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14 May 1959

Albinism in the California Hagfish Eptatretus stoutii

Abstract. The discovery of an albino specimen of the hagfish, *Eptatretus stoutii* (Lockington), is reported, together with general notes regarding collection of this species for research purposes. The extreme abundance of hagfish in the area described refutes the generally accepted notion that they are of infrequent occurrence in southern California waters.

True albinism occurs but rarely among fishes, and it is believed that this is the first record of such a natural freak to be reported among the most primitive of craniate chordates, the hagfish (Fig. 1). There is a report of an albino lamprey (*Petromyzon marinus*), the other division of the class Cyclostomata, which was collected some years ago at the mouth of the Salmon River on Lake Champlain in New York (*1*).

During a routine collection for specimens of *Eptatretus stoutii* (=*Polistotrema stoutii*) on 5 May 1959, using 5-gallon-can traps, I obtained an albino individual in a total collection of some 500 fish. Over the past 2 years I have taken about 5900 hagfish of this species from the same general vicinity (latitude, 32° 31'N; longitude, 117° 18'W). This area is the floor of a submarine canyon, a tributary of the San Diego Trough, where the bottom is green mud and the depth is 210 fathoms. The traps remained on the bottom for 7 hours and they were baited with fish oil and mackerel.

Normally, the body color of this species is pinkish to purple grey, lighter ventrally than dorsally. Of the several thousand hagfish studied, about 20 were piebald (Fig. 2), with certain areas pale pink because of reduced pigmentation.

The skin of the albino is white save for a pinkish cast imparted by the blood. This coloration is most pronounced in the eye regions, the barbels, and the caudal area. The pulsations of the caudal heart are plainly visible beneath the translucent skin.

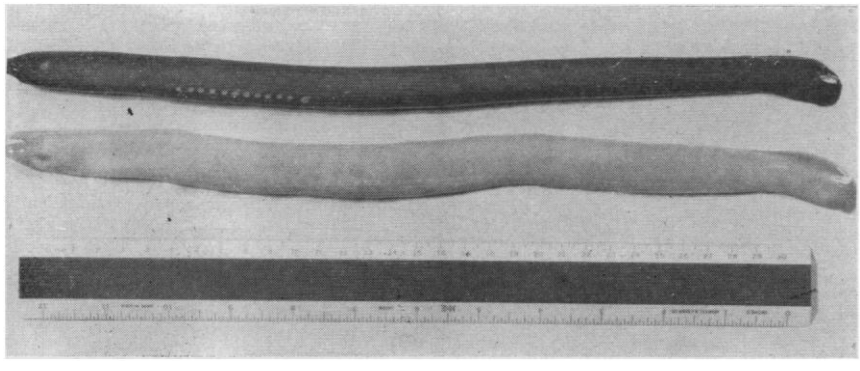

Fig. 1. Living albino *Eptatretus stoutii* with normally colored individual for comparison (anesthetized). Note the slime glands beneath the skin in the albino (especially prominent posterior to the gill openings), not readily evident in the pigmented specimen.

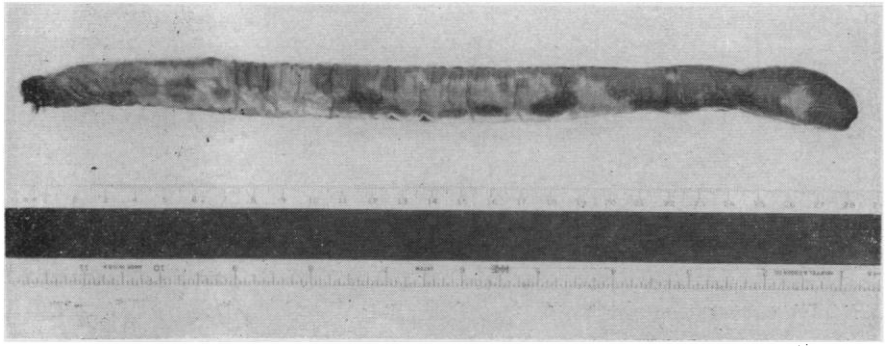

Fig. 2. Piebald specimen of *Eptatretus stoutii* (preserved), collected 7 Nov. 1958.

Measurements of the albino specimen (*2*) were as follows: body length, snout to tip of tail, 38.1 cm; weight, 55 g; external gill openings, 12 left, 12 right. Measurements of the piebald specimen were as follows: body length, 28.3 cm; weight, 36 g; gill openings, 11 left, 11 right.

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2. This specimen was obtained during the course of research supported by a grant from the San Diego County Heart Association, to which grateful acknowledgment is made.

10 June 1959

Identification of a Growth Inhibitor from Extracts of Dormant Peach Flower Buds

Abstract. A growth inhibitor in dormant peach flower buds was identified as naringenin (5,7,4'trihydroxyflavanone) from infrared and ultraviolet spectra, through determination of melting point, through paper chromatography, and from activity in the bioassay test.

A growth-inhibiting substance has been reported to occur in resting peach flower buds by a number of investigators (*1–3*). This substance has been closely correlated with the emergence of peach buds from rest (*1*); however, its identity is unknown.

In an investigation of the seasonal fluctuation in quantity of growth substances in resting peach flower buds, several attempts were made to obtain a purification step which would separate the inhibiting substance from oils, fats, and waxes present in the plant extract. The inhibitor could not be separated by means of a sodium bicarbonate solution; therefore, the ether or methanol extract was dissolved in 0.1*N* NaOH. The *p*H was adjusted downward, one unit at a time, with 0.1*N* HCl, and after each adjustment of *p*H the aqueous solution was extracted with peroxide-free diethyl ether and the ether extract was assayed for activity. It was noted that the intense straw color at *p*H 9.0 changed to a less intense straw color at *p*H 8.0. It was found that the inhibitor was very soluble in the ether phase at *p*H 8.0. There was a small amount of the inhibitor in the ether phase at *p*H 9.0 and 7.0. This provided a means whereby the inhibitor could be recovered free from fats, oils, and waxes and from many of the acidic substances which still remained in the salt form at this *p*H.

A number of attempts were made to crystallize the inhibitor from resting

peach buds. The procedure described below was successful.

On 1 Dec. 1958, 221 g (fresh weight) of buds were collected and extracted for 48 hours with four changes of peroxide-free diethyl ether at approximately 12-hour intervals. The ether extracts were combined and purified with *n*-hexane and acetonitrile. The acetonitrile fraction was evaporated to dryness, and the residue was dissolved in aqueous 0.1*N* NaOH. The *p*H of the aqueous NaOH solution was adjusted to *p*H 10 with 1*N* HCl and extracted with ether. This ether, containing the ether-soluble materials (fats, waxes, and so on), was discarded. The aqueous solution was adjusted to *p*H 8.0 and extracted with four 50-ml aliquots of fresh peroxide-free ether. This ether fraction, containing the growth-inhibiting substance, was concentrated and chromatographed on Whatman No. 1 filter paper in a water-saturated *n*-butanol-ammonia (100:8 vol./vol.) solvent. The chromatograms were dried and sprayed with 0.1*N* NaOH, and the inhibiting zone, bright yellow in color, was eluted with distilled water. The aqueous fraction was retained and adjusted to *p*H 10 and extracted with ether, and the ether was discarded. The *p*H was then adjusted to 8.0 and extracted with five 25-ml aliquots of fresh peroxide-free ether. This ether fraction was evaporated to dryness, and the residue was dissolved in approximately 10 ml of absolute methanol. The methanol was concentrated under a jet of compressed air, and when approximately 3 ml remained, about 15 ml of distilled water was added. The substance which precipitated strongly inhibited the growth of coleoptiles in the biological assay test.

The precipitate formed was a bright yellow compound having a very sharp melting point at 244°C (uncorrected). This substance was slightly soluble in water and very soluble in alcohol and ether. The original 221 g of buds produced about 140 mg of the substance, or 0.63 mg per gram of buds. Recrystallization of the inhibitor from 95 percent ethanol resulted in long needle-shaped crystals which exhibited considerable inhibition in the bioassay test.

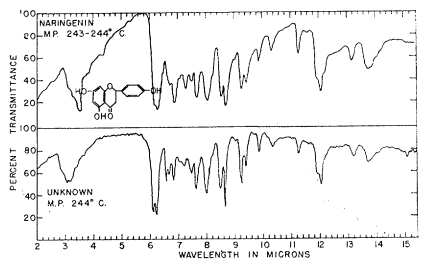

Fig. 1. Infrared absorption spectra of authentic naringenin (top) and of material crystallized from dormant peach buds (bottom).

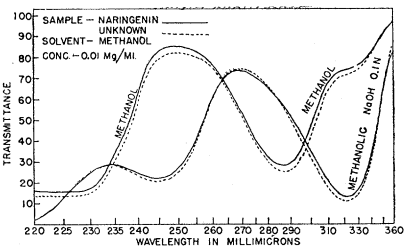

Fig. 2. Ultraviolet absorption spectra of authentic naringenin and of material crystallized from dormant peach buds, determined in methanol and methanolic NaOH, 0.1*N*.

The same procedure was followed in a second isolation, from 120 g (fresh weight) of buds collected 2 Feb. 1959. This isolation yielded 17 mg of material having the same melting point and inhibitory characteristics as the previous isolate. This was equivalent to 0.142 mg per gram (fresh weight) of buds. This difference in yield could have been due to a difference in concentration of the substance in the buds on the two dates.

The crystalline material obtained from the second isolation was used in determining the identity of the growth inhibitor, from infrared and ultraviolet spectra and through chromatography, color tests, and bioassay.

An infrared spectrum of the unknown was obtained from Sadtler Research Laboratories, Philadelphia, Pa. This spectrum resembled very closely the spectrum of naringenin (5,7,4′tryhydroxyflavanone), the aglycone of naringin (Fig. 1). One major shift in the peak at 3.45 microns was noted. Also, the unknown had somewhat less absorption in the region of 4 to 6 μ. Very minor differences can be noted in other areas of the spectrum.

Further evidence that the inhibitor was naringenin was obtained by comparing the ultraviolet spectrum of the unknown with that of authentic naringenin, both in absolute methanol and in methanolic 0.1*N* NaOH. The comparison was made with a concentration of both materials of 0.01 mg/ml. The results (Fig. 2) show that the absorption curves in methanol and in methanolic 0.1*N* NaOH are almost identical. In absolute methanol, maximum absorption occurred at 287 mμ, with minima at 249 mμ, for both the unknown and naringenin. The shift in peaks as a result of the change in the *p*H of the solvent was the same for both the naringenin and the unknown. The maximum absorption occurred at 322 to 323 mμ, with a minima at 269 mμ.

Descending chromatography on Whatman No. 1 filter paper was conducted with the inhibitor and naringenin in a water-saturated *n*-butanol-ammonia (100:8 vol./vol.) solvent.

In this solvent, the inhibitor had an R_f of 0.47, identical with the growth-inhibiting area of the naringenin (obtained from Delta Chemical Works, New York). A second spot on the chromatogram, above naringenin, appeared with the naringenin sample. This spot was not active in the bioassay test.

Paper chromatograms of the unknown crystalline material were sprayed with diazotized sulfanilic acid, ferric chloride, sodium hydroxide, and Ehrlich's reagent, and colors identical to those given by authentic naringenin were obtained. Naringenin and the inhibitor absorbed in ultraviolet light, and when the chromatograms were sprayed with 1-percent alcoholic $AlCl_3$, an intense greenish-white fluorescence was obtained.

Paper chromatograms of the unknown crystalline material at various concentrations were sectioned and placed with etiolated wheat coleoptiles, and the amount of inhibition obtained was the same as that obtained with the authentic naringenin at a concentration of $1.84 \times 10^{-3}M$. When the pure substance was not chromatographed but was assayed directly, the concentration required to give the same degree of inhibition that was found when the material was chromatographed was $4.6 \times 10^{-4}M$. This discrepancy in effective concentration between the chromatographed and nonchromatographed material may have indicated that the substance was not completely eluted from the paper, or that part of the substance remained in the salt form and was not effective when the material was chromatographed in ammonia solvent.

The results obtained from this investigation (*4*) strongly indicate that the inhibitor obtained from methanol or ether extracts of dormant peach flower buds was naringenin (5,7,4′trihydroxyflavanone), the aglycone of naringin. The glycoside, naringin, occurs abundantly in grapefruit (*Citrus paradisi*) (*2*), but to our knowledge neither the glycoside nor the aglycone has been reported to occur in peach (*Prunus persica*).

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4 May 1959

Inhibitor of O-Methylation of Epinephrine and Norepinephrine in vitro and in vivo

Abstract. Pyrogallol inhibits the O-methylation of epinephrine and norepinephrine by catechol-O-methyl transferase in vitro as well as the metabolism of these catecholamines, and the formation of their O-methylated metabolites, in the intact mouse. Since pyrogallol also prolongs the physiological effects of epinephrine, it is suggested that catechol-O-methyl transferase terminates the actions of the catecholamine hormones.

Previous work in this laboratory has shown that catechol-O-methyl transferase (*1*) is mainly responsible for the metabolism of epinephrine and norepinephrine (*2*). Whether this enzyme inactivates these hormones has not been established. A classical procedure showing a physiological role of an enzyme involves a demonstration that inhibition of the enzyme prolongs the actions of its substrates.

Many years ago Bacq demonstrated that pyrogallol and other catechols markedly increased the duration of response to epinephrine and sympathetic nerve stimulation in vivo (*3*). This potentiation was attributed to the antioxidant properties of the catechols (*3*). Since catechol-O-methyl transferase methylates all types of catechols (*1*), it appeared to us that pyrogallol might prolong the responses of epinephrine by competing for this enzyme.

The action of pyrogallol on catechol-O-methyl transferase was examined in vitro. Incubating epinephrine ($1\times10^{-5}M$) with O-methyl transferase obtained from rat liver, with magnesium chloride, and with the methyl donor S-adenosylmethionine resulted in the formation of metanephrine (3-O-methylepinephrine). In the presence of pyrogallol at a concentration of $1\times10^{-5}M$, the formation of metanephrine was inhibited approximately 50 percent. When the concentration of the substrate was increased 100-fold, the inhibition by pyrogallol was abolished, and thus the competitive nature of the reaction was indicated. Essentially the same results were obtained when norepinephrine was used as a substrate.

The effect of pyrogallol on the metabolism of epinephrine and the formation of metanephrine in vivo was then studied. Mice were given H^3-epinephrine intravenously and were killed 10 minutes later. The whole animal was homogenized in 0.1*N* HCl in a Waring blender, and an aliquot of the homogenate was examined for remaining H^3-epinephrine and for total (free and conjugated) H^3-metanephrine formed (*4*). It was found that about 70 percent of the administered epinephrine was metabolized in this time (Fig. 1). Almost all of the catecholamine that disappeared could be accounted for as metanephrine. Pretreating the mice with pyrogallol dramatically blocked both the metabolism of epinephrine and the formation of metanephrine (Fig. 1), indicating that pyrogallol is an effective inhibitor of catechol-O-methyl transferase in vivo. Pyrogallol also inhibited the disappearance of norepinephrine in the intact mouse. The metabolism of epinephrine was found to be blocked after the intravenous administration of 500 to 10 mg of the catechol-O-methyl transferase inhibitor per kilogram to mice. Epinephrine levels after pyrogallol treatment were elevated for many hours, suggesting that binding of the catecholamine might be an alternate mechanism for the inactivation of this hormone. Although pyrogallol exerts its effects at a relatively low concentration, it is likely that there are more potent inhibitors of catechol-O-methyl transferase. Many flavonoids (rutin, quercitrin) at low concentrations have been shown to potentiate the actions of epinephrine in vivo (*5*). Since flavonoids possess a catechol nucleus, this effect might be due to their ability to inhibit catechol-O-methyl transferase.

For many years monoamine oxidase was considered to be the enzyme chiefly concerned with the metabolism and inactivation of catecholamines. Recent work, however, has shown that the inhibition of monoamine oxidase in vivo did not prolong the physiological effects of exogenous (*6*) and endogenous (*7*) catecholamines. Monoamine oxidase was shown to be mainly involved in the deamination of the O-methylated metabolites of epinephrine and norepinephrine, rather than of the catecholamines themselves (*2*). Treatment with iproniazid, a monoamine oxidase inhibitor, did not affect the rate of metabolism of epinephrine in mice (Fig. 1). These observations provide further evidence for the negligible role of monoamine oxidase in the inactivation of epinephrine.

It has been shown that O-methylation is the principal pathway for the metabolism of epinephrine and norepinephrine (*2*). More recently we have found that within 2 minutes after the administration of epinephrine to cats, more than half of the catecholamine has been converted to metanephrine (*4*). From the observations of Bacq, showing that pyrogallol prolongs the physiological actions of epinephrine (*3*), and from findings that this compound inhibits the O-methylation of the epinephrine and norepinephrine in vitro and in vivo, it can be concluded that catechol-O-methyl transferase is the enzyme primarily concerned with terminating the action of these hormones. The prolongation of responses to sympathetic nerve stimulation after pyrogallol administration (*3*) points to catechol-O-methyl transferase as the enzyme mainly involved in the inactivation of the neurohumor norepinephrine in the sympathetic nervous system.

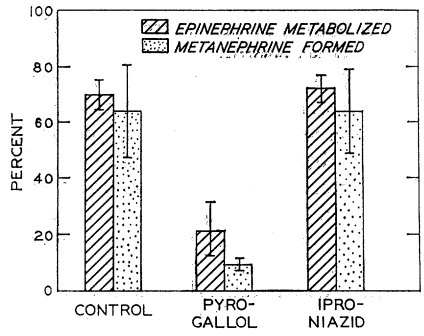

Fig. 1. Inhibition of epinephrine metabolism by pyrogallol. All mice received 3 μg of β-H^3-epinephrine (1.5 μc) in the tail vein and were killed 10 minutes later. Each animal was assayed for H^3-epinephrine and total (free and conjugated) H^3-metanephrine. Pyrogallol was given intravenously (100 mg/kg) 2 minutes before the injection of the epinephrine. Iproniazid (100 mg/kg) was given intraperitoneally 4 hours prior to the administration of the catecholamine. Ten mice were used in each group. The vertical bracketed lines represent the standard deviation of the mean.

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7 May 1959