Studies on Flavonoid Metabolism

IDENTIFICATION OF THE METABOLITES OF (+)-CATECHIN IN RAT URINE

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(*Received* 25 *November* 1963)

Although the catechins and their derivatives are known to occur in many foodstuffs of plant origin, in tea and in cocoa (Roberts & Wood, 1953; Forsyth, 1955; Griffiths, 1960), the metabolic fate of ingested (+)-catechin in the mammal has hitherto remained largely unknown.

Studies on the related compound epicatechin by Gero (1946) showed that in the human 20% of the orally administered epicatechin was excreted unchanged, whereas in the guinea pig 65% of the epicatechin administered by intraperitoneal injection was excreted. No urinary metabolites of epicatechin were reported.

Since recent studies (Booth, Murray, Jones & DeEds, 1956; Booth, Jones & DeEds, 1958; Masri, Booth & DeEds, 1959) have shown that several of the phenolic constituents of normal urine originate from the flavonoid constituents of the diet, it was decided to determine whether administration of (+)-catechin to rats on a standardized diet caused any modification in the urinary phenolic excretion pattern. Further, since the studies of Booth *et al.* (1956, 1958) and Masri *et al.* (1959) were limited to flavonoid compounds of the flavonol, flavanone or flavone type, it was hoped that the present study would throw some light on the type of ring-cleavage occurring in a flavonoid molecule lacking an oxo group at $C_{(4)}$. A preliminary study describing the isolation and identification of one of the major metabolites of (+)-catechin in rat urine has already been reported (Griffiths, 1962).

EXPERIMENTAL

Animals. Male albino rats, weighing approx. 250 g., were used. The animals were housed in separate metabolism cages, designed to permit the collection of urine separately from faeces. The animals were fed at 24 hr. intervals before the period of urine collection but unrestricted water intake was allowed throughout the experimental period.

Diet. The animals were fed on a standardized powder-type diet of the following composition: white flour, 600 g.; dried milk, 200 g.; dried yeast powder, 30 g.; sodium chloride, 10 g. Approx. 15 g. of the food powder was given to each animal per day during the period of the experiment.

In designing this diet, an attempt was made to formulate a natural diet in which the content of flavonoid and other plant phenolic constituents could be restricted to a low level without diminishing the normal aromatic-amino acid content of the diet.

Test substance. (+)-Catechin was obtained from L. Light and Co., Colnbrook, Bucks. After recrystallization, the substance was shown to be chromatographically pure, m.p. 219°.

For oral administration, the test substance was given in admixture with the diet at the doses stated.

For intraperitoneal administration, the test substance was dissolved in 5 or 10 ml. of 0·9% sodium chloride and the solution sterilized by means of a bacterial filter before injection.

Collection of urine. The 24 hr. urine samples were collected over a period of 3–7 days, as stated in the text. A few drops of conc. HCl were added to the urine during the period of collection to stabilize the alkali-labile phenolic compounds and to decrease bacterial activity in the urine.

Preparation of urinary extracts for chromatography. Each of the 24 hr. urine samples was filtered and made up to 50 ml. with distilled water, sufficient conc. HCl being added to adjust the pH of the diluted urine to about pH 1.

A 12·5 ml. portion of each sample was extracted in a liquid extracter with diethyl ether (analytical reagent) for about 6 hr., after which the ether extracts were evaporated to 2 ml. in a vacuum oven at 60°.

Acid hydrolysis of urine. The routine procedure followed that of Booth *et al.* (1956) and Armstrong, Shaw & Wall (1956). Unhydrolysed urines were extracted to avoid the possible destruction of acid-labile compounds, but acid-hydrolysed urines were also examined for the presence of conjugated metabolites. Conc. HCl (2 ml.) was added to 10 ml. of fresh urine, and the mixture was boiled under reflux for 30 min., cooled and filtered. The hydrolysate was then extracted with ether by the standard procedure.

Preparation of faecal extracts for chromatography. The 24 hr. samples were macerated in hot methanol (10 ml.), a further 10 ml. of methanol was added, and the mixture stirred and then left for 1 hr. The suspension was filtered and the extract dried at 60° in a vacuum oven. After cooling, the residue was shaken with 10 ml. of ether and allowed to stand for 30 min. The ethereal extract was filtered off and evaporated to 2 ml. Portions (0·2 ml.) were applied to the chromatogram.

Preparation of chromatography paper. Whatman no. 1 paper was employed for normal qualitative chromatography. When ultraviolet-absorption spectra were to be determined on the separated metabolites, Whatman no. 3 MM paper was used; this was pre-washed successively in (*a*) propan-2-ol–aq. ammonia (sp.gr. 0·88)–water (8 : 1 : 1, by vol.), (*b*) benzene–acetic acid–water (6 : 7 : 3, by vol.), (*c*) methanol and (*d*) distilled water.

Solvent systems. Two sets of solvent systems were used: system A, propan-2-ol–aq. ammonia (sp.gr. 0·88)–water (8:1:1, by vol.) (Armstrong *et al.* 1956) followed by benzene–acetic acid–water (6:7:3, by vol.) (Griffiths, 1957); system B, chloroform–acetic acid–water (2:1:1, by vol.) followed by 20% (w/v) KCl (Booth *et al.* 1956).

Although system A gave better results, system B was of value in the identification of alkali-labile compounds, including (+)-catechin, which underwent decomposition in system A.

A third two-dimensional solvent system (C), butan-1-ol–acetic acid–water (4:1:5, by vol.) and 2% (v/v) acetic acid (Roberts & Wood, 1953), was used to confirm the presence of (+)-catechin in urinary extracts. All identifications were based on the use of markers and co-chromatography rather than recorded R_F values.

Spray reagents. The reagent of Barton, Evans & Gardner (1952) normally was used for the detection of phenolic compounds, but duplicate chromatograms were sprayed with diazotized *p*-nitroaniline (Swain, 1953) or diazotized sulphanilic acid (Smith, 1960). The diazotized *p*-nitroaniline reagent gives a wide range of colours with the phenolic acids, and closely running compounds could be distinguished by differences in colour and shade.

A *p*-dimethylaminobenzaldehyde reagent prepared as described by Smith (1960) was used for the detection of benzoylglycines. Free glycine was detected on the chromatogram by the use of the *o*-phthalaldehyde reagent of Patton & Foreman (1949).

Colorimetric determination of phenolic acids. For the determination of phenolic acids a modification of the method of Bray & Thorpe (1954) was employed. Rat urine (100 ml.), after filtration and acidification with conc. HCl (3 ml.), was extracted continuously for 5 hr. with diethyl ether. The ether extract (50 ml.) was evaporated *in vacuo* and the residue redissolved in about 2 ml. of diethyl ether–ethanol (1:1, v/v). This value was applied to Whatman no. 3 MM paper as a band. Markers of the appropriate phenolic acid were applied at each side of the main band. The paper was developed (40 cm.) with the propan-2-ol–aq. ammonia (sp.gr. 0·88)–water solvent and, after being dried, the marker strips were sprayed with diazotized *p*-nitroaniline to locate the main band. The paper strip containing the phenolic acid was cut into small fragments and eluted with methanol (3 × 10 ml.). The extract was evaporated to 2 ml. *in vacuo* and applied to a second chromatogram which was developed with benzene–acetic acid–water. The appropriate band was eluted with 3 × 7 ml. of diethyl ether–ethanol (1:1, v/v). After evaporation *in vacuo* the residue was dissolved in 25 ml. of distilled water and this solution used for the colorimetric determination. Folin–Ciocalteu reagent (1 ml.) and 2 ml. of 20% (w/v) sodium carbonate were added to appropriate samples, and the volume was adjusted to 25 ml. Colours were measured after 2 hr. in an EEL photoelectric colorimeter with a Chance OR2 orange filter. Standard curves over the range 0–200 μg. were prepared for each of the phenolic acids determined.

The efficiency of the method was examined by adding appropriate phenolic acid samples to specimens of urine, when recoveries of about 96% of the phenolic acid were obtained.

Determination of ultraviolet-absorption spectra of phenolic acids. The aqueous eluates (about 20 ml.) obtained from band chromatograms were made alkaline by the addition of an equal volume of 0·1 M-sodium carbonate and shaken with 3 × 10 ml. of diethyl ether to remove any remaining neutral ultraviolet-absorbing impurities. (If necessary, the washing procedure was repeated.) The alkaline solution was then acidified to pH 1–2 with conc. HCl and re-extracted with 3 × 10 ml. of diethyl ether. The pooled ethereal extracts were dried *in vacuo* and the residues redissolved in 25 ml. of 0·1 N-NaOH or in 25 ml. of 0·1 N-HCl. The ultraviolet-absorption spectra were determined in a Spectronic 505 recording spectrophotometer.

Determination of (+)-catechin. The volume of the urine was made to 100 ml. with distilled water and extracts were prepared by the standard technique. The extracts were fractionated by band chromatography in (1) chloroform–acetic acid–water and (2) butan-1-ol–acetic acid–water. The appropriate band was located by spraying side strips with diazotized *p*-nitroaniline, and the (+)-catechin was eluted with ethanol. A sample of the ethanolic solution was used for the determination of the ultraviolet-absorption spectrum. A further sample was dried and made up to 25 ml. with distilled water for colorimetric determination by the procedure used for the phenolic acids, catechin being used to prepare the standard curve.

RESULTS

Phenolic constituents of urine of rats given the standard diet. A group of six rats were given the standard diet over a period of 3 weeks, and during the third week the urine was collected at 24 hr. intervals. Examination of the extracts by chromatography indicated the presence of several phenolic substances reacting with both the phenolic reagent of Barton *et al.* (1952) and the diazotized *p*-nitroaniline reagent (Fig. 1). The major phenolic constituent was provisionally identified as *p*-hydroxyphenylacetic acid. Smaller amounts of substances showing similar chromatographic behaviour to *p*-hydroxyphenylpropionic acid, *p*-hydroxybenzoic acid and *m*-hydroxyhippuric acid were also present. An unidentified compound (spot 6) giving a strong yellow colour with diazotized *p*-nitroaniline was found to be a constant constituent of the urine under these conditions. In view of the low content of phenolic compounds in the diet, it is thought likely that most of these compounds are metabolites of dietary tyrosine. The identification of *p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpropionic acid, *p*-hydroxybenzoic acid and *m*-hydroxyhippuric acid, and trace amounts of *m*-hydroxyphenylpropionic acid, was subsequently confirmed by examination in solvent system B (Fig. 2).

Extracts from both hydrolysed and unhydrolysed urine were then examined by paper chromatography. The acid-hydrolysed urines contained larger amounts of *p*-hydroxybenzoic acid, indicating that this substance was present in a conjugated form. A new spot (spot 14) appearing on the chro-

matograms of the hydrolysed extract was identified as *m*-hydroxybenzoic acid. No other conjugated phenolic acids were present.

Metabolites of (+)-catechin

In the first series of experiments a group of 12 rats maintained for the preceding 14 days on the standard diet were divided into two groups. A single oral dose of 50 mg. of catechin was given to each rat of the first group only, in admixture with the standard diet. Both groups were then maintained on the standard diet. The 24 hr. urine samples collected over 4 days were extracted by the standard procedure. Examination of the extracts in solvent system A followed by spraying with the reagent of Barton *et al.* (1952) showed the presence of spots 2, 3, 4, 5 and 10 on the chromatograms of the catechin-fed rats; these spots were absent or present in greatly diminished amounts on the control chromatograms. Of these compounds, spot 2 has been identified as free *m*-hydroxyphenylpropionic acid and spot 3 as *m*-hydroxyhippuric acid. Spots 4, 5 and 10 were not identified.

Identification of m-*hydroxyhippuric acid.* Co-chromatography in systems A and B showed that the chromatographic behaviour of the compound corresponding to spot 3 was identical with that of *m*-hydroxyhippuric acid (Figs. 1 and 2). Both gave an orange–yellow colour with the *p*-dimethylaminobenzaldehyde reagent (which is specific for benzoylglycines; Smith, 1960) and a positive reaction with the phenolic reagent of Barton *et al.* (1952). Identical colour reactions were also obtained with the diazotized reagents. Both compounds showed characteristic fluorescence in ultraviolet light.

Supporting evidence was obtained by the determination of the ultraviolet-absorption spectra. Solutions of the metabolite were prepared for

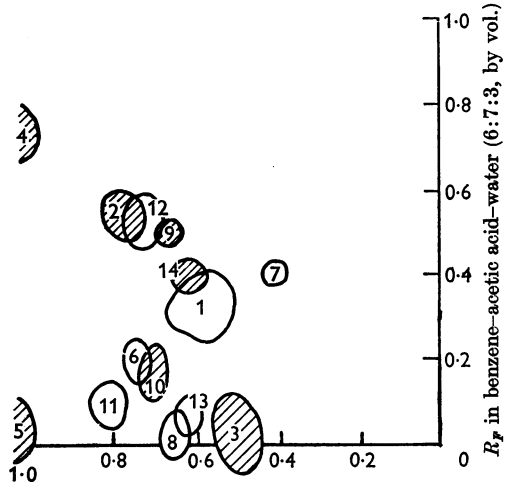

Fig. 1. Composite diagram showing positions of the phenolic metabolites of (+)-catechin and other phenolic constituents of rat urine in solvent system A. Experimental details are given in the text. Spot 1 (*p*-hydroxyphenylacetic acid): purple with diazotized *p*-nitroaniline, purple with diazotized sulphanilic acid. Spot 2 (*m*-hydroxyphenylpropionic acid): red with diazotized *p*-nitroaniline, orange with diazotized sulphanilic acid. Spot 3 (*m*-hydroxyhippuric acid): red with diazotized *p*-nitroaniline, yellow with diazotized sulphanilic acid. Spot 4 (unidentified): red with diazotized *p*-nitroaniline. Spot 5 (unidentified): red with diazotized *p*-nitroaniline. Spot 6 (unidentified): yellow with diazotized *p*-nitroaniline. Spot 7 (*p*-hydroxybenzoic acid): red with diazotized *p*-nitroaniline, yellow with diazotized sulphanilic acid. Spot 8 (unidentified): purple with diazotized *p*-nitroaniline. Spot 9 (unidentified): pink with diazotized *p*-nitroaniline. Spot 10 (unidentified): red with diazotized *p*-nitroaniline. Spot 11 (unidentified): orange with diazotized *p*-nitroaniline. Spot 12 (*p*-hydroxyphenylpropionic acid): purple with diazotized *p*-nitroaniline, purple with diazotized sulphanilic acid. Spot 13 (unidentified): orange with diazotized *p*-nitroaniline. Spot 14 (unidentified): pink with diazotized *p*-nitroaniline. Spots shown shaded were formed wholly or in part from administered (+)-catechin. (See also note on R_F values in the Experimental section.)

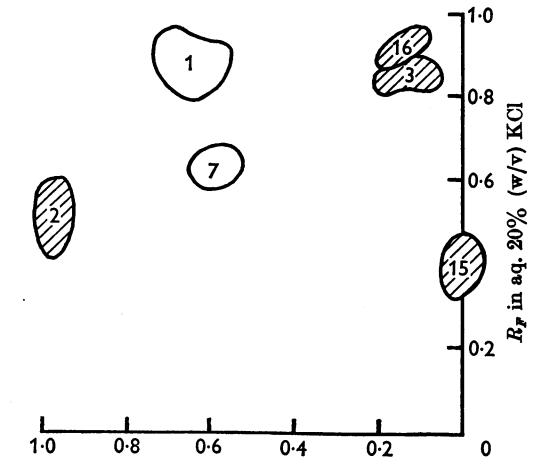

Fig. 2. Composite diagram showing the positions of the identified phenolic metabolites of (+)-catechin and other phenolic constituents of rat urine in the confirmatory solvent system B. Experimental details are given in the text. Spots 1, 2, 3 and 7: as in Fig. 1. Spot 15 [(+)-catechin]: brown with diazotized *p*-nitroaniline, brown with diazotized sulphanilic acid. Spot 16 (3,4-dihydroxyphenylacetic acid): purple with diazotized *p*-nitroaniline, red–grey with diazotized sulphanilic acid. (Unidentified compounds are omitted from this diagram.) Spots shown shaded were formed wholly or in part from administered (+)-catechin. (See also note on R_F values in the Experimental section.)

ultraviolet spectroscopy by the procedure described in the Experimental section.

Examination of the ultraviolet-absorption spectra of the compound corresponding to spot 3 revealed an absorption peak at 295 mμ in 0·1N-hydrochloric acid. A characteristic bathochromic displacement to 315 mμ was observed when the compound was examined in 0·1N-sodium hydroxide. These spectral characteristics were identical with those shown by pure solutions of *m*-hydroxyhippuric acid. After hydrolysis with 20% (v/v) hydrochloric acid, *m*-hydroxybenzoic acid was identified by chromatographic comparison with authentic material. The ultraviolet-absorption spectral characteristics were also shown to be identical, absorption maxima being present at 293 mμ in 0·1N-hydrochloric acid and at 312 mμ in 0·1N-sodium hydroxide. Similar ultraviolet fluorescence was shown by both preparations. Glycine was detected in the hydrolysate by ninhydrin treatment of chromatograms developed in phenol–water (4:1, w/v) and butan-1-ol–pyridine–water (1:1:1, by vol.). On spraying with the *o*-phthalaldehyde reagent, a yellow–green coloration was observed, which is known to be a specific colour reaction for glycine (Patton & Foreman, 1949).

Identification of m-*hydroxyphenylpropionic acid.* It was shown previously (Griffiths, 1962) that the chromatographic properties of the compound corresponding to spot 2 were identical with those of *m*-hydroxyphenylpropionic acid (Figs. 1 and 2).

Supporting evidence was obtained by the determination of the ultraviolet-absorption spectra. Solutions of the metabolite were prepared for ultraviolet spectroscopy by the standard procedure. Examination in 0·1N-hydrochloric acid revealed an absorption maximum at 272 mμ, whereas in 0·1N-sodium hydroxide a bathochromic displacement of the peak to 290 mμ occurred and a second maximum was seen at 239 mμ. These values were identical with those obtained with solutions of pure *m*-hydroxyphenylpropionic acid.

With solvent system B the presence of two further compounds was shown. One (spot 15) gave a strong brown colour with diazotized *p*-nitroaniline and a positive reaction with the reagent of Barton *et al.* (1952). This compound had chromatographic properties identical with those of an authentic specimen of (+)-catechin. The identification was confirmed by examination in solvent system C (R_F 0·75 in butan-1-ol–acetic acid–water; R_F 0·40 in 2% acetic acid). The ultraviolet-absorption spectrum of the eluate was identical with that of a pure specimen of (+)-catechin, giving a well-defined maximum at 282 mμ.

The second compound (spot 16) gave a purple colour with diazotized *p*-nitroaniline and a red colour (rapidly darkening) with diazotized sulphanilic acid. This compound was tentatively identified as 3,4-dihydroxyphenylacetic acid. This compound was found in very small amounts and was not formed by all rats examined. Failure to detect this compound with solvent system A is attributable to its lability in the presence of ammonia.

Although the spot due to unchanged (+)-catechin was maximal in the first 24 hr. sample, the metabolic products of catechin appeared later, reaching maximal amounts on the second and third day. Examination of the hydrolysed urine samples from catechin-fed rats showed that *m*-hydroxyphenylpropionic acid was present not only in the free form but also as a conjugate. Two catechin metabolites (spots 9 and 14) not detected in the unhydrolysed urine were also released by acid hydrolysis. Spot 14 was identified as *m*-hydroxybenzoic acid.

The amounts of *m*-hydroxyphenylpropionic acid, *m*-hydroxyhippuric acid and (+)-catechin in the urine of catechin-fed rats were next determined by the colorimetric procedure. The urines obtained on each day of the experiment were pooled and submitted to the quantitative procedure. The results of this and similar experiments (Tables 1 and 2), in agreement with the two-dimensional chromatographic method, show that excretion of *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid did not reach a maximum until the second or third day. This late appearance may suggest that breakdown of the (+)-catechin is dependent on the activities of the intestinal flora. The amounts of

Table 1. *Excretion of* m-*hydroxyphenylpropionic acid,* m-*hydroxyhippuric acid and* (+)-*catechin in urine after the oral administration of* (+)-*catechin in the rat*

Experimental details are given in the text. The results are mean values obtained with 3 rats.

Day	(+)-Catechin administered (mg.)	*m*-Hydroxyphenylpropionic acid excreted (μg./rat/24 hr.)	*m*-Hydroxyhippuric acid excreted (μg./rat/24 hr.)	(+)-Catechin excreted (μg./rat/24 hr.)
0	—	19·0	15·1	0
1	50	37·7	30·3	65·1
2	—	75·0	101·6	9·5
3	—	20·5	27·0	0
4	—	18·5	14·3	0

Table 2. *Effect of antibacterial compounds on the formation of* m-*hydroxyphenylpropionic acid and* m-*hydroxyhippuric acid from* (+)-*catechin in the rat*

Experimental details are given in the text. The results are mean values obtained with three rats in each group.

	Day	(+)-Catechin administered (mg.)	Phthaloylsulphathiazole administered (mg.)	Aureomycin administered (mg.)	*m*-Hydroxyphenylpropionic acid excreted (μg./rat/24 hr.)	*m*-Hydroxyhippuric acid excreted (μg./rat/24 hr.)
Group A	0	—	—	—	20·3	14·5
	1	20	—	—	109·3	96·8
	2	20	—	—	222·7	218·1
	3	20	—	—	238·1	250·0
	4	20	—	—	218·1	243·5
	5	20	—	—	205·6	255·0
	6	20	—	—	222·7	265·5
Group B	0	—	—	—	19·7	15·1
	1	20	—	—	102·1	90·1
	2	20	—	—	215·3	209·5
	3	20	—	—	250·7	240·0
	4	20	70	50	209·3	191·0
	5	20	70	50	87·3	82·4
	6	20	70	50	10·3	5·0
Group C	0	—	—	—	19·2	19·7
	1	—	—	—	20·1	20·4
	2	—	—	—	19·3	22·5
	3	—	—	—	19·7	19·4
	4	—	70	50	12·0	8·7
	5	—	70	50	0·5	0·7
	6	—	70	50	0·5	1·2
Group D	0	—	—	—	19·5	19·9
	1	—	—	—	19·7	19·8
	2	—	—	—	19·7	19·9
	3	—	—	—	19·5	20·2
	4	—	—	—	19·8	20·0
	5	—	—	—	19·6	19·7
	6	—	—	—	19·5	19·9

phenolic acids formed are small in relation to the quantity of (+)-catechin fed. Excretion of unchanged (+)-catechin in the urine is likewise small in amount, but occurs mainly within 24 hr. of administration.

Participation of the intestinal microflora in the metabolism of (+)-*catechin in the rat*

Experiments with orally administered antibiotics. Of 12 animals maintained for the preceding 14 days on the standard diet, six were given (+)-catechin (20 mg. daily) for 7 days. From the fourth day, in addition to the catechin, 70 mg. of phthaloylsulphathiazole and 50 mg. of aureomycin were added daily to the powder diet of three of these animals (group B), and the other three (group A) were given the antibiotic-free catechin-supplemented diet. A third group C, was given the catechin-free diet to which the antibacterial compounds were added from the fourth day, and a fourth group, D, received the basal diet without supplement. During the first 3 days of the experiment the formation of *m*-hydroxyphenylpropionic acid, *m*-hydroxyhippuric acid and the compounds corresponding to spots 4, 5 and 10 were demonstrated chromatographically in all the animals receiving catechin. On the addition of phthaloylsulphathiazole and aureomycin to the diet of group B, a decrease in the size and intensity of these spots occurred. By the seventh day, *m*-hydroxyphenylpropionic acid and spots 4, 5 and 10 had completely disappeared from the chromatograms, and the amount of *m*-hydroxyhippuric acid had fallen to the level of that present on the control chromatograms. No decrease in the amount of these phenolic acids occurred on the chromatograms from the control group A. In group D, only trace quantities of these phenolic acids were present. Spots 11 and 13 were detected only on the chromatograms from antibiotic-fed rats. Since they were present in the urine of rats (group C) in which the antibiotics were given in the absence of (+)-catechin it was evident that they were metabolites of one or other of the antibacterial compounds. Separate feeding of each of the antibacterial compounds showed that compounds 11 and 13 are metabolites of phthaloylsulphathiazole.

The effect of antibacterial compounds on the formation of *m*-hydroxyhippuric acid and of *m*-hydroxyphenylpropionic acid was next studied by the colorimetric technique. The results are shown in Table 2. The marked elevation in the values of *m*-hydroxyhippuric acid and *m*-hydroxyphenyl-

propionic acid after the administration of catechin is completely abolished in group B by the administration of the antibacterial compounds, and a decrease in the formation of these compounds on the unsupplemented standard diet is also seen in group C in the presence of the antibacterial substances. In group D, the amounts of *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid remained low throughout the experiment.

Examination of urinary phenolic compounds after the intraperitoneal administration of (+)-catechin

A group of six rats was taken, three of which were injected intraperitoneally with 20 mg. of (+)-catechin in 5 ml. of 0·9 % sodium chloride, and the urines were collected for 3 days. Under these conditions, no phenolic metabolites of catechin were found in the urine. The only difference between chromatograms of the experimental and the control group was the presence of a large amount of (+)-catechin on the former, which appeared during the first 24 hr. after injection.

Examination of faeces for phenolic metabolites of orally administered (+)-catechin

Chromatographic examination of the faeces of catechin-fed rats [50 mg. of (+)-catechin per rat] revealed trace amounts of *m*-hydroxyphenylpropionic acid and compounds 4 and 5.

In an attempt to increase the amount of catechin metabolites detected in the faeces, a high dosage rate of 200 mg. of (+)-catechin per rat was employed but no marked increases were noted.

No phenolic metabolites were detected in the faeces of the control group receiving the standard diet without catechin supplement.

DISCUSSION

The present study shows that dietary (+)-catechin gives rise to metabolites, including *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid, in the urine when given orally to rats. Formation of these compounds is clearly dependent on the action of the gut microflora, as high oral doses of the antibacterial compounds phthaloylsulphathiazole and aureomycin suppressed their formation. When (+)-catechin was parenterally administered, no metabolites of catechin were detectable in the urine. Unchanged (+)-catechin was shown to be present.

Additional support for the intestinal origin of *m*-hydroxyphenylpropionic acid and compounds 4 and 5 was provided by the detection of small amounts of these substances in the faeces of catechin-fed rats. In view of the very small amounts found in the faeces, compared with the amounts detected in the urine, it is evident that they are readily absorbed through the intestinal wall. The absence of *m*-hydroxyhippuric acid in faecal extracts would be expected since conjugation of aroyl metabolites with glycine is known to occur in the liver. Detection of *m*-hydroxyphenylpropionic acid in the faeces of catechin-fed rats is in agreement with the findings of Booth & Williams (1963), who reported that faecal extracts of the rat were able to metabolize (+)-catechin to *m*-hydroxyphenylpropionic acid *in vitro*.

A possible pathway for the formation of *m*-hydroxyphenylpropionic acid from (+)-catechin has already been advanced (Griffiths, 1962). It appears likely that the *m*-hydroxyhippuric acid is formed in the tissues of the rat from the *m*-hydroxyphenylpropionic acid by a process of β-oxidation followed by conjugation with glycine. Formation of both of these catechin metabolites would thus depend on the action of the gut microflora at a stage before the formation of *m*-hydroxyphenylpropionic acid.

Although substantial amounts of *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid were found in the urine of most of the rats receiving catechin, other catechin metabolites (spots 4, 5, 10 and 16) were sporadic in occurrence and variable in amount. This may reflect the presence or absence of less commonly found bacterial strains or species.

SUMMARY

1. The metabolism of (+)-catechin in the rat has been studied after oral administration and intraperitoneal administration of the compound.

2. *m*-Hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid were shown to be formed from orally administered (+)-catechin. These substances were not formed when (+)-catechin was administered in the presence of high doses of phthaloylsulphathiazole and aureomycin or when administered by intraperitoneal injection.

3. The presence of unchanged (+)-catechin was shown in the urines of rats receiving (+)-catechin orally or by intraperitoneal injection.

4. The presence of trace amounts of *m*-hydroxyphenylpropionic acid and the unidentified compounds corresponding to spots 4 and 5 were detected in the faeces of (+)-catechin-fed rats.

Thanks are due to Dr M. D. Armstrong for the gift of several phenolic acids and to Mr Oh Swee Huat for skilled technical assistance.

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Tissue Fractionation Studies

17. INTRACELLULAR DISTRIBUTION OF MONOAMINE OXIDASE, ASPARTATE AMINOTRANSFERASE, ALANINE AMINOTRANSFERASE, D-AMINO ACID OXIDASE AND CATALASE IN RAT-LIVER TISSUE*

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(*Received* 23 *September* 1963)

Conflicting reports exist in the literature with respect to the intracellular localization of catalase, D-amino acid oxidase and monoamine oxidase in liver.

Studies performed by a number of different authors indicate that catalase is associated partly with cytoplasmic particles and partly with the supernatant fraction, the ratio of particulate to soluble activity depending on the species and sex of the animal and on the nature of the medium adopted for preparing the homogenate (Ludewig & Chanutin, 1950; Nyberg, Schuberth & Änggård, 1953; Thomson & Mikuta, 1954; Greenfield & Price, 1956; Thomson & Klipfel, 1957; Adams & Burgess, 1957; Adams, 1959; Datta & Shepard, 1959; Mason, Chin, Li & Ziffren, 1960; Miller, 1962; Higashi & Peters, 1962; Peters & Higashi, 1963). Most workers believe that the particles that contain catalase are mitochondria, but this view has been brought into question by the results of Thomson & Klipfel (1957), who have shown that catalase sediments closely together with urate oxidase in homogenates of mouse liver centrifuged through a stabilizing density gradient. In view of this observation and of the finding that the activity of particulate catalase is enhanced by Triton X-100 (Adams & Burgess, 1957), Feinstein (1959) has put forward the hypothesis that the enzyme may be a component of the lysosomes.

A mitochondrial localization has also been reported for D-amino acid oxidase in rat liver (Dianzani, 1954), but Paigen (1954) has found in subfractionation experiments that this enzyme comes down predominantly with the lighter mitochondrial fractions, in contrast with succinoxidase which is more abundant in the heavier fractions.

According to Cotzias & Dole (1951) and Zile & Lardy (1959), monoamine oxidase belongs essentially to the mitochondria in rat liver, whereas Hawkins (1952) and Zeller, Barsky & Berman (1955) have found the enzyme to be associated also partly with the microsomal fraction.

In view of these discrepancies, it was decided to re-investigate the distribution of these three enzymes, by using the centrifugation scheme specially developed by Appelmans, Wattiaux & de Duve (1955) for the purpose of unravelling complex enzyme distributions.

In two experiments, assays were also made of aspartate aminotransferase and of alanine aminotransferase. The former enzyme has been found to be unevenly distributed between the mitochondrial fraction and the final supernatant (Müller & Leuthardt, 1950; Hird & Rowsell, 1950; Rowsell, 1956; May, Miyazaki & Grenell, 1959; Gaull & Villee, 1960; Rosenthal, Thind & Conger, 1960;

* Part 16: Lejeune, Thinès-Sempoux & Hers (1963).

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