Mutation of the Blue-Green Alga, Anacystis nidulans

Abstract. *Cells of* Anacystis nidulans *in the logarithmic phase of growth were briefly treated with the mutagen,* N-*methyl*-N′-*nitro*-N-*nitrosoguanidine, and plated under conditions suitable for single-cell growth. Selection of aberrant colonies and examination of the cultural characteristics of these clones suggest that mutants of at least several types are easily derivable in* Anacystis.

Most of the accumulated information on blue-green algae, aside from comparative studies on photosynthesis, pertains to their growth and nutritional characteristics (*1*, *2*). Little is known of their biochemistry, and even less of their genetics. Kumar (*3*) attempted recombination experiments using penicillin- and streptomycin-resistant strains of *Anacystis nidulans*, but resistant clones were not isolated and the data are therefore only tentative.

Development of methodology for producing mutants is essential for investigation of blue-green algal genetics. Auxotrophic and pigment mutants can be expected to aid in investigating biochemical problems and details of reaction mechanisms in photosynthesis.

I now report data on the effect of the chemical mutagen, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) on *Anacystis nidulans*; these data suggest that mutants of blue-green algae can be obtained.

Anacystis nidulans used for inoculum was routinely grown in batches on Medium C (*1*) at 39°C, 8 cm from two fluorescent lamps; carbon dioxide (1 percent in air) was bubbled through the cultures. Single cells were plated on a modified Medium C, manganese chloride was omitted from the A-5 microelements, and A-5 was used at one-half the usual concentration (*1*). The medium was solidified with 1 percent Difco agar (Difco No. 0140), and the plates were sealed with Scotch tape. They were incubated at 32° to 35°C, 26 cm from a linear bank of 60-watt tungsten lamps. Under these conditions single cells quantitatively gave rise to pinpoint colonies after 3 days (*4*).

With *Anacystis* and several marine coccoids as test organisms, previous attempts to produce mutants with standard methods such as ultraviolet irradiation (2537 Å), heat, or nitrous acid have not been successful. However, clearly recognizable variant colonies were obtained in the following way. Cells of *Anacystis* in the logarithmic phase of growth were exposed to NTG (200 μg/ml) in Medium C for 2 to 10 minutes; the cells were separated by centrifugation and resuspended in fresh Medium C and placed for 3 hours under optimum conditions for growth. Samples were then removed and plated on modified Medium C appropriately enriched (Table 1).

Table 1. Specific growth-rate constant (k) for mutants of *Anacystis nidulans*. Medium C prepared according to method of Kratz and Myers (*1*) with no modifications. C + 50 and C + 100 indicate Medium C plus 50 mg and 100 mg of NH_4Cl per liter, respectively. Illumination provided by two 20-watt fluorescent lamps 8 cm from the growth tubes; CO_2 (1 percent in air) bubbled through the tubes. Each of the mutants has been serially transferred in liquid culture at least three times with no change in characteristics.

Stock designation of mutant	Growth temperature (°C)	k ($\log_{10}$ unit/day)		
		Med. C	Med. C + 50	Med. C + 100
Wild-type	39	1.92	1.92	
Wild-type	30	1.58		
330y	39	0.00	1.68	1.68
2a	39	0.52	1.96	2.00
2b	39	0.26	1.50	1.40
Pt6i	39	1.15		
Hpur	39	1.50		
Bpur	39	0.00		
Bpur	30	0.80		

Mutants 330y, 2a, and 2b represent commonly occurring types blocked at different stages in nitrate reduction. Mutant Pt6i was somewhat impaired in growth rate and the pigment ratio was permanently altered. The ratio OD_{678}/OD_{620} calculated from whole cell spectra, determined according to the method of Shibata (*5*), was 0.39. The ratio approaches that reported for wild-type *Anacystis* grown under predominately red illumination (*6*). So far no mutants have been found in which either chlorophyll or phycocyanin was entirely absent or in which a change in structure of the phycobilin was indicated. Mutant Hpur represents another common type in which cell division was apparently impaired. The colony morphology was altered from the normal circular, convex form to forms with an irregular or filamentous appearance. Microscopically the cells were normal in width but 12 to 15 times as long as normal, and the filaments were distorted and twisted. Similar mutant types are found in *Escherichia coli* (*7*). Only occasional cross walls were found. This aberrant morphology was not altered by the presence of vitamins, purines, or amino acids during growth. Mutant Bpur is apparently a temperature mutant. It did not grow at 39°C, and even at 30°C growth was slow. The growth rate at 30°C was stimulated slightly by vitamin B_{12} and thiamine, but not by exogenous purines or amino acids.

From the foregoing results there should be no great hindrance to eventual development of specific mutants as experimental tools for photosynthetic studies and studies on problems in blue-green algae.

CHASE VAN BAALEN
Institute of Marine Science, University of Texas, Port Aransas

References and Notes

1. W. A. Kratz and J. Myers, *Amer. J. Bot.* **42**, 282 (1955). A-5 microelements stock solution is composed of (per liter): H_3BO_3, 2.86 g; $MnCl_2 \cdot 4H_2O$, 1.81 g; MoO_3, 0.0177 g; $CuSO_4 \cdot 5H_2O$, 0.079 g. One milliliter of stock solution is used per liter of Medium C.
2. C. Van Baalen, *Bot. Marina* **4**, 129 (1962).
3. H. D. Kumar, *Nature* **196**, 1121 (1962).
4. C. Van Baalen, *J. Phycol.* **1**, 19 (1965).
5. K. Shibata, *J. Biochem.* **45**, 599 (1958).
6. L. Jones and J. Myers, *J. Phycol.* **1**, 7 (1965).
7. J. Curry and J. Greenberg, *J. Bacteriol.* **83**, 38 (1962).
8. Supported by NSF grant GB 2006. The technical assistance of Rita O'Donnell is gratefully acknowledged.

17 May 1965

Nature of the Excitatory Sarcoplasmic Reticular Junction

Abstract. *The appositional regions between the surface membrane and sarcoplasmic reticulum in insects, decapod crustaceans, and barnacles are largely diadic and show a four-layered structure which is roughly circular in surface view. Each consists of the 56-angstrom double-membrane of the intermediary (here called excitatory) element, and the 75-Å double-membrane of the cisternal element of the reticulum, separated by a space of about 100 Å. A sheet of electron-dense material is found between the two elements, giving the superficial appearance of an additional membrane. The orbits of thin filaments around the thick filaments adjacent to both excitatory and reticular elements are incomplete on the contact side. Regularly spaced bridges connect the thick filaments with both the excitatory elements and cisternal elements and hold the diads in place during stretch and contraction.*

One of the most important unresolved problems in muscle physiology is the nature of the coupling processes between the electrical changes at the

surface-membrane and the contractile machinery. Of great significance in relation to these processes is the nature of the junction between the recently discovered invaginations of the surface membrane and the sarcoplasmic reticulum.

Fahrenbach recently concluded (*1*) that a "tight junction" exists between the intermediate element and adjacent cisternum of the sarcoplasmic reticulum in striated muscles from humans, copepods, ostracods, and barnacles. In this laboratory we have examined extensively the corresponding junctions in striated muscles of the insects *Schistocerca gregaria* (extensor tibiae, anterior coxal adductor, and spiracular muscle) and *Periplaneta americana* (extensor tibiae); the crabs *Cancer magister* (main flexor, accessory flexor) and *Paralithodes* (closer of walking leg); the crayfish *Cambarus clarkii* (fast-abdominal flexors); the lobster *Homarus americana* (slow abdominal flexor); and the barnacle *Balanus nubilus* (scutal depressors). Several thousand junctions have been seen.

In each of these muscles the junctions were examined in material fixed first in glutaraldehyde and then in osmium tetroxide, embedded in epon, and double-stained with lead citrate and uranyl acetate; the electron microscope used was a Siemens Elmiskop 1A.

The junctions are formed from the apposition of two elements. One is part of the sarcoplasmic reticulum enveloping the myofibrils longitudinally and is not open to the exterior. The other is formed by the terminations of clefts continuous with the surface membrane invaginated along the axis of the fiber and open to the exterior.

These elements may run longitudinally as well as transversely, so they will be referred to as E (excitatory or external) rather than T (transverse) as is customary with vertebrate material, but they are clearly analogous to vertebrate T-elements. They were referred to by Smith (*2*) as intermediary tubules (IT), but in our experience they are always flattened, not tubular. The locations differ from those of the triadic contacts found in ordinary vertebrate muscle, which run transversely, at the outer margins of the Z-discs (*3*) or, rarely, at the edges of the A-bands (*4*). Instead, the points of contact occur at the outer ends of the A-bands; several are present round each fibril. They are similar in appearance in both longitudinal and transverse sections and so consist of flattened, probably roughly circular, or oval, patches. Examples are given in Fig. 1, and a three-dimensional drawing of the region is shown in Fig. 2. The contribution from the enclosed sarcoplasmic reticulum is cysternal (*C*) and clearly analogous to the cysternal element of vertebrate muscle. The vast majority of the invertebrate contacts are diadic, though some triadic forms, in which the E-element is shared with C-elements from

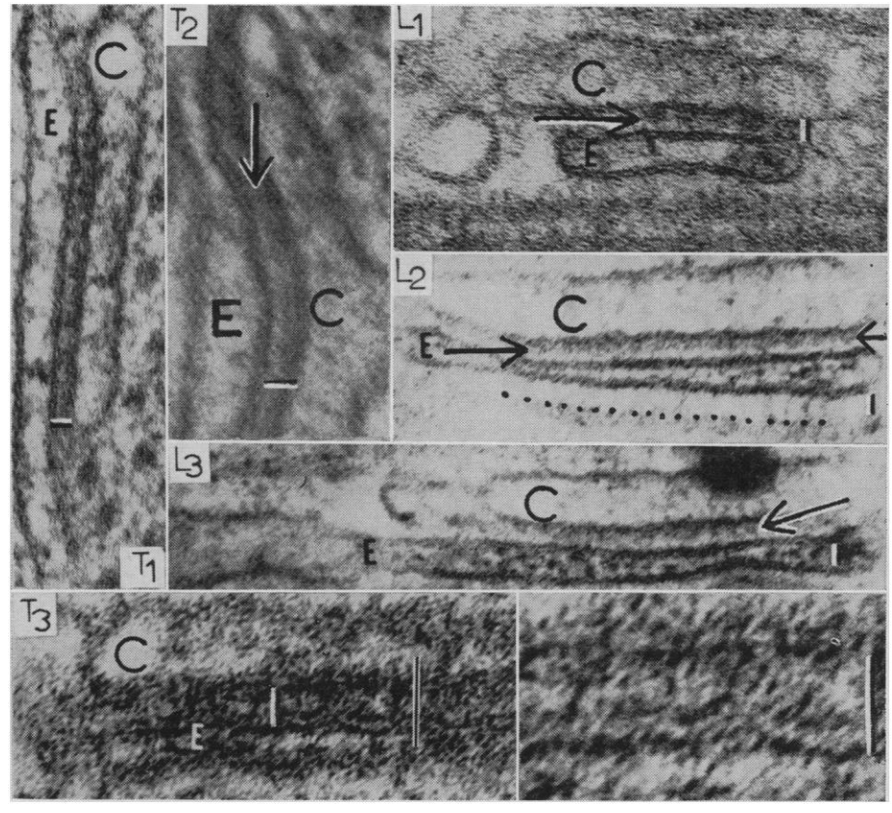

Fig. 1. Insect diadic junctions, fixed in glutaraldehyde and OsO_4, embedded in epon, sectioned at 200 to 500 Å, and stained with lead citrate and uranyl acetate. T_1, T_2, Transverse sections of right metathoracic anterior coxal adductor of male *Schistocerca gregaria*. T_3, Transverse section of right metathoracic extensor tibiae of male *Periplaneta americana*. L_1–L_3, Longitudinal sections of same block as T_1. Dots in L_2 indicate positions of bridges between thick filament, lying just below line of dots, and E-element. Arrows point to line of fine granular material between *E* and *C*. The portion to the left of the vertical marker line in T_3 has been further enlarged in the right-hand portion, where its total magnification is 720,000. Calibration marks in all represent 200 Å.

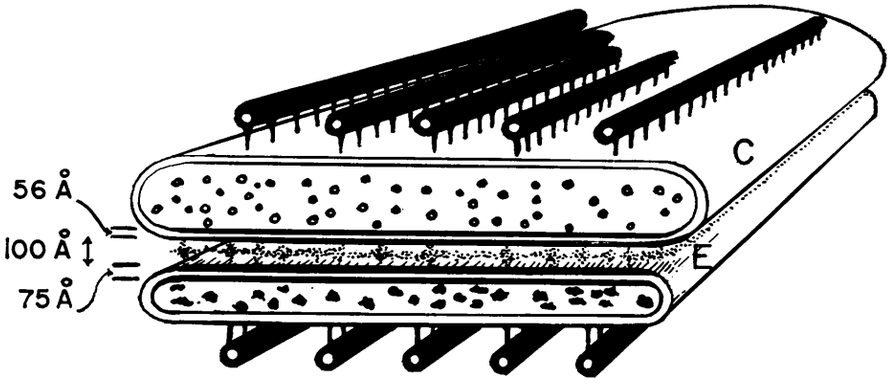

Fig. 2. Drawing to show the general structure and cross-section of an appositional region between an invaginated element continuous with the sarcolemma and the believed excitatory (*E*) and cysternal (*C*) elements of the sarcoplasmic reticulum.

two neighboring fibrils, have been found occasionally in all the material examined.

At the appositional region a space of from 60 Å to 140 Å (usually about 100 Å) separates the 75-Å double-membrane of the E-element from the 56-Å double-membrane of the C-element. Smith (*5*) stated that in *Tenebrio* flight muscle the reticular and cysternal wall is "clearly" not a "double membrane" but is simple, single, and only 50 Å thick. The membrane surrounding these elements does not take the stain or differentiate as clearly as it does in the E-elements, but in our best sections it is always distinctly a three-layered, normal-looking "double membrane" (*6*), though thinner than its apposed neighbor. Its thickness in these sections is 56 Å. This remarkably thin membrane is composed of an outer osmiophilic line (the appositional one) 20 Å thick, a clear middle line 20 Å wide, and an inner line 16 Å thick. The two apposed membranes are heavily osmiophilic and stand out sharply. The outer wall of the E-element is usually also strongly osmiophilic, but not the outer wall of the C-element.

Between the two elements a line of fine granules is located, often with regularly spaced thickenings, giving a scalloped appearance. At the thickenings bridges link the two elements.

This appearance is substantially similar to that reported by Fahrenbach (*1*), but the interpretation is different. He interprets the line of granules as a fusion of the outer membranes of the two elements, whereas it is quite clear in our material that it cannot be so interpreted. However, the "inner membrane" of the C-element illustrated by Fahrenbach is dense and quite thick (60 Å) and could therefore be an unresolved double membrane.

Of some interest is the nature of the anchoring devices which keep the diads in position during contraction. In vertebrate muscle positioning is taken care of by the close association of the triads with the Z-discs, which with their lateral connections form an effective structural framework. In the arthropods examined the diadic or triadic elements were found to be linked by a series of cross bridges, presumably elastic, but about 200 Å long as seen in rest-length fibrils, between the adjacent thick filaments and the E- or C-elements (Figs. 1 and 2). They occur with a regular periodicity of about 180 Å. In these regions the orbits of thin filaments are incomplete, those on the E- or C-side being absent.

In the appositional region granules are present in both the E- and the C-elements. In the former, they are larger and stain more heavily.

It is unlikely that a simple form of electrical transmission could occur at such a junction.

GRAHAM HOYLE

Department of Biology,
University of Oregon,
Eugene 97403

References and Notes

1. W. H. Fahrenbach, *Science* **147**, 1308 (1965).
2. D. S. Smith, *Rev. Can. Biol.* **21**, 279 (1962).
3. K. R. Porter and C. Franzini-Armstrong, *Sci. Amer.* **212**, 73 (Mar. 1965).
4. P. Revel, *J. Cell Biol.* **12**, 571 (1961).
5. D. S. Smith, *ibid.* **10**, 123 (1961).
6. J. D. Robertson, *Biochem. Soc. Symp. Cambridge, Engl. No. 16* (1959), p. 3.
7. It is a pleasure to thank Patricia Ann Hyde for much assistance and Allen Selverston, Benjamin Walcott, and Shokrollah S. Jahromi for collaboration in examining many muscles. Supported by grant GB2609 from NSF.

9 April 1965

Lipase: Localization in Adipose Tissue

Abstract. *Certain problems usually associated with the histochemistry of lipases are obviated by a technique that utilizes the endogenous blood chylomicrons and the cellular stores of triglyceride as substrates for the histochemical demonstration of lipolytic enzyme activity* in situ. *In spreads of mesenteric adipose tissue, the technique makes it possible to distinguish between lipoprotein lipase activity at sites in the capillaries and lipolysis occurring in the adipocytes. The selective anatomic localization of the lipase reaction correlated with the functional state of the tissue, and the absence of reaction product in control mesenteries from starved mice or in heat-inactivated controls, support the validity of this histochemical reaction.*

Adipose tissue contains at least two distinct lipolytic enzyme systems (*1*): (i) an alkaline lipase (*2*) that preferentially catalyzes the hydrolysis of triglyceride present in chylomicrons and low-density lipoproteins; and (ii) an acid lipase that facilitates the hydrolysis of triglyceride stored in adipocytes (*3*).

On the basis of its wide distribution in the body tissues and its very rapid appearance in the plasma after intravenous injection of heparin, it has generally been assumed that lipoprotein lipase activity occurs at the luminal surface of capillaries, but recently Rodbell (*4*) failed to find any lipoprotein lipase associated with his stromal-vascular fractions of adipose tissue. A specific histochemical method for demonstrating the localization of lipoprotein lipase activity has therefore been needed.

There are histochemical methods in existence that are claimed to demonstrate "lipase" (*5*), but results yielded by such methods are difficult to assess because they use synthetic esters in water-soluble form, which are not specific for the fat-splitting enzymes that act on particulate triglyceride substrates (*6*). Emulsified triglycerides are desirable substrates for these lipases but obviously it is not feasible to permeate tissues in vitro with particulate, nondiffusible substances. Alternatively, we took advantage of the fact that a natural triglyceride emulsion (chylomicrons) is plentiful in the circulation of animals after feeding. We reasoned that if lipoprotein lipase activity occurs at the endothelial surface of capillaries, and if chylomicrons are the natural substrate for the enzyme, then under appropriate conditions the enzymic activity should be demonstrable histochemically by application of the classical metal-salt precipitation principle (*7*). This hypothesis was tested and the method and results are herein described.

Adipose tissue was chosen for this demonstration because of its ample supply of the enzyme (*2*). Initial technical difficulties in processing adipose tissue for histology were finally obviated by the use of murine mesenteric spreads that contain discrete patches of adipose tissue and yet are thin enough for microscopic examination without embedding or sectioning. Segments of mesentery were excised from decapitated mice and gently spread over coverslips. The spreads were immediately fixed in cold 4-percent formaldehyde for 10 to 20 minutes to help prevent subsequent detachment of the tissue. After thorough rinsing to remove excess formalin, the coverslip mounts were transferred to a solution containing 0.1*M* tris buffer (*p*H 8.6) and 0.02*M* calcium chloride and incubated overnight at 37°C. Such conditions of incubation favor the hydrolytic activity of lipoprotein lipase, and the enzymically released fatty acids are captured by the available Ca^{++} and