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APPLICATION OF NUCLEAR MAGNETIC RESONANCE AND OPTICAL ROTATORY DISPERSION TO STUDIES IN DRUG METABOLISM—STEREOSELECTIVITY IN THE METABOLISM OF *trans*-2-*p*-TOLYL-CYCLOHEXANOL AND *trans*-2-*o*-TOLYLCYCLOHEXANOL IN THE RAT*

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Abstract—Metabolic studies have been undertaken in male Holtzman rats on *trans*-2-*p*-tolylcyclohexanol (I) and *trans*-2-*o*-tolylcyclohexanol (II). Nuclear magnetic resonance and vapor phase chromatography were used for the detection and identification of urinary metabolites and optical rotatory dispersion was used for assignment of absolute configurations of optically active metabolites. About half of the urinary metabolites recovered from i.p. administration of the racemic *para* isomer I had undergone aromatic methyl group oxidation, while there was no evidence of methyl group oxidation in the *ortho* isomer II. The other major metabolite from racemic I was optically active I having the absolute configuration (1S,2R)-(+)-*trans*-2-*p*-tolylcyclohexanol. The predominant metabolite of the *ortho* isomer II was shown to be the compound which had undergone ring hydroxylation in the axial position on C-5. The metabolite obtained from racemic II was optically active with positive rotation.

IN THE STUDY of drug metabolism, the determination of the stereochemistry of metabolites, including the absolute configuration of asymmetric molecules, makes it possible to investigate the stereospecificity of the enzymatic reactions associated with biotransformation processes. Recent advances in instrumentation have greatly facilitated this type of investigation. Most classical methods of metabolite identification, such as the various chromatographic techniques, are indirect and require a comparison with a known compound whose structure is suspected to be that of a metabolite. Although these methods are very valuable for the detection of metabolites, they provide little information in themselves concerning molecular structure and may be particularly misleading when used in studying compounds which give rise to isomeric metabolites. It is not uncommon for two isomers to migrate identically on more than one chromatogram. Consequently, the elucidation of the structure of unknown metabolites must depend upon more rigorous and direct information.

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Two very powerful techniques for the investigation of molecular structure and geometry are nuclear magnetic resonance (n.m.r.) and optical rotatory dispersion (o.r.d.). These methods provide knowledge of chemical structure not readily available by other means. The power of n.m.r. in solving stereochemical problems is well established. As applied to problems of drug metabolism, n.m.r. has the additional advantage of often providing extremely valuable information on material of low purity, o.r.d. (and its accompanying phenomenon of circular dichroism) is a powerful tool for the determination of absolute configuration.[1]

By using these techniques along with vapor phase chromatography (v.p.c.), we undertook the study of the metabolism of *trans*-2-*p*-tolylcyclohexanol (I) and *trans*-2-*o*-tolylcyclohexanol (II). We were interested in determining if any stereoselectivity existed in the biotransformation of the enantiomers of a given isomer, particularly in view of the results of Elliott *et al.*[2] on the metabolism of the 2-methylcyclohexanols, compounds stereochemically related to I and II. In addition, the influence of the position of the methyl group (whether *ortho* or *para*) was to be examined. Since the absolute configuration of II was known,[3] a reference was available for the assignment of absolute configuration to the metabolites. Only urinary metabolites were investigated. The work of Elliott *et al.*[2] showed that metabolites of methylcyclohexanols were eliminated almost exclusively in the urine as glucuronides.

EXPERIMENTAL

Animals and administration of compounds

The animals used were male Holtzman rats weighing initially 190 ± 10 g. The animals about doubled their weight over the approximate 3-week duration of an experiment. The diet consisted of ground rat Purina lab chow and the animals were allowed free access to food and water throughout the course of an experiment. They were placed in pairs in metabolic cages designed to separate urine from feces and the same pair was always kept in the same cage. Each pair of animals was used in only one experiment.

The compounds were administered i.p. three times daily at 5- to 7-hr intervals in the form of an emulsion or a suspension in 5% gum acacia. The dosage forms were prepared fresh daily, stored at 5° and warmed just prior to injection. All animals received 20 mg of compound per dose. In each experiment a total of 1 g of compound was given collectively to 6 animals. Control animals received injections of 5% gum acacia. All animals receiving II underwent a short period of ataxia. In some cases there was an initial short period of convulsions characterized by arching of the back and neck, and a running motion of the front legs. No untoward effects were noted in animals receiving I at this dose level.

Collection and treatment of urine

The urine was collected every 12 hr, pooled, frozen and stored at dry-ice temperature until thawed for analysis. The urine samples were collected for two periods of 7 days and the two batches were analyzed separately. Control samples of urine were collected for a minimum period of 3 days prior to the start of an experiment; the animals served as their own controls.

The 7-day urine collection was thawed, filtered, acidified to 1 N with concentrated HCl and boiled under reflux for ½ hr. Additional heating did not increase the recovery

of metabolites. After chilling, the hydrolyzed urine was extracted several times with ethyl ether. The combined ether extracts were reduced in volume, extracted with 10% sodium carbonate solution and washed with a small portion of water. After filtering through a small amount of anhydrous sodium sulfate, the solvent was removed with a rotary evaporator and any residual water was azeotroped with absolute ethanol or benzene under reduced pressure at 50°. This first extract, containing the neutral substances, was dark red and generally weighed about 100–120 mg; it showed no tendency to crystallize. The corresponding extract from the control urine was the same color but weighed considerably less.

The acidified basic washes were extracted with ether and the combined ether extracts were washed once with water. After filtration through anhydrous sodium sulfate, the solvent was removed and the dark oily residue of this extract, containing organic acids and possibly phenols, solidified on standing. The weight of this extract was usually about 1 g.

Analysis of crude extracts

The crude extracts were analyzed by v.p.c. and n.m.r. Comparison was made with the extracts obtained from control urine and it was found that the metabolites were present exclusively in the first 7-day collection fraction. The crude extracts of neutral substances, obtained as described in the preceding section, were dissolved in about 0·5 ml deuteriochloroform and the n.m.r. spectra obtained. A small sample of this solution was analyzed by gas chromatography on Carbowax 20 M and Silicon QF1 columns at 190°. The Carbowax column gave better separation of the various components. The base-soluble extract was dissolved in a solution of 10% sodium carbonate in deuterium oxide and the n.m.r. spectrum was obtained with sodium-2,2-dimethyl-2-pentasilane-5-sulfonate (DSS) as an internal reference. Because the signal of the aromatic hydrogens and that of the hydrogens of the aromatic methyl group could be readily seen, the n.m.r. spectra of the crude extracts were extremely useful in the detection of metabolites.

The o.r.d. curves were obtained in methanol at 25° on a Cary 60 recording spectropolarimeter using a 250 W xenon lamp as the light source in a 1·0-cm cell and employing a fixed slit width of 0·3 mm from 600 to 450 mμ and the manufacturer's preprogrammed slit width from 450 to 230 mμ.

The n.m.r. spectra were determined at 60 MHz on a Varian Associates A-60 spectrometer in the solvents specified.

RESULTS

Experiments on urine

The pH of each 12-hr urine sample from each cage (2 animals) was determined by using short-range pHydrion paper. One sample of urine from each cage was tested daily with Hema-Combistix for urinary blood, protein, glucose and pH. No deviations from control values were noted in any animals receiving the compounds.

Isolation and characterization of metabolites

The main urinary metabolic products recovered from racemic I and racemic II are shown in Fig. 1. Both metabolites arising from I were characterized. At least three metabolites were detected as arising from II, but only one was isolated and characterized.

trans-2-p-*Tolylcyclohexanol I.* After the administration of 1·0 g of racemic I to six animals, the 7-day urine yielded a crude extract of neutral substances weighing approximately 100 mg. Analysis of this extract by v.p.c. on both a Carbowax 20 M and a Silicon QF1 column indicated the presence of only a single component which could be considered as a metabolite. The retention time for this component was identical to that of the administered alcohol. The n.m.r. spectrum from this extract confirmed the presence of a metabolite.

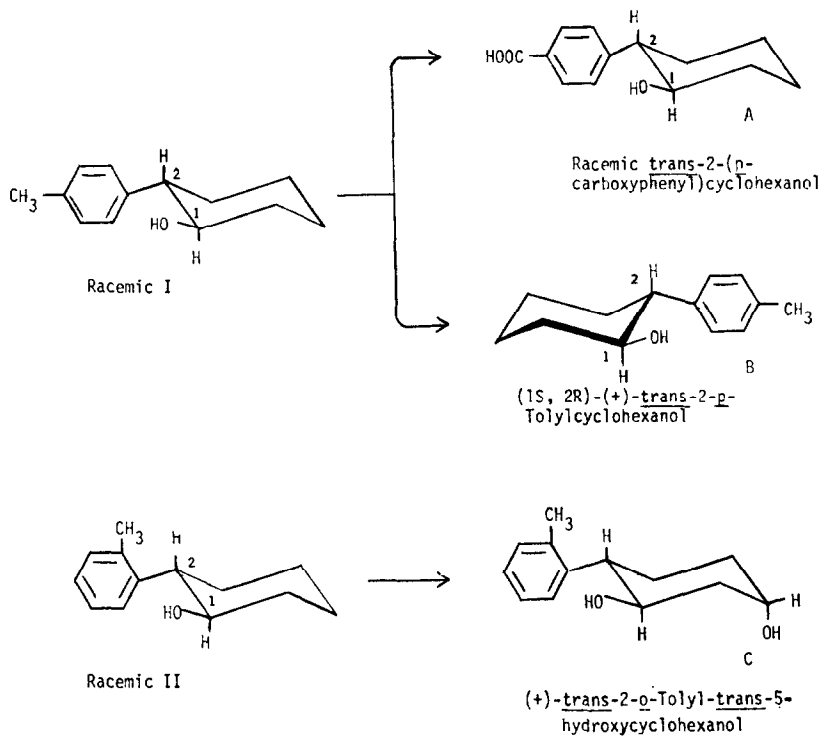

FIG. 1. Major urinary metabolites isolated from the administration of racemic I and racemic II.

The n.m.r. spectrum of this crude extract showed the presence of a metabolite from clearly discernible singlets at τ 2·90 and 7·70, indicative of aromatic hydrogens and tolyl methyl hydrogens respectively. The metabolite was isolated by column chromatography of the extract over silica gel with petroleum ether and benzene as eluents. Figure 2 shows the n.m.r. spectrum of authentic *trans*-2-*p*-tolylcyclohexanol I (upper curve) and that of metabolite B (lower curve). The n.m.r. spectra of *trans*- and *cis*-2-arylcyclohexanols have been reported and interpreted previously.[4,5] The important signals in the spectrum of I are the singlet caused by the 4 aromatic hydrogens at τ 2·90, the singlet of the aromatic methyl hydrogens at τ 7·70 (the small blips at τ 2·40, 3·30 and 7·30 are rotational side bands of these 2 main singlets), and the signal of H-1, the axial hydrogen on the hydroxyl-bearing carbon, which appears as a broad multiplet centered at about τ 6·45. The width of this multiplet is typical of signals of axial hydrogens coupled with 2 adjacent axial hydrogens and 1 equatorial hydrogen

in *trans*-2-arylcyclohexanols.[4,5] The signal of the hydroxyl hydrogen appears as a sharp singlet at τ 8·34. The spectrum of metabolite B was obtained on a smaller quantity of material of lower purity than that of authentic I. With the exception of the position of the signal of the hydroxyl hydrogen, which appears at about τ 8·08 in the spectrum of metabolite B, the spectra are essentially identical. Chemical shifts of

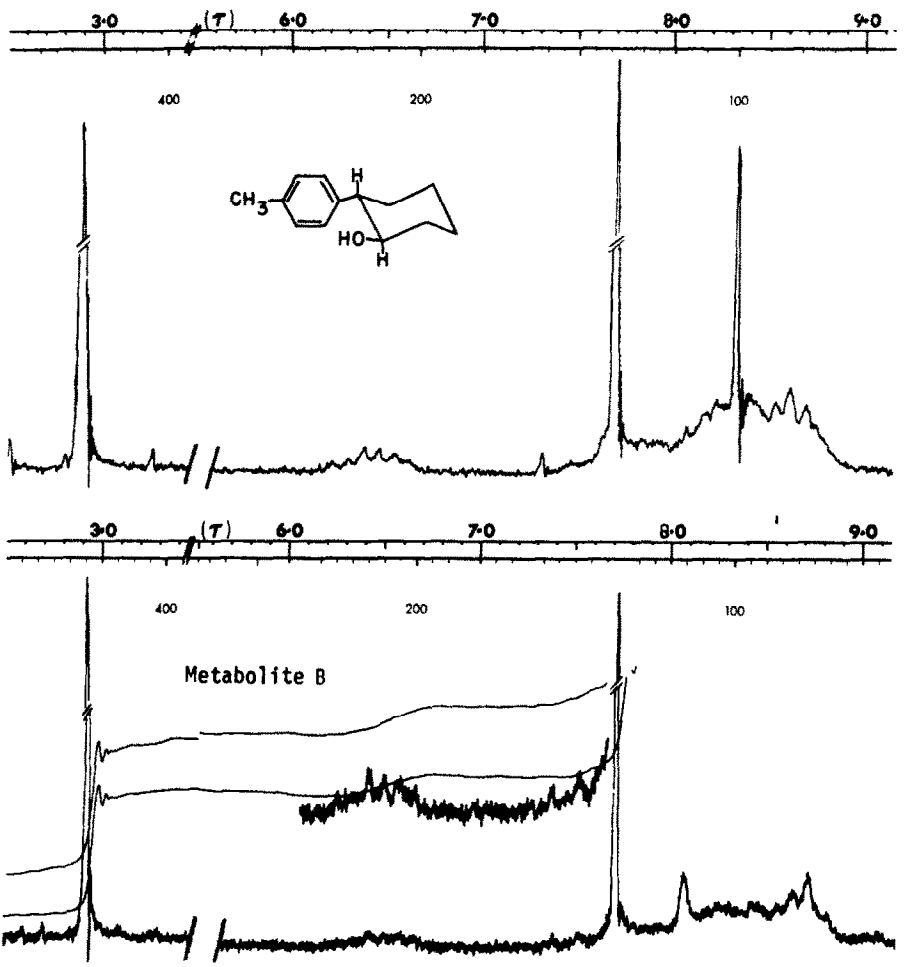

FIG. 2. The n.m.r. spectra in deuteriochloroform of *trans*-2-*p*-tolylcyclohexanol (upper) and of the neutral urinary metabolite B from the administration of racemic I (lower).

hydroxyl hydrogens are concentration dependent and are also affected by small amounts of impurities which can undergo hydrogen exchange. The shape of the signal is also affected by the rate of exchange. Differences in the signals of hydroxyl hydrogens are therefore not meaningful. Integration of the spectrum of metabolite B gave a ratio of 1:3:4 for signals of H-1, aromatic methyl, and aromatic hydrogens respectively.

The neutral metabolite B was found to be optically active and dextrorotary. Its o.r.d. curve is given in Fig. 3 and compared with that of authentic levorotary

(1R,2S)-(-)-*trans*-2-*p*-tolylcyclohexanol,* of known absolute configuration,† obtained by resolution of racemic I. Comparison of specific rotations indicates that the metabolite is approximately 60 per cent optically pure, i.e. 80 per cent enantiomer. With the exception of slightly lower intensity of the metabolite curve, the two curves are mirror images of each other. The o.r.d. curve of metabolite B was also similar in sign and shape to that of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol, for which the absolute configuration has been established and published.[3] The assignment of the absolute configuration of metabolite B can therefore be made as (1S,2R)-(+)-2-*p*-tolylcyclohexanol.

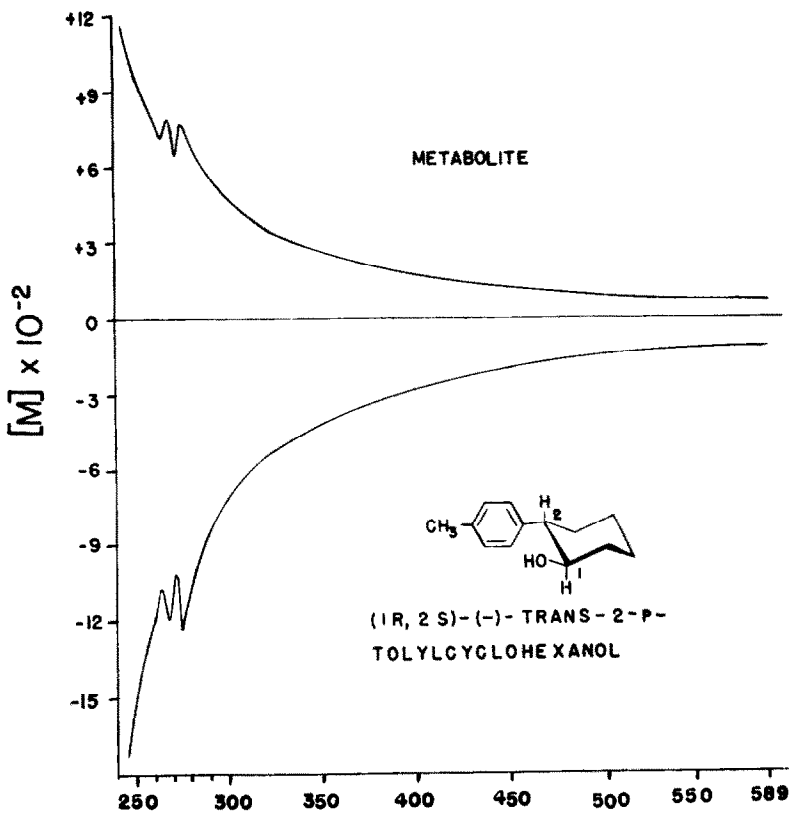

FIG. 3. Optical rotatory dispersion curves of the neutral metabolite B from administration of racemic I and of the levorotary enantiomer obtained by resolution of racemic I.

From the extract for acidic substances, about 100 mg of optically inactive *trans*-2-(*p*-carboxyphenyl)cyclohexanol was obtained, m.p. 204–205·5°. The acidic metabolite was characterized primarily from the n.m.r. spectrum of its sodium salt measured in D_2O, shown in Fig. 4. The pair of doublets centered at τ 2·01 and τ 2·71, showing a value of J = 8·0 cps, and the lack of a methyl signal in the region of τ 7·7 clearly show that the aromatic methyl group has been oxidized to the carboxyl group. The

* The configuration is designated according to the sequence rule of Cahn *et al.*[6,7]

† The absolute configuration of this enantiomer has been established by X-ray crystallography (A. C. Huitric, unpublished data) and will be reported in a separate publication.

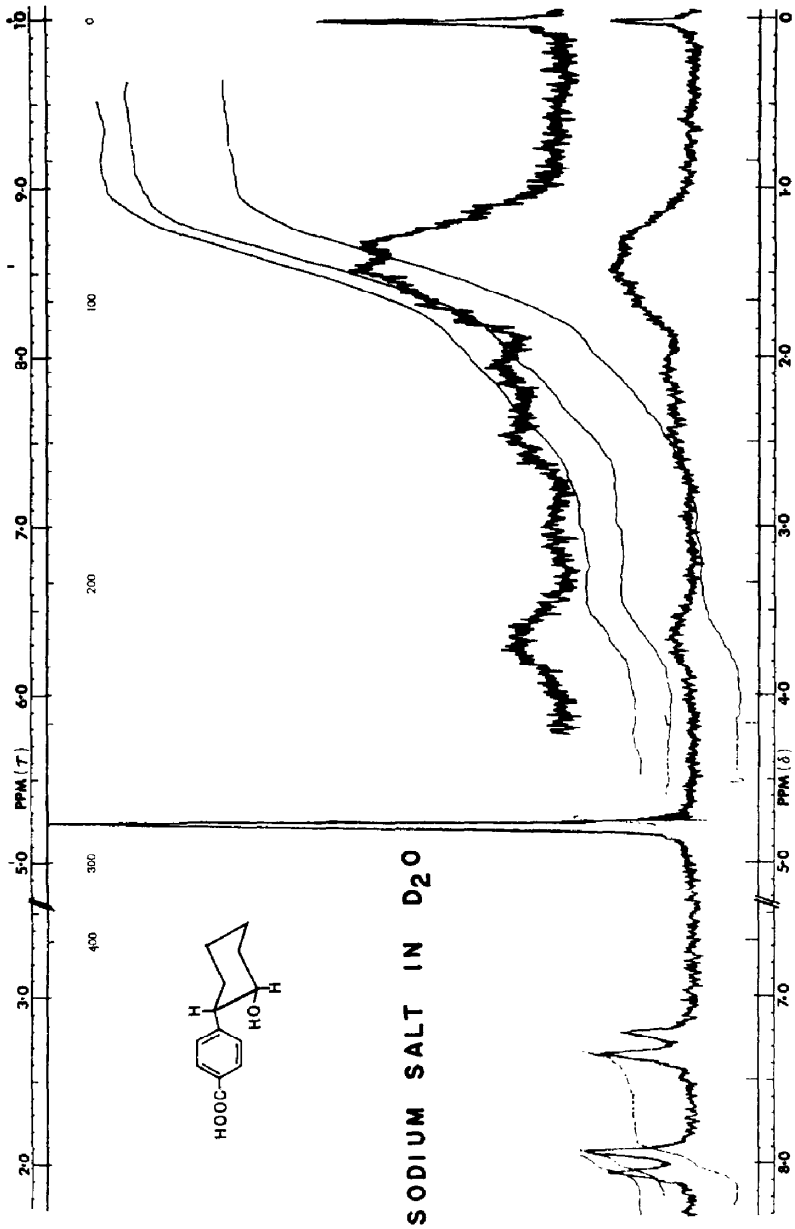

FIG. 4. The n.m.r. spectrum in D_2O of the sodium salt of the acidic urinary metabolite A from the administration of racemic I.

aromatic hydrogens of the starting material gave a single peak (see Fig. 2). The doublets with coupling constants of 8·0 cps give the typical A_2B_2 pattern of a *para*-substituted benzene ring having substituents of different electronegativities and/or different long-range shielding effects, such as a *para*-substituted benzoic acid. The broad unresolved multiplet of half-width of 18·0 cps centered at τ 6·32 is consistent with the signal of axial H-1 and the broad multiplet at τ 7·5 is consistent with the signal of axial H-2. The integration of the spectrum is consistent with the proposed structure. The sharp singlet at τ 5·23 is due to water. The i.r. spectrum (potassium bromide disc) indicated a broad band in the region of OH stretching and an intense carbonyl band at 1685 cm^{-1}, typical of carboxylic acids.

trans-2-o-*Tolylcyclohexanol II.* From a total of 1·0 g of this alcohol administered to 6 rats, approximately 125 mg of an extract of neutral substances was obtained. Analysis of this extract by gas chromatography showed four peaks not present in the control urine extract. One of these peaks, of very low amplitude, had a retention time identical to that of the parent alcohol on both columns employed. The 3 major peaks had considerably longer retention times than either the parent alcohol or any component of the control urine extract. The n.m.r. spectrum of this extract indicated that the metabolites were hydroxylated derivatives of the parent alcohol. After column chromatography on neutral alumina, with benzene and petroleum ether as eluents, the major component was isolated and characterized as the metabolite resulting from axial hydroxylation of II at C-5 (metabolite C). Metabolite C was optically active.

Attempts to isolate the other components in sufficient quantities for identification were not successful. The compound having the same effluent time as the parent alcohol was concentrated in the fractions containing the less polar substances of the extract. The amount of this compound was extremely low. Further attempts to isolate the other two metabolites by column chromatography yielded only partial separation. The n.m.r. and v.p.c. data on these partially purified fractions suggest that these components are also diols resulting from ring hydroxylation.

The n.m-r. spectrum of metabolite C suggested the axial C-5 ring hydroxylated product. The identity of the metabolite was established primarily by comparison of its n.m.r. spectra in two different solvents with those of authentic *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol previously synthesized in this laboratory.[8] The n.m.r. spectra in deuteriochloroform and in pyridine are given in Figs. 5 and 6 respectively. Unequivocal chemical proof of the stereochemistry of the authentic diol has been reported.[8] The stereochemistry is further substantiated by the spectrum of the diol in pyridine (Fig. 6). The signal of axial H-1 appears as a sextet centered at τ 5·25. The upfield portion of the signal is partially overlapped by the much narrower signal of equatorial H-5 centered at τ 5·50. The sextet at τ 5·25 results from coupling of axial H-1 with adjacent axial H-2 and axial H-6, plus equatorial H-6. The apparent coupling constants measured from the spectrum are Jaa = 10·5 and Jae = 4 cps. The half-height width of the signal of H-5 is about 9–10 cps and this is consistent with H-5 being equatorial and coupled with 2 adjacent axial and 2 equatorial hydrogens. The spectrum of the corresponding tetradeuterated diol with deuteriums on C-3 and C-6 has been measured in pyridine and reported.[9] In this spectrum the signal at τ 5·25 gave a doublet with separation of 10 cps resulting from coupling of axial H-1 with axial H-2, and the signal at τ 5·50 gave a triplet with separations of about

3 cps, resulting form coupling of equatorial H-5 with the equatorial and axial hydrogens on C-4. The equatorial orientation of the hydroxyl group on C-1 and the axial orientation on C-5 are therefore completely substantiated by n.m.r. spectra of the authentic diol and its 3,3,6,6-tetradeuterated analog. The observed axial–axial and axial–equatorial coupling constants are within the normal ranges.[10] The n.m.r. spectra

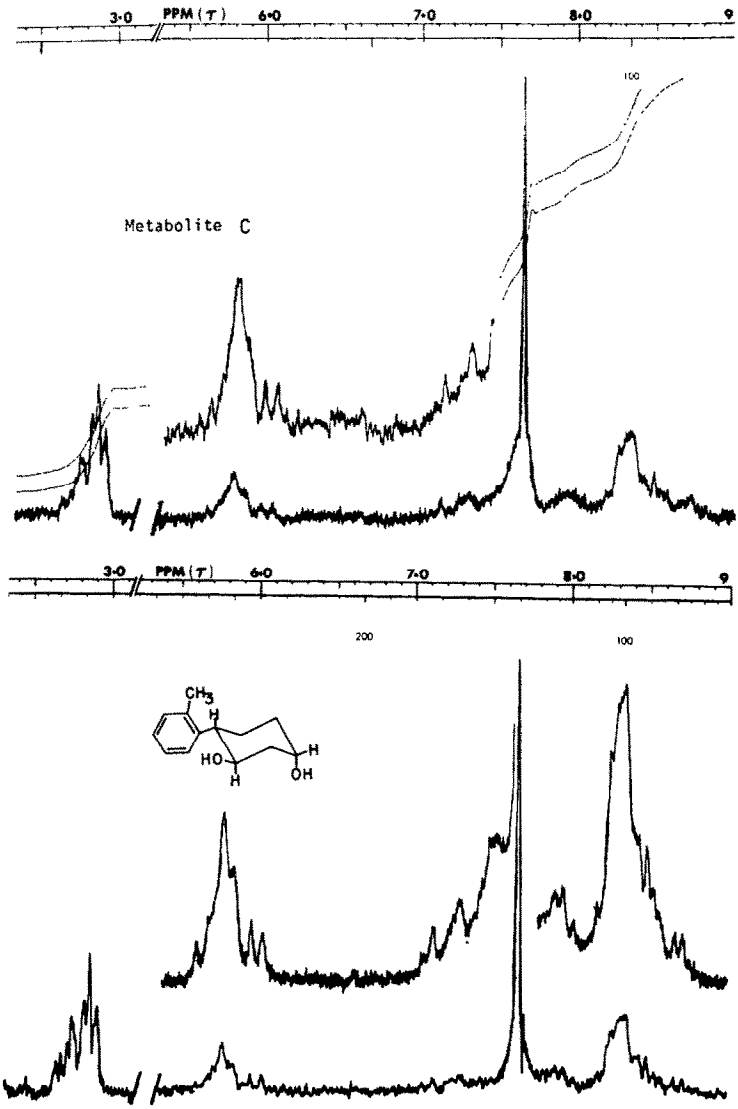

FIG. 5. The n.m.r. spectra in deuteriochloroform of the neutral urinary metabolite C from the administration of racemic II (upper) and of *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol (lower).

of metabolite C were obtained on less pure and more dilute samples than those of the authentic diol, but comparison of the spectra leaves very little doubt regarding the identity of the metabolite. The identical solvent effects on chemical shifts for the two samples in going from chloroform to pyridine are very significant. Solvent effects on

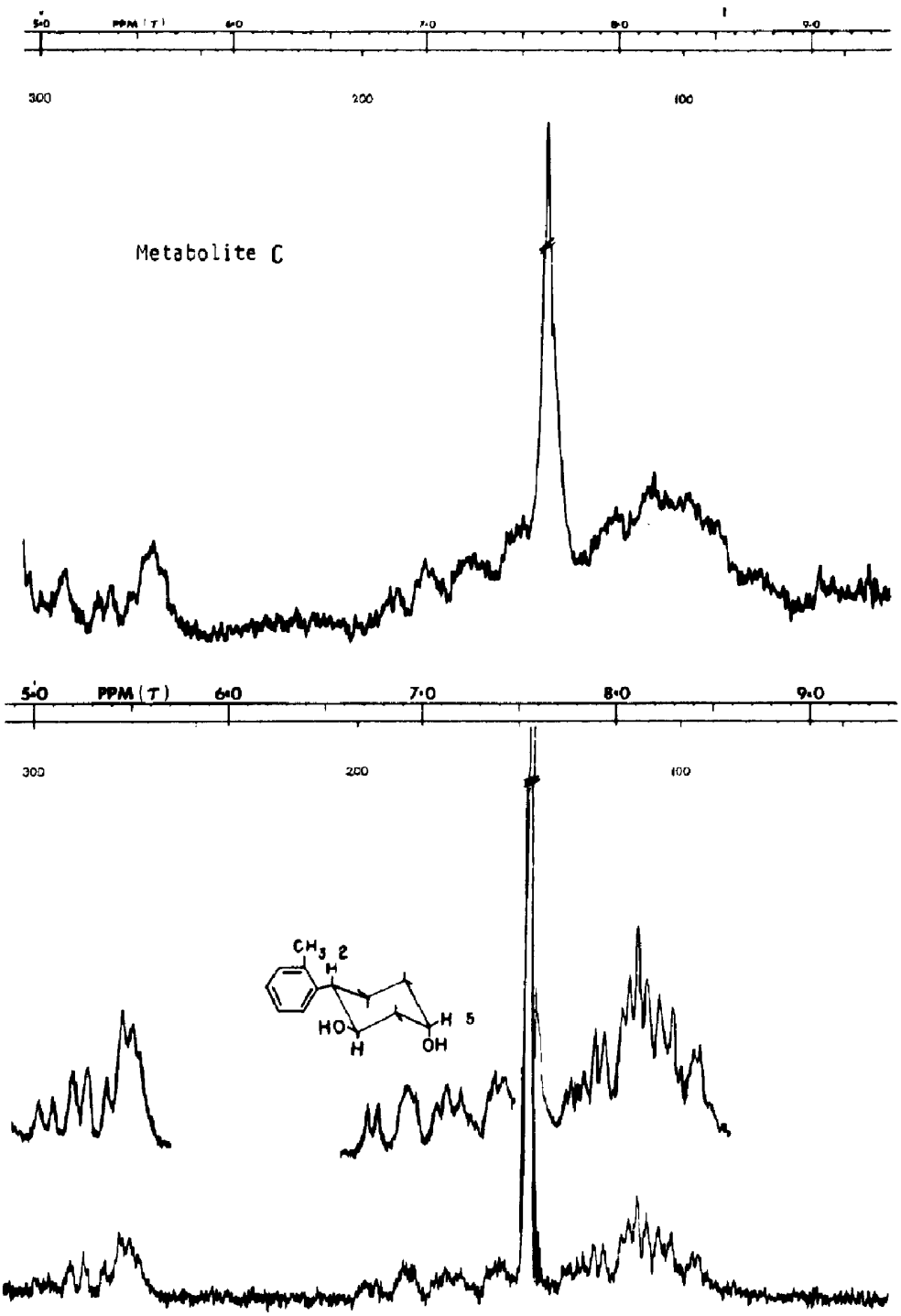

FIG. 6. The n.m.r. spectra in pyridine of the neutral urinary metabolite C from the administration of racemic II (upper) and of *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol (lower).

chemical shifts of H-1, H-2 and H-5 of the authentic diol have been reported.[9] Metabolite C and the authentic diol were also shown to have identical retention times on two different columns in gas chromatography.

The optical purity of the metabolite is unknown. The o.r.d. curve is shown in Fig. 7. The similarity of the curve in the region of the aromatic chromophore absorption,

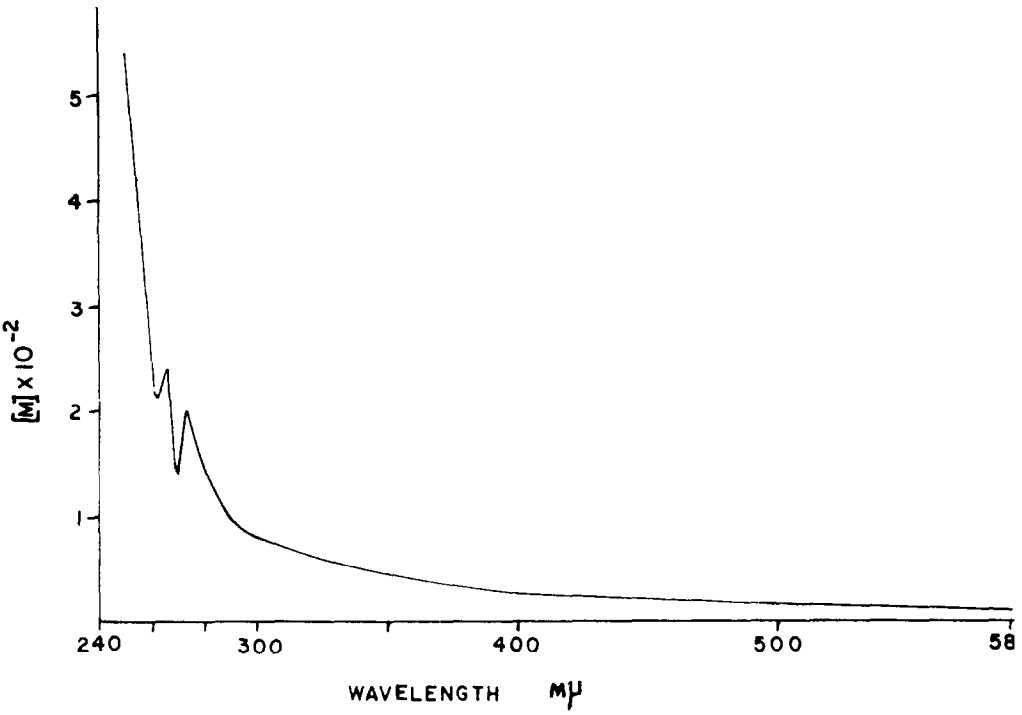

FIG. 7. Optical rotatory dispersion curve in methanol of the neutral urinary metabolite C from the administration of racemic II.

around 260 mμ, with the o.r.d. curve of (1S,2R)-(+)-*trans*-2-*o*-tolylcyclohexanol,[3] and comparison with the curves in Fig. 3, suggest that metabolite C has the S configuration at C-1 and the R configuration at C-2. This would require the R configuration at C-5, since the axial orientation of the hydroxyl group at C-5 has been established by n.m.r.

No acidic fractions could be found among the urinary metabolic products of II. The n.m.r. spectrum of the base-soluble extract was the same as the spectrum of the corresponding extract of control urine.

DISCUSSION

On the assumption that the metabolites of the present study would be eliminated as glucuronides, the isolation procedure was initiated with acid hydrolysis of the urine. The heating period was arbitrarily chosen at 30 min. The ½-hr reflux was found to be sufficient, since an additional reflux period of 1 hr and subsequent extractions failed to produce more metabolites as indicated by v.p.c. analysis. Gas chromatography analysis indicated that the hydrolytic conditions did not cause elimination reactions.

Extensive elimination would also have been detected in the n.m.r. spectra, but no signals were found in the region of absorption of vinylic hydrogens. The metabolites were excreted as conjugates, since no metabolites could be detected by ether extraction of the urine prior to hydrolysis.

The recovery of metabolites from the urine was low, ranging from 10 to 20 per cent. This recovery suggests that biliary excretion may also be involved in their elimination, but the fecal matter was not examined. The uptake of these compounds in fat depots followed by a slow release over many days seems unlikely, since in no case was any metabolic product detected in the second 7-day urine sample.

A large amount of benzoic acid was found in the base-soluble extracts of hydrolyzed urine. This was assumed to arise from dietary sources. In one instance the actual elimination product, hippuric acid, was isolated and identified. It was also found in control urine extracts. Cleavage of the tolyl ring from the rest of the molecule would lead to derivatives of either toluic acid or phthalic acid, but could not lead to benzoic acid.

The reproducibility in the n.m.r. spectra of the control urine extracts suggests that examination of crude extracts for drug metabolites is amenable to n.m.r. analysis. Although there were small deviations in peak intensities in the n.m.r. spectra of the corresponding extracts from various groups of control animals (and also from the second-week test urine samples), these differences were minor.

In studying the metabolism of the 2-methylcyclohexanols and 2-methyl-cyclohexanones, Elliott *et al.*[2] showed that racemic *trans*-2-methylcyclohexanol was excreted unchanged (via the urine as glucuronides), but that racemic *cis*-2-methylcyclohexanol and racemic 2-methylcyclohexanone were both converted to (+)-*trans*-2-methylcyclohexanol. This indicates that the *cis* alcohol is metabolized to the (+) enantiomer of the *trans* isomer through the ketone as an intermediate and that the reduction of the ketone is stereospecific. They found no evidence for ring hydroxylation to diols or polyols. This mechanism requires that only 50 per cent of the racemic ketone should be reduced to the dextrorotary alcohol unless the ketone isomerizes through enolization. Their v.p.c. evidence indicated the *cis* alcohol was a urinary metabolite of the ketone, but it was not isolated. Consequently, the optical activity of this metabolite is unknown. Cheo *et al.*[11] have obtained (+)-*cis*-2-*t*-butylcyclohexanol from the urine of rabbits fed racemic 2-*t*-butylcyclohexanone.

In the light of Elliott's report and of the fact that the 2-*o*-tolylcyclohexanols are configurationally related to the 2-methylcyclohexanols,[3,12] we also conducted some preliminary metabolic studies on *cis*-2-*o*-tolylcyclohexanol and 2-*o*-tolylcyclohexanone. After administration of the racemic *cis* alcohol to rats, the crude extract of neutral substances showed (by v.p.c. analysis) at least 5 metabolic products. One peak of very small amplitude had the same retention time as that of the administered alcohol and the other components all had longer retention times. No evidence for aromatic methyl group oxidation was found. The v.p.c. and n.m.r. data suggest that the major urinary metabolites are diols resulting from ring hydroxylation, but these products have not been isolated and characterized.

The urinary extract after administration of the ketone contained two, or possibly three, metabolites having longer retention times in gas chromatography than the administered ketone, but shorter than the retention times of the metabolites of either the *cis* or *trans* alcohol. No unchanged ketone nor products of aromatic methyl

group oxidation were found. The n.m.r. spectrum of the mixture showed signals of the aromatic hydrogens and of the tolyl methyl hydrogens of the ketone. It appears that the metabolites may be monohydroxy derivatives of the ketone.

In contrast to the results of Elliott, we found no evidence for oxidation of either alcohol to the ketone or of reduction of the ketone to either alcohol. In the *para*-tolyl series only the *trans* alcohol was investigated and it is not possible to give a definite explanation for the fact that the (+)-enantiomer was recovered from the administration of the racemic mixture, especially since the other metabolite (the carboxylic acid) was not optically active. The optical inactivity of the acid metabolite means that the oxidation process proceeds with equal facility in both enantiomers.

Our results also show that the *ortho* and *para* isomers I and II are metabolized by entirely different routes. A methyl group in the *ortho* position appears to inhibit conjugation of a *trans* vicinal OH function, presumably by steric effects. Such effects are also evident in methyl group oxidation. Only the nonhindered *para* methyl group was oxidized. It is also of interest that only the *ortho* isomer underwent ring hydroxylation and this process was, at least, stereoselective.

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