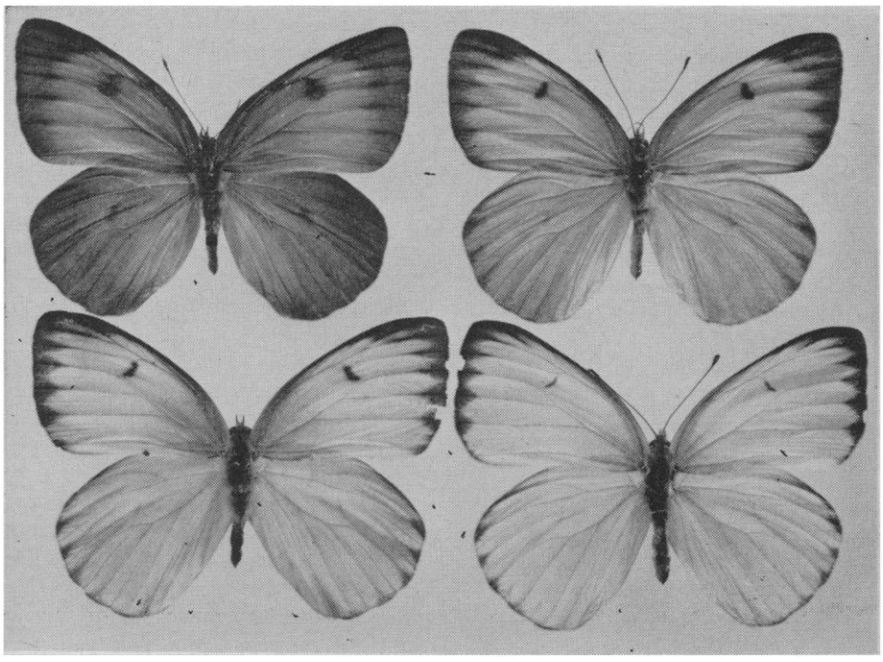

Fig. 1. Variation in pigmentation of female *Ascia monuste*. (Top row) black (16 hours light) and gray (16 hours light). (Bottom row) light gray on left (16 hours light) and white on right (8 hours light).

butterflies that were produced, all 16L/-8D females were black (nine individuals), gray (five), or light gray (three), while all 8L/16D females (16 individuals) were white (Fig. 1). (All males—28 under 16L/8D and 16 under 8L/-16D—were white.) Eight of the nine black females were offspring of female XI and have a violet cast which the remaining female lacks. Segregation for two categories occurred among offspring of two parents. The black, gray, and light gray categories intergrade.

Not all of each seven-egg lot survived to produce pupas. Crowding is apparently not a factor in this experiment, since white females emerged from lots with seven, five, four, and one larvae surviving, while black females emerged from lots with seven, six, and four survivors.

A qualification must be made in evaluating the experiment. The light unit in the 8L/16D box caused the daytime temperature to reach a peak of 87°F even with the fan and system of baffles. A higher temperature was thus associated with the shorter day. In the field, darker females are frequent in the spring, summer, and fall, but some white females are present during these seasons. A sample of 99 females taken at Jupiter Island, Florida, on 30 July 1961 by Thomas Pliske of Amherst College included 56 melanics, 33 intermediates, and 10 whites. White females are the only type found from November to February (*5*). Since long day and high temperature are associated with the same effect under natural conditions as long day and lower temperature in the laboratory, day length seems a more likely cause of the response than temperature.

A single experiment with only two alternative conditions will not establish whether photoperiod, intensity of light, or total amount of light per day is decisive in causing dark and white forms. Therefore, the contribution of each of these factors cannot be separated from the others on the basis of this experiment.

Dark females are not restricted to Florida, but they are more common there at the proper time than in any other area (*6*). South American populations do not have the dusky females. The environmental conditions required for production of the dark form certainly should occur in the Southern as well as the Northern Hemisphere. It is suggested that the South American subspecies lacks the capacity to produce it.

A hypothesis explaining both the experimental results and the occurrence of melanics in Florida is the following. The capacity to produce melanic females in response to the proper environmental stimulus is conferred by a gene or genes. The gene frequency is high in Florida but not at fixation, since a few white females occur at all times. Light is implicated as a factor stimulating the dimorphism, but the experiment does not exclude the possibility that other factors such as starvation, crowding, or condition of food plant may be important, although they are not relevant to the described experiment. Thus, the question may be asked whether the dark pigment is laid down in response to a single factor (an environmental induction) or in response to any factor which influences development sufficiently.

The adaptive significance of the dark and white forms remains obscure. The critical stage in development during which the organism is most sensitive to light effects is not known. However, since the adult wing pigment is laid down in the pupa, this stage is suspect (*7*).

ROGER W. PEASE, JR.
Department of Biology,
Yale University,
New Haven, Connecticut

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Photoautotrophy in Gymnodinium breve Davis

Abstract. Pure cultures of the Florida "red-tide" flagellate required light and carbon dioxide for growth. Multiplication in darkness was not supported by any of a number of organic compounds and mixtures. The ecological importance of micronutrients is suggested.

The catastrophic mortalities of marine animals associated with dense populations of the dinoflagellate, *Gymnodinium breve* Davis, have occasioned considerable interest in the biological requirements of this organism (*1*). Sev-

eral environmental factors have been suggested as agents contributing to "red-tide" outbreaks which recur along the Florida Gulf coast. Such phenomena have been more closely associated with extended periods of heavy rain than with any other condition (*2*). In view of the prevalence of organic soils in this locality (*3*), and the fact that aqueous extracts of organic soil promoted growth in the first successful cultures of *G. breve,* it seemed that river discharge, representing soil drainage, might be "instrumental in initiating" blooms of this organism (*4*). Wilson and Collier also suggest that decaying red-tide-killed fish "imparted nutrients that helped to perpetuate the bloom" (*4*). In the light of references to the widespread occurrence of heterotrophy among dinoflagellates (*5*), it seemed appropriate to determine whether the organic content of Florida river waters or coastal fishes might provide a direct source of energy for multiplication in *G. breve.* Accordingly, my report represents an attempt to define the heterotrophic capacities of this organism.

Axenic, 10-ml cultures of the flagellate were prepared in screw-capped tubes, using about 200 motile cells for inoculum, and a virtually inorganic, artificial sea water medium (*6*). Since the basic medium permits neither multiplication nor survival of *G. breve* in darkness, it was assumed that the degree to which added substances could serve as energy sources would be related to their abilities to support multiplication in the dark. The extreme sensitivity of this organism renders the incidence and rate of multiplication somewhat unpredictable, even in replicate cultures. This type of variation in results was avoided by starting all cultures with the basic medium only and illuminating them (500 ft-ca) until successes and failures were obvious at 10 × magnification. Cultures showing comparable growth (at least a tenfold population increase within 4 weeks) were then selected for use in this work. Aseptic additions of the organic compounds were made to all except control tubes, and the cultures were placed in darkness. An identical series of enriched and unenriched cultures was illuminated as above. All cultures were maintained at 25° ± 1°C. Estimates of population growth and survival were made microscopically.

One of the Florida soil extracts tested (prepared as described in (*4*) from soil obtained in the Fort Myers area) had also been used in natural Florida sea water media, in which it aided multiplication of *G. breve* in illuminated cultures. Each soil extract was tested at concentrations of 10 and 100 ml/liter. The fish extracts represented a filtered, 10-percent composite homogenate of muscle tissue from anchovy (*Anchoa mitchilli*), sand seatrout (*Cynoscion arenarius*), spot (*Leiostomus xanthurus*), Atlantic croaker (*Micropogon undulatus*), and striped mullet (*Mugil cephalus*), all common inhabitants of Florida west coast waters. This type of extract (either fresh or aged 3 days with uninhibited bacterial growth) was tested at a concentration of 5 ml/liter. The other organic substances were tested at several concentrations (0.1, 1.0, and 10.0 mg/liter) chosen to include and exceed the usual range of carbohydrate and total tyrosine concentrations found in Florida coastal water (*7*).

Table 1. Organic substances tested as potential energy sources for *Gymnodinium breve.*

Substance	Substance
Carbohydrates and related compounds	*Lipids (Contd.)*
Arabinose	Butyrate
Ribose	Valerate
Xylose	Palmitate
Galactose	Stearate
Glucose	Oleate
Mannose	Linoleate
Fructose	
Sorbose	*Nitrogen compounds*
Rhamnose	Urea
Cellobiose	Alanine
Lactose	Aspartate
Maltose	Glutamate
Melibiose	Serine
Sucrose	Asparagine
Trehalose	Creatine
Melezitose	Adenine
Raffinose	Gelatin
α-methyl glucoside	
Glycogen	*Growth factors*
Inulin	Ascorbic acid
Pectin	Dehydroascorbic acid
Soluble starch	Biotin
	Cobalamin
Alcohols	Thiamine
Ethanol	Kinetin
Butanol	
Dulcitol	*Miscellaneous compounds and mixtures*
Mannitol	Beef blood serum
Sorbitol	Casamino acids
Ethylene glycol	Casein hydrolyzate
Glycerol	Coconut milk
	Egg yolk
Glycolytic and citric acid cycle constituents	Fish extract
Glucose-1-phosphate	Liver concentrate
Fructose-6-phosphate	Malt extract
Phosphoglycerate	Mangrove extract
ATP	Milk
Citrate	Milk protein hydrozyate
Pyruvate	Oxalate
Isocitrate	Peptone
α-ketoglutarate	Peptone-iron
Succinate	Thioglycollate
Fumarate	Tartrate
Malate	Tryptone
Oxaloacetate	Florida soil extracts (Ft. Myers) (St. Petersburg)
Lactate	Texas soil extract (San Antonio)
Lipids	Yeast extract
Formate	
Acetate	
Propionate	

None of the organic compounds or mixtures (Table 1) supported multiplication or permitted extended survival in darkness. The longest survival was in the valeric acid cultures, which showed a 90-percent population reduction in 2½ weeks. No population increase was observed, however, in six replicate newly inoculated cultures containing valeric acid. All other cultures, both control and enriched, lost at least 90 percent of their initial populations in 1 to 2 weeks. Most of the identically enriched illuminated cultures did not decrease in population size during the same period, thus indicating no inhibitory effect for most of the compounds tested. The four exceptions contained 10 mg of linoleic acid, sodium oleate, sodium stearate, or kinetin per liter. Attempts to facilitate gas exchange by using very loosely packed sterile cotton plugs in place of the regular screw caps failed to extend survival without light. Attenuation of light over a period of several days, when introducing experimental cultures to darkness, also failed to alter these results. Furthermore, removal of CO_2 from the medium by suspending 0.2 ml of 10 percent KOH in the gas phase of established cultures and sealing the tubes with paraffin prevented multiplication or survival of *G. breve* in illuminated cultures. (Similarly sealed cultures with 0.2 ml of distilled water suspended in the gas phase showed no change in population.)

One must conclude that this dinoflagellate is primarily photoautotrophic, requiring light and CO_2 for growth and survival. The present findings parallel those of Barker and of Provasoli and co-workers, who did not find organic metabolic substrates capable of supporting growth of photosynthetic dinoflagellates in darkness (*9*). The growth-stimulating effects of thiamine, biotin, and cobalamin in illuminated cultures (*8*) represent the only known deviation from strict photoautotrophy in *G. breve.* Provasoli also noted the importance of B vitamins to the multiplication of such organisms.

A compound capable of supporting growth in darkness can be overlooked in this type of study, as Lewin has pointed out (*10*). However, the present findings suggest that heterotrophy is not an important consideration in the devel-

opment of the astronomical *G. breve* populations involved in Florida red-tide outbreaks.

If Florida west coast rivers do not provide direct energy sources for multiplication of *G. breve,* the organism's vitamin, trace-metal, and chelator requirements assume added ecological significance as factors potentially limiting population growth. Similar indirect evidence may be seen in extensive field data (*11*), which suggest that phosphate and nitrate are not usually growth restricting to this form in nature.

Vitamins, trace metals, and chelators may be introduced by river waters into coastal environments. Provasoli (*12*) has pointed out broad ecological implications: "It is well known that the waters near estuaries, inlets, and inshore are fertile areas; this fertility should now be considered not only in terms of phosphates and nitrogenous compounds but also of vitamins, trace metals, and chelating agents." The natural fluctuations in levels of such substances may also be a fruitful area for future study of *G. breve* and red-tide ecology.

DAVID V. ALDRICH

Bureau of Commercial Fisheries, Biological Laboratory, Galveston, Texas

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4 May 1962

Convenient Assay of Xanthine Dehydrogenase in Single Drosophila melanogaster

Abstract. Each fruit fly is homogenized in buffer and treated with charcoal. The enzyme is subsequently assayed by following the conversion of 2-amino-4-hydroxypteridine to isoxanthopterin in a sensitive fluorometer.

Many methods have been devised for the assay of xanthine dehydrogenase (*1*). The most convenient way is to determine the increase in fluorescence which occurs during the oxidation of 2-amino-4-hydroxypteridine to 2-amino-4,7-dihydroxypteridine (isoxanthopterin), a reaction catalyzed by this enzyme (*2*). Interest in xanthine dehydrogenase of *Drosophila melanogaster* stems from the fact that mutants at two loci (maroon-like eye color and rosy eye color) lack this enzyme (*3*). As a consequence, these abnormal flies accumulate the enzyme substrates (hypoxanthine and 2-amino-4-hydroxypteridine) and show no trace of the products (uric acid and isoxanthopterin) formed from these compounds (*4*). Enzyme activity has not been detected in partially purified extracts of the mutants (*5*). The following assay in single flies, weighing 0.7 to 0.8 mg, was developed to avoid the necessity for separating large numbers of the various genotypes of flies derived from crosses (*6*). This procedure is not a microassay; convenient macro volumes are used.

All procedures except the assay are carried out at temperatures of less than 5°C. A single fly is homogenized in 4 ml of 0.1*M* Tris buffer, *p*H 7.5, containing 10 mg of crystalline bovine albumin (Armour) per milliliter. Approximately 10 mg of Norite-A is added, and the mixture is allowed to stand for 60 minutes with occasional stirring. The solution is then centrifuged for 15 minutes at 30,000*g*, and then the supernatant is filtered through sintered glass to remove the last remnants of charcoal. One milliliter is placed in a fluorometer cuvette, which is then kept in a dry bath (Thermoline) until it reaches a constant temperature of 30°C (3 to 5 min), after which 0.02 ml of 0.001*M* methylene blue and 0.01 ml of 0.001*M* 2-amino-4-hydroxypteridine (*7*) are added. The mixture is stirred, and readings are taken at 1-minute intervals for 10 minutes in a fluorometer (Photovolt No. 540) equipped with a 340-mμ primary filter and a 405-mμ secondary filter. The fluorometer is set at maximum sensitivity; thus a solution of $1.4 \times 10^{-6}M$ quinine in 0.1*M* sulfuric acid has a reading of 100. The high blank of the reaction mixture is adjusted to zero with the "zero control" on the fluorometer. During the assay, the cuvette is always returned to the dry bath between readings. The entire procedure can also be used on eggs, larvae, and pupae.

The use of albumin in the homogenizing fluid and the use of Norite-A during the processing of the extracts are important. If albumin is omitted, then the activity of xanthine dehydrogenase in the extracts is lost during filtering. The use of albumin prevents this and has, so far as we can tell, little effect on the rate of the reaction. Norite-A is used to remove endogenous "inhibitors" (such as purines and pteridines) from the extract. By means of experiments involving the addition of various amounts of Norite-A to extracts containing known amounts of *Drosophila* xanthine dehydrogenase, it has been clearly demonstrated that no loss of enzyme activity occurs during the processing of these extracts for assay.

Figure 1 shows that the reaction rate is linear for at least 20 minutes. Other data indicate that the enzyme activity is proportional to the number of flies used in the assay. Thus, in one experiment, one fly had 2.1 units of xanthine dehydrogenase, two flies yielded 4.4

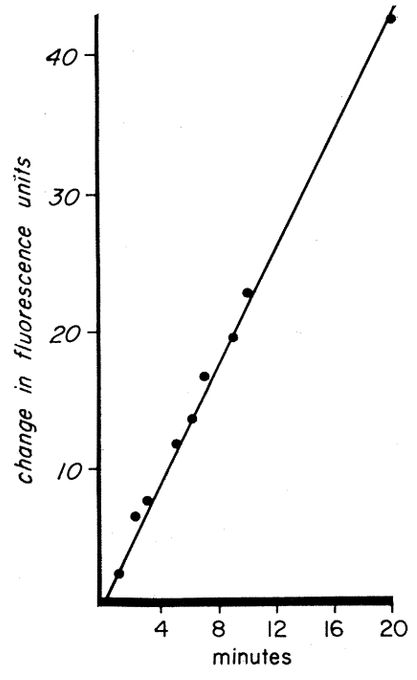

Fig. 1. Linear change in fluorescence units during assay of xanthine dehydrogenase in a single fruit fly.