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INDUCTION OF BIOLUMINESCENCE CAPABILITY IN THE MARINE FISH, *PORICHTHYS NOTATUS*, BY *VARGULA* (CRUSTACEAN) [^{14}C]LUCIFERIN AND UNLABELLED ANALOGUES

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Summary

Along the Pacific coast of North America, the marine fish, *Porichthys notatus*, is distributed as a northern nonluminescent population and a southern luminescent one. Bioluminescence capability is inducible in nonluminescent *P. notatus* by a single oral or intraperitoneal administration of a small amount of luciferin, a compound used by the marine ostracod crustacean, *Vargula*, as substrate for light emission. Once induced, the fish is capable of luminescing for more than 2 years. The results of the present study show that: (1) *P. notatus* photophores incorporate ^{14}C-labelled *Vargula* luciferin: (2) the specific activity of the incorporated label is undiluted when recovered from the photophores 7 weeks later; (3) two chemical analogues of *Vargula* luciferin do not induce bioluminescence capability in the fish; (4) luciferinol, another close analogue of *Vargula* luciferin, induces a very weak bioluminescent capability in the fish; and (5) two key intermediates in the degradation of *Vargula* luciferin, oxyluciferin and etioluciferin, are ineffective in inducing bioluminescence capability. The conclusions to be drawn from this study are that *P. notatus* recycles luciferin and that, in the uninduced fish, the recycling does not involve the resynthesis of luciferin from oxyluciferin or etioluciferin.

Introduction

The marine toadfish, *Porichthys notatus*, presents an interesting example of the dependence of one animal on a substrate (luciferin) used in the bioluminescence reaction of another unrelated organism (McCapra & Hart, 1980; Shimomura,

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Inoue, Johnson & Haneda, 1980; Herring, 1982). The toadfish is commonly found along the Pacific coast of North America as a southern luminescent population and a northern nonluminescent one (Strum, 1969; Warner & Case, 1980). *P. notatus* is characterized by more than 700 small (≈1 mm diameter) dermal photophores that are arranged serially in a species-specific pattern over the head and trunk (Greene, 1899). In the southern fish, subcutaneous injection of norepinephrine causes whole-body photophore luminescence that is clearly visible to the dark-adapted eye. The photophores of southern *P. notatus* show a strong greenish fluorescence in near-ultraviolet light, due to the presence of luciferin (Baguet & Zietz-Nicolas, 1979), whereas those of the northern form are nonfluorescent. The bioluminescence and fluorescence characteristics distinguish the two populations of *P. notatus* even though, ultrastructurally, the photophores of the northern fish are indistinguishable from those of the southern form (Strum, 1968).

Previous studies with *Porichthys notatus* from California and Puget Sound, Washington, have shown that the inability of the Puget Sound fish to luminesce is due to a lack of luciferin from all developmental stages of the animal (Tsuji, Barnes & Case, 1972; Barnes, Case & Tsuji, 1973). However, nonluminescent *P. notatus* may be induced to develop luminescence capability by an intraperitoneal or oral administration of luciferin from the tiny (3 mm long) marine ostracod crustacean, *Vargula hilgendorfii* (Tsuji *et al.* 1972; Barnes *et al.* 1973; Tsuji *et al.* 1975), whose luciferin and luciferase show reciprocal light-emitting cross-reactions with those of *P. notatus* (Cormier, Crane & Nakano, 1967; Tsuji, Haneda, Lynch & Sugiyama, 1971). Because *Vargula* luciferin is similar, if not identical in structure, to that of *P. notatus* luciferin (Cormier *et al.* 1967; Tsuji *et al.* 1971), it has been postulated that *P. notatus* obtains its luciferin from *Vargula* either by ingesting these crustaceans or by an indirect route through the food chain (Tsuji *et al.* 1972). Support for this dietary hypothesis is provided by an apparent sympatric distribution of luminescent *Vargula tsujii* with that of southern *P. notatus* (Warner & Case, 1980).

Although it is certain that only a small amount of *Vargula* luciferin is required to induce long-term bioluminescence capability in *Porichthys notatus*, nothing is known about the mechanism of luminescence induction, especially regarding the 3- to 4-day interval between the time luciferin is administered and the time luminescence capability develops. The great susceptibility of *Vargula* luciferin to autoxidation by molecular oxygen (Tsuji, 1955; Shimomura, Goto & Hirata, 1957; Haneda *et al.* 1961) seems to rule out any simple incorporation and utilization of luciferin by the photophores. Recently we found that bioluminescence capability, once induced, lasts for more than 2 years, and that it is due either to *de novo* synthesis or to recycling of luciferin (Thompson, Nafpaktitis & Tsuji, 1987). In an attempt to decide between the two mechanisms, ^{14}C-labelled *Vargula* luciferin was synthesized and administered to nonluminescent Puget Sound *P. notatus*. Luciferin that was subsequently recovered from the photophores showed no change in specific activity, indicating that luciferin recycles, but the inability of *Vargula* oxyluciferin and etioluciferin to induce luminescence capability suggests that the

induction and persistence of bioluminescence capability involves more than a recycling of these compounds.

Materials and methods

Synthesis of [^{14}C]luciferin

^{14}C-labelled *Vargula* luciferin, 2-(2′-butyl)-6-(3′-indolyl)-8-(1′-guanido-3′-propyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, (IV), was chemically synthesized according to the scheme shown in Fig. 1. [^{14}C]Thiourea (specific activity 10·2 mCi mmol^{-1}) (Amersham) was reacted with C_2H_5Br to give [^{14}C]S-ethylisothiourea-HBr (I) (Brand & Brand, 1955). *Vargula* etioluciferamine (II), prepared as described by Kishi *et al.* (1966), was converted by treatment with I to give [^{14}C]etioluciferin-2HBr (III) which was purified by silica gel column chromatography with 20 % methyl alcohol in CH_2Cl_2: concentrated NH_3 (aqueous) (20:1, v/v) as the mobile phase. Racemic *Vargula* [^{14}C]luciferin-2HBr (IV) was then obtained by treatment of III with sec-butylglyoxal in methyl alcohol containing concentrated HBr (Sugiura, Inoue & Goto, 1970).

The analogue of luciferin, 8-(3-aminopropyl)-2-(2-butyl)-6-(3-indolyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (V) (Fig. 2), was prepared by treating *Vargula* etioluciferamine (II, Fig. 1) with sec-butylglyoxal in methyl alcohol containing a 2·4-fold excess of HBr over that used in preparing *Vargula* [^{14}C]luciferin. The

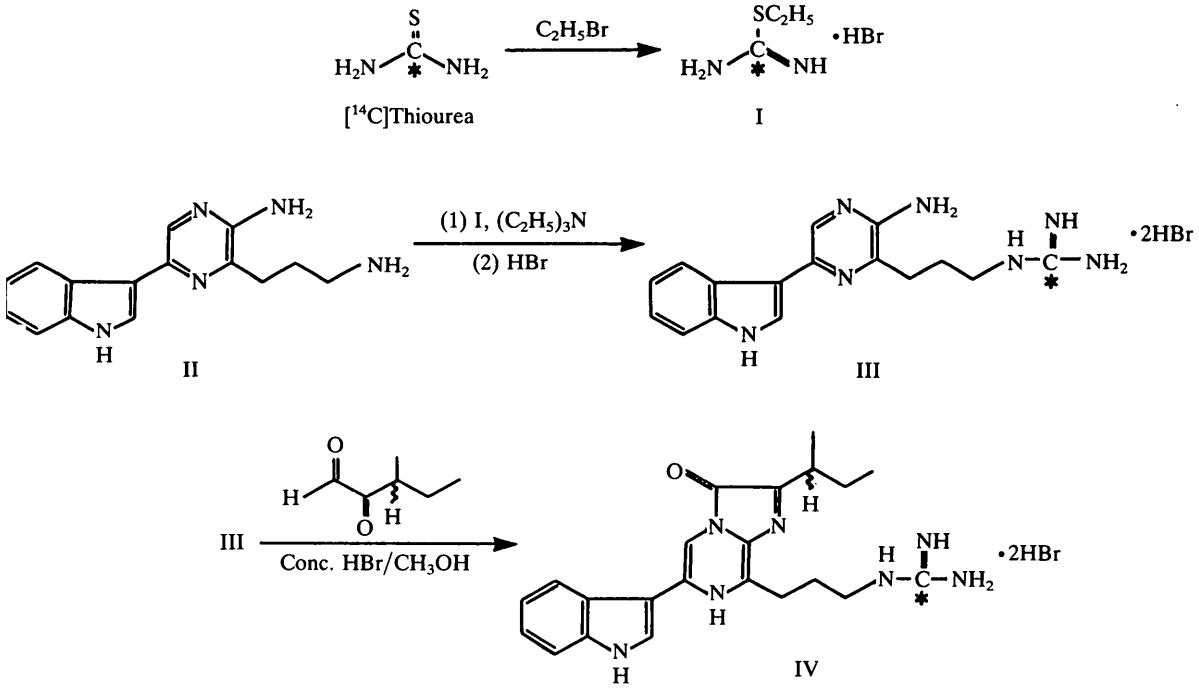

Fig. 1. Scheme for the synthesis of *Vargula* [^{14}C]luciferin. Asterisk indicates position of label. Other details are described in the text.

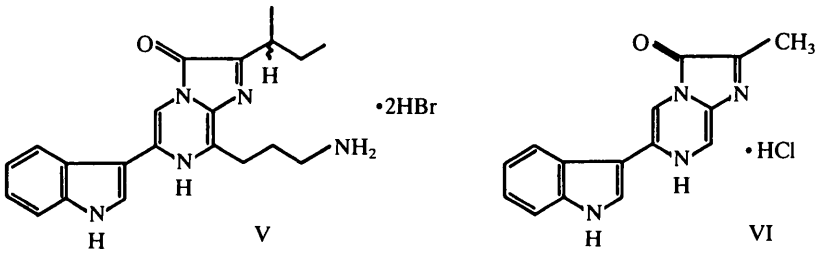

Fig. 2. Structures of synthetic *Vargula* luciferin analogues: V, 8-(3-aminopropyl)-2-(2-butyl)-6-(3-indolyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one; VI, 6-(3-indoyl)-2-methyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one.

luciferin analogue, 6-(3-indolyl)-2-methyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (VI, Fig. 2), was prepared by the method of Goto, Sugiura & Inoue (1970). *Vargula* oxyluciferin, *Vargula* etioluciferin and *Vargula* luciferinol were prepared by the methods of Sugiura *et al.* (1970), Kishi *et al.* (1966) and Toya, Nakatsuka & Goto (1983), respectively.

Determination of specific activity of [^{14}C]luciferin

Determination of specific activity was carried out using a Beckman LS8000 liquid scintillation counter. Samples were counted in 20 ml glass scintillation vials with Aquasol-2 (New England Nuclear) as scintillation cocktail. An optimal counting volume of 10 ml was used for all measurements. On dissolving luciferin in Aquasol-2, an intense light emission resulted, due to the chemiluminescence of luciferin in the hydrophobic solvent (Goto, Inoue & Sugiura, 1968). The chemiluminescence disappeared after several days, so these samples were allowed to stand for 5 days before counting. The specific activity of the synthetic [^{14}C]luciferin was $6{\cdot}13 \pm 0{\cdot}05$ mCi mmol^{-1}, determined by counting samples of known luciferin concentration (calculated from $E = 5700\,M^{-1}\,cm^{-1}$ after measuring absorbance in methyl alcohol at 430 nm) (Sugiura *et al.* 1970).

Administration of compounds

Nonluminescent *Porichthys notatus* were collected using an 8-m otter trawl at Port Orchard, Puget Sound, Washington, and shipped by air to Scripps Institution of Oceanography. The fish were maintained in a running seawater aquarium at 12–15°C and were allowed a 1-week acclimation period prior to experimental treatments. All fish were fed a diet of nonluminescent squid during the course of the study. Specimens of *P. notatus* used in the experiment ranged in standard length from 12·8 to 23·8 cm.

Following the acclimation period, 10 fish received *Vargula* [^{14}C]luciferin according to the schedule in Table 1, six fish were given 20 μg each of *Vargula* oxyluciferin, six received 20 μg each of *Vargula* etioluciferin, four were given 20 μg each of luciferinol, four were administered 25 μg each of analogue V (Fig. 2), and

Table 1. *Schedule of oral administration of* Vargula *[^{14}C]luciferin*

Day	[^{14}C]luciferin/fish (μg)
1	7·5
5	7·5
9	7·5
14	7·5
18	7·5
22	7·5
26	15·0
30	15·0
47	killed

four received 25 μg each of analogue VI (Fig. 2). In all cases, except for *Vargula* [^{14}C]luciferin, the compounds were administered as a single dose, with half of the fish receiving the dosage orally and the other half by intraperitoneal injection. The required dosages of each compound were prepared by dissolving the compound in methyl alcohol and drying the samples *in vacuo* in 1·5-ml Eppendorf tubes. The compounds were administered to *Porichthys notatus* anaesthetized with tricaine methanesulphonate (Sigma, 200 mg l^{-1} sea water). For oral administration, the dried compound was dissolved in 0·15 ml of filtered (0·45 μm pore diameter) sea water and delivered into the stomach *via* intramedic tubing. The Eppendorf tube was rinsed once with 0·15 ml of filtered sea water and this was introduced into the fish's stomach. For intraperitoneal administration, the dried compound was dissolved in 0·15 ml of filtered (0·45 μm pore diameter) sea water and injected using a 1-ml syringe and 25 gauge needle.

In the *Vargula* [^{14}C]luciferin experiment, seven control *Porichthys notatus* received 0·30 ml of filtered sea water *via* intramedic tubing. For each of the remaining compounds, four control fish were used and in each case two received 0·30 ml of filtered sea water orally and two by intraperitoneal injection.

Assay of luciferin

An excess of *Vargula* luciferase, prepared as previously described (Tsuji, 1978), was used for determining luciferin. For each assay, a 20-μl sample of purified luciferase (1·3 mg ml^{-1}) was mixed with 2 ml of 0·20 mol l^{-1} Tris-HCl, pH 7·60, in a 20-ml scintillation vial. The vial was placed in a holder in the photomultiplier housing of a Mitchell–Hastings photometer (Mitchell & Hastings, 1971) and injected with 10–50 μl of the luciferin solution to be assayed, mixed with 2 ml of 0·20 mol l^{-1} Tris-HCl, pH 7·60. The resultant light was integrated with a Hewlett–Packard 3392A integrator. Total light, directly proportional to the amount of luciferin present, was used to determine luciferin concentration.

With the above technique, a standard curve was constructed over a range of 0·20 to 90 ng *Vargula* luciferin. At the lower end of this range, where luciferin becomes especially susceptible to autoxidation, dilute solutions were prepared from a concentrated stock solution of luciferin immediately prior to each assay. Under these conditions, the standard curve obtained was highly reproducible, deviating only slightly from linearity through the origin at these lower luciferin concentrations.

HPLC of luciferin

High-performance liquid chromatographic (HPLC) analysis of *Vargula* luciferin was carried out using an Altex Ultrasphere ODS (5 μm diameter) column under conditions described in Fig. 3. *Vargula* luciferin was prepared for analysis as previously described (Tsuji, 1978).

Recovery and analysis of [^{14}C]luciferin

The [^{14}C]luciferin-treated fish, anaesthetized with tricaine methanesulphonate (200 mg l^{-1} sea water), were killed by freezing rapidly over dry ice. All subsequent manipulations were carried out near 0°C unless otherwise specified. The ventral photophores were quickly removed with a pair of scissors by cutting strips of skin with the photophores attached. The strips were cut into pieces a few millimetres in length and ground in absolute methyl alcohol (250 photophores/5 ml) using a PCU-2 Polytron (Kinematica) homogenizer (30 s ×2). The homogenates were combined, acidified with a few drops of concentrated HCl, and placed in a sealed extraction vessel equipped with an inlet tube, for bubbling argon into the extract, and an outlet port. The extraction was continued while bubbling with 99·999 % argon, passed over hot copper filings to remove the last traces of molecular oxygen, for an additional 2 h. The extract was centrifuged at 5000 ***g*** in a Beckman J-21B refrigerated centrifuge for 5 min. After removing a small sample for luciferin assay, the supernatant (no. 1) was concentrated *in vacuo* until a whitish precipitate appeared. The extract was placed in an ethyl alcohol/dry ice bath for 5 min at −70°C to allow additional precipitate to form and then centrifuged at 5000 ***g*** for 5 min at −10°C. The clear yellow supernatant (no. 2) was immediately transferred to another extraction vessel of the same design and flushed with 99·999 % argon as before. The same procedure was followed for the control fish.

For ^{14}C analysis, replicate background samples were made by mixing 8·5 ml of Aquasol-2 with 1·5 ml of the photophore extract from the control fish. A quench curve was plotted using the H-number method (Price, 1978), by preparing a series of scintillation vials containing a known amount of [^{14}C]toluene standard (New England Nuclear) in Aquasol-2, selecting for uniformity of counts, and adding various amounts of extract from the control fish, in replicate, at a constant volume of 10 ml. To analyse photophore extracts from the [^{14}C]luciferin-treated fish, 1·5 ml samples of extract were mixed with 8·5 ml of Aquasol-2.

Monitoring of fish fed unlabelled compounds

On days 7, 14, 21 and 42 after feeding of the various compounds, the fish were tested for photophore fluorescence in near-ultraviolet light and for bioluminescence capability by subcutaneous injection, under the branchiostegal photophores, of 30 μl of 0·001 mol l^{-1} DL-norepinephrine. Those fish which had received *Vargula* luciferinol continued to be monitored for photophore fluorescence and bioluminescence capability at monthly intervals for 1 year.

Results

In the HPLC elution profiles of *Vargula* luciferin and its oxidation products, oxyluciferin, etioluciferin and luciferinol (Fig. 3), the instability of *Vargula* luciferin is apparent with virtually complete loss of activity taking place by autoxidation within 72 h.

On completion of the feeding regimen (Table 1), the photophores of the Puget Sound *Porichthys notatus* that were fed *Vargula* [^{14}C]luciferin showed a greenish-yellow fluorescence in near-ultraviolet light characteristic of southern *P. notatus* and indicative of the presence of luciferin. None of the control fish developed fluorescence and extracts of their photophores did not contain luciferin or ^{14}C activity.

The concentration and total amount of luciferin present in supernatant no. 1 of the fish fed labelled luciferin were 7·4 ng ml^{-1} and 1·05 ± 0·06 μg, respectively, or 0·11 μg per fish. The corresponding values for supernatant no. 2 were 38·6 ng ml^{-1} and 1·03 ± 0·04 μg, respectively. Thus, 98 % of the luciferin activity in the initial photophore extract was accounted for in the final supernatant. The luciferin concentrations were determined from the standard curve described in Materials and methods and from the same curve extrapolated linearly through the origin. The luciferin concentration determined using the nonlinear portion of the curve gave a specific activity of 5·64 ± 0·11 mCi $mmol^{-1}$, whereas that estimated from the linear portion gave a value of 6·91 ± 0·14 mCi $mmol^{-1}$. Considering the instability of luciferin in dilute solutions, the two values represent the lower and upper limits, respectively, for the true specific activity of the recovered luciferin. These values ranged from 92 to 113 % of the original specific activity of 6·13 ± 0·04 mCi $mmol^{-1}$ for the ingested *Vargula* [^{14}C]luciferin, indicating that no dilution of specific activity occurred over the 7-week experimental period.

The results of the administration of oxyluciferin, etioluciferin, luciferinol and two luciferin analogues to nonluminescent Puget Sound *Porichthys notatus* are summarized in Table 2. Only the administration of luciferinol resulted in the development of photophore fluorescence and bioluminescence capability. The bioluminescent response to the 20 μg dose of luciferinol was of very weak intensity (mean = $3·4\times10^{9}$ quanta s^{-1}), considerably lower than that resulting from a single feeding of 6·4 μg of purified *Vargula* luciferin (mean = $2·2\times10^{10}$ quanta s^{-1}) (Thompson *et al.* 1987).

Discussion

It seems remarkable that a small amount (6·4 μg) of luciferin from a wholly unrelated organism should induce bioluminescence capability in nonluminescent

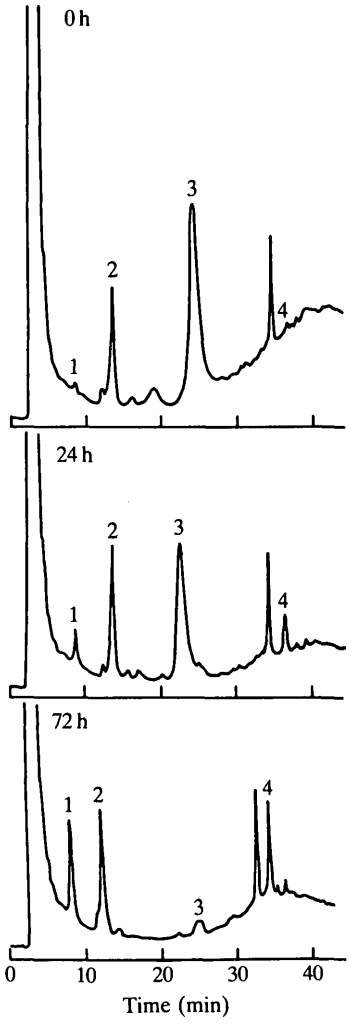

Fig. 3. HPLC elution profiles showing the formation of air-oxidation products at room temperature of *Vargula* luciferin with time. At time zero, luciferin was dissolved in absolute methanol and placed in a closed vial; subsequently, identical samples containing 1·0 μg of the initial concentration of luciferin were injected into the column at time 0, 24, and 72 h. 1, etioluciferin; 2, oxyluciferin; 3, luciferin; 4, luciferinol. The identity of each peak was confirmed by running authentic samples using the same column. Column, 25 cm × 4·6 mm i.d.; Altex Ultrasphere ODS (5 μm); temperature, ambient; flow rate, 1 ml min^{-1}; detector, 254 nm. A gradient elution programme was used: 0–10 min, MeOH–H_2O (1:1); 10–40 min, linear gradient to MeOH–H_2O (9:1); 40–42 min, MeOH–H_2O (9:1). Ammonium acetate was held constant at 0·2 mol l^{-1} throughout.

Table 2. *Administration of* Vargula *luciferin oxidation products and analogues to nonluminescent Puget Sound* Porichthys notatus

Compound	No. of fish*	μg/fish	Days after administration† 7 F	7 B	14 F	14 B	21 F	21 B	42 F	42 B
Oxyluciferin	6	20	−	−	−	−	−	−	−	−
Etioluciferin	6	20	−	−	−	−	−	−	−	−
Luciferinol	4	20	+	+	+	+	+	+	+	+
Analogue (V)‡	4	25	−	−	−	−	−	−	−	−
Analogue (VI)‡	4	25	−	−	−	−	−	−	−	−

*For each compound tested, half of the Puget Sound *Porichthys notatus* received the compound orally and half by intraperitoneal injection. Response to the compounds was the same by either method.

†F and B indicate tests for photophore fluorescence and bioluminescence capability, respectively. Fish receiving luciferinol, when tested monthly, measured positive for a period of 1 year.

‡Structures of the analogues are shown in Fig. 2.

Porichthys notatus lasting undiminished for more than 2 years (Thompson *et al.* 1987). No other compound tested thus far, except *P. notatus* luciferin itself, has ever induced bioluminescence capability in nonluminescent *P. notatus*. The result is all the more impressive since *Vargula* luciferin is highly unstable (Fig. 3) and is rapidly autoxidized in aqueous solutions with loss of activity occurring in a matter of days (Tsuji, 1955; Shimomura *et al.* 1957; Haneda *et al.* 1961). The finding that more light is produced in *P. notatus* than can be accounted for by the amount of luciferin administered suggests that luciferin either recycles or is synthesized *de novo* in the fish (Thompson *et al.* 1987).

Vargula luciferin was labelled in the guanido group (R_2, Fig. 4) because it is essential for full activity with *Vargula* luciferase (Goto, Isobe, Coviello & Kishi, 1973) and is not altered on oxidation of luciferin. The present observation of an absence of dilution of [^{14}C]luciferin during the 7-week experimental period suggests that a recycling mechanism, rather than *de novo* synthesis, is responsible for the long-lasting bioluminescence capability of *Porichthys notatus*. Alternatively, it could be argued that the administered luciferin is incorporated and stored in the photophores and that the specific activity of the recovered luciferin simply reflects that of the administered luciferin. Considering the above-mentioned instability of luciferin, this seems unlikely, unless a mechanism is present to stabilize the luciferin from autoxidation. The recent observation that luciferin remains in the blood of the induced fish at a steady level for many months (Thompson, Nafpaktitis & Tsuji, 1988) suggests that blood luciferin is in equilibrium with photophore luciferin and that luciferin turns over in *P. notatus*. Studies by Cormier *et al.* (1967) and our own studies have shown that there are no large stores of luciferin in the liver or any other tissue of *P. notatus*. When 200 μg of

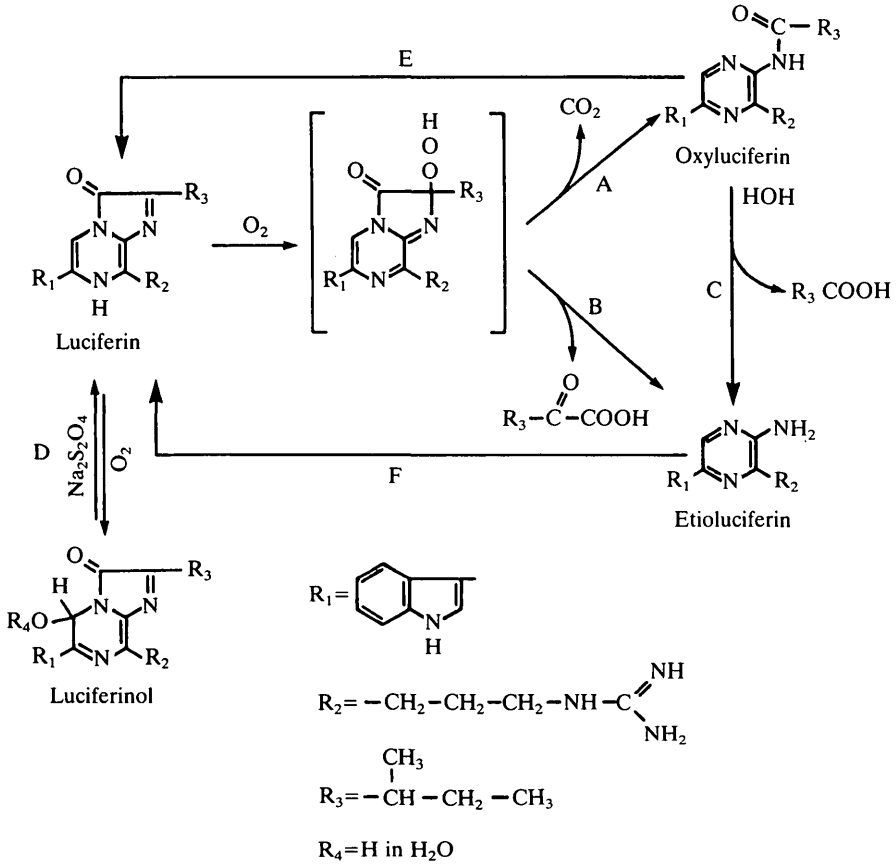

Fig. 4. Pathways for the oxidation of *Vargula* luciferin and hypothetical routes for its resynthesis. The ratio of luciferin oxidized by pathways A and B is approximately 7:1 for the *in vitro* bioluminescence reaction, compared with 1:1 for the chemiluminescence reaction in diglyme or acetone (Shimomura & Johnson, 1971). Further details are discussed in the text.

TLC-purified native *Vargula* luciferin (Tsuji, 1978) was administered *via* the gut to each of three Puget Sound *P. notatus*, the amounts of luciferin found 34 days later in pooled tissues were as follows: liver, undetectable; subocular gel, undetectable; gonads, 8·9 ng; and gall bladder, 4·5 ng (E. M. Thompson & F. I. Tsuji, unpublished observations).

The pathways involved in the bioluminescent and chemiluminescent oxidation of *Vargula* luciferin are shown in Fig. 4. Pathway A is the luciferase-catalysed bioluminescent reaction leading to the formation of oxyluciferin and CO_2 (McCapra & Chang, 1967; Stone, 1968), whereas pathway B is the chemiluminescence reaction in a hydrophobic medium such as diglyme leading to the formation of etioluciferin and α-keto acid (Shimomura & Johnson, 1971). Pathway C is the hydrolytic reaction in which luciferase, acting as a hydrolase, slowly hydrolyses oxyluciferin to etioluciferin and the corresponding aliphatic acid (Shimomura, Johnson & Masugi, 1969). Pathways E and F are possible routes

whereby luciferin could be resynthesized from either oxyluciferin or etioluciferin. Pathway E was previously investigated by administering air- and luciferase-oxidized *Vargula* luciferin to Puget Sound *Porichthys notatus* with negative results (Barnes *et al.* 1973). The present finding of a lack of response to synthetic oxyluciferin confirms these results and indicates that induction and maintenance of bioluminescence capability cannot be the simple recycling of oxyluciferin. Recycling by pathway F also does not appear to take place since synthetic etioluciferin did not induce bioluminescence capability.

The observation that neither oxyluciferin nor etioluciferin induced bioluminescence capability seems paradoxical. It is conceivable that the relatively long latency period (3–4 days) (Tsuji *et al.* 1972; Barnes *et al.* 1973) that exists between the administration of the luciferin and the first sign of luminescence capability in *Porichthys notatus* is involved in turning on the production of enzymes required for resynthesizing luciferin from either oxyluciferin or etioluciferin; that is, *P. notatus* must first be 'primed' with luciferin if the administered oxyluciferin or etioluciferin is to be recycled. The failure of *P. notatus* to utilize oxyluciferin and etioluciferin could also mean that the molecules are not able to penetrate to the site of luciferin resynthesis. Since oxyluciferin, etioluciferin and luciferin analogues V and VI (Fig. 2) are fluorescent compounds, the absence of photophore fluorescence also means that these compounds are not incorporated by the photophores. Moreover, the lack of uptake of analogue V, which differs from *Vargula* luciferin only by a guanido group, further indicates that the fish possesses a high degree of specificity for *Vargula* luciferin. Administration of 20 μg of luciferinol did induce bioluminescence capability, but this response was 10 times less intense than that produced by a single feeding of 6·4 μg of *Vargula* luciferin (Thompson *et al.* 1987). This suggests that some of the luciferinol may have been reduced to luciferin (pathway D) in the fish and then incorporated into the photophores since luciferinol itself is nonfluorescent and inactive with *Vargula* luciferase.

A further possibility that cannot be ruled out by the present results is that ingested luciferin somehow triggers *de novo* synthesis of luciferin, for example by gene activation, but because *de novo* synthesis may be a very slow process, or may only be turned on at certain seasons, such as during reproductive activity, it was not detectable by the present method.

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