Mean activity for red cell glutathione reductase (moles of NADPH oxidized per milliliter per minute) of 11 individuals homozygous for the variant is 9.50×10^{-8} with S.D. 1.71×10^{-8}; for 17 homozygous for the usual form, mean activity is 7.44×10^{-8} with S.D. 1.31×10^{-8}, referred to a hemoglobin concentration of 1 g per 100 ml of hemolyzate. Enzyme assays were performed as described (*5*) except that final hemoglobin concentration of reaction mixtures was 0.048 instead of 0.105 g per 100 ml of hemolyzate. The increased mean activity of the enzyme variant compared to the activity of enzyme from normal individuals is statistically significant by analysis of variance; F is 11.3793, and P is smaller than .01 (one degree of freedom).

Because of our previous finding of elevated activity of red cell glutathione reductase in a group of Caucasians with untreated primary gout (*6*) and because a majority of the 28 Negro males with primary gout in the G-6-PD study (*1*) appeared to have the glutathione reductase variant on initial electrophoresis, electrophoresis of the stored samples from the gout patients was repeated for a 14-hour period instead of the former 12-hour period. After assay, the samples were adjusted to the same enzyme activity for application to gels, where they were alternated with samples from homozygotes with the usual form of the enzyme. All samples had been stored in the cold room with acid-citrate-dextrose solution for a similar period of time. Each sample was run in duplicate on separate gels. There was a strong association between the glutathione reductase variant and primary gout (Table 1). Chi-square with Yates' correction was 42.6126, and P was smaller than .001 (two degrees of freedom).

Potentially, increased glutathione reductase activity, which appears to be characteristic of the variant enzyme, could result in greater availability of NADP, stimulation of glucose metabolism over the NADP-dependent pentose phosphate pathway, increased ribose production via that pathway, and eventually increased uric acid production from ribose. Excess ribose production via this pathway has been postulated as a possible mechanism for increased uric acid production in persons with gout (*7*).

In the United States, gout in Negroes and in Caucasians appears to occur with similar frequency (*9*). Differences of method between this study, where primary gout was found in 1.9 percent of 1473 Negro male general medical and medical specialty clinic outpatients, and other surveys of gout among Negroes (*9*) make it impossible to determine whether the frequency of gout in this outpatient group is similar to that found by others in Negro populations.

Table 1. Frequency of glutathione reductase (GSSG-R) phenotypes among gout patients and general medical patients. Chi-square with Yates' correction is 42.6126; P is less than .001 (two degrees of freedom).

GSSG-R phenotype*	Patients	
	Gout (No.)	General medical (No.)
S	5	150
FS	15	40
F	8	6

*S indicates the usual electrophoretically slower type; F indicates the electrophoretically fast variant.

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Metabolism of Rotenone in vitro by Tissue Homogenates from Mammals and Insects

Abstract. *Hydroxylation of rotenone in vitro in the enzyme system composed of microsomes and reduced nicotinamide-adenine dinucleotide phosphate, and in living mice and houseflies, yields products tentatively identified as rotenolone I; rotenolone II; 8′-hydroxyrotenone; 6′,7′-dihydro-6′,7′-dihydroxyrotenone; two rotenolones of each of the last-mentioned two compounds; and uncharacterized polar materials. The toxicity of certain of these rotenoids to mice is of the same order as that of rotenone.*

Rotenone, in the form of ground derris roots, is used as an insect poison for the control of pests on plants and animals; as a fish poison to manage fish populations in reservoirs, lakes, and streams; and to eliminate undesirable species. As an insecticide chemical, rotenone has a short residual action and is exempt from the requirement of a residue tolerance when applied to growing crops in accordance with good agricultural practice. The fact that the metabolic fate of rotenone in insects and mammals is incompletely understood, although it has been rated as moderately to highly toxic to mammals, caused us to undertake the work reported here.

The enzyme system involved in the coupled oxidation of reduced nicotinamide-adenine dinucleotide ($NADH_2$) and reduction of cytochrome *b* is inhibited by very low concentrations of rotenone, whether or not this enzyme system is derived from species that are highly susceptible or resistant to rotenone poisoning (*1–3*). Thus, selective toxicity probably results from species

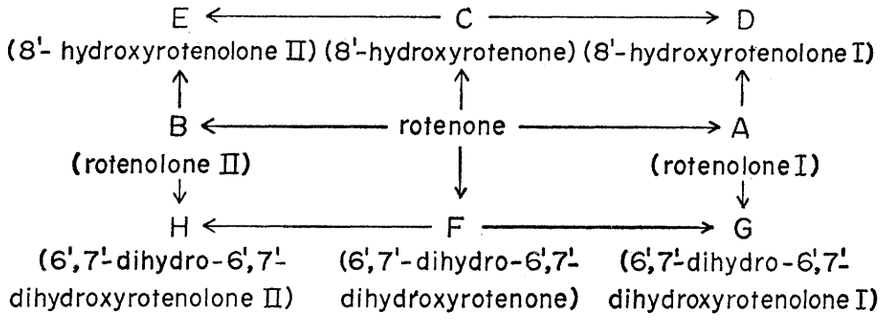

Fig. 1. Rotenone metabolites.

differences in localization or ease of degradation of rotenone, and not from differences in the $NADH_2$ oxidation systems in susceptible and resistant species (*2*). Slight modifications in the rotenone structure reduce or destroy both the insecticidal activity and potency for inhibition in vitro of enzymes involved in $NADH_2$ oxidation (*3*). On this basis, metabolism of rotenone is expected to yield products of reduced biological activity. Definitive data are lacking on this subject because available analytical methods have not been appropriate. This limitation was remedied by the recent preparation of rotenone-6a-C^{14} (*4*). We now report the initial reactions involved in metabolism of rotenone-6a-C^{14} as applied to the enzyme system consisting of the microsome fraction and reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$), and to living mice and houseflies (*Musca domestica* L.).

The sources of enzyme were homogenates of housefly abdomens, mouse livers, or rat livers. Preparation of homogenates, centrifugal separation into microsome and soluble fractions, and incubation with substrate have been described (*5*). Rotenone-6a-C^{14} (97 percent radiochemical purity) was added to each incubation mixture, in ethanol solution, to yield final concentrations of 3 μg of rotenone per milliliter and 1.5 percent ethanol. After incubation for 2 hours at 37°C, each reaction mixture was extracted with ether, and the metabolites recovered in the ether were resolved by thin-layer chromatography (TLC). The aqueous phase was acidified with hydrochloric acid and reextracted with ether, and then extracted with *n*-butanol. The sequence of metabolic reactions was examined by repeating the experiment with 6a,12a-dehydrorotenone-6a-C^{14} (*4*) or one of several rotenone-6a-C^{14} metabolites (recovered in radiochemically pure form from the chromatoplates) as substrates in the place of rotenone-6a-C^{14}. In these sequence studies, the incubation mixture contained $NADPH_2$ and the microsome fraction or the combined microsome and soluble fractions from rat liver. Metabolites formed from each product were compared by TLC to ascertain whether there was a common intermediate in the metabolism.

In the studies in vivo, male white mice were treated by stomach tube with 12 μg of rotenone-6a-C^{14} dissolved in 50 μl of dimethyl sulfoxide. After 48 hours the expired air, urine, feces, and tissues were examined for radioactivity. Urine was extracted and analyzed by the procedure used in the enzyme-incubation experiments. In certain cases, extracts of the liver and small intestine, taken 4 hours after the treatment, were analyzed for metabolites by TLC. Each of a group of houseflies was injected with 0.1 μg of rotenone-6a-C^{14} in 1.0 μl of a mixture of acetone and water (40 : 60); after 4 hours, the flies were extracted with acetone, and the recovered labeled compounds were analyzed by TLC. The interference of certain substances in extracts of flies, liver, and intestine was minimized by developing the chromatoplates, in the first direction, with benzene to move the interfering materials (but not the metabolites) from the origin and, in the second direction, with a mixture of benzene and methanol (90 : 10) to resolve the metabolites.

Metabolites of rotenone-6a-C^{14} and their degradation products were resolved by TLC and compared with 24 different unlabeled rotenone derivatives (*6*). The chromatoplates, coated with 0.25 mm of silica gel G, were developed with benzene-methanol (90 : 10) for compounds related to rotenone, and with a mixture of ethyl acetate (which had been saturated with 30 percent aqueous ammonium hydroxide) and methanol (5 : 1) for tubaic acid and related carboxylic compounds. The chromatographic position of labeled metabolites was determined by radioautography, and each unlabeled metabolite or known rotenone derivative was developed as a colored spot with either phosphomolybdic acid—sprayed on as a solution (20 g per 100 ml of ethanol) and fixed by heat (5 minutes at 110°C) —or 2,6-dichloroquinonechlorimide reagent, or iodine vapor (*7*).

Rotenone metabolism occurs more rapidly with the microsome–$NADPH_2$ enzyme system than with other combinations of cofactors and individual fractions from homogenates of rat livers. About 70 percent of the rotenone-6a-C^{14} is metabolized by this system; 60 percent of the metabolite mixture is recovered by ether extraction, and 10 percent is obtained as more polar metabolites. Addition of the soluble fraction from liver to this microsome–$NADPH_2$ system enhances metabolism so that 97 percent of the rotenone-6a-C^{14} is metabolized to yield 22 percent as ether-extractable products and 75 percent as more polar products. The five major metabolites (designated as metabolites A, C, D, F, and G) are always the same and they occur in the ether-extractable fraction from the microsome and $NADPH_2$ system and the microsome, soluble fraction, and $NADPH_2$ system. The corresponding system from housefly abdomens is comparable in activity to that of the liver microsomes, and yields the same five major metabolites. Both systems yield three additional, minor products (metabolites B, E, and H) which apparently occur in larger amounts in the fly system than in the liver system. The R_f values for rotenone and its metabolites, in the benzene-methanol system, are as follows: rotenone, 0.85; A, 0.73; B, 0.70; C, 0.48; D, 0.34; E, 0.31; F, 0.24; G, 0.15; and H, 0.11. The more polar metabolites, not recovered by ether extraction, are present in larger amounts in the mixture from the liver microsome, the soluble fraction, and $NADPH_2$ than in that from the fly abdomen–$NADPH_2$ enzyme system; these products appear largely in the fraction extractable by *n*-butanol, and their chemical nature remains undetermined.

Sequence studies with the microsome fraction and $NADPH_2$ from rat liver homogenates or the microsome and the soluble fraction plus $NADPH_2$ demonstrate that metabolites A, B, C, and F arise from rotenone but not from any other intermediate; metabolite A

yields D and G; metabolite C yields D and E; metabolite F yields G and H. In combination with other results, which are discussed later, this indicates a sequence for the metabolite formation as shown in Fig. 1.

Cochromatography studies with many rotenone derivatives are the basis of tentative characterization of several metabolites. Alkaline oxygenation (*8*) of rotenone results in introduction of a hydroxyl group at the 12a position of the B-C ring juncture, a mixture of rotenolone I and rotenolone II being formed; this characteristic reaction also occurs with related compounds (metabolites C and F), and is useful for considering the structures of the rotenone metabolites (*8*, *9*). The experimental data show that 6a,12a-dehydrorotenone is not a metabolite and that it does not give metabolites having R_F values equal to any of the rotenone metabolites. Rotenolone I cochromatographs with metabolite A, rotenolone II with metabolite B, and 8′-hydroxyrotenone (*10*) with metabolite C. Alkaline oxygenation of radioactive metabolite C yields products with R_F values of 0.34 and 0.31. The products of alkaline oxygenation of 8′-hydroxyrotenone cochromatograph with metabolites D and E (R_F values of 0.34 and 0.31, respectively), indicating that metabolite D is 8′-hydroxyrotenolone I and metabolite E is 8′-hydroxyrotenolone II. The nature of these chemical modifications is shown in Fig. 2.

Metabolite F, the major metabolite in the experiments in vitro, was purified and subjected to spectral examination and degradation studies, and attempts were made to synthesize it. In order to obtain a larger amount of metabolite F, the liver microsome-plus-soluble–$NADPH_2$ enzyme system was used with the addition of ethylenediaminetetraacetic acid (EDTA) at 0.01*M*, and the rotenone-6-a-C^{14} substrate level was increased to 1 mg in each of several flasks. The incubation mixture was extracted with ether, the ether was removed by evaporation, and the resultant product was washed with hexane to remove interfering lipids. The product was then purified by TLC, elution of metabolites from the gel with methanol, two additional series of TLC purifications and elutions, evaporation of the methanol, addition of water and ether, and, finally, ether extraction. The ether was removed by evaporation, and the resultant oil was washed with carbon tetrachloride, yielding the insoluble residue containing metabolite F as the major constituent.

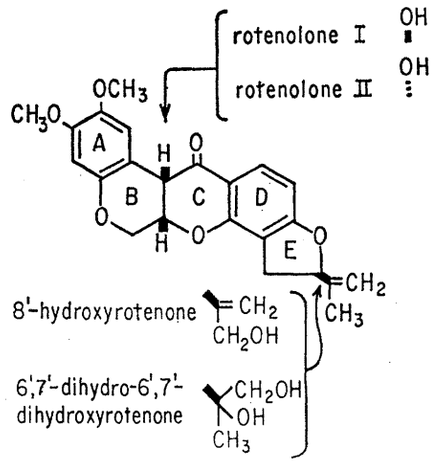

Fig. 2. Structures of rotenone and four compounds tentatively identified as its initially formed metabolites: rotenolone I, rotenolone II, 8′-hydroxyrotenone, and 6′,7′-dihydro-6′,7′-dihydroxyrotenone.

The infrared and ultraviolet absorption spectra for metabolite F are similar to those of rotenone, except for infrared bands associated with the hydroxyl group. The metabolite reacts with methyl isocyanate but not with diazomethane. It is stable in aqueous acid but decomposes on alkaline oxygenation to give products of R_F 0.15 and 0.11. Under degradation conditions suitable for converting rotenone to tubaic acid (*11*), metabolite F gives a phenolic product which is more polar than tubaic acid. A mass spectrogram of metabolite F indicates a molecular weight of 428, a value equal to that produced by the introduction of two oxygen and two hydrogen atoms into the rotenone molecule. The mass spectrographic fragmentation pattern shows that the moiety made up of the A, B, and C rings is the same for the metabolite as for rotenone (*12*). The structure most consistent with these observations is 6′,7′-dihydro-6′,7′-dihydroxyrotenone. The major product from reaction of rotenone with osmium tetroxide is 6′,7′-dihydro-6′,7′-dihydroxyrotenone, m.p. 202°C. Identification of this rotenone derivative is based on elemental analyses, infrared and ultraviolet spectra, nuclear magnetic resonance peaks, mass spectroscopy (*13*), and on the fact that it cochromatographs with metabolite F.

Another major metabolite, G, and the minor metabolite, H, are formed by enzymatic attack on metabolite F, but, under the conditions used, metabolites G and H do not undergo further degradation. Alkaline oxygenation of metabolite F gives products which have R_F values corresponding to metabolites G and H. A mass spectrogram of metabolite G, examined as a mixture with metabolite F, indicates a molecular weight of 444, a value equal to the introduction of three oxygen and two hydrogen atoms into the rotenone molecule. The most conspicuous mass-spectrogram fragmentation peaks, derived from the A-B-C ring moiety of metabolite G, are the same as those derived from rotenolone I, indicating that formation of metabolite G from metabolite F involves introduction of one oxygen at the B-C ring juncture. Therefore, metabolite G probably is 6′,7′-dihydro-6′,7′-dihydroxyrotenolone I, and metabolite H seems to be 6′,7′-dihydro-6′,7′dihydroxyrotenolone II.

Rotenone metabolites produced in living mice and flies are, at least in part, the same as those formed by the enzymatic systems in vitro. Forty-eight hours after treating mice with rotenone-6a-C^{14}, 20 percent of the C^{14} was in the urine, 0.3 percent was expired, 5 percent remained in the body, and the rest was in the feces. Certain of the metabolites in urine are the same as those resulting from the microsome-plus-soluble–$NADPH_2$ enzyme system; in both cases, metabolites F and G predominate in the initial ether extract, and the radioactivity which remains in the aqueous phase, after extraction with ether, is subsequently extracted by *n*-butanol. Before the urine is acidified, 18 percent of the radiocarbon is recoverable by ether extraction; after acidification an additional 15 percent is recoverable. The major ether-extractable metabolites in the liver and small intestine, 4 hours after the animals had been treated, are similar in chromatographic characteristics to those formed by the microsome–$NADPH_2$ enzyme system. With living houseflies, the products formed include metabolites A, C, and F, as judged by cochromatography, and included are products that chromatograph in the regions of metabolites D, E, G, and H.

When assayed for inhibition of the L-glutamic dehydrogenase system (*2*, *4*), the mixture of rotenone metabolites, produced by incubation with the rat liver microsome-plus-soluble–$NADPH_2$ enzyme system, is less potent than rotenone. On intraperitoneal administration of the pure, authentic rotenoids as solutions in dimethyl sulfoxide, the

LD_{50} (lethal dose for 50 percent) values found with male mice are as follows (mg/kg): rotenone, 2.8; 8′-hydroxyrotenone, 2.6; 6′,7′-dihydro-6′,7′-dihydroxyrotenone, 10; rotenolone I, 4.1; rotenolone II, > 25.

The biological instability of rotenone in insects and mammals is attributable to its susceptibility to attack, by the microsome–$NADPH_2$ enzyme system, at the positions indicated in Fig. 1. In addition to the eight metabolites discussed here, at least two additional minor ether-extractable metabolites exist, but their structure is not yet known. These metabolites represent only the initial steps in rotenone metabolism because more polar products are subsequently formed. There is reason to believe that the more polar products comprise, in part, conjugates of the hydroxylated rotenone derivatives. It is interesting that 8′-hydroxyrotenone is a natural constituent in some plants in the form of a glycoside (amorphin) (*10*) and 11-hydroxyrotenone (sumatrol) also occurs in certain plants; however sumatrol is not one of the rotenone metabolites found in animals.

Our results suggest that variation in the ease of biodegradation of rotenone in different species may be one factor in its selective toxicity. Also, they suggest that certain of the metabolites are toxic and, therefore, need to be considered in further studies on the toxicology of rotenone and in biochemical studies where rotenone is used as a specific inhibitor of the electron-transport system.

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Conditioning with Delayed Vitamin Injections

Abstract. *Rats deficient in thiamine were allowed to drink saccharin-flavored water. They were then given an injection of thiamine which caused their intake of the nonnutritive fluid to increase. Delay of the intramuscular injection up to 30 minutes had no effect upon the acquisition of this conditioning. Presumably, this delay reflects specialization in the central integrative mechanisms which serve these afferent modalities.*

Lorenz has proposed that an animal samples small amounts of novel foods and then forms an engram of its reaction to each. The fact that omnivorous animals eat only small amounts of novel foods supports this hypothesis (*1*). Animals tend to select beneficial diets. However, feeding behavior is a complex sequence in which many events (licking, chewing, and stomach distention) intervene between the discrimination of food by taste and smell and the correction of the homeostatic imbalance. Such intervening events can serve to mediate learning as they demonstrably reduce hunger (*2*).

Factors such as novelty and palatability of the food and dietary deficiencies also have a regulatory effect on food preference and intake; thus, the precise role of learning and memory is difficult to assess. For example, Rodgers and Rozin have demonstrated that rats deficient in thiamine will invariably select novel diets, and they will develop a stable preference for this novel diet if it contains the needed thiamine. Exclusive ingestion of the novel food for several days provides the opportunity for the development of a stable preference for the vitamin-rich diet, presumably by means of causal sequence of mediating events (*3*).

One way to circumvent some of these complexities which obscure the role of memory is to follow the ingestion of a discriminable nonnutritive substance with an intramuscular injection that introduces the dietary requirement directly into the blood stream. Patients with a thiamine deficiency often report that injections of thiamine make them "feel better" soon afterward. Therefore, we tested the effect of conditional associations of ingestion of a mixture of saccharin and water and a subsequent injection of thiamine in rats deficient in thiamine.

Forty-day-old male rats were placed on an unrestricted thiamine-deficient diet (Nutritional Biochemicals, Cleveland). They were given access to water for a 10-minute period at the same time each day. After 18 days their water intake declined to less than 50 percent of initial measures; this decline indicated the symptomatic loss of appetite. Food consumption and body weight declined similarly. Fifty percent intake was thus designated as the criterion of deficiency, and treatment was begun.

Two studies were conducted. In experiment A, the experimental group ($N = 19$) was given water flavored with saccharin (0.5 g/liter) for 10 minutes at the usual drinking time, then given an intramuscular injection of 200 mg of thiamine hydrochloride per kilogram of body weight. The injection was followed by the characteristic increase in appetite, which in turn was reflected in increased water consumption on the next day. Subsequently, water intake steadily declined so that in