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STUDY OF THE INTESTINAL TYROSINE METABOLISM USING STABLE ISOTOPES AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Deuterated tyrosine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylpropionic acid, 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid were incubated under anaerobic conditions with human faecal specimens for the *in vitro* study of their respective metabolisms. After 1 week, aromatic acids and phenols were extracted and analyzed by gas chromatography–mass spectrometry.

[3′,5′-$^2H_2$]Tyrosine produced 4-hydroxyphenyllactic acid, 4-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid; [3′,5′-$^2H_2$]-4′-hydroxyphenyllactic acid produced 4-hydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, 4-hydroxyphenylacetic acid and phenylpropionic acid; [3′,5′-$^2H_2$]-4′-hydroxyphenylpropionic acid produced 3-hydroxyphenylpropionic acid and phenylpropionic acid; [3′,5′,2,2-$^2H_4$]-4′-hydroxyphenylacetic acid produced *p*-cresol; and [3′,5′-$^2H_2$]-4′-hydroxybenzoic acid produced phenol.

Thus the intestinal flora showed activities for decarboxylation leading to phenol and *p*-cresol, dehydroxylation leading to phenylpropionic acid and rearrangement leading to 3-hydroxyphenylpropionic acid. Retention of both deuterium labels was observed in the rearrangement reaction.

INTRODUCTION

Deuterated amino acids and steroids have been used for *in vivo* studies of their respective metabolisms in man. These labelled compounds have been given orally to healthy subjects as well as to patients, and the metabolites and their isotope content have been analyzed in urine using combined gas chromatography–mass spectrometry (GC–MS). This technique elucidated the metabolic pathways *in vivo* without risk to the subjects. We previously reported our results from patients with irregularities in the tyrosine degradation pathway, one patient with phenylketonuria, one with hyperphenylalanineamia and the third with oligophrenia of unknown genesis[1]. The patients were dosed orally with 150 mg/kg of deuterated L-tyrosine. GC–MS investigations of

the urine revealed that the same metabolites were deuterated as in normal controls, but in addition deuterated benzoic acid, hippuric acid, 3-hydroxyhippuric acid and 3-hydroxyphenylhydracrylic acid were found deuterated and excreted in increased concentrations. When neomycin was given during 3 days for partial sterilization of the intestine before the dosing, tyrosine was no longer converted into the four additional compounds mentioned above. No excretion of the ordinary urinary metabolites *p*-cresol and phenol was found after admission of neomycin.

Bakke[2] reported similar results after treating rats orally with neomycin. When experimenting with rats, the level of urinary phenols was reduced to almost zero.

It must be concluded that benzoic acid, hippuric acid, 3-hydroxyhippuric acid, 3-hydroxyphenylhydracrylic acid, phenol and *p*-cresol should be derived from the degradation of tyrosine or tyrosine metabolites by the intestinal flora. We were interested, therefore, to study the metabolic activity of the human intestinal flora with tyrosine and its metabolites. Drasar and Hill[3] described the production of phenolic acids and phenols from tyrosine. Bacteria desaminate tyrosine by reduction to 4-hydroxyphenylpropionic acid, which is decarboxylated to 4-ethylphenol or oxidized to 4-hydroxyphenylacetic acid. These compounds are either decarboxylated to *p*-cresol or oxidized to *p*-hydroxybenzoic acid. The latter is further decarboxylated to phenol. Bakke[2] demonstrated the decarboxylation of 4-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid by rat caecal content. When tyrosine was incubated with rat caecal content, he found 4-hydroxyphenylacetic acid, *p*-cresol and phenol. Scott *et al.*[4] demonstrated that 4-hydroxyphenylpropionic acid was dehydroxylated to phenylpropionic acid by sheep rumen content. Studies of bacterial metabolism were often carried out with radioactive test substances in connection with thin-layer chromatography, high-voltage electrophoresis or GC[5]. *In vitro* studies of the tyrosine metabolism using gut bacteria are handicapped by the bacteriostatic action of some aromatic acids. Hence, only small amounts of labelled substances could be used for incubation with human faecal content.

For *in vitro* studies with labelled precursors, the stable isotope GC–MS technique seems to be very advantageous. Apart from physiological safety, there are analytical advantages in using stable isotopes. The components can be separated by GC and their isotope content can be directly analyzed by mass spectrometry. The GC–MS combination is superior in its separating capacity to conventional methods of determining radioactive isotopes, where the components are usually separated by paper, thin-layer or column chromatography and then measured off-line with a liquid scintillation counter.

For these reasons and in analogy with our previously published *in vivo* experiments, we used deuterated compounds for incubation.

EXPERIMENTAL

Collection of samples

Two normal persons were dosed with 150 mg/kg of deuterated tyrosine and 500 mg of carmine red, the latter serving as a coloured marker, which could be easily re-found in the faeces. A 50-g amount of the first red-coloured faeces was suspended in 100 ml of a sterile phosphate buffer of pH 7 which was flushed with nitrogen. The urine of these persons was also collected from the time of the dosing until 2 days after-

wards. Another faecal specimen of 50 g suspended in 100 ml of phosphate buffer was delivered by a patient with phenylketonuria. The patient had been dosed with phenylalanine 2½ days before collection of the sample.

Materials

The following chemicals were used: 4-coumaric acid (Fluka 28190; Fluka, Buchs, Switzerland), 3-coumaric acid (Fluka 28180), 3-(4-hydroxy)phenylpropionic acid (Fluka 56190), *p*-cresol (Fluka 61030), 4-ethylphenol (Merck 821290; Merck, Darmstadt, G.F.R.), phenol (Merck 822296), hydrochloric acid-d_1, 20% in D_2O (Merck 2900), phenylpropionic acid (Fluka 56670), deuterium oxide (Ciba-Geigy 10690; Ciba-Geigy, Basel, Switzerland), bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Regis 270112; Regis, Morton Grove, Ill., U.S.A.), 3% XE-60 liquid phase on 80–100-mesh Gas-Chrom Q (Supelco, Bellefonte, Pa., U.S.A.), DL-4-hydroxyphenyllactic acid (Sigma H-3253; Sigma, St. Louis, Mo., U.S.A.) and DL-4-hydroxymandelic acid (Sigma H-0502). 3-Hydroxyphenylpropionic acid was synthesized from 3-coumaric acid by catalytic hydrogenation with platinum in glacial acetic acid. All other reagents and solvents were of analytical-reagent grade.

Deuterated compounds

3,5-Dideutero-L-tyrosine and 3,5-dideuterobenzoic acid were synthesized by A. Wessiak and P. Hemmerich, Department of Biology, University of Konstanz, G.F.R.

By analogy with the method of Jacobsen[6], 4-hydroxyphenylacetic acid and 4-hydroxyphenylpropionic acid were deuterated with hydrochloric acid-d_1 and deuterium oxide. The aromatic acid (0.5 g) was suspended in 0.5 ml of 20% deuterohydrochloric acid and 1 ml of deuterium oxide. The test-tube containing the reaction mixture was sealed under atmospheric pressure. After incubation at 120° for 24 h, the mixture was evaporated to dryness. The deuteration was controlled by nuclear magnetic resonance (NMR) spectroscopy and GC–MS.

For the NMR spectroscopy, 30 mg of substance were dissolved in 0.5 ml of acetone-d_6. As the internal standard we used tetramethylsilane. The NMR spectrum was obtained with a Hitachi Perkin-Elmer R-24 high-resolution NMR spectrometer. For the MS, a few milligrams of substance were dissolved in 0.5 ml of ethyl acetate and the derivatization was carried out as described below. 4-Hydroxyphenylpropionic acid was deuterated twice in the aromatic ring. 4-Hydroxyphenylacetic acid had two deuterium atoms in the aromatic ring and two in the side-chain in the α-position to the carboxyl group. 4-Hydroxyphenyllactic acid and coumaric acid were completely destroyed under the same conditions.

4-Hydroxyphenyllactic acid could be deuterated by a modified deuteration procedure. The test-tube, containing the same amounts of aromatic acid, deuterohydrochloric acid and deuterium oxide as above, was sealed under high vacuum and incubated at 100° for 24 h. The slightly red-coloured reaction mixture was extracted three times with ethyl acetate and evaporated to dryness. NMR spectroscopy and GC–MS were carried out as above. 4-Hydroxyphenyllactic acid was deuterated twice in the aromatic ring. Coumaric acid was again destroyed under these incubation conditions.

METHODS

Incubation of faecal specimens with deuterated aromatic acids

Clostridial medium (12.7 g) was dissolved in 300 ml of water. The test substances tyrosine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylpropionic acid, 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid were dissolved in water to give a concentration of 10 μmole/ml and 10.8 ml of clostridial medium and 1.2 ml of the test substances were placed in test-tubes with screw-caps. The test-tubes were heated in a boiling water-bath for 15 min and cooled under nitrogen–carbon dioxide (1:1). After sterilization (20 min at 120°), the ten tubes (two of each test substance) were inoculated with faeces in a glove bag under nitrogen–carbon dioxide (1:1). The test-tubes were placed in serum bottles which were closed under nitrogen–carbon dioxide (1:1). After incubation at 37° for 1 week, all of the test-tubes were closed with screw-caps and stored at —4°. In order to control the anaerobic conditions an oxygen indicator according to Willis[7] accompanied the test-tubes through the whole process.

Extraction of urine, faeces and cultures of faecal specimens

The well established extraction method for aromatic acids in urine described by Völlmin *et al.*[8] was also applied to faecal suspensions and cultures of faecal specimens.

A 10-ml volume of urine, faecal suspension or culture of faecal specimens was acidified with 6 *N* hydrochloric acid to pH 1 saturated with sodium chloride and extracted three times with 20 ml of ethyl acetate. The organic phase was then brought to pH 1 with 6 *N* hydrochloric acid, saturated with sodium chloride and again extracted three times with 20 ml of ethyl acetate. The organic phase was dried with sodium sulphate and the solvent evaporated to 0.1 ml in a vacuum rotary evaporator. This extract was used for GC–MS analysis.

The phenols were extracted from 10 ml of urine, faeces or cultures of faecal specimens. The samples were acidified with 2 ml of 6 *N* hydrochloric acid and steam distillation by use of a Kjeldahl apparatus was performed. This procedure resulted in acid hydrolysis of the phenolic sulphate esters and in extraction of the free phenols. The pH of the solution of phenols in water was adjusted to 7.5 with 0.1 *N* sodium hydroxide solution. The solution was saturated with sodium chloride and extracted three times with 20 ml of diethyl ether. The combined extracts were dried with sodium sulphate, cooled in an ice-bath for 10 min and evaporated to 0.5 ml at room temperature using a vacuum rotary evaporator. The sample was then transferred to a test-tube. After addition of 1 μmole of 4-ethylphenol in 0.5 ml of ethyl acetate as an internal standard, the sample was evaporated to 0.2 ml.

Derivatization

The methyl esters were formed by adding an excess of diazomethane in diethyl ether. After reducing the volume to 0.2 ml under a stream of nitrogen, 0.1 ml of BSTFA was added for silylation. Undecylenic acid methyl ester (1 μmole in 0.1 ml of ethyl acetate) was added as an internal standard. For the GC–MS analysis of the phenols, no derivatization was carried out.

Gas–liquid chromatography with flame-ionization detection

The quantitative determination of aromatic acids and phenols in urine, faeces and faecal cultures was carried out with a Hewlett-Packard Model 7620A gas chromatograph with a dual flame-ionization detector. The glass column (3.3 m × 2 mm I.D.) was packed with 3% XE-60 on Gas-Chrom Q (80–100 mesh). The flow-rate of the carrier gas (nitrogen) was 30 ml/min, and the temperature of the injector block and the detector was 250°.

For the chromatography of the aromatic acids, we used the following temperature programme: 10 min isothermal at 80°, then heating to 220° at the rate of 2°/min. The chromatography of the phenols started isothermally at 70° during 10 min, followed by heating to 130° at the rate of 2°/min. The oven was heated to 170° and kept isothermal for 10 min before starting a new programme. Volumes of 1–2 μl of the samples were injected.

Gas–liquid chromatography–mass spectrometry

A combination of a Carlo Erba Fractovap 2101 gas chromatograph coupled to a vacuum generator mass spectrometer (Micromass 16F) and a vacuum generator data system (Type 2000) was used.

The GC column (2 m × 3 mm I.D.) was filled with 3% XE-60 on 80–100-mesh Gas-Chrom Q. The flow-rate of the carrier gas (helium) was 30 ml/min and the flash heater temperature was 250°. The chromatography of phenol and *p*-cresol was carried out with an oven temperature of 105°, increased to 150° at the rate of 4°/min; for the aromatic acids the initial temperature of 90° was maintained for 3 min and then increased to 200° at the rate of 2.5°/min. The temperature of the single jet separator and the ion source was 230°. The accelerating voltage was 4 kV, the electron multiplier voltage 3 kV and the trap current 20 μA. The mass spectra were obtained at 20 eV.

RESULTS

In vivo studies

From the two normal persons who had been orally dosed with deuterated L-tyrosine, we determined the aromatic acids and phenols in urine and some faecal specimens. In faeces we did not find any aromatic acids or phenols up to 72 h after the dosing test. After these dosing tests we found the following deuterated urinary tyrosine metabolites: 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 4-hydroxymandelic acid, homovanillic acid, 4-hydroxyphenyllactic acid, vanillylmandelic acid, 4-hydroxyhippuric acid, phenol and *p*-cresol.

In vitro studies

Incubation with dideutero-L-tyrosine. The incubation of faecal specimens from the two normal controls with dideutero-L-tyrosine showed the following deuterated aromatic acids, which contained two deuterium atoms: 4-hydroxyphenylacetic acid, 4-hydroxyphenylpropionic acid and 4-hydroxyphenyllactic acid. Fig. 1 shows the gas chromatogram of the faecal aromatic acids after incubation with tyrosine.

Only very small amounts of 4-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid could be detected after incubation of the faecal specimen from the

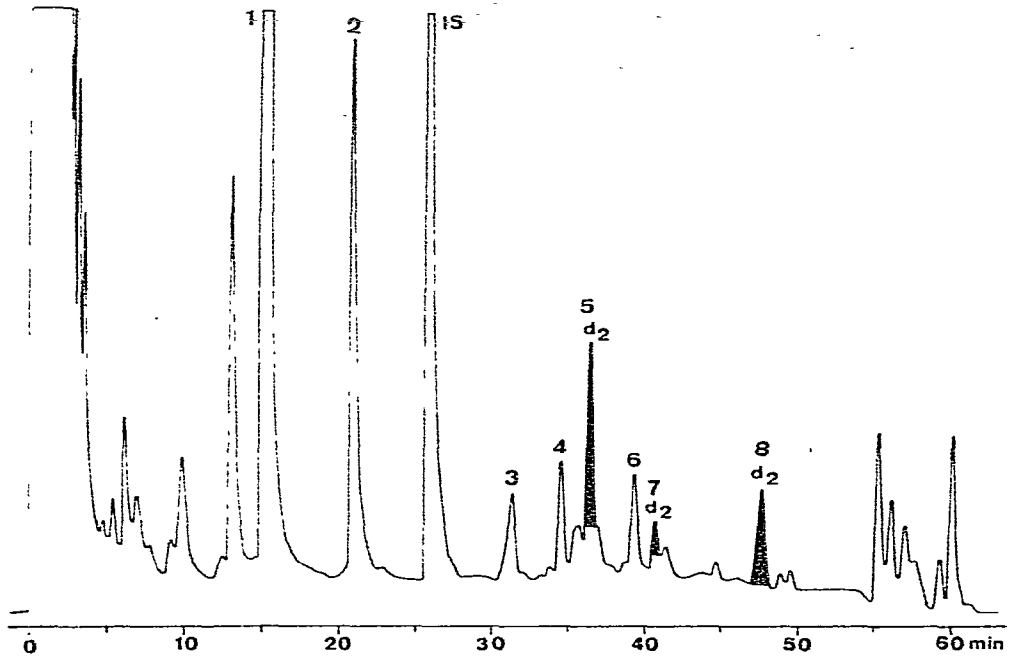

Fig. 1. Gas chromatogram of the aromatic acids extracted from incubation of tyrosine with a faecal specimen from a normal person. The peaks marked in black signify deuterated aromatic acids. Peaks: 1 = phenylacetic acid; 2 = phenylpropionic acid; 3 = phenyllactic acid; 4 = 3-hydroxyphenylacetic acid; 5 = 4-hydroxyphenylacetic acid; 6 = 3-hydroxyphenylpropionic acid; 7 = 4-hydroxyphenylpropionic acid; 8 = 4-hydroxyphenyllactic acid; IS = internal standard.

phenylketonuric patient with tyrosine. Phenols were not found in any of faecal cultures with tyrosine as a test substance.

Incubation with dideutero-4-hydroxyphenyllactic acid. Different kinds of deuterated aromatic acids were detected after incubation of the faecal specimens from the three subjects with 4-hydroxyphenyllactic acid. Phenylpropionic acid was found in the highest concentration, followed by 4-hydroxyphenylpropionic acid and 3-hydroxyphenylpropionic acid and 4-hydroxyphenylacetic acid in smaller amounts. All compounds still contained two deuterium atoms, also 3-hydroxyphenylpropionic acid.

Fig. 2 shows the gas chromatogram of the faecal aromatic acids after incubation with deuterated 4-hydroxyphenyllactic acid. 3-Hydroxyphenylpropionic acid was identified by its retention time and mass spectrum using a reference compound synthesized from 3-coumaric acid (see *Materials*). The mass spectra of the deuterated and non-deuterated 3-hydroxyphenylpropionic acid are shown in Figs. 3 and 4.

Incubation with dideutero-4-hydroxyphenylpropionic acid. After incubation of 4-hydroxyphenylpropionic acid with faeces from one of the normal controls, deuterated phenylpropionic acid was found in large amounts. After incubation of faeces from the second normal subject we detected smaller amounts of deuterated phenylpropionic acid and, in addition, dideutero-3-hydroxyphenylpropionic acid. The faecal specimen from the phenylketonuric patient showed the presence of phenyl-

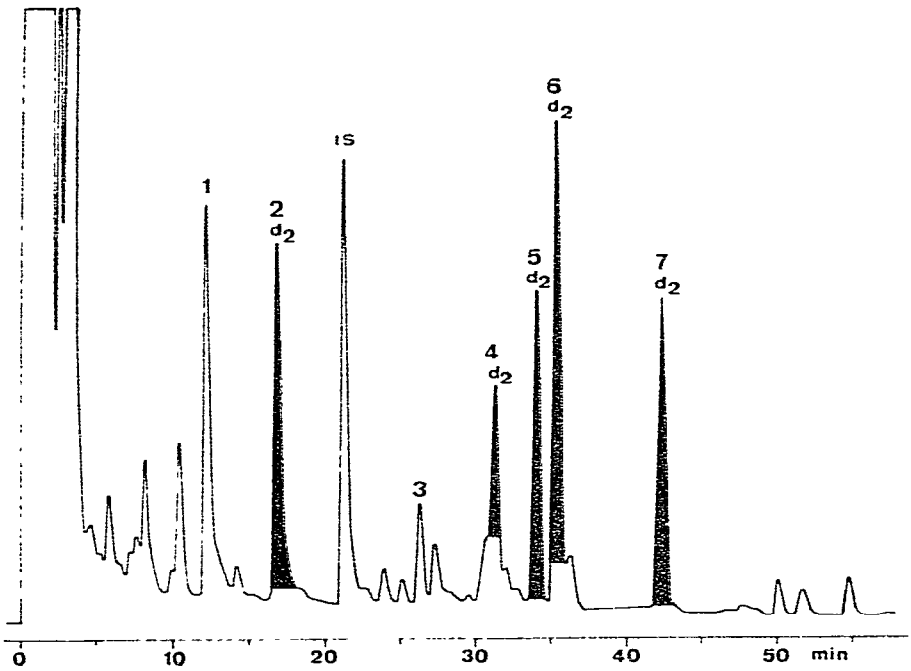

Fig. 2. Gas chromatogram of the aromatic acids extracted from incubation of 4-hydroxyphenyllactic acid with a faecal specimen from a normal person. The peaks marked in black signify deuterated aromatic acids. Peaks: 1 = phenylacetic acid; 2 = phenylpropionic acid; 3 = phenyllactic acid; 4 = 4-hydroxyphenylacetic acid; 5 = 3-hydroxyphenylpropionic acid; 6 = 4-hydroxyphenylpropionic acid; 7 = 4-hydroxyphenyllactic acid.

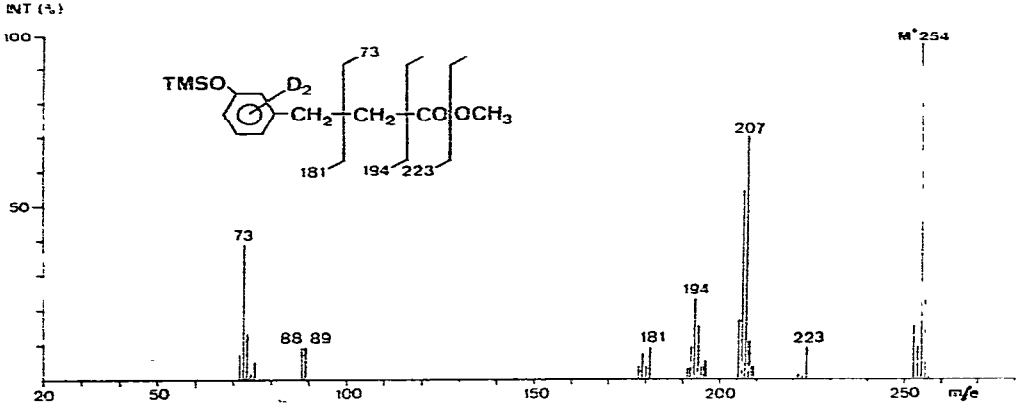

Fig. 3. Mass spectrum of partly dideuterated 3-hydroxyphenylpropionic acid obtained after incubation of a faecal specimen with [3′,5′-$^2H_2$]-4′-hydroxyphenyllactic acid.

propionic acid, 3-hydroxyphenylpropionic acid and 4-hydroxyphenyllactic acid. Deuterated phenols were not detected in any incubation with faecal specimens.

Incubation with dideuterophenylacetic acid and dideutero-4-hydroxybenzoic acid. Further incubations with faeces from the three different test persons with deuterated

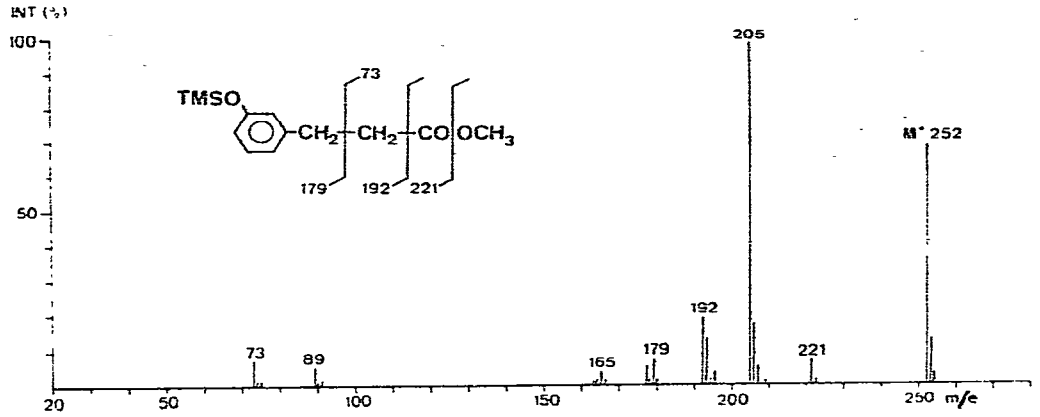

Fig. 4. Mass spectrum of non-deuterated 3-hydroxyphenylpropionic acid.

4-hydroxyphenylacetic acid yielded large amounts of *p*-cresol, and incubations with 4-hydroxybenzoic acid showed large amounts of phenol.

The mass spectrum of *p*-cresol, which was obtained after incubation with tetradeutero-4-hydroxyphenylacetic acid, is shown in Fig. 5. In comparison, Fig. 6 shows the mass spectrum of non-deuterated *p*-cresol.

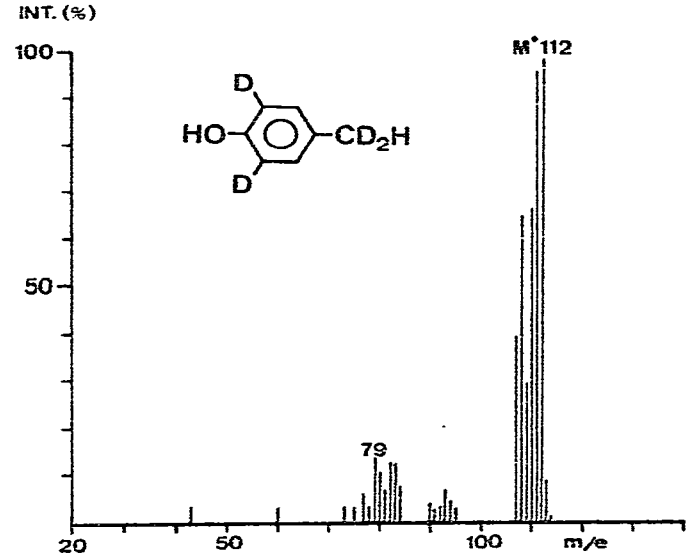

Fig. 5. Mass spectrum of partly tetradeuterated *p*-cresol, a metabolic product of [3′,5′,2,2-$^2H_2$]-4′-hydroxyphenylacetic acid.

Fig. 7 shows the metabolic pathways of the tyrosine metabolism, referring only to the monohydroxylated and non-hydroxylated aromatic acids and to the phenols.

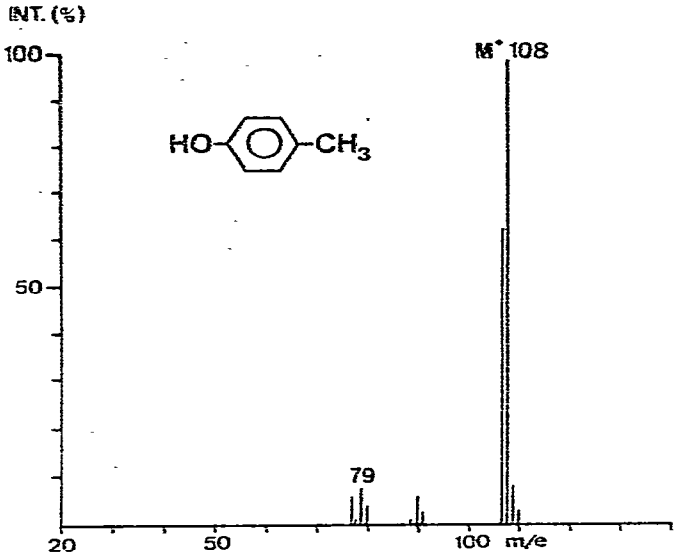

Fig. 6. Mass spectrum of non-deuterated *p*-cresol.

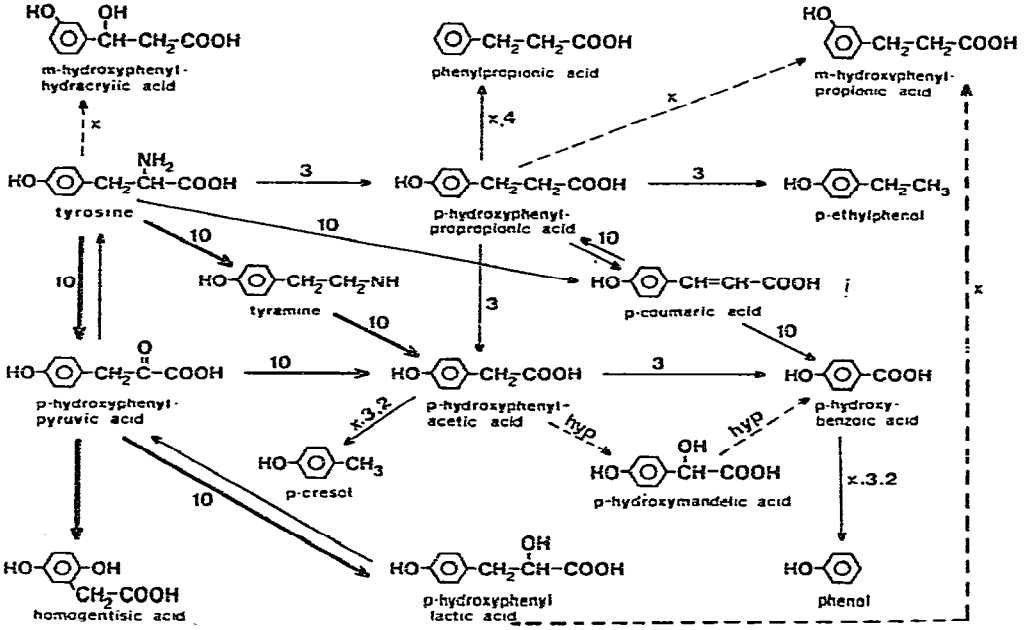

Fig. 7. Tyrosine metabolic pathways. The numbers on the arrows refer to the references; × refers to our results obtained by incubation of faecal specimens with tyrosine metabolites; hyp is a hypothetical route for 4-hydroxybenzoic acid.

DISCUSSION

Our studies of the correlation of human endogenous metabolism and intestinal bacterial flora showed that stable isotope techniques combined with GC–MS are

suitable for the investigation of metabolism both *in vivo* and *in vitro*. In order to obtain comparable results, we applied incubation methods similar to those reported by Drasar and Hill[3] and Bakke[2] for intestinal flora. The isolation of the aromatic acids from faecal specimens and urine was performed according to our earlier method[8]. Further purification of the extract, including high-voltage electrophoresis, active charcoal and chromatography on Kieselguhr, resulted in the loss of some of the aromatic acids.

In normal human urine, there is usually a relatively large amount of conjugated phenol and *p*-cresol which makes it difficult to determine small amounts of the deuterated compounds. The same difficulties occurred in faecal incubations, where sometimes large amounts of free phenol and *p*-cresol were detected. In a few instances it was difficult to differentiate the labelled isotope from natural isotope content.

To the faecal suspension we added 10% of glycerol in order to avoid bursting of the bacteria and stored the suspension in a deep-freeze.

After dosing two normal persons with dideuterotyrosine, we found decarboxylated products, *viz.*, phenol and *p*-cresol, in their urine. In earlier dosing tests with three patients having irregularities in their tyrosine metabolism we were able to find a dehydroxylation activity leading to benzoic and hippuric acid as well as to a rearrangement activity leading to 3-hydroxyphenylhydracrylic acid and 3-hydroxyhippuric acid[1]. These products were absent after disinfection of the intestine with neomycin. The result is in agreement with the statement of Drasar and Hill[3] that the dehydroxylation activity is destroyed by neomycin.

Dehydroxylation reactions

In the *in vitro* studies with human faecal specimens, we found dehydroxylation products only after incubation with two test substances. The incubation with 4-hydroxyphenylpropionic acid resulted in large amounts of phenylpropionic acid; 4-hydroxyphenyllactic acid yielded 4-hydroxyphenylpropionic acid and, in smaller amounts, phenylpropionic acid. The influence of neomycin on these incubations will be studied later.

None of the test substances used in the incubations with faecal specimens resulted in benzoic acid or hippuric acid, which we have found in large amounts in the urine of several persons after dosing tests with tyrosine.

Decarboxylation reactions

With respect to decarboxylation reactions, the faecal incubation with deuterated 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid showed large amounts of phenols. On the other hand, the incubation of faeces with 4-hydroxyphenylpropionic acid never resulted in 4-ethylphenol. In the urine of all of the test persons we found, after dosing with dideuterated tyrosine, large amounts of phenol and *p*-cresol, except after medication with neomycin. In contrast to Drasar and Hill[3], we never detected 4-ethylphenol.

It is possible that urinary phenols play a role in human cancer, and this aspect was discussed by Drasar and Hill[3].

Rearrangement reactions

We previously reported on three patients with the abnormal urinary tyrosine metabolites 3-hydroxyphenylhydracrylic and 3-hydroxyhippuric acid[1]. Either migra-

tion of the side-chain or of the hydroxyl group might be caused by the intestinal flora. We cannot exclude the possibility that neomycin interferes with the enzyme which is responsible for the migration.

The *in vitro* incubation studies with deuterated tyrosine metabolites showed analogous results. While using 4-hydroxyphenyllactic acid or 4-hydroxyphenylpropionic acid for incubation, we obtained 3-hydroxyphenylpropionic acid which was still dideuterated.

There are three possibilities for the rearrangement reaction.

(1) One possibility is a rearrangement of the hydroxyl group. We cannot describe any pathway for such a reaction because *m*-hydroxylphenylpropionic acid still contains two deuterium atoms to a large extent and therefore one deuterium atom must have shifted with the hydroxyl group (Fig. 8).

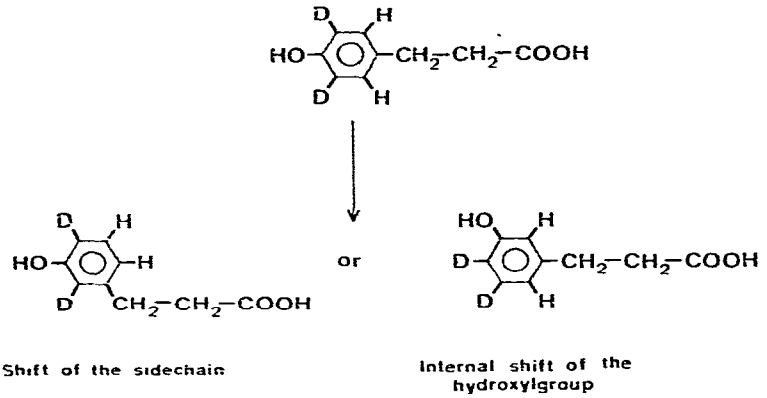

Fig. 8. The incubation of faecal specimens with 4-hydroxyphenylpropionic acid or with 4-hydroxyphenyllactic acid resulted in 3-hydroxyphenylpropionic acid; either the side-chain or the hydroxyl group may have been shifted, as shown.

(2) A second possibility is a shift of the side-chain without introduction of a further hydroxyl group (Fig. 8), as known in the case of the formation of homogentisinic acid.

(3) The third possibility is a *meta*-hydroxylation by tyrosine 3-hydroxylase followed by a *para*-dehydroxylation. This reaction is improbable because 3-hydroxyphenylpropionic acid is still dideuterated after incubation with 4-hydroxyphenylpropionic acid and an NIH shift is not possible in phenols, as described by Massey and Hemmerich[9]. Further, we performed our incubation studies under anaerobic conditions, excluding hydroxylation by tyrosine 3-hydroxylase, which requires molecular oxygen for its activity.

We intend to carry out further studies on the reaction mechanism of the above-mentioned rearrangements.

The induction of bacterial enzymes is a very important source of artefacts, which we cannot eliminate and which we have to consider by faecal incubation and loading tests. The test substances can induce the bacterial enzyme system, resulting in a much higher bacterial activity compared with the non-induced normal gut flora. We chose the long incubation time of 1 week in order to obtain enough metabolic

products for a qualitative analysis. For further experiments, we should assimilate our incubations to physiological conditions by reducing the incubation time. In order to minimize induction, we plan to reduce the amount of the test substances used to a minimum.

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