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Daily Growth Increments in Otoliths of Starry Flounder (*Platichthys stellatus*) and the Influence of Some Environmental Variables in Their Production

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Tetracycline injected into juvenile starry flounders (*Platichthys stellatus*) was incorporated into the periphery of the sagittal otoliths within 24 h. The resulting band, visible under ultraviolet light, was used as a dated mark on the otolith growth increments. This technique was used to verify that increments were laid down on a daily basis, both in field and laboratory environments. Subdaily increments were visible in otoliths of fishes reared under most environmental conditions. The production of daily increments in juvenile starry flounders preconditioned to a natural environmental regime was unaffected by photoperiod or temperature fluctuation, suggesting the presence of an internal circadian rhythm.

Key words: starry flounder, *Platichthys stellatus*; otoliths, daily rings, growth increments, circadian, tetracycline

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La tétracycline injectée à de jeunes plies du Pacifique (*Platichthys stellatus*) pénètre à la périphérie de la sagitta de l'otolithe en moins de 24 h. La bande qui en résulte et qui est visible à la lumière ultraviolette a servi de marque de date connue sur les zones de croissance de l'otolithe. Grâce à cette méthode, on a pu vérifier que les zones de croissance sont déposées sur base quotidienne, tant sur le terrain qu'en laboratoire. Chez des poissons élevés dans la plupart des milieux, on observe des zones de croissance presque quotidiennes. La production de zones de croissance quotidienne chez des plies du Pacifique acclimatées au préalable à un milieu naturel n'est pas influencée par la photopériode ou des variations de température, ce qui laisse supposer un rythme circadien.

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THE existence of daily growth increments on otoliths of marine and freshwater fishes has been both suggested (Schmidt and Fabrizio 1980; Steffensen 1980; Townsend 1980) and confirmed (Pannella 1971; Brothers et al. 1976; Struhsaker and Uchiyama 1976; Taubert and Coble 1977; Radtke 1978; Wild and Foreman 1980; Wilson and Larkin 1980). These concentrically formed increments have considerable potential for the exact determination of hatching dates and early life history growth rates of wild fish populations. However, daily increments are not always produced in some species (Wild and Foreman 1980) and at some ages (Pannella 1971), making the verification of the increment to age relationship essential before its application to the aging of wild populations.

Most daily increment verification studies to date have employed laboratory-reared fish of known hatching date. However, many species, including the starry flounder (*Platichthys stellatus*), are not easily reared from hatching in a laboratory environment. In addition, cyclical laboratory events may influence growth increment formation. As a result, laboratory results may not be applicable to wild populations. In a series of experiments, Taubert and Coble (1977) concluded that an internal biological clock entrained by a 24-h light–dark cycle was responsible for daily increment formation in young mouthbrooders. In contrast, Brothers (1978) regarded temperature fluctuation as the key factor to the timing of increment deposition in temperate stream fishes. Photoperiod and feeding were considered to be less significant as increment inducers. The possibility of an endogenous rhythmicity was not discussed.

Tetracycline application to a fish at a known date can be used in age validation studies (Holden and Vince 1973) and as a temporal mark in growth studies (Wild and Foreman 1980). Tetracycline is known to be incorporated into calcifying tissues in a fish during growth, depositing a band fluorescent under ultraviolet light (Weber and Ridgway 1967; Meunier and Boivin 1974). We used the tetracycline band as a temporal mark, first of all to verify the existence of daily growth increments in juvenile starry flounders, both in the laboratory and in situ, and secondly, to test the current suggestions on the role of possible environmental modifiers on the deposition of otolith daily growth increments.

Materials and Methods

Intraperitoneal injection of oxytetracycline hydrochloride (100 mg tetracycline/kg fish) resulted in deposition of a UV-fluorescent band on the sagittae of the flounders. Injected volume was 0.025 mL. Although similar results were achieved by immersing the fish in a 0.02% oxytetracycline/saline solution for 1 d, the experiments described herein were carried out by means of an injection. All experimental fish in this study were 4–8 cm in standard length and ~ 8 mo old.

To be effective as an accurately dated marker, the tetracycline must be incorporated into the otolith soon after application. To determine how quickly tetracycline was incorporated into the otolith, 12 flounders were injected and killed after 0, 6, 10, 24, and 48 h. Left- and right-hand-side sagittae were removed immediately and processed (see following paragraphs).

For the in situ experiment, 25 flounders were collected at the mouth of the Nooksak River in Bellingham Bay, Washington, on October 10, 1980. The fish were injected and placed immediately in a 3.1 × 3.1 × 1 m mesh enclosure in a tidal channel of the estuary. Five control fish were collected by seine and eight fish were sampled from the enclosure on October 24, 1980; further sampling was impossible because of flood waters. Unmarked control fish served to check for naturally occurring fluorescence not induced by tetracycline. Left and right sagittae were removed from the fish the same day, brushed clear of tissue, and attached sulcus-side down with instant glue onto a standard microscope slide. Slides were stored in darkness until processing. Dark storage for up to 4 mo resulted in no reduction of fluorescence intensity.

For the laboratory experiments, wild-collected flounders were first reared in the laboratory for 2 mo under a natural light–dark cycle. Five aquaria in separate, light-proof cubicles were stocked with 20 flounders each. Experimental conditions for each aquarium were designed to test the effect of photoperiod and temperature fluctuation upon increment deposition. Temperatures were selected to approximate those measured in situ. Twenty-four and 36-h photoperiods were tested at constant temperature, with the latter also tested in conjunction with a 36-h temperature cycle. Constant light conditions with a 36-h temperature cycle were used to examine the effect of temperature alone. If the periodicity of either variable is related to increment timing, the 36-h cycle should result in fish with 67% of the increments visible under a 24-h cycle. Fish were also exposed to constant conditions for each variable, in the event that fluctuation of either environmental variable entrained an endogenous circadian rhythm. All experimental conditions are detailed below:

1) photoperiod of 13 h light:11 h dark (i.e. a natural light regime); constant temperature at 15°C (= 13L:11D/CT).

2) 24 h light; constant temperature (= 24L/CT).

3) 24 h light; 24 h at 15°C:12 h at 18°C (= $24L/24T_1:12T_2$).

4) 24 h light:12 h dark; constant temperature (= 24L:12D/CT).

5) 24 h light:12 h dark; 24 h at 15°C:12 h at 18°C (= $24L:12D/24T_1:12T_2$).

Temperatures were monitored with a continuous temperature recorder accurate to 0.25°C. During temperature changes, the final temperature was reached less than 2 h after initiation. All lighting was fluorescent. One satiation feeding with live *Tubifex* and frozen zooplankton was given at a random time during a 24-h period, with some periods omitted totally to avoid any entrainment of daily growth patterns due to feeding periodicity. All fish were acclimated to experimental conditions for 2 wk prior to tetracycline injection. Five fish from each aquarium were sampled within 1 d of injection, with the remainder of the fish sampled 25–26 d and 43–47 d after injection.

Mounted sagittae were ground to a plane where the peripheral growth increments were most visible. Otolith slides were mounted on a grinding apparatus (Neilson and Geen 1981) coupled to an automated rotater and ground on metallurgical lapping film (3 μm). Final preparations were superior to those made by hand on sintered glass with aluminum oxide, as the grind was finer and on a more even plane.

All prepared otoliths were examined and photographed at 500×–1250× under both white and ultraviolet light on a

Leitz Orthoplan fluorescence microscope with a 35-mm camera attachment. We used excitation filter bands from 450 to 490 nm and barrier filters at 510 and 515 nm.

To determine the position of the fluorescent band relative to the growth increments visible under white light, we photographed each otolith in a paired sequence: once under bright-field illumination and once under UV light. The otolith position and focus were not adjusted within a photograph pair sequence. Developed negatives were mounted directly into projector slides. Increment counts were made from the projected image. We defined an increment as a bipartite structure. Under transmitted light, it consisted of a narrow, opaque band and an adjacent wider, translucent region. Both major and total increment counts were made along the long axis of the sagittae. Major increments were generally easily distinguished on the basis of their continuity, intensity, and regularity of spacing. Minor increments differed, in that they often merged with other increments or were significantly lighter and thinner in appearance than the surrounding increments. The presence and spacing of increments in some otoliths was verified with a Perkin–Elmer Autoscan scanning electron microscope after etching the otoliths with 1% HCl for 90 s and coating with gold.

All counts were replicated at least three times in a random sequence by each author individually. Agreement within 10% between readers was considered acceptable and the mean taken; all questionable counts and/or preparations were removed from further analysis. These rejections comprised 3% and 9%, respectively, of the total number of otoliths.

Otolith anomalies that could conceivably affect the final increment count were noticeable in 10–20% of the light and electron microscopy photographs. These anomalies included the existence of multiple-growth foci leading to growth increments that merged with others and discontinuous increments. In two cases, these anomalies could have significantly altered the eventual increment count; these otoliths were rejected.

Results and Discussion

Tetracycline incorporation into the otolith was evident in 97% of the flounders injected. In each of those left alive at least a month after injection, the resulting fluorescent band spanned a minimum of five major increments and generally 14–20.

Incorporation into the otolith began in less than a day. A fluorescent band was visible in 50% of the fish, 10 h after injection, and in 100% of the fish, 24 h after injection. Therefore, the proximal edge of the band was considered to be located within one or two increments of the otolith periphery at the time of injection. A thin fluorescent line often associated with the ground otolith/glue interface was easily distinguishable from the fluorescence due to the tetracycline.

Both major and total increment counts were made from the proximal edge of the tetracycline band to the periphery of the otolith. Growth increments in left and right sagittae were counted separately for each fish. However, the mean of the two sagittae was used in the data analysis, as the two sides were not significantly different (95% confidence level). The width of the major increments varied between 0.9 and 3.4 μm, depending on the individual fish and location on the

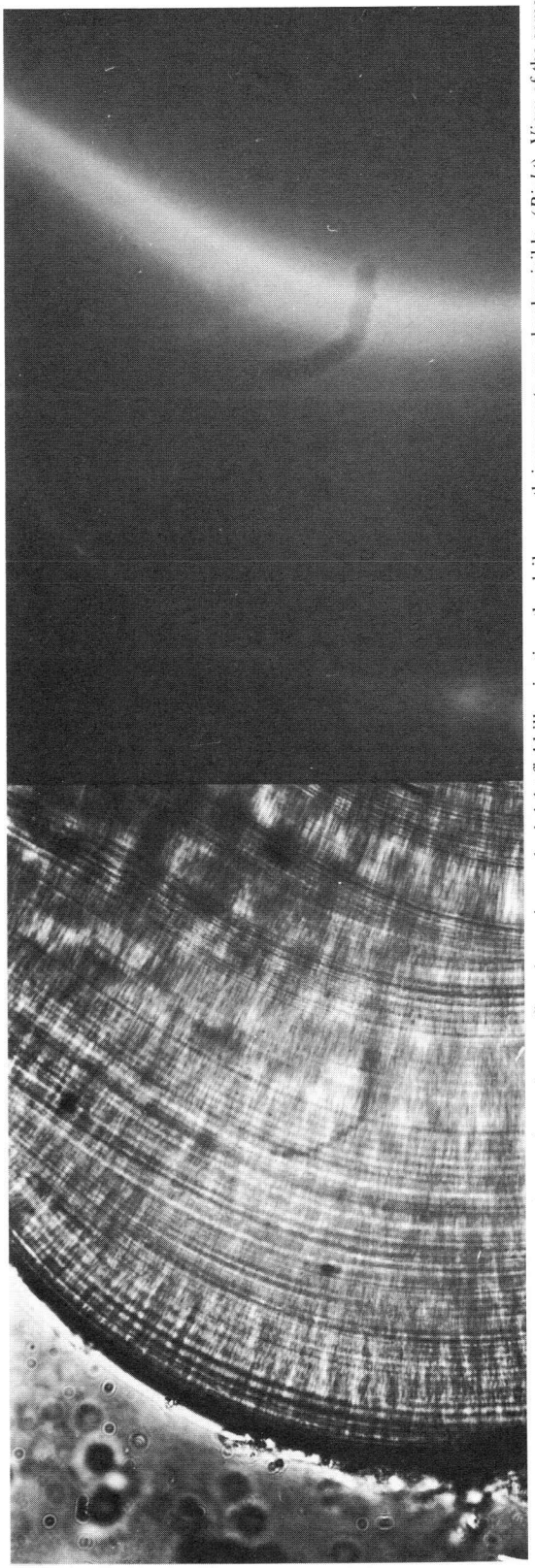

FIG. 1. (*Left*). Peripheral view of a ground, experimental starry flounder sagitta under bright field illumination; the daily growth increments are clearly visible. (*Right*). View of the same sagitta under ultraviolet light. The fluorescent band is due to incorporated tetracycline, whose location can now be associated with specific increments visible in *left* view. The flounder was sampled 45 d after tetracycline injection. Bar = 20 μm.

TABLE 1. Regression coefficient (b), standard error (SE), and corresponding R^2 value for the regression of major increment count on the number of days elapsed, for each of the experimental and in situ environments.

Expt.[a]	No. of fish	b	SE	R^2
In situ	13	1.06	0.06	0.96
13L:11D/CT	10	1.04	0.07	0.96
24L/CT	20	0.90	0.08	0.89
$24L/24T_1:12T_2$	16	0.90	0.06	0.93
24L:12D/CT	15	0.99	0.09	0.90
$24L:12D/24T_1:12T_2$	10	0.96	0.06	0.97

[a]L = light hours; D = dark hours; CT = constant temperature; $T_1 = 15°C$; $T_2 = 18°C$.

otolith. Minor increments numbered up to three between adjacent major increments; scanning electron microscope measurements indicated that these could be as narrow as 0.25 μm.

If the major growth increments on the otolith are laid down on a daily basis, a slope (b) of one should be obtained in the regression of major increment count on the number of days elapsed since tetracycline injection. All experimentally conditioned fish, as well as the fish maintained in situ, produced otoliths with a day:major increment slope not significantly different from one at the 95% confidence level (Table 1). We conclude that daily increments were laid down both in situ and in the experimental situations studied. Daily increments can be seen in the sagitta of Fig. 1. The major increments are daily, making the minor increments subdaily in nature. The laboratory data were then pooled as the slopes were not significantly different and Bartlett's test indicated homogeneity of variance between the experiments. Figure 2 shows the pooled laboratory daily increment data. The field data could not be pooled with the laboratory results, despite their similar slopes, because of the significantly lower variance of the former. The decreased variance was at least partially due to the relatively short time period between injection and sampling in situ. Both the field and laboratory results confirm a previously unverified observation of daily growth increments in starry flounders (Wilson and Larkin 1980).

Our results indicate that daily increments are formed in the sagittae of juvenile starry flounders under a variety of experimental conditions, as well as under a natural environment. This study also supports the view that unnatural light or temperature stimuli are not likely to induce a significant deviation from a natural daily increment pattern in postlarval fish preconditioned to a natural environmental regime. In starry flounders at least, laboratory observations of daily increment patterns in juveniles can probably be extrapolated to the natural situation.

The variance associated with the increment counts is somewhat higher than that reported in most previous publications. Although some of this may be attributable to reader error and poor otolith preparation, there appears to be a large species effect present in the integrity of increment deposition rate. Identical preparatory and reader counts made on threespine stickleback (*Gasterosteus aculeatus*) sagittae resulted in a much lower level of intraspecific variance (Campana unpublished data). This fact indicates that flounders produce an average of one increment per day, but large individual variations in production rates do occur. The reason for this variation is not clear, but does not appear to be common to many species.

Suggestions that temperature fluctuation (Brothers 1978) or photoperiod (Taubert and Coble 1977; Radtke 1978) might be the major factors controlling increment periodicity were not supported by our results. Thirty-six-hour "days" of the two conditions, even in conjunction, were ineffective in altering the production of one major otolith growth increment per 24 hours, averaged over a number of individuals. Similarly, constant temperatures and photoperiods had no visible effect on daily increment production indicating that neither environmental stimulus is mandatory for the maintenance of a 24-h periodicity in otolith growth, at least in flounders of several months of age. This is not to say that light or temperature can have no effect on increment deposition, but that a circadian rhythm is maintained despite their influence. It is possible that larval fish would be more susceptible to abnormal photoperiod or temperature cycles, as all of the flounders studied here were juveniles and therefore preconditioned to a natural light–dark cycle. If such were the case, these results would not be inconsistent with Taubert and Coble's (1977) observations. However, J. D. Neilson and G. H. Geen (1982, unpublished data) have reared chinook salmon (*Oncorhynchus tshawytscha*) eggs and alevins in total darkness and observed clear daily increment production in all cases.

The possibility that fish possess an internal, diel clock has been suggested before (Gibson et al. 1978; Eriksson and van Veen 1980). In mammals, endogenous 24-h rhythms are often entrained by a cyclic photoperiod, although a circadian rhythm persists under constant light or dark (Kramm 1980; Lynch et al. 1980). Taubert and Coble (1977) noted that larval sunfish (*Lepomis*) responded to an unnatural photoperiod by the production of nondaily growth increments that were not correlated with the relative "day" length. Therefore, they concluded that an endogenous biological clock, using photoperiod as a zeitgeber, was in operation. The results of our study differ, in that a 24-h periodicity in increment production was continued despite a 36-h photoperiod or constant light conditions. This inconsistency may be due to the different ages of the fishes used. However, our evidence does support the hypothesis that certain fish species possess an internally regulated circadian rhythm. The diurnal feeding and locomotory behavior in starry flounders supports this suggestion (Campana unpublished data). In addition, an endogenous periodicity is not contrary to the concept of variance associated with increment counts. Large individual variations in the expression of endogenous rhythms have been demonstrated in other fish species (Godin 1981).

A measure of the incidence of subdaily increments was given by total increment counts. A high degree of variability was associated with total counts, possibly resulting from the difficulty of viewing the subdaily increments and potential confusion with optical artifacts (Fig. 3). The slopes for the various experimental conditions using total counts were not significantly different (95% confidence level). However, the results did seem to indicate a high incidence of subdaily increments under a natural photoperiod regime (13L:11D/CT) ($b = 1.80$: SE = 0.24) and virtually none under conditions of constant light and temperature (24L/CT)

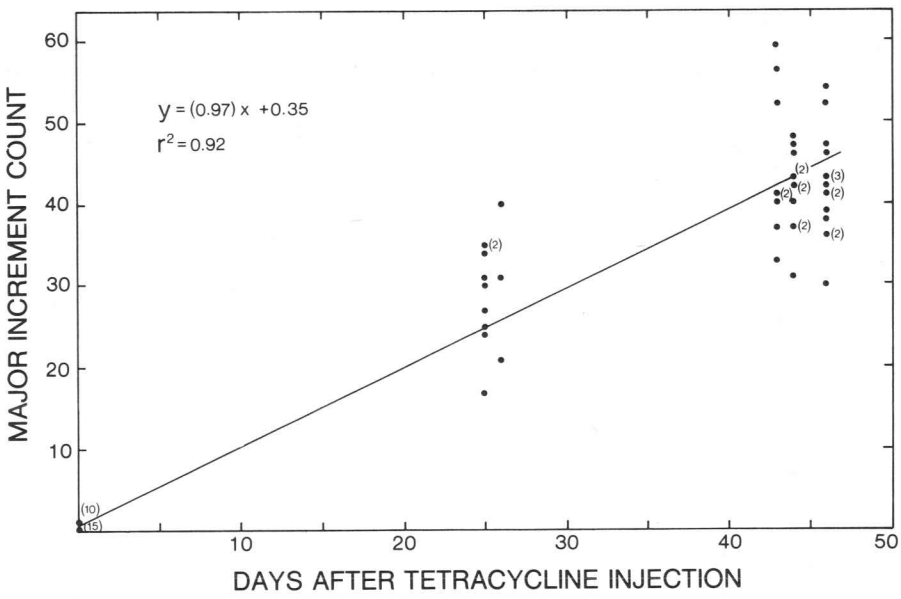

FIG. 2. Counts of major otolith growth increments distal to the origin of the fluorescent tetracycline band. Data are pooled from all laboratory environments.

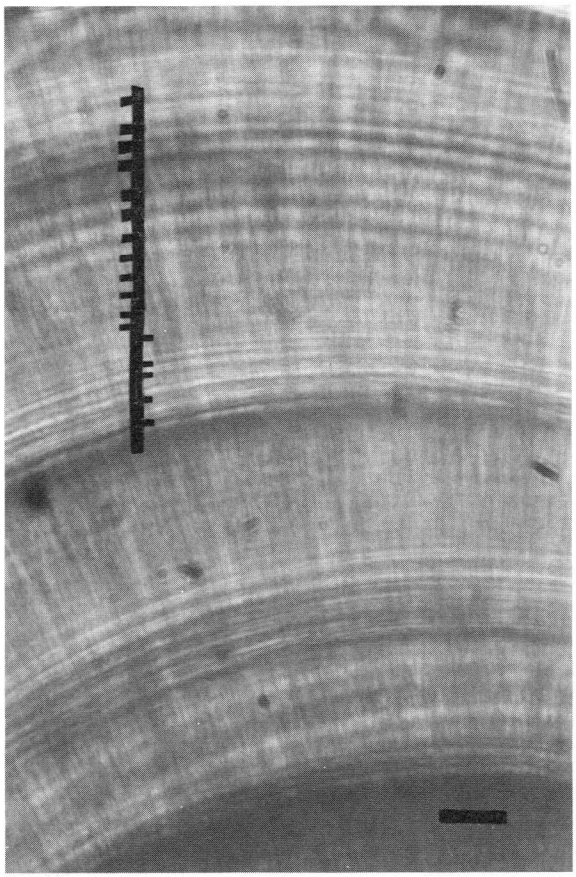

FIG. 3. Ground sagitta from an experimental starry flounder. Fine subdaily increments can be seen between the indicated daily increments. Bar = 10 μm.

(b = 0.99 : SE = 0.17). The in situ and other experimental fish were intermediate in their incidence of subdaily increments. Growth rates did not vary between experiments and therefore could not be implicated in the incidence of subdaily increments.

The variation in number of subdaily increments between experimental treatments was of interest. Although the difference was not significant by t-test ($0.05 < P < 0.10$), constant light conditions produced only 13% of the subdaily increments produced under a normal photoperiod. Subdaily increments were also observed in the fish kept in the outdoor enclosure. Subdaily patterns have been noted by other researchers (Taubert and Coble 1977; Brothers 1978), but the reasons behind their formation remain obscure. Both temperature cues (Brothers 1978) and feeding frequency (Neilson and Geen unpublished data) have been implicated in their formation.

The value of tetracycline for growth increment studies is enhanced by its rapid incorporation into the otolith, as reported here and elsewhere (Meunier and Boivin 1974). However, our results were not consistent with other observations of total incorporation over a single day in tuna (Wild and Foreman 1980). Tetracycline bands were generally 14−20 increments wide in this study; as sagittal growth occurs concentrically with increments added through time, tetracycline incorporation must be occurring over a 14- to 20-increment time period. Kobayashi et al. (1964) also noted that dual tetracycline injections remained distinct only when a month or more was alloted between applications. The discrepancy may be due to the higher metabolic rate of tuna relative to flounders and goldfish (*Carassius auratus*). Nevertheless, if the proximal edge of the band is accepted as corresponding to the date of injection, the width of the band is irrelevant, as the origin is accurately dated.

The tetracycline technique we used is one approach to confirming daily increment existence in wild fish, or in fish

of unknown age. Daily growth increments have been verified in only a few fish species. However, their occurrence has been suggested in numerous other species (Pannella 1971, 1974; Brothers et al. 1976; Wilson and Larkin 1980). The method outlined here provides a means of corroborating the occurrence of daily growth increments.

In those fish species where otolith daily increments are shown to exist, counts should prove useful in confirming the nature of annuli or spawning checks on scales or otoliths. Similarly, they can be used to determine the hatching dates of natural populations. Such information is valuable for the study of many species where spawning grounds or times are unknown. A precaution is necessary in that daily increment production may not persist in otoliths of older fish (Pannella 1974; Campana unpublished data). Also, the time of first increment formation in relation to the hatching date would have to be determined. Perhaps more importantly, the close correspondence between otolith length and fish length (Struhsaker and Uchiyama 1976; Taubert and Coble 1977; Wild and Foreman 1980) suggests that the distance between increments may be an accurate measure of daily growth in some cases. Such a measure would prove invaluable to the study of larval fish populations and is a suitable topic for future research.

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