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THE METABOLISM OF ETHYL CARBONATE ESTER OF p-METHOXY-[U-^{14}C] PHENOL IN THE RAT

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The metabolism of ethyl carbonate ester of *p*-methoxy-[U-^{14}C] phenol in the rat has been investigated. Ethyl carbonate ester of *p*-methoxy-[U-^{14}C] phenol was synthesized from [U-^{14}C] phenol and purified by thin layer chromatography on silica gel and finally by chromatography on silicic acid-celite column. The radioactive ester was fed to the rats and various tissues examined. About 0.08% of the administered dose was excreted in bile collected for 5 hr via a cannulation. *p*-Methoxy-[U-^{14}C] phenol was very poorly absorbed in the animal tissues and also very quickly removed from them. Liver and intestine contained appreciable amount of radioactivity while heart and kidney did not contain much. Ubliquinone isolated from tissue lipids did not contain any radioactivity. The trapped carbon dioxide from the expired air was found to be nonradioactive. *p*-Methoxy-[U-^{14}C] phenol was mainly excreted in a conjugated form in the urine. About 50% of the administered dose was found in the urine 9 hr after dosing.

In the animal body phenols undergo two main reactions, namely conjugation of the hydroxyl group with glucuronic acid to form aryl glucuroindes and with sulphuric acid to form ethereal sulphates.[1–3] Other minor reactions include insertion of another hydroxyl group and in some cases the methylation of this group has also been observed. Aromatic ethers can undergo several possible reactions in the animal body, but the oxygen–aromatic carbon link appears to be stable. They get metabolized by hydroxylation of the aromatic ring[4] or the ether link gets split up with the loss of alkyl group. Sometimes a hydroxylation of the aromatic ring as well as dealkylation occurs.[5]

The aim of the present experiments was firstly to check if any of the *p*-methoxyphenol could be metabolized to form an intermediate in the biosynthesis of ubiquinone(Q) nucleus and secondly to check on the mode of its excretion and the possibility of its metabolism to smaller fragments which might be utilized for the synthesis of the Q side chain. For this, ethyl carbonate ester of *p*-methoxy-(U-^{14}C) phenol was synthesised from (U-^{14}C) phenol. Three experiments were carried out. In the first two experiments only bile and the major body tissues were examined but in order to investigate the metabolism of ethyl carbonate ester of *p*-methoxyphenol in more detail, the third experiment was conducted whereby the expired carbon dioxide and urine as well as the various organs of the rat dosed with radioactive ester were examined. Previously it has been reported that in rabbits, *p*-methoxyphenol is mainly excreted in the urine as the glucuronide or sulphate.[5] The presence of *p*-methoxyphenol in beaver sweat gland secretion has already been reported.[6]

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Materials and Methods

Synthesis of Ethyl Carbonate Ester of p-Methoxy-(U-^{14}C) phenol

Preparation of ^{14}C-hydroquinone.—The starting material was (U-^{14}C) phenol. Phenol was converted to *p*-benzoquinone which was then reduced to hydroquinone. The hydroquinone was methylated to *p*-methoxyphenol which was finally esterified with ethyl chloroformate.

A very satisfactory method of preparation of *p*-quinones is by coupling a monohydric phenol with diazotized sulphanilic acid. The azo compound is first reduced to *p*-aminophenol with sodium hydrosulphite, and this is subsequently oxidized to quinone. The method is particularly satisfactory for the preparation of alkylated quinones.[7–11] The method was first standardized with a cold run of phenol and the conditions for maximum yield in each step were determined.

1 mg (100 μc) of uniformally labelled ^{14}C-phenol was made up to 94 mg with carrier phenol and converted to *p*-^{14}C-benzoquinone by the above hydroquinone method. *p*-^{14}C-benzoquinone was reduced to hydroquinone with sodium hydrosulphite, and 39 mg of the ^{14}C-hydroquinone was obtained.

Methylation of Hydroquinone.—The ^{14}C-hydroquinone (39 mg) was made up to 50 mg by adding another 11 mg of carrier hydroquinone and methylated according to the method of Mauthner with dimethyl sulphate.[12] After the completion of reaction the contents were transferred to a small separating funnel and extracted several times with ether. The ether extract yielded 16.2 mg of the product.

The aqueous alkaline solution was acidified with HCl and then extracted three times with benzene. 16.9 mg of mainly monomethylated product was obtained from benzene extract.

The UV absorption of the above two extracts were taken in ethanol and found to be very similar. That of the ether extract was similar to the spectrum of *p*-methoxyphenol, but the presence of a little impurity was indicated from the spectrum taken after the addition of alkali. There was a slight shoulder in the absorption band at 293 mμ, corresponding to unchanged *p*-dimethoxybenzene. The spectrum of the benzene extract was, however, clear. Therefore, both these extracts were bulked and esterified with ethyl chloroformate as described below. 41 mg of the crude ester was obtained. The esters howed $E^{1\%}_{1cm}$ 90 at 277.5 mμ and its specific activity was found to be 4.08×10^5 cpm/mg.

Purification of the Radioactive Ester.—The crude ester as obtained above was found to have a peculiar smell. Frurthermore when it was fed to rats (Experiment 1) and the animal tissue lipids then examined, anisole was detected in kidney and liver lipids. The counts were, however, not proportional to the amount of anisole present (see below). The ester was, therefore, purified further by chromatography on a thin layer of silica gel and finally on a silicic acid–Celite column.

(i) Thin Layer Chromatography. A suitable solvent system was found by running the spots of the authentic compounds which might be present as impurities on thin layer of silica gel. Ethylene dichloride was found to be a suitable solvent for the separation of phenol, hydroquinone, ester of *p*-methoxyphenol and anisole. Phenol, ester of *p*-methoxyphenol, and anisole were found to run with R_f values 0.25, 0.44 and 0.61 respectively, while the hydroquinone remained at the origin. The radioactive ester of *p*-methoxyphenol was removed from the plate at R_f 0.44. The ester showed $E^{1\%}_{1cm}$ 97 at 277.5 mμ.

(ii) Silicic Acid–Celite Chromatography. To raise the $E^{1\%}_{1cm}$ of the ester further, it was chromatographed on a small column of silicic acid–Cellite (2g+1g), and the fractions collected in 10-ml graduated tubes. Light petroleum and 2% ether in light petroleum fractions did not show any characteristic spectrum and radioactivity. 4% ether in light petroleum showed absorption at λ_{max} 290 mμ and shoulder at 298 mμ, and also contained radioactivity. There was no effect of alkali on the spectrum. The spectrum was found to be similar to that of authentic *p*-dimethoxybenzene. The ester of *p*-methoxyphenol was eluted in 6% ether in light petroleum fraction. 17 mg of the ester of *p*-methoxyphenol was obtained. The $E^{1\%}_{1cm}$ of the ester was found to be 101 at 277.5 mμ. The IR spectrum of the pure ester showed the presence of an ester band at 1215 cm^{-1} and the absence of hydroxyl group band at 3440 cm^{-1} which is present in *p*-methoxyphenol. The spectrum was very similar to that of ester made from authentic (B.D.H.) *p*-methoxyphenol (Fig. 1).

Administration of Ester to Rats.—Ester of *p*-methoxyphenol was dissolved in a minimum amount of arachis oil and then mixed with about 3 g of cellulose–starch mixture (50/50) and a little bit of icing sugar. To the above mixture a small volume of water was added and the whole made into small pellets. The rats were kept without food for 16–18 hr before dosing.

Collection of Bile.—After 3–4 hr of dosing, the rats were anaesthsized with Nembutal (approximately 0.25 ml for a 230-g rat) by interaperitoneal injection and the abdominal cavity opened up. The bile duct was located in the pancreas and cannulated with fine nylon tubing. The bile was collected in a graduated tube. The body fluid was kept up to the volume by injecting 0.9% saline into the abdominal cavity to replace that lost in the bile. After the collection of bile, the rats were killed with chloroform and the tissues removed.

Collection of Expired Carbon Dioxide, Urine and Faeces.—The rat immediately after dosing was placed in a metabolic chamber. This consisted of a small desiccator, in which a piece of circular wire gauze was placed well above the bottom. On the wire gauze a pad of Whatman No. 3MM paper (5 thicknesses) rested over which urine and faeces were collected. A supply of air was drawn through the desiccator by connecting the outlet tube to a water pump. Before the air entered the desiccator, carbon dioxide was removed by a series of absorbing towers containing 50% caustic soda, and finally through a tower containing 50% lactic acid. The expired carbon dioxide was collected by bubbling the expired air through a set of two towers containing 10% caustic soda. The sets of absorbing towers were changed at regular intervals. The contents of each set of towers were bulked and placed in an airtight flask and kept in cold room until analysed. The rat was kept in the metabolic chambers for about 9 hr during which time the expired carbon dioxide was collected. The faeces and urine were also

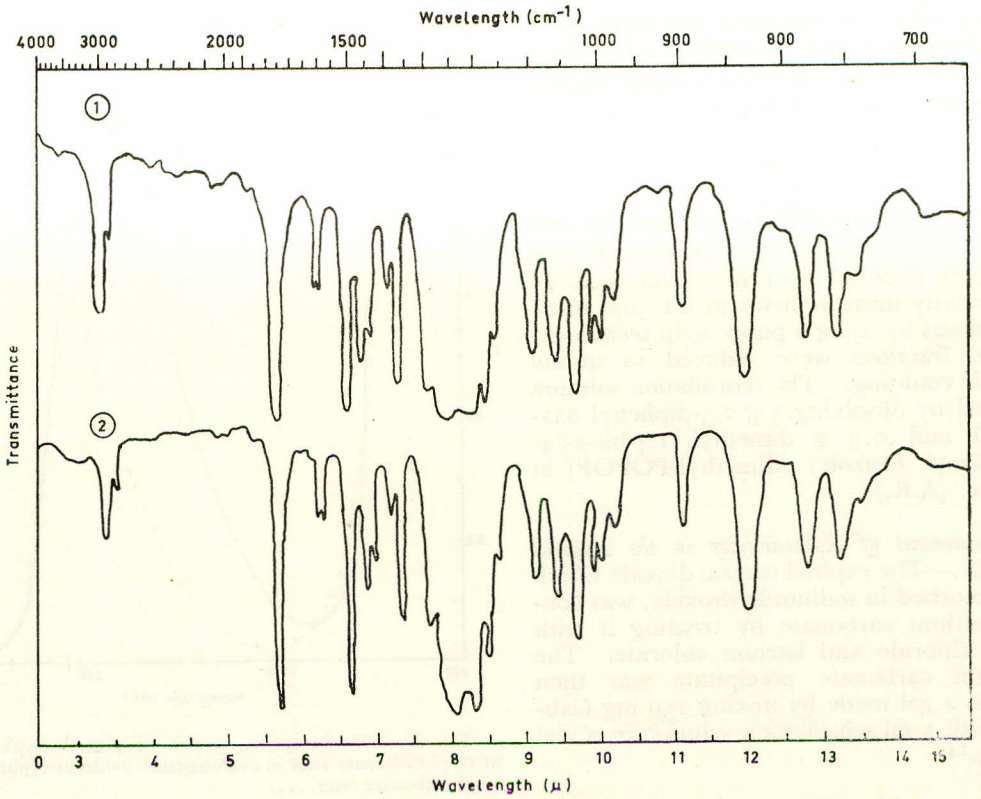

Fig. 1.—IR spectra of ethyl carbonate ester of *p*-methoxyphenol: (1) ester made from synthesized radioactive *p*-methoxyphenol; (2) ester made from authentic *p*-methoxyphenol (B.D.H).

collected on paper and analysed for radioactivity. After the experiment, the rat was chloroformed and killed. The intestine, liver, kidney and heart were removed and their lipids extracted by the procedure described below.

Extraction of Urine from Paper.—The urine was extracted thrice from the paper on which it had been absorbed using boiling aqueous ethyl alcohol (ethyl alcohol–water, 80:20) The solvent was removed under reduced pressure with a stream of nitrogen. The residue was then dissolved in a minimum volume of water.

Direct Ether Extraction of the Urine.—An aliquot from the urine extract as collected from the paper was adjusted to pH 6.5 and then extracted with ether.[5]

Hydrolysis of the Urine.—An aliquot from the urine extract was hydrolysed with 10N H_2SO_4 in the presence of a known weight of Carrier *p*-methoxyphenol and the contents then extracted with ether.[5]

Extraction of Lipids from Tissues.—The tissue was cut into a number of small pieces and ground up with a little silver sand to a homogenous mass. To this, anhydrous sodium sulphate was added gradually and ground until a dry powder was obtained. The powder was then extracted with boiling ether.

Chromatography on Alumina.—The tissue lipids were fractionated by chromatography on alumina (Grade P, O. Spence & Co., Widnes) partially deactivated with water to Brockman grade 3.

Chromatography on Silicic acid–Celite.—The crude ester of *p*-methoxy (U-^{14}C) phenol was purified by chromatography on silicic acid–Celite column. Two portions of silicic acid (100 mesh, A.R., Mallinckordt Chemical Work) and one portion of Celite (Celite 503, Johns-Manville Co. Ltd.) were used to make the column.

Thin Layer Chromatography.—A thin film of uniform thickness (200 μ) of silica gel G (E. Merck) was obtained using the Shandon apparatus. The positions of the lipid spots on the developed chromatogram were ascertained by spraying with a reagent prepared by dissolving 2 g iodine in 100 ml chloroform. Emmerie and Engel reagent[13] was used for the identification of phenols.

Spectroscopy.—UV spectra were recorded on a Unicam SP800 flat bed recording instrument. IR spectra were recorded on Perkin Elmer Model 237 Grating Spectrophotometer and the IR prism Model 37. The IR spectra were usually determined in a solvent-free film.

Assay of Radioactivity.—The radioactivity was assayed by Tricarb Scintillation Counting System (Model 314 ex Packard Instrument Co., U.S.A.) The radioactivity measurements in bile and urine were carried out by using a paper strip technique. Ubliquinone fractions were reduced to quinol prior to their counting. The scintillation solution was prepared by dissolving 5 g 2,5-diphenyl oxazole (PPO) and 0.3 g dimethyl, 1,4-bis-2-(4-methyl-5-phenyl oxazole) (dimethyl-POPOP) in 1 l. toluene (A.R.).

The Measurement of Radioactivity in the Expired Carbon Dioxide.—The expired carbon dioxide which had been absorbed in sodium hydroxide, was converted to barium carbonate by treating it with ammonium chloride and barium chloride. The dried barium carbonate precipitate was then suspended in a gel made by mixing 150 mg Cab-O-Sil M5 with 5 ml scintillation solution in a vial for counting.[14]

Esterification with Ethyl Chloroformate.—*p*-Methoxyphenol is labile so it was esterified with ethyl chloroformate to stabalise it.[15] *p*-Methoxyphenol was dissolved in 5 ml acetone in a small conical flask and treated with twice molar quantity of ethyl chloroformate. The flask was swirled round for a few seconds and then 1 ml 1N sodium hydroxide solution added. The flask was placed in an ice-cold water bath and shaken for about 5 min. It was then taken out of the cold water, about 3–4 ml acetone added and the reaction mixture was once again treated with the same quantity of ethyl chloroformate and sodium hydroxide as above to ensure the complete esterification of *p*-methoxyphenol. About 50 ml water was added to the reaction mixture and the ester extracted with light petroleum. An oily liquid substance was obtained which showed UV absorption maxima at 277.5 and 283.5 mμ in cyclohexane (Fig. 2). An ester made from authentic *p*-methoxyphenol showed $E^{1\%}_{1cm}$ 105 at 277.5 mμ.

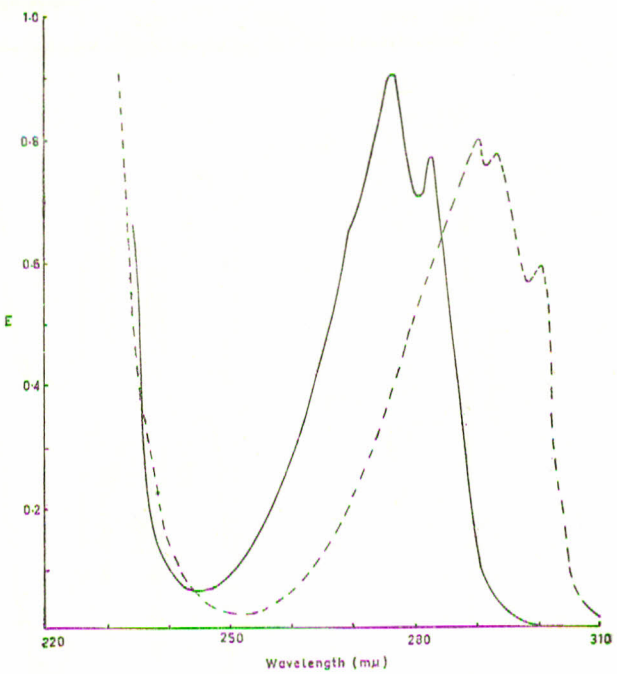

Fig. 2.—UV absorption spectra of *p*-methoxyphenol and its ethyl carbonate ester in cyclohexane: *p*-Methoxyphenol——; ethyl carbonate ester.....

Results and Discussion

Experiments 1 and 2

Examination of Bile.—In both these experiments one rat died during the process of bile collection. Hence the bile was collected from only one rat. Table 1 shows the details of experiments. It is clear from the results that part of the ester has been hydrolysed and a tiny amount of *p*-methoxy (U-^{14}C) phenol is excreted in the bile. In the second experiment as the bile was collected from one rat only, about 0.08% of administered dose was found in the bile collected for 5 hr.

Examination of Tissue Lipids.—Table 2 records the amount of radioactivity in the lipids from tissues. Out of the various tissues examined, liver and intestine contained appreciable amounts of radioactivity, while kidney and heart did not show much. The results show that *p*-methoxyphenol is either very poorly absorbed or not retained in animal tissues. The values for radioactivity in the tissues together with the previous results on bile indicate that the absorption of *p*-methoxy (U-^{14}C) phenol was appreciably slower in the second experiment. The *p*-methoxy-(U-^{14}C) phenol or its metabolites, however, were absorbed into the various tissue lipids examined. These were then resolved further by chromatography to determine the distribution of radioactivity among the components of the mixture. The results show that the absorbed *p*-methoxy (U-^{14}C) phenol must be quickly removed from these tissues. This is clear from the third experi-

ment where only a trace amount of radioactivity was found in the tissue lipids 9 hr after dosing.

Alumina Chromatography of Tissue Lipids.—In case of first experiment the fraction 10%, 15%, 20%, ether in light petroleum and 100% ether from alumina column showed UV absorption at λ_{max} 261, 271, 278 mμ in cyclohexane, characteristic of anisole. However, radioactivity measurements of these fractions showed that counts were not proportional to the quantity of the anisole present. The 10% ether in light petroleum fraction which contained the main quantity of anisole had the least counts.

In the second experiment as the more purified ester was used no such UV absorption spectrum was observed. The chromatography of the tissue

TABLE 1.—EXCRETION OF RADIOACTIVITY IN RAT BILE DOSED WITH ETHYL CARBONATE ESTER OF *p*-METHOXY-(U-^{14}C) PHENOL

Expt. No.	No. of rats	Dose given (c.p.m.)	Time of collection of bile (hr)	Bile collected (ml)	Radioactivity excreted in bile (cpm)	% Dose excreted
1	2	4.098×10^6	1*	0.40	1740	0.085
2	2	2.34×10^6	5†	2.60	909	0.079

*One rat died during the process of cannulation and no bile collected.

†One rat died just after cannulation and no bile collected.

TABLE 2.—DISTRIBUTION OF RADIOACTIVITY IN THE TISSUE LIPIDS OF GROUPS OF 2 RATS DOSED WITH ETHYL CARBONATE ESTER OF *p*-METHOXY-[U-^{14}C] PHENOL

Expt. No.	Time examined (hr)	Dose given (cpm)	Tissue	Tissue (g)	Lipids (mg)	Total activity (c.p.m.)
1	1	4.098×10^6	Liver	12.5	323.0	2529
			Intestine	8.7	695.3	12909
			Kidney	3.7	108.6	1115
			Heart	1.5	23.6	32
2	5	2.34×10^6	Liver	13.8	289.1	1099
			Intestine	7.2	560.2	1080
			Kidney	3.1	120.6	280
			Heart	1.8	20.3	20

TABLE 3.—ALUMINA CHROMATOGRAPHY OF LIVER AND KIDNEY LIPIDS (EXPERIMENT 2).

Lipids=360.0 mg. Total activity=1250 c.p.m.

Solvents	Fraction (mg)	UV spectrum (mμ)	Comments	Total radioactivity (c.p.m.)
Light petroleum	1.9	inflexion 230	Hydrocarbon	25
2% E/P*	6.1	λ_{max} 315 inflexion 297 & 327	Vit. A ester	27
4% E/P	1.9	λ_{max} 275	Ubiquinone	—
6% E/P	17.5	λ_{max} 275		20
8% E/P	1.8	λ_{max} 275		10
10% E/P	6.9	λ_{max} 325	Vit. A sterol	15
15% E/P	26.4	λ_{max} 235, 325 inflexion 280		Cartenoid 15
20% E/P	4.9	λ_{max} 235, 325		Carotenoid 39
Ether	12.0	λ_{max} 275, inflexion 235	Pigment	940
Methyl alcohol	73.0	λ_{max} 252, 262 inflexion 268		72

*E/P, solution of diethyl ether in light petroleum.

lipids from the second experiment is given in Table 3 as an example of the procedure used. The spectra of the fractions were recorded in cyclohexane. There were very few counts present in the light petroleum fraction which did not give any characteristic spectrum. The 2% ether in light petroleum fraction showed absorption at λ_{max} 315 mμ and inflexions at 297 and 327 mμ; it contained only a few counts. The 4%, 6% and 8% ether in light petroleum fractions showed spectra characteristic of ubiquinone, but did not possess many counts. The fractions 10%, 15% and 20% ether in light petroleum containing vitamin A (λ_{max} 235, 325 and inflexion 280 mμ) and some carotenoids and sterol, possessed very few counts. Nearly all of the radioactivity recovered from the column was found in 100% ether fraction, which showed absorption at λ_{max} 275 mμ and an inflexion at 235 mμ. This shows the presence of some more polar substance which was only eluted from the column by elution with more polar solvent. The washing of the column with methyl alcohol brought off most of the lipids but little or no radioactivity. The recovery of lipids from the column was poor (38%), but nearly 92% of the radioactivity put on the column was recovered.

Further Examination of Ubiquinone Fractions.—The alumina column fractions containing ubiquinone were bulked and the material was chromatographed on thin layers of silica gel using 1% ethyl alcohol in benzene as a developing solvent. Ubiquinone was removed from the plate at R_f 0.45, mixed with some carrier ubiquinone-45 and crystallized twice from hot ethyl alcohol. The crystallized ubiquinone did not contain radioactivity. Thus *p*-methoxyphenol is probably not a precursor of ubiquinone ring and is also not broken down to smaller fragments which can be utilised for the synthesis of side chain of ubiquinone.

Experiment 3

Examination of the Expired Carbon Dioxide.—The absorbed carbon dioxide from the expired air was analysed for radioactivity as given above. No radioactivity was found in any of the sample collected. Thus showing that the *p*-methoxyphenol is not broken down to an appreciable extent to small fragments at this level of dosing in the animal body.

Examination of Tissue Lipids.—Table 4 records the amount of radioactivity present in the lipids extracted from the tissues. Lipids from the heart and kidney did not contain any radioactivity. Very few counts were present in liver, intestine and faeces lipids. These results indicate that *p*-methoxyphenol is also poorly absorbed into the animal tissues. As the tissue lipids did not contain any appreciable amount of radioactivity, therefore, they were not examined further.

Examination of Urine.—The urine showed considerable radioactivity, 1.16×10^6 c.p.m. The direct ether extract was found to contain about 0.54% of radioactivity excreted in the urine (Table 5). As only a very small amount of radioactivity was extractable from the urine with ether at pH 6.5. Therefore, this step was omitted and instead an aliquot of the urine was hydrolysed with H_2SO_4 in the presence of carrier *p*-methoxyphenol. The lipid extract from the urine hydrolysate was found to contain nearly all the radioactivity excreted in the urine.

The above ether extract was esterified with ethyl chloroformate and the ester then chromatographed on a small column of silicic acid–Celite and the fractions eluted by light petroleum, 2%, 4%, 6%, 50% ether in light petroleum and 100% ether were collected in 10 ml graduated tubes. The fractions were examined in SP800 spectrophotometer and the tubes containing the ester of *p*-metoxyphenol were bulked and the ester recovered. The ester showed $E^{1\%}_{1cm}$ 103 at 277.5 mμ in cyclohexane. The ester of *p*-methoxyphenol was found to contain nearly all the radioactivity excreted in the urine (Table 5). About 90%

TABLE 4.—DISTRIBUTION OF RADIOACTIVITY IN THE TISSUE LIPIDS OF RAT AFTER THE ADMINISTRATION OF ETHYL CARBONATE ESTER OF *p*-METHOXY-(U-^{14}C) PHENOL.

Dose=2.34×10^6 c.p.m.

Tissue	Wt of tissue (g)	Wt of lipids (mg)	Total radioactivity (c p.m.)
Liver	6 8	189.1	96
Intestine	4.7	219.2	102
Kidney	1.8	33.5	—
Heart	0.8	6.6	—
Faeces	1.5	18.0	126

TABLE 5.—DISTRIBUTION OF RADIOACTIVITY IN THE RAT URINE DOSED WITH ETHYL CARBONATE ESTER OF *p*-METHOXY-(U-^{14}C) PHENOL.

Dose=2.34×10^6 c.p.m.

Sample	Total radioactivity (whole urine) c.p.m.
Direct ether extract from urine (pH 6.5)	6.24×10^6
Ether extract from urine hydrolysate	1.07×10^5
Pure ester of *p*-methoxyphenol as obtained from the column	9.60×10^3

of the radioactivity put on the column was eluted in the ester of *p*-methoxyphenol. The other fractions were however not examined.

It is clear from the above results that about 50% of the administered dose was found in the urine 9 hr after dosing. Only a negligible amount of the total radioactivity excreted in the urine at pH 6.5 was extractable with ether before the hydrolysis. However, all the radioactivity was extractable with ether when the urine was hydrolysed with acid. Thus showing that nearly all of the *p*-methoxy-(U-^{14}C) phenol excreted in the urine was in conjugated form. These results quite agree with those of previous workers,[5] who found that on feeding *p*-methoxyphenol to rabbits after 24 hr about 82% of the administered dose was excreted in the urine. Further they observed that *p*-methoxyphenol excreted in the urine was almost all conjugated with glucuronic and sulphuric acids.

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