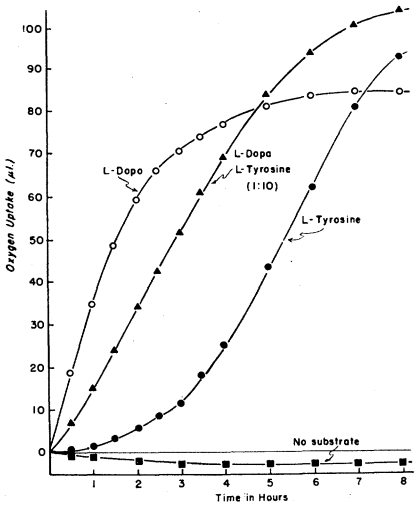

Fig. 1. Enzymic oxidation of L-tyrosine and L-3, 4, dihydroxyphenylalanine (L-DOPA) by homogenates of the retinal pigment epithelium of Rhode Island Red chick embryos. Total amount of substrate, 1.77 µmole.

to form melanin in plants, insects, and chordates (*3*).

For our studies, we used retinal pigment epithelium dissected from eyes of the Rhode Island Red and Black Australorp chick embryos. Homogenates of pigment epithelium from approximately 20 eyes of 9- to 12-day embryos were used in each Warburg flask. In the presence of the homogenates from both species, L-3,4-dihydroxyphenylalanine (L-DOPA) was immediately oxidized without any detectable induction period (Fig. 1. The homogenates catalyzed the oxidation of L-tyrosine only after an induction period which was shortened by the addition of a small amount of L-DOPA (Fig. 1). Four atoms of oxygen and 5 atoms of oxygen were consumed per molecule of L-DOPA and L-tyrosine, respectively. The pigment epithelium homogenates did not catalyze the oxidation of *p*-cresol, catechol, or D-tyrosine. Tyrosinase activity was completely inhibited by the addition of 0.001*M* 4-chlororesorcinol or sodium diethyldithiocarbamate.

By a technique similar to that of Kertesz (*4*), copper was removed by dialysis in the presence of potassium cyanide. The metal-free enzyme was without activity on L-tyrosine or L-DOPA, but addition of trace amounts of copper restored the catalytic activity against L-DOPA but not against L-tyrosine. The failure of the reconstituted enzyme to catalyze the oxidation of L-tyrosine is similar to the behavior of mammalian tyrosinase following removal and addition of copper (*4*). The trace element, copper, which is necessary for formation of melanin, has been found in the retina of mammals, amphibians, and fish (*5*).

A study of pigment granules isolated from the retinal pigment epithelium of Rhode Island Red embryos at different embryonal stages revealed a change in the level of tyrosinase activity during differentiation. Tyrosinase activity was first detectable on the sixth day and gradually increased, reaching a maximum on the tenth day. Enzyme activity fell abruptly after the 12th day, and no tyrosinase activity was present on and after the 14th day of development (Fig. 2).

With a histochemical autoradiographic technique in which DL-tyrosine-2-C^{14} was used as a substrate (*6*), tyrosinase activity was shown to be present in the retinal pigment epithelium of both a C-57 15-day mouse embryo and a 6-month human fetus (*7*). However, no tyrosinase activity was detectable in the retinal pigment epithelium of adult Rhode Island Red and Black Australorp chickens, the C-57 mouse, and the adult human being.

Avian tyrosinase present in the retinal pigment epithelium appears to be similar to plant, insect, and mammalian tyrosinase in its requirement for copper and in exhibiting an induction period in the oxidation of monophenols which can be abolished by addition of small amounts of diphenols (*3*). The specificity of avian tyrosinase for tyrosine and DOPA places it in the class of tyrosinases found in mammalian malignant melanomas (*8*). Plant tyrosinases, being less specific than mammalian tyrosinase, readily catalyze the oxidation of cresol and catechol as well as of tyrosine and DOPA.

It appears from these biochemical studies that the pigment in the retinal pigment epithelium is formed during differentiation by the enzymic oxidation of tyrosine to melanin, catalyzed by tyrosinase attached to cytoplasmic pigment granules. Since avian retinal tyrosinase exhibits some similarities to mammalian tyrosinase present in mouse and human malignant melanomas, it may be useful in elucidating some of the biochemical factors that regulate tyrosinase activity in normal mammalian melanoblasts during differentiation.

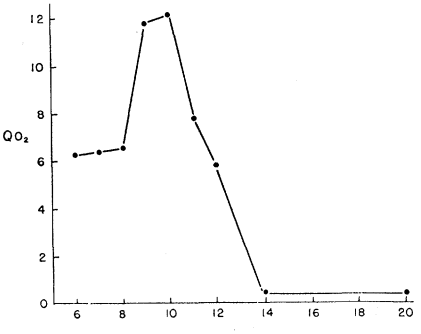

Fig. 2. Changes in tyrosinase activity of melanin granules isolated from the retinal pigmented epithelium of the Rhode Island Red chick during embryonal development.

MASAMITSU MIYAMOTO
THOMAS B. FITZPATRICK
Division of Dermatology, University of Oregon Medical School, Portland

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Kojic Acid—a Convulsant

The mode of formation of kojic acid by microorganisms (*1*), its great chemical reactivity (*2*), and the fact that it is further metabolized by microorganisms suggested to one of us (T.E.F.) that kojic acid might be metabolized by multicellular organisms and, perhaps, also by the diabetic animal (*3*). In the dogs studied by him, the intravenous injection of 150 mg/kg of body weight produced a marked toxic response, including convulsions.

In our studies, mice, guinea pigs, and rabbits, as well as dogs, were employed. Three routes of administration were utilized: subcutaneous, peritoneal, and intravenous. Mice were employed in the intravenous survey experiments (to determine the effective convulsive dose) and in the anticonvulsive studies (*4*). Aqueous solutions adjusted to *p*H 7.0 were employed in all experiments and, in the toxicity experiments, all doses were injected within 20 seconds (*5*).

The administration of large doses of kojic acid (1000 mg/kg) to two dogs caused death to both, yet evidence of kojic acid excretion in saliva and urine, even if indirect, was thereby secured. The nonspecific ferric chloride reaction was used. The CD_{50} and the LD_{50} in mice are approximately 350 and 500 mg/kg, respectively.

The gross external manifestations of kojic acid administered to mice, guinea pigs, rabbits, or dogs are generally alike and are similar to the well-known effects of Metrazol.

Because in dogs the toxic manifestations of kojic acid in dosages of 200 to

500 mg/kg are similar in some aspects to the clinical phenomena of epilepsy, the effects in dogs are dealt with in some detail.

Early effects were alike in all the dogs, regardless of dose or route of administration. All appeared irritable and all urinated, defecated, and vomited, and some became restless. Three dogs that were passing into the second stage of action seemed dazed, panted a great deal, and salivated to some extent. Panting and salivation became more marked as the second stage progressed. Walking was associated with head held low, attempts to lie down, and finally a collapse. Feces were mixed with considerable mucus. The remarkable thing about these second-stage dogs was that they recovered several times and seemed to behave normally for intervals of 5 to 10 minutes, after which they again relapsed. In every instance where coma did not develop, symptoms disappeared within 3 hours.

Late stages of toxicity were always ushered in with considerable excitability. Barking, panting, and salivation were very marked. Convulsions of the clonic-tonic type occurred at intervals, between which the dogs seemed stuporous. Continuous salivation and occasional twitching, particularly of the extremities, were observed between the seizures.

Dogs given kojic acid intravenously exhibited a more severe degree of toxicity than those given the acid by another route. None of the dogs died, although the canine that received 500 mg/kg of kojic acid intravenously exhibited a very stormy convulsive stage. All of the dogs given kojic acid intravenously developed third-stage toxicity, and the two dogs given the higher doses of kojic acid had convulsions of the clonic-tonic type. Effects, regardless of the dose, occurred within 15 minutes and, with the largest dose, within 5 minutes. None of the dogs recovered completely until approximately 24 hours after injection, and the dog given the largest dose, 500 mg/kg, was not well for about 48 hours. The dog given the smallest dose of kojic acid intravenously, 200 mg/kg, recovered sufficiently in about 8 hours to drink small quantities of water.

The reaction patterns observed in the dogs given kojic acid subcutaneously and peritoneally were similar to those observed after intravenous administration. What differences were noted could be ascribed mainly to the route.

If the response of the dogs differs in any manner from the manifestations of the other animal species, it is in the retching and vomiting observed early, and in what may be described as intervals of relative normalcy between periods of seizure and later effects. Since deaths, with the doses used, occurred in the other animals and not among the dogs, it is possible that the latter are better able to detoxify kojic acid.

In the evaluation of the protective ratio (PR) of barbital and phenobarbital (50 mg/kg each) by the Orloff method (*4*), kojic acid was substituted for, and compared with, Metrazol. The average dose of Metrazol for a single twitch was 50 mg/kg and for a persistent convulsion, 150 mg/kg. To produce comparable effects with kojic acid took 135 and 400 mg/kg, respectively. Average dosages were obtained from 20 mice weighing 18 to 20 g. The protective ratios are tabulated in Table 1. The ratios for barbital-Metrazol and for barbital-kojic acid are very much alike.

Metrazol is a much more toxic substance per unit weight than kojic acid, for the latter required approximately 400 mg/kg of body weight to produce a persistent convulsion, whereas Metrazol required approximately one-third of this amount. It is of interest to note that when kojic acid was administered to mice in the dose of 400 mg/kg of body weight by way of the rapid infusion technique (effective convulsive dose studies), two out of ten mice died, whereas all recovered from an equal amount of kojic acid administered by the divided-infusion method. The detoxification mechanism probably is given a longer opportunity to act in the divided-infusion method.

Table 1. Evaluation of the anticonvulsive properties of barbital and phenobarbital—comparison of kojic acid and Metrazol. Twenty mice were used in each experiment.

Group	Time (min)	Dose (avg.) for one twitch (mg/kg)	PR for one twitch	Dose (avg.) persistent convulsions	PR for persistent convulsions
Phenobarbital-Metrazol	5	83.00	1.66	240.00	1.60
	60	116.50	2.33	180.00	1.20
	120	100.00	2.00	255.00	1.70
Phenobarbital-kojic acid	5	236.25	1.75	664.00	1.66
	60	337.50	2.5	415.00	1.00+
	120	286.20	2.12	580.00	1.45
Barbital-Metrazol	5	83.00	1.66	195.00	1.30
	60	100.00	2.00	225.00	1.50
	120	66.50	1.33	180.00	1.20
Barbital-kojic acid	5	218.70	1.62	440.00	1.10
	60	270.00	2.00	600.00	1.50
	120	236.25	1.75	420.00	1.00+

S. C. Werch
Department of Obstetrics-Gynecology, University of Miami School of Medicine, Miami, Florida

Y. Thomas Oester
Department of Pharmacology, Stritch School of Medicine, Loyola University, Chicago, Illinois

Theodore E. Friedemann
U.S. Army Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colorado

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Detection of Rare-Earth Ions as Oxalates and Cupferrates

Although a great amount of work has been published on the rare-earth oxalates, little is known about the sensitivity of the reaction between the metal ions and oxalic acid. In 0.5*N* mineral acid medium at 60°C, the sensitivity limit has been set as 700 ppm (*1*). This, however, is for the rare-earth group and not for the individual rare-earth metal ions.

Cupferron (ammonium nitrosophenylhydroxylamine) also forms insoluble precipitates with the rare-earth metal ions (*2, 3*). However, nothing is known concerning the sensitivity of the precipitation reaction.

A modification of the method of Irving, Butler, and Ring (*4*) was used to determine the reaction sensitivity. Tests were conducted in 1- by 6-in. test tubes containing a known amount of the 0.01*M* or 0.001*M* rare-earth metal chloride solution and 0.2 ml of 0.1*M* precipitating agent solution in a total volume of 7.0 ml. The test solutions were heated for 10 minutes at 80°C, allowed to cool to room temperature, and then observed visually for the presence of a precipitate.