The Metabolism of 2:4:5-Trihydroxybutyrophenone in the Rat and Dog

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The widespread use of phenolic antioxidants as stabilizers in foodstuffs raises the question of the manner of their disposal after intake by the animal body. It is intended to investigate the metabolism of several phenolic antioxidants, and in this paper some aspects of the metabolism in the rat and dog of 2:4:5-trihydroxybutyrophenone are discussed. This study was made during chronic-toxicity studies designed to test the suitability of 2:4:5-trihydroxybutyrophenone for use as an antioxidant in foodstuffs. The metabolism of the pure compound in rats and dogs, its excretion in urine and faeces, the extent of its storage in tissues at various dose levels, and the period of retention of a single dose as 2:4:5-trihydroxybutyrophenone and any metabolic products have been examined. The metabolism of 2:4:5-trihydroxybutyrophenone presents several potentially interesting features arising from the presence of a polyhydric phenolic nucleus and a butyryl side chain. Although the metabolism of phenols has been extensively studied (Williams, 1949) and the principal routes (ethereal sulphate and glucuronide conjugation, oxidation to polyhydric phenols) are well known, much less information is available about the metabolism of polyhydric phenols. The problem of the orientation of conjugation has been discussed (Dodgson, Smith & Williams, 1950) for phenols having more than one hydroxyl available for conjugation. It seemed of particular interest to see whether 2:4:5-trihydroxybutyrophenone underwent conjugation, whether more than one conjugate was produced and whether conjugates of different orientation were formed.

MATERIALS AND METHODS

Animals, dosage and diet

Urine, faeces and tissues from dogs and rats used in chronic studies were kindly supplied by Dr H. C. Hodge, Division of Pharmacology and Toxicology, University of Rochester, School of Medicine and Dentistry, Rochester, N.Y., to whom we are indebted for the following information.

Dogs used in a feeding study for 1 year were beagles and beagle-type mongrels on a basal diet of Purina Chow. Dogs nos. 1-12 were maintained separately in metabolism cages and fed on daily doses of 0.1, 0.3 and 0.5 g. of 2:4:5-trihydroxybutyrophenone (THBP)/kg., three dogs to each dose and three controls being used. The dose was mixed

with the diet; water was supplied *ad lib.* and for the metabolism studies urine and faeces were collected daily. For the single-dose studies mongrel dogs, nos. 13-16, were used and kept separately in metabolism cages. A single dose of 300 mg./kg. was given to each dog by meat capsule, and urine and faeces were collected daily for 6 days.

Rats used in a study for 2 years were weanling male and female albino rats, Rochester strain (ex-Wistar, 1923), in groups of 30 males and 30 females on a diet of Purina Fox Chow Meal. THBP was mixed with the diet and given at dose levels of 0.1, 0.3, 1.0 and 3.0 % of the diet. Faeces and urine were collected as required from metabolism cages. Single-dose studies were performed with female albino rats, Sprague-Dawley strain, kept singly or doubly in metabolism cages on a Purina Chow diet. THBP was administered by an oral injection of 400 mg. in 10 % (w/v) corn oil suspension/kg., and urine and faeces were collected daily for 6 days. Extended metabolism studies with rats were carried out similarly with a daily dose of 400 mg./kg., and the urine and faeces were pooled.

Materials

THBP was kindly supplied by the Tennessee Eastman Co., Kingsport, Tennessee, U.S.A. The following were purchased: 2:2'-dipyridyl, *n*-butyric anhydride, *n*-butyronitrile, 2:5-dimethoxyaniline, quinol dimethyl ether, *D*-glucuronolactone (Eastman Organic Chemicals, Rochester, N.Y.) and β -glucuronidase (ox-liver, Ketodase; Warner-Chilcott, New York, N.Y.). Substances synthesized are described below.

Methods

Paper chromatography. Separation of THBP and its metabolites, compounds A, B and C, in experimental urine was carried out on strips of Whatman no. 1 paper by the descending method. Table 1 records their R_f values and colour reactions in various solvent systems. Quantities of THBP down to 5 μ g. in a 0.5 cm. diameter spot were detectable. Ultraviolet spectra of materials on the paper were obtained by cutting out appropriately located zones and inserting them in the cell holder of a Beckman model DU spectrophotometer.

Examination of urine. (i) Paper chromatography. A volume (10 μ l.) of each test and control urine from rats or dogs was chromatographed in solvent I (Table 1), and THBP and its phenolic metabolites were located by their ferric colours. THBP and compound A, located by their fluorescence in u.v. light, were estimated by cutting out the zones, extracting these spots with aq. 80 % (v/v) ethanol and estimating the reducing power of the solution by the FeCl_3 -2:2'-dipyridyl reaction (see below). Compounds B and C are non-reducing, and the acid hydrolysis of eluates seemed to decompose any phenols which may have been liberated. No direct estimate of THBP thus combined

could be made. (ii) THBP content and free phenols. When method (i) failed to detect THBP, urine at pH 6.0 was extracted with ether and the dried ether extract evaporated; the residue in aq. 80% ethanol was tested with FeCl_3 -2,2'-dipyridyl reagent. Since paper chromatography of the ether extract did not reveal the presence of free phenols other than those present in normal urine, any excess of reducing power of the experimental ether extract over control ether extract was expressed as THBP. (iii) Glucuronides. Total glucuronides were estimated by a modified naphtharesorcinol method (Bray & Thorpe, 1954). A 1% (w/v) solution of naphtharesorcinol in aq. 20% (v/v) ethanol replaced the aq. 0.375% solution previously used; it was found that inconsistencies caused by interfering substances were thus minimized. Urine was diluted to a concentration of 5–100 μg . of glucuronides/ml., and readings were made at 615 $m\mu$; a standard curve was prepared from D-glucuronolactone. (iv) Ethereal sulphates. Total and inorganic sulphate was estimated gravimetrically by Folin's procedure (Bray & Thorpe, 1954).

Examination of faeces. Faeces (110 g.) after 4 days of collection from four rats in the single-dose study were extracted with benzene (Soxhlet) for 24 hr. The benzene extract, diluted with 200 ml. of ether, was shaken with 2N-NaOH (2 $\times$ 50 ml.). On acidification and ether extraction an amorphous residue resulted. A portion of an ethanolic solution of this was tested with FeCl_3 -2,2'-dipyridyl reagent and the excess of reducing power over that of control faeces was expressed as THBP. Faeces from one dog in a single-dose study were collected for 4 days; the 24 hr. benzene extract was similarly treated. Faeces from rats and dogs in chronic studies were extracted with benzene, and the benzene extract, after concentration, was examined chromatographically for THBP as in the procedure for urines.

Estimation of 2:4:5-trihydroxybutyphenone and compound A in animal tissues. A modification of the Emmerie & Engel FeCl_3 -2,2'-dipyridyl method as used for fat-soluble phenolic antioxidants (Mahon & Chapman, 1951) was employed. A weighed quantity of fresh tissue from a recently killed animal was macerated with anhydrous Na_2SO_4 or anhydrous MgSO_4 and stirred with CHCl_3 for 0.5 hr. The mixture was left overnight, the solution removed, the residue washed and the combined solutions were evaporated to dryness and the residue was dissolved

in 30 ml. of light petroleum (b.p. 66–75°). This was extracted with aq. 80% (v/v) ethanol (3 $\times$ 4 ml.), and on concentration *in vacuo* an amorphous residue resulted, which was dissolved in CHCl_3 -water-ethanol (1:1:1, by vol.) to a volume of about 1.2 ml. A portion (0.1 ml.) of the tissue extract was run on the same chromatogram, in solvent I, with 10 μl . portions of 'standard' rat urine known to contain compounds A, B and C, and to which 1 vol. of ethanolic 1% (w/v) THBP had been added. The zones in the tissue chromatogram in which THBP and compound A should occur were thus located, cut out and left for 20 min. in aq. 80% (v/v) ethanol (10 ml.). The paper was removed and washed with ethanol (5 ml.) and to the combined ethanolic solutions were added ethanolic 0.1% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (4 ml.) and ethanolic 0.1% (w/v) 2,2'-dipyridyl (4 ml.). All mixing was done in weak artificial light; after 40 min. in the dark, absorption intensities at 515 $m\mu$ were measured in an Evelyn colorimeter against a reagent blank. A calibration curve was prepared from known amounts of THBP equivalent to quantities of 0–100 μg . on the chromatogram. Recoveries of known amounts of THBP from tissues were $75 \pm 10\%$. Reducing power in the compound A zone was expressed as THBP; no estimate of the accuracy of recoveries of this substance was possible.

RESULTS

Excretion of free reducing phenols by control animals

The mean daily excretion per rat of free reducing phenols, estimated chromatographically as THBP on sixteen undosed rats, was 2.2 (0–2.8) mg. in the urine and 0.41 (0–1.3) mg. in the faeces, and per dog on seven undosed dogs was 9.5 (0–26.0) mg. in the urine, and 2.5 (0–2.8) mg. in the faeces.

Paper chromatography of 2:4:5-trihydroxybutyphenone and its metabolites

By paper-chromatographic examination of experimental rat urine in solvent I (Table 1) three new substances, which were not present in control urines, were located by the fluorescence of their spots in u.v., and by the colour reactions with

Table 1. R_F values and colour reactions of 2:4:5-trihydroxybutyphenone and its metabolites in the urine of experimental animals

Solvent mixtures: I, organic phase of freshly mixed butanol-acetic acid-water (4:1:5, by vol.); II, organic phase of benzene-acetic acid-water (2:2:1, by vol.). Detecting reagents: A, aq. 1% (w/v) AgNO_3 soln.-aq. 10% NH_3 soln. (1:1, v/v); B, 1% of FeCl_3 in aq. 50% (v/v) ethanol; C, freshly mixed 1% (w/v) sulphonic acid in 3N-HCl-aq. 5% (w/v) NaNO_2 (1:1, v/v). The dried paper was suspended in an atmosphere of NH_3 .

Substance	R_F in solvent mixture		Colours given by reagents			Fluorescence of spot in u.v. light
	I	II	A	B	C	
THBP*	0.92–0.95	0.42–0.44	Brown	Rose-pink	Rose-pink	Pale yellow
Compound A	0.75–0.81	0.14	Brown-yellow	Green, fades rapidly to yellow	Blue	Light blue
Compound B	0.68–0.72	0.12	None	Purple	Yellow-brown	Orange
Compound C	0.45–0.55	0.05	None	Purple	None	Weak grey

* R_F values unchanged for the pure substance in ethanolic soln. THBP had R_F 0.33–0.35 in aq. 15% (w/v) KCl soln.

ferric chloride, ammoniacal silver nitrate and diazotized sulphanilic acid solutions (Table 1). The substance with R_f 0.92–0.95 (solvent I) was shown to be THBP. The spot with R_f 0.78 $\pm$ 0.03 was attributed to a new substance, compound A; its decreased R_f in solvents I and II (Table 1) suggested the attachment of a hydrophilic moiety to THBP, the coupling reaction suggested phenolic character and the ferric and reducing reactions the retention of a 1:2- or 1:4-dihydroxybenzene system. Since *O*-substitution seems more probable at the 4-hydroxyl group, compound A was formulated as a 4-conjugate of THBP. Compound B, with R_f 0.70 $\pm$ 0.02, was formulated as a 5-conjugate of THBP from a similar consideration of its properties. The close similarity of the u.v.-absorption spectra of THBP, compound A and compound B on the chromatogram confirmed their relationship (Table 2). Compound C, with R_f 0.50 $\pm$ 0.05, was detected on some chromatograms of experimental urine from rats, and in fewer cases from dogs. This was clearly a metabolite of THBP,

whose colour reactions suggested similar substitution to compound B, but it was not produced consistently by either species, and all attempts to characterize or to isolate it were unsuccessful.

Metabolism of 2:4:5-trihydroxybutyphenone

A single dose of 400 mg. of THBP/kg. was given to each of sixteen rats in four groups of four rats, and urine and faeces, pooled according to group, were collected daily. A single dose of 300 mg./kg. was given to each of four dogs, and urine and faeces were collected daily. The excretion of THBP and its metabolites in the urine was examined chromatographically and the daily free THBP, glucuronide and ethereal sulphate outputs were estimated. The quantity of THBP remaining unabsorbed was estimated in the total faeces. The results are summarized in Table 3.

In both species, after a single dose was given, there was an increase in ethereal sulphate and glucuronide output which accounted for about 75 % of the given dose; the path of metabolism

Table 2. *Absorption spectra of some 2:4:5-trisubstituted butyphenones*

Substance	Medium	$\lambda_{\max}$ (m μ)	log ϵ	$\lambda_{\max}$ (m μ)	log ϵ	$\lambda_{\max}$ (m μ)	log ϵ	$\lambda_{\max}$ (m μ)	log ϵ
THBP	Propan-2-ol	213	4.09	243	4.07	282	4.05	350	3.86
	Whatman no. 1 paper*	—	—	237	—	281	—	347	—
Potassium 5-butyryl-2:4-dihydroxyphenyl sulphate	Ethanol	—	—	235	4.02	274	4.05	328	3.81
	Whatman no. 1 paper*	—	—	231	—	276	—	322	—
5-Butyryl-2:5-dihydroxyphenyl glucosiduronic acid	Whatman no. 1 paper*	—	—	237	—	274	—	336	—
5-Hydroxy-2:4-dimethoxybutyphenone	Propan-2-ol	—	—	235	4.17	270	3.92	332	3.84
4-Hydroxy-2:5-dimethoxybutyphenone	Propan-2-ol	—	—	234	4.09	272	4.04	330	3.91

* Absorption of the appropriate spot after paper chromatography of urine of animals dosed with THBP.

Table 3. *Excretion of 2:4:5-trihydroxybutyphenone and metabolites after a single dose*

Results are expressed as percentage of dose-fed: 0.4 g./kg. was given orally to each of 16 rats in four groups of four; 0.3 g./kg. was given orally to each of four dogs. Urine and faeces were collected daily for 6 days. Values for rat groups were obtained from the pooled daily outputs of the rats in each group. Metabolites were detected chromatographically. Means are given in parentheses.

Rat group*	Dose (g.)	Urinary THBP	Gluc-urionide (G)	Ethereal sulphate (ES)	Total conjugates (G + ES)	Period of excretion (days)	Metabolites detected	Faecal THBP
1	0.13	7.8	21	44	65	3-4	Compounds A, B, C	1.8
2	0.13	6.3	23	66	89	3-4	Compounds A, B, C	1.5
3	0.15	8.0	15	50	65	4-5	Compounds A, B	2.2
4	0.15	3.1 (6.3)	32 (23)	47 (52)	79 (75)	3-4	Compounds A, B	1.3 (1.7)
Dog no.								
13	2.85	2.2	36	30	66	4-5	Compounds A, B, C	4.9
16	4.34	1.3	41	31	72	4-5	Compounds A, B, C	6.2
14	4.2	2.2	19	65	74	3-4	Compounds A, B	4.8
15	3.64	3.6 (2.3)	24	54	78 (73)	4-5	Compounds A, B	5.5 (5.3)

* The mean dose/rat in each group is recorded.

was largely by conjugation, and no evidence of reduction or oxidation was found. Chromatographic examination of the urine showed that, in both species, THBP and the metabolites were almost completely excreted by the third or fourth day. Glucuronide and ethereal sulphate output had returned to pre-dose levels usually by the third or fourth day in the rat, and the fourth or fifth day in the dog; in all rat groups and in dogs nos. 13 and 14 conjugate output was greatest on the first day, and in dogs nos. 15 and 16 on the second day, after dosing. In dogs there was a variation in glucuronide and ethereal sulphate excretion which appeared to be correlated with the presence (dogs nos. 13 and 16) or absence (dogs nos. 14 and 15) of compound C. In both species little of the THBP was unabsorbed.

Table 4 shows the effect of dose variation on urinary and faecal THBP excretion of selected dogs in chronic studies.

The tissues of nine experimental and three control dogs were examined; samples of pooled perirenal, omental and subcutaneous fat, brain, liver and kidney were obtained and whenever possible examined immediately or refrigerated until examination. Brain, pooled perirenal, omental and subcutaneous fat, liver and kidney samples from the surviving rats at the conclusion of the study were pooled according to tissue, dose level and sex and immediately examined. Results are presented in Tables 5 and 6.

Metabolites of 2:4:5-trihydroxybutyphenone

Compound B was obtained pure from an experimental dog urinary sediment by acetone extraction, and gave on methylation and mild hydrolysis 5-hydroxy-2:4-dimethoxybutyphenone identical with a synthetic specimen; it was shown to be potassium 5-butyryl-2:4-dihydroxyphenyl sulphate. Amorphous compound A was obtained by partition-column chromatography of a 'glucuronide gum' from experimental rat urine (Kamil, Smith & Williams, 1951), and on methylation and prolonged

incubation with β -glucuronidase gave 4-hydroxy-2:5-dimethoxybutyphenone identical with a synthetic specimen. Compound A was formulated as 4-butyryl-2:5-dihydroxyphenyl glucosiduronic acid. The same partition yielded a gum which gave, on methylation and mild hydrolysis, the *OO*-dimethyl ether derived from compound B.

ISOLATION OF METABOLITES

All m.p.'s are corrected.

Source of metabolites. Compound B was obtained from the sediment from urine of dog no. 8 (fed with 0.3 g. of THBP/kg. in the diet/day) collected in a metabolism cage. The urine used in the separation of compounds A and B was collected in metabolism cages from eight female albino rats, Sprague-Dawley strain, given a daily oral injection of 400 mg. of THBP in 10% corn oil suspension/kg. A chromatographic examination of pooled daily urines determined whether they could be used as a rich enough source of compounds A and B.

Isolation of potassium 5-butyryl-2:4-dihydroxyphenyl sulphate (compound B). Clarified dog urine (80 ml.), containing compounds A and B but no THBP or compound C, deposited a sediment on standing. This was collected by centrifuging to give a dry powder (450 mg.). Extraction with hot acetone afforded a solid residue which on crystallization from aq. ethanol gave colourless plates (150 mg.). Potassium 5-butyryl-2:4-dihydroxyphenyl sulphate had m.p. 230–232° (inserted at 215°) (Found: C, 38.4; 38.2; H, 3.2; 3.3; S, 9.5; K, 12.5. $C_{10}H_{11}O_7SK$ requires C, 38.2; H, 3.5; S, 10.2; K, 12.4%). A chromatogram of 50 μ g., under the usual conditions, compared with experimental rat urine, showed a trace of THBP (R_F 0.95), and a spot at R_F 0.71 which gave silver nitrate, diazotized sulphanilic acid and ferric colours identical with those due to compound B, R_F 0.68, in the rat urine. The u.v. absorption (Table 2) showed similarity with that of THBP, and infrared absorption showed a band at 9.5 μ (S–O stretching vibration). The acetone-insoluble portion of the urinary deposit was identified as calcium oxalate. The residue on ignition of compound B showed a characteristic K-emission spectrum with strong lines at 3447.7 and 3446.7 Å.

Hydrolysis of potassium 5-butyryl-2:4-dihydroxyphenyl sulphate. Compound B (70 mg.) in 3N-HCl (10 ml.) was warmed to 95° for 0.5 hr. The cooled solution was extracted with ether and the residue after evaporation of the ether extract crystallized from benzene-hexane as yellow prisms of THBP (25 mg.), m.p. 147–148°, unchanged by admixture with an authentic sample. The aqueous phase from the ether extraction gave a strong positive test for SO_4^{2+} ions with 5% $BaCl_2$ soln.

Conversion of compound B into 5-hydroxy-2:4-dimethoxybutyphenone. Compound B (200 mg.) in methanol (100 ml.) was treated with an excess of ethereal diazomethane. After 12 hr. a test portion gave no reaction with Fe^{3+} ions, and the solution was evaporated to a discoloured gum, which was heated for 30 min. on a steam bath with ethanol (5 ml.) and N-HCl (20 ml.). The ether extract of the cooled solution was washed with 0.5N-NaOH soln. (3 $\times$ 20 ml.) and the acidified washings were extracted with ether (3 $\times$ 20 ml.). The dried ethereal solution left a gummy residue which was extracted with boiling hexane, to give

Table 4. Net daily excretion of 2:4:5-trihydroxybutyphenone by dogs in chronic studies at various dose levels

Results are expressed as mean percentage of daily dose for three dogs at each dose level; urine and faeces were collected over several days and the total THBP content of each was expressed as a daily excretion.

Daily dose level (g./kg.)	Urinary THBP	Faecal THBP
0.1	0.7	0.8
0.3	0.3	3.5
0.5	0.6	5.8

Table 5. *Storage of 2:4:5-trihydroxybutyrophenone and compound A in organs of experimental dogs*

Dogs were maintained at the appropriate dose level for 1 year; the animals were then killed and the organs analysed for THBP and compound A. Results are expressed as THBP.

Dog no.	Body wt. (kg.)	Daily dose (g./kg.)	Total dose (kg./1 year)	Substance	Net organ storage ($\mu\text{g./organ}$)			Fat storage ($\mu\text{g./g. of fat}$)
					Liver	Brain	Kidney	
1 (♀)	13.1	0	0	THBP* Compound A*	90	25	30	0.7
2 (♂)	11.8	0	0		450	90	200	1.1
3 (♀)	12.2	0	0					
4 (♂)	10.4	0.1	0.38	THBP	40	0	90	0.1
				Compound A	850	0	0	4.5
5 (♀)	13.6	0.1	0.49	THBP	0	145	0	1.5
				Compound A	2000	550	400	5.0
6 (♂)	11.8	0.1	0.43	THBP	240	75	20	1.3
				Compound A	0	40	0	1.3
7 (♀)	12.7	0.3	1.38	THBP	0	45	0	0.2
				Compound A	1000	260	150	5.0
8 (♂)	10.9	0.3	1.19	THBP	0	15	270	0.1
				Compound A	700	470	200	1.5
9 (♂)	12.2	0.3	1.33	THBP	0	55	0	0
				Compound A	700	360	40	1.5
10 (♂)	10.4	0.5	1.89	THBP	370	5	80	1.5
				Compound A	0	0	0	1.0
11 (♀)	9.7	0.5	1.76	THBP	1400	5	20	2.2
				Compound A	2000	0	0	1.0
12 (♂)	7.7	0.5	1.02	THBP	360	15	120	4.0
				Compound A	0	210	50	7.0

* Mean control values.

Table 6. *Storage of 2:4:5-trihydroxybutyrophenone and compound A in organs of surviving experimental rats*

Rats were maintained at the appropriate dose for 2 years, when the survivors were killed, the tissues pooled according to dose and sex and the organs analysed. Number of survivors are given in parentheses. Results are expressed as THBP.

Percentage of THBP in diet	Sex	Average body wt. (g.)	Average daily dose (mg.)	Total dose (g./2 years)	Substance	Net organ storage ($\mu\text{g./organ}$)			Fat storage ($\mu\text{g./g. of fat}$)
						Liver	Brain	Kidney	
0	♂ (1)	398	0	0	THBP Compound A	3.5	*	*	0.8
0	♀ (6)	279	0	0		19	*	*	4.2
0.1	♂ (5)	404	21	15.3	THBP	0	4	20	1.5
					Compound A	3	15	35	4.5
0.1	♀ (6)	285	15	11.0	THBP	0	15	0	0.5
					Compound A	0	15	12.5	2.0
0.3	♂ (3)	399	60	43.8	THBP	12	5	*	0.0
					Compound A	20	15	*	3.5
0.3	♀ (5)	262	39	28.5	THBP	1	10	4	1.0
					Compound A	10	0	40	2.5
1.0	♂ (2)	412	206	150	THBP	1	0	8	2.0
					Compound A	10	12	50	1.0
1.0	♀ (4)	251	125	91	THBP	0	7	8	0.0
					Compound A	0	2	7	0.2
3.0	♂ (3)	357	535	390	THBP	1	7	3	0.0
					Compound A	0	8	25	1.0
3.0	♀ (5)	268	402	290	THBP	3	6	15	9.5
					Compound A	11	20	20	13.0

* Insufficient material.

pink plates (32 mg.) of 5-hydroxy-2:4-dimethoxybutyrophenone, m.p. 87–88° (Found: C, 64.2; H, 7.2; OCH₃, 27.1. C₁₂H₁₄O₄ requires C, 64.3; H, 7.2; (OMe)₂, 27.6%). The substance was readily soluble in ethanol and the ethanolic solution gave a faint-green with FeCl₃ soln. The u.v.-absorption spectrum closely resembled that of THBP (Table 2).

Isolation of compounds A and B from experimental rat urine. The urine (250 ml.), known to contain THBP, compounds A, B and C, was adjusted to pH 6.0 and gently washed with ether (3 × 200 ml.). The urine was adjusted to pH 4.5 and an excess of saturated lead acetate soln. added with stirring (Kamil *et al.* 1951). The precipitate was collected after 1 hr. by centrifuging and washed with water (2 × 100 ml.) by centrifuging and decantation. The precipitate from the combined filtrate and washings at pH 8.0 was similarly collected and washed. Lead was removed by precipitation with H₂S and the residual solutions yielded two 'glucuronide' gums. Chromatographic examination showed that no metabolites remained in the treated urine, that the first glucuronide gum contained only a trace of compound B and that the second contained all three metabolites. The second gum (800 mg.), in aqueous solution, was mixed with Whatman no. 1 powdered cellulose (5 g.) and the mixture evaporated and dried *in vacuo*. The well-mixed powder was tamped on to the top of a column of powdered cellulose (32 cm. × 31 cm.) previously equilibrated with the eluent, the organic phase of butanol-acetic acid-water (8:1:10, by vol.). Fractions (5 ml.) of eluate were collected under a slight positive pressure by means of a fraction collector; each fraction was examined chromatographically in solvent I. The fractions were pooled according to the metabolites they contained. Compound B was found in fractions 4–15, compound A in fractions 17–28 and compound C in fractions 34–50. The first group had few, the second some and the third many phenolic contaminants. The groups were evaporated to give gums B, A and C respectively.

Gum A. On drying *in vacuo* at 65° a brown gum (350 mg.) resistant to crystallization was obtained. This was dissolved in water (7.5 ml.) and saturated lead acetate soln. was added; the pale-yellow precipitate was washed and triturated in aqueous suspension while H₂S was passed; the solution after removal of PbS gave an amorphous powder (120 mg.). This was insoluble in ethanol, and in water gave a deep-green ferric colour which changed slowly to bright yellow as the solution was made progressively more alkaline, thus excluding a 1:2-dihydroxybenzene system (Porteous & Williams, 1949); in the absence of alkali the colour changed slowly to pale orange. The *R_F* and colour reactions on a chromatogram were identical with those of compound A. An aqueous solution gave a strong positive naphtharesorcinol reaction. This material was the purest obtainable specimen of compound A.

Methylation of compound A. Compound A (120 mg.) in methanol (30 ml.) was treated with ethereal diazomethane and left for 12 hr. The excess of solvent was removed to give a pale-yellow gum (115 mg.), which resisted crystallization. No ferric colour was obtained, and a strong positive naphtharesorcinol test was given. Warming or boiling with *n*-HCl gave decomposed products.

Acetylation of methylated compound A. Methylated compound A (100 mg.) was dissolved in acetic anhydride (2 ml.) and 70% perchloric acid (0.02 ml.) was added.

After 2 hr. the mixture was poured into water to yield an amorphous solid (120 mg.). Attempted purification on acid-washed alumina afforded by elution with ethanol-acetic acid (1:1, v/v) an amorphous solid (100 mg.), insoluble in water, readily soluble in benzene and hexane, giving a strong positive naphtharesorcinol test and decomposing on attempted acid hydrolysis.

Hydrolysis of the methylated compound A by β-glucuronidase. Methylated amorphous compound A (100 mg.) was dissolved in water (5 ml.); 0.2*N*-acetic acid (5 ml.), 0.2*N*-sodium acetate (to pH 4.5) and 10 ml. of Ketodase [equivalent to 60 000 Fishman units (Talalay, Fishman & Huggins, 1946) of β-glucuronidase] were added. After incubation for several days at 37.5° the mixture was extracted with ether (3 × 30 ml.). The ether extract was shaken with *N*-NaOH (3 × 20 ml.), and the aqueous layer acidified and re-extracted with ether. The dried ether solution gave a residue of semi-crystalline material (20 mg.) which, on sublimation at 1 mm. Hg and crystallization from hexane, gave elongated plates of 4-hydroxy-2:5-dimethoxybutyrophenone, m.p. 86–88° (Found: C, 63.8; H, 7.4; C₁₂H₁₄O₄ requires C, 64.3; H, 7.2%). The product dissolved readily in ethanol, ether and benzene and gave a faint blue colour with ethanolic FeCl₃ soln. The u.v. spectrum closely resembled that of THBP (Table 2).

Gum B. The gum (150 mg.) was dissolved in methanol, and three successive portions of ethereal diazomethane solution were added until no ferric colour was obtained. The solutions were evaporated and the residue was heated in *n*-HCl (60 ml.) for 0.5 hr. on the steam bath. The mixture was extracted with ether (3 × 70 ml.), the ether solution extracted with *N*-NaOH, the alkaline solution acidified and extracted again with ether (3 × 70 ml.). Extraction of the gummy residue with hexane gave pink plates (25 mg.) of 5-hydroxy-2:4-dimethoxybutyrophenone, m.p. 85–86°, not depressed on admixture with that derived from compound B from dogs.

Gum C. The gum (200 mg.) was shown chromatographically to contain contaminants, and gave gummy or decomposed residues on methylation, acetylation, warming and boiling with dilute acid. It gave a strong naphtharesorcinol reaction. From this and the behaviour on chromatography compound C is possibly the 5-*O*-glucuronide of THBP.

Synthesis of 5-hydroxy-2:4-dimethoxybutyrophenone

2:4-Dihydroxybutyrophenone. Resorcinol (14 g.) was dissolved in freshly purified and distilled nitrobenzene (100 ml.). Aluminium chloride (19 g.) was added, and the mixture stirred and heated at 80° while freshly distilled butyric anhydride (10 g.) was added dropwise. After stirring at 80° for 6 hr. and cooling, water (150 ml.) was added and the mixture stirred. After washing with water (150 ml.) again the combined aqueous washings were washed with ether (3 × 100 ml.) and then extracted with 10% (w/v) NaOH soln. (3 × 70 ml.) and water (70 ml.). The combined aqueous and alkaline solutions were washed with ether (2 × 50 ml.) and acidified with 6*N*-HCl. Ether extraction (3 × 250 ml.) afforded a black oily phenolic residue from which repeated hexane extractions gave colourless needles of the ketone (9 g.; 39%). 2:4-Dihydroxybutyrophenone had m.p. 56–57°; Nakazawa (1954) reported m.p. 73° for this compound prepared from resorcinol and butyric acid in the presence of polyphosphoric acid (Found:

C, 66.3; H, 6.5. Calc. for $C_{10}H_{11}O_3$; C, 66.6; H, 6.7%. The spectrum had $\lambda_{\max}$ at 233, 278 and 315 $m\mu$, and strong bands at 3.1 μ ($-\text{OH}$) and 6.09 μ ($-\text{C}=\text{O}$) were found. The product gave a port-wine colour with ethanolic FeCl_3 soln. The 2:4-dinitrophenylhydrazones had m.p. 245° (Found: C, 52.9; H, 4.8; $C_{16}H_{15}O_6N_4$ requires C, 53.3; H, 4.4%) and occurred as red needles from ethanol.

2:4-Dihydroxy-5-nitrobutyrophenone. 2:4-Dihydroxybutyrophenone (4 g.) was added in small portions with stirring to 20 ml. of HNO_3 (sp.gr. 1.42) cooled in ice. The mixture was allowed to attain room temperature and was poured into water (50 ml.), when a red solid separated which was collected and twice recrystallized from aq. ethanol to yield yellow needles (4.6 g.; 92%). 2:4-Dihydroxy-5-nitrobutyrophenone had m.p. 121.5–122° (Found: C, 53.2; H, 5.0; N, 6.3. $C_{10}H_{11}O_5N$ requires C, 53.3; H, 4.8; N, 6.2%). An orange-yellow ferric colour was given in ethanolic solution. (Nitration in the expected 5-position was shown by methylation of the final product of the synthesis to 2:4:5-trimethoxybutyrophenone.)

2:4-Dimethoxy-5-nitrobutyrophenone. 2:4-Dihydroxy-5-nitrobutyrophenone (5.5 g.), anhydrous K_2CO_3 (20 g.) and methyl sulphate (20 ml.) were refluxed with acetone (250 ml.). After 12 hr. no reaction was given with ethanolic FeCl_3 soln. and the mixture was filtered, the residue washed with acetone and the combined filtrate and washings were concentrated. Water (25 ml.) and an excess of aq. 10% NH_3 soln. were added, and, on cooling, a crystalline solid separated which yielded from aq. ethanol rhombic plates (4.3 g.); 2:4-dimethoxy-5-nitrobutyrophenone had m.p. 113–114° (Found: C, 56.9; H, 5.9. $C_{12}H_{13}O_5N$ requires C, 56.9; H, 5.9%).

5-Amino-2:4-dimethoxybutyrophenone. 2:4-Dimethoxy-5-nitrobutyrophenone (3.8 g.), 25 ml. of HCl (sp.gr. 1.19) and clean mossy zinc (10 g.) were heated together on the water bath; H_2 was evolved. After complete solution the cooled mixture was made strongly alkaline and extracted with ether (3×100 ml.), which gave a residue from which hexane extraction afforded yellow elongated prisms (1.7 g.) of 5-amino-2:4-dimethoxybutyrophenone, m.p. 75–76° (Found: C, 64.7; H, 7.7; N, 6.4. $C_{12}H_{17}O_3N$ requires C, 64.5; H, 7.6; N, 6.2%).

5-Hydroxy-2:4-dimethoxybutyrophenone. 5-Amino-2:4-dimethoxybutyrophenone (350 mg.) was dissolved in aq. 50% (v/v) H_2SO_4 (10 ml.) and cooled in ice. 1.5M-Sodium nitrite soln. (2 ml.) was added dropwise with mechanical stirring, and the diazonium sulphate soln. then added dropwise to stirred 0.3M- CuSO_4 soln. (100 ml.) at 95°. Stirring was maintained for 0.5 hr. after the addition. Ether extraction (3×75 ml.) of the cooled solution yielded a gummy residue which crystallized from hexane in pink prisms (50 mg.). 5-Hydroxy-2:4-dimethoxybutyrophenone had m.p. 86–88° (Found: C, 63.8; H, 7.2. $C_{12}H_{14}O_4$ requires C, 64.3; H, 7.2%). The m.p. was not depressed on admixture with specimens of 5-hydroxy-2:4-dimethoxybutyrophenone prepared from compound B obtained from both rat and dog urine. The natural and synthetic substances gave identical ferric colours and had identical u.v. and i.r. spectra.

2:4:5-Trimethoxybutyrophenone. THBP (500 mg.) in methanol was incompletely methylated by ethereal diazomethane; the ether was removed and the residue dissolved in acetone to which anhydrous K_2CO_3 (10 g.) and methyl sulphate (5 g.) were added. The mixture was refluxed

overnight, the product was isolated as described for 2:4-dimethoxy-5-nitrobutyrophenone, and crystallized from hexane to yield yellow hexagonal prisms (250 mg.) of 2:4:5-trimethoxybutyrophenone, m.p. 75–76° [Found: C, 65.3; H, 7.4; OCH_3 , 29.3. $C_{13}H_{16}O_4$ requires C, 65.5; H, 7.6; (OCH_3)₃, 26.0%].

Synthetic 5-hydroxy-2:4-dimethoxybutyrophenone was treated with diazomethane to yield yellow hexagonal prisms from hexane, m.p. 76–78°, not depressed on admixture with an authentic specimen of 2:4:5-trimethoxybutyrophenone. The identity of these substances confirms the orientation of the synthetic product.

Synthesis of 4-hydroxy-2:5-dimethoxybutyrophenone

2:5-Dimethoxyphenol. 2:5-Dimethoxyaniline (12.5 g.) was diazotized in aq. 50% (v/v) H_2SO_4 (100 ml.) at 0–5° with 5M- NaNO_2 soln. (20 ml.). Excess of NaNO_2 was decomposed with urea (2 g.) and the chilled diazo solution added dropwise to boiling aq. 50% H_2SO_4 (400 ml.) down a water-cooled condenser tube terminating at the liquid surface (Lambooy, 1950). The product was steam-distilled as it was formed. The distillate (1.5 l.) was extracted with ether to yield an oil, distillation of which gave a fraction (4.5 g.) b.p. 75–80°/12 mm. Hg [Found: C, 61.8; H, 6.7; OCH_3 , 42.5. Calc. for $C_8H_{10}O_3$; C, 61.9; H, 7.1; (OCH_3)₂, 39.9%]. In ethanolic solution a pale blue, fading rapidly, was given by FeCl_3 . These properties accord with those reported previously for 2:5-dimethoxyphenol (Gilman & Van Ess, 1939). The *p*-nitrobenzyl ether had m.p. 118–120° (Found: C, 62.4; H, 5.2; $C_{15}H_{15}O_3N$ requires C, 62.2; H, 5.5%).

4-Hydroxy-2:5-dimethoxybutyrophenone. Freshly distilled 2:5-dimethoxyphenol (5.5 g.) and freshly distilled *n*-butyronitrile (3 g.) were dissolved in dry ether (250 ml.) and freshly fused and powdered ZnCl_2 (5 g.) was added. Hydrogen chloride gas was passed into the cooled stirred solution to saturation and stirring continued for 4 hr. The mixture was then left at 5° for 2 days; water (50 ml.) was added and the aqueous phase was collected and heated at 90° for 2 hr. with 6N- HCl (6 ml.). Ether extraction gave a discoloured oil (1.50 g.). On distillation a fraction was collected at 78–80°/0.1 mm. Hg, identified as 2:5-dimethoxyphenol by its *p*-nitrobenzyl ether, and at 120–140°/0.1 mm. Hg a fraction distilled which solidified on cooling. This crystallized from benzene to yield elongated plates (300 mg.) showing strong absorption at 2.8 and 6.09 μ . 4-Hydroxy-2:5-dimethoxybutyrophenone had m.p. 85–86°, not depressed on admixture with 4-hydroxy-2:5-dimethoxybutyrophenone obtained from compound A [Found: C, 63.8; H, 7.4; OCH_3 , 26.8. $C_{12}H_{14}O_4$ requires C, 64.3; H, 7.2; (OCH_3)₂, 27.6%]. Infrared and u.v. spectra and ferric colours of the natural and synthetic phenols were identical.

The ethereal phase of the reaction mixture after washing with 2.5N- NaOH yielded colourless plates of a neutral substance (1.8 g.) which was shown to be quinol dimethyl ether, m.p. 57–59°, not depressed on admixture with an authentic specimen [Found: C, 69.5; H, 7.3; OCH_3 , 44.2. Calc. for $C_8H_{10}O_2$; C, 69.6; H, 7.2; (OCH_3)₂, 44.9%]. Synthetic 4-hydroxy-2:5-dimethoxybutyrophenone was treated with diazomethane to yield yellow prisms from hexane, m.p. 76–77°, not depressed on admixture with an authentic specimen of 2:4:5-trimethoxybutyrophenone. The third possible isomeric *OO*-dimethyl ether of THBP,

2-hydroxy-4:5-dimethoxybutyrophenone, has been reported to have m.p. 81° and to give a blue-green ferric colour (Jones, Mackenzie, Robertson & Whalley, 1949).

DISCUSSION

The amounts of glucuronide and ethereal sulphate excreted by both species in these studies indicate that the path of metabolism must be largely by conjugation. The small quantities of 2:4:5-trihydroxybutyrophenone excreted unchanged in urine and faeces show that the large doses administered in these studies do not exceed the capacity of the organism to absorb and conjugate the dose. In all cases fairly rapid elimination of the administered dose is found; in both rats and dogs the last detectable metabolite is eliminated by the third or fourth day after dosing and glucuronide and ethereal sulphate levels approach normal values by the fourth or fifth day after dosing. It seems safe to conclude that 2:4:5-trihydroxybutyrophenone and its metabolites do not circulate for any length of time after dosing.

Tissue storage is of a low order (Tables 5 and 6). Little correlation can be detected between dose and amount stored, however. Since, in general, experimental values do exceed control values, it is inferred that some deposit of THBP in tissues has taken place, but in proportion to the total dose and the time of ingestion this storage is negligible.

The path of metabolism of 2:4:5-trihydroxybutyrophenone appears to be that frequently found for phenolic substances. The absence of detectable amounts of free phenols formed by oxidation is not surprising in view of the high degree of hydroxylation. The absence of readily detectable substances arising from oxidation of the butyryl side chain or reduction of its carbonyl group accords with the observation that hydroxylation of aromatic ketones usually results in the side chain remaining intact on metabolism (Williams, 1949). The presence in the molecule of three phenolic hydroxyl groups leads to a number of possibilities in conjugation orientation, two of which are realized by the formation of a 5-sulphate and of a 4-glucuronide as shown in Scheme I. Conjugation of the 2-hydroxyl group seems unlikely because of its proximity to the bulky 1-butyryl group and

because of hydrogen bonding with the carbonyl group, and it does not seem to be found here.

The influence of substituents on the glucuronide conjugation of polyphenols has been discussed (Dodgson *et al.* 1950). The formation of a 4-glucuronide of THBP is in accord with the general observation that glucuronide formation in phenols takes place with the hydroxyl least subject to steric hindrance: cf. 4-chlorocatechol, 4-chlororesorcinol (Williams & Dodgson, 1949); 4-chloroquinol (Dodgson *et al.* 1950) and resacetophenone (Dodgson, 1950). A Fisher-Taylor-Hirschfelder model (Fisher Scientific Co., Pittsburgh, Pa., U.S.A.) of 2:4:5-trihydroxybutyrophenone shows that the 4-hydroxyl group is subject to much less hindrance than is the 2-hydroxyl group and to somewhat less hindrance than is the 5-hydroxyl group. (The less-frequent formation of compound C, which may be the 5-O-glucuronide, is not in conflict with this observation.)

A correlation between sulphate conjugation and dissociation constant of phenols has been pointed out; the more acidic the phenol, the less the extent of sulphate conjugation (Robinson, Smith & Williams, 1953). It seems probable that the 5-hydroxyl group of 2:4:5-trihydroxybutyrophenone is somewhat less acidic than the 4-hydroxyl group, from the directional influence of the 1-butyryl and 2-hydroxyl groups. If sulphate conjugation with the less acidic hydroxyl group occurs the conjugate would be the observed 5-sulphate.

The extent to which the two metabolites characterized above contribute to the total quantity of metabolites is not entirely clear, since their quantitative isolation was not achieved. The analytical data for the rat, however, suggest that they are the principal metabolites in this species, and in both species examined they are the principal detectable metabolites.

SUMMARY

1. The fate of 2:4:5-trihydroxybutyrophenone in the rat and dog has been studied.

2. At the dose levels studied, 2:4:5-trihydroxybutyrophenone is almost completely absorbed; about 75% of a single dose of 400 mg./kg. in the rat, and 300 mg./kg. in the dog, is excreted as conjugates, and the path of metabolism is largely by

etheral sulphate and glucuronide conjugation. The butyryl side chain appears to survive intact, and no evidence of oxidation to more complex phenols was obtained.

3. Etheral sulphate conjugation at the 5-hydroxyl group leads to 5-butyryl-2:4-dihydroxyphenyl hydrogen sulphate, isolated as its potassium salt. This was converted into 5-hydroxy-2:4-dimethoxybutyrophenone, and the synthesis of this substance is described.

4. Glucuronic acid conjugation at the 4-hydroxyl group leads to 4-butyryl-2:5-dihydroxyphenyl glucosiduronic acid, which was not isolated but was converted into 4-hydroxy-2:5-dimethoxybutyrophenone. The isolation and synthesis of this substance is described.

5. Dogs readily tolerate a daily dose level of 0.5 g./kg. for 1 year, and rats a daily dose level of 1.5 g./kg. for 2 years, with negligible storage of 2:4:5-trihydroxybutyrophenone in fat, liver, brain and kidneys.

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Metabolism of the Developing Retina

2. THE EFFECT OF AN INHERITED RETINAL DEGENERATION ON THE DEVELOPMENT OF GLYCOLYSIS IN THE RAT RETINA*

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An inherited retinal degeneration which bears a striking resemblance to human retinitis pigmentosa has been described in rats (Bourne, Campbell & Tansley, 1938) and other animals (Tansley, 1933; Parry, 1953). The defect is inherited as a simple Mendelian recessive, and histologically the condition is characterized by the degeneration of the whole of the outer retina between the inner nuclear layer and the choroid.

Graymore (1959) observed a dramatic increase in glycolysis in the retina of the developing rat, towards the end of the second week of life, coinciding approximately with the time at which the

eyes opened. Since this surge in activity did not appear to be light-dependent (Graymore, 1958*a*), it was suggested that it might be a reflexion of the stage of histological development, possibly being a manifestation of the differentiation of the visual cells. This idea gains support from the observation that subsequent destruction of the visual cells by intravenous injection of iodoacetate leads to a much reduced rate of retinal glycolysis (Graymore & Tansley, 1959*a, b*), and it has been suggested that the visual cells may make a major contribution to the high rate of retinal glycolysis.

The development of glycolysis in rats exhibiting the inherited retinal degeneration has been studied to determine whether or not metabolic changes accompanied the cellular breakdown. Such studies

* Part 1: Graymore (1959).

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