statistically significant flavor changes in the potatoes were used on one light soil, one heavy soil, and two muck soils. However, a chi square test (*6*) showed no significant relationship when the soil types and the number of locations that produced significant mean scores were compared ($p = 0.900$).

A chi square test was also made to determine whether there was any association between the use of over 100 pounds per acre of pentachloronitrobenzene and the presence of a significant flavor change, as evidenced by a high mean flavor score. The test proved not to be significant ($p = 0.990$), and hence the use of over 100 pounds per acre of pentachloronitrobenzene is not associated with a flavor change in this study.

Again using chi square, the possibility was investigated that there might be some relationship between the methods of applying pentachloronitrobenzene and the following variables: types of soil, the treatments yielding significant flavor differences, and the amounts of pesticide used per acre. In none of these tests was $p$ found to be significant at the 5% level—i.e., there was no statistically significant relationship between methods of application of pentachloronitrobenzene (spray, band, or broadcasting) and the three other factors enumerated above.

The term flavor change rather than off-flavor has been used in this report, purposely, because it was not clear at the conclusion of the study that the change in flavor of some of the potato samples was to the detriment of the product. Some of the tasters reported that they felt the change was an improvement in the flavor—i.e., that on some days the potatoes marked with five-digit numbers tasted less watery and were of better texture than the samples marked R. This was not, however, uniformly true at all times. On some occasions, some tasters described the change in the samples as a definite off-flavor.


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**FEEDSTUFFS ANTIOXIDANTS**

# Toxicity Studies on the Antioxidant 6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline


**ROBERT H. WILSON and FLOYD DeEDS**

**Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, Calif.**



**The effects on rats of the antioxidant 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline have been investigated. The following aspects are emphasized: acute toxicity, chronic toxicity, skin toxicity, weight of vital organs, and reproductive ability.**


STABILIZATION OF CAROTENE, provitamin *A*, in dehydrated alfalfa and other forage products has been a problem for many years. Many antioxidants have been tried, but 6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline (EMQ) has proved to be the most active and usable compound found to date (*1*). When added to alfalfa meal at a level of 0.015% (150 p.p.m.) it affords considerable protection to carotene. Use in feeds should be permitted only if no chronic toxicity were apparent at the proposed levels. Acute toxicity tests and tests on skin reactions are important, because the results might be used to prevent undesirable reactions in individuals applying the materials to alfalfa, or handling the treated material as such, or incorporating it into finished feeds.

### Acute Toxicity

Acute oral toxicity was determined in rats. Twenty and 50% solutions of EMQ in cottonseed oil were given by stomach tube at dosage levels of 125 to 1000 mg. per kg. Five of seven rats receiving 1000 mg. per kg. died, as did two out of five receiving 800 mg. per kg., and one of five receiving 640 mg. per kg. Symptoms noted before death or recovery were loose stools attributable to the oil, and a depression developing on the second day and lasting 4 or 5 days.

Acute intraperitoneal and intravenous toxicity was determined on mice. The EMQ was mixed with half its volume of Tween 80 and suspended in physiological saline to produce 2 or 5% emulsions. Control animals receiving corresponding amounts of the emulsifying agent in saline showed no symptoms. The depression noted in rats given EMQ orally was much more apparent in mice receiving the material intraperitoneally or by vein. At a dosage level of 1000 mg. per kg. intraperitoneally, there was excitement and uncertain gait, followed by prostration within 5 minutes. Within a few minutes, the animals were comatose. Five of seven mice died. With a dose of 800 mg. per kg. or less, all animals survived, being free of symptoms the following day. A mild depression lasting a few hours was seen with a dose as low as 400 mg. per kg. Following intravenous injection of lethal dosages, there was immediate convulsive jumping followed within seconds by prostration and coma. Death occurred in 2 minutes to a few hours. With nonlethal injections, there was a depression lasting less than a day. The $LD_{50}$ was 178 mg. per kg. [19/20 confidence limits were 152 to 208 mg. per kg. as calculated previously (*5*)].

### Chronic Toxicity

The chronic toxicity tests were made on 270 weanling albino rats from the colony, described previously in detail (*8*), by incorporation of EMQ into the diet and feeding *ad libitum* for considerable periods. Thorough mixing was ob-

tained by dissolving the oily EMQ in two volumes of ethyl alcohol and adding this to the basal diet drop by drop while stirring in a mechanical mixer. Diets containing lower concentrations were made by dilution with basal diet. The basal diet used in this experiment was that designed by Addis, and has been described in several publications (*7*). Because of spontaneous oxidation of EMQ, the quantity of food offered to the rats was limited to a week's supply or less. The diets were prepared at frequent intervals and stored at 4° C. until used. Body weights, food intakes, and general appearance were noted weekly. Some of the animals were autopsied after 200 days, with determination of organ weights, and with gross and microscopic examination of the tissues. Certain groups were autopsied after 430 days, the remainder were continued for 715 days.

A preliminary short-term study (20 days, with a different strain of rats from that used for the main body of the work) suggested a lack of toxicity when the EMQ content of the diet was as high as 0.4% (4000 p.p.m.). This was the highest concentration used in subsequent studies and it was fed to the first of two groups autopsied after 200 days and to some of the animals autopsied after 400 days. Other concentrations ranged down to 0.0062%. The highest concentration regularly used was 0.2%, and the tables are constructed using this concentration as the high level. The animals receiving EMQ appeared healthy except for poor growth, for the first 2 to 4 weeks, of the rats eating diets containing 0.2% or more of EMQ. These animals subsequently grew nearly as rapidly as the controls (Table I).

Hemoglobin values for both sexes eating the 0.2 and 0.4% diets were normal 100 and 300 days after the start of the experiment. A modification of urinary constituents either in amount or kind was also considered, especially in view of the finding of renal lesions described. Urine was examined at various periods in rats at the 0.2% level for nearly 2 years, and in those eating the 0.4% concentration for 400 days. Neither male nor female rats on these diets excreted hemoglobin or unusual amounts of protein. Because of EMQ metabolites (*9*), experimental urines were darker than normal, blackened on standing, and fluoresced brilliantly.

Mortalities during the feeding experiment are summarized in Table II. This table was constructed with data from groups of 10 or 11 rats each. A few of the animals were sacrificed prior to the 2-year termination date for reasons other than extreme illness—e.g., for tissue examination. When this occurred, mortality calculations on the remaining rats were weighted. There is no indication that the time of death was influenced by the EMQ in the diets.

**Table I. Body Weights in Grams at Selected Times of Rats Receiving Diets Containing EMQ**

| Concn. in Diet, % | Days from Start | | | |
|---|---|---|---|---|
| | 0 | 21 | 34 | 225 |
| | *Males* | | | |
| 0 | 46.4 ± 0.96 | 117 ± 4.7 | 162 ± 6.5 | 382 ± 16 |
| 0.1 | 46.2 ± 0.56 | 117 ± 2.4 | 165 ± 3.1 | 352 ± 11 |
| 0.2 | 46.5 ± 0.60 | 108 ± 4.4 | 149 ± 5.7 | 325 ± 11[a] |
| | *Females* | | | |
| 0 | 45.3 ± 1.09 | 110 ± 2.7 | 140 ± 3.7 | 260 ± 6.0 |
| 0.1 | 45.3 ± 0.79 | 103 ± 2.7 | 130 ± 3.6 | 266 ± 4.8 |
| 0.2 | 44.2 ± 0.95 | 94 ± 1.8[b] | 123 ± 2.0[b] | 232 ± 6.7[a] |

[a] Statistically different from controls. $P = <0.05$. [b] Statistically different from controls. $P = <0.01$.

At autopsy, close inspection revealed shallow pitting of the renal cortex of male animals receiving the higher dosage levels for 200 or more days. How early the lesions developed was not determined. Males receiving the 0.1% diet had a minimal amount of grossly observable kidney damage, so slight that the lesions probably would be missed in a careful routine autopsy. The lesions in males on the 0.2 and 0.4% diets were somewhat more apparent. Two male rats on the 0.4% diet had stones in the renal pelvis. All other organs in the males, and all tissues in the females, appeared normal.

Organ weights were determined at the 200-day autopsies. In the female, livers of animals receiving the 0.2% diet were normal in weight on one occasion, while there was a statistically significant increase in liver weight in a repeat experiment (Table III). The kidneys of the females were significantly heavier at the 0.2 and 0.4% levels. In the males, significantly heavy livers were found in animals on the 0.1% and higher levels, and heavier kidneys were noted on levels from 0.025% up. In the males eating the 0.2% diet, liver and kidney weights were 25 to 30% greater than tissues in control animals of equal body weight.

Microscopical examination, by Cox (*2*), of tissues from the 400-day animals (0.2 and 0.4% EMQ) disclosed no distinct lesions in the females, but clear lesions in the kidneys, livers, and thyroid glands were present in many male rats.

The most striking changes were in the kidneys from males which had received EMQ. All of these showed irregular zones of fibrosis, tubular atrophy, focal tubular dilatation, and lymphocytic infiltration characteristic of chronic pyelonephritis. The degree of change was related to the amount of administered EMQ. Two kidneys presented deposits of calcific material in the medulla and in one of these there was a recognizable zone of necrosis of renal tissue in the same region.

Groups of hepatic cells from most of the treated male rats contained rounded eosinophilic hyaline inclusions of different sizes up to the diameter of the cell nuclei. The nature of these bodies has not been determined further. Their frequency was related to the dose of EMQ. Other alterations in the involved cells were not apparent, so the observed changes may represent stored foreign material rather than significant hepatic cell injury.

In the thyroid glands of the male rats on the larger dose of EMQ there was decrease in stored colloid, with diffuse increase in height of the follicular epithelium, indicating mild hyperplasia. One gland presented a well-defined adenoma. The glands from the animals on the 0.2% EMQ level were not distinctly different from controls.

In male animals fed 0.2% EMQ for longer periods, up to 717 days, lesions were like those in comparable animals of the 400-day group, and in female rats there were patchy changes in the kidneys similar to, but less marked than, those from the male animals.

With smaller doses of EMQ there were milder changes in the male group, but none in the females. No well-defined results of EMQ administration could be detected after feeding of 0.0062%.

Similar, though less marked, changes were found in the animals exposed to EMQ for 200 days. Minute lesions were present in the kidneys of two of five males receiving the 0.05% diet. Because of their more advanced age, changes associated with senility increased the number of pathological lesions in the 700-day animals. The lesions associated with the treatment again were limited to kidney, liver, and thyroid. The changes in the latter two were less marked than had been seen in the 400-day rats. The kidney changes were no worse than those in the younger animals, except that some lesions now appeared in the kidneys of females receiving the 0.2% diet.

Occasional tumors were present in the 700-day animals. These included six hypophyseal adenomas, six adrenal adenomas, two adrenal carcinomas, three mammary tumors of different types, three adenomatous uterine tumors, and

**Table II. Mortality of Rats Eating EMQ Diets**

| EMQ in Diet, % | Days on Diet | | | |
|---|---|---|---|---|
| | 200 | 400 | 600 | 715 |
| | Males, % | | | |
| 0 | 10 | 28 | 64 | 82 |
| 0.0062 | 0 | 20 | 60 | 60 |
| 0.0125 | 10 | 10 | 30 | 66 |
| 0.025 | 0 | 0 | 0 | 20 |
| 0.05 | 0 | 0 | 40 | 80 |
| 0.1 | 0 | 20 | 20 | 60 |
| 0.2 | 10 | 10 | 46 | 82 |
| | Females, % | | | |
| 0 | 0 | 0 | 40 | 80 |
| 0.0125 | 0 | 0 | 0 | 25 |
| 0.025 | 0 | 0 | 40 | 40 |
| 0.05 | 0 | 0 | 20 | 90 |
| 0.1 | 0 | 17 | 33 | 83 |
| 0.2 | 0 | 20 | 20 | 20 |

one thyoma. Two of the tumors appeared in control animals and the remainder were distributed without relation to the dose of EMQ.

### Skin Toxicity

Rabbits and guinea pigs were used for the study of skin irritation. A single drop of the oily undiluted EMQ was gently rubbed over an area of skin 2 cm. in diameter, daily, except for the weekend, for 2 weeks. Repeated applications produced a slight erythema, followed by a papular eruption and in some instances scab formation. When treatment was stopped, the lesions gradually disappeared, leaving a normal appearing skin after a few weeks.

Skin sensitization was studied by the method of Draize, Woodard, and Calvery (*3*). The EMQ was mixed with an equal amount of Tween 80 and suspended in saline for the injections. No sensitization developed.

### Reproduction

The recent discovery (*4*, *6*) that diphenyl-*p*-phenylenediamine (DPPD) led to a major reproductive disturbance in rats, although a battery of tests had shown it to be innocuous in other ways, reemphasizes the importance of reproductive studies as part of chronic toxicity testing. The following experiment was done with rats eating EMQ diets.

For greatest sensitivity, it seems desirable to have a stock diet which is marginal in its ability to support reproduction. If a highly fortified diet is used, a slight detrimental effect of the substance under study might be obscured. If a protective action is anticipated, a diet slightly lacking in reproductive efficiency is desirable. The diet used in this experiment was the same as that used in the continued feeding studies. It had been found to be marginal in tocopherol.

The rats used, for the most part, were of the Sprague-Dawley strain, obtained from a commercial animal supply house which claims good reproductive history. Seven rats of another strain, which had been fed the above diet, were included only for the first breeding at the 0.05% EMQ level. EMQ was added to the diet to give concentrations of 0, 0.025, 0.05, and 0.1%. The highest concentration has been shown to damage the kidney of male rats, and all concentrations are many times greater than that proposed for feeds containing alfalfa meal treated as described earlier.

The animals were placed on their respective diets when 60 days old, and received them continuously thereafter. The rats were mated when 100 days old, with four females and one male to a cage and rotation of the males once a week. After this mating, the rats receiving 0.1% EMQ were discarded, because this concentration seemed unnecessarily high. After all litters were weaned, the rats were remated, and following weaning of these litters, a third mating was initiated. In addition to the above, offspring of animals receiving the 0, 0.025, and 0.05% EMQ diets were saved from the first mating, continued on their respective diets, and mated when 100 days old for a second generation study (Table IV).

The animals receiving the experimental diets produced young and raised them more successfully than the controls. The stress of successive matings was apparent with each remating of the control animals. Control young from the first mating were particularly unproductive. Unlike the original breeders, which had come from a colony with adequate vitamin E intake, these offspring presumably started life with low vitamin E resources. They grew well, but were unable to stand the stresses of gestation and lactation. On the other hand, both the production and the successful rearing of young were possible in those rats receiving EMQ in their diets. The more concentrated diet was more effective than the lower. It would appear that the antioxidant, EMQ, protected the marginal amount of tocopherol enough to cause the observed differences.

**Table III. Organ Weight Differences of Rats Receiving Diets Containing EMQ for 200 Days**

(Differences are presented as % of values expected in control rats of equal body weight)

| Concentration of EMQ, % | Difference from Theoretical Weight, % | | | |
|---|---|---|---|---|
| | Males | | Females | |
| | Liver | Kidney | Liver | Kidney |
| 0.025 | 9.8 ± 6.0[a] | 12.5 ± 3.8[a,c] | | |
| 0.05 | 12.4 ± 6.7[a] | 13.5 ± 3.1[a,b] | | |
| 0.1 | 18.0 ± 5.9[a,c] | 16.3 ± 4.0[a,b] | 3.6 ± 6.4[a] | 4.0 ± 4.2[a] |
| 0.2 | 28.8 ± 6.0[a,b,c] | 29.3 ± 6.3[a,b] | 26.6 ± 5.7[a,b] | 18.4 ± 4.4[a,b] |

[a] Experimental values above control values. [b] Statistically different from controls. $P = <0.01$ [c] Statistically different from controls. $P = 0.02$

**Table IV. Reproduction of Rats Fed EMQ Diets**

| EMQ Concn., % | Females Mated, No. | Live Litters: Born, No. litters | Live Litters: Born, Av. No. in litter | Live Litters: Weaned, No. litters | Live Litters: Weaned, Av. No. in litter |
|---|---|---|---|---|---|
| | First Mating | | | | |
| 0 | 12 | 9[e] | 6.8 | 8 | 7.0 |
| 0.025 | 12 | 12 | 6.7 | 10 | 6.9 |
| 0.05 | 19[a] | 15 | 6.8 | 15 | 6.2 |
| 0.1 | 12 | 12 | 7.5 | 11 | 6.5 |
| | Second Mating | | | | |
| 0 | 11[b] | 7 | 9.1 | 5 | 6.8 |
| 0.025 | 12 | 11 | 8.1 | 8 | 7.5 |
| 0.05 | 12 | 12 | 7.8 | 9 | 7.0 |
| | Third Mating | | | | |
| 0 | 8[c] | 6 | 7.7 | 5 | 5.8 |
| 0.025 | 11 | 8 | 7.5 | 6 | 7.5 |
| 0.05 | 12[a,c] | 10 | 7.7 | 8 | 4.4 |
| | Second Generation | | | | |
| 0 | 12 | 2 | 8.0 | 2 | 2.5 |
| 0.025 | 12[d] | 6 | 7.7 | 4[f] | 5.0 |
| 0.05 | 12[b] | 10 | 6.6 | 8 | 5.3 |

[a] One litter stillborn. [b] Two females died during delivery. [c] One female died with full-term fetuses. [d] Two females died with full-term fetuses. [e] One mother died 3 days after delivery. [f] A fifth litter lost through accident.


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**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.

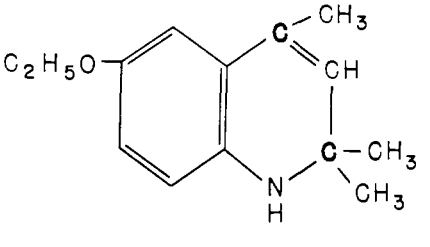


Figure 1. Positions labeled with carbon-14 are indicated by heavy type in EMQ formula

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