Stanford University Medical School, personal communication.
(3) Draize, J. H., Woodard, G., Calvery, H. O., *J. Pharmacol. Exptl. Therap.* **82,** 377 (1944).
(4) Draper, H. H., Goodyear, S., Barbee, K. D., Johnson, B. C., *Proc. Soc. Exptl. Biol. Med.* **93,** 186 (1956).
(5) Litchfield, J. T., Jr., Wilcoxon, F., *J. Pharmacol. Exptl. Therap.* **96,** 99 (1949).
(6) Oser, B. L., Oser, M., J. AGR. FOOD CHEM. **4,** 796 (1956).
(7) Wilson, R. H., DeEds, F., *Arch. Ind. Hyg. Occupational Med.* **1,** 73 (1950).
(8) Wilson, R. H., DeEds, F., Cox, A. J., Jr., *Cancer Research* **1,** 595 (1941).
(9) Wilson, R. H., Thomas, J. O., Thompson, C. R., Launer, H. F., Kohler, G. O., J. AGR. FOOD CHEM. **7,** 206 (1959).

Received for review July 25, 1958. Accepted January 12, 1959.

FEEDSTUFFS ANTIOXIDANTS

Absorption, Metabolism, and Excretion of the Antioxidant, 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline

ROBERT H. WILSON, JOHN O. THOMAS, C. RAY THOMPSON, HERBERT F. LAUNER, and GEORGE O. KOHLER

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.

The metabolism and excretion of tagged ethoxydihydrotrimethylquinoline (EMQ-C^{14}, Santoquin-C^{14}) was studied in the rat and cow. EMQ is rapidly and nearly completely excreted in urine and feces. There is little breakdown to carbon dioxide, indicating stability of the ring system. Distribution in tissues suggests a modification of the molecule to make it more water-soluble. Traces of radioactivity remain in tissues for as long as 4 weeks. Continued ingestion by the rat of a diet containing 0.005% EMQ for 10 days produced tissue concentrations, as EMQ, ranging from 0.04 to 0.3 p.p.m. in muscle to 2.1 to 4.8 p.p.m. in kidney and liver. Milk from rats eating the 0.005% EMQ diet for 10 days contained 0.12 to 0.19 p.p.m. of activity as EMQ. A single dose of 155 mg. of EMQ per cow produced a maximum milk concentration of EMQ of 0.036 p.p.m. A small degree of placental transfer was found in rats.

FRESHLY DEHYDRATED ALFALFA or grass is the most economical source of carotene and many other labile nutrients available to the mixed feed industry. However, many of these components are destroyed by oxidation during storage, if consumption by farm animals is delayed. A number of antioxidants have been tried to prevent this loss. 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) has the highest activity and most desirable physical properties of any compound found to date (*1*). Before it could be considered as a feed additive its effect on animals had to be determined. Various toxicity studies on small animals were previously reported (*4*). This paper considers the metabolic fate of ingested EMQ.

Experimental Procedure

In a preliminary study, it was found that when EMQ migrated on filter paper with the lower phase of a chloroform–acetic acid–water mixture (2:1:1) it moved with the solvent front. It was found by its intense fluorescence, and when in sufficient concentration, by a color reaction with diazotized sulfanilic acid. It did not migrate in 20% potassium chloride. On the other hand, if an ether extract of a 24-hour urine sample from an animal which was given a single oral dose of EMQ was chromatographed in the same way, no fluorescent spot moved in the first direction, but several fluorescent spots with differing R_f values were obtained with the potassium chloride solution. Only after very heavy dosing was unchanged EMQ found in small amounts in the urine. This indicates rapid absorption, modification, and excretion of the EMQ. That the modification is not drastic enough to cleave the ring structure is suggested by the fact that fluorescence persists.

A more detailed study of excretion and metabolism was made possible by use of EMQ tagged as shown with carbon-14 in the heterocyclic ring (Figure 1). Chromatographic migration of this compound in the system mentioned above, followed by scanning the paper for radioactivity, was used as a test of purity. Most of the activity moved to the EMQ position. However, about 5% of the applied activity remained at the point of application and must be considered an impurity.

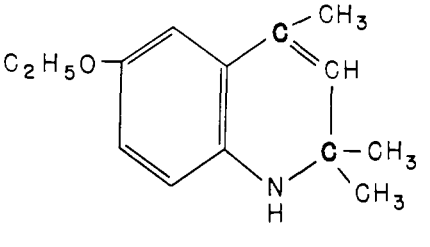

Figure 1. Positions labeled with carbon-14 are indicated by heavy type in EMQ formula

The more extensive portion of this study used albino rats as test animals. This was followed by a single administration to a lactating cow. The rat studies involved: balance studies and tissue concentrations in animals given a single oral dose of test material; tissue concentrations in animals receiving the material regularly in the ration; concentration in milk and placental transfer following continued oral administration. The cow experiment, designed primarily to indicate concentrations in milk, was used incidentally for an approximate balance study. All measurements of excretion and tissue concentration have been calculated in terms of EMQ, although probably little or no unmodified EMQ was present.

Methods. Except for tissues, all estimations of radioactivity were made on barium carbonate samples measured in a Tracerlab SC-16 windowless flow counter. Oxidation of the samples to

carbon dioxide was done by wet combustion, according to the method of Weisburger *et al.* (*3*). Under the conditions of use, and after appropriate corrections, 1 count per minute represented 1.6×10^{-6} mg. of EMQ. In the case of tissues, readings were made on dried aliquots of tissue homogenates. Here 1 count per minute represented 6.3×10^{-7} mg. of EMQ.

For the acute single-dose studies, the rats were conditioned by being placed for several weeks on a diet containing 0.005% of untagged EMQ. This is a concentration below what is proposed for alfalfa meal (0.015%), but above what would be in the total diet. After several weeks on this diet, the animals were given a single dose by stomach tube of 1.5 mg. of tagged EMQ. Collections of respired air, urine, and feces were made periodically and, at the end of varying periods, the animals were sacrificed for tissue examination. Females weighing 159 to 167 grams when dosed were sacrificed after 1, 2, and 7 days, and males weighing 182 to 238 grams when treated were sacrificed after 7, 14, and 28 days.

From the information furnished by the first three of the above animals, it was decided that, roughly speaking, 10 days was the turnover time of a single dose of EMQ. Therefore, two male rats conditioned to a diet containing 0.005% unlabeled EMQ and weighing 166 and 225 grams, respectively, were given the same diet with the EMQ enriched with radioactive material. Respiratory samples were obtained after 10 days. The animals then were killed with ether, freed of hair with clippers, and washed with ethyl alcohol to avoid contaminating the tissues with feed, and the desired tissues were obtained for analysis. Urine and feces were not collected because of the impossibility of avoiding contamination with the enriched feed.

Two pregnant rats, which had been eating a diet containing 0.005% EMQ, were changed to the labeled diet described above. Both animals delivered 9 days later. Before the appearance of milk in the stomach, one rat from each litter was taken for analysis for indication of placental transfer of EMQ or its metabolites. Next day, when stomaches were visibly filled, the remaining pups were sacrificed and stomach contents pooled for analysis of EMQ concentration of milk. Again, one rat from each litter was saved for analysis after first removing the gastrointestinal tract. The mothers were sacrificed for tissue examination.

Use of the cow was a less drastic, but more practical experiment. The animal was conditioned, as well as could be, to alfalfa meal treated with molasses and 0.015% of EMQ. This animal, and another, refused to eat this meal. On test day, the cow was force-fed a meal containing radioactive EMQ. This test material consisted of 1575 grams of a mixture containing alfalfa meal mixed with 17.5% molasses and 1% cottonseed oil to which were added 155 mg. of the EMQ-C^{14}. Urine, feces, milk, and respired air were obtained for analysis for the next $3^1/_2$ days.

Results

Excretion. EMQ labeled in the heterocyclic ring as used here yielded very little respiratory $C^{14}O_2$. Indeed, the small amount of impurity shown to be present, by paper chromatography, could account for this excretion. The rapid excretion of the $C^{14}O_2$ in three rats which were given a single oral dose is shown in Figure 2. Rats receiving a smaller amount of EMQ-C^{14} daily in the diet showed so little radioactivity in the respired air that the counts were barely above background. This also was true in the cow experiment.

Practically all of the administered radioactivity appeared within one or two days in urine and feces. Table I presents the individual protocols for the rats studied, and a summary which also includes amounts in the tissues is shown in Table II. That part of the administered EMQ not accounted for in those animals studied for several days probably was in the urine, because to avoid contamination of the urines with extraneous food, thorough washing of collecting funnels was not attempted. The cow also, although a ruminant and with a different rate of movement through the gastrointestinal tract, excreted the administered radioactivity early and practically completely in urine and feces (Figure 3). Of the 155 mg. of EMQ-C^{14} administered, 45.3 mg. (as EMQ) was found in the feces and 107.9 mg. in the urine, a total of 153 mg. This very close correlation of intake and output is undoubtedly partly chance, because it would be practically impossible to grind and mix the feces so thoroughly that an aliquot of 14 mg. taken for combustion would be truly representative. There is no question, however, that in the cow as well as the rat, there is a rapid and nearly complete excretion of EMQ.

Tissue Concentrations. To determine the tissue concentrations of EMQ, and to estimate the time required for complete elimination of this material, three rats equilibrated on a diet containing 0.005% untagged EMQ were given a single dose of EMQ-C^{14} and autopsied 1, 2, and 7 days later. The results suggested that the turnover time might be about 10 days. However, because the tissue concentrations had not reached zero, three more rats were treated in the same way and autopsied after 7, 14, and 28 days. The concentrations found in the various tissues are tabulated in Table III. Liver and kidney had the highest concentrations of radioactivity, which

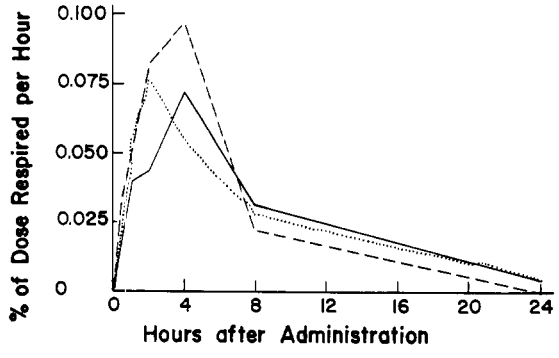

Figure 2. Respiratory excretion of $C^{14}O_2$ after a single oral dose to each of three rats

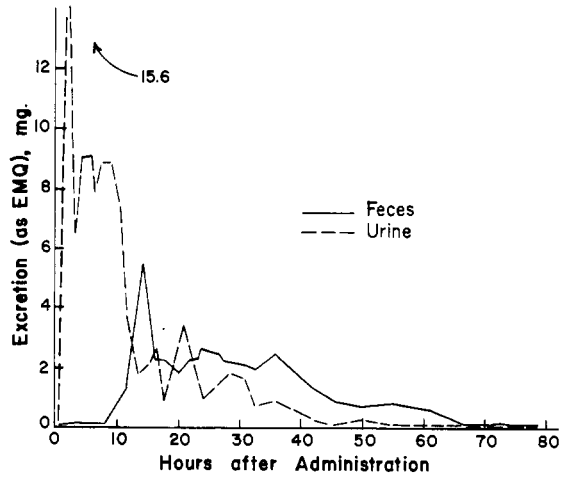

Figure 3. Urinary and fecal excretion of EMQ and metabolites after single oral dose of 155 mg. of labeled EMQ to a cow

they lost progressively with time. These tissues still contained the highest concentrations after a week (0.3 to 0.4 p.p.m.). At the end of 4 weeks, the concentrations had dropped to a thirtieth or less of the amount present after one day. Heart, skeletal muscle, and brain, starting with much lower concentrations, also lost the material rapidly and progressively. Spleen, blood, and abdominal fat had intermediate concentrations at the start, and eliminated the material at slower rates than did the other tissues.

Undoubtedly, the most important tissue, from the standpoint of food, is skeletal muscle. In an EMQ-equilibrated rat, only 0.09 p.p.m. of tagged EMQ was found in muscle one day after administration of 1.5 mg., and after 2 weeks, the concentration was practically zero. Fat retained its radioactive material more strongly. After 1 to 4 weeks, it contained 0.1 to 0.2 p.p.m. of radioactivity measured as EMQ.

In the balance studies reported, it was desirable to estimate the amount of EMQ in the total tissues (Table III). Actual weights of tissues were used where possible. It was assumed that muscle, storage fat, and blood accounted for 45, 7, and 5%, respectively, of the body weight (*2*). Because of their greater mass, muscle and fat become important along with the liver as EMQ depositories; spleen and the other organs become less important. Concentrations were shown to decrease with time; total content also decreases.

In livestock feeding, alfalfa meal treated with EMQ would not be given just once, but daily. Therefore, tissue concentrations of EMQ or its metabolites in animals receiving a known daily amount of EMQ are of greater importance. It was assumed that in the rat the turnover time of a single dose was about 10 days. While this is not strictly true, it is a fair approximation. Two male and two female rats were given diets containing 0.005% of enriched EMQ for 10 to 11 days. Their daily intakes of EMQ averaged from 2.3 to 3.7 mg. per kg. over the 10-day period. Concentrations in the tissues, calculated as EMQ, at the end of this period are presented in Table IV. Again, the liver and kidney showed the highest concentrations of EMQ (2.1 to

Table I. Excretion of EMQ-C^{14} by Rats after a Single Oral Dose

Rat	Sex	Day of Collection	Dose Excreted, %		
			Urine	Feces	Respiration
1	F	1	56.5	13.5	0.70
		2–3	1.66	21.6	0
		4	0.011	0.89	0
		5–7	0.010	0.051	0
		1–7	58.2	36.04	0.7
2	F	1	28.6	33.1	0.68
		2	1.26	1.06	0
		1–2	29.9	34.2	0.7
3	F	1	34.6	16.0	0.67
8	M	1	59.4	24.9	
		2	2.95	3.06	
		3	0.329	0.539	
		4	0.213	0.539	
		1–4	62.9	29.0	
9	M	1	43.8	30.5	
		2	2.28	2.90	
		3	0.651	1.38	
		4	0.280	0.359	
		1–4	47.1	35.1	
10	M	1	33.3	21.6	
		2	4.60	15.7	
		3	1.19	1.83	
		4	0.540	0.61	
		1–4	39.6	39.7	

Table II. Recovery of Single Oral Dose of EMQ-C^{14}

	Rat					
	1	2	3	8	9	10
	Days Observed					
	7	2	1	4	4	4
	Dose Recovered, %					
Urine	58.2	29.9	34.6	62.9	47.1	39.6
Feces	36.0	34.2	16.0	29.0	35.1	39.7
Respiration	0.70	0.68	0.67			
Tissues	0.57	1.09	1.86	0.53	0.21	0.21
Total	95.5	65.9	53.1	92.4	82.4	79.5

Table III. EMQ-C^{14} and Metabolites in Tissues after Single Oral Dose[a]

	Days after Administration									
	1	2	7	14	28	1	2	7	14	28
	Concentration EMQ, P.P.M.					Dose Found in Total Tissue, %				
Liver	2.04	1.32	0.34 0.32	0.11	0.038	0.95	0.54	0.17 0.19	0.067	0.017
Kidney	1.36	0.80	0.41 0.34	0.099	0.048	0.111	0.062	0.036 0.040	0.013	0.005
Fat	0.45	0.28	0.19 0.14	0.083	0.13	0.34	0.21	0.15 0.14	0.09	0.12
Spleen	0.30	0.31	0.32 0.062	0.045	0.063	0.0061	0.0057	0.0066 0.0029	0.0020	0.0020
Heart	0.23	0.072	0.075 0.031	0.015	0.014	0.0117	0.0033	0.0036 0.0019	0.0008	0.0006
Muscle	0.089	0.055	0.038 0.017	0	0.006	0.44	0.27	0.20 0.12	0	0.034
Brain	0.045	0.031	0.018 0.011	0	0.003	0.0021	0.0026	0.0017 0.0011	0	0.0003
Blood			0.048	0.044	0.044			0.038	0.034	0.029

[a] The dosage for individual rats ranged from 6.7 to 9.2 mg. of EMQ per kg.

Table IV. Concentrations of EMQ and Metabolites in Rat Tissues after 10 to 11 Days of Continued Feeding

(Diets contained 0.005% EMQ)

Tissue	Females		Males	
	No. 4	No. 5	No. 6	No. 7
	Parts Per Million			
Liver	2.17	2.16	2.35	4.83
Kidney	2.28	2.16	2.55	2.65
Heart	0.44	0.32	0.20	0.33
Fat	0.50	0.25	0.27	0.77
Muscle	0.18	0.04	0.28	0.14
Brain	0.16	0.14	0.03	0.04
Spleen	0.98	0.46	0.10	0.47
Blood	0.60	0.45	0.65	0.73
Milk	0.19	0.12		

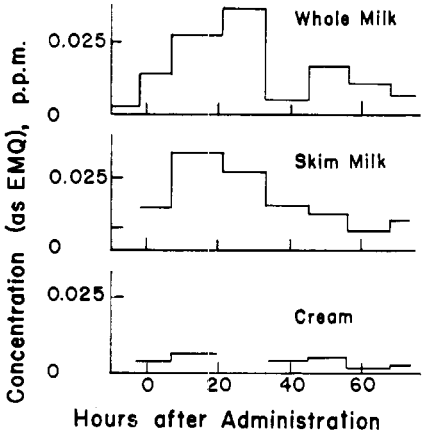

Figure 4. Concentration of EMQ and metabolites in milk after single oral dose of 155 mg. of labeled EMQ to a cow

4.8 p.p.m.). Spleen was fairly high, 1 p.p.m. in one of the four rats. The highest concentration in muscle was 0.3 p.p.m., and in fat 0.8 p.p.m. It seems likely that meat animals, ingesting a smaller amount of EMQ per kilogram, would have less accumulation in the tissues than found in these rats.

Milk. The concentration of a foreign substance in milk is of major importance. Coagulated rat milk from rats of mothers eating a diet containing 0.005% labeled EMQ for 10 days was obtained. The concentrations of EMQ or of its metabolites in the two samples of milk were 0.19 and 0.12 p.p.m., respectively.

From the cow, two daily milkings furnished a total of seven samples following the administration of a single dose of 155 mg. of EMQ-C^{14} in an animal equilibrated with unlabeled EMQ. The concentrations found in the milk are presented in Figure 4. The highest concentration was found in the third milk sample, obtained 33 hours after dosing. This sample contained 0.036 p.p.m. of EMQ. The concentration rose rapidly to this peak and then declined, as was seen even more strikingly in urine and feces. Of the material in the milk, nearly all was in the skim milk, very little in the cream.

For a better picture of distribution, whole milk sample 2 was treated with ethyl alcohol and extracted with ethyl ether for fat. The residue was adjusted to pH 4.6 and heated to precipitate protein. The remainder was called whey. These fractions were dried, subjected to combustion, and the barium carbonate from them was measured for radioactivity. The following counts per minute per milligram of carbon were obtained: fat, 0.69; protein, 1.36; whey, 1.24. This shows clearly that the small percentage in milk of the administered activity is not exclusively in the form of EMQ, because EMQ would appear in the fat fraction only.

Next, the whey sample was further fractionated, first by crystallization and then by paper chromatography, to give a chromatographically pure lactose. The carbon dioxide from combustion of the lactose was counted as a carbon-dioxide-methane mixture. The result showed 1.3 millimicrocuries per millimole of lactose. A very small part of the administered radioactivity was thus found in a normal milk constituent.

Placental Transfer. In obtaining the rat milk by the method described, the opportunity arose of determining something of the placental transfer of EMQ and its metabolites. From each of the two litters, a pup was removed and killed shortly after birth. At this time, there was no visible food in the stomach. This does not prove that the animals had had no food, but the amount, if any, must have been small. The intestinal tracts were removed, and the entire remaining animal was homogenized. Counts were made on the dried homogenates. Two more pups were treated similarly on the following day. The newly born animals from rats 4 and 5 contained 0.15 and 0.21 p.p.m. of EMQ. A day later, and after a day's feeding, the concentrations were 0.12 and 0.20 p.p.m., an insignificant drop. We now know that only a small fraction of ingested EMQ appears in the milk, and that this amount could hardly sustain body levels of measurable size. Therefore, these activities in the pups represent a transfer occurring *in utero*.

Discussion

The most outstanding finding concerning the fate of oral EMQ is its rapid absorption and the rapid, nearly complete excretion of its metabolites. In view of the ease and rapidity of absorption, it seems probable that the portion of administered material which is eliminated in the feces is modified (perhaps by digestive juices or the microflora) to some form which is not readily adsorbed. Material in urine and feces accounts for almost all of the ingested EMQ.

The material in urine is not EMQ. This was clearly demonstrated by paper chromatography after substantial doses of untagged EMQ and after small doses of EMQ-C^{14}. The material cannot have been extensively degraded, however. Because the excreted material still fluoresces strongly, it seems probable that the ring structure has remained intact. A further indication of this is seen in the respiratory study. Had the heterocyclic ring been broken, a considerably increased excretion of $C^{14}O_2$ could have been anticipated.

A very small portion of the administered EMQ has been found as respired carbon dioxide or temporarily stored in body tissues, or excreted in milk, and has been incorporated, at least in part, into normal body constituents. Solubility characteristics again indicate that the stored material is not unchanged EMQ. It is even possible that this material is not derived from EMQ. Approximately 5% of the radioactivity of the sample of tagged EMQ was an impurity. This could more than account for the respiratory excretion and the tissue deposits. This question is unresolved.

The experiment with the cow was less detailed than with the rats, but it was more practical, because of the economic importance of this animal, and because an alfalfa meal containing EMQ will not be fed until it undergoes weeks or months of storage. During this storage, EMQ disappears, as judged by extractable fluorescence, although preservation of easily oxidizable alfalfa constituents remains. In this cow experiment, the EMQ-C^{14} was added to the meal several weeks before the animal was to be fed, so as to allow this change to occur. The animal handled this modified EMQ in the same way that rats handled EMQ itself.

Acknowledgment

The authors are indebted to the Monsanto Chemical Co. for both tagged and untagged EMQ (Santoquin), to the University of California, at Davis, for the cow, to Jack Luick and Max Kleiber for the use and care of the animal, to L. M. White for the carbon dioxide oxidation; and to F. A. Loewus for the final purification of the lactose and measurement of its radioactivity.

Literature Cited

(1) Bickoff, E. M., Livingston, A. L., Guggolz, J., Thompson, C. R., J. AGR. FOOD CHEM. **2,** 1229 (1954).
(2) Caster, W. O., Poncelet, J., Simon, A. B., Armstrong, W. D., *Proc. Soc. Exptl. Biol. Med.* **91,** 122 (1956).
(3) Weisburger, J. H., Weisburger, E. K., Morris, H. P., *J. Am. Chem. Soc.* **74,** 2399 (1952).
(4) Wilson, R. H., DeEds, F., J. AGR. FOOD. CHEM. **7,** 203 (1959).

Received for review July 25, 1958. Accepted January 12, 1959.