ISOLATION, CHARACTERIZATION, AND PHYSIOLOGY OF THE CYANOPHYCIN GRANULES FROM THE BLUE-GREEN ALGA ANABAENA CYLINDRICA LEMM.

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
ROBERT DAVID SIMON
1971

This is to certify that the

thesis entitled

ISOLATION, CHARACTERIZATION, AND PHYSIOLOGY OF THE CYANOPHYCIN GRANULES FROM THE BLUE-GREEN ALGA ANABAENA CYLINDRICA LEMM.

presented by

ROBERT DAVID SIMON

has been accepted towards fulfillment
of the requirements for

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Coleman Peter Wolk
Major professor

Date August 11, 1971

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ABSTRACT

ISOLATION, CHARACTERIZATION, AND PHYSIOLOGY OF THE CYANOPHYCIN GRANULES FROM THE BLUE-GREEN ALGA ANABAENA CYLINDRICA LEMM.

By

Robert David Simon

Cyanophycin granules are subcellular structures characteristic of the blue-green algae, and have been seen in most species investigated, both uni- and multicellular. They are present in older cultures and are particularly abundant in spores, but are lacking in actively growing cultures. Histochemical evidence suggests that they are proteinaceous and contain arginine. They thus appear to represent a form of storage protein. However, nothing was known about them biochemically. This thesis is concerned with a study of the composition and physiology of the cyanophycin granule.

The cyanophycin granules from two-week-old cultures of the blue-green alga Anabaena cylindrica Lemm. were isolated by disruption of the cells followed by differential centrifugation. The isolated particles were shown to be pure and composed of a single type of protein.

This protein contain only two amino acids, aspartic acid and arginine, in a 1:1 molar ratio. The polypeptide chains have a molecular weight between about 25,000 and 100,000.

Methods are given for the qualitative and quantitative assay of the amount of cyanophycin granules in the blue-green alga Anabaena cylindrica. It is shown that the maximal rate of synthesis of cyanophycin granules occurs after the transition from exponential to stationary phases of growth. Under the experimental conditions used the stationary phase cultures contain high amounts (4%-9% of the total cell dry weight) of the granules. The granules are reutilized when a stationary culture is diluted with fresh medium and active growth resumes.

Data are presented which suggests that phycocyanin also disappears during the transition from stationary to exponential growth and that this chromo-protein may also serve as a protein reserve.

The problems involved in setting up a reliable system of flask cultures which will produce cyanophycin granules are discussed. The most important problem to overcome is that of pH control.

Preliminary data suggest that three factors are important in controlling the production of cyanophycin

granules--the amount of nitrate in the medium, a high rate of aeration, and deficiency of some nutrient(s).

It is shown that neither trypsin, pepsin, or pronase will cleave the protein of the cyanophycin granules of the blue-green alga Anabaena cylindrica under any of the experimental conditions examined. Within the cells of rapidly growing cultures of this alga, the cyanophycin granules are degraded. Such cells possess a soluble enzyme which cleaves the protein of cyanophycin granules. Reaction rate is linear with respect to time, concentration of substrate, and concentration of enzyme. The pH optimum is broad and in the range from pH 8-10. Product analysis and the use of artificial substrates for proteases suggest that more than one protease activity is present in the cell free extracts.

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By

Robert David Simon

A THESIS

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TO MY WIFE, SHERI

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ABBREVIATIONS

AA/16	medium of Allen and Arnon (1) diluted sixteen-fold
BAPNA	benzoly-arginine-p-nitroaniline
BICINE	N,N-bis(2-hydroxyethyl)glycine
DMSO	dimethyl sulfoxide
EDTA	disodium (ethylenedinitrilo)tetraacetate
MES	2-(N-morpholino)ethanesulfonic acid
OD	optical density
PSI	pounds per square inch
Renographin	N,N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate
SDS	sodium dodecyl sulfate
Super	supernatant liquid
TAPS	tris(hydroxymethyl)methylamino-propanesulfonic acid
TCA	trichloroacetic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TRICINE	N-tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminomethane
Triton-X-100	octyl phenoxy-polyethoxyethanol

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INTRODUCTION

The Cyanophyta (blue-green algae) have the subcellular structure of the procaryotes (3, 23, 50). At the same time, they obtain their carbon and energy by photosynthetic mechanisms which are similar to those found in higher plants (42). The eucaryotes have membrane-bounded organelles (chloroplasts, mitochondria, peroxisomes, etc.) which contain sets of enzymatic systems. Electron microscopic studies of the Cyanophyta have shown that this group of algae lacks the membrane-bounded organelles which are found in the eucaryotes (50). The blue-green algae do, however, contain a number of distinctive subcellular particles which are regularly seen in most species--cyanophycin or structured granules, α-granules or polyglucoside bodies, polyhedral bodies, polyphosphate bodies, lipid droplets, etc. (50). There are also present in blue-green algae unique structures which can be seen with the electron microscope and which occur in relatively few species or are found under unusual growth conditions--gas vacuoles, mesosome-like structures, whorls of membranes, etc. (22, 43, 51). Each subcellular

structure of the Cyanophyta must eventually be studied in detail in order to obtain a complete understanding of the biology of the whole organism.

The composition of some of the subcellular particles and their function in the physiology of the organism is only beginning to be understood. Chao and Bowen (15) have recently isolated the α-granule and have shown it to consist of highly branched polyglucosyl units with short external chain lengths. Jones and Jost (44, 45) isolated gas vacuoles and demonstrated that this structure is composed of a single type of protein subunit. The α-granule, cyanophycin granule, lipid droplets, and polyphosphate bodies all probably represent aggregations of reserve material (27, 42, 50). None of the subcellular particles so far examined appear to contain enzymatic systems. And, although there has been much speculation, structures which are analogous to the membrane bounded organelles of eucaryotes have not been demonstrated in the blue-green algae.

The present thesis is concerned with the study of the composition and physiology of one of the subcellular particles of the blue-green algae--the cyanophycin or structured granule.

As will be evident from the following literature survey, all previous knowledge about the cyanophycin granule came from microscopic observations. It seemed

desirable, therefore, to isolate the granules and to determine their exact composition as well as to attempt to reach an understanding as to what role the cyanophycin granule plays in the "life process" of the blue-green algal cell. The first objective therefore was to isolate, purify, and characterize the granule. The second objective was to study the physiology of the granules, particularly the conditions under which the granules form. If the granules are reserve substances, it seemed desirable to know something about how the cells utilize this reserve. The third objective was, therefore, to study the enzymatic system which the alga uses to digest the granules.

LITERATURE REVIEW

Light Microscopy and Early Observations

The cyanophycin granules (cianoficina--Borzi, 1886) are highly refractile granules located in the cells of the blue-green algae. The granules vary in shape and in size. They are most often located at the cell periphery, and in some species are characteristically found along the transverse cell walls. The structures were described by early light microscopists as being soluble in dilute acids and as possessing a definable set of histochemical staining properties (27, 33, 41). Particles with these characteristics were first described by Schmitz in 1879 (79) and were given the name "Schleimkugeln." Many authors have since observed cyanophycin granules in the light microscope and have described these structures under a variety of different names (Table 1). Since 1936 (33), the accepted name for the structure as seen in the light microscope has been cyanophycin granule.

The older literature concerning the properties of the granules has been reviewed by several authors (7, 27, 30, 33, 41), and will not be dealt with here in great

Table 1. A list of early light microscopists and the names they chose for the subcellular structure of the blue-green algae which is now known as the cyanophycin granule. This subcellular structure has a particular set of histochemical properties (cf. Table 2).

AUTHOR	YEAR	NAME	REFERENCE
Schmitz, F.	1879	Schleimkugeln	(79)
Borzi, A.	1886	Cianoficina	(10)
Zacharias, E.	1889	Körner	(91)
Nadson, G.	1895	Reservekörner	(63)
Bütschli, O.	1896	Farblose Körner	(13)
Phillips, O.P.	1904	Slime-balls	(72)
Gardner, N.L.	1906	β-Granules	(31)
Dehorne, A.	1920	Mitochondrien	(17)
Baumgärtel, O.	1920	Ektoplasten	(7)

detail. Also, histochemical staining of the granules will be discussed here only insofar as it defines the particles or elucidates their composition or function. Table 2 lists some of the characteristic staining properties of the granules which clearly distinguishes them from other subcellular particles in the blue-green algae. The discussion as to the implications that the staining properties have with regard to composition and function will follow in a later section (p. 13).

Electron Microscopy

With the advent of the technique of thin sectioning, the procaryotic nature of the blue-green algae was clearly shown and numerous subcellular particles were identified (66, 69, 76). Drews and Niklowitz (20, 21) in 1956 were the first to report particles in electron micrographs, which were thought to be equivalent to the cyanophycin granules in the light microscope. The reasoning was that the particles were large (about 0.3μ in diameter) and irregular in shape, as were the cyanophycin granules. Also, the particles in the electron micrographs were located at the transverse cell walls, the same position at which large cyanophycin granules were seen. Fuhs, in 1958 (28), saw particles in Oscillatoria amoena (Kütz) Gom. up to 0.5μ in diameter which he believed to be cyanophycin granules. These particles stained heavily

Table 2. Characteristic staining properties of the cyanophycin granule.

STAIN	REACTION[a]	REFERENCE
Acetocarmine	+	(27)
Neutral Red	+	(27)
Methylene Blue	-	(27)
Haematoxylin	+	(13)
Sakaguchi Reagent for Arginine	+	(26)
Millions Reagent for Tyrosine	-	(41)
Xanthroproteic Reaction	-	(41)

[a] + indicates that the cyanophycin granules take up stain. - indicates no staining.

with osmium, and this correlated with an earlier claim of Neugnot (65) that the cyanophycin granule could reduce OsO_4. After permanganate fixation, areas of low to medium electron density were found in the vegetative cells of Cylindrospermum. These areas were identified as cyanophycin granules because of their absence in young cells, peripheral position in old cells, and great accumulation in spores (57).

Drews and Niklowitz (21) observed a limiting membrane around the cyanophycin granule. Pankratz and Bowen (69) found dense granules (osmium stained) up to 0.5μ in diameter near the crosswalls of the blue-green alga Symploca muscorum. These granules showed irregular internal patterns of dense and less dense regions. However, no limiting membrane was found and there was no evidence of internal membranous structure. The absence of a limiting membrane around the cyanophycin granule was confirmed by Wildon and Mercer (88). In no electron microscopic studies of the blue-green algae other than that of Drews and Niklowitz (21) can one find evidence of a limiting membrane around the cyanophycin granule (20, 37, 50, 52, 76).

As with the light microscopists, the electron microscopists gave different names to the same structure, depending on what characteristic they considered most important. Drews and Niklowitz (20, 21), on the basis of histochemical evidence, thought the cyanophycin granule to be a

mitochondrial equivalent and thus named the particle "fermentative Granula." Fuhs (29) and Ris and Singh (76), who saw in their electron micrographs evidence of sub-structure, called their particles "geschichtete" (which may be translated literally as stratified or laminated) and "structured" granules respectively. Maugini (58) merely called the particle Type I granule. The name structured granule has become the accepted name for the particle as seen in the electron microscope (50).

One of the difficulties in comparing the structures of particles from different electron micrographs has been the wide range of electron opacity seen for apparently the same structure. After permanganate fixation the granules appear as empty holes, while osmium fixation leads to a wide range of staining. The recent work of Lang and Fisher (52) has clearly shown that the nature of the staining of the particle, and as a result the substructure seen, is a function not only of the type of stain but also of the fixation and staining procedures used.

Localization and Occurrence

The occurrence of cyanophycin granules has been used as a taxonomic characteristic (27). They occur in most blue-green algae although they are not indispensable since they appear to be lacking in Spirulina (19, 41).

Hegler (41) pointed out that the cyanophycin granules are missing in actively growing cells. Tischer (85)

examined eighteen different blue-green algae and came to the conclusion that the cyanophycin granules were present in older cultures, while they were absent or scarce in rapidly growing cultures. These observations are supported by the finding of large Sakaguchi-positive granules in old cultures of Anabaena cylindrica (26), but their absence in young cultures.

Cyanophycin granules are present in large amounts in the spores (akinetes) and hormogonia of certain blue-green algae (16, 27, 41, 61). Wildon and Mercer (89) suggested that the large particles in spores represent an additional type of granule. However, Clark and Jensen (16) considered that there was no reason to make this distinction. Madsen (57) found large numbers of granules in the spores and aged cells of Gloeotrichia.

The cyanophycin granules in the vegetative cells disappear as the cells differentiate into heterocysts (49, 89). Heterocysts have large refractile polar nodules which may possibly have the same composition as cyanophycin granules. Palla (68) considered cyanophycin granules and polar nodules to be made out of the same material, but this was disputed by Geitler (32) and Baumgärtel (7). Although the polar nodules are Sakaguchi-positive like the cyanophycin granules, they are still present after two hours of extraction with 1N HCl, and Fogg (26) took this

to indicate that the two structures are made out of different material.

In litchens containing blue-green algae, the algal cells usually contain few or no cyanophycin granules, although the amount of granules seems to depend on the degree of contact between the alga and the fungus (27). In Collema sp. such granules are always present, although fewer in number than in free Nostoc, while in Ephebe sp. the number of granules depends on the amount of fungal hyphae.

The blue-green pigmented plastids (cyanelles) of Glaucocystis nostochinearum (39) and the Cyanophora paradoxa (38) have been examined in the electron microscope. The micrographs show that the plastids are similar to free-living blue-green algae except that they appear to lack an outer sheath and cell wall. These cyanelles contain polyphosphate and lipid droplets, but lack cyanophycin granules. Hall and Claus (39) rationalize the absence of such granules by arguing that the cyanelle does not need storage material since a continuous supply of food is available from the host.

Function

It is generally theorized that the cyanophycin granule is a form of reserve substance, probably proteinaceous (27, 33, 50). The basic evidence for this theory is the occurrence of granules in aged cultures and spores,

and the disappearance of granules under conditions where reserves would be expected to be used up. Hegler (41) reports the gradual disappearance of cyanophycin granules in darkness. The cyanophycin granules in akinetes disappear when the spores germinate (61). The granules are absent during rapid cell growth (as in exponentially growing cultures [26, 85]), and are also reported to be lacking in the growing apical cells of the alga Stigonema (27).

Origin

Pankratz and Bowen (69) found that the structured granules of Symploca muscorum are usually surrounded by polyglucoside bodies and that this complex is surrounded by a dome shaped lamellar unit. Lamont (47, 48) also made this observation in two Oscillatoria species, and suggested that the structured granules represent a tight aggregate of polyglucoside bodies.

Brown and Bisalputra (12), on the basis of electron microscopic evidence, suggested that structured granules are formed by aggregation of irregular membranous bodies which originate from the photosynthetic lamellae. They consider the disappearance of cyanophycin granules during the germination of spores and the concomitant increase of photosynthetic lamellae (61) as supporting this hypothesis.

Composition

The only studies on the composition of the cyanophycin granules which had been carried out prior to the work reported in this thesis consisted of the examination of their histochemical staining properties and the treatment of intact cells with various reagents to see how these chemicals affected the cyanophycin granules.

Macallum (55) demonstrated histochemically that the granules did not contain phosphate, thus distinguishing them from polyphosphate bodies. Zastrow (92) showed that the granules were Feulgen negative and would not stain with reagents specific for DNA and RNA. She concluded that the granules do not contain nucleic acids. The granules are not soluble in normal lipid solvents such as ethanol, ethyl ether, or chloroform (10, 41, 91) and do not stain with lipid stains. Fuhs (28) did find, however, that they stain with the phospholipid stain of Baker (6).

Millons' reagent and the xanthoproteic reaction (boiling HNO_3) are both tests for aromatic amino acids. Hegler (41) found that the granules would not stain with Millons' reagent and did not give a positive test using the xanthoproteic reaction, suggesting that the granules did not contain protein. However, since the granules could be dissolved with pepsin-HCl, an observation confirmed by Baumgärtel (7), he concluded that they were protein crystals. Baumgärtel (7), Guilliermond (36), and Tischer

(85) came to the same conclusion and considered the cyanophycin granule to be composed of lipoprotein. Fogg (26) found that the cyanophycin granules of the blue-green alga Anabaena cylindrica stained strongly with Sakaguchi reagent. This reagent identifies arginine-containing proteins (59). Fogg concluded that the stained granules were cyanophycin granules for the following reason: after the alga had been extracted with 1N HCl for one hour at room temperature, the staining reaction could no longer be demonstrated. Until the present time, this reaction was the best indication that the cyanophycin granule is proteinaceous.

A number of investigators observed that the cyanophycin granule could be stained with reactions that were characteristic for mitochondrial oxidations, and as a result concluded that the cyanophycin granule was a mitochondrial equivalent. Dehorne (17) found that the cyanophycin granule would stain with Altman's mitochondrial stain. Drews and Niklowitz (20) found granules at the transverse cell walls of Phormidium uncinatum which would reduce tetrazolium dye to formazan. The same structure was deeply stained with Janus Green B. Tischer (85) also found tetrazolium dye reduction, while Fuhs (28) showed that the granules would stain with Hartmans mitochondrial stain. However, Ris and Singh (76) and Wildon and Mercer (88) questioned the hypothesis that the cyanophycin granule

was a mitochondrial equivalent since in their electron micrographs (76, 88), they found that the granules had neither a limiting membrane nor an internal membranous structure. Bisalputra, Brown, and Weier (8) using potassium tellurite and tetranitroblue tetrazolium were able to demonstrate at the electron microscopic level that respiratory reduction was not associated with any granular inclusion, but occurred instead at sites on the photosynthetic lamellae. If the granules are not the sites of respiratory oxidation, then there is a problem in explaining why many investigators found that the granules would stain with reagents which were characteristic for mitochondria. A discussion of this problem will be presented later (p. 98).

Lang and Fisher (52) studied the effects of trypsin, acid phosphatase, and lipase in different buffer systems on thick sections of Anabaena sp. which had been prepared by techniques modified from Feder and O'Brian (25). Cyanophycin granules in both vegetative cells and akinetes were examined, but none of these tests for protein, polyphosphate, or lipid proved positive. The authors concluded that the final proof of the composition of the cyanophycin granule must await more precise biochemical investigations.

MATERIALS AND METHODS

Culture and Harvest

Anabaena cylindrica Lemm. was grown axenically in thirteen liter batches in fourteen liter New Brunswick fermentors as previously reported (90). The fermentors were illuminated with twelve cool white (ITT F15T8/CW) lamps and were gassed with air at a flow rate of four liters per minute. The growth medium consisted of the medium of Allen and Arnon (1) diluted sixteen-fold and modified by the addition of one gram of $NaNO_3$ per liter. Cultures were grown to stationary phase (ca. two weeks) because cyanophycin granules were formed after exponential growth had stopped (see Results section B). Cultures were harvested after examination with a light microscope revealed the presence of refractile granules. The algal cells were collected either with a Szent-Györgyi and Blum continuous flow centrifugation system (Ivan Sorvall Inc. KSB-R) at 48,000 x g, or by transferring the culture to four-liter flasks and allowing them to stand in a cold room (4°C) overnight. In the cold room the cells would settle out, and the bulk of the medium could be decanted. The remaining cell suspension was further concentrated by

low speed centrifugation. Harvested cells were either used immediately, or were frozen and stored at -20°C until use.

Isolation of Cyanophycin Granules

Figure 1 illustrates the fractionation scheme used for the isolation of cyanophycin granules. The harvested cells were resuspended in distilled water and were concentrated to a cell density corresponding to about 0.2 mg chlorophyll per ml. The cells were then broken by one passage through a Sorvall RF-1 cell fractionator at 4,000 psi (with maximum pressure set at 7,000 psi). Alternatively, the cells could be disrupted by cavitation in a Branson Sonifier (Heat Systems Co. Model S-125) as follows: Twenty-five ml portions of the algal suspension were placed in a continuous flow head (stainless steel) which had the bottom part sealed off with a rubber serum cap and which was cooled to 12°C by flowing tap water. The suspension was cavitated at the lowest setting for fifteen minutes. The sonicate was removed through the bottom serum cap with a syringe. Processing of the suspension could be continued by adding another 25 ml batch to the sonicator. Cell suspensions broken either with the cell fractionator or the sonicator were then treated similarly.

The crude cell lysate was diluted 1:1 with distilled water and centrifuged for ten minutes at 27,000 x g in the

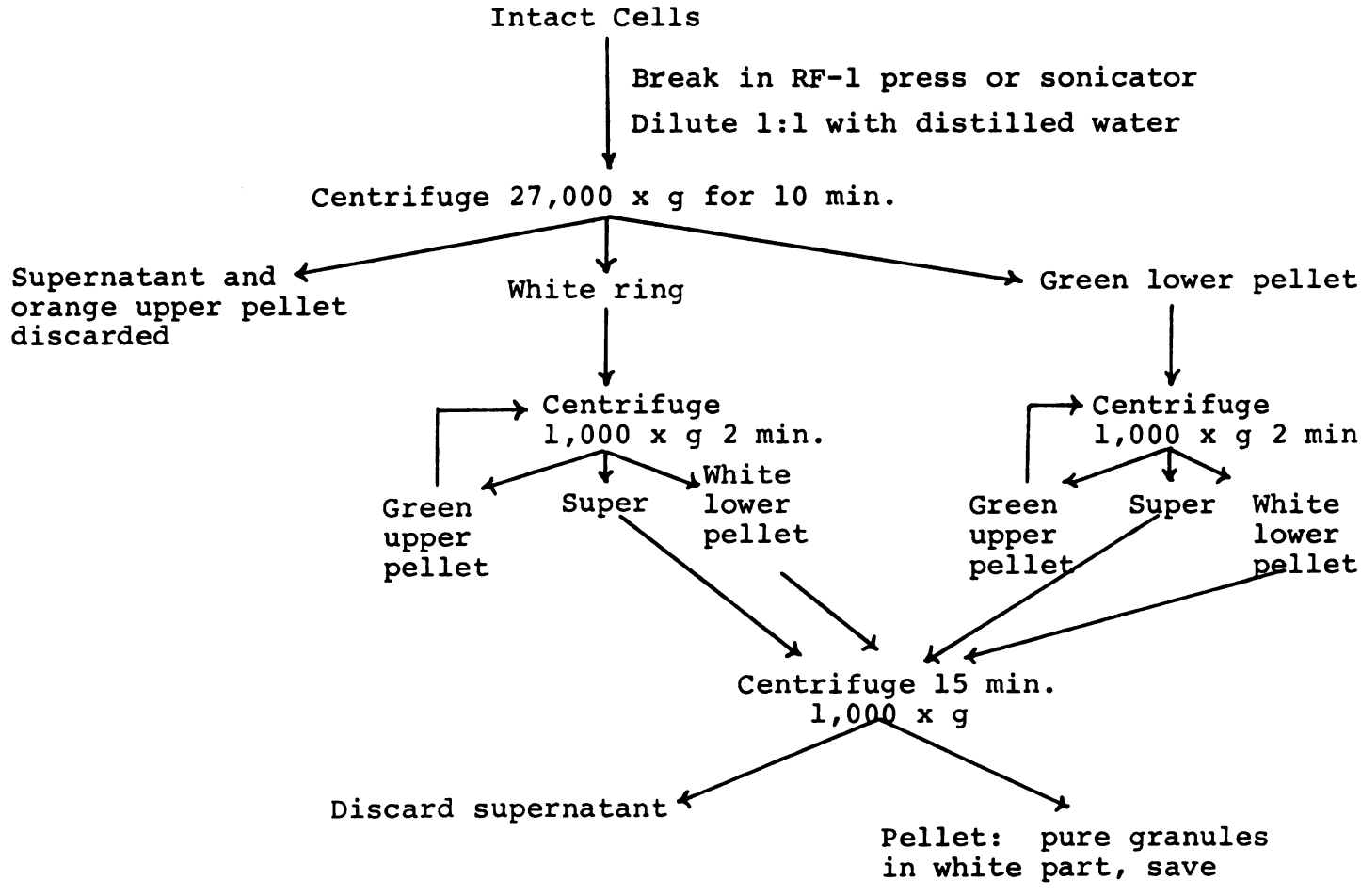


Figure 1.--Fractionation scheme for the isolation of cyanophycin granules.

SS-34 rotor of a Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded. The pellet consisted of three parts: an orange-colored upper part which consisted of broken fragments of vegetative cell walls; a white ring of cyanophycin granules around the edge of the pellet; and the lower green part, which comprised the bulk of the pellet and which consisted of heterocysts and intact vegetative cells. The orange upper layer was carefully removed using a paint brush and streams of water from a wash bottle, and was discarded. The white ring and lower green pellet were separated by use of a paint brush. The white ring and green pellet were individually resuspended in distilled water and were centrifuged at top speed (1,000 x g) in fifteen-ml conical tubes in the swinging bucket rotor of a clinical centrifuge (International Eqpt. Co.) for two minutes. The supernatant was saved. The pellet from each fraction consisted of a lower white part and an upper green part. The upper green part was removed, resuspended in distilled water, and reprocessed in the same manner as the original white ring and green pellet. The lower white pellet, which consisted mainly of cyanophycin granules was also resuspended in distilled water. The supernatant and resuspended white pellet were centrifuged for fifteen minutes at 1,000 x g in the swinging bucket rotor of a clinical centrifuge. The supernatant from this step was discarded, and the white

pellet was removed and saved. The white pellet consisted of highly purified granules and was resuspended in distilled water for analysis, or was frozen and lyophilized for later study.

If the granules at this step were not pure white, they were further purified by resuspending them in water and centrifuging them in a SS-34 fixed angle rotor of a Sorvall RC2-B centrifuge at 48,000 x g for twenty minutes. After centrifugation, contaminants usually layered out on top of the pellet and were removed by placing the tube in a test tube rack such that the tube was at a 45° angle with the rack and the pellet was vertical to the rack. The tube and rack were allowed to sit in the refrigerator until the loose upper part of the pellet (contamination) had slipped off the lower well-packed pellet (cyanophycin granules). The loose contamination was then easily removed using a Pasteur pipette. The remaining granules were then resuspended in water for analysis.

General Chemical Assays

Protein was determined by measuring free α-amino groups (77) after hydrolysis in 6 N HCl at 105°C for 24 hours, and by the Lowry procedure (54) or the Biuret method (53) using bovine serum albumin as a standard. Carbohydrate was measured either by the anthrone procedure (40) or by measuring the appearance of reducing

sugars (70) after hydrolysis in 2 N H_2SO_4 at 100°C. Lipid was determined gravimetrically after extraction of lyophilized granules with chloroform-methanol (2:1 v/v). Phosphate was measured after wet ashing (71). Moisture content was taken as the difference in weight of material dried first at 50°C and then at 105°C. Chlorophyll was measured by the method of Mackinney (56).

Measurement of Arginine

Arginine was measured by a modified Sakaguchi reaction using a procedure similar to that reported by Messineo (60). The composition of the reagents for the assay and the procedure for arginine measurement will be given in detail since they differ from those reported (60).

Reagents:

a. 0.9 gm potassium iodide made up to one liter with distilled water.

b. 84.1 gm potassium hydroxide made to 300 ml with distilled water + 6.0 gm potassium sodium tartrate + 1.2 gm 2,4-dichloro-1-naphthol (Eastman 3704) + 540 ml ethanol + 28.0 ml full strength bleach (5.25% sodium hypochlorite by weight--Big Chief Bleach).

c. Full strength bleach diluted with distilled water 1:4 (v/v) (must be made up fresh each time used).

Assay:

1. 1.0 ml sample with between 0 and 40 µgm arginine or between 0 and 400 µgm cyanophycin granules.

2. Add 1.0 ml reagent a.

3. Add 3.0 ml reagent b. and mix on vortex mixer.

4. Wait one hour.

5. Add 1.0 ml freshly made reagent c. and mix well.

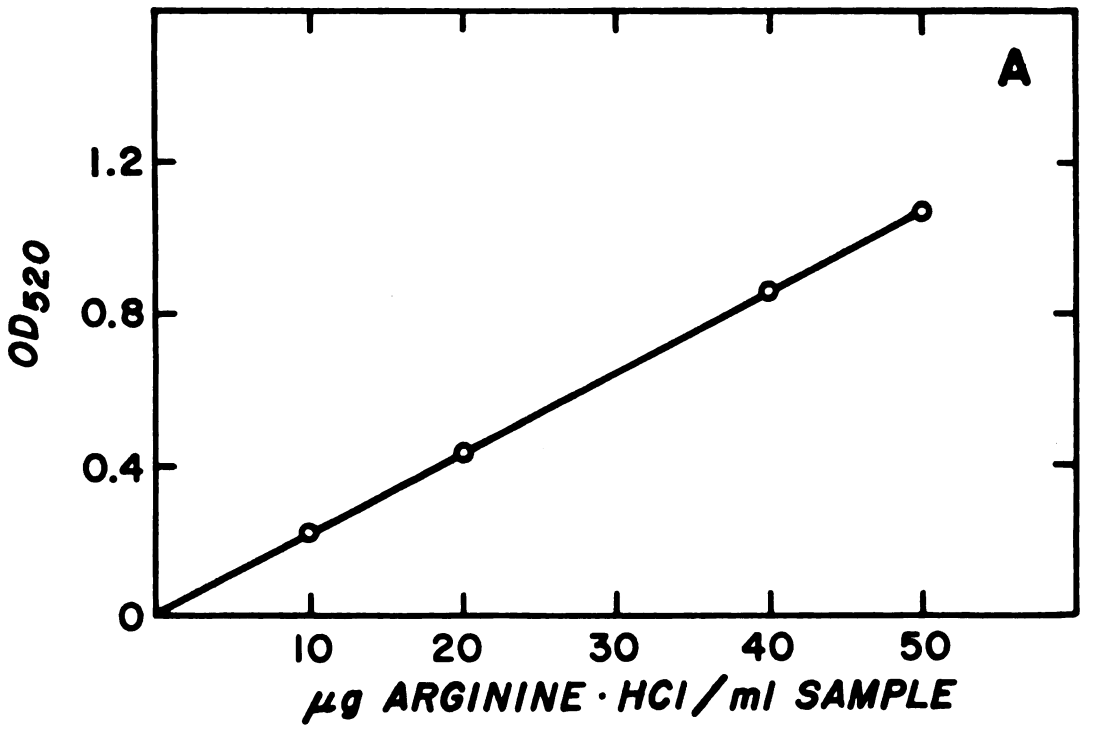
6. Wait 1/2 hour and read optical density at 520 nm.

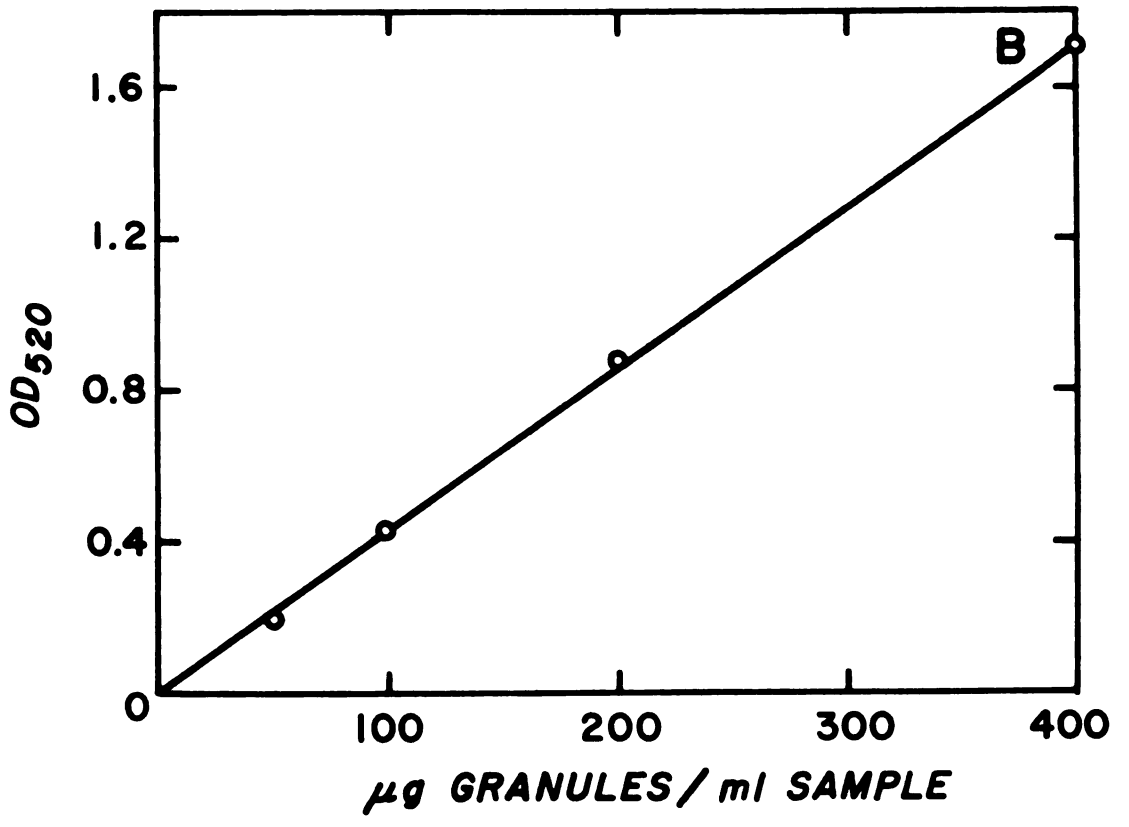
Figure 2A shows a standard curve using arginine ·HCl salt. The assay works as well with arginine ·HCl as with the free base. One of the advantages of the Messineo procedure (60) is that arginine within proteins can be assayed directly without having first to hydrolyze the protein to amino acids. Although Messineo claimed that the reaction was quantitative, my experiments showed that although the amount of color at 520 nm was proportional to the amount of unhydrolyzed protein assayed, the amount of color which developed was less than would be expected from calculations based on the amount of arginine in the protein. Using bovine serum albumin and protamine sulfate as standards less than 40% of the arginine could be accounted for. Even without this quantitative relationship, the assay could be used to quantitate granule amounts since the amount of color was proportional to the amount of granules present: cf. Figure 2B.

Amino Acid Analysis

For amino acid analysis, the granules were hydrolyzed at 105°C in 6 N HCl in sealed vials that had been flushed with nitrogen gas. After hydrolysis the material was dried in a dessicator over NaOH pellets to remove the HCl,

Figure 2A and B.--Assay for arginine and cyanophycin granules--the optical density obtained at 520 nm using an arginine assay modified from that given by Messineo (60). Graph A gives the correlation between the amount of arginine ·HCl present and the OD_{520}. Graph B shows the correlation between the concentration of cyanophycin granules (in µg/ml) and the OD_{520}.


A
1.2
0.8
0.4
0
OD_{520}
10
20
30
40
50
μg ARGININE · HCl / ml SAMPLE


B
1.6
1.2
0.8
0.4
0
OD_{520}
100
200
300
400
μg GRANULES / ml SAMPLE

and analyzed on an automatic amino acid analyzer as previously described (45).

Granule hydrolysates and aspartic acid standards were silylated for gas chromatography by heating one hour at 50°C in the presence of N,O-bis-(trimethylsilyl)-acetamide in pyridine (2:1 v/w). Gas chromatography was performed on an SE-30 column at 130°C. Mass spectra from the peaks of the gas chromatograph were obtained using an LKB-9000 gas chromatograph--mass spectrometer.

Determination of Configuration of Amino Acids

The configuration of the granule amino acids was determined by hydrolyzing 75 mg dry weight of granule protein in 6N HCl for twenty-four hours at 105°C. The solution was then taken to dryness in a vacuum dessicator over NaOH pellets and resuspended in 5 N HCl to 0.5% (w/v) final concentration. The optical rotation was determined at room temperature in a Perkin-Elmer model 141 Polarimeter using the mercury line at 546 nm. The observed rotation was compared to the expected rotation for various combinations of D- and L-aspartic acid and arginine using the previously determined specific rotations of Otey *et al.* (67).

Partial Acid Hydrolysis and Identification of the Products

Granules were resuspended at 1 mg/ml in 6 N HCl and hydrolyzed for various lengths of time at 150°C.

After hydrolysis the samples were taken to dryness in a vacuum dessicator over NaOH pellets. The dried residues were then resuspended in distilled water and assayed for free α-amino groups (77). A sample hydrolyzed for twenty-four hours was considered 100% hydrolyzed. Therefore, the per cent hydrolysis at any given time was determined by taking the ratio, free α-amino groups at the given time/free α-amino groups at twenty-four hours, and multiplying this ratio by 100.

The products of the hydrolysis were separated by electrophoresis on Whatmann #4 paper at pH 1.9 using a buffer system of acetic acid-formic acid-water (150:50:800 v/v/v) as previously described (44). After completion of electrophoresis, the paper was dried at room temperature overnight. Spots containing amino acids were located by dipping the paper in ninhydrin-acetone-acetic acid-pyridine (0.3:100:1:1 w/v/v/v) and heating at 70°C. Arginine-containing spots were identified by using a modified Sakaguchi reagent (5).

The amino acid-positive and/or arginine-positive spots which ran between free arginine and free aspartic acid were eluted from the electropherogram with distilled water and were rehydrolyzed in 6 N HC 1 at 105°C for twenty-four hours. The hydrolysates were dried, and the residues redissolved in distilled water and spotted on 21 cm x 21 cm sheets of Whatmann #1 paper. The hydrolysates

were then chromatographed using ascending chromatography with a solvent of butanol-acetic acid-water (3:1:1 v/v/v). The chromatograms were sprayed with ninhydrin reagent (Nutritional Biochemical Co.--Ninspray) to identify the amino acid-containing spots. The R_f's of spots in the samples were compared with the R_f's of aspartic acid and arginine standards.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed on an apparatus which was similar to that described by Scandalios (78) for acrylamide gels. The dimensions of the slabs of 7.5% polyacrylamide used were 20 x 18 x 0.3 cm. Samples were applied to the gels by cutting the gel with a nylon knife and inserting into the slit strips of filter paper which had been wetted with the sample and blotted dry. Gels were prepared at room temperature but run at 4°C. Electrophoretic separation was achieved using 50 milliamps per slab. The sample paper wicks were removed one hour after the gels were started, and the current was continued until a marker (either bromphenol blue or methylene blue) had reached about 10 cm from the origin. Gels were stained for protein with 1% Amido Black 10B in methanol-water-acetic acid (4:5:1 v/v/v) for one hour and were then destained by washing with methanol-water-acetic acid (4:5:1 v/v/v) over a period of several days.

Since the granules are insoluble in water or dilute buffer, they were first dissociated with sodium dodecyl sulfate (SDS), urea, or phenol-acetic acid-water (2:1:1 w/v/v). The recipes for the gels, which also contained these dissociating agents, are as follows:

The gel system with 0.1% SDS, pH 8.2, is a modification of the acrylamide system suggested by Scandalios (78).

a. Buffer I--pH 8.2 citric acid (anhydrous) 1.6 gm; Tris 4.8 gm; water to one liter.

b. Buffer II--pH 8.7 lithium hydroxide (anhydrous) 1.44 gm; boric acid 11.8 gm; water to one liter.

Running gel: 108 ml Buffer I + 12 ml Buffer II + 0.12 gm SDS + 8.4 gm Cyanogum-41 (Fisher Chemical Company C-588) are mixed and then filtered through moistened Miracloth. Then, 1.2 ml of 10% ammonium persulfate and 0.24 ml TEMED (N,N,N',N',-tetramethylethylenediamine Eastman 8178) is added and the mixture poured into the slab frame and covered with a glass plate. After 15 minutes the gel hardens.

Buffer for Electrodes: Anode buffer is Buffer II, while cathode buffer is Buffer II containing 0.1% (w/v) SDS.

The granule protein is dissolved in 1.0% SDS in water prior to being applied to the sample paper.

The gel system containing 4.0 M urea was the same as described above for SDS except that the gel contained 28.8 gm urea instead of 0.12 gm SDS, and the tank buffers for both electrodes was Buffer II only. The sample was dissolved in 4.0 M urea and was boiled over a flame briefly to assure better dissociation of the granules. The granules

in urea solution were allowed to cool before being applied to the gel.

The electrophoresis system containing phenol-acetic acid-water (2:1:1 w/v/v), pH 2.0, followed the method of Takayama et al. (83). In order to get the acidic polyacrylamide to solidify, the freshly poured gel was heated by placing a 500 watt lamp five inches from the covered slab surface.

Molecular Weight Determinations

For dialysis experiments the granules were dissolved in 0.1 N HCl and were dialyzed against 0.1 N HCl for eighteen hours. Protein in the dialysis bag was determined by the Lowry procedure (54) or by measuring total α-amino groups with ninhydrin (77) following hydrolysis of the dialysate, adjusted to 6 N HCl, for twenty-four hours at 105°C.

Analytical sodium dodecyl sulfate-acrylamide gels were run according to Shapiro et al. (81) except that a 20 x 18 x 0.3 cm slab of 5% acrylamide was used instead of individual discs.

For gel exclusion chromatography, the cyanophycin granules were dissolved in 6.0 M urea--0.1 M H_3PO_4 (pH 2.7) and run on a Sephadex G-200 column which had been equilibrated with the same buffer. Granule protein in the column eluate was assayed by determination of the amount of arginine present (see above). Blue Dextran--2,000 was

used to determine the void volume, and molecular weight was estimated according to the method of Andrews (4).

Density Determination

The density of the isolated granules was determined by centrifugation in gradients of Renographin (Methylglucamine Diatrizoate-Squibb 7494). This compound, which is non-ionic and gives solutions of high density and low viscosity, was previously used to determine the density of bacterial spores (84). Since Renographin-60 (60% w/v), which has a density of 1.355, was found in preliminary experiments to be below the density of the granules, denser solutions were made by lyophilizing Renographin-60 and adding water to the dry powder to give solutions with a density ca. 1.49. A gradient-maker was used to prepare continuous gradients between density 1.25 and 1.40. The granules in water (2 mg/ml) were mixed with an equal volume of Renographin-60 and layered on top of the gradients which had been prepared in nitrocellulose tubes. The tubes were centrifuged at 27,000 x g for one hour in the HB-4 swinging bucket rotor of a Sorvall RC2-B centrifuge. Following centrifugation, samples were collected from the gradient by puncturing the bottom of the centrifuge tube and collecting 10-drop fractions. The density of each fraction was determined by measuring the optical density at 260 nm and transforming the OD_{260} into density (ρ) by the use of the formula $\rho = 1 + 0.385\ (OD_{260} \times 10^{-4})$

(84). The position of the granule band was determined by assaying each fraction for arginine (see above).

Solubility and Staining

The solubility of the cyanophycin granules was determined by mixing lyophilized isolated granules with various reagents (at 1.0 mg/ml) and allowing these mixtures to stand for at least twelve hours. The granules were considered as being soluble in a reagent if the originally milky-white suspension turned clear or if when the mixtures were centrifuged at 48,000 x g for 30 minutes, no arginine-containing pellets were found. For more quantitative studies of solubility, the amount of granules solubilized was determined. This was done by centrifuging the mixtures at 48,000 x g for one hour and measuring the amount of arginine in the supernatant.

Staining was determined by mixing granules resuspended in distilled water (1.0 mg/ml) with various stains, allowing them to stand for one hour, and centrifuging the granules in a clinical centrifuge (1,000 x g) for five minutes. The pellet of granules was then examined to see if it was stained.

Microscopy

Intact cells with an without cyanophycin granules were examined in the phase contrast microscope (Carl Zeiss Co.), and were photographed using Kodak PAN-X film.

Dr. Norma Lang (Department of Botany, University of California, Davis) examined in the electron microscope isolated cyanophycin granules as well as intact cells which contained cyanophycin granules. The cells were stained with both osmium and $KMnO_4$, and the electron microscopy carried out by the procedure of Lang and Fisher (52).

Measurement of the Production of Cyanophycin Granules

The production of cyanophycin granules may be assayed in two different ways, one qualitative and one quantitative. Because cyanophycin granules are refractile and sufficiently large, their presence may be easily discerned with the light microscope. With phase contrast microscopy, the granules stand out even more clearly (Figure 3). With practice there was little difficulty in determining whether or not a culture has produced extensive numbers of granules. A cautionary note must be added here. The fact that the cells in a culture possessed large refractile bodies was no proof that these bodies were cyanophycin granules. The culture conditions could have induced the alga to form other subcellular particles. The only absolute proof that a structure was a cyanophycin granule was to isolate and chemically characterize the particle.

The amount of cyanophycin granules in a culture could be determined quantitatively by taking advantage of

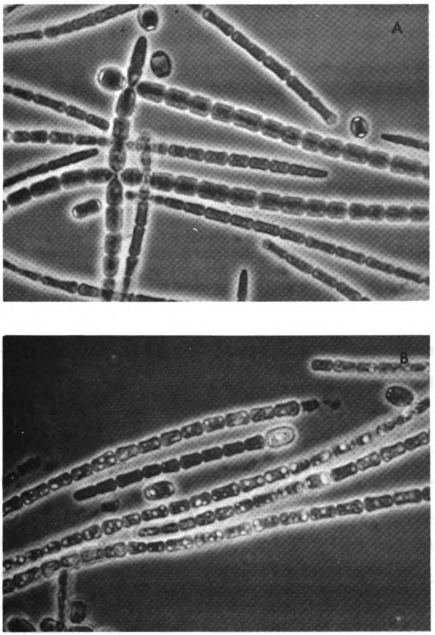


Figure 3 A and B.--Phase contrast light micrographs of filaments of Anabaena cylindrica with and without extensive formation of cyanophycin granules. Picture A shows cells from exponentially growing cultures; the cells contain few granules. Picture B shows cells from a stationary culture; these cells have formed many granules. The magnification in both pictures is 400 X.

their size and unusual solubility properties. Granules in a broken cell suspension pelleted in fifteen minutes at 27,000 x g. Also, since the granule protein was insoluble in distilled water and 2% Triton X-100 but soluble in 0.1 N HCl, it could be easily separated from the bulk of the cellular protein. Figure 4 shows the flow sheet for the quantitative assay of cyanophycin granules. Twenty-five ml portions of a cell suspension (with known dry weight or chlorophyll content) were put in the continuous flow head of a Branson Sonifier (S-125). The suspension was then sonicated at setting three for four minutes, which broke almost all of the vegetative cells. The resulting suspension was then centrifuged for fifteen minutes at 27,000 x g. The supernatant was discarded and the pellet was washed first with distilled water, then with 2% Triton X-100, and finally two additional times with distilled water. Next, 1.0 ml of 0.1 N HCl was added to the pellet. After one-half hour at room temperature, the tube containing the resuspended pellet was centrifuged at 27,000 x g for 15 minutes. The supernatant was saved. The pellet was reextracted with 1.0 ml of 0.1 N HCl and the supernatant from this step added to the supernatant from the first extraction. The amount of cyanophycin granules could be determined by two methods. The HCl extract was directly assayed for arginine by the method described above. This value gave the amount of cyanophycin granules when compared to a standard curve which was

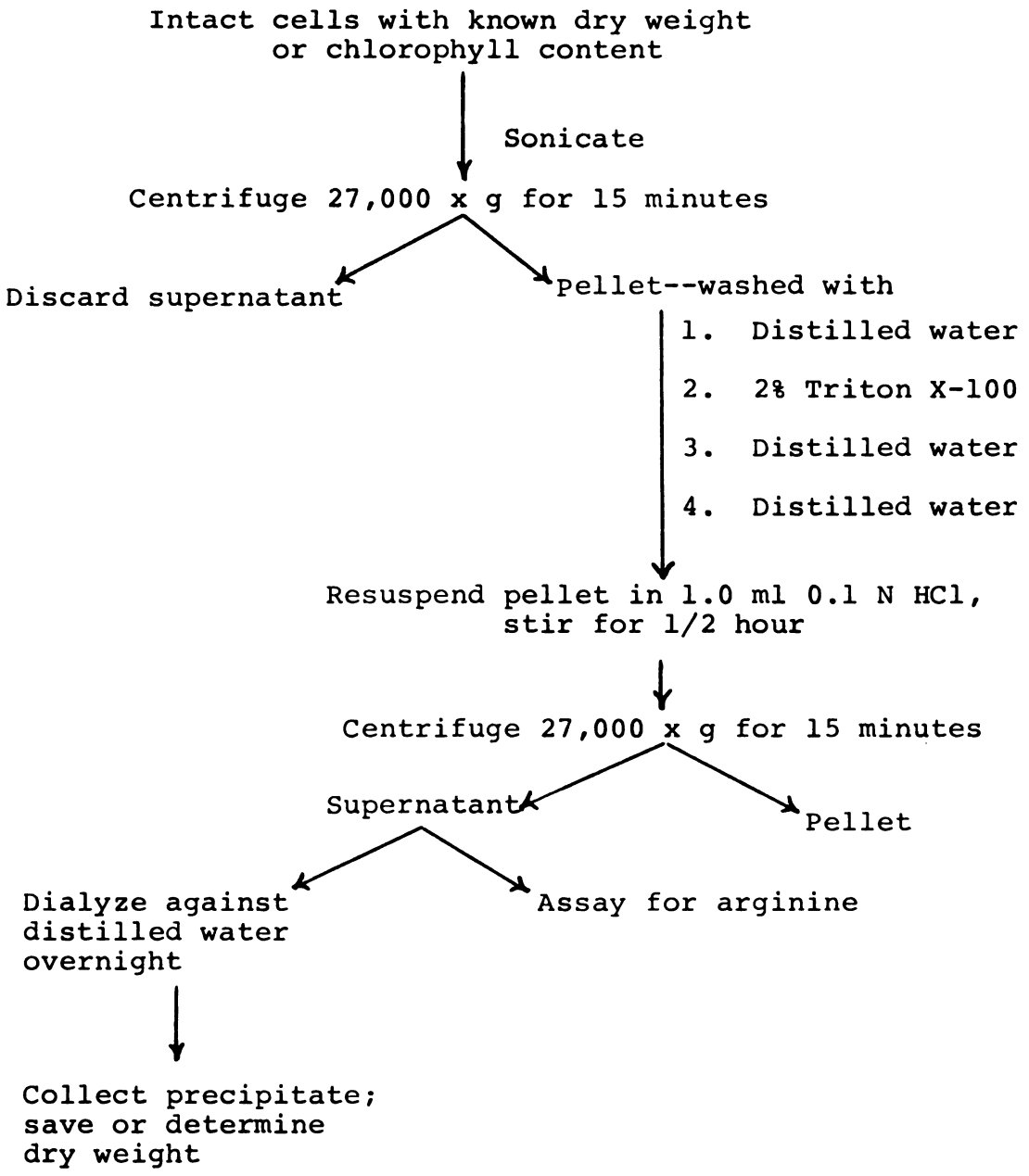


Figure 4.--Fractionation scheme for the quantitative assay of cyanophycin granules.

determined using isolated cyanophycin granules. Alternatively, the HCl extract was dialyzed against distilled water overnight. This caused the granule protein to precipitate. This protein was then saved, or its dry weight determined by the same methods used to determine the dry weight of the alga (see below).

Culture Conditions for Granule Production

Anabaena cylindrica was grown as already described (p. 16). For experiments in which the production of cyanophycin granules as a function of culture age was measured, 75 ml samples of the culture were taken every day and fresh medium was added to the culture to keep the volume constant. The 75 ml samples were analyzed for the following:

1. The amount of cyanophycin granules in the culture (as % dry weight of the cells) was determined as described above.

2. The dry weight of the alga in the culture (in mg/ml of culture) was determined by filtering aliquots of the culture through preweighed 0.45μMillipore filters (HAWG 013). The filters were dried at 50°C and reweighed. The difference in weight with and without the alga yielded the mg dry weight/ml of culture.

3. Chlorophyll content of the culture was determined by the method of Mackinney (56).

4. Phycocyanin content of the culture was determined by taking the OD_{620} of an algal suspension which had been sonicated for four minutes at setting three of a Branson Sonifier (S-125). The broken cell suspension was centrifuged at 27,000 x g for fifteen

minutes prior to the determination of the optical density. The OD_{260} was transformed into mg Phycocyanin/ml using the extinction coefficient given by Allen and Smith (2).

5. Cell protein was measured by applying the Lowry procedure (54) to the cell-free sonicates from part four above.

6. The pH of the culture was measured with a Leeds-Northrop pH meter.

Flask cultures (50 ml of medium per 125 ml Pyrex Erlenmeyer flask) were used to determine the effect of various buffering agents on growth and on the production of cyanophycin granules. Compounds to be tested were added to the standard medium (AA/16 + 1.0 gm $NaNO_3$/liter), the pH was adjusted to the desired level, and the flasks were autoclaved. Flasks were inoculated with 1.0 ml of an exponentially growing culture (seven-day-old culture in AA/16 without combined nitrogen). The cultures were grown on a rotary shaker in a Sherer-Gillett mobile greenhouse at 24°C with 700 foot-candles of light from cool-white fluorescent tubes.

Cultures used to test the effect of air flow rate upon the production of cyanophycin granules consisted of 1.5 liters of AA/16 + 1.0 gm $NaNO_3$/liter in 2.0 liter Pyrex Erlenmeyer flasks. Each flask was fitted with a rubber stopper and inserted into this stopper was a glass tube which ran to the bottom of the flask. Air was filtered through a 0.45μ filter (Millipore HAWG 013) prior to being passed through the culture. The cultures

were inoculated with 50 ml from an exponentially growing culture (seven-days-old) and were grown at room temperature under 700 foot-candles of light from cool white fluorescent tubes.

In experiments with aged medium, the aged medium was obtained from stationary fermentor cultures which had produced granules. The cells from such cultures were allowed to settle overnight at 4°C. The decanted medium was then centrifuged at 48,000 x g for fifteen minutes and the supernatant from this centrifugation step was passed through a 0.45μ Millipore filter and then through a 0.22μ Millipore filter (Millipore HAWP 047 and GSWP 047 respectively). Cells from exponentially growing cultures in AA/8 without combined nitrogen were added to the aged medium and the cells were examined at various times thereafter for the production of granules.

Assay for the Enzymatic Hydrolysis of Granule Protein

Trypsin, pepsin, and pronase were tested for their ability to cleave the cyanophycin granule protein. Isolated, lyophilized cyanophycin granules were resuspended in various incubation media (see Table 7, p. 85) at 1mg/ml, and commercial enzyme was added in amounts ranging from 1%--2.5% of the amount of cyanophycin granules present. The suspensions were incubated at 30°C for up to twenty-four hours, and 0.2 ml aliquots were taken at various

times and assayed for α-amino acids by the procedure of Rosen (77). Any significant increase in the α-amino acids compared with the 0-time control was considered to be a positive indication of enzymatic degradation of the granules.

Methylated cyanophycin granule protein (methylated at the free carboxyl of aspartic acid) was prepared by the method of Riehm et al. (74) using methanolic HCl.

Culture and Harvest of Cells Containing Protease Activity

Cultures were grown as previously described (p. 16). When a culture contained significant amounts of granules as determined by examination of the cells under the light microscope, twelve of the thirteen liters in the fermentor were removed and replaced with fresh medium. After two days, when rapid growth had resumed, as measured by increases in chlorophyll and dry weight, the culture was harvested. Cells were harvested at 48,000 x g using a Szent-Györgyi and Blum continuous flow centrifugation system (Ivan Sorvall Inc., KSB-R). From this point on, the cells were kept at 0°C. The pellet was resuspended in 0.1 M TES, pH 7.8, and was either used immediately for enzyme preparations or was frozen with dry ice plus acetone and stored at -20°C until use.

Preparation of Cell-Free Extracts

Cell-free extracts were prepared in two ways, with all operations performed at 4°C. The first method was as follows: The cells from rapidly growing cultures which had been resuspended in 0.1 M TES, pH 7.8, were cavitated in the continuous flow head of a Branson Sonifier (S-125) for one minute at setting three. The suspension was centrifuged at 27,000 x g for fifteen minutes to remove unbroken vegetative cells, heterocysts, and large fragments. The supernatant from this step was then centrifuged at 48,000 x g for thirty minutes and the membranous pellet discarded. The supernatant, which was blue-green in color and which still contained membrane fragments, was used as the crude extract.

The second method for preparation of cell-free extracts used a Ribi Cell Fractionator (Ivan Sorvall Co.). The cells were broken at 7,000 psi. The broken cell suspension was centrifuged at 27,000 x g for fifteen minutes and then at 48,000 x g for thirty minutes. The supernatant from the second centrifugation was clear blue and contained no membrane fragments. Because the resulting extracts were free of membranes, the second method was preferred to the first.

Partial Purification of the Granule Protease

Crude extracts prepared from sonicated cell suspensions were centrifuged at 103,000 x g for ninety

minutes in a Beckman L2-65B ultracentrifuge. All of the membranes in the extract are removed by this procedure. The clear blue supernatant was then fractionated by ammonium sulfate precipitation. The precipitates from the following ammonium sulfate fractions (as % of saturation at room temperature) were collected: 0-30%, 30-60%, 60-90%, 90% supernatant liquid. The precipitate from each fractionation step was resuspended in a minimal amount of 0.05 M TES, pH 7.8, and dialyzed overnight against 0.01 M TES, pH 7.8. All of the above procedures were carried out at 4°C.

Assay for Granule Protease

Assays were run in 75 x 100 mm disposable culture tubes (Becton, Dickinson and Co. 7810) and contained the following: cell extract (usually 25 µl); 100 µl containing 0.5 mg lyophilized granules in 0.1 M TES, pH 7.8; and additional 0.1 M TES, pH 7.8, to give a total volume of 0.2 ml. The reactions were initiated by the addition of the cell-free extracts. The tubes were covered with parafilm (American Can Co.) and allowed to shake at setting seven on a New Brunswick water bath shaker at 30°C. At the end of one hour (longer for experiments measuring protease activity as a function of time), 1.0 ml of 10% TCA was added. This stopped the reaction. The tube was then put on an ice bath for one hour after which it was centrifuged at 11,000 x g for fifteen minutes. The entire

supernatant was decanted into a clean tube and assayed for arginine by the procedure described above. Protease activity was thus measured as the release of TCA soluble arginine from the originally insoluble granules.

Alternatively, protease activity could be measured as the release of TCA soluble free α-amino groups. The supernatant decanted from the 11,000 x g centrifugation was extracted three times with ethyl ether to remove TCA which was present and which would interfere with the assay for α-amino groups. The supernatant was then assayed by the method of Rosen (77).

In some experiments the reaction mixtures consisted of 1.0 mg granules added to 1.0 ml cell-free extract. In these cases 0.2 ml of 50% TCA was added to stop the reaction. After centrifugation at 11,000 x g, 0.25 ml samples were taken for arginine assay and the remainder of the supernatant extracted three times with ethyl ether to remove the TCA. Fifty microliter aliquots were used for the α-amino acid assays.

One unit of enzyme activity is defined as the amount of enzyme that will release 1.0 nmole arginine/ hour when the substrate concentration is 1.0 mg in 1.0 ml total volume.

Analysis of Protease Product

One ml of 0.05 M TES, pH 7.8, containing 2.0 mg of lyophilized cyanophycin granules was mixed with 0.25 ml of

protease which had been purified by ammonium sulfate fractionation (1,710 units/ ml) and with 0.75 ml 0.05M TES pH 7.8. The reaction mixture was incubated at 30°C in a New Brunswick waterbath-shaker. Samples of 0.4 ml were taken at 0, 1/2, 1, and 2 hours. The reaction was stopped by the addition of 2.0 ml of TCA. After sitting on an icebath for one hour, the reaction mixture was centrifuged at 27,000 x g for fifteen minutes. The supernatant was decanted and then extracted three times with ethyl ether to remove the TCA. The supernatant was then frozen and lyophilized. The dry residue was taken up in 0.25 ml of distilled water and spotted on a sheet of Whatmann #4 paper. Electrophoresis was carried out at pH 1.9 in a buffer containing acetic acid-formic acid-water (150:50:800 v/v/v) as previously described (44). Upon completion of electrophoresis the paper was allowed to dry overnight at room temperature. The areas of the electropherogram containing amino acids were then located by dipping the paper in ninhydrin-acetone-acetic acid-pyridine (0.3:100:1:1 w/v/v/v) and heating at 70°C.

Other Assays of Protease Activity

"Trypsin-like" activity was measured using the artificial substrate benzoyl-arginine-p-nitroaniline (BAPNA) (24). Leucine amino peptidase was measured qualitatively after starch gel electrophoresis of protease

extracts with tris-citrate buffer, pH 7.0, using the artificial substrate l-leucyl-beta-naphthylamide (78).

RESULTS

Part A: Composition, Identification, and Characterization of Isolated Cyanophycin Granules

This section reports the isolation and characterization of the cyanophycin granules from Anabaena cylindrica. It will be shown that the granules consist of a unique polypeptide of heterogeneous molecular weight, and that this polypeptide is composed of only two amino acids, aspartic acid and arginine.

Composition

Table 3 shows the composition of the granules. With the Lowry procedure (54) only 20% of the granule weight could be accounted for as protein, while analysis of free amino groups after hydrolysis (77) indicated that 86% of the granule weight (98% of the granule dry weight) was protein. This apparent contradiction became understandable when an acid hydrolysate of the granules was put through an amino acid analyzer. Only two amino acids could be detected, aspartic acid and arginine, in a molar ratio of 1:1. Only the normal background of ammonia was detected on the amino acid analyzer, indicating that

Table 3. Composition of isolated cyanophycin granules.

COMPONENT	COMPOSITION (%)
Protein	86[a] (20%)[b]
Carbohydrate	1
Lipid	0
Phosphate	1
Moisture	10
TOTAL	98

[a]Measured as free amino groups after acid hydrolysis (77).

[b]Measured by the Lowry procedure (54).

none of the aspartic acid in the granules was present as the amide.

The unusual amino acid composition of the granules explains why only 20% of the dry weight could be accounted for as protein by means of the Lowry procedure (54). This procedure is sensitive to both the amount of peptide bonds and the amount of tyrosine in a protein. Since the granules contain no tyrosine, the amount of protein is underestimated. The Biuret procedure, which is sensitive to peptide bonds only (53), accounts for essentially all of the granule dry weight as protein.

Because of the unique composition of the granules, it was felt desirable to confirm the presence of aspartic acid and arginine. Trimethysilylated (TMS)-amino acids from the hydrolysate were run on a gas chromatograph-mass spectrometer along with a TMS-aspartic acid standard. The retention times on the gas chromatograph were the same for the hydrolysate and standard, and the mass spectra were identical. TMS-arginine, which is known to break down during gas chromatography (73), was not detected.

The Sakaguchi reaction is a relatively specific test for arginine (59, 60). The isolated granules gave a red color with the Sakaguchi reagent, confirming the presence of arginine.

The specific rotation determined for a hydrolysate of the granules was +29.4. This compared with a calculated

specific rotation of +31.2 if both the arginine and the aspartic acid in the granules were of the L-configuration (67). If one amino acid was D- and the other L- the specific rotation would be near zero. If both amino acids were D- it would be -31.2. Therefore, both the aspartic acid and the arginine in the granules were of the L-configuration.

Characteristics of the Protein of Cyanophycin Granules

Figure 5 shows the patterns obtained after polyacrylamide gel electrophoresis of granules which had been dissociated in three different solvent systems: SDS, urea, and phenol-acetic acid-water (2:1:1 w/v/v). The bands were visualized by staining with Amido Black 10B. Only one protein-containing band can be seen in each gel.

The granules are composed of high molecular weight polymers and not of free amino acids or short peptides. This is shown most simply as follows. They are insoluble in distilled water but can be solubilized in dilute acid or base. Granules that have been solubilized in 0.1 N HCl and then dialyzed against 0.1 N HCl do not pass out of the dialysis bag, indicating that the granule polypeptide has a high molecular weight. On analytical acrylamide gels containing sodium dodecyl sulfate, the granule material moves as a single band. The limits of the band indicate a molecular weight between about 25,000 and

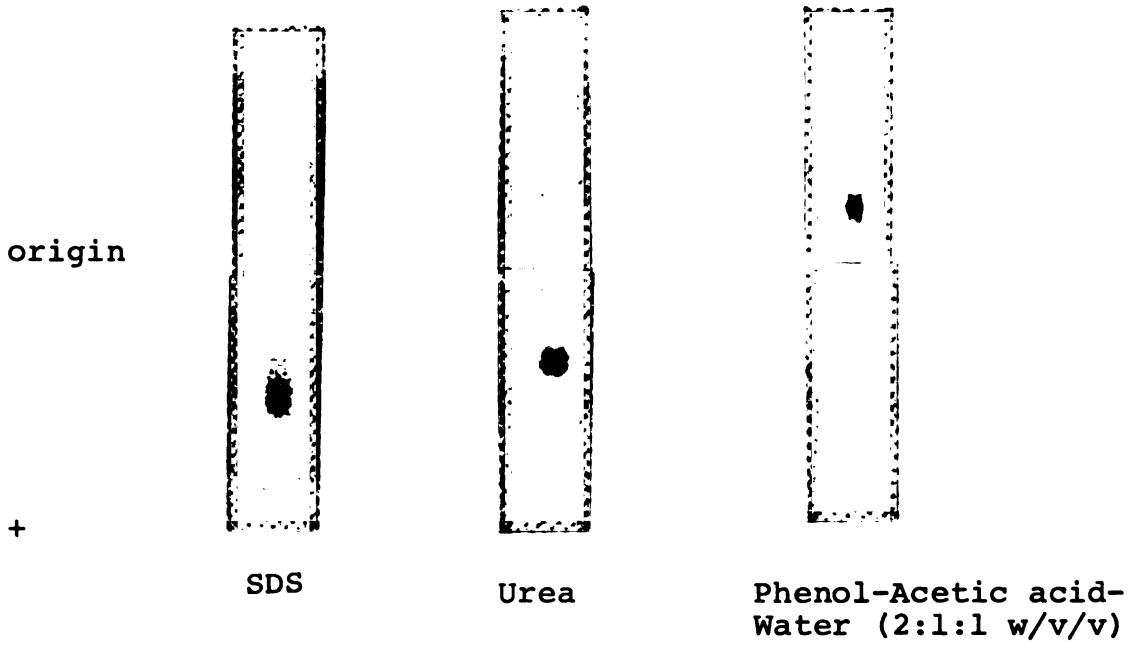


Figure 5.--Polyacrylamide gel electrophoresis of cyanophycin granule protein solubilized in three dissociating systems: SDS, urea, and phenolacetic acid-water (2:1:1 w/v/v).

100,000 when compared with proteins of known molecular weight run on the same gel (Figure 6).

Figure 7 shows a plot of elution volume (V_e)/void volume (V_o) versus arginine content of the column eluate, for granules solubilized in 6.0 M urea-0.1 M H_3PO_4 (pH 2.7). Since the pK of the ß-carboxyl of aspartic acid was 3.9, and the pH of the buffer was 2.7, ionic bonding within the protein should be neutralized. 6.0 M urea would eliminate hydrogen bonding. Thus, the granule protein should be completely dissociated. The broad band on Sephadex G-200 indicated a population of molecules with heterogeneous molecular weight. From the V_e/V_o values for standard proteins, the molecular weight limits were found to be similar to those determined on analytical acrylamide gels.

Partial Acid Hydrolysis

Figure 8 shows the per cent hydrolysis of the isolated granules in 6 N HCl at 105°C as a function of time of hydrolysis. The hydrolysis was initially very rapid, with the material being 50% hydrolyzed after only about forty minutes. Rapid hydrolysis was expected since aspartic acid is usually the first amino acid which is cleaved during the acid hydrolysis of a protein (9, 80, 86).

Figure 9 shows an electropherogram of a granule hydrolysate (about 60% hydrolyzed) stained with both

Figure 6.--Semilogarithmic plot of molecular weight versus relative distance of migration in sodium dodecyl sulfate-containing acrylamide gels for protein standards and for the polypeptides from cyanophycin granules. Granule polypeptides migrate as a single broad band, the limits of which are indicated. Protein standards include: cytochrome c (12,400), chymotrypsinogen A (25,000), ovalbumin (45,000), bovine serum albumin (67,000; 134,000; 201,000), and α-globulin (160,000; 55,000; 25,000).

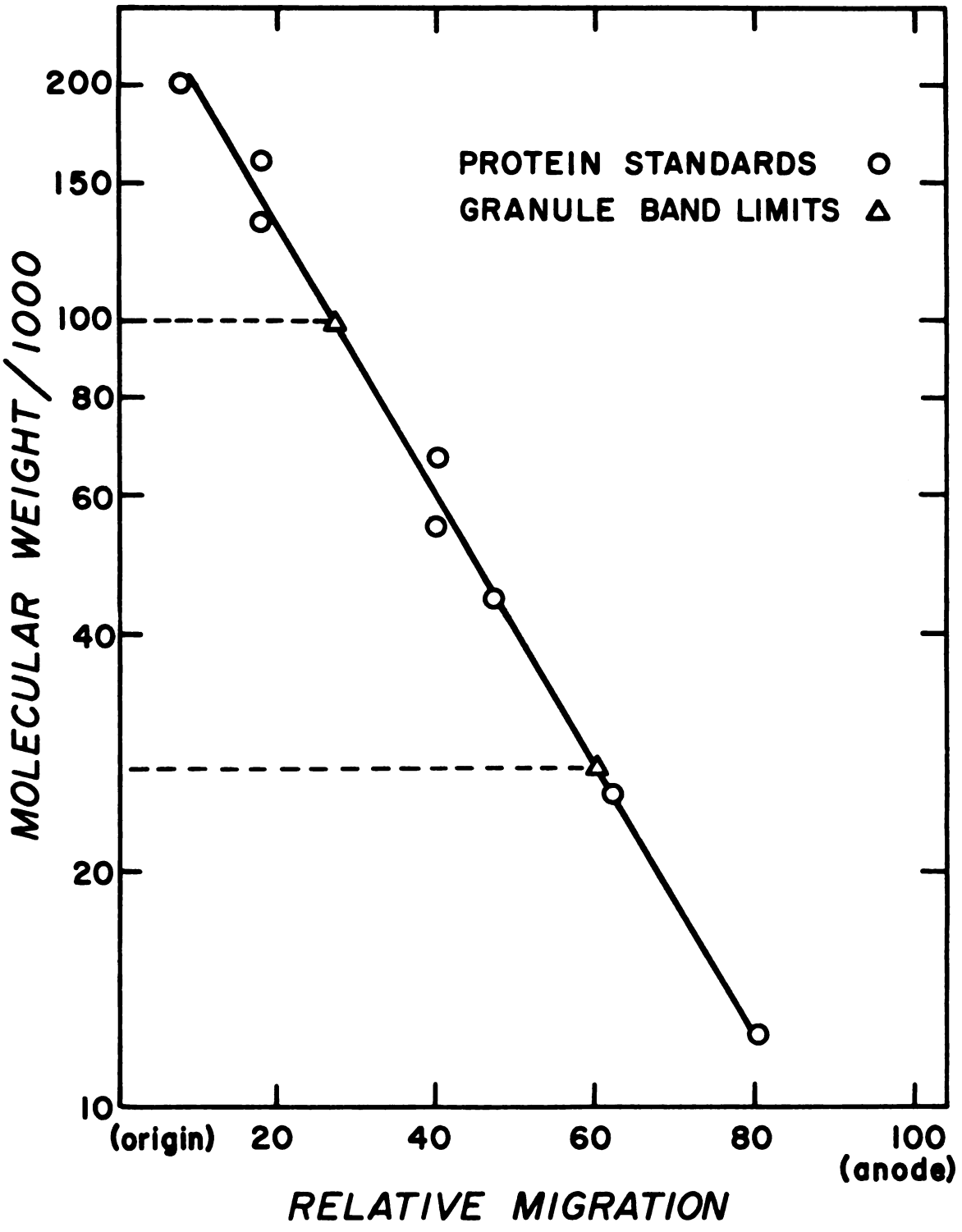

200
150
100
80
60
40
20
10
MOLECULAR WEIGHT / 1000
PROTEIN STANDARDS
GRANULE BAND LIMITS
(origin)
20
40
60
80
100
(anode)
RELATIVE MIGRATION

Figure 7.--Sephadex G-200 separation of granule polypeptides dissociated in 6.0 M urea-0.1 M H_3PO_4, pH 2.7. The ratio of elution volume (V_e) to void volume (V_o) is a measure of the molecular size (4). The amount of granule protein at each value of V_e/V_o is determined by measuring the arginine content (see Materials and Methods, p. 21), which is plotted as the absorbance determined at 520 nm.

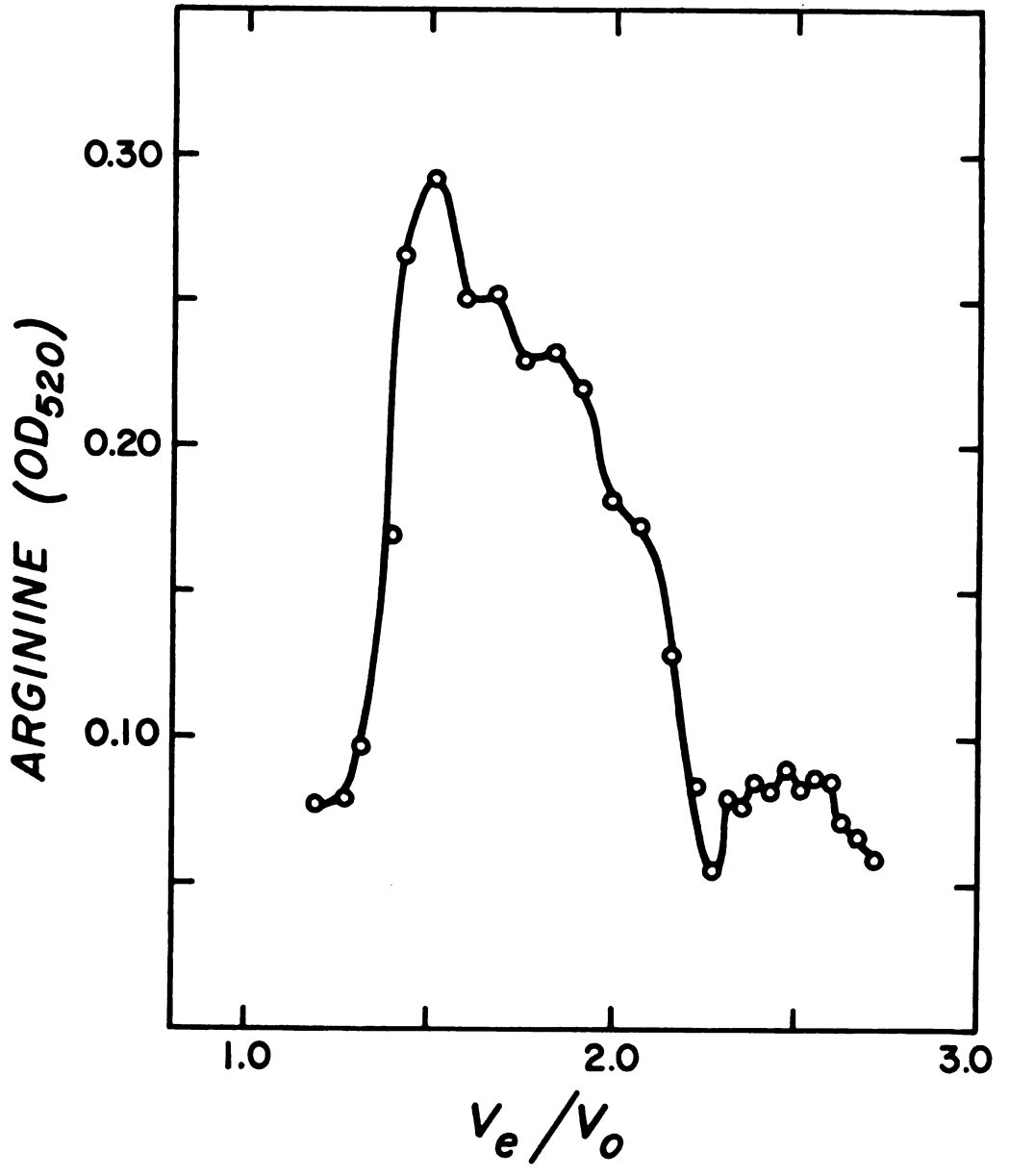

0.30
0.20
0.10
ARGINE (OD520)
1.0
2.0
3.0
Ve/Vo

Figure 8.--Per cent hydrolysis of cyanophycin granules in 6 N HCl at 105°C as a function of time of hydrolysis. The amount of hydrolysis was determined by measuring α-amino groups released (77). A sample hydrolyzed for twenty-four hours was considered 100% hydrolyzed. Therefore, the per cent hydrolysis at any given time was determined by taking the ratio, free α-amino groups at the given time/free α-amino groups at twenty four hours, and multiplying this ratio by 100.

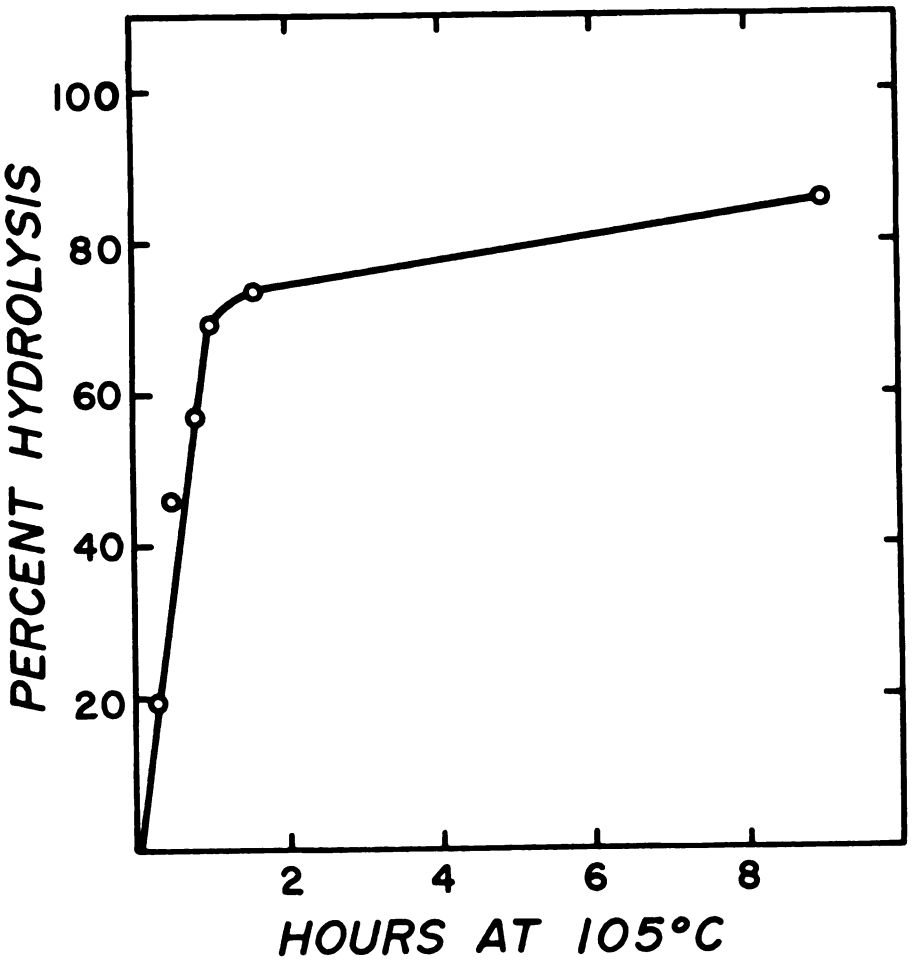
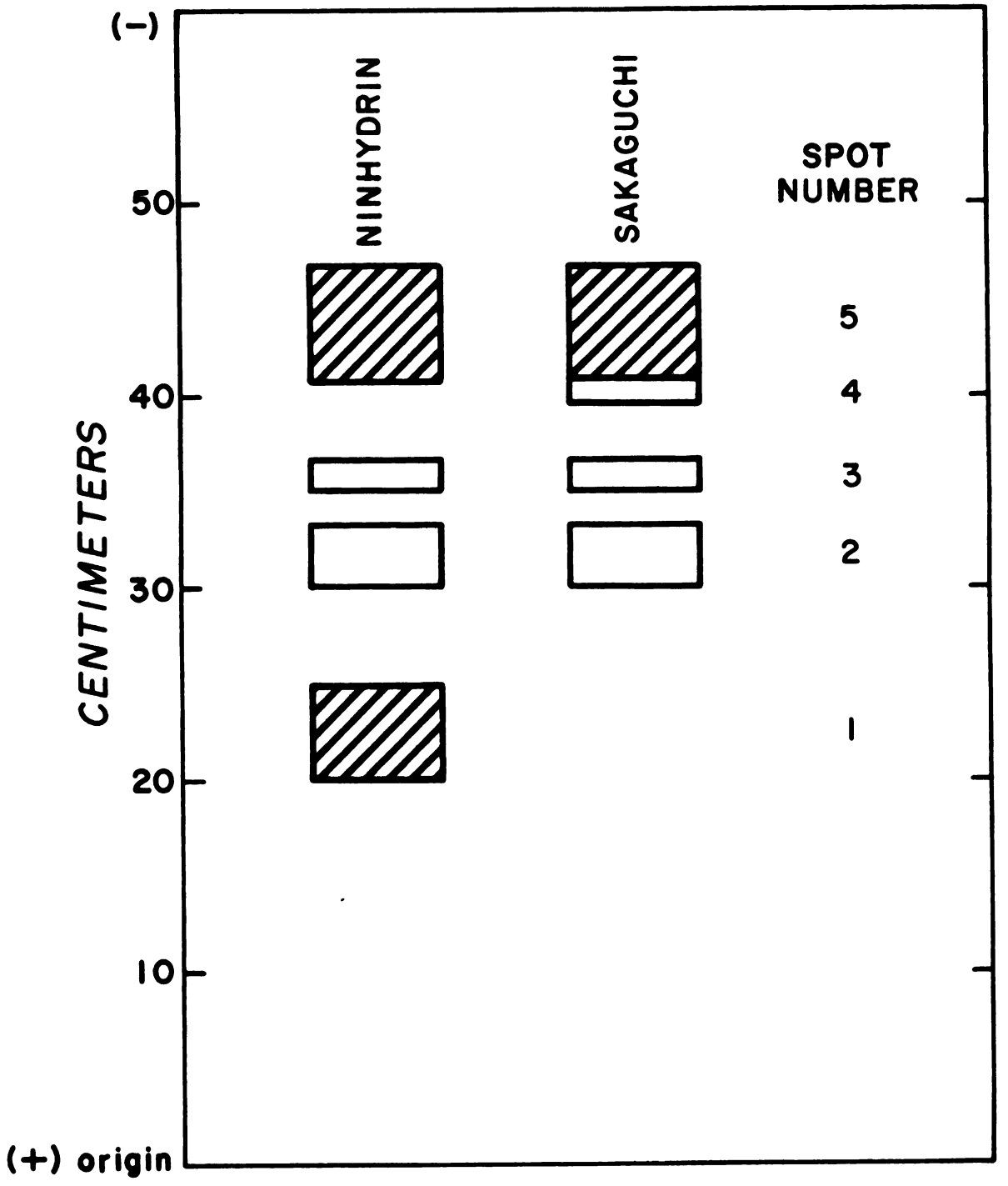

PERCENT HYDROLYSIS
100
80
60
40
20
2
4
6
8
HOURS AT 105°C

Figure 9.--Electropherogram of cyanophycin granules which had been partially hydrolyzed (60%) in 6 N HCl at 105°C for one hour. Ninhydrin stains areas of the paper containing free α-amino groups, while Sakaguchi reagent stains areas containing arginine. The cross-hatched spots represent areas of heavy staining while the open spots represent areas of light staining. Spot 1 co-electrophoresed with free aspartic acid and spot 5 co-electrophoresed with free arginine.


(-)
NINHYDRIN
SAKAGUCHI
SPOT NUMBER
50
40
30
20
10
CENTIMETERS
5
4
3
2
1
(+) origin

ninhydrin and Sakaguchi reagent. The bulk of the material in the residue of the hydrolysate was free aspartic acid (spot 1) and free arginine (spot 5), but a few peptides are present (spots 2, 3, and 4). When the peptide spots was further hydrolyzed and chromatographed, all the peptides were found to contain both aspartic acid and arginine.

Density

The position of the band of cyanophycin granules after centrifugation for one hour at 27,000 x g in a density gradient of Renographin is shown in Figure 10. The density of the peak of the band was determined to be 1.36. Upon centrifugation for a second hour at 27,000 x g the position of the granule band did not change, indicating that it had come to equilibrium within the first hour.

Solubility and Staining

Table 4 lists the solubility properties of the isolated granules. The granules are not soluble in distilled water, but are soluble in acidic or basic solutions and in solutions which are highly ionic. A non-ionic detergent such as Triton-X-100 did not solubilize the granules.

Figure 11 shows the solubility of the isolated cyanophycin granules in increasing concentrations of trichloroacetic acid (TCA). The granules are soluble at

Figure 10.--Density gradient centrifugation of isolated cyanophycin granules. The gradient is composed of Renographin solutions from density 1.25 to density 1.49. The gradient was centrifuged for one hour at 27,000 x g, and fractions collected by puncturing the bottom of the tube with a needle. The density was determined by measuring the optical density of each fraction at 260 nm and transforming the OD_{260} into density (ρ) by the use of the formula $\rho = 1 + 0.385\ (OD_{260} \times 10^{-4})$.

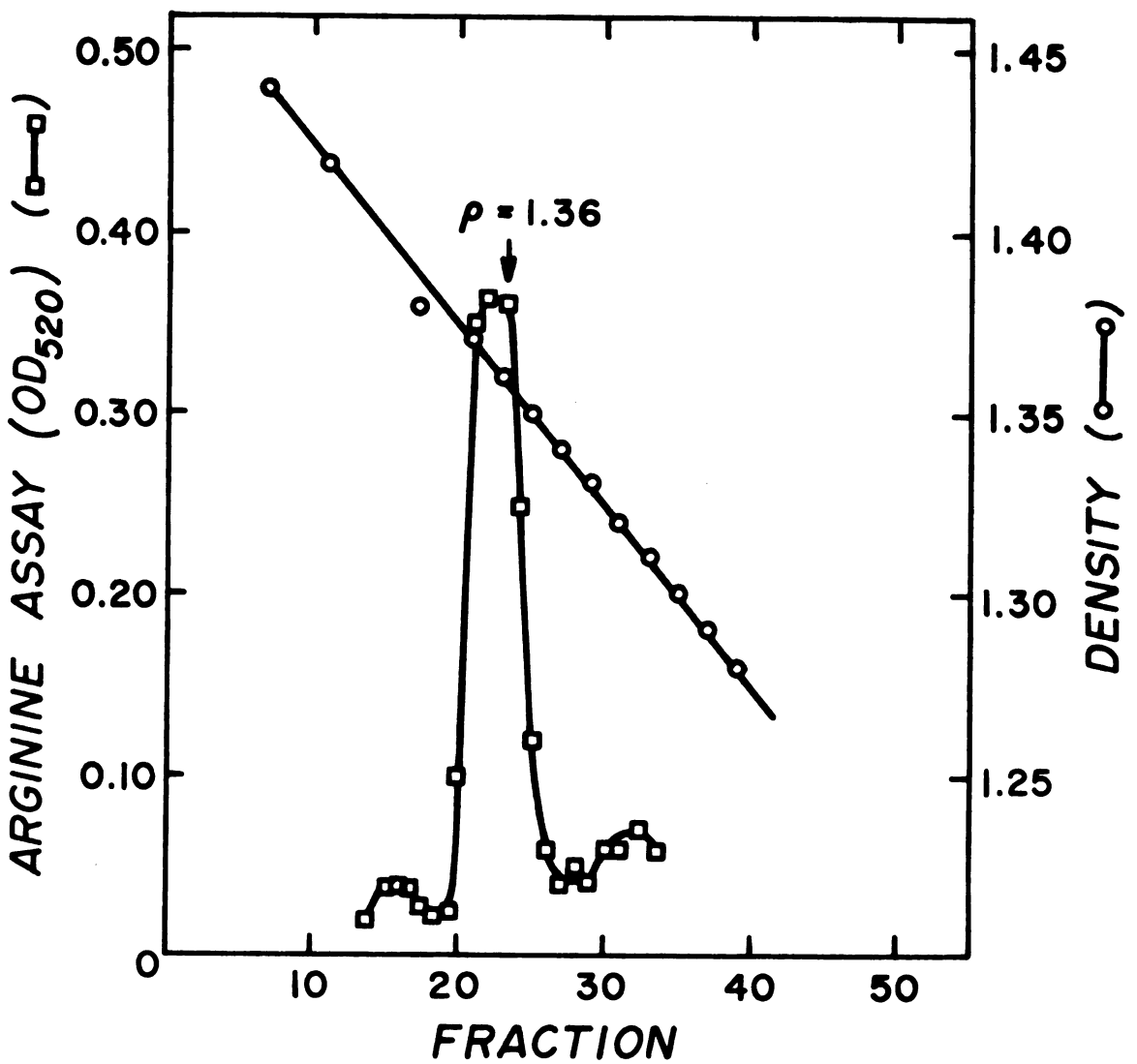
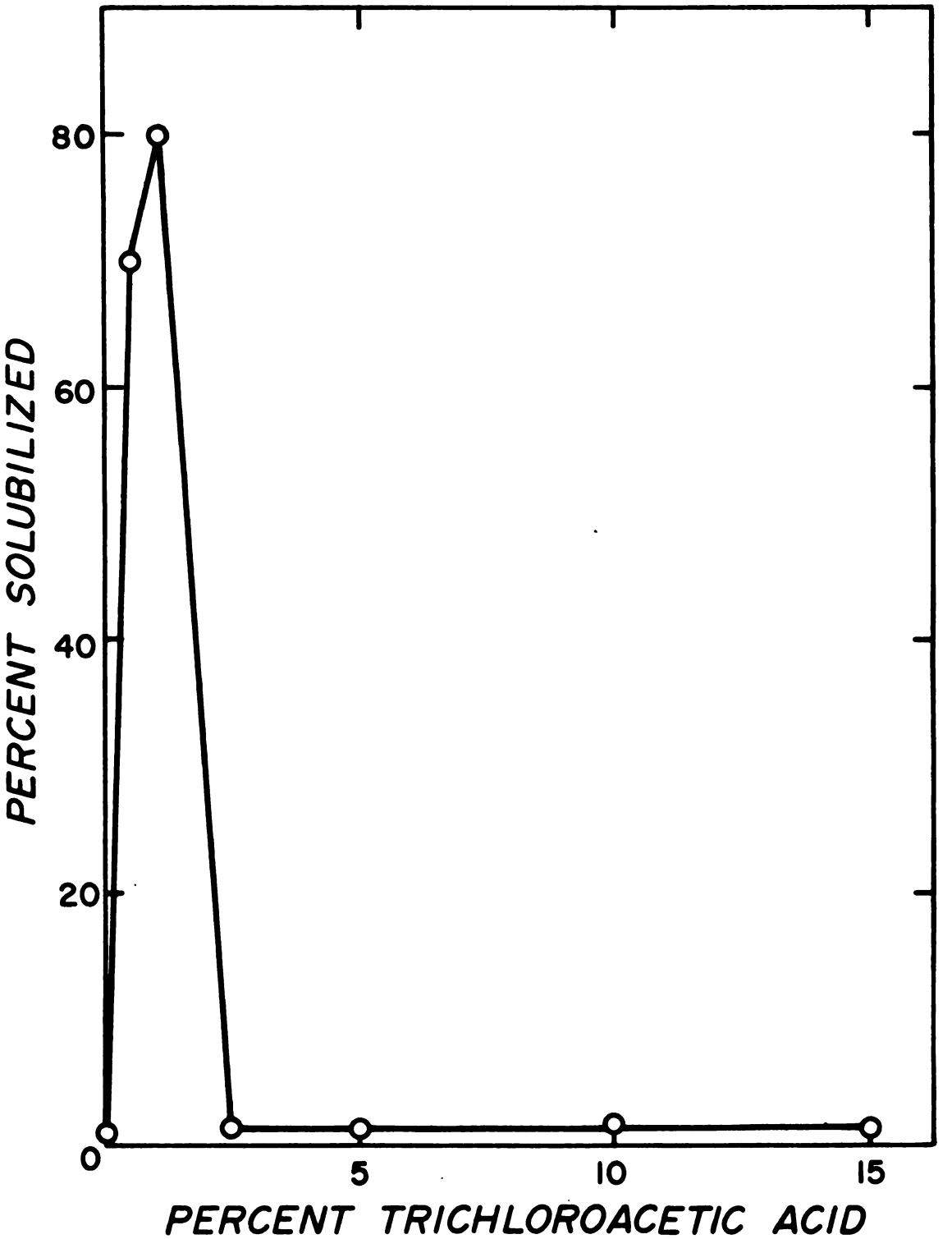

0.50
0.40
0.30
0.20
0.10
0
1.45
1.40
1.35
1.30
1.25
ρ = 1.36
ARGININE ASSAY (OD520) (□—□)
DENSITY (○—○)
10
20
30
40
50
FRACTION

Table 4. Solubility of isolated cyanophycin granules in different solvents. The granules were considered soluble if twelve hours after the addition of the reagent to the lyophilized granules the originally milky-white suspension had turned clear.

REAGENT	SOLUBILITY[a]
Distilled Water	-
0.05 N HCl	+
3.0 N NaOH	+
0.5% Glycine	-
0.01 M EDTA	-
Methanol	-
Ethylene Glycol	-
DMSO	-
Formamide	-
Dimethylformamide	-
1% SDS	+
2% Triton X-100	-
1% Deoxycholate	-
4.0 M Urea	+
Cesium Chloride (density = 1.4)	+
Renographin-60 (density = 1.355)	-

[a]"+" means soluble and "-" means insoluble.

Figure 11.--The solubility of isolated cyanophycin granules in various concentrations of trichloroacetic acid (TCA).


80
60
40
20
0
PERCENT SOLUBILIZED
5
10
15
PERCENT TRICHLOROACETIC ACID

low concentrations of TCA, but are insoluble at high concentrations of TCA.

Table 5 lists the staining properties of the isolated granules. Besides stains which characterize lipid, starch and protein, several stains classically used to identify cyanophycin granules (acetocarmine, neutral red and methylene blue [27]) are listed.

Electron Microscopy

Figure 12 shows intact filaments from a two-week-old culture of Anabaena cylindrica which have been stained with osmium tetroxide and examined in the electron microscope. The dark areas (labeled--cy) represent structured granules. Figure 13 shows intact cells from the same culture stained with $KMnO_4$. The clear areas represent structured granules. The patterns of staining with both osmium and potassium permanganate are similar to those reported by Lang and Fisher (52).

Figures 14 and 16 show isolated cyanophycin granules stained with osmium, while Figure 15 shows isolated granules stained with potassium permanganate. Comparison of Figure 12 with Figures 14 and 16 and of Figure 13 with Figure 15 shows that the isolated cyanophycin granules have the same morphology as structured granules.

Figure 16 shows an isolated granule stained with osmium, at a higher magnification than Figure 13. The large granule in the center shows detailed substructure.

Table 5. The staining properties of isolated cyanophycin granules.

STAIN	REACTION[a]	TEST FOR
Acetocarmine	+	-
Neutral Red	+	-
Methylene Blue	+	-
Sudan Red	-	Lipid
Alkaline Bromphenol Blue	-	Lipid
I_2-KI	-	Starch
Sakaguchi Reagent	+	Arginine
Conc. HNO_3	-	Protein-tyr., tryp., phenylalanine
Amido Black 10B	+	Protein

[a]Granules in distilled water (1.0 mg/ml) were mixed with various stains, allowed to stand for one hour, and centrifuged at 1,000 x g for five minutes. "+" indicates that the granules in the pellet had taken up the stain, while "-" means that there was no staining.

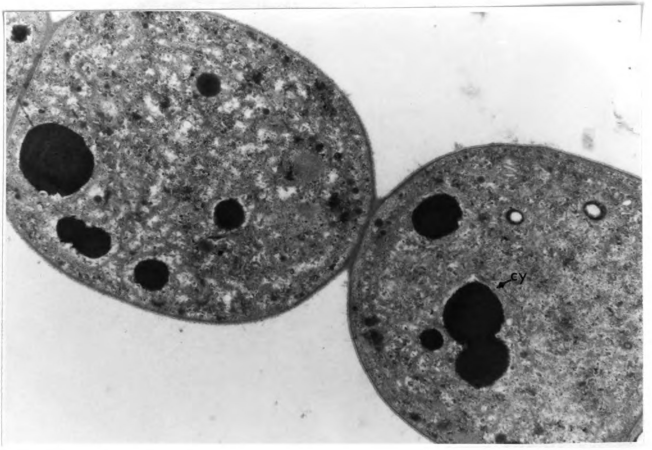


Figure 12.--Electron micrograph of intact filaments stained with osmium. Both cells in the picture are vegetative cells. cy. = cyanophycin granule.

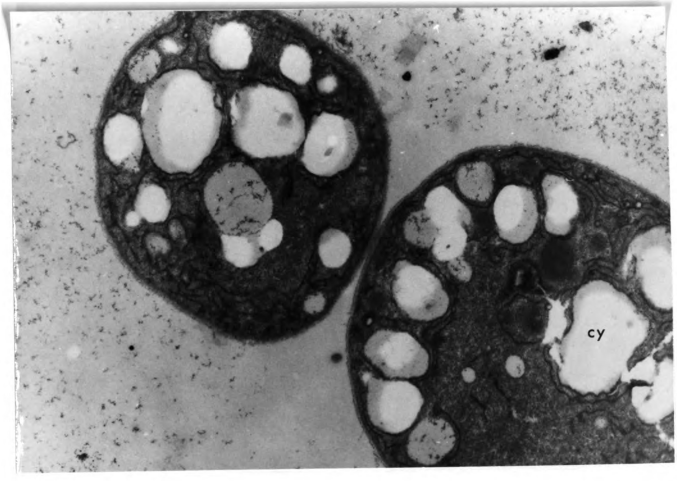


Figure 13.--Electron micrograph of intact filaments stained with $KMnO_4$. Both cells in the picture are vegetative cells. cy. = cyanophycin granule.

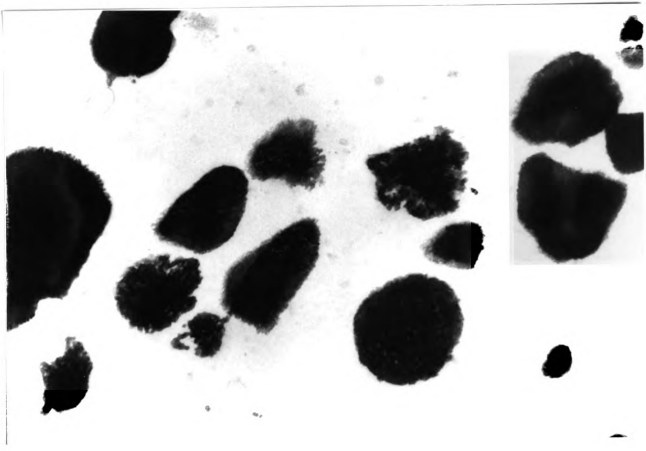


Figure 14.--Isolated cyanophycin granules stained with osmium.

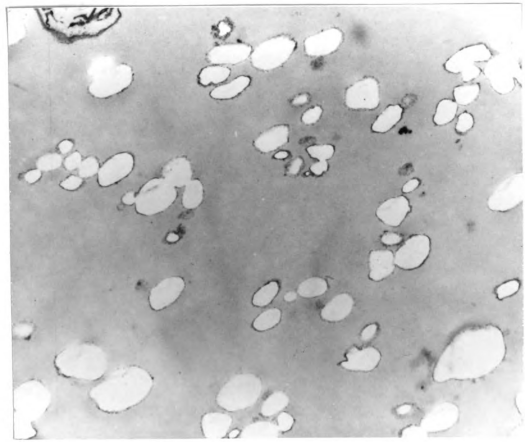


Figure 15.--Isolated cyanophycin granules stained with $KMnO_4$.

Figure 16.--Isolated cyanophycin granules stained with osmium. This picture was taken at higher magnification than Figure 13, and the large granule in the center of the picture shows substructure.

The substructure seen in the picture is closely similar to but not identical to that seen in a number of published electron micrographs of cyanophycin granules. That is, there are more tiny holes than are usually seen. The differences in detailed substructure may be due to the isolation procedure, or may be the result of small differences in the staining procedure (52).

Part B: The Physiology of the Production of Cyanophycin Granules

This section is concerned with the results of experiments on the production of cyanophycin granules in Anabaena cylindrica.

Figure 17 shows the amount of granules (in per cent dry weight of the cells) at different culture ages, and compares this with the dry weight (mg/ml) and the amount of chlorophyll in the culture (µg/ml). At day 13 the culture is just leaving the phase of exponential growth and entering the stationary phase. Granule synthesis begins at 14 days and proceeds throughout stationary phase, and reaches a maximum at about 18 days.

In one experiment it was found that in rapidly growing cells (specific growth rate $k_{10} = 0.35$ (46) as measured on the basis of chlorophyll content, where $k_{10} = 0.301$ indicates one doubling/day) the amount of cyanophycin granules present represented 0.3% of the total cell dry weight. In some stationary cultures up to 9% of the dry weight could be cyanophycin granules.

Figure 17A and B.--The amount of cyanophycin granules as a function of the culture age. In graph A are presented the following parameters of growth: chlorophyll in µg/ml and dry weight of the alga in mg/ml of culture. Graph B shows the amount of cyanophycin granules as a per cent of the cell dry weight. The arrow indicates that at day 18, eleven of the twelve liters in the fermentor culture were removed and eleven liters of fresh medium were added to reinitiate growth.

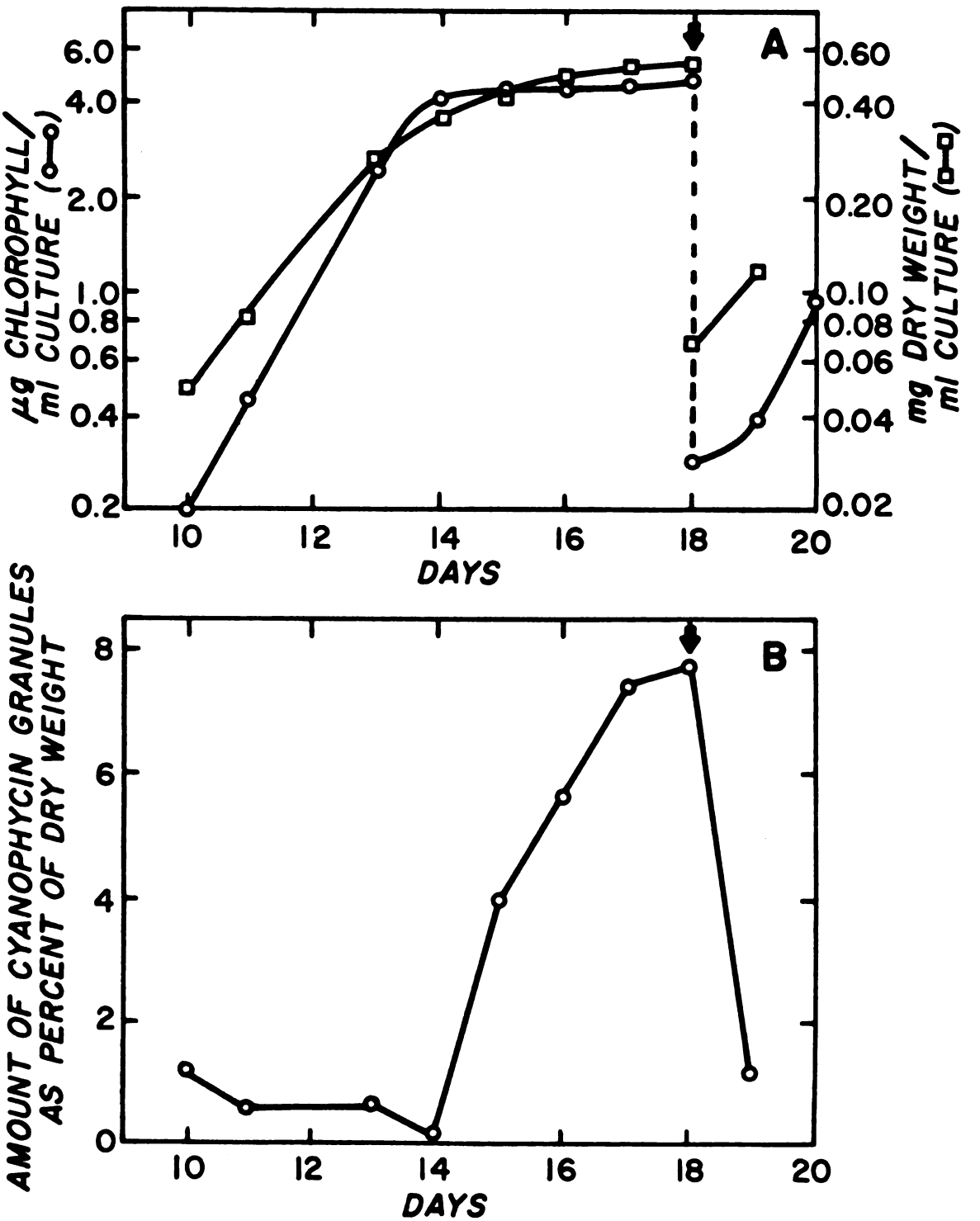
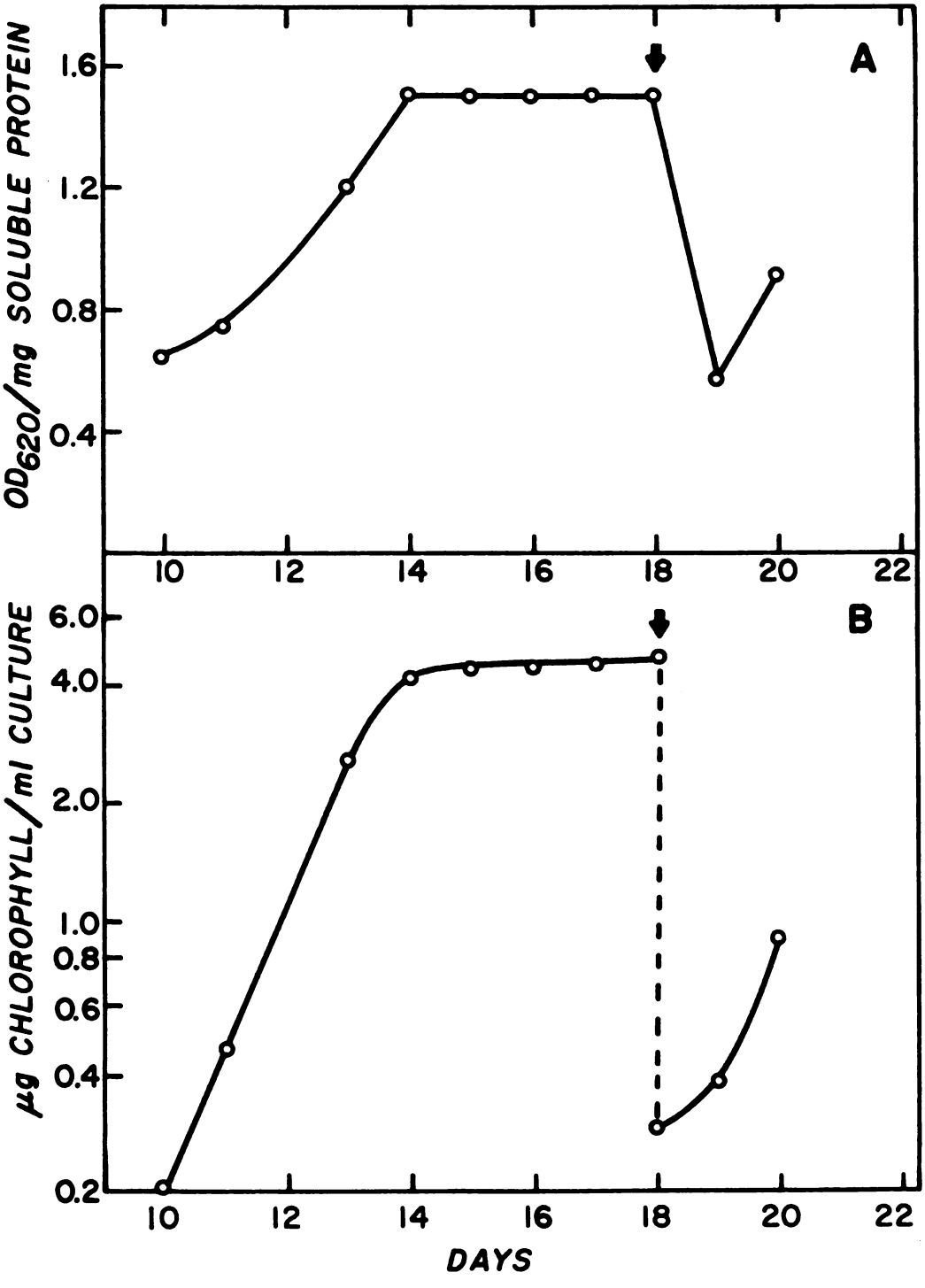

A
6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
µg CHLOROPHYLL/ ml CULTURE (o—o)
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
mg DRY WEIGHT/ ml CULTURE (□—□)
10
12
14
16
18
20
DAYS
B
AMOUNT OF CYANOPHYCIN GRANULES AS PERCENT OF DRY WEIGHT
8
6
4
2
0
10
12
14
16
18
20
DAYS

Figure 17 also shows that when a culture in stationary phase is diluted, and growth recommences, the granules are rapidly broken down (7.8% of the dry weight to 1.3% of the dry weight in one day). Figure 18 shows the specific phycocyanin content in terms of the ratio OD_{620}/mg cytoplasmic protein) of the vegetative cells at different stages in the growth of a culture. The ratio increases during the exponential growth of the culture but remains a constant 1.5 after the cells enter stationary phase. When the culture is diluted and growth resumes, the ratio falls rapidly (1.5 to 0.58 in one day) indicating either that there is some effect of culture conditions on the phycocyanin chromophore (for which no precedent is known) or that the phycocyanin protein is broken down and used as a reserve. If the phycocyanin protein is being broken down, the decrease in the specific phycocyanin content from 1.5 to 0.58 indicates that in one day 61% of the phycocyanin is used up. Since Anabaena cylindrica growing with $NaNO_3$ in the medium was found to have 0.29 mg of phycocyanin/mg of protein and 0.51 mg of protein/mg of dry weight, the first day after the transition from stationary phase to exponential growth the alga uses up 0.09 mg phycocyanin/mg dry weight. In the same period the amount of granules drops from 7% of the dry weight to 1.3% of the dry weight indicates that 0.057 mg of granules/mg of dry weight is utilized. Therefore, when

Figure 18A and B.--Concentration of phycocyanin as a function of culture age. Graph A gives the ratio of OD_{620}/mg solubilized protein over the growth period from 10-20 days. Graph B shows the chlorophyll content of the culture (μg chlorophyll/ml of culture) and is used to indicate the growth stage of the culture. The arrow at day 18 indicates when eleven of the twelve liters in the fermentor culture were removed and eleven liters of fresh medium were added to reinitiate growth.

vanin as a
o of
od from
t of the
d to indi-
at day 18
fermentor
edium were

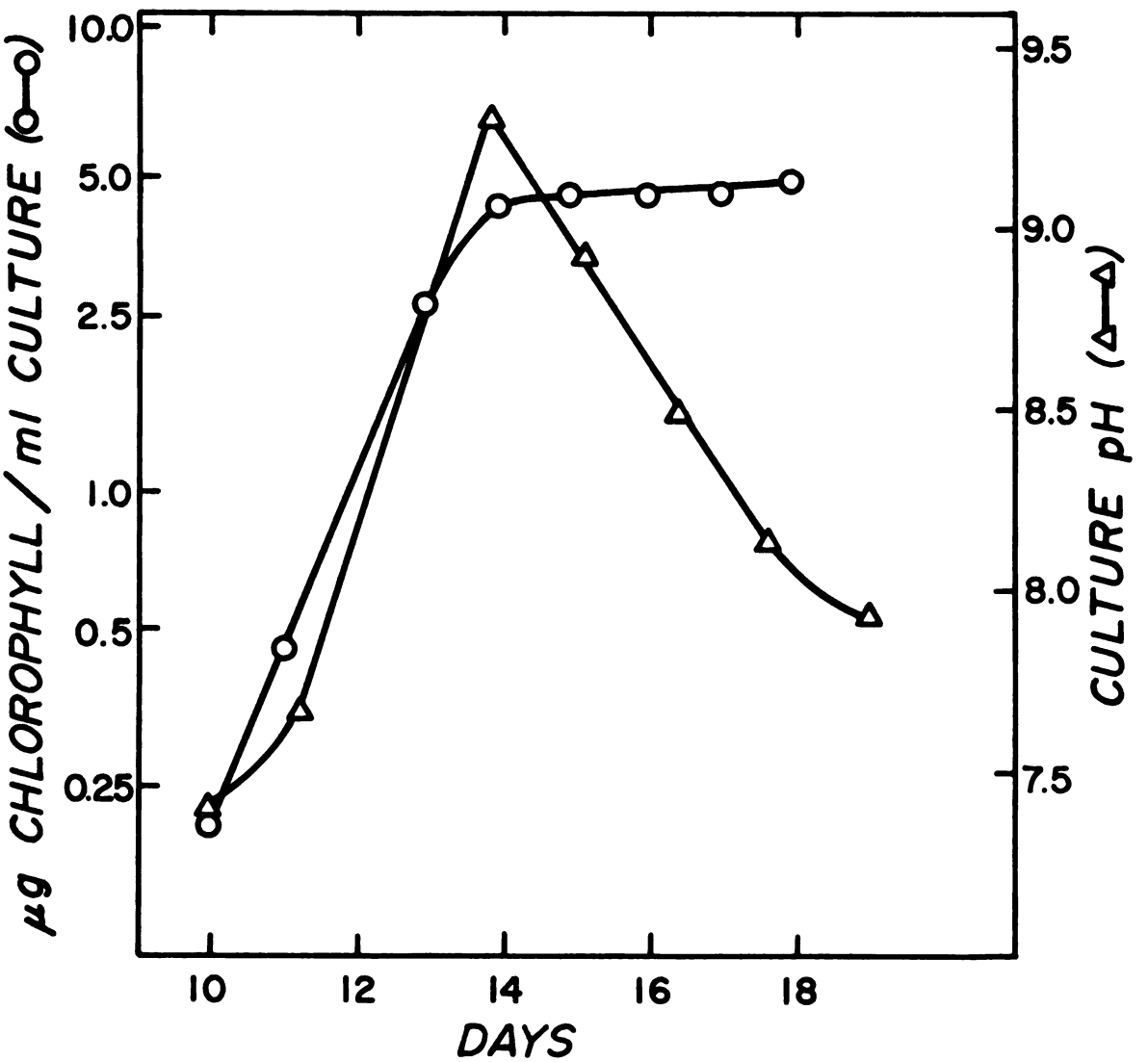


exponential growth is resumed after stationary phase, similar amounts of cyanophycin granules (0.06 mg/mg of dry weight) and phycocyanin (0.09 mg/mg of dry weight) are utilized as reserve materials.

Figure 19 shows the pH of the fermentor medium as a function of culture age. The pH rises from an initial value of 7.42 to a high of 9.32 at the end of exponential growth. After reaching the maximum, it falls slowly. The rise in pH is apparently due to the uptake of NO_3^- which is present in the medium as 1.0 gm $NaNO_3$/liter. The alga apparently cannot take up enough cations to keep ionic balance and as a result excretes OH^- which causes the pH to rise. Uptake of 1.0 gm $NaNO_3$/liter (about 0.01 M) in the lightly buffered medium used (AA/16) could theoretically raise the pH to 12 if there were uptake of NO_3^- and no compensatory uptake of Na^+. The fall in pH is probably due to the fact that the carbon dioxide in the air that is bubbling through the culture at four liters/minute acts a weak acid ($CO_2 + OH^- \rightarrow HCO_3^-$) and brings the pH down slowly. Replacing $NaNO_3$ with 1.0 gm KNO_3/liter did not affect the initial rise in pH.

Flask cultures in AA/16 did not form large numbers of granules even when the cultures reached stationary phase. Cultures with added nitrate (1.0 gm/liter) grew to less than one-fifth of the final dry weight of cultures not containing nitrate and also did not form granules.

Figure 19.--The pH of the culture medium as a function of the culture age. The chlorophyll content (μg chlorophyll/ml culture) of the culture is given as a parameter of culture growth.


10.0
5.0
2.5
1.0
0.5
0.25
µg CHLOROPHYLL / ml CULTURE (o—o)
9.5
9.0
8.5
8.0
7.5
CULTURE pH (Δ—Δ)
10
12
14
16
18
DAYS

The poor growth of the cultures containing nitrate is due to the rise in pH. Because there is much less gas exchange in a flask culture than in a fermentor being bubbled with air, the carbon dioxide in the air cannot serve efficiently to neutralize base formed, and the pH of the medium rises until it reaches a point where the cells will no longer grow. Therefore, since it was felt desirable to develop a flask culture system that would form significant amounts of cyanophycin granules (fermentor cultures not being applicable to large numbers of experiments) the effects of various buffering agents on growth and granule production were examined. The buffers were used at a concentration of 0.01 M. The pH at the end of the experiments was measured to make sure that the pH was being held constant. Table 6 gives the results of these experiments. Although a number of Good buffers (34) would support growth in the presence of 1.0 gm $NaNO_3$/liter, only Bicine allowed the formation of granules. Bicine, however, was not used routinely in the following experiments because in its presence many of the cells were misshapen and on occasion large vacuolated areas were observed in the cells. Instead, the pH problem was overcome by bubbling air through the flask cultures. Cultures in 2-liter flasks were used for measuring the effect of air flow rate on growth and granule production. Preliminary results indicate that whereas growth occurs with air flow rates of

Table 6. The effects of various buffering agents on the growth of Anabaena cylindrica and on the production of granules.

BUFFER[a]	pH[b]	GROWTH[c]	GRANULE FORMATION[d]
None	7.3 → 9.9[e]	+	-
TES	7.8	+++	-
TRICINE	8.2	++	-
BICINE	8.4	++	+
TAPS	8.6	++	-
Glycyl-glycine	8.4	-	-
Alanyl-glycine	8.4	+	-
Alanyl-alanine	8.5	++	-
Phosphate	7.2	-	-

[a]Buffer concentration if 0.01 M in the medium of Allen and Arnon (1) diluted sixteen times, plus 1.0 gm $NaNO_3$/liter.

[b]Initial and final pH unless otherwise noted.

[c]"-" = no growth "+" = fair growth "++" = good growth "+++" = excellent growth.

[d]"-" = no granules "+" = granules form as scored at the stationary phase of growth.

[e]The initial pH of 7.3 increased to 9.9 at the end of growth.

0.25 liters/min and 3.0 liters/min, cyanophycin granules form only in cultures bubbled with air at 3.0 liters/minute. These experiments suggest that aeration of the culture may be important in controlling the production of cyanophycin granules.

When an exponentially growing culture is centrifuged and resuspended in aged medium, large numbers of granules form in about two days. If a cell suspension is mixed 1:1 (v/v) with aged medium, it takes two weeks for granules to form. Also, concentrates of aged media prepared by lyophilization of aged media and resuspension in small volumes of water fail to induce granule formation upon addition to exponentially growing cultures of Anabaena cylindrica. These data indicate that the depletion of certain nutrient(s) is necessary for granule formation to occur.

Part C: Partial Purification and Characterization of a Protease from Anabaena Cylindrica Which Degrades the Protein of Cyanophycin Granules

Data already presented show that when a culture in stationary phase is diluted and growth resumes there is a rapid degradation of cyanophycin granules (Figure 17B). These data suggest that young, rapidly growing cultures possess an enzymatic system capable of degrading the protein of cyanophycin granules. This system and its control are important in the reinitiation of growth

following dilution. This section of the thesis will describe the protease, isolated from rapidly growing cultures, which hydrolyzes the granule protein. The data presented here serve as a basis for the further purification and study of the protease.

Under no conditions examined would trypsin, pepsin, or pronase digest the protein of the cyanophycin granule. Table 7 indicates the various incubation conditions examined. Trypsin would not cause cleavage even after 2 M NaCl or 0.1% SDS were added to solubilize the cyanophycin granule protein, even though trypsin is known still to be active under these conditions (62). The free carboxyls of aspartic acid residues were methylated (74) to prevent intramolecular ionic bonding in the protein. However, methylated protein was also resistant to the action of trypsin.

Cell-free extracts from recently diluted rapidly growing cultures were examined for a protease which would hydrolyze the protein of cyanophycin granules. Figure 20 shows that a cell-free extract would release TCA-soluble arginine. Such release of TCA-soluble arginine could not be found in either the reaction mixture without substrate or in the reaction mixture which contained boiled extract. Figure 21 confirms that this release phenomenon is one of hydrolysis and not simply one of making the intact granule protein soluble in the presence of TCA. Free α-amino

Table 7. Incubation conditions for the hydrolysis of cyanophycin granule protein with trypsin, pepsin, and pronase.

ENZYME	pH	INCUBATION CONDITIONS[a]	% ENZYME[b]	HYDROLYSIS[c]
TRYPSIN	8.0	0.05M Tris 0.01M $CaCl_2$	1%, 2%	-
	8.0	0.05M Tris 0.10M $CaCl_2$	1%	-
	8.0	0.05M Tris 0.01M $CaCl_2$ 2.0 M NaCl	2%	-
	8.0	0.05M Tris 0.01M $CaCl_2$ 0.1% SDS	1%	-
PRONASE	8.0	0.05M Tris	2.5%	-
	6.7	0.10M MES	2%	-
PEPSIN	2.0	0.01N HCl	2%	-
		4.5% Formic Acid	2%	-

[a]Cyanophycin granule protein is present at a concentration of one mg/ml.

[b]Amount of enzyme relative to substrate.

[c]Measured as release of free α-amino groups using the procedure of Rosen (77). Granules were incubated for twenty-four hours at 30°C. Any amino acid release is taken as an indication of hydrolysis (+) and no amino acid release indicates no hydrolysis (-).

Figure 20.--TCA-soluble arginine released from cyanophycin granules by cell-free extracts of rapidly growing cultures. One mg of dry granules was suspended in 1.0 ml cell-free extract and was assayed at various times for the release of TCA-soluble arginine (O-O). "No substrate" indicates arginine released in reaction mixtures lacking cyanophycin granules (Δ-Δ). "Boiled" indicates that the cell-free extract was heated at 100°C for one minute prior to being assayed for arginine-releasing activity (□ - □).

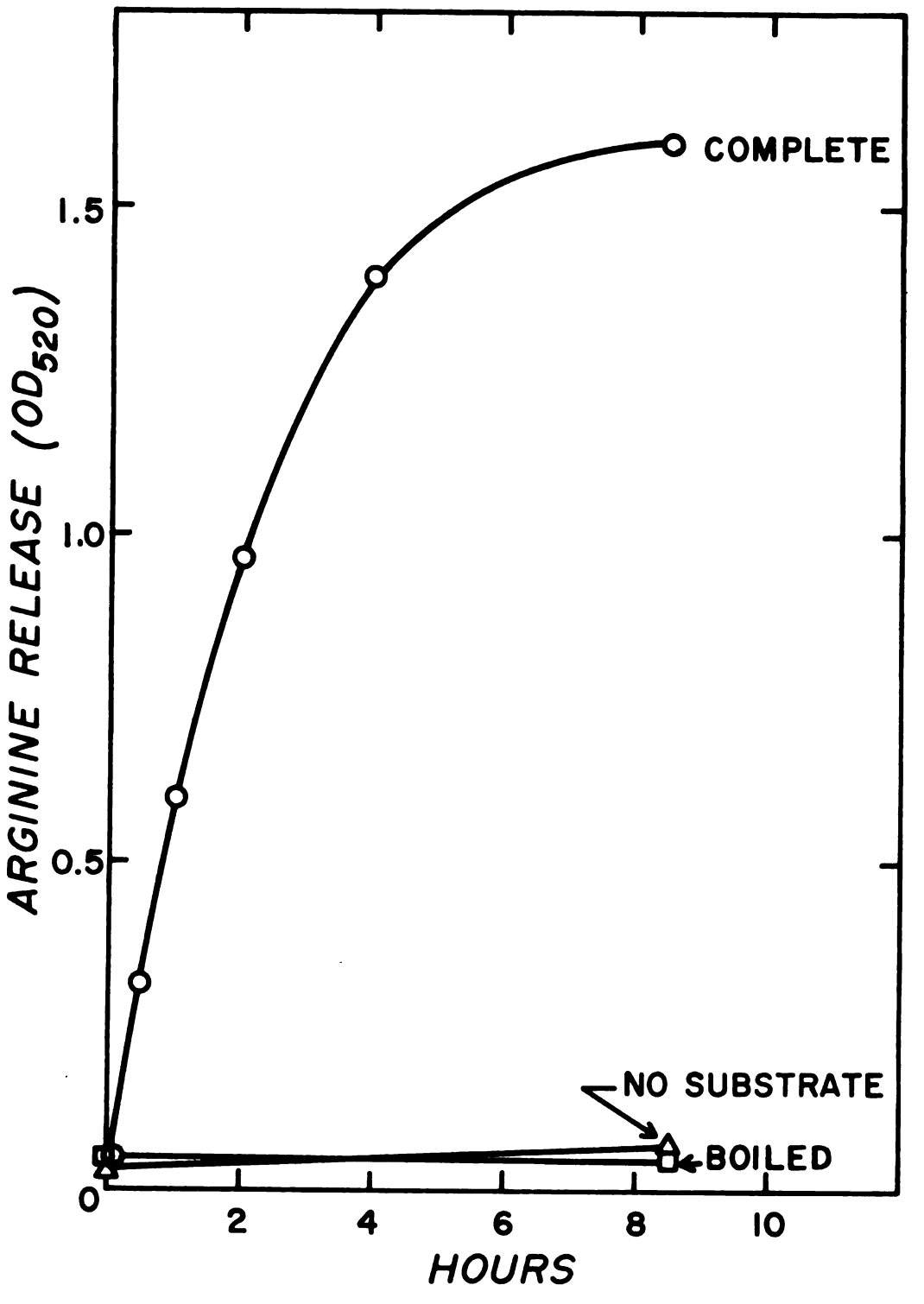
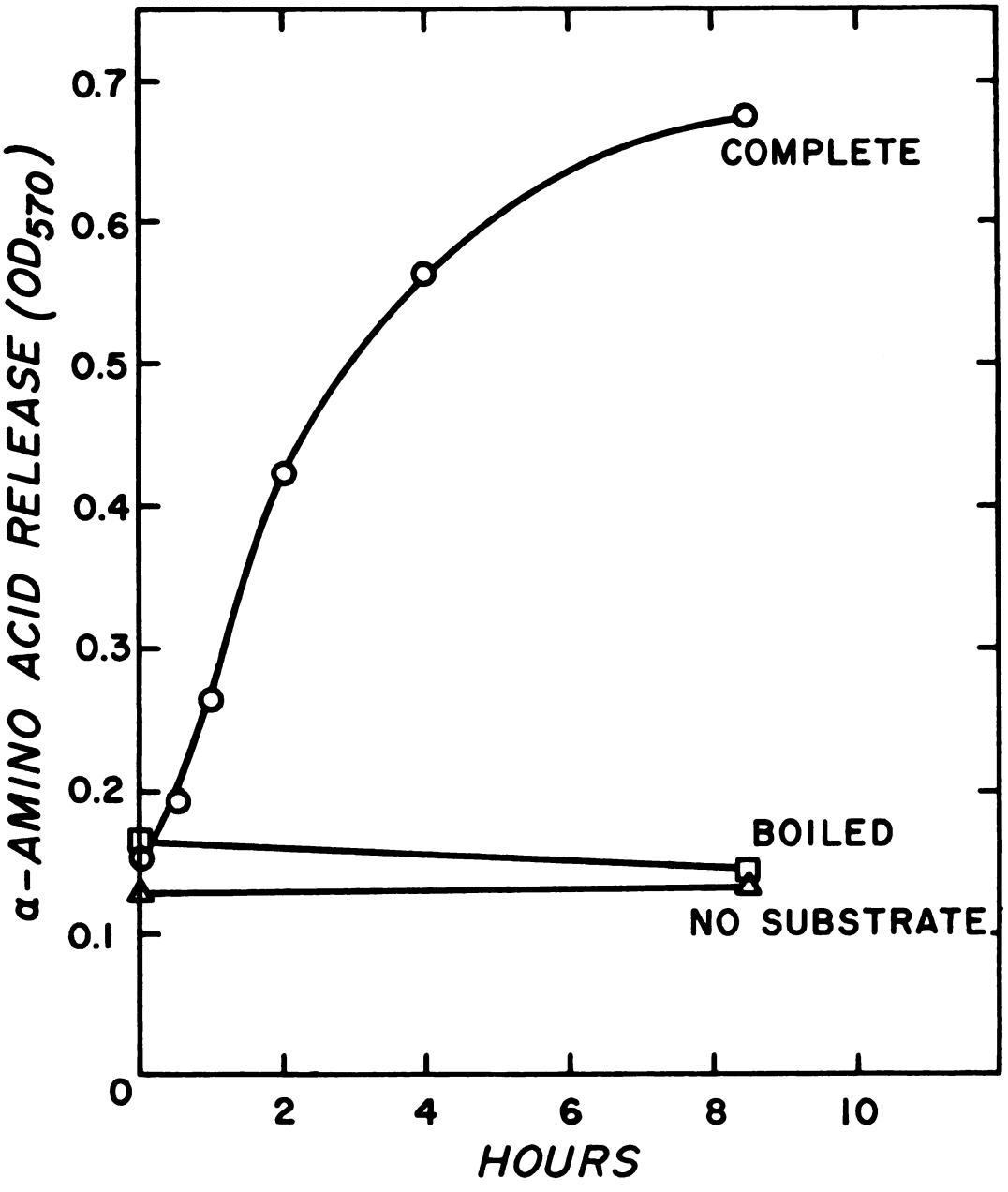

COMPLETE
NO SUBSTRATE
BOILED
ARGININE RELEASE (OD_{520})
HOURS
0
0.5
1.0
1.5
2
4
6
8
10

Figure 21.--TCA-soluble α-amino acids released from cyanophycin granules by cell-free extracts of rapidly growing cultures. One mg of dry granules was suspended in 1.0 ml of cell-free extract and was assayed at various times thereafter for the release of TCA-soluble amino acids (O-O). "No substrate" indicates amino acid release in reaction mixtures lacking cyanophycin granules (Δ-Δ). "Boiled" indicates that the cell-free extract was heated at 100°C for one minute prior to being assayed for amino acid-releasing activity (□ - □).


α-AMINO ACID RELEASE (OD_{570})
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
HOURS
COMPLETE
BOILED
NO SUBSTRATE

groups are released by the crude extract while no such release occurs in the absence of substrate or in the presence of boiled enzyme. In the experiments reported in Figures 20 and 21 the molar ratio of α-amino groups released to arginine released varied with time from 0.82 to 2.2. At the end of four hours of incubation, 53% of the original cyanophycin granule material had been solubilized.

When the crude cell-free extract prepared by cavitation was centrifuged at 103,000 x g for ninety minutes, all of the protease activity was found in the membrane-free supernatant. The extract could be further fractionated by use of ammonium sulfate precipitation. Table 8 shows the per cent activity in the various ammonium sulfate fractions. The bulk of the activity was in the 30%-60% fraction, the same fraction which contained the majority of the soluble protein. Ammonium sulfate precipitation would not be the method of choice for purification since some activity is lost using the procedure. In one experiment, although the specific activity (units/mg protein) increased from 119 in the crude extract to 205 after removal of the membrane fragments by ultracentrifugation, it decreased to 162 after ammonium sulfate precipitation. Also, the yield for the ammonium sulfate step was only 42%.

Table 8. Per cent protease in various ammonium sulfate fractions of crude[a] cell-free extract from a rapidly growing culture of Anabaena cylindrica.

FRACTION	ACTIVITY[b]	ml FRACTION	TOTAL ACTIVITY	%
0-30%	0.029	8.0	2.32	7.0
30% - 60%	0.269	11.0	29.60	86.0
60% - 90%	0.003	4.0	0.10	0.3
90% supernatant	0.006	43.0	2.58	7.0

[a]Crude cell-free extract was prepared using a Sorvall RF-1 cell fractionator.

[b]Measured as arginine released: OD_{520}/hr/0.1 ml sample.

Figure 22 shows that the rate of hydrolysis is linear over a narrow range with respect to both the amount of granules and the amount of cell-free extract in the reaction mixture.

In Figure 23, enzyme activity is plotted as a function of pH. The enzyme has no activity below pH 6.0, indicating that it is not an acid protease. There is a broad peak of activity with the optimum being between pH 8.0 and pH 10.0.

The analysis of the product released by the protease showed only free aspartic acid and arginine. The electropherograms showed no peptides in any of the TCA soluble fractions examined. Even at short times of hydrolysis no peptides could be seen together with the faint spots of the free amino acids.

Finally, the ammonium sulfate-purified extracts were examined for other protease activities. "Trypsin-like" activity which resulted in the hydrolysis of BAPNA was detected, and was found to be linear with the amount of extract in the reaction mixture. Also, upon starch gel electrophoresis (12% gel with tris-citrate, pH 7.0 [78]) of the extract, one band of leucine amino peptidase activity could be detected.

Figure 22A and B.--Protease activity plotted as a function of the amount of cell-free extract in the reaction mixture and as a function of the amount of substrate present in the reaction mixture. Graph A shows the µmoles of arginine released per hour when the amount of the cell-free extract in the 0.2 ml reaction mixture is varied. In graph B the granule substrate is varied from 10µg-100µg in a 0.2 ml reaction mixture which contains 100 µl of enzyme extract.

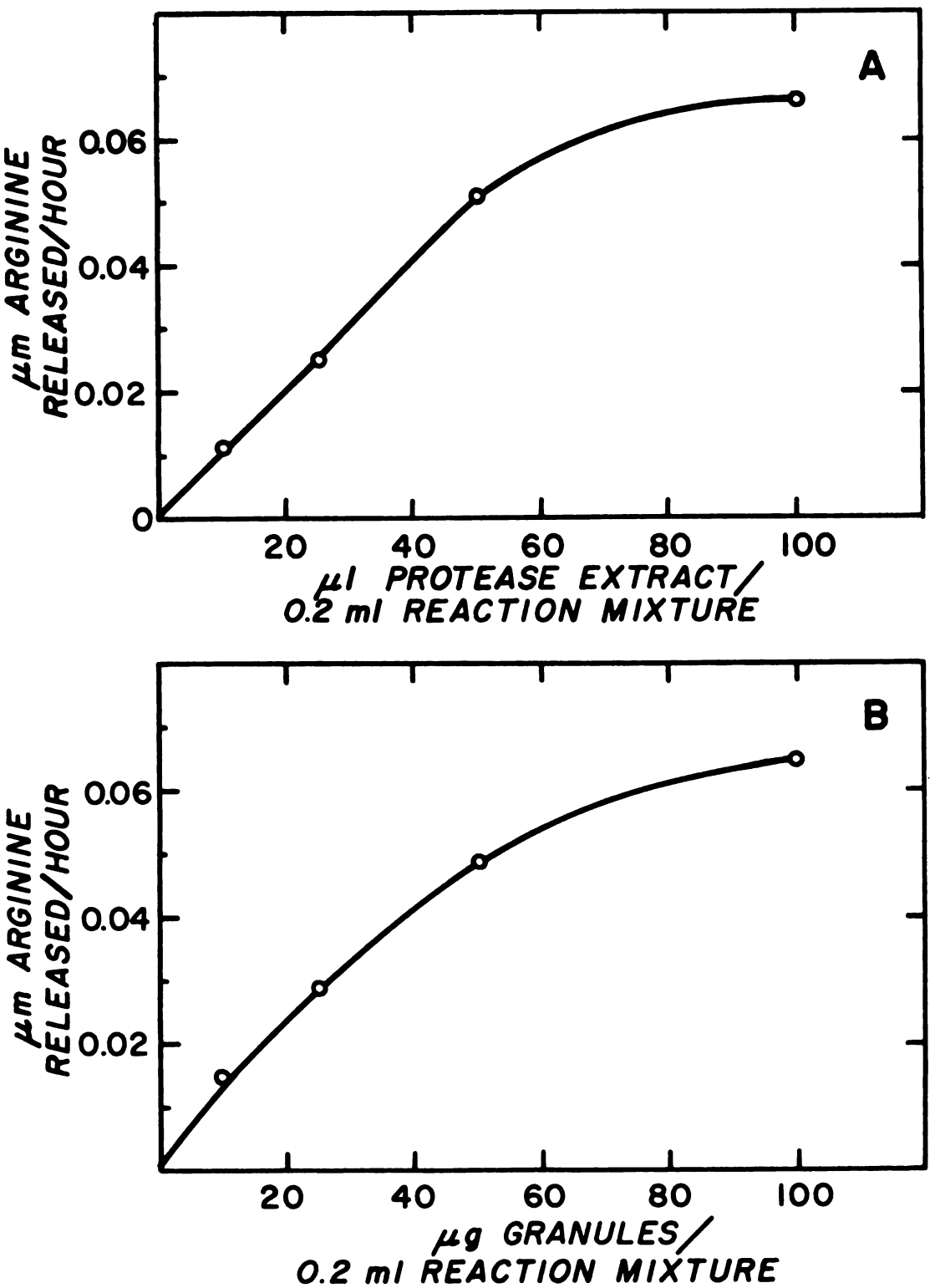
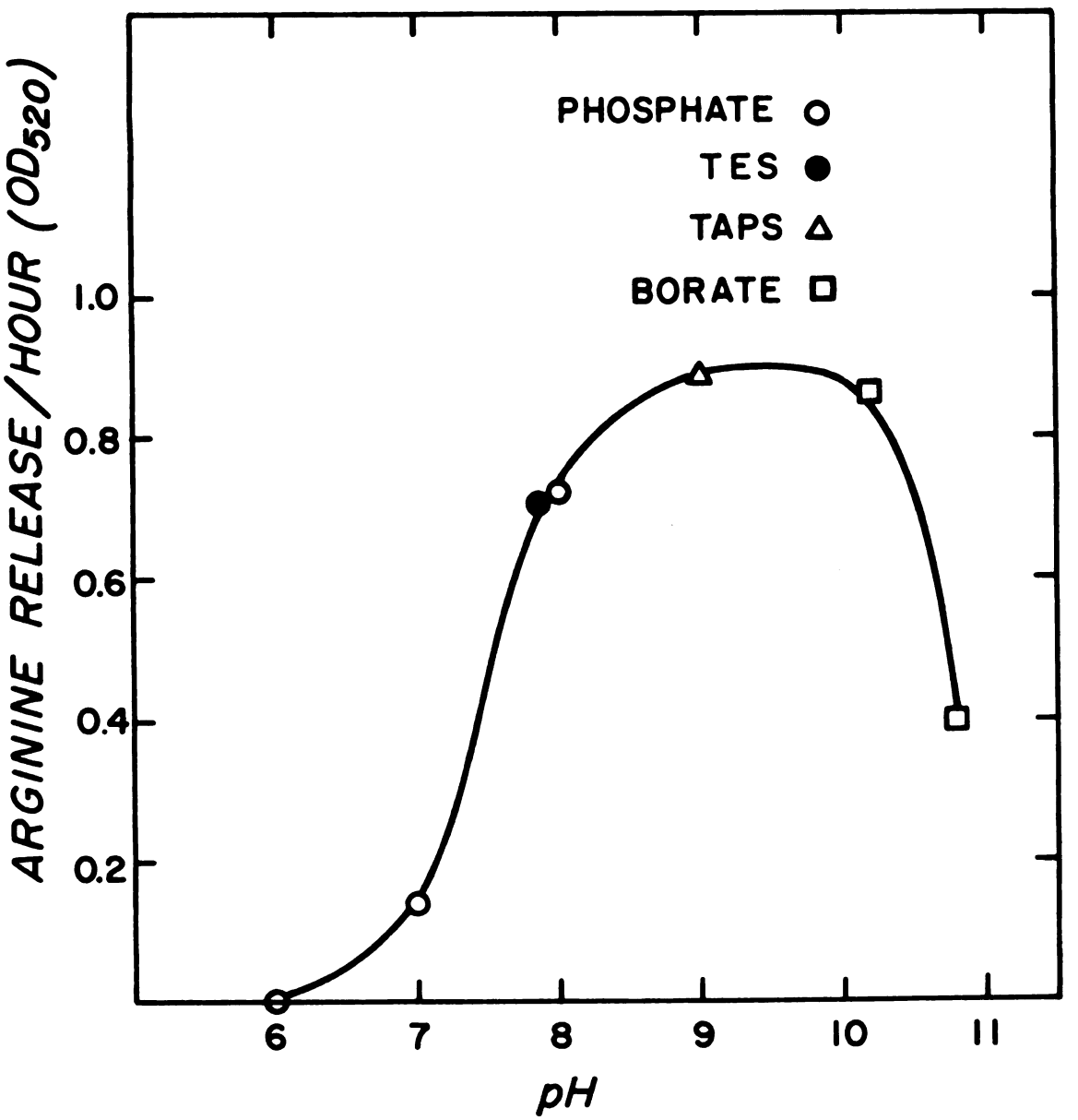

A
μm ARGININE RELEASED/HOUR
0.06
0.04
0.02
0
20
40
60
80
100
μl PROTEASE EXTRACT/ 0.2 ml REACTION MIXTURE
B
μm ARGININE RELEASED/HOUR
0.06
0.04
0.02
20
40
60
80
100
μg GRANULES/ 0.2 ml REACTION MIXTURE

Figure 23.--Protease activity as a function of the pH of the reaction mixture. Buffers used in the range from pH 6.0 to pH 10.8 were: phosphate (O), TES (●), TAPS (Δ), and borate (□).


PHOSPHATE
TES
TAPS
BORATE
ARGININE RELEASE/HOUR (OD520)
1.0
0.8
0.6
0.4
0.2
6
7
8
9
10
11
pH

DISCUSSION

This is the first reported isolation of the cyanophycin granule of the blue-green algae. The granules are relatively pure as indicated by the low percentage of carbohydrate, lipid, and phosphate present; by the fact that only two amino acids are present after hydrolysis with 6 N HCl for twenty-four hours at 105°C; and by the fact 98% of the weight of the isolated granules can be accounted for in terms of the two amino acids.

The solubility and staining properties of the isolated granules correlate well with the previously described histochemical properties of the cyanophycin granules except for the staining of the granules with methylene blue. Although it was reported that cyanophycin granules in intact cells would not stain with methylene blue (27), isolated granules do accumulate the stain. The reason for this discrepancy in staining properties *in vivo* and *in vitro* is not evident. The electron micrographs of the isolated cyanophycin granules show particles which are similar to those previously described as structured granules (52, 76). Thus, evidence from this

thesis supports the equivalence of the electron microscopists' structured granule with the light microscopists' cyanophycin granule.

The unusual composition of the granules explains why early microscopists found that they would stain with some protein reagents (Sakaguchi for arginine [26]) but not with others (Millon's reagent or the xanthoproteic reaction for aromatic amino acids). Also, the absence of lipids in the granules, as well as the lack of any other types of protein, is strong evidence that the granules are not membranous in nature. They are not composed of condensed membrane subunits as has been suggested (12) and, as isolated, they cannot serve as mitochondrial equivalents. If there was originally a membrane surrounding them (not seen in the electron micrographs), it has been lost during isolation.

There are many reports in the literature where it is claimed that the cyanophycin granules take up mitochondrial stains (17, 20, 28). In some reports the granules accumulate dye which must be reduced before it can be seen. In light of what is known about the composition of the granules the only possible explanations for such observations are either that there is another particle localized in the same region within the cells or that because of the unusual composition of the granules,

they become stained with reagents thought to be specific for mitochondria.

The protein of the cyanophycin granule is composed of chains of aspartic acid and arginine linked together. This is indicated by the fact that the protein migrates as a single band in several polyacrylamide gel electrophoresis systems. If there were separate chains of polyaspartic acid and polyarginine, one would expect two bands, one moving to the anode and the other moving to the cathode. Another proof that aspartic acid is linked to arginine is that peptides isolated from partial acid hydrolysates of the granules contain both amino acids.

The configuration of both amino acids in the granules is L- as shown by the optical rotation of granule hydrolysates. The amino acids are probably α-linked, because one can account entirely for the granule protein by using the Biuret reaction for peptide bonds. Arginine β-linked to aspartic acid or most combinations of α and β linkages would not be expected to give a complete reaction. The α-linked poly-D-glutamic acid polymer from Bacillus anthracis does not react at all with the Biuret reagent (11).

The fact that aspartic acid and arginine are present in a 1:1 molar ratio in the granules suggests that there may be an ordered sequence of amino acids in the polypeptide. Attempts to examine this possibility by end group

analysis have thus far failed to give definitive results.

Several high molecular weight polymers of a few amino acids have been found in biological material. As noted above, Bacillus anthracis makes a polymer of α-linked poly-D-glutamic acid. Also, the blood of certain arctic fish contains a freezing point-depressing glycoprotein whose peptide backbone contains repeating units of the tripeptide alanine-alanine-threonine (18). However, to my knowledge, the protein of the cyanophycin granule is the first large polymer of two amino acids isolated from biological material.

The composition of the granules correlates well with the proposed function as a protein reserve. The storage of one molecule of arginine corresponds to the storage of four atoms of nitrogen. Also, aspartic acid need only be reductively deaminated to form fumarate, an important compound in intermediary metabolism (35).

The quantitative data presented agree with the earlier observations (26, 41, 85) that cyanophycin granules are found mainly in old cultures and only to a much lesser extent in young growing cultures. The maximal rate of cyanophycin granule production occurs after exponential growth of a culture stops, and the maximal amount of granules is found in stationary phase cells. When an old culture is diluted and growth resumes, the

granule protein largely disappears. These results support the concept that the granules are a reserve that is built up at the end of the growth phase. The reserve is presumably utilized in building new cellular components when growth recommences.

The protein of cyanophycin granules is not the only reserve material to be utilized upon transition from stationary phase cultures to exponentially growing cultures. The ratio OD_{620}/mg vegetative cell protein drops significantly during the first day after the resumption of growth and this indicates either that there is some culture effect upon the chromophore of phycocyanin or that the protein as a whole is being degraded. Degradation of the protein would seem the more probably event. This concept of phycocyanin as a reserve protein has been suggested by Allen and Smith (2) and by Neilson *et al.* (64) who showed that the pigment disappears under conditions of nitrogen starvation, while resynthesis occurs when starved cells are furnished with a nitrogen source.

The data cannot distinguish whether the aspartic acid and arginine used in biosynthesis of the protein of the cyanophycin granule comes (1) from the fixation of CO_2, which is channeled into a new type of product, or (2) from old cell constituents which are being broken down. Measurements of rates of CO_2 fixation in exponential and stationary cultures as well as investigations of the

early products of such fixation might suggest the source of the aspartic acid and arginine. The question is one that deserves attention since if at the end of exponential growth new synthetic pathways are turned on, this system would represent a simple example of physiological differentiation which is amenable to study at the biochemical level.

Preliminary data indicate that three factors are involved in controlling the production of cyanophycin granules: nitrogen source, deficiency of some other component(s) in the nutrient medium, and aeration rate. These experiments must be repeated in more detail, and the production of cyanophycin granules under different growth conditions determined quantitatively. Laboratory cultures grown in various culture media or at relatively low levels of combined nitrogen rarely form large amounts of cyanophycin granules. The conditions under which large amounts of granules form in fermentor cultures are highly unusual and probably have little relation to conditions controlling granule formation in nature. Few algae are exposed to so high a concentration of inorganic nitrogen. Studies of these unusual "forcing" conditions may possibly have some relevance to studies on sporulation. In the process of sporulation the same proliferation of cyanophycin granules occurs (16, 27, 41, 57, 61). Canabaeus (14) observed in 1929 that in the presence of "too high"

nitrate or sulfate almost all of the cells of Anabaena laxa would sporulate. It is possible that she had observed the forcing of the production of large numbers of cyanophycin granules as reported in this thesis and took the presence of large numbers of storage granules to mean that the cells had sporulated.

The establishment of methods both for measuring cyanophycin granules and for culturing cells which will produce cyanophycin granules lays the foundation for answering some of the basic questions about the metabolism of the granules. An example of such a basic question is whether the granule protein is made via the usual routes of protein synthesis or whether the peptide bonds are formed enzymatically in a non-ribosomal system.

Neither trypsin, pepsin, nor pronase cleaved the protein of the cyanophycin granule under any experimental conditions examined. The lack of hydrolysis by trypsin was not unexpected. Although the enzyme will cleave polyarginine to monomers and dimers (87), it will not hydrolyze polyaspartic acid (82). Also, increasing percentages of glutamic acid are found to inhibit hydrolysis by trypsin of the heteropolymer glutamic acid-lysine. At high lysine-glutamic acid ratios the polymer is cleaved by trypsin, but when the ratio is one there is no hydrolysis (75). Even after methylating the carboxyl groups of the granule protein to remove the effect of intramolecular

bonding, there is no cleavage by trypsin. Although pepsin-HCl had previously been reported to dissolve the cyanophycin granules (7, 41), there was no indication of hydrolysis of the protein by pepsin. The earlier observations were perhaps due to solubilization of the granule protein by dilute acid.

Purification of the components of an enzymatic system which would degrade the usual protein of the cyanophycin granule would aid studies of the amino acid sequence of the protein. Since trypsin, pepsin, or pronase do not degrade the protein of the cyanophycin granule, it would be desirable to isolate and to characterize a protease which would degrade the protein. Cells from recently diluted cultures contain a hydrolytic system for the breakdown of the protein of cyanophycin granules. The heat lability of the activity, the linearity of the reaction rate with respect to substrate concentration and with respect to the amount of cell-free extract employed, and the concentration of the activity in a specific ammonium sulfate fraction all point to the fact that the degradation is enzymatic in nature. No blue-green algal protease has been purified and studied in detail. The purification of the granule protease and the examination of its properties should therefore be of interest from the standpoint of comparative biochemistry.

The enzyme will probably have to be purified further before it will be of any value in sequencing the protein of the granules, because the data suggest that following ammonium sulfate fractionation other types of protease activity are still present. These activities were measured with artificial substrates (BAPNA and L-leucyl-beta-naphthylamide). It is not clear if the granule protease can hydrolyze these artificial substrates or if their hydrolysis is due to other proteases. Since the products found following enzymatic hydrolysis of the granules are the free amino acids, either the granule protease is an exopeptidase or other peptidases present cleave the product(s) of the granule protease into free amino acids.

How is the cyanophycin granule utilized? I.e., by what steps is the storage protein degraded, and what controls these steps? The data presented about the protease serve as a basis for the study of the system which degrades cyanophycin granules in vivo. The rate of granule degradation may be controlled by the quantity of protease, or through regulation of the activity of a constant amount of protease. Measurement of the protease activity as a function of culture age, and studies on the effects of various compounds (e.g., aspartic acid and arginine) on the protease activity in vivo and in vitro might distinguish between the two possible levels of control.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Allen, M. B., and D. I. Arnon, 1955. Studies on nitrogen-fixing blue-green algae-I. Growth and nitrogen fixation by Anabaena cylindrica Lemm. Plant Physiol. 30:366-372.

2. Allen, M. M., and A. Smith, 1969. Nitrogen chlorosis in blue-green algae. Arch. Mikrobiol. 69:114-120.

3. Allsopp, A., 1969. Phylogenetic relationships of the procaryota and the origin of the eucaryotic cell. New Phytol. 68:591-612.

4. Andrews, P., 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96;595-606.

5. Bailey, J. L., 1967. Detection of amino acids. In Techniques in Protein Chemistry, p. 23, Elsevier, Amsterdam, Netherlands.

6. Baker, J. R., 1946. The histochemical recognition of lipid. Quant. J. Micr. Sci. 87:441.

7. Baumgärtel, O., 1920. Das Problem der Cyanophyzeenzeele. Arch. Protistenk. 41:50-148.

8. Bisalputra, T., D. L. Brown, and T. E. Weier, 1969. Possible respiratory sites in a blue-green alga Nostoc sphaericum as demonstrated by potassium tellurite and tetranitro-blue tetrazolium reduction. J. Ultrastruct. Res. 27:182-197.

9. Blackburn, S., and G. R. Lee, 1954. The liberation of aspartic acid during acid hydrolysis of proteins. Biochem. J. 58:227-231.

10. Borzi, A., 1886. Le comunicazioni intracellulari delle Nostochinee. Malpighia 1:28-74.

11. Bovarnick, M., 1942. The formation of extracellular D(-)-glutamic acid polypeptide by Bacillus subtilis. J. Biol. Chem. 145:415-424.

12. Brown, D. L., and T. Bisalputra, 1969. Fine structure of the blue-green alga Nostoc sphaericum: the structured granule. Phycologia. 8:119-126.

13. Bütschli, O., 1896. Über den Bau der Bacterien und verwandter Organismen. Leipzig, Germany.

14. Canabaeus, L., 1929. Über die Heterocysten und Gasvakuolen der Blaualgen und ihre Beziehung zueinanden. Pflanzenforschung. 13:1-48.

15. Chao, L., and C. C. Bowen, 1971. Purification and properties of glycogen isolated from a blue-green alga, Nostoc muscorum. J. Bact. 105: 331-338.

16. Clark, R. L., and T. E. Jensen, 1969. Ultrastructure of akinete development in a blue-green alga. Cylindrospermum sp., Cytologia. 34:439-448.

17. Dehorne, A., 1920. Contribution à l'étude comparée de l'appareil nucléaire des Infusoires ciliés, des Euglènes et des Cyanophycées. Arch. Zool. exper. et gen. 60:47-176.

18. DeVries, A. L., and J. Vandenheede, and R. E. Feeney, 1971. Primary structure of freezing point depressing glycoproteins. J. Biol. Chem. 246:305-308.

19. Dobell, C. C., 1912. Researches on the Spirochaetes and related organisms. Arch. Protistenk. 26:117-240.

20. Drews, G., and W. Niklowitz, 1956. Beiträge zur Cytologie der Blaualgen II Zentroplasma und granuläre Einschlüsse von Phormidium uncinatum Gom. Arch. Mikrobiol. 24:147-162

21. Drews, G., and W. Niklowitz, 1957. Beiträge zur Cytologie der Blaualgen III Untersuchungen über die granularen Einschlüsse der Hormogonales. Arch. Mikrobiol. 25:333-351.

22. Echlin, P., 1964. Intra-cytoplasmic membranous inclusions in the blue-green alga, Anacystis montana. Arch. Mikrobiol. 49:267-274.

23. Echlin, P., and I. Morris, 1965. The relationship between blue-green algae and bacteria. Biol. Rev. 40:143-187.

24. Erlanger, B. F., N. Kokowsky, and W. Cohen, 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95:271-278.

25. Feder, N., and T. P. O'Brien, 1968. Plant microtechnique: some principles and new methods. Amer. J. Bot. 55:123-142.

26. Fogg, G. E., 1951. Growth and heterocyst production in Anabaena cylindrica Lemm. III. The cytology of heterocysts. Ann. Bot., n.s. 15:23-35.

27. Fritsch, F. E., 1945. The structure and reproduction of the algae, v. 2, Cambridge University Press, Cambridge, England.

28. Fuhs, G. W., 1958. Über die Natur der Granula in Cytoplasma von Oscillatoria amoena (Kütz) Gom. Österr. Botan. Zeitschrift. 104:531-551.

29. Fuhs, G. W., 1958. Untersuchungen an Ultradunnschnitten von Oscillatoria amoena (Kütz) Gom. Protoplasma, 49:523-540.

30. Fuhs, G. W., 1968. Cytology of the blue-green algae: light microscopic aspects. In Algae, man and the environment, (ed. D. F. Jackson), pp. 213-233, Syracuse University Press, Syracuse, New York.

31. Gardner, N. L., 1906. Cytological studies in Cyanophyceae. University Calif. Publ. Bot. 2:237-296.

32. Geitler, L., 1922. Zur Cytologie der Blaualgen. Eine Kritik der Arbeit O. Baumgärtels: Das Problem der Cyanophyceenzelle. Arch. Protistenk. 45:413-418.

33. Geitler, L., 1960. Schizophyzeen, 2. Aufl. (Handbuch der Pflanzenanatomie, Band 6, Teil 1), Borntraeger, Berlin, Germany.

34. Good, N. E., C. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh, 1966. Hydrogen ion buffers for biological research. Biochem. 5:467-477.

35. Greenberg, D. M., 1969. Catabolism of amino acids. In Metabolic pathways, v. III, (ed. D. M. Greenberg), pp. 95-190, Academic Press, N.Y., N.Y.

36. Guilliermond, A., 1926. Contribution à l'étude cytologique des Cyanophycées. Ebenda 38:129 et seq.

37. Hall, W. T., and G. Claus, 1962. Electronmicroscope studies on ultrathin sections of Oscillatoria chalybea Mertens. Protoplasma 54:355-363.

38. Hall, W. T., and G. Claus, 1963. Ultrastructural studies on the blue-green algal symbiont in Cyanophora paradoxa Korschikoff. J. Cell Biol. 19:551-563.

39. Hall, W. T., and G. Claus, 1967. Ultrastructural studies on the cyanelles Glaucocystis nostochinearum Itzigsohn. J. Phycol. 3:37-51.

40. Hassid, W. Z., and S. Abraham, 1957. Chemical procedures for analysis of polysaccharides. In Methods in enzymology v. III, pp. 34-50, Academic Press, N.Y., N.Y.

41. Hegler, R., 1901. Untersuchungen über die Organisation der Phycochromaceenzellen, Jahrb. wiss. Botanik. 36:229-354.

42. Holm-Hensen, O., 1968. Ecology, physiology, and biochemistry of blue-green algae. Ann. Rev. Microbiol. 22:47-70.

43. Jensen, T. E., and C. C. Bowen, 1960. Cytology of blue-green algae. II Unusual inclusions in the cytoplasm. Cytologia. 35:132-152.

44. Jones, D. D., 1970. Isolation and characterization of gas-vacuole membranes from Microcystis aeruginosa Kuetz. emend Elenkin. Ph.D. thesis, Michigan State University.

45. Jones, D. D., and M. Jost, 1970. Isolation and chemical characterization of gas-vocuole membranes from Microcystis aeruginosa Kuetz. emend Elenkin. Arch. Mikrobiol. 70:43-64.

46. Kratz, W. A., and J. Myers, 1955. Nutrition and growth of several blue-green algae. Amer. J. Bot. 42:382-389.

47. Lamont, H. C., 1968. Trichome breakage in Oscillatoriacean blue-green algae. Abstract of Ph.D. thesis, Harvard University, Cambridge, Massachusetts.

48. Lamont, H. C., 1968. Shear-oriented microfibrils in the mucilaginous investments of two motile Oscillatoriacean blue-green algae. J. Bact. 97:350-361.

49. Lang, N. J., 1965. Electron microscopic study of heterocyst development in Anabaena azollae Strasburger. J. Phycol. 1:127-134.

50. Lang, N. J., 1968. The fine structure of blue-green algae. Ann. Rev. Microbiol. 22:15-46.

51. Lang, N. J., and P. M. M. Rae, 1967. Structures in a blue-green algae resembling prolamellar bodies. Protoplasma. 64:67-74.

52. Lang, N. J., and K. A. Fisher, 1969. Variation in the fixation image of "structured granules" in Anabaena. Arch. Mikrobiol. 67:173-181.

53. Layne, E., 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In Methods in enzymology, v. III, pp. 447-458, Academic Press, N.Y., N.Y.

54. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

55. Macallum, A. B., 1899. On the cytology of non-nucleated organisms. Trans. Canadian Inst., Toronto. 6:439-506.

56. Mackinney, G., 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140:315-322.

57. Madsen, M. J., 1966. Electron microscopy of the blue-green algae Cylindrospermum and Gloeotrichia. Master's theis, University of California, Davis, California. In Lang, N. J., 1968. The fine structure of blue-green algae. Ann. Rev. Microbiol. 22:33.

58. Maugini, E., 1966. Contributo alla conoscenza citologica die Nostoc commune Vaucher con particolare riguardo all'ultrastruttura. Caryologia. 19:267-298.

59. McLeish, J., and H. S. A. Sherrat, 1958. The use of the Sakaguchi reaction for the cytochemical determination of combined arginine. Expt. Cell Res. 14:625-629.

60. Messineo, L., 1966. Modification of the Sakaguchi reaction: spectrophotometric determination of arginine in proteins without previous hydrolysis. Arch. Biochem. Biophys. 117: 534-540.

61. Miller, M. M., and N. J. Lang, 1968. The fine structure of akinete formation and germination in Cylindrospermum. Arch. Mikrobiol. 60: 303-313.

62. Miller, W. G., 1964. Degradation of synthetic polypeptides. III. Degradation of poly-α, L-lysine by proteolytic enzymes in 0.20 M sodium chloride. J. Amer. Chem. Soc. 86:3918-3922.

63. Nadson, G., 1895. Ueber den Bau des Cyanophyceen--Protoplastes. Script. Bot. Hort. Univ. Petropolitani. 4:157-232.

64. Neilson, A., R. Rippka, and R. Kunisawa, 1971. Heterocyst formation and nitrogenase synthesis in Anabaena species. A kinetic study. Arch. Mikrobiol. 76:139-150.

65. Neugnot, D., 1950. Contribution a l'étude cytochimique des cyanophycées par application des techniques de mise en évidence de l'appareil nucléaire chez les bactéries. Compt. rend. Acad. Sci. Paris. 230:1311.

66. Niklowitz, W., and G. Drews, 1956. Beiträge zur Cytologie der Blaualgen I. Untersuchungen zur Substruktur von Phormidium uncinatum Gom. Arch. Mikrobiol. 24:134-146.

67. Otey, M. C., J. P. Greenstein, M. Winitz, and S. Birnbaum, 1955. Studies on the diastereoisomeric α-amino acids and corresponding α-hydroxy acids. IV. Rotatory dispersion of the asymmetric α- and ω-carbon atoms of several diastereoisomeric amino acids. J. Amer. Chem. Soc. 77: 3112-3114.

68. Palla, E., 1893. Beitrag zur Kenntnis des Baues des Cyanophyceen-Protoplastes. Jahrb. wiss. Bot. 25:511-562.

69. Pankratz, H. S., and C. C. Bowen, 1963. Cytology of blue-green algae, I. The cells of Symploca muscorum. Amer. J. Bot. 50:387-399.

70. Park, J. T., and M. J. Johnson, 1949. A submicrodetermination of glucose. J. Biol. Chem. 181:149-151.

71. Parvin, R., and R. A. Smith, 1969. Determination of inorganic phosphate in the presence of labile organic phosphates. Anal. Biochem. 27:65-72.

72. Phillips, O. P., 1904. A comparative study of the cytology and movements of the Cyanophyceae. Contr. Bot. Lab. Univ. Penn. 2:237-335.

73. Pierce, A. E., 1968. Silylation of organic compounds, Pierce Chemical Company, Rockford, Illinois.

74. Riem, J. P., C. A. Bloomfield, and H. A. Scheraga, 1965. The abnormal carboxyl groups of ribonuclease. II. Position in the amino acid sequence. Biochem. 4:760-771.

75. Rigbi, M., 1957. Abstract of Ph.D. thesis, Hebrew University, Jerusalem, Israel.

76. Ris, H., and R. N. Singh, 1961. Electron microscope studies on blue-green algae. J. Biophys. Biochem. Cytology. 9:63-80.

77. Rosen, H., 1957. A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys. 67:10-15.

78. Scandalios, J. G., 1969. Genetic control of multiple molecular forms of enzymes in plants: a review. Biochem. Genetics. 3:37-79.

79. Schmitz, F., 1879. Über die Zellkerne des Thallophyten. Sitz-Ber. d. Niederrhein. Ges. F. Naturheilk. Bonn. In Baumgärtel, O., 1920. Das Problem der Cyanophyzeenzelle. Arch. Protistenk. 41:98.

80. Schultz, J., 1967. Cleavage of aspartic acid. In Methods in enzymology, v. XI, pp. 255-263, Academic Press, N.Y., N.Y.

81. Shapiro, A. L., E. Vinuela, and J. V. Maizel, 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Comm. 28:815-820.

82. Simons, E. R., and E. R. Blout, 1964. The effect of proteolytic enzymes on synthetic polypeptides. Biochem. Biophys. Acta. 92:197-199.

83. Takayama, K., D. H. MacLennan, A. Tzagoloff, and C. D. Stoner, 1966. Studies on the electron transfer system. LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. 114:223-230.

84. Tamir, H., and C. Gilvarg, 1966. Density gradient centrifugation for the separation of sporulating forms of bacteria. J. Biol. Chem. 241:1085-1090.

85. Tischer, I., 1957. Untersuchungen über die granulären Einschlüsse und das Reduktions-Oxydations-Vermögen der Cyanophyceen. Arch. Mikrobiol. 27:400-428.

86. Tsung, C. M., and H. Fraenkel-Conrat, 1965. Preferential release of aspartic acid by dilute acid treatment of tryptic peptides. Biochem. 4:793-804.

87. Wilchek, M., S. Ariely, and A. Patchornik, 1966. Synthesis of poly-L-arginine. Biopolymers. 4:91-96.

88. Wildon, D. C., and F. V. Mercer, 1963. The ultra-structure of the vegetative cell of blue-green algae. Aust. J. Biol. Sci. 16:585-596.

89. Wildon, D. C., and F. V. Mercer, 1963. The ultrastructure of the heterocyst and akinete of the blue-green algae. Arch. Mikrobiol. 47:19-31.

90. Wolk, C. P., 1968. Movement of carbon from vegetative cells to heterocysts in Anabaena cylindrica. J. Bact. 96:2138-2143.

91. Zacharias, E., 1889. Ueber die Zellen der Cyanophyceen. Deutsch. Bot. Ges. 7:(31)-(34).

92. Zastrow, E. M. von, 1953. Über die Organization der Cyanophyceenzelle. Arch. Mikrobiol. 19:174-205.