NaOH was added to the combined ethereal extract. The alkaline layer was heated on a steam bath and then cooled and HCl was added. The benzoic acid that separated was recrystallized from hot water (charcoal) and its activity became constant after the second recrystallization (m.p. and mixed m.p. 122°). Conversion into the silver salt and back into the free acid did not alter the activity.

Hippuric acid. This acid (1g.) was added to 5ml. of urine containing approx. 1μc of activity. To the mixture were added 3ml. of acetic acid and 10ml. of water, and the solution was kept for 1hr. at room temperature. The mixture was then extracted with ether continuously for 16hr., and the hippuric acid recovered from the ethereal extract was washed with ether and recrystallized from hot water to constant activity (m.p. and mixed m.p. 188–189°).

Free benzoic acid. The procedure used was similar to that described for hippuric acid except that 0·5g. of benzoic acid was added as carrier and the mixture was extracted with ether for 5hr. The recovered benzoic acid was recrystallized from hot water to constant activity (m.p. and mixed m.p. 121–122°). It was then converted into its sodium salt and reprecipitated as the free acid again, as described for total benzoic acid. No change in activity was observed after recrystallization from water.

Infrared spectroscopy. The infrared-absorption spectrum (in CCl_4) of the steam-distilled acid hydrolysate of the glucuronide isolated from urine of rabbits receiving methylcyclohexane revealed in the 'finger-print' region the diagnostic bands (Eliel & Lukach, 1957) of 943 and 1139 cm.$^{-1}$ for *trans*-3-methylcyclohexanol, of 1010cm.$^{-1}$ for *trans*-4-methylcyclohexanol, of 925, 986 and 1030cm.$^{-1}$ for *cis*-4-methylcyclohexanol, and of 1065cm.$^{-1}$ for *trans*-2-methylcyclohexanol.

Gas–liquid chromatography (on a diglycerol–Celite column at 118°) carried out on the crude alcohol mixture resulting from acid hydrolysis of the glucuronides isolated from urine of rabbits dosed with methylcyclohexane revealed two significant peaks. By comparison with reference materials, the retention time of the major peak corresponded to a mixture of *trans*-4- and *cis*-3-methylcyclohexanol, whereas the smaller peak corresponded to a mixture of *trans*-2-, *trans*-3- and *cis*-4-methylcyclohexanol.

RESULTS AND DISCUSSION

After the administration of methylcyclohexane (4·4m-moles/kg. body wt.) to rabbits, 42% of the dose was excreted as glucuronides and 2% as ethereal sulphate, but there was no increase in mercapturic acid excretion. The small excretion of ethereal sulphate is similar to that found by Elliott *et al.* (1959) after the administration of cyclohexane to rabbits. Treon *et al.* (1943*a*,*b*) noticed a decrease in the normal excretion of inorganic sulphate in relation to the total sulphate after the administration of methylcyclohexane.

Urine after the administration of the hydrocarbon had pH8 and gave an intense naphtharesorcinol reaction. It did not reduce Benedict's solution or give a precipitate with Brady's reagent, and there was no blue colour with 2,6-dichloroquinonechloroimide in sodium hydrogen carbonate solution or a red colour with ferric chloride, these tests showing the absence of ketones and phenols. From the same urine, methyl (*trans*-4-methylcyclohexyl tri-*O*-acetyl-β-D-glucosid)uronate was isolated and characterized both by comparison with an authentic specimen and by identification of the hydrolysis products.

Paper chromatography of the fresh urine revealed only one glucuronide spot, whose R_F values (0·27 in solvent *A* and 0·79 in solvent *B*) were identical with those of *trans*-4-methylcyclohexyl glucuronide. However, since isomeric 2-, 3- and 4-methylcyclohexyl glucuronide have virtually identical R_F values, the presence of these other glucuronides could not be excluded. The absence of any significant amount of 1-methylcyclohexyl glucuronide could nevertheless be established (Elliott *et al.* 1965).

The radioautographs revealed the presence of six radioactive spots, only one of which was intense, and this was the only spot (R_F 0·57 in solvent *I* and 0·72 in solvent *II*) that gave a positive reaction for glucuronides. None of the spots reacted with ninhydrin, indicating that amino acids that might have been formed by transamination were absent. The spray for carboxylic acids gave a positive reaction with two spots, one of which corresponded to the glucuronide metabolite and the other (R_F 0·84 in both solvent systems) to hippuric acid. However, the presence of the small amount of free benzoic acid and the possibility of the presence of traces of 1-methylcyclohexanol and cyclohexylmethanol could not be demonstrated conclusively by this method.

Distribution of radioactivity. The general distribution of radioactivity after administering [U-^{14}C]-methylcyclohexane is shown in Table 2. The comparatively low (about 10%) excretion of unchanged methylcyclohexane in the breath as compared with cyclohexane, where 40% of unchanged hydrocarbon was found in the breath (Elliott *et al.* 1959), is in accordance with the known relative volatility of the compounds. The small amount of $^{14}CO_2$ (about 5%) detected probably arises from oxidative cleavage of the methylcyclohexane ring, possibly through intermediary dicarboxylic acids. However, none of these substances was detected in the urine by radioautography.

The distribution of radioactivity occurring in the tissues is small (3·4%), and none of the values calls for special comment. Faecal excretion is unimportant (0·5%).

Table 2. *Distribution of* ^{14}C *after a single dose of* [U-^{14}C]*methylcyclohexane in rabbits*

Experimental details are given in the text.

	Expt. 1	Expt. 2	Expt. 3
Dose:			
(m-moles/kg. body wt.)	2·26	2·10	2·41
(μc/animal)	83	82·3	81·9
Radioactivity found (% of dose) in:			
Expired air as CO_2	3·3	5·0	8·6
Expired air as methylcyclohexane	1·3	15·9	4·4
Tissues	5·9	3·8	2·8
Faeces	0·5	0·4	0·7
Urine	54·2	64·5	77·4
Total radioactivity accounted for	65·2*	89·6	93·9

* The low value was due to incomplete recovery of radioactivity from the expired air.

Table 3. *Metabolites of* [U-^{14}C]*methylcyclohexane in the urine of rabbits after hydrolysis*

Experimental details are given in the text.

	Expt. 1	Expt. 2	Expt. 3
Dose:			
(m-moles/kg. body wt.)	2·26	2·10	2·41
(μc/animal)	83	82·3	81·9
Duration of experiment (hr.)	60	68	58
Radioactivity found (% of dose) in metabolite sought:			
1-Methylcyclohexanol	—	—	—
(±)-*cis*-2-Methylcyclohexanol	0·3	0·5	0·7
(±)-*trans*-2-Methylcyclohexanol	1·1	1·2	1·2
(±)-*cis*-3-Methylcyclohexanol	8·9	10·5	15·0
(±)-*trans*-3-Methylcyclohexanol	8·5	11·1	11·9
cis-4-Methylcyclohexanol	2·8	2·4	2·0
trans-4-Methylcyclohexanol	13·1	11·6	19·4
Cyclohexylmethanol	<0·2	0·3	<0·3
(±)-2-Methylcyclohexanone*	0	0	0
(±)-3-Methylcyclohexanone*	0	0	0
4-Methylcyclohexanone*	0	0	0
Cyclohexanecarboxylic acid	0	0	0
Total benzoic acid	1·6	1·8	2·2
Cyclohexanol†	2·4	2·6	—
Sum of radioactivities of metabolites sought in the urine	39·1	42·0	52·7
Total radioactivity in urine	54·2	64·5	77·4

* The hydrolysis procedure was omitted.

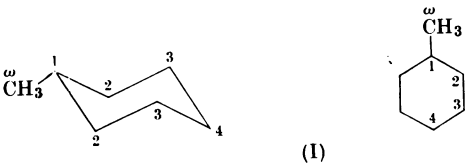
† Cyclohexanol is derived from cyclohexane contained in the [U-^{14}C]methylcyclohexane sample.

Most of the radioactivity (about 65%) was eliminated in the urine, and the single glucuronide spot revealed by paper chromatography was shown, by isotope-dilution methods (Table 3) supported by infrared spectroscopy and gas–liquid chromatography, to consist of a mixture of the glucuronides of the isomeric endocyclic alcohols. The cyclohexanol reported in Table 3 arises from [U-^{14}C]-cyclohexane, which was a contaminant of the [U-^{14}C]methylcyclohexane. The principal metabolite of cyclohexane is known to be cyclohexanol (Elliott *et al.* 1959).

Mechanism of hydroxylation. In the related field of steroid metabolism it is generally believed (e.g. Hayano, Gut, Dorfman, Sebek & Peterson, 1958; Bergstrom, Lindstredt, Samuelson, Corey & Gregoriou, 1958; Corey, Gregoriou & Peterson, 1958) that the biological hydroxylation of alicyclic rings occurs through direct replacement of a ring hydrogen by a hydroxyl group, but whether the

reaction involves an electrophilic or a free-radical substitution has not been decided and, further, the nature of the enzyme system involved appears to be unknown.

The methylcyclohexane molecule (I) has five positions at which hydroxylation could occur, namely at the tertiary carbon at position 1, at the secondary carbons at positions 2, 3 and 4, and at



(I)

the primary carbon in the ω-position. No evidence was obtained for hydroxylation in position 1, which is sterically hindered. Hydroxylation, however, occurred at all the other positions, the total hydroxylation at each position being 1·7% of the dose at position 2, 22·0% at position 3, 17·1% at position 4 and not less than 0·3% at the ω-position (see Table 4). Thus hydroxylation occurs most readily at positions 3 and 4.

The extent of hydroxylation at the ω-position is probably about 1%, because it has to be assessed not only from the amount of cyclohexylmethanol found but also from the total benzoic acid excreted. Part of the benzoic acid (not more than 1%) is probably derived from the toluene in the methylcyclohexane administered, but since the total benzoic acid is about 1·9% of the dose some of it must have been derived from methylcyclohexane via cyclohexanecarboxylic acid (see Table 3). The low value for the ω-position suggests that the methyl group of methylcyclohexane is relatively resistant to attack *in vivo*. Hydroxylation at the 2-position (and there are two of these) is just under 2%, and this low value may be due to steric hindrance. However, there is excreted some two to three times as much *trans*-2-alcohol as *cis*-2-alcohol, and this is not unexpected since the *trans*-2-alcohol is the thermodynamically more stable form of 2-methylcyclohexanol. The total hydroxylation in the 3-position is 22%, but since there are two 3-positions the average hydroxylation for each 3-position is 11%. The thermodynamically more stable form of 3-methylcyclohexanol is the *cis*-form, but Table 4 shows there is almost as much *trans*-3-alcohol excreted as *cis*-3-alcohol. The main position of hydroxylation of methylcyclohexane is the 4-position (17·1%), and the major metabolite of the hydrocarbon is conjugated *trans*-4-methylcyclohexanol (14·7%). The *trans*-4-alcohol is also the thermodynamically more stable form of 4-methylcyclohexanol.

The hydroxylation of methylcyclohexane *in vivo* thus shows a relatively non-specific pattern, although the actual extent of hydroxylation at each position (i.e. relative proportions 0:0·9:11:17:1 for positions 1, 2, 3, 4 and ω respectively) appears to be controlled by steric factors. There is of course the possibility that different enzymes are needed for the hydroxylation of the different positions of the methylcyclohexane molecule, particularly as primary, secondary and tertiary carbon atoms are involved.

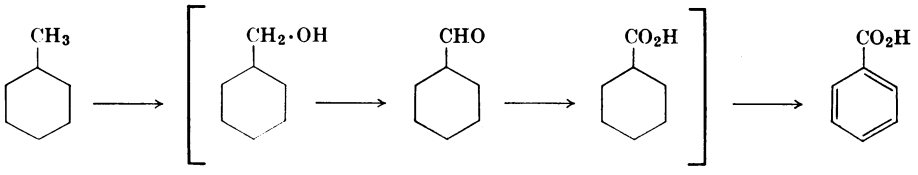
Aromatization. Table 3 shows that there is an excretion of labelled free and conjugated benzoic acid amounting to about 2% of the dose. Most of this (1·5%) was hippuric acid, 0·5% being free benzoic acid. Gas–liquid chromatography had shown that the [U-^{14}C]methylcyclohexane used contained not more than 1% of toluene, and it is likely that some of the benzoic acid found arose by direct oxidation of toluene (El Masri, Smith &

Table 4. *Extent of hydroxylation* in vivo *of the various carbon atoms of methylcyclohexane*

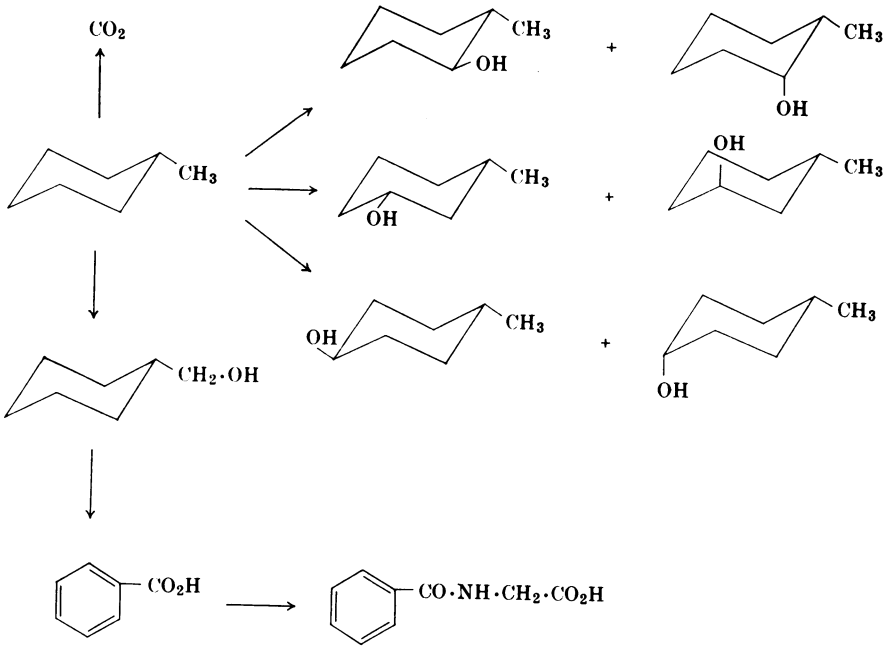
Experimental details are given in the text. The results given are average values for three determinations (see Table 3).

Position	Hydroxylation (% of dose)		
	Total	*cis*-Isomer	*trans*-Isomer
1	0	0	0
2	1·7*	0·5	1·2
3	22·0*	11·5	10·5
4	17·1	2·4	14·7
ω	$<$0·3	—	—

* There are two 2- and two 3-positions.



Scheme 1. Route of conversion of methylcyclohexane into benzoic acid.



Scheme 2. Metabolism of methylcyclohexane in the rabbit.

Williams, 1956). Approx. 0·3% of cyclohexylmethanol was detected in the urine, and this suggests that a small amount of methylcyclohexane had been metabolized to benzoic acid via cyclohexylmethanol and cyclohexanecarboxylic acid by the route shown in Scheme 1.

The metabolism of methylcyclohexane based on the findings in the present paper is shown in Scheme 2.

We gratefully acknowledge the generosity of the Wellcome Foundation in giving the [U-^{14}C]methylcyclohexane for this work, and the kind help of Professor Carl Djerassi who determined the purity of this material by gas–liquid chromatography. This investigation was supported in part by U.S. Public Health Service Research Grant GM 10042-02 from the Institute of General Medical Sciences.

REFERENCES

Bergstrom, S., Lindstredt, S., Samuelson, B., Corey, E. J. & Gregoriou, G. A. (1958). *J. Amer. chem. Soc.* **80**, 2337.

Bernhard, K. (1937). *Hoppe-Seyl Z.* **248**, 256.

Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. & Yankwich, P. F. (1949). *Isotopic Carbon*, p. 104. New York: John Wiley and Sons Inc.

Corey, E. J., Gregoriou, G. A. & Peterson, D. H. (1958). *J. Amer. chem. Soc.* **80**, 2338.

Eliel, E. L. & Lukach, C. A. (1957). *J. Amer. chem. Soc.* **79**, 5986.

Elliott, T. H., Parke, D. V. & Williams, R. T. (1959). *Biochem. J.* **72**, 193.

Elliott, T. H., Tao, R. C. C. & Williams, R. T. (1965). *Biochem. J.* **95**, 59.

El Masri, A. M., Smith, J. N., & Williams, R. T. (1956). *Biochem. J.* **64**, 50.

Gerarde, H. W. (1962). In *Patty's Industrial Hygiene and Toxicology*, vol. 2, p. 1211. Ed. by Irish, D. D. & Fassett, D. W. New York: Interscience Publishers Inc.

Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.

Hayano, M., Gut, M., Dorfman, R. I., Sebek, O. K. & Peterson, D. H. (1958). *J. Amer. chem. Soc.* **80**, 2336.

Kamil, I. A., Smith, J. N. & Williams, R. T. (1951). *Biochem. J.* **50**, 235.

Kingsbury, F. B. & Swanson, W. W. (1921). *J. biol. Chem.* **48**, 13.

Parke, D. V. (1956). *Biochem. J.* **62**, 339.

Parke, D. V. & Williams, R. T. (1950). *Biochem. J.* **46**, 236.

Parke, D. V. & Williams, R. T. (1953). *Biochem. J.* **54**, 231.

Paul, J. (1951). Ph.D. Thesis: University of Glasgow.

Quick, A. (1926). *J. biol. Chem.* **68**, 477.

Sperber, I. (1948). *J. biol. Chem.* **172**, 441.

Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.

Tipson, R. S. (1950). In *Technique of Organic Chemistry*, vol. 3, p. 426. Ed. by Weissberger, A. New York: John Wiley and Sons Inc.

Treon, J. F., Crutchfield, W. E., jun., & Kitzmiller, K. V. (1943*a*). *J. industr. Hyg.* **25**, 199.

Treon, J. F., Crutchfield, W. E., jun. & Kitzmiller, K. V. (1943*b*). *J. industr. Hyg.* **25**, 323.

Van Slyke, D. D., Plazin, J. & Weisiger, J. R. (1951). *J. biol. Chem.* **191**, 299.