Studies on Flavonoid Metabolism

METABOLISM OF (+)-[¹⁴C]CATECHIN IN THE RAT AND GUINEA PIG

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1. The fate of (+)-[U-¹⁴C]catechin and (+)-[*ring A*-¹⁴C]catechin has been studied in the guinea pig and rat. 2. (+)-[U-¹⁴C]Catechin was shown to give rise to labelled phenolic acids, labelled phenyl-γ-valerolactones and $^{14}CO_2$. 3. (+)-[*ring A*-¹⁴C]-Catechin did not give rise to labelled phenolic acids, but labelled phenyl-γ-valerolactones were detected together with a higher proportion of $^{14}CO_2$. 4. Administered [¹⁴C]δ-(3-hydroxyphenyl)-γ-valerolactone gave rise to labelled *m*-hydroxyphenylpropionic acid in the rat whereas administered [¹⁴C]*m*-hydroxyphenylpropionic acid gave rise to a compound yielding labelled *m*-hydroxybenzoic acid on hydrolysis. 5. The distribution of radioactivity in the urine and faeces of (+)-[¹⁴C]catechin-fed animals is described; a high proportion of residual radioactivity was found in urine that had been exhaustively extracted with diethyl ether.

In earlier investigations on the metabolism of (+)-catechin in the rat, *m*-hydroxyphenylpropionic acid and *m*-hydroxyhippuric acid were shown to be the main phenolic acid metabolites arising from orally administered (+)-catechin (Griffiths, 1964). The nature of these metabolites suggested that they were derived from ring B of the flavan molecule, but it was not possible on the basis of these studies to account for the metabolic fate of the A ring. Other studies, on the rabbit (Oshima, Watanabe & Isakari, 1958; Oshima & Watanabe, 1958; Watanabe, 1959a,b) and on the guinea pig and rat (Das & Griffiths, 1968), showed that, in addition to phenolic acid metabolites of (+)-catechin, phenyl-γ-valerolactone derivatives are also excreted. Although the major portion of the phenyl-γ-valerolactone molecule (II) is thought to be derived from the B ring of the catechin molecule (I), it has been suggested by Oshima & Watanabe (1958) that two carbon atoms (marked with *) of the lactone ring may arise from the A ring.

Parallel studies on the metabolic fate of the related flavonoid molecule, quercetin (III), in the rat produced similar results in that all of the phenolic acid metabolites identified were derived from the B ring of the molecule (Booth, Murray, Jones & DeEds, 1956; Nakagawa, Shetlar & Wender, 1965). The claim that the A ring of quercetin was metabolized to phloroglucinol and phloroglucinolcarboxylic acid in the rat (Kallianos, Petrakis, Shetlar & Wender, 1959) was vitiated by the observation of

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(I)

(II)

(III)
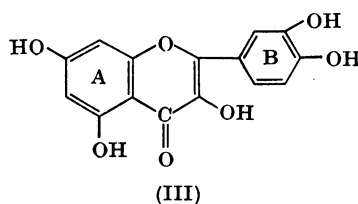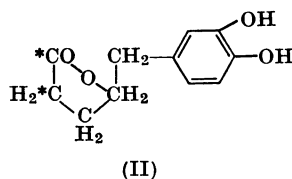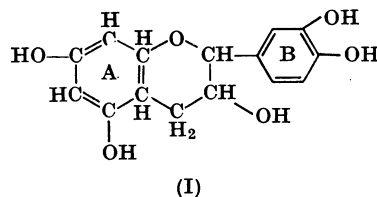
Masri, Booth & DeEds (1959) that these products were formed when pure quercetin was directly submitted to the acid extraction procedure employed by the former workers.

In view of the limited information on the metabolic fate of the A ring of flavonoid molecules, consideration was given to the possible use in animal-metabolism studies of (+)-[¹⁴C]catechin selectively

labelled in the A ring. A suitably labelled [^{14}C]-catechin was prepared by the administration of sodium [1-^{14}C]acetate to the plant *Uncaria gambir* Roxb. (Das & Griffiths, 1967). In accordance with the hypothesis of Birch & Donovan (1953), it was found that selective labelling of the A ring of (+)-catechin occurred with this precursor.

Since earlier investigations (Griffiths, 1964; Das & Griffiths, 1968) had shown that a relatively small amount of the orally administered (+)-catechin could be accounted for as free phenolic metabolites or as unchanged (+)-catechin it was evident that a considerable part had been metabolized to un-identified products. To investigate this problem (+)-[U-^{14}C]catechin was biosynthesized by the administration of ^{14}CO$_2$ to *Uncaria gambir* Roxb. The resulting (+)-[^{14}C]catechin was shown to be equally labelled in both rings (Das & Griffiths, 1967).

Administration of (+)-[U-^{14}C]catechin to the rat and guinea pig has shown that radioactivity is present in phenolic acids, lactones and lactone con-jugates and in respired carbon dioxide. (+)-[*ring A*-^{14}C]Catechin has been shown to give rise mainly to large amounts of ^{14}CO$_2$. Labelled phenolic lactones and lactone conjugates were also found but not labelled phenolic acids. These observations are in accordance with the pathway of (+)-catechin catabolism put forward by Watanabe (1959b) and extended by Das & Griffiths (1968).

EXPERIMENTAL

Animals. Male guinea pigs (approx. 300g.) and male rats (approx. 250g.) were used. The former were maintained on a standard diet described earlier (Das & Griffiths, 1966) and the latter on a diet described by Griffiths (1964).

Test substances. (+)-[U-^{14}C]Catechin and (+)-[*ring A*-^{14}C]catechin (sp. radioactivities 16·3mc/mole and 42mc/mole respectively) were obtained from young shoots of *Uncaria gambir* Roxb., to which ^{14}CO$_2$ and sodium [1-^{14}C]-acetate respectively had been administered (Das & Griffiths, 1967). The compounds were recrystallized to constant specific radioactivity, were shown to be chromato-graphically pure and possessed i.r. spectra identical with that of an authentic specimen of pure (+)-catechin.

Administration of test substances and collection of labelled metabolites. The labelled catechins were given to the animals by stomach tube at a dose of 50mg./animal. After adminis-tration of the labelled compound, the rats were maintained in a metabolism chamber (Jencons Scientific Ltd., Hemel Hempstead, Herts.) and the guinea pigs in a similar chamber constructed of sealed Perspex. Both chambers were designed to permit the separate collection of expired air, urine and faeces. The current of air passing into the chamber was dried and freed from atmospheric CO$_2$ by passage through CaSO$_4$ (Drierite, 6 mesh) and soda lime (Indicarb, 8–14 mesh). After passage through the chamber the air contain-ing the respired ^{14}CO$_2$ was passed through 3 × 50ml. of 4M-NaOH contained in Dreschel bottles or coiled columns. The containers were recharged with fresh alkali at 6hr. or 12hr. intervals over 3 days. The urine was collected over a period of 4 days and each 24hr. sample submitted to separate extraction with diethyl ether under standard conditions (Griffiths, 1964). Faeces collected separately over a 2-day period were homogenized with ethanol.

Paper chromatography. Whatman no. 1 paper was employed for routine qualitative chromatography, but for the isolation of labelled metabolites by band chromato-graphy either Whatman 3MM or Whatman 17MM paper was used. The solvent mixtures were: *A*, propan-2-ol–aq. NH$_3$ (sp.gr.0·88)–water (8:1:1, by vol.); *B*, benzene–acetic acid–water (6:7:3, by vol.); *C*, chloroform–acetic acid–water (2:1:1, by vol.); *D*, aq. 20% (w/v) KCl. The spray reagents were described by Griffiths (1964). R_F values of identified (+)-catechin metabolites in these solvents were reported earlier (Das & Griffiths, 1968).

Acid hydrolysis of conjugates in urine. Conc. HCl (1 vol.) was added to urine (2 vol.) and the mixture heated under reflux in a boiling-water bath for 3hr.

Radioassay. Radioactive spots on chromatograms were located by radioautographs on X-ray film (Kodirex, Kodak Ltd.) with exposure for 3–4 weeks, or by a Packard 7200 scanner.

Gas-flow Geiger counting of the guinea-pig preparations was carried out in a Nuclear–Chicago D47 gas-flow window-less counter, by a procedure described earlier (Das & Griffiths, 1967). Liquid-scintillation counting of radio-activity of rat preparations was carried out in an automatic scintillation counter (Nuclear-Chicago) as described by Das & Griffiths (1967).

Radioactivity in urine. The total radioactivity of the urines of animals receiving labelled (+)-catechin was determined either by direct scintillation counting of the urine samples (for rat) or by counting in the gas-flow Geiger counter of the solid residues obtained by evaporating suit-able volumes of urine on planchets under an infrared lamp (for guinea pig), as described by Parke & Williams (1950).

Ether extracts. Each 24hr. urine sample was made up to 25ml. and divided into two equal fractions. The first was adjusted to pH1 and submitted to continuous extraction with diethyl ether for 24hr. The second was subjected to acid hydrolysis under the standard conditions and then extracted with ether for 24hr. Each urine extract was evaporated to dryness and the residue dissolved in 3ml. of methanol. Samples (0·1ml.) were taken for scintillation counting.

Phenolic metabolites. For the determination of the radio-activity of the metabolites of (+)-catechin, extracts of unhydrolysed urine were used. Separation of each meta-bolite was effected by band chromatography on Whatman 3MM or Whatman 17MM paper, with the solvents described in a preceding section. The metabolites were further puri-fied by repeated chromatography with two or three different solvent systems. The final methanolic eluate was made up to a suitable volume and 0·1ml. taken for scintillation counting.

Faeces. Each faecal sample was separately homogenized with ethanol and portions were either dried on planchets for gas-flow Geiger counting (guinea pig) or samples (0·1ml.) were directly submitted to liquid-scintillation counting (rat). For radioautography, particle-free extracts were obtained by low-speed centrifugation.

Respired ^{14}CO$_2$. The ^{14}CO$_2$ respired was measured by the method described by Olson, Lindberg & Bloch (1957).

The 4M-NaOH solutions through which respiratory CO_2 had been passed were pooled and 0·1 ml. samples taken, to which saturated $BaCl_2$ solution (1 ml.) and 10% $(NH_4)_2CO_3$ (1 ml.) were added. The precipitated $BaCO_3$ was then plated on aluminium planchets (3 cm. diam.) and its radioactivity was measured in the gas-flow Geiger counter.

For scintillation counting, samples (0·1 ml.) of the pooled 4M-NaOH solution were taken and to each was added 0·5 ml. of water followed by 10 ml. of the phosphor (Das & Griffiths, 1967). Ethanol (0·5 ml.) was added to produce a homogeneous suspension, which was then counted in the scintillation counter.

In the above methods of counting, a count of 10 000 was made for each sample, to give a standard deviation of 1% (Faires & Parkes, 1960).

RESULTS

After the oral administration of 50 mg. of (+)-[U-^{14}C]catechin (sp. radioactivity 16·3 mc/mole) to each of two rats and two guinea pigs, respired carbon dioxide, urine and faeces were collected and their radioactivity was determined as described in the Experimental section. Parallel experiments were carried out with a further two rats and two guinea pigs, which each received 50 mg. of (+)-[ring A-^{14}C]catechin (sp. radioactivity 42 mc/mole).

Radioactivity in respired carbon dioxide. In both rat and guinea pig (Figs. 1 and 2) a considerably higher percentage of the administered dose of [ring A-^{14}C]catechin than of the [U-^{14}C]catechin was converted into $^{14}CO_2$. In all animals the highest rates of $^{14}CO_2$ production occurred within 24 hr. of administration and after 30 hr. fell to a low value.

Radioactivity in urine. Examination of the radioactivity of the urines of both rats and guinea pigs showed that the total radioactivities over the 4-day period of urine collection amounted to 50–63% of the administered dose of (+)-[U-^{14}C]catechin. Of this radioactivity only 8–11% was present in the form of ether-extractable substances in unhydrolysed urine (Table 1), although 21–28% was recoverable after hydrolysis. After the administration of (+)-[ring A-^{14}C]catechin, urinary excretion accounted for 53–56% of the dose, of which 3·3–5·6% was present in the ether-extractable fraction, which increased to 7·9–11·6% after acid hydrolysis of the urine.

The ether extracts obtained from the unhydrolysed urine of each animal dosed with (+)-[U-^{14}C]catechin were submitted to two-dimensional chromatography in solvents A and B and in solvents C and D. The positions of the radioactive metabolites
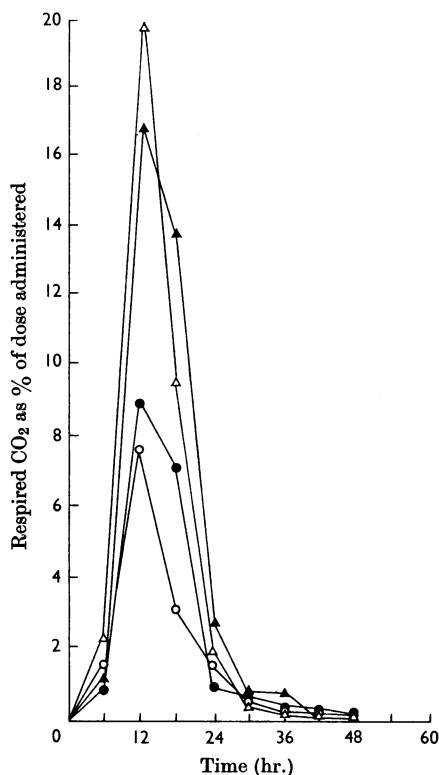

Fig. 1. $^{14}CO_2$ respired by guinea pigs given [^{14}C]catechin. Guinea pig given [U-^{14}C]catechin: ○, animal A; ●, animal B. Guinea pig given [ring A-^{14}C]catechin: △, animal A; ▲, animal B.
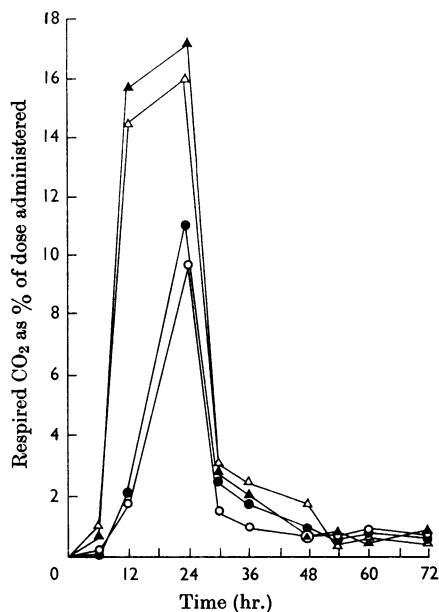
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Fig. 2. $^{14}CO_2$ respired by rats given [^{14}C]catechin. Rat given [U-^{14}C]catechin: ○, animal A: ●, animal B. Rat given [ring A-^{14}C]catechin: △, animal A; ▲, animal B.

Table 1. *Radioactivity in urine of guinea pigs and rats excreted over 4 days after administration of [^{14}C]catechin*

Results are expressed as the mean values of two experiments.

Animal	Total radioactivity of 24 hr. urine			Radioactivity of ether-extractable constituents of unhydrolysed urine			Radioactivity of ether-extractable constituents of hydrolysed urine		
	(c.p.m.)	(d.p.m.)	(% of dose)	(c.p.m.)	(d.p.m.)	(% of dose)	(c.p.m.)	(d.p.m.)	(% of dose)
[U-^{14}C]Catechin (50 mg.; sp. radioactivity 16·3 mc/mole)									
Guinea pig	277007	—	51·6	55463	—	10·2	120740	—	22·5
Rat	—	3518394	63·4	—	517763	9·3	—	1595133	28·7
[*ring A*-^{14}C]Catechin (50 mg.; sp. radioactivity 42 mc/mole)									
Guinea pig	1196790	—	54·5	109273	—	4·9	244932	—	11·1
Rat	—	7041817	55·1	—	516751	4·1	—	1089518	8·6

were then determined by radioautography. By subsequent spraying with the diazotized *p*-nitro-aniline or ferric chloride–potassium ferrocyanide reagents, it was shown that the positions occupied by the previously characterized phenolic metabolites of (+)-catechin (Griffiths, 1964; Das & Griffiths, 1968) coincided with areas of high radioactivity. Evidence was thus obtained of the formation of labelled δ-(3,4 dihydroxyphenyl)-γ-valerolactone, δ-(4-hydroxy-3-methoxyphenyl)-γ-valerolactone, δ-(3-hydroxyphenyl)-γ-valerolactone, *m*-hydroxy-hippuric acid, *m*-hydroxyphenylpropionic acid (rat only) and *m*-hydroxybenzoic acid (guinea pig only) from (+)-[U-^{14}C]catechin.

By similar methods, after the administration of (+)-[*ring A*-^{14}C]catechin, labelled δ-(3,4-dihydroxy-phenyl)-γ-valerolactone, δ-(4-hydroxy-3-methoxy-phenyl)-γ-valerolactone and δ-(3-hydroxyphenyl)-γ-valerolactone were detected on the chromatograms. Although *m*-hydroxyhippuric acid, *m*-hydroxyphenylpropionic acid (rat only) and *m*-hydroxybenzoic acid (guinea pig only) were detected by the use of the diazotized *p*-nitroaniline reagent, no detectable radioactivity was shown by radioautography in these positions.

The ether extracts obtained from the unhydro-lysed urine of each animal dosed with (+)-[U-^{14}C]-catechin were submitted to band chromatography with solvents *A*, *B*, *C* and *D*, and the metabolites, separated and identified as described earlier (Griffiths, 1964; Das & Griffiths, 1968), were assayed for radioactivity by the standard liquid-scintillation method. The results (Table 2) show that the total radioactivity possessed by the identified uncon-jugated phenolic metabolites of (+)-catechin was in the range 3·5–4·4% of the administered dose.

Radioactivity in faeces. The faeces of rats given (+)-[U-^{14}C]catechin contained 1·3–1·5% of the administered dose over the 2-day period of collec-tion. Radioautography of faecal extracts revealed radioactive areas in positions corresponding to δ-(3-hydroxyphenyl)-γ-valerolactone and the δ-(3,4-dihydroxyphenyl)-γ-valerolactone. Adminis-tration of (+)-[*ring A*-^{14}C]catechin to rats resulted in the elimination of 0·56–0·61% of the dose in the faeces over a 2-day period. By radioautography the presence of labelled δ-(3-hydroxyphenyl)-γ-valero-lactone and δ-(3,4-dihydroxyphenyl)-γ-valero-lactone was shown in faecal extracts.

The faeces of guinea pigs given (+)-[U-^{14}C]-catechin showed low total radioactivity, namely 0·07–0·12% of the administered dose, although higher radioactivities were detected after giving [*ring A*-^{14}C]catechin. No phenylvalerolactones were detected by the diazotized *p*-nitroaniline reagent on the chromatograms prepared from faecal extracts, nor was radioactivity detected in the cor-responding positions by autoradiography.

Table 2. Radioactivity in the unconjugated phenolic metabolites of [U-14C]catechin isolated from guinea-pig and rat urine

Results are expressed as the mean values from two experiments.

Animal	δ-(4-Hydroxy-3-methoxy-phenyl)-γ-valerolactone		δ-(3-Hydroxy-phenyl)-γ-valero-lactone		δ-(3,4-Dihydroxy-phenyl)-γ-valerolactone		m-Hydroxy-benzoic acid		m-Hydroxy-hippuric acid		m-Hydroxy-phenyl-propionic acid		(+)-Catechin		Total
	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(d.p.m.)	(% of dose)	(% of dose)
Guinea pig	8704	0·15	66314	1·19	23120	0·41	77625	1·39	68144	1·23	—	—	—	—	4·40
Rat	4657	0·08	86803	1·56	29276	0·53	—	—	19393	0·35	45938	0·82	6626	0·12	3·43

[U-14C]Catechin (50mg.; sp. radioactivity 16·3mc/mole)

Interrelationships of (+)-catechin metabolites. [14C]-δ-(3-Hydroxyphenyl)-γ-valerolactone obtained from the urine of [U-14C]catechin-fed rats by the procedure of Das & Griffiths (1968) was given to three rats under the standard conditions and the urine was collected for 48hr. The urines were then hydrolysed under conditions described in the Experimental section and extracted with ether for 24hr. The ether extracts were then examined by two-dimensional chromatography with solvents A and B and solvents C and D. By radioautography it was shown that radioactive areas, corresponding to m-hydroxyphenylpropionic acid, m-hydroxybenzoic acid (derived from hydrolysed hippuric acid), an artifact (compound F; Das & Griffiths, 1968) and δ-(3-hydroxyphenyl)-γ-valerolactone were present.

[14C]m-Hydroxyphenylpropionic acid isolated from the urine of [U-14C]catechin-fed rats by a similar procedure was found, on administration to rats under the conditions described above, to give rise to radioactive areas on chromatograms prepared as described above, corresponding to m-hydroxybenzoic acid and m-hydroxyphenylpropionic acid.

DISCUSSION

The results not only confirm that the phenolic acids (Griffiths, 1964; Das & Griffiths, 1968) and substituted phenyl-γ-valerolactones (Oshima & Watanabe, 1958; Watanabe, 1959a,b; Das & Griffiths, 1968) excreted after the administration of (+)-catechin are derived from the carbon skeleton of the catechin molecule, but also lend support to the postulated pathway for the catabolism of catechin in the mammal (Watanabe, 1959b; Das & Griffiths, 1968). The phenolic acid metabolites of catechin (Griffiths, 1964; Das & Griffiths, 1968) are shown to arise from the B ring of catechin, since radioactivity was demonstrated in these metabolites after giving [U-14C]catechin, but not after giving [ring A-14C]catechin. The phenyl-γ-valerolactones, on the other hand, which were postulated to arise in part from the A ring (Watanabe, 1959b; Das & Griffiths, 1968), are shown to possess radioactivity derived from [ring A-14C]catechin. In addition, a higher production of $^{14}CO_2$ from [ring A-14C]catechin than from [U-14C]catechin (Figs. 1 and 2) indicates a selective breakdown of the A ring of catechin to carbon dioxide and accounts for the absence of metabolites containing the intact A ring in the urine.

Further evidence in support of the proposed pathway was obtained by administration to the rat of [14C]δ-(3-hydroxyphenyl)-γ-valerolactone, which was shown, by radioautography, to give rise to labelled m-hydroxyphenylpropionic acid as postulated by Das & Griffiths (1968). Administered

Table 3. *Distribution of radioactivity in the excretory products of the guinea pig and rat*

Results are expressed as the mean values of two experiments.

	[U-^{14}C]-Catechin (mean %)	[*ring A*-^{14}C]-Catechin (mean %)	[U-^{14}C]-Catechin (mean %)	[*ring A*-^{14}C]-Catechin (mean %)
CO_2	17·5	35·1	18·5	41·5
Urine	51·6	54·5	63·4	55·1
Faeces	0·1	1·0	1·4	0·6
Total	69·2	90·5	83·3	97·2

[^{14}C]*m*-hydroxyphenylpropionic acid was also shown to give rise to labelled *m*-hydroxybenzoic acid, which, as shown earlier (Griffiths, 1964), is derived from *m*-hydroxyhippuric acid under the conditions of acid hydrolysis used.

Although the free phenolic metabolites of [U-^{14}C]-catechin (Table 2) account for only one-third to one-half of the radioactivity of the ether-soluble fraction of urine (Table 1), it is known (Das & Griffiths, 1968) that certain of these phenolic compounds also occur as conjugates possessing R_F 0 in the benzene–acetic acid–water solvent (*B*). It was not possible to determine the radioactivity of the latter constituents as their separation on paper chromatograms presents considerable difficulty (Das & Griffiths, 1968). The radioactivity of the non-ether-soluble fraction may also in part be attributable to the presence of phenolic conjugates, although it is notable that even after ether extraction of the hydrolysed urine (Table 1) a considerable proportion of radioactivity remains in the aqueous fraction. These observations suggest the presence in urine of non-phenolic metabolites, possibly aliphatic intermediates in the production of the $^{14}CO_2$ detected.

It is evident (Table 3) that the major portion of the ^{14}C administered to both guinea pig and rat is excreted in the urine and breath over the period of examination. As determinations of the radioactivity of the faeces were carried out for only a restricted period, namely 48hr. after administration of [^{14}C]catechin, the radioactivity reported (Table 3) is thought to represent a relatively small amount of the total faecal excretion. The balance of radioactivity not accounted for (Table 3) may be present not only in the intestinal tract but also as tissue constituents derived from $^{14}CO_2$ and the postulated aliphatic intermediates.

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