BLUE-GREEN ALGAL (ANABAENA FLOS-AQUAE) PROTEIN AS HUMAN FOOD

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
YOUNG RACK CHOI

1976

This is to certify that the

thesis entitled

BLUE-GREEN ALGAL (ANABAENA FLOS-AQUAE)
PROTEIN AS HUMAN FOOD

presented by

Young Rack Choi

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Food Science

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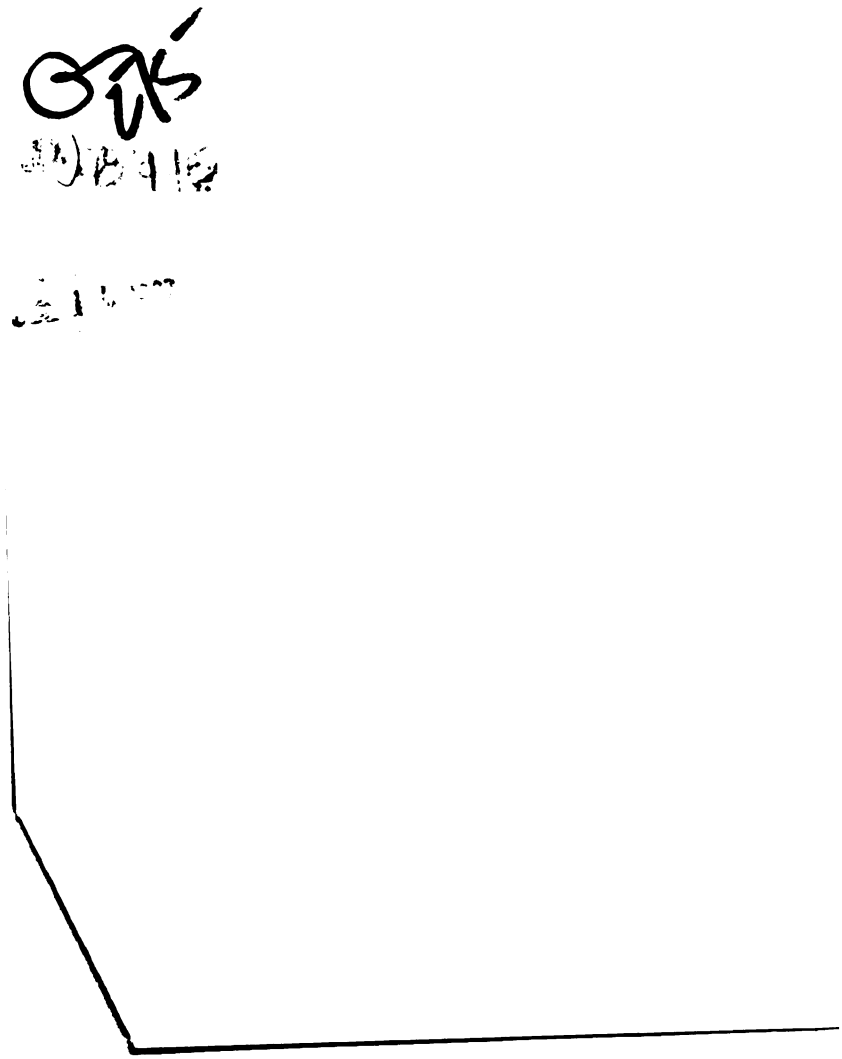
Date May 13, 1976

O-7639



ABSTRACT

BLUE-GREEN ALGAL (ANABAENA FLOS-AQUAE) PROTEIN AS HUMAN FOOD

By

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Although the nitrogen-fixing and photosynthesizing blue-green algae are an attractive possibility as a source of human food, attempts to render their protein content available by classical extraction methods have failed. Extracts thus prepared possess a grassy flavor and an undesirable green color and odor. The objective of the present study was to devise a simple method of extraction to yield a digestible, colorless and odorless protein isolate of high nutritive value.

The blue-green algae Anabaena flos-aquae was grown usually at 32 C both in an autotrophic medium (control) without nitrogen (Kratz and Myers medium) and in a heterotrophic medium containing the following levels of additives: urea (0.01% w/v), $NaNO_3$ (0.01%), $NaNO_2$ (0.01%), peptone (0.05%), glucose (1%), and glucose plus urea (0.02% each). Addition of $NaNO_3$, $NaNO_2$, urea, peptone, and the mixture of glucose plus urea did not increase the cell yield and protein content. With glucose alone

however, the blue-green algae showed good heterotrophic growth with increased yield. Changes in the concentration of glucose above or below 1% resulted in decreased yield. The theoretical yield and optimum harvesting rate of the cells were calculated on the basis of an equation from the growth curve.

It was found that the green color of the algae was removed completely by illuminating with 15,064 lux fluorescent light for 8 hours at 32 C (algal suspension of 50 mg/100 ml). Heating, ultraviolet light, and high pH accelerated the bleaching reaction.

Extraction after treatment with HCl, single alkali extraction, and mechanical extraction were tried and compared on the basis of protein yield and quality. It was found that the highest yield of protein was obtained by the HCl-pretreatment method. Colorless and odorless protein could easily be obtained by photolyzing the protein extract after HCl-pretreatment.

The composition and digestibility of the whole cells and isolated protein were determined in vitro. The digestibility of a protein isolate with pepsin was much greater than that of the protein in the intact cell in vitro.

An attempt was made to fractionate successively the protein of algae with water, 0.8% NaCl, and 0.2% NaOH. Most of the protein was extracted with 0.2% NaOH. Only a

small part of the protein was water extractable. The sulfur-containing amino acids were limiting in the biological evaluation of the protein. The HCl-pretreatment extraction caused loss of histidine and tyrosine. The chemical score of the whole cells was 44, and that of the protein isolate was 46.

Polyacrylamide disc gel and SDS gel electrophoresis analyses were performed on the proteins successively fractionated with water, 8% NaCl and 0.2% NaOH, and on the protein from the HCl-pretreatment extraction. Optimum separation was obtained by using a 13% gel concentration on polyacrylamide disc gel electrophoresis. Whereas the water-extractable fraction revealed numerous separate bands, there were 4 bands in the NaCl-extractable protein, and one band in the alkali-extractable protein. By SDS gel electrophoresis, the molecular weights of the protein monomers of algal protein were found to be in the range of 18,000 to 30,000 daltons.

BLUE-GREEN ALGAL (ANABAENA FLOS-AQUAE) PROTEIN AS HUMAN FOOD

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1976

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. P. Markakis, my graduate research advisor, for valuable guidance, suggestions and invaluable spiritual support during the course of this study, and for his assistance in the preparation of this manuscript. I am also grateful to the other members of the guidance committee, Drs. G. A. Borgstrom, J. R. Brunner, D. D. Dilley, and H. A. Lillevik for their efforts in the direction of the graduate program and thesis preparation.

Special thanks are also due the Department of Food Science and Human Nutrition for providing the facilities and financial support. Additionally I wish to thank Dr. F. B. Shorland (visiting professor) for his discussion and assistance in the preparation of this manuscript.

Finally, I thank my wife, Jung Ja, for her love, understanding and assistance during the course of this study.

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INTRODUCTION

Food deficiency, especially shortage of protein, exists in many parts of the world. One of the novel food supplements which has shown potential in alleviating world shortages is the protein of single cells. Microbial cells can reproduce themselves much faster than conventional food sources. They have high protein content and can be produced without the limitation of available farm land. Moreover their production can be easily controlled independently of climate (Mitsuda et al., 1969).

Although algae as a source of food or feed proteins is well known and techniques for large scale production have been developed, consideration of the algae for this purpose involves several problems. The products from the algae should: (1) be free from agents of acute, subacute and chronic toxicity as well as from tetratogenic and carcinogenic factors, (2) not contain excessive indigestible matter, such as cell walls, (3) be organoleptically acceptable. Excessive quantities of colored substances, such as chlorophylls and xanthophylls, and unpleasant flavors should be removed.

Research has been done on green algae, especially Chlorella for human food. However, the potential of blue-green algae as a foodstuff is unexplored. Blue-green algae are microorganisms of particular interest because they can both photosynthesize and fix atmospheric nitrogen.

The purpose of this work was to study (1) the growth of blue-green algae in defined media, (2) the extraction of acceptable protein from the cells, and (3) the chemical and nutritional aspects of isolated protein.

LITERATURE REVIEW

General Algal Protein

There is a shortage of food and specially of protein in the world today, which is associated with widespread malnutrition (Pirie, 1971); Borgstrom, 1967; Brown, 1968). As the population grows, the problem of providing sufficient food becomes more difficult. Population distribution does not correspond to regional food productivity (FAO, 1965). More than 50% of the world population is crowded in Asia, only 28% of the world's food is produced in this region. Ten to fifteen percent of the world's population is undernourished, and up to half is suffering from some kind of malnutrition (FAO, 1963, 1973).

The possibilities for improving the protein food supply include (1) increasing conventional agricultural production, (2) importing and exporting food to improve the regional food situation, (3) developing novel protein. The first approach has limited potential. The second approach may facilitate an even distribution of food, but it will not solve the worldwide shortage. Recent interest has been placed on the third approach. Most authors agree that the problem will not be solved without the use of some

supplementary protein sources. Several sources which deserve investigation include the soybeans, cotton seed, peanut, coconut, fish protein concentrate, microorganisms cultured in petroleum products, synthetic nutrients, and the recovery of protein from leaves (Byers et al., 1965; Parpia, 1967; Russo, 1969; Anderson, 1971). A single source is unlikely to solve the protein shortage. Therefore, all potential sources of supplementary protein must be explored and evaluated (Oke and Tella, 1969; Kisella, 1970; Abbott, 1974).

Microbial cells are attractive as possible food sources because of their higher reproduction rates than plants and animals, their high protein content, their limited space requirement and ease of production (Bhattacharjee, 1971).

Green algae have always been important in the food chain, but have only recently been investigated as possible source of food for man. Black (1958), Burlew (1953), Fisher (1965) listed the advantages of micro algae as follows:

(1) The calculated annual yield is higher than that of conventional crops.

(2) Algae require only light, water, carbon dioxide and inorganic salts for growth.

(3) Algae yield more protein, fat and vitamins than most conventional crops except certain seeds such as soybean and cotton seed.

(4) Most of the solar energy absorbed by unicellular algae is converted to useful food.

(5) Because algae tolerate high temperature and require little water for cultivation they can be grown in otherwise useless areas.

(6) Algae are amenable to mass cultivation and processing.

Mass cultured micro algae produce annually up to 40 tons per acre of dry matter containing 20 tons of protein compared with 0.75 tons dry matter as seed containing 0.25 tons protein obtainable from soybeans which require more effort in their production (Anonymous, 1959, 1963; Black, 1958). Animal sources such as chicken, hog and cattle grow even more slowly taking 2-10 weeks to double their weight compared with 2-6 hours for green algae (Vilenchich and Akhtar, 1971).

Culture of Algae

The blue-green algae photosynthesize from carbon dioxide and fix atmospheric nitrogen (Allison and Morris, 1930; Drewes, 1929; Fogg, 1942, 1974; Allen and Arnon, 1955, 1960; Allen, 1969). Nitrogen fixation by *Anabaena flos-aquae* has been demonstrated in a bacteria-free culture (Davis *et al.*, 1969). The elements molybdenum (Fogg,

1954; Wolfe, 1954), calcium (Allen and Arnon, 1956) and sodium (Brownell and Nicholas, 1967) were shown to be especially important for the growth of blue-green algae in nitrogen gas.

In blue green algae, vegetative cells can differentiate into heterocysts and sometimes into spores (Fogg, 1944, 1949). Differentiation involves major changes in certain of the cytochemical and physiological characteristics of the vegetative cells. The position of the heterocysts and spores along the algal filaments form well-defined patterns. These developmental patterns are dependent upon interactions between cells, as well as intracellular changes (Wolk, 1973). The blue-green algae are the only known oxygen producing and photosynthesing prokaryotes.

Allen (1952) described media used up until about 1950 for autotrophic growth of blue-green algae. Since then, the following variations have been introduced (Healey, 1973):

(1) The calcium concentration was decreased in order to prevent precipitation during autoclaving, but the increased levels of phosphate were required for optimum buffering conditions (Kratz and Myers, 1955).

(2) All compounds of nitrogen were omitted from the medium.

(3) Microelements were included.

(4) A chelating agent was provided.

Many laboratories now make use of the media of Allen and Arnon (1955), Kratz and Myers (1955). Carbon dioxide is frequently supplied to cultures at concentrations in excess of the concentration in air (0.03%) (Allison and Morris, 1937), to overcome the rate limiting process of diffusion of carbon dioxide into culture medium. Alternatively, a rapid flow of air well dispersed in a culture, can provide carbon dioxide sufficiently rapidly that its rate of dissolution is not limiting for algae growth.

Culture experiments with bacteria-free blue-green algae failed to elicit growth in the dark on organic substrates (Kratz and Myers, 1955). However, there are now increasing indications that the ability to grow heterotrophically is widespread among the blue-green algae (Henderson and Wyatt, 1971). Numerous investigators have found that sugars increase the growth of blue-green algae in light (Allison and Morris, 1930, 1937; Allen, 1952). This work culminated in the demonstration (Baalen, 1967; Kiyohara et al., 1962; Khoja and Whitton, 1971) that Anabaena quadruplicatum grows in the presence of glucose in dim light which is itself insufficient to support growth; glucose supported only marginal growth in the dark. Glucose, however, can contribute up to 46% of the dry weight of A. variablilis without affecting the growth

rate (Pearce and Carr, 1969). Glycolate, another carbon source, is rapidly assimilated and respired to the extent about 90% by A. flos-aquae in dark. Light increases the amount of glycolate metabolized by about 40%, and greatly decreases the amount respired, so that about 50% of glycolate can be assimilated in one hour in light (Miller et al., 1971).

Concerning nitrogen sources, extracellular proteolysis, manifested by blue-green algae (Allen, 1952), is the most probably explanation of the ability of egg albumin, peptone and casein to supply the nitrogen requirement for growth of these algae (Pringsheim, 1913). By supplying amino acids (Leu, Met, Phen, Ala, Val and Tyr) to cultures of Fremyella diplosiphen, which were deuterated amino acid feed, Crespi et al. (1971) demonstrated the uptake of these amino acids and their incorporation into phycocyanin. Asparagine can serve as a nitrogen source for certain blue-green algae but not for others (Baalen, 1962; Pringsheim, 1913).

The occurrence of urease in blue-green algae (Berns et al., 1966) explains the ability of various of these algae to utilize urea as nitrogen source (Allen, 1952; Baalen, 1962; Cobb and Myers, 1964; Kratz and Myers, 1955; Wyatt et al., 1971). The metabolic products of ^{14}C-urea resemble those of $^{14}CO_2$, except for lesser

labeling of glutamate and aspartate and greater labeling of glutamine and asparagine (Allison et al., 1954).

In autotrophic cultures of blue-green algae, the availability of light limits growth in all but dilute suspensions (Crespi et al., 1970; Lyman et al., 1967; Wolk, 1968). Both fluorescent and incandescent illumination can support maximum growth rates (Kratz and Myers, 1955). High intensity Lucalox lamps also support rapid growth, provided that the time-average light intensity to which individual cells are exposed is not excessive (Wolk, 1973). The spectrum of incident light can affect the pigmentation of the algae (Fritsch, 1945).

Growth rates of the order of a one to two days doubling time have frequently been found for blue-green algae (Kurz and Larue, 1971; Gerloff et al., 1950). An approximate 6 hours doubling time has been reported for A. cylindrica (Cobb and Myers, 1964) and for Nostoc sp. (Hoare et al., 1971), whereas coccoid blue-green algae double in as little as two hours. Cell densities achieved range from about 0.2 (Gerloff et al., 1950, 1952) to 7 grams (dry weight) per liter (Allen and Arnon, 1955).

Temperatures appropriate for culture of thermophilic blue-green algae are discussed by Castenholz (1969, 1970). Many nonthermophilic blue-green algae are normally grown at room temperature (20-25 C), but show increased

growth rates as the temperature is raised to 30 C (Hoogenhout and Amesz, 1965; Kratz and Myers, 1955).

William et al. (1952) demonstrated nitrogen fixation by Chlorella, but a medium for its growth must include fixed nitrogen, the element already most commonly limiting the world's agricultural production. Chemical fixation of nitrogen requires expenditure of energy, and there is a question whether our fossil fuels should be dissipated in the fixation of nitrogen by Chlorella, when the blue-green algae, capable of utilizing solar energy for nitrogen fixation, might be exploited.

The yields reported for blue-green algae are slightly lower than those for Chlorella. However, less effort has been expended in studying the optimum conditions of culture for blue-green algae than for Chlorella.

Large Scale Culture of Algae

Controlled large scale microalgal culture began in the late 1940s and 1950s with the pioneering work of Jorgenson and Convit (1961), and Ketchum and Lillich (1949). Their cultures varied from one to 1000 liters in volume. During 1951, the Arther D. Little Company experimented with Chlorella cultures varying from 2000 to 5000 liters (Ludwig and Oswald, 1952). The University of California operated cultures of 10,000 liters (Gotaas, Oswald and Golueke, 1957). In the ensuing years many large scale cultures have been grown. Units of 1000 liters were

operated in Japan (Mituya, Nyuonoya and Tamiya, 1961) and these were expanded to units in excess of 100,000 liters by Tamiya (1955, 1959). In eastern Europe, a 10,000 liters unit described by Enebo (1967) has been operated in Trebon, Czechoslovakia and according to Gromov (1967) cultures of algae at Leningrad, USSR, are of the same order of magnitude. During 1967 in Condord, California and in St. Helena, California and in Chandler, Arizona, a new generation of controlled microalgal cultures of 10^7 liter magnitude was brought into operation, not primarily for protein production, but for oxygen production and a harvesting method of microalgae was described by Clement et al. (1967).

Abott (1974) has recently presented a detail analysis of the cost of protein in various foods. Based on published costs of protein per acre, energy content per unit of yield, and sunlight energy data, photosynthetic efficiencies can be computed for each protein source. With these photosynthetic efficiencies and Abott's data, one can plot protein cost versus photosynthetic efficiency. Although the high degree of accuracy of known food costs cannot be ascribed to estimate photosynthetic efficiencies, nonetheless the fundamental relationships are quite apparent. As light energy conversion efficiency increases, the price of protein decreases. The most basic way to lower the cost of protein without sacrificing quality is to improve photosynthetic

efficiency. An enormous increase in efficiency is easily attained in large scale production of algae. The reasons for low photosynthetic efficiencies in agriculture are well known.

Decolorization of Algae

The only chlorophyll found in blue-green algae is chlorophyll a. (Myers and Kratz, 1955; Seybold and Egle, 1938). It has been confirmed that the chlorophyll from Phormidium luridum contains phytol (Souza and Nes, 1969). The representative values of chlorophyll as percent of algae (dry weight) are 0.2% to 1% (Stransky and Hager, 1970). It is known that α-carotene, echinenone, myxoxanthophyll and zeaxanthin are most frequently the major carotenoids present, although in exceptional cases cathaxanthin, caloxanthin, nostoxanthin, and oscillaxanthin have been found to account for 10% or more of the total carotenoid present (Stransky and Hager, 1970; Hertzber et al., 1971; Wolk, 1973). All or almost all, of the cellular chlorophyll, and much or all of the carotenoids, are localized in the lamellae (Thylakoids) (Allen, 1968; Skatkin, 1960).

Other pigments of blue-green algae are the phycocyanin, allophycocyanin, and phycoerythrin which are water soluble (Emerson and Lewis, 1942; Jone and Myers, 1965). Phycocyanin and allophycocyanin are blue and phycoerythrin is red. These three pigments are attached to proteins and

are bile pigments of linear tetrapyroles (O'hEocha, 1958, 1965). They are, therefore, referred to as phycobiliproteins of simply biliproteins. In vivo aggregates of biliprotein molecules are called phycobilisomes (Wolk, 1973).

Dried algae powder is accepted poorly as human food due to its unpleasant odor, flavor and bright green color, and low digestibility in vivo. Decolorizing the dried algae with methyl alcohol not only reduced the odor and color, but also improved its digestibility. Weanling rats fed dried Chlorella or Scenedesmus grew poorly when compared with similar rats fed casein. Decolorized algae improve the growth of the rats but the products continued to be inferior to casein. Improved growth is related to the partial destruction of the cell wall by the decolorizing process (Miller, 1959).

Mitsuda et al. (1970) reported that when acetone, methanol, mixtures of acetone and methanol, and mixtures of methanol and hexane were compared regarding decolorization of Chlorella ellipsoida, the rate of decolorization of the cells was highest with the mixed solvent of methanol/hexane:4/3. Another approach to bleaching is to change the composition of the medium after maximum growth. By increasing the concentration of glucose to reach a carbon/nitrogen mole ratio of 20/1, bleached cells are obtained after 5 days culturing. In cells grown on a

medium supplemented with 0.2% glucose at the start of the culture, both chlorophyll and carotene contents were highest at the optimum temperature for growth, whereas total xanthophylls were at the highest level at higher temperatures. At a temperature over 40 C, cells were bleached and most of the pigments were decomposed. However, the nitrogen content was lower in bleached cells than in normal cells.

The breakdown of chlorophyll under various conditions of light and darkness was studied in a mixotrophic mutant of Chlorella pyrenoidosa. In cultures exposed to 25,000-30,000 lux light intensity the compounds lacking phytol, pheophorbide of protochlorophyllide-like compounds degraded faster than the chlorophylls, but they did not disappear completely, unless all chlorophyll was broken down and chlorophyllase activity increased during the bleaching process (Ziegler and Schandler, 1969). The reaction was slightly enhanced if the culture was mixed with pure oxygen. In low light intensity (500 lux) formation and degradation of the pheophorbide were superimposed. It is concluded that removal of the phytol group is one of the primary steps in the chlorophyll degradation.

Pringsheim (1952) found that certain strains of Euglena gracillis were devoid of chloroplasts when grown at a temperature just below maximal multiplication (34-35 C), and that a sensitive strain lost its chloroplasts

irreversibly but multiplied as the bleached form under heterotrophic conditions. A number of studies revealed that bleaching and greening of Euglena could be effected by U. V. light (Lyman et al., 1961) and by streptomycin treatment (Provasoli et al., 1948).

Extraction of Protein

The structural features of the cells of blue-green algae include a central region ("centroplasm, nucleoplasm"), which is rich in nucleic acid and which interdigitates with a peripheral region ("chromatoplasm") containing the photosynthetically active pigments, various inclusions, and circumferential layers including plasmalemma, a pellicula wall, and often, a layer of mucilagenous material (Fritsch, 1945). Between the plasmalemma and the extracellular mucilage is a wall layer, termed the "inner invertment," which has been resolved by the electron microscope into four layer (L_I through L_{IV}) (Jost, 1965). Layers L_I and L_{III} are electron transparent, and vary from about 3 nm each in thickness to about 10 nm. Blue-green algae can be lysed by growing them in penicillin (Foster et al., 1953; Fuhs, 1958; Kumar, 1962). Moreover, lysozyme, which breaks down bacterial cell walls, can cause lysis of blue-green algae (Fuhs, 1958), a fact which has permitted the isolation of protoplasts or spheroplasts from these algae (Fulco et al., 1967; Pegott and Carr, 1971). The sensitive layer to lysozyme is not directly at

the cell surface. It has been clearly demonstrated that the layer destroyed by lysozyme is L_{II} (Jensen and Sicko, 1971).

It was found that the lysozyme sensitive layer of the cell walls contain peptidoglycan (Salton, 1960; Stainier, 1962; Work and Dewey, 1953). Cellulose has not been detected in the isolated wall. L_{IV} layer is not always seen and is approximately 75 A to 80 A thick, external to the peptidoglycan layer. This layer consists of lipopolysaccharide of which about 60% is carbohydrate, principally mannose but with glucosamine, 2-amino-2-deoxyheptose, 2-keto-deoxyoctonate, and other sugars also present (Weise et al., 1970). The end walls of unicellular blue-green algae do not differ in ultra structure from the side walls (Carter et al., 1939; Echlin, 1963; Ingram and Thurston, 1970). The end walls are formed by irislike ingrowth of the side walls, beginning with plasmalemma. A layer of mucilage surrounds the cells. The mucilage has a fibrillar appearance and it is a polysaccharide.

The dried protococcal algae are bright green. They have a specific odor and flavor, which renders them rather difficult to use in human diets, and their digestibility is low. According to Mitsuda et al. (1969) protein isolated from green algae is better digested by trypsin, pepsin in vitro than the protein in the intact cell. The problem with protein availability seems to be

related to the durable cell wall of microalgae. Enebo (1967) recently discussed the methods of breaking the algae wall and releasing the protein, using mechanical breakdown with a ball-mill, and enzymatic cell wall breakdown using the stomach juice of the snail Helix poatia.

In a number of papers, whose authors tried to increase the digestibility of protein from protococcal algae, efforts were made to destroy the cell membranes by means of cellulolytic enzymes (Boyko and Klyshkina, 1964; Toyama et al., 1960). However, no encouraging results were obtained as cell membrane does not respond to cellulases. Mitsuda et al. (1969) have presented an excellent review on the extraction of Chlorella protein, using a urea soaking method and various salt solutions. Tamura and Baba (1960), Fowden (1951, 1952) extracted the protein from Chlorella using detergents, a high pressure method, and a mixture of citrate (5%) and malate (5%). It was found that water and inorganic salt solutions extracts very few nitrogen compounds from Chlorella cells, while butanol or a combination autolysis-butanol treatment was highly satisfactory for the complete extraction of protein.

Treatment of Chlorella cells with NaOH or β-glycosidic enzyme was rather disappointing, since the nonprotein nitrogen of the extracts increased (Mitsuda et al., 1969). Klyushkina and Fofanov (1967) studied the extraction of protein from a lyophilized mixture of

Chlorella and Scenedesmus using a homogenizer with a motor rotation rate of 7000 to 14000 r.p.m., glass beads, 0.15 mm in diameter, and 0.5% NaOH solution; 63% nitrogen extraction was obtained. The proteins were completely precipitated from the extract by acidification to a pH of 4.5 to 5.0.

Baba et al. (1960) used a 10% solution of caustic soda to extract protein from fresh Chlorella. They precipitated the extracted protein at a pH of 4.5. The protein preparation obtained contained 13.7% nitrogen, which corresponds to extraction of 65% of the protein from the original Chlorella. According to these authors, the preparation was contaminated by cell fragments and was green. The same authors were able to extract 78% of algae nitrogen as a result of 48 hours extraction at room temperature by treatment with 6% NaOH solution and then treatment with 2% solution of the sodium salt of a alkyl-aryl-sulfo acid.

Hayami et al. (1959) used osmotic shock to disintegrate Chlorella cells. They obtained plasmolysis of cell contents in minerals or saccharose solution. After that, the concentration of solution was rapidly decreased to induce osmotic shock. The extraction of chlorophyll with ethanol after osmotic shock was high, but the protein extraction was low.

Northcote and Goulding (1958) obtained 81% of the nitrogenous material by vibrating 50 mg of dry Chlorella cells for 90 minutes with 10 ml water, 4 g glass beads 0.05 mm diameter in a Mike cell disintegrator. The degree of extraction of algal protein from Chlorella was increased by raising the pH to 11 (Mitsuda et al., 1969) and by urea treatment of preferably the bleached cell (Mitsuda et al., 1970). A combined method of urea soaking and alkali or acid pretreatment was recommended by Mitsuda et al. (1969) but it was found that urea disrupted intermolecular bonds in the spatial configuration of protein, denaturing the proteins, and dispersing protein aggregates by forming a urea-protein complex of lower molecular weight.

Hendenskog and Enebo (1969) showed that mechanical treatment of Scenedesmus (the use of a ball mill) caused 70-90% disintegration of the cells. The enzyme method involving treatment with cellulolytic enzyme gave a slight increase in pepsin digestibility of the extracted protein but chemical treatment with hydrogen peroxide gave no increase in digestibility.

Nutrition Value

The chemical composition of blue-green algae varies with the condition of culturing. Extracellular mucilage can account for a large part of the dry weight under poor condition for growth (Wolk, 1973).

Cobb and Myers (1964) found that blue-green algae grown in the presence of NO_3^-, urea or nitrogen gas did not differ in any of the following analytical parameters: nitrogen (10.4%), carbon (48%), hydrogen (7%), phosphorus (2%), chlorophyll a (2%), phycocyanin (15%). The numbers in parenthesis indicate content as a dry basis.

Collyer and Fogg (1955) determined the fatty acid, unsaponifiable lipid, total cell nitrogen and hydrolysable polysaccharide contents of several kinds of algae at various stages of growth in pure culture. For A. cylindrica, he found 43% protein, 25% carbohydrate and 4% lipid on a dry weight basis after 23 days of cultivation. For Spirulina Clement et al. (1971) reported 64-73% protein, 12-27% carbohydrate and 5-7% lipid on dry weight.

Lewis and Gonzalves (1960) determined the amino acid composition of the unicellular marine algae, Rhosymenia palmata, using paper chromatography. Clement et al. (1967a) determined the nitrogenous constituents and amino acid composition of Spirulina maxima, and reported that dried algae, more than 60% of which was protein-like material, contained all the essential amino acids except the S-containing amino acid in sufficient quantity for humans. The chemical score was 43.5 and the biological value was 48-54. Clement et al. (1967a) emphasized the low digestibility of whole cells and the

need to extract the protein in order to increase the digestibility.

Leveille et al. (1961, 1962, 1962a) reported that all the algae tested (Chlorella, Scenedesmus strains) were inferior to either casein or the soybean protein controls used with the rat and chicken studies. They concluded that the algae were deficient in methionine for the rat and chick, and Chlorella pyrenoidosa appeared to be deficient in histidine (in addition of methionine) for the rat.

Japanese investigators (Hayami et al., 1960, 1960a; Nakamura, 1961) have contributed greatly in both nutritional studies and cultural techniques. In a representative experiment with rats they reported that when dried green algae was the source of protein the weight gain was only 66% of that for a skin-milk diet. Digestibility coefficient of absorption was only 57.5%. A considerably higher digestibility coefficient for the protein of dried Chlorella pyrenoidosa was reported by Lubitz and Casey (1963), yet the body weight gains for the Chlorella fed rats was also much less than those fed egg or casein, and food consumption was less.

Lee and Fox (1967) studied the supplementary value of the algal protein in human diets and reported that algae was considered excellent source of both lysine and threonine and the beneficial effect of these amino acids

in improving the protein quality of rice had been demonstrated. Since cereal proteins in general are deficient in lysine, algae protein may be useful in supplementing other cereal protein. However, in view of the work of Mickelsen et al. (1974) it may well be that the requirements for lysine are lower in humans compared with rats.

Leveille et al. (1961) studied the addition of enzymes on the digestibility of algae and reported that supplementation of algae diets with diastase, alpha-amylase and cellulase, resulted in improved protein digestibility in young rats. Hemicellulase or hydrase (mixture of diastatic enzymes also possessing protease, cellulase, lipase, pectinase and hemicellulase activity), did not influence digestibility.

Human subjects tolerated diets supplemented with algae in amounts up to 100 g per man per day. Some gastrointestinal symptoms (abdominal distention, accompanied by increased eructation and flatulence) appeared. At feeding levels above 100 g per day, the algae were very poorly tolerated because these symptoms increased along with nausea and some subjects experienced diffuse abdominal pain, vomiting, malaise, and headache. There was no diarrhea, instead the stools became bulky and dry, and some times bowel evacuation caused rectal pain. Physical examination failed to show abnormalities other than those associated with the gasterointestinal tract (McDowell and

Leveille, 1963; Powell et al., 1961). Leveille et al. (1963) concluded that algae can be tolerated in limited quantities as a food supplement, but further processing such as an isolation of the protein will be necessary if algae are to be useful as a major food source. Cooks (1962) studied the nutritive value of a mixture of Scenedesmus and Chlorella, and determined the protein efficiency ratio (PER) of these algae for rats (PER is the grams of weight gained by test organisms per gram of protein consumed). The PER was 1.62 for algae, and 2.31 for casein. When a diet was fed in which casein furnished 75% of the protein and algae 25% of it, the PER equalled that of casein alone.

Dillenberg and Dehenel (1960) reported that some of the blue-green algae killed dogs, fish, cattle and horses, but the cases of human poisoning were not fatal. Vanderveen et al. (1962) believed there was good evidence that some of the toxicity associated with algae as food was caused by bacterial contamination. This toxicity may result from secondary causes such as decomposition of algae by bacteria.

EXPERIMENTAL

Chemicals and Materials

Chemicals. The chemicals used in this study and their sources are listed in the Appendix, Table 1. All were reagent grade unless otherwise specified. Distilled or deionized water was used in the preparation of buffer and chemical solution.

Blue-green algae. The experimental organism was an axenic culture of Anabaena flos-aquae. This culture was obtained from the Department of Botany at Michigan State University. It was cultivated in the Kratz-Myers medium, which was free from any sources of combined nitrogen. EDTA, as chelating agent, was used to maintain iron and manganese in solution at the alkaline pH at which blue-green algae normally grow. The composition of Kratz-Myers medium is given on Table 1.

Table 1.--Standard growth medium (Kratz-Myers).

Water	965 ml
K_2HPO_4 (1%)	10 ml
$CaCl_2$ $2H_2O$ (0.31%)	5 ml
$MgSO_4$ $7H_2O$ (1.5%)	16.5 ml
FeA_6	20 ml
pH	7.6
Where FeA_6 solution is	
H_2O	95 ml
$Na_2EDTA.$ $2H_2O$	200 mg
$FeCl_3.$ $6H_2O$	21.5 mg
A_6Mel	5 ml
Where A_6 Mel solution is	
H_2O	1000 ml
H_3BO_3	2.86 g
$MnCl_2$. $4H_2O$	1.81 g
$ZnSO_4$. $7H_2O$	0.222 g
MoO_3 (85%)	0.0177 g
$CuSO_4$ $5H_2O$	0.079 g
$CoCl_2$ $6H_2O$	0.042 g
Na_2MoO_4 . $2H_2O$	0.983 g

Methods

(1) Physical Methods

(A) Effect of Carbon and Nitrogen Sources on the Growth

Experiments were conducted with the following concentrations of various sources of nitrogen expressed in mg per liter of the standard medium; 100 mg each of urea, sodium nitrate, sodium nitrite, and 50 mg of peptone. As a carbon sources, glucose (0-2.5% w/v) was added to the media. In one experiment equal amounts of glucose and urea were used. The sterilized medium (250 ml) in a 500 ml Erlenmeyer flask was innoculated with 10 ml of the culture (20 mg dry matter/liter). The flask was incubated at 30-32 C for 11 days by shaking under 15,064 lux fluorescent light. The cells were centrifuged at 10,000 x g for 10 minutes, washed with about 50 ml water and centrifuged again. The washing was repeated twice. The cells were dried at 100 C for 6 hours. The nitrogen content of the dried cells was determined by micro-Kjeldahl method. The light intensity was measured in foot candles and converted to international lux.

(B) Mass Culture of Cell and Growth Rate

A nineteen-liter bottle was used for mass production of the cells. The light intensity on the bottle surface facing the fluorescent light was 15,064 lux. The medium

was aerated by means of compressed air passing through a sterilized cotton filter at the rate of about 2.5 liters per minutes (Fig. 1). The inoculum was introduced under aseptic conditions into 16 liters of the medium which had been sterilized at 100 C for one hour.

Ten ml of the cell suspension was periodically taken out from the culturing bottle and centrifuged at 10,000 x g for 20 minutes, washed carefully with water twice and resuspended in 10 ml water. The absorbance of the suspension was determined at 420 nm in Beckman DU spectrophotometer. The growth curve of algae was determined from a standard curve obtained by plotting absorbance at 420 nm against dry weight of cells (Fig. F_1 in Appendix). After cell concentration in the incubator reached maximum (about 650 mg dry cell per liter) the cells were centrifuged, washed with water twice and lyophilized. The lyophilized algae were kept in a desiccator.

(C) Decolorization

(C-1) Absorption Spectra of Cell Suspension

Freeze dried algae were suspended in water and the absorption spectrum of the suspension measured in the visible region using the opal paper technique (Shibata, 1958), 1 cm path-length cuvettes, and a recording spectrophotometer (Bausch and Lomb 505). The principle of the method is illustrated in Fig. 1. The sample and blank cell

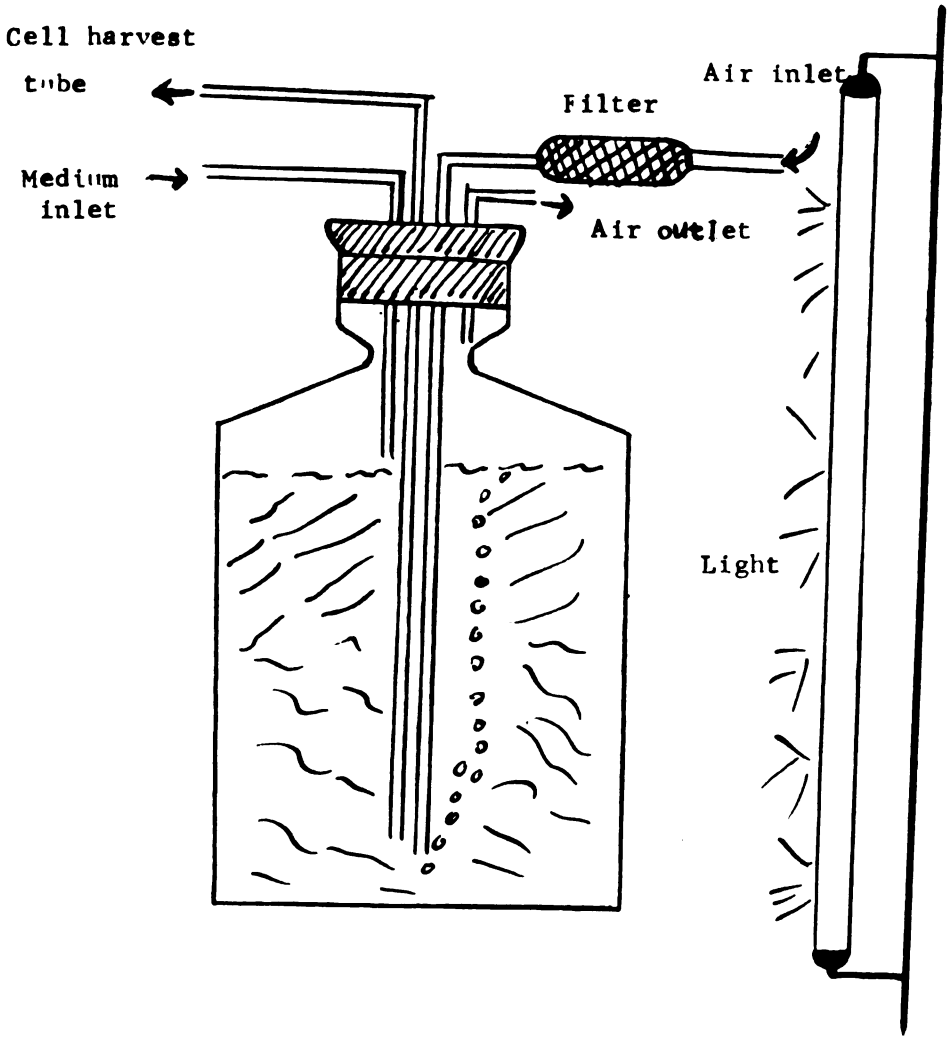

Fig. 1.--Diagram of cell culturing.

compartments were both provided on the sides of emerging light with identical opalescent paper which diffuses uniformly the light as it leaves the cuvette.

The paper (Whatman 3 MM) was prepared by dipping into paraffin oil and allowing to drain overnight to remove excess paraffin. Two sheets of oil impregnated filter paper were sandwiched between cuvette and cuvette holder. Only one side of the cuvette had oil paper (Fig. 2).

(C-2) Decolorization of the Cells by Photolysis

Fifty mg of freeze-dried algae were suspended in 100 ml of water and stirred. Ten ml of the cell suspension were placed in a 15 ml glass ampule and few drops of toluene were added as preservative. The sealed ampules were photolyzed at 32 C under 15,064 lux of fluorescent light. The absorption spectrum of the photolyzed cell suspension was measured by the opal paper method. The effects of heat, time, pH, ultraviolet light on the photolysis were studied.

(C-2-1) Heat.--The ampules containing the cell suspension were heated in a water bath (90 C) for various time periods and cooled to room temperature. The spectrum was measured without photolysis.

(C-2-2) Length of Time of Photolysis.--Two ampules containing the cell suspension were periodically removed

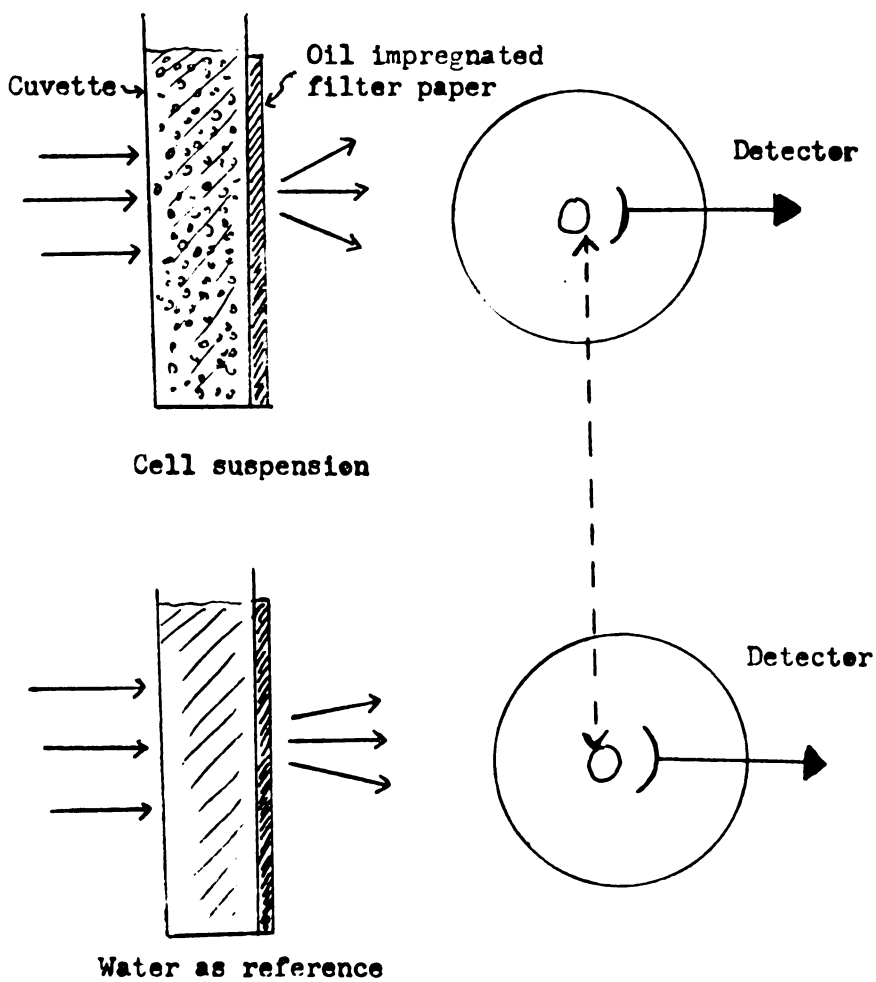

Fig. 2.--A method of measuring the absorption spectrum of a cell suspension by using paraffin-impregnated Whatman 3 MM paper screens.

from the photolysis chamber and the spectrum of the cell suspension was measured in the visible region (400-700 nm).

(C-2-3) pH.--Fifty mg of freeze-dried cells were suspended in 100 ml of various pH buffers (pH 1, 3, 5, 7, 8, 11, 12). Ten ml of these suspensions were pippetted into 15 ml glass ampules containing a few drops of toluene and sealed on the flame. The spectra of the cell suspensions were measured before and after photolysis.

(C-2-4) Ultraviolet Light.--Twenty ml of the cell suspension were placed in a Petri dish. The thickness of the cell suspension in the dish was about 0.5 cm. The distance from the surface of cell suspension to ultraviolet lamps was about 7 cm. One lamp emitted at 253.7 nm, and the other at 366.0 nm. The illumination was performed at room temperature for 1 hour.

(C-3) Decolorization of the Protein Extract of HCl-Pretreatment

The cell suspended in 3 N HCl were heated at 90 C for 10 minutes and the protein of the cells extracted at pH 11 (Fig. 4). The spectrum of the extract was measured before and after photolysis.

(D) Protein Extraction

(D-1) Determination of Optimum Conditions for Protein Extraction

(D-1-1) pH Effect of Extracting Solution.--One hundred twenty five mg of freeze-dried algae were suspended in 100 ml of water in which the pH was adjusted at various levels by adding 1 N HCl or 1 N NaOH solution with constant stirring. Ten ml of each suspension were placed in 15 ml glass ampules containing a few drops of toluene. The ampules were sealed in the flame and divided into 3 groups: One group was exposed to 15,064 lux fluorescent light for 8 hours in order to accomplish the extraction and bleaching of protein simultaneously. The second group kept in the dark, and third group was only heated at 95 C for 20 min. Triplicate samples were prepared for each combination of pH treatment.

The cell suspensions were centrifuged at 50,000 x g for 10 min. The amount of protein extracted was determined by the Folin-Ciocalteau method (Lowry et al., 1951). A standard curve was prepared using bovine serum albumin.

(D-1-2) Effect of Temperature and of Algal Concentration.--Freeze-dried algae (44 mg to 480 mg in 100 ml) were suspended in 0.05 phosphate buffer pH 11, using a magnetic stirrer. Ten ml of each cell suspension were

pippetted in the ampule and sealed. The sealed ampules were heated in a water bath at various temperature for 30 min. After photolysis the amount of protein extracted was determined.

(D-1-3) Heating Time Effect at 90 C.--Ampules containing a cell suspension (pH 11) of 400 mg dry algae/ 100 ml were heated at 95 C for various periods (10, 20, 30 minutes etc.). After cooling to room temperature, the protein in the extract was determined.

(D-2) Determination of Optimum Condition for HCl-Pretreatment of Cells

(D-2-1) Effect of HCl Concentration.--Four hundred mg of freeze-dried algae were suspended in 100 ml of either 1.5 N, 3.0 N, or 6.0 N HCl for 30 minutes with magnetic stirring. Ten ml of each suspension were pippetted in each ampule, sealed and heated in a 95 C water bath for 5 min. The pH of the cell suspension was then adjusted to 11 with 6 N NaOH and the final volume was made up to 25 ml with water. The amount of protein extracted was determined after filtering the cell suspension (Lowry et al., 1951).

(D-2-2) Optimum Length of Time for Protein Extraction After the HCl-Pretreatment.--Four hundred mg of freeze-dried algae were suspended in 100 ml of 3 N HCl

with stirring at room temperature for 20 min. An Erlenmeyer flask (125 ml) containing 50 ml of the suspension was heated in a water bath of 95 C for 10 min. with occasional shaking. The heated flask was cooled to room temperature using tap water. The pH of the flask contents was adjusted to 11 with 3 N NaOH and the mixture was left at room temperature for (a) 40 min. in the dark or (b) 72 hours in the dark, or (c) 72 hours under 15,064 lux light.

All samples were centrifuged at 10,000 x g for 20 min. The amount protein in the supernatant was determined in triplicate by the micro-Kjeldahl method.

(D-2-3) Optimum pH for Precipitating Protein from the Extract after the HCl-Pretreatment.--Cell suspension was hydrolyzed as previously and extracted at room temperature for 2 hours. The supernatant solution was photolyzed at 32 C under 15,064 lux fluorescent light for 10 hrs. The bleached supernatant solution was precipitated at various pH: pH 3, pH 4, pH 5, pH 6, and then centrifuged at 50,000 x g for 20 min. The precipitates were washed twice and then lyophilized. Their nitrogen content was determined (Kjeldahl method).

The protein which was not recovered by pH adjustment was precipitated with zinc acetate (2 g per 100 ml of supernatant) and pH adjustment of 7.0 with 1% NaOH solution. After centrifugation, the precipitates were lyophilized.

(D-3) Protein Extraction and Recovery Methods

(D-3-1) Methods.--The extraction and recovery methods are illustrated in Figures 3, 4, and 5, respectively. "HCl-pretreatment extraction method" was a method according to which the protein of the cells was extracted at high pH after pretreatment of HCl. "Single alkali extraction" meant that the cells were directly extracted with alkali solution without any pretreatment. "Mechanical extraction" meant that the cells were extracted in alkali solution with mechanical disintegration of the cells.

(D-3-2) Effect of Photolysis on TCA-Precipitable Protein Yield in the Single Alkali Method or the HCl Pretreatment Method.--After extracting the protein by either the HCl-pretreatment method (Fig. 3) or the single alkali method (Fig. 4), the extracts were treated with 30% trichloroacetic acid (final concentration: 18%) before or after photolysis.

(E) Fractionation of Algal Protein (Fig. 6)

(E-1) Removal of Fat and Pigments

Twenty grams of freeze-dried algae were ground with 20 g of purified sea sand and acetone in a mortar. This procedure was repeated until the acetone extract was

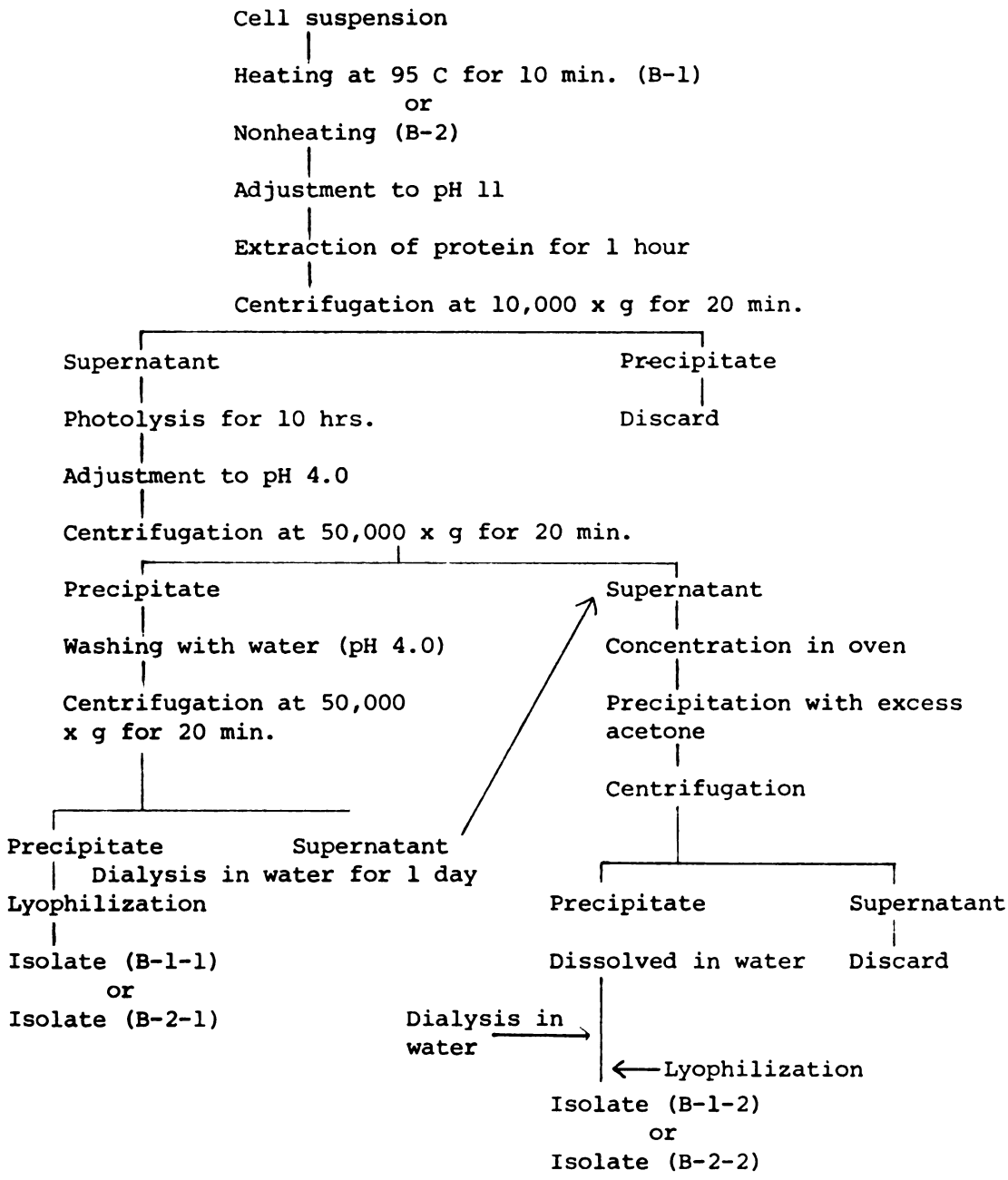

Fig. 3.--Flow diagram of the HCl-pretreatment extraction method.

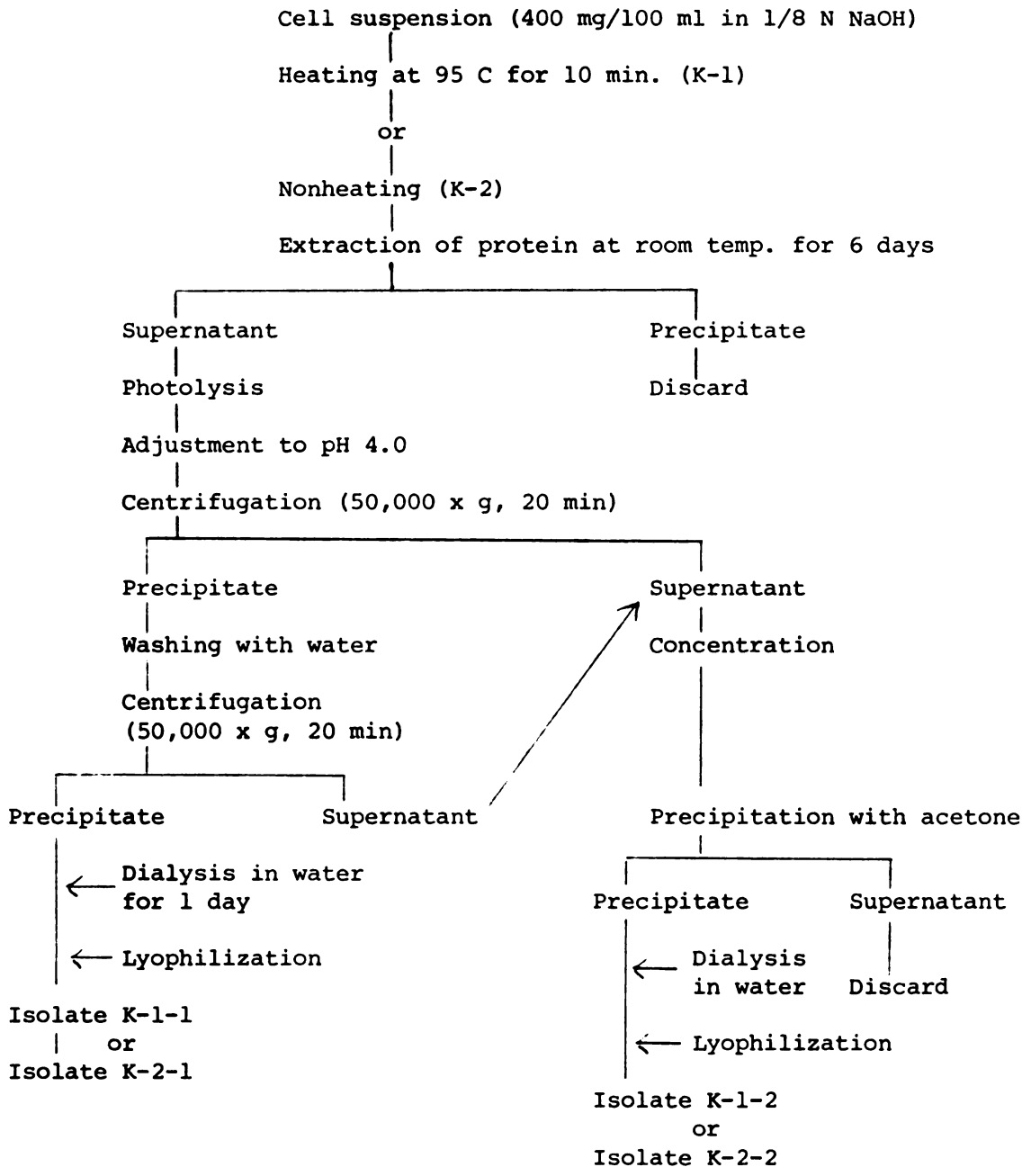

Fig. 4.--Flow diagram of the single alkali extraction method.

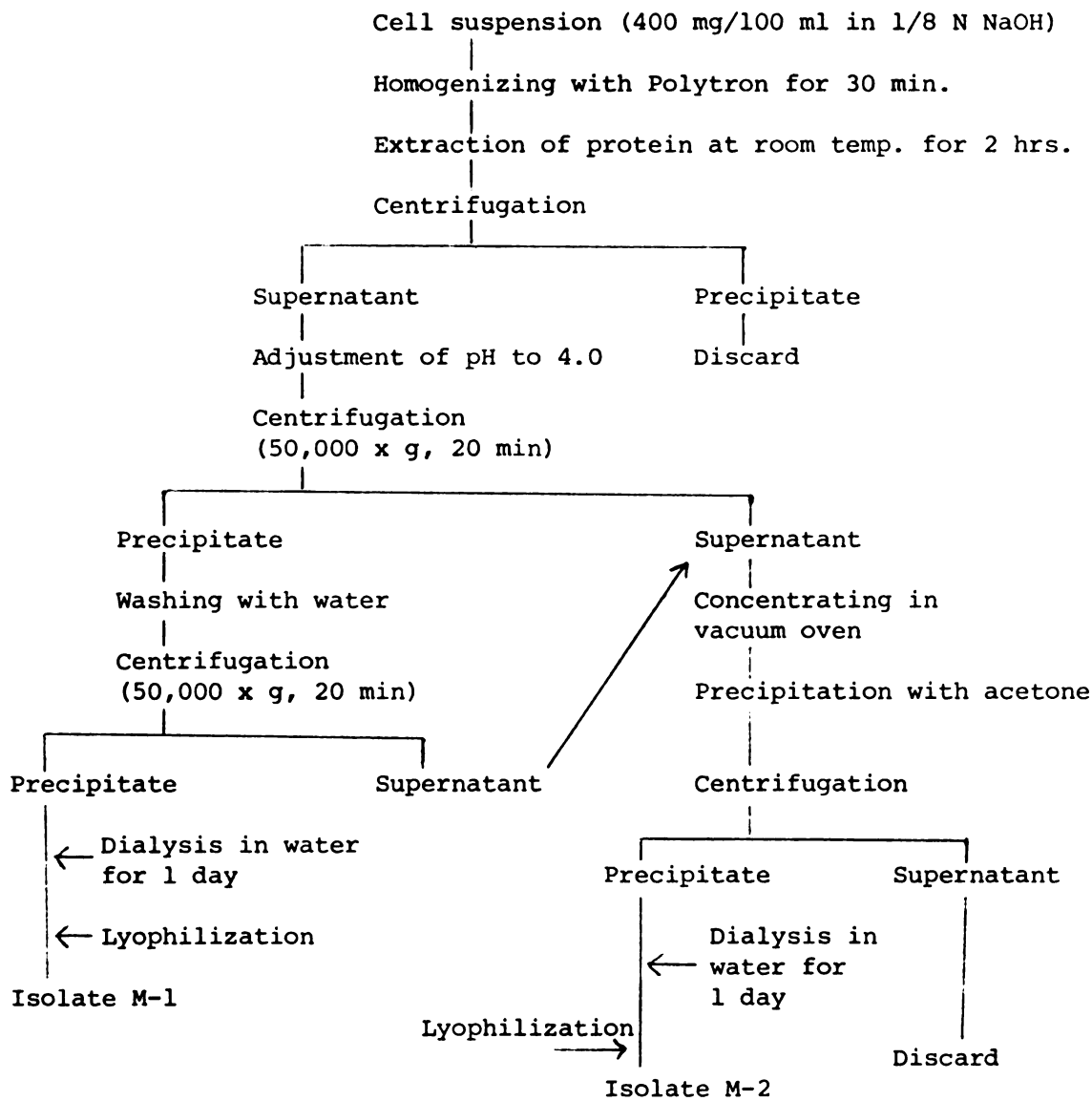

Fig. 5.--Flow diagram of the mechanical extraction.

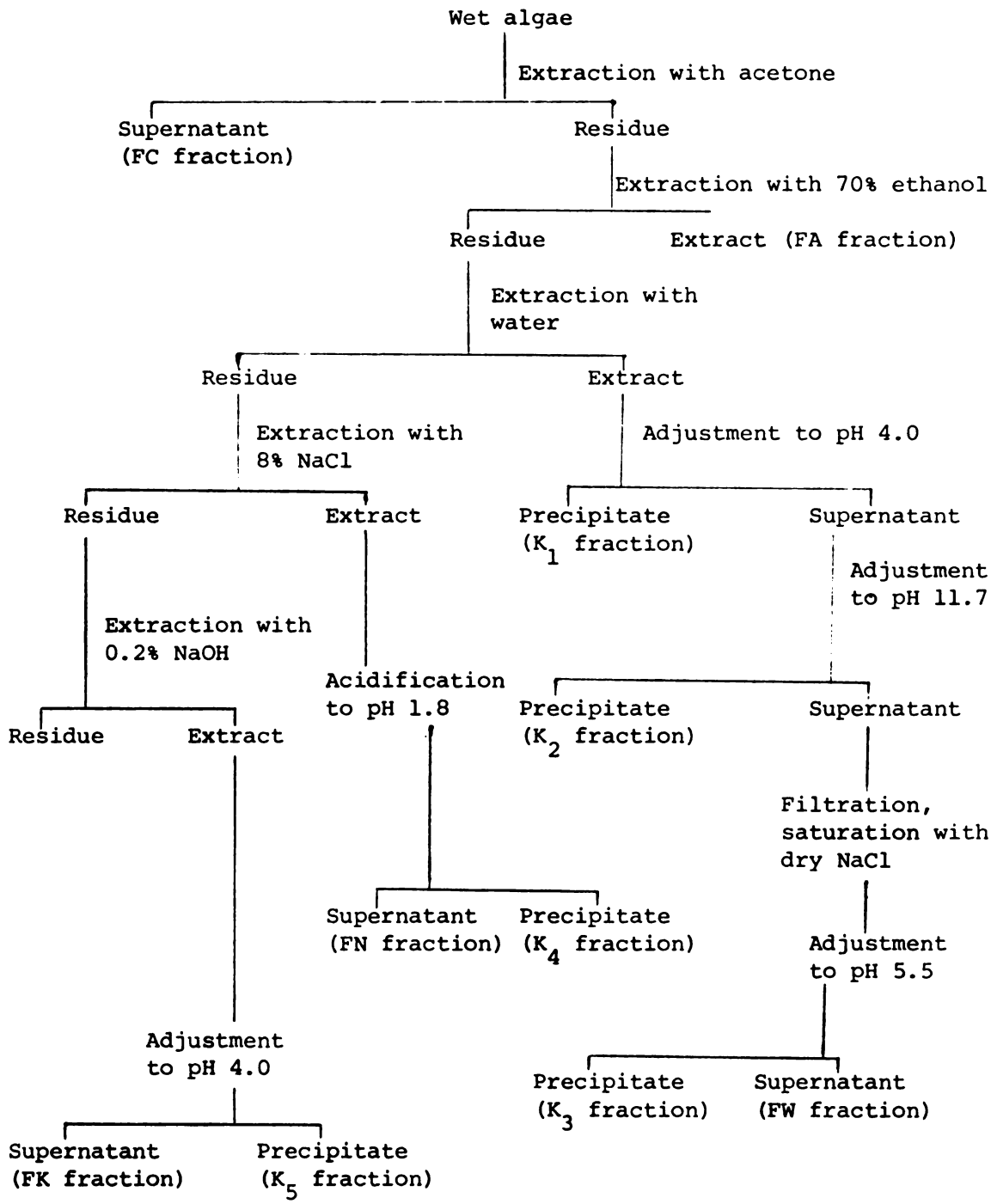

Fig. 6.--Flow diagram of fractionation of algal proteins.

was colorless. After evaporation of the solvent a dark brown residue was left from the extract.

(E-2) Isolation of Amino Acids and Peptide Fractions

After the acetone extraction, the cell residue was ground with 70% ethanol in a mortar. This procedure was repeated six times with fresh ethanol. The ethanol extracts were combined, evaporated to dryness in a vacuum oven at 27 C. The nitrogen content of the dried residue was determined by the micro-Kjeldahl method.

(E-3) Isolation of Water Extractable Protein

(E-3-1) Isolation of Acid-Precipitable Proteins.-- The algal residue remaining after alcohol extraction was thrice extracted with water (pH 6.6) using the solid-water ratios of 1:5, 1:4, 1:3, in a mortar for 3 hrs., at 25-28 C. The suspensions were centrifuged and combined supernatants were filtered through a ground paper-asbestos bacterial filter.

By acidification to pH 4.0 with hydrochloric acid (4% v/v), the water soluble protein (Fraction K_1) was precipitated. The precipitate was removed by centrifugation at 50,000 x g for 20 minutes and washed with water acidified to pH 4.0 and lyophilized. The superantant solution after obtaining fraction K_1 was filtered through filter paper.

(E-3-2) Isolation of Alkali-Precipitated Proteins.--
The addition of alkali to the supernatant solution from (E-3-1) led to the formation of small quantities of loose, slowly settling flakes, which were collected by centrifugation. The pH of precipitation was 11.7 (Fraction K_2). The supernatant was filtered and the filtrate was saturated with NaCl. The insignificant deposit that precipitated was removed by centrifugation. On acidification with 4% HCl to pH 5.5 a copious deposit (Fraction K_3) precipitated from the supernatant.

(E-4) Isolation of Salt Extractable Protein

The residue of algae after water extraction was three times extracted in a mortar with 8% NaCl solution in the solid-solution ratios of 1:5, 1:4, 1:3. The suspension was centrifuged each time for 20 min. The salt extracts were combined and filtered. The filtrate was acidified with 4% HCl to pH 1.8 and the precipitate (Fraction K_4) was separated by centrifugation. The supernatant solution was concentrated in a vacuum evaporator for protein determination.

(E-5) Isolation of Alkali-Soluble Protein

The residue of algae left after obtaining fraction K_4 was thrice extracted with a 0.2% solution of NaOH at the ratios of solid to liquid 1:5, 1:4, 1:3. The extracts

were filtered and precipitated by acidification with 4% HCl. Maximum precipitation was obtained at pH 4.0. The protein precipitated was separated by centrifugation (Fraction K_5).

The residue left after the separation of the alkali-soluble protein was transferred to a 500 ml Kjeldahl flask for nitrogen determination.

(F) Digestibility of Protein Isolates and Whole Cells In Vitro

Two hundred mg of the protein isolates, whole cells or casein were suspended in 20 ml of 0.2 M KCl buffer, pH 1.8. A few drops of toluene and 5 mg of pepsin were added. The mixtures were incubated at 38 C. Periodically 5 ml of these solutions were pipetted into 5 ml of 20% TCA and centrifuged. Nitrogen in the supernatant solution was determined by the micro-Kjeldahl method.

The percent digestibility is the percent of nitrogen in supernatant solution after enzyme digestion vs. the total nitrogen in the sample.

(2) Chemical Methods

(A) Protein Determination

The amount of protein extracted was determined by the Folin-Ciocalteau method (Lowry et al., 1951). Nitrogen content of the solid materials was determined by the micro-Kjeldahl method (A.O.A.C., 1971).

(B) Carbohydrate Determination

Carbohydrates were determined by the method of Dubois et al. (1965). The absorbance at 490 nm was measured in a Beckman DU spectrophotometer. A standard curve was made over the range of 0.0 to 0.2 mg carbohydrate using a standard solution of galactose-mannose mixture (1:1).

(C) Total Lipid Determination

Total lipid was determined by a micro-modification of the method described by Mojonnier and Troy (1925). A sample of 50-100 was taken into a conical centrifuge tube and 1.5 ml of 2% KCl was added and agitated. Then, 1.0 ml of 95% ethanol was added. The tube was sealed with a stopper wrapped in saran wrap, shaken for 30 seconds, and centrifuged for 1 min. in a clinical centrifuge. The upper layer was carefully removed with a syringe and placed in a previously tared dish on a hot plate. The tube was washed with 1.0 ml of ethyl ether:petroleum ether mixture (1:1). The extraction procedure was repeated. The extracts were evaporated to dryness, placed in 110 C vacuum oven for 30 min., cooled in a desiccator and reweighed.

(D) Nucleic Acid Determination

The cell suspensions were precipitated in the cold with 5% trichloro acetic acid (TCA). After washing with cold 5% TCA, the precipitate was extracted three successive

times with 2.0 ml of 5% TCA at 90 C for 10 min. About 90-95% of nucleic acid was extracted in the first two treatments and no further extraction took place after the third treatment. The extracts were collected, combined and made up to 10 ml with water. Ten ml of the extract was then diluted to 20 ml with water and the absorption at 260 nm, determined in a Beckman DU spectrophotometer against a blank containing an equivalent amount of TCA. The extinction coefficient used in this procedure was 16 for 0.1% nucleic acid. The extinction coefficient was calculated from absorbance of 0.1% solution of standard RNA (Gale et al., 1953).

(E) Ammonia Nitrogen

The algal suspension was distilled in a basic solution, using a micro-Kjeldahl distillation. The ammonia was trapped in saturated boric acid and titrated with standard HCl.

(F) Humin Nitrogen

Freeze-dried algae were hydrolyzed in 6 N HCl at 110 C for 22 hrs., and the hydrolyzate was centrifuged at 50,000 x g for 20 min. The nitrogen content in the residue was determined by Kjeldahl and was considered to be the humin nitrogen.

(G) Amino Acid Determination

Amino acid analysis was performed on 22 hrs. and 72 hrs. acid hydrolysates of all samples. The amino acids were quantitatively determined by automatically recording the intensity of the color produced by their reaction with ninhydrin (Moore and Stein, 1963; Spackman and Stein, 1958).

Samples containing 15 mg of protein were placed into 10 ml glass ampules. To each ampule 5 ml once distilled 6 N HCl were added. Prior to deoxygenation the ampules were placed in Sonogen ultrasonic cleaner by Branson ultrasonic Corp. for 1-2 hours to break up the insoluble sample residues. Then the sample in the ampule was frozen in a dry ice-ethanol bath. Using a high vacuum pump, each umpule was carefully evacuated and warmed until all dissolved gases were removed from the liquid sample-acid mixture. It was necessary to add some anti-foam to the neck of the ampule to prevent excessive foaming during this step. The ampule contents were then refrozen and sealed in an air-propane flame.

The sealed ampules were placed in an oil bath set in a 110 + 0.1 C for 22 hrs. or 72 hrs. They were then opened and 1 ml of norleucine standard (2.5 mol/ml) was added to each as an internal standard to measure transfer losses. The HCl was removed from the hydrolysate in a pear shaped 25 ml flask, connected to Rinco rotary evaporator

under vacuum, and partly immersed in a 50-55 C water bath. Antifoam in the stem of the connector prevented the sample from foaming out of the flask. The residue was washed with about 10 ml water and re-evaporated. This was done three to four times in succession until HCl could no longer be detected. The acid-free hydrolysate residue of each sample was dissolved in 3-4 ml of dilute buffer (0.067 M sodium citrate-HCl, pH 2.2). Each sample solution was quantitatively transferred to a 5 ml volumetric flask and diluted to volume with buffer. To rid the samples of interfering humin they were centrifuged 10-15 minutes in an International clinical centrifuge. Aliquots (0.1 ml) of the supernatant solution were applied to the Backman 120 C automatic amino acid analyzer columns. The resulting chromatograms were compared to those of standard amino acid calibration mixtures. The ratio of areas under the curve of each amino acid for the samples and the standard were compared and converted to give the amino acid composition of the sample. Corrections for losses of threonine, serine, and tyrosine during acid hydrolysis were made using the equation given by Hirs, Stein and Moore (1954). The other amino acid were determined as the simple average of the 22 and 72 hrs. results.

(G-1) Methionine and Cystine

Methionine and cystine undergo variable destruction during acid hydrolysis. Therefore, they were oxidized to

methionine sulfone and cysteic acid by the procedure of Lewis (1966) using the performic acid reagent of Schram, Moore, and Bigwood (1954). Approximately 60-65 mg samples of protein were weighed into small sample bottles. These were placed in an ice bath and cooled to 0 C.

Ten ml of 30% hydrogen peroxide and 90 ml of 88% formic acid were mixed to make performic acid and allowed to stand at room temperature for 1 hr. Ten ml of the precooled performic acid were then added to each sample. The sample bottles were kept in an insulated crushed ice bath inside a 4 C cold room for 17 hrs. Twenty ml of ice cold water were added to each sample. Each sample was then diluted with an additional 180 ml of water at room temperature. The samples were freeze-dried.

The freeze-dried protein samples were hydrolyzed by the procedure described for amino acid analysis. Two tenths ml of the final supernatant solution were applied to the analyzer column. Standards containing cysteic acid, methionine sulfone and methionine sulfoxide were analyzed. Normalized values corrected for sample weight, norleucine recovery, volume on the column, and incomplete oxidation for cysteic acid and methionine sulfone of the oxidized sample were substituted for cystine and methionine, respectively, of the original unoxidized sample.

(G-2) Tryptophan

As tryptophan is destroyed by acid hydrolysis, the procedure of Spies (1970) was used for its chemical determination. One to five mg of cells ground with sand were weighed into 2 ml glass vials with screw caps and 0.1 ml of pronase solution was added to each vial. The pronase solution was made by adding 10 ml of 0.1 M sodium phosphate buffer, pH 7.5, to 100 mg pronase. The suspension was shaken gently for 15 min. and clarified by centrifugation for 5 min. in an International clinical centrifuge. The pronase solution was freshly prepared each day. The closed vials were incubated 24 hours in a 20 ± 1 C water bath. The incubated vials were quickly cooled to room temperature in a crushed ice bath. To each vial 0.9 ml of 0.1 M sodium phosphate buffer, pH 7.5, was added. In 50 ml Erlenmeyer flasks 30 mg of p-dimethylamino-benzaldehyde and 9.0 ml of 21.2 N sulfuric acid were mixed quickly by gentle swirling. The flasks were stoppered and placed in the dark at 25 C for 6 hrs. Then, 0.1 ml 0.045% (w/v) sodium nitrite was added to each flask. After 30 min., the absorbance at 590 nm was measured in a Beckman DU spectrophotometer. Simultaneously, duplicate samples of the pronase solution, without protein, were treated and analyzed as above. The tryptophan content of the pronase solution was subtracted from the total tryptophan value of the protein sample.

A standard curve covering the range of 0-200 mg of tryptophan was made according to procedure E of Spies and Chambers (1948). Four mg of tryptophan were weighed into a 200 ml volumetric flask which was brought to volume with 19 N sulfuric acid containing 3 mg p-dimethyl-aminobenzaldahyde per ml. Zero, 2, 4, 6, 8, and 10 ml aliquots were then added to 25 ml Erlenmeyer flasks. The total volume was brought to 10 ml with 19 N sulfuric acid. The stoppered flasks were placed in the dark at 25 C for 6 hrs. Then 0.1 ml 0.045% sodium nitrite was added and the absorbance at 590 nm read after 30 min. (Fig. F_2 in Appendix).

(H) Polyacrylamide Disc Gel Electrophoresis

Disc electrophoresis was performed as described by Davis (1964) with two modifications: (1) No sample gel was used; and (2) The stock reservoir buffer was diluted 1:1 with deionized water instead of 1:9. Electrophoresis was conducted with a laboratory apparatus having a buffer capacity of 250 ml in each of the electrode tanks. Electrophoresis was conducted initially at 2.5 mAmp per tube and increased to 5 mAmp per tube when the tracking dye entered the running gel.

Gels were stained for 1-2 hrs. in a solution of Amido black 1 OB, containing 250 ml water, 250 ml methanol, 50 ml glacial acetic acid and 5 g Amido black as described

by Weber and Osborn (1969). Amido black-treated gels were electrically destained in 7% glacial acetic acid.

(I) Polyacrylamide-SDS Disc Gel Electrophoresis for Estimation of Polypeptide Molecular Weight

Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was conducted according to the procedure of Weber and Osborn (1969). Protein samples ranging from 1-4 mg were weighed into 5 ml vials and 1 ml of 0.01 M phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% -mercapto-ethanol (ME) was added. These mixtures were permitted to equilibrate for 2 hrs. at room temperature, then a 50 µl aliquot was diluted with 50 µl of the same phosphate buffer, 5 µl of ME, 1 drop of glycerol and 3 µl of tracking dye (0.05% Bromphenol Blue in water). A thirty microliter portion was applied to the top of the gel column and electrophoresis was conducted for 4 hrs. at 6 mAmP per tube. The gels were removed from the tubes and stained according to the method described by Weber and Osborn (1969).

Molecular weight for the subunits were estimated from a plot of relative mobility versus the log the molecular weight for standard proteins. Standards used in this study were Cytochrome (11,700), Trypsin (23,300), Pepsin (35,000), Catalase (60,000). Relative mobilities

were evaluated from measurements of the gel column, dye zone and protein zones as follows:

$$\text{Relative mobility} = \frac{\text{distance protein migrated}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

RESULTS AND DISCUSSION

(A) The Effect of Nitrogen Sources and Glucose on Cell Growth

Many types of blue-green algae fix molecular nitrogen and carbon dioxide. Thus these algae are among the most completely autotrophic organisms (Fogg, 1974).

The primary purpose was to maximize the yield of algae, and to determine their heterotrophic properties, since the yield of algae fluctuates from 0.2 to 7 g dry cells per liter of culture depending upon the culturing conditions (temperature, light intensity, nutrients, pH, etc.). One of the difficulties in maximizing the protein yield is an extracellular mucilage which is a polysaccharide, amounting up to 40% of the dry weight in a nitrogen deficient medium. Combined nitrogen sources inhibit the formation of the mucilage, heterocysts, and spores, thereby increasing the amount of protein in the harvested dry cell (Fogg, 1949, 1962).

In the case of one blue-green algae, Anabaena doliolum, vegetative growth increases directly with an increase in the concentration of nitrate or nitrite nitrogen ($NaNO_3$ or $NaNO_2$) up to 0.02 M, above which growth is retarded. However, with increased levels of nitrate

or nitrite, especially of ammonia, the formation of spores and heterocysts decrease (Singh et al., 1967). These nitrogen sources also delay sporulation.

Urea, peptone, sodium nitrate, and sodium nitrite, at the concentration of 10 mg % (w/v), were added to the basal medium. The medium was incubated for 11 days with aeration under aseptic conditions. Addition of urea or peptone as organic nitrogen source resulted in no appreciable increase in cell production and protein content in comparison with the controlled culture which was just supplied with atmospheric nitrogen (Table 2). However, adding sodium nitrate or sodium nitrite decreased the

Table 2.--Yield of A. flos-aquae culture harvested after 11 days of growth in media containing various sources of nitrogen.

Nitrogen sources	Concentration of N source mg/l	Mg dry cell matter per liter medium	Crude protein (% N x 6.25), dry basis
Urea	100	263 ± 19	63 ± 1.6
Sodium Nitrate	100	155 ± 11	50 ± 1.2
Sodium Nitrite	100	140 ± 8	44 ± 0.9
Peptone	50	245 ± 20	63 ± 1.1
Control (Atmosphere N)	--	256 ± 22	63 ± 1.7

Each value is the average of three replicates, each replicate subjected to two determinations.

yield as well as the protein content. The retardation of growth with sodium nitrate or nitrite as N sources may be principally related to their sodium concentration rather than nitrate or nitrite. Use of organic nitrogen by blue-green algae has been reported, but the responses to nitrogen sources are entirely different for different species. According to Wyatt et al. (1971), aerobically grown, nitrogen-fixing blue-green algae are not known to synthesize nitrogenase (the nitrogen fixing enzyme) in a cultural medium containing ample supplies of readily available combined nitrogen.

If ample atmospheric nitrogen is not supplied to the cell, mucilage is formed; consequently the protein content per dry weight appears to be lower than that of cells cultured in the presence of combined nitrogen in the medium. In these experiments no significant differences in protein content between the control culture and the culture containing organic nitrogen sources were observed.

The total concentration of NO_3 or NO_2 used in the present investigation was about 74 mg/l or 67 mg/l respectively, which is well below the level of 1.4 g per liter of medium required to retard vegetative growth (Kratz et al., 1955). Most blue green marine algae require a concentration of 1-5 mg per liter of medium of sodium ion for effective growth (Allen, 1956), but some blue-green

algae require a higher concentration (about 4-40 mg/l) (Kratz and Myers, 1955). In the present work the $NaNO_3$ medium contained 26 mg/l of sodium ion and the $NaNO_2$ medium contained 33.3 mg/l. These concentrations were high enough to retard vegetative growth. It suggested that A. flos-aquae may be relatively sensitive to sodium in comparison with other blue-green marine algae.

As indicated by Kratz and Myers (1955), the concentration of carbon dioxide in air is a limiting factor in the growth of blue-green algae. He found that the concentration of carbon dioxide of 0.03% normally found in air was not enough for optimum growth. Inadequate aeration or diffusion of air into the culture media is occasionally a limiting factor for algal growth because of an insufficient supply of carbon dioxide. It has also been demonstrated that glucose can increase the marginal growth in insufficient light, but it has been questioned whether glucose can overcome the rate limitation resulting from insufficient carbon dioxide. Supplying glucose up to 1% concentration to the basal medium increased the vegetative growth approximately four times (Fig. 8). However, addition of urea (above 0.03%) decreased vegetative growth markedly to about 1/10 that of the control medium (Fig. 7). The mixture of glucose and urea at equal concentrations resulted also in retardation of cell production. It appears that the presence of glucose alleviated somewhat

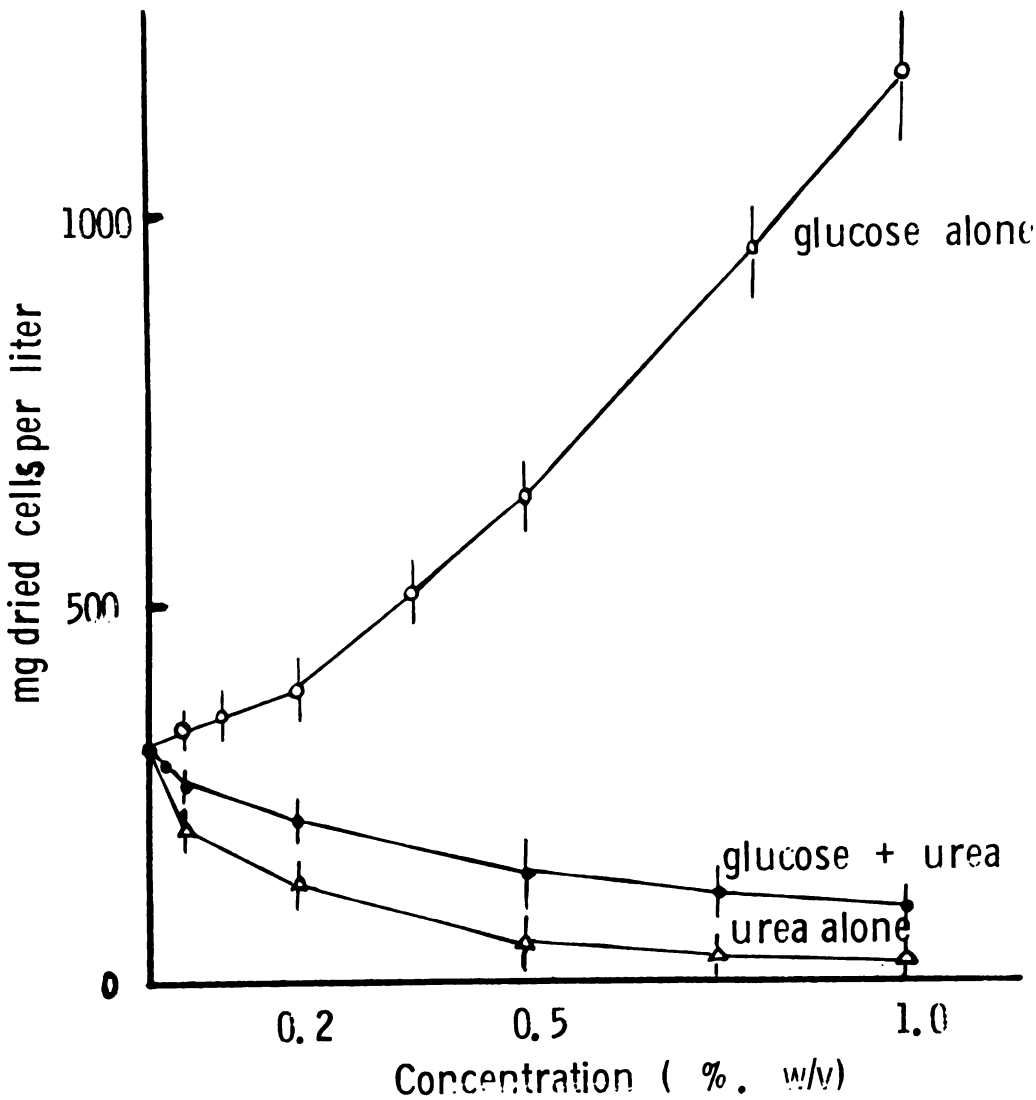

Fig. 7.--Effect of glucose, urea, and a mixture of glucose and urea (1:1) on the growth of A. flos-aquae. Means and standard errors are shown. The cells were cultured at 30 C under 15,064 lux fluorescent light for 11 days.

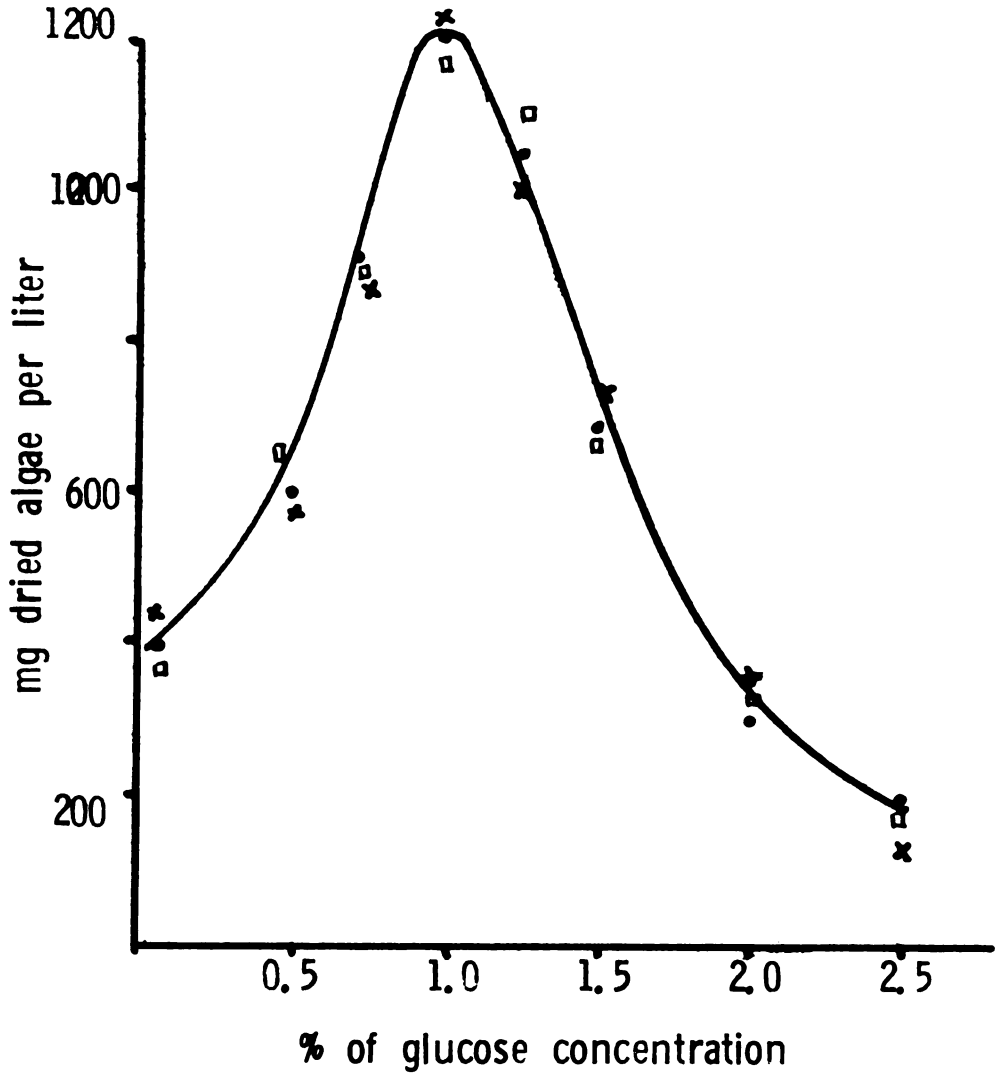

Fig. 8.--Effect of glucose concentration on the growth of A. flos-aquae. Triplicate values are shown.

the depressing effect of urea. Enrichment of the carbon source is required for maximum yield of the cell. The optimum concentration of glucose was about 1%, further increase in glucose concentration decreased cell production (Fig. 8). Similar enhancement of growth with addition of glucose to the medium has been obtained by other investigators (Kiyohara et al., 1960). In contrast to earlier concepts according to which autotrophic algae can utilize only CO_2 for biosynthesis of cellular material, it is becoming clear that other sources of carbon such as glucose may also be utilized (Khoja and Whitton, 1971). The Cyanophyceae have previously been generally described as obligate autotrophs (Kratz and Myers, 1955). However, recent work has demonstrated by using labeled carbon substrate that some of the Cyanophyceae possess heterotrophic activity (Henderson and Wyatt, 1971). A. flos-aquae metabolized an exogenous organic carbon source showing heterotrophic property. However, the mechanism of dissimilation of glucose in blue-green algae is still unknown. Among the many hypothesis, the pentose phosphate shunt has been suggested as the major route of glucose dissimilation. But indubitably CO_2 contributes significantly to the metabolism of blue-green algae and it still forms the major source of cell carbon (Pearce and Carr, 1969).

(B) Population Growth of A. flos-aquae and Theoretical Yields

The theory of "optimum catch" developed by Ketchum and Redfield (1938) states that every population has an optimum density for the production of new individuals. If the population is too dense, competition or other adverse factors limit the multiplication rate so that fewer surviving individuals are produced. The maximum harvest can be achieved at a level at which the product of the multiplication rate times the size of the population is at a maximum. If the harvesting rate is kept within the limits of the capacity of the population to reproduce, the population will approach a concentration at which its multiplication rate just balances the rate of harvest. If the harvesting rate is excessive, the population will approach extinction.

In the present work it was found that the growth of a population of A. flos-aquae was characterized by an initial lag period, followed by a period of logarithmic increase in which the rate of increase of the cell concentration was proportional to its size, and a period of declining multiplication rate when growth was limited by competition or other factors (Fig. 9). Using an initial cell concentration of 30 mg/l in a 16 liter capacity culture, a maximum cell concentration was obtained in 12 days of incubation. The concentration was in the range 630-660 mg dry algae per liter.

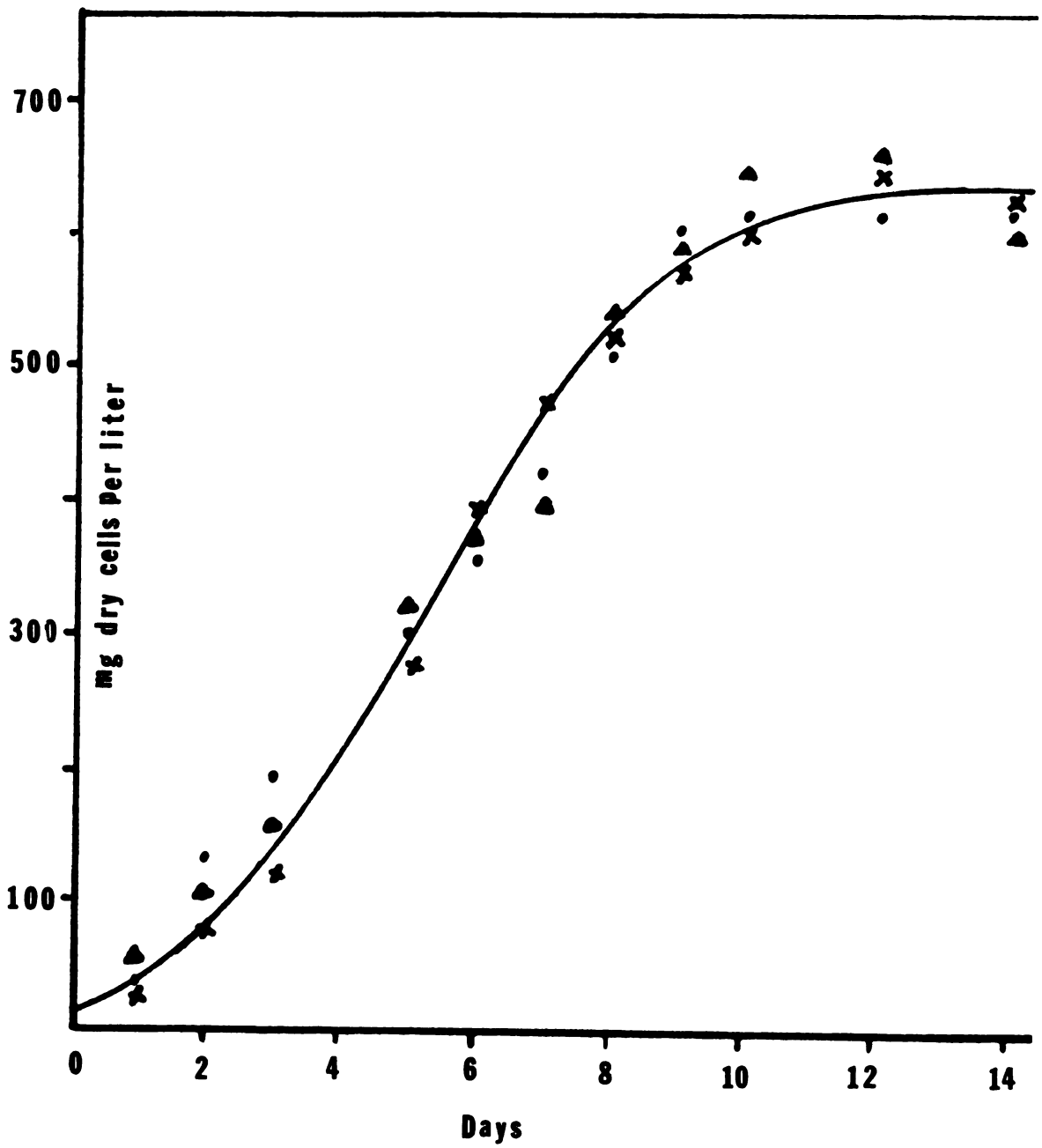

Fig. 9.--Growth of the three cultures of A. flos-aquae in 16 liters of medium at 32 C under 15,064 lux fluorescent light, and bubbling of 2.5 l of air per minutes.

It was also shown in the present work (Fig. 9) that the division rate was a function of time, t, and the division rate K could be obtained from the following equation modulated from the growth curves: The fitness of the equation to the growth curve was determined by computer simulation.

$$\ln \frac{C_2}{C_1} = K\ (t_2 - t_1)$$

in which C_2 and C_1 are respectively the cell concentrations at the end and the beginning of the time period under consideration, $(t_2 - t_1)$. The division rate of these cultures increased at first from a low value, characterizing the initial lag period, to a briefly maintained maximum value, and then declined progressively throughout the remainder of the period as shown in Fig. 11, in which the calculated division rate is plotted against the cell concentration. The maximum division rate was about 0.69 per day and was obtained at initial cell population.

The total daily production of cells, or yield of the culture, was compared to size of the population in Fig. 10. Under the conditions prevailing in our culture method, the yield first increased as the cell concentration increased. Between about 250 and 350 mg dry algae per liter, a uniform, theoretical maximum yield of 90 mg dry cells per liter per day was obtained. The total production

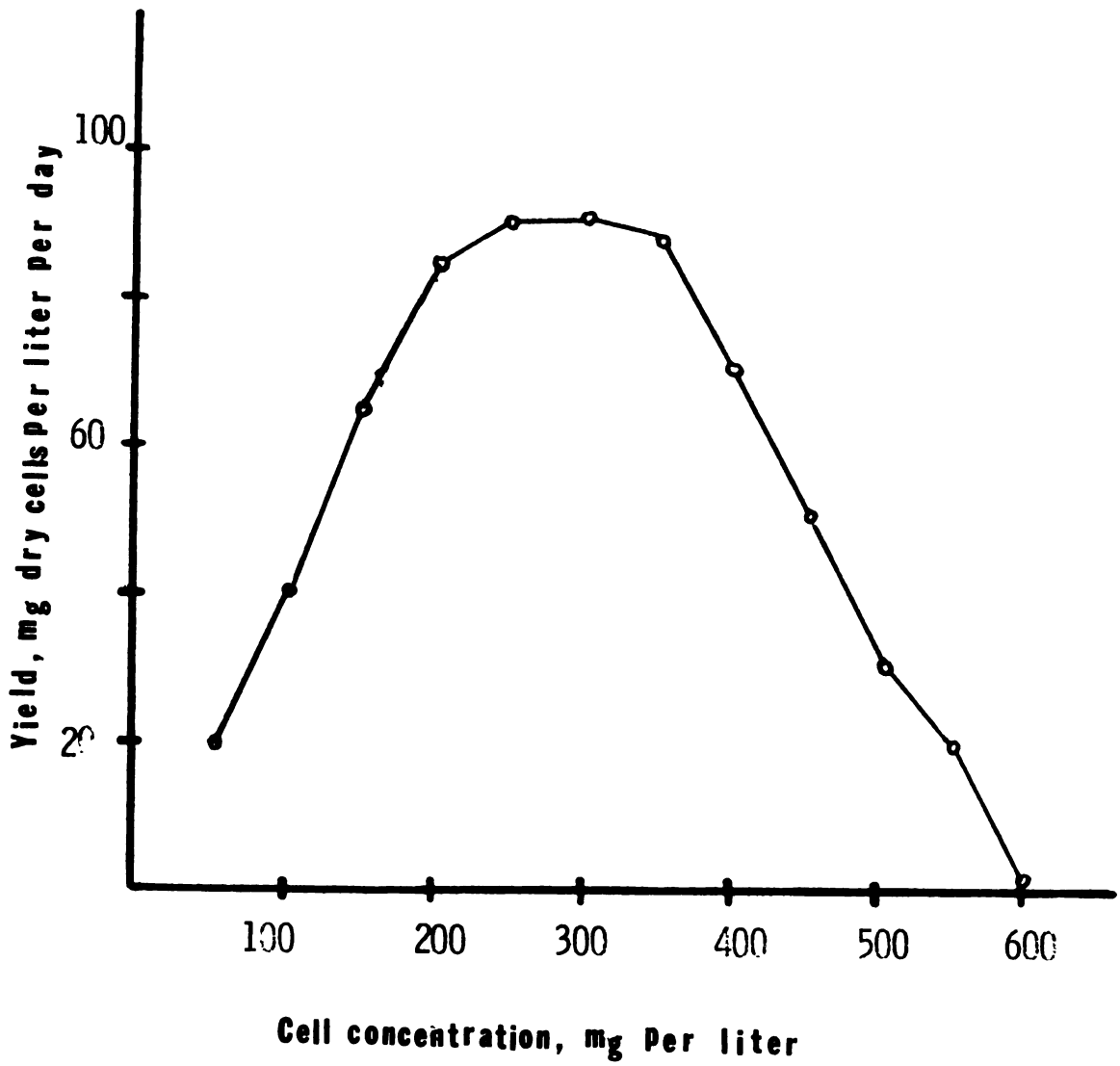

Fig. 10.--The yield in mg of dry matter per liter per day of A. flos-aquae cultures at different population densities. The values are calculated from the growth curve in Fig. 9.

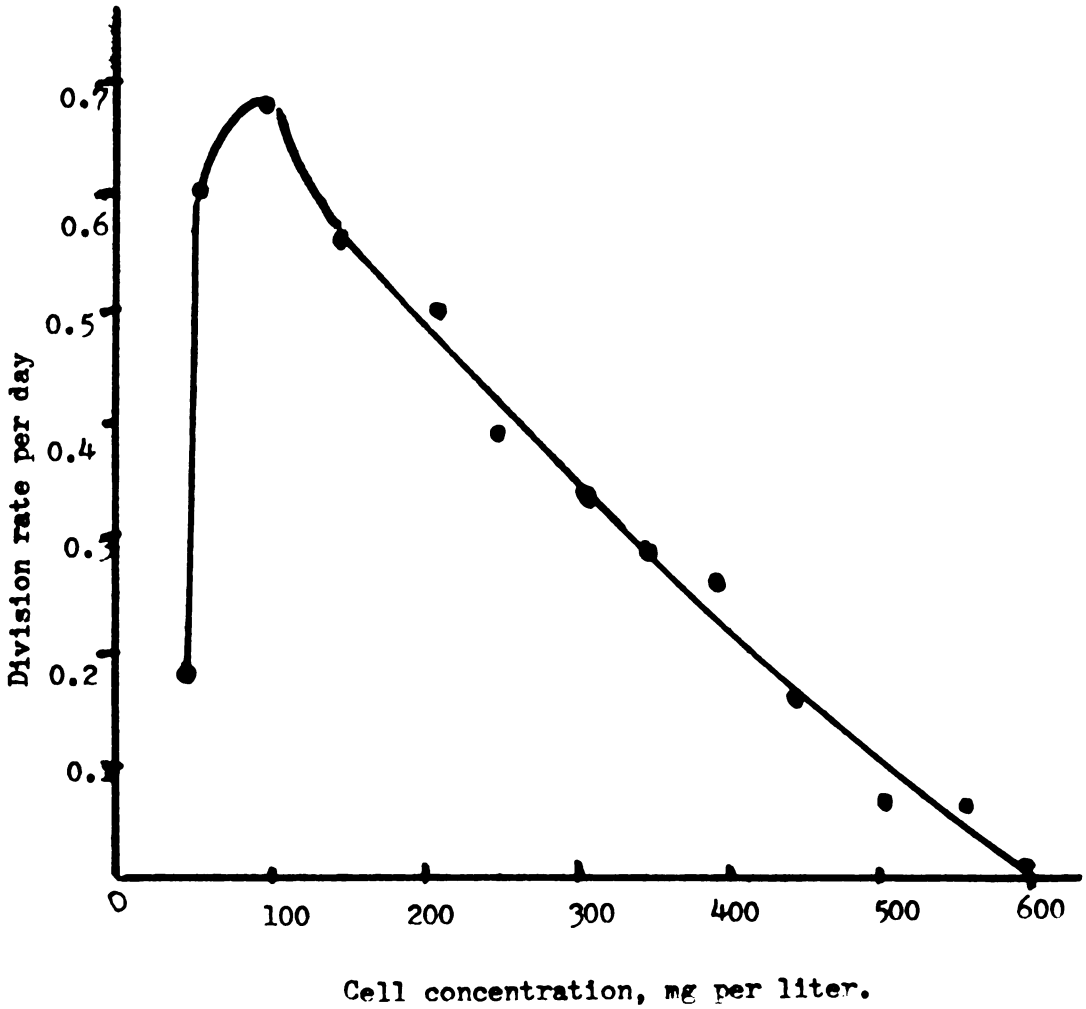

Fig. 11.--The division rate per day of A. flos-aquae culture at different population densities.

per day then decreased gradually, in spite of increasing cell concentration, as the multiplication rate decreased. Both the division rate and the yield were zero at a cell concentration of about 580 mg per liter.

The results indicate that the interval between harvests and the appropriate densities of populations can be determined graphically from the growth curve. The optimum yield can also be deduced. It remains to be determined whether such a yield can repeatedly be obtained from the same culture by removing a portion of the culture at intervals and replacing the harvest with an equivalent volume of culture solution.

However, the optimum harvesting conditions under various systems of harvest can be predicted from doubling the time and initial cell concentration obtained from Fig. 10. Much reliance may be placed on the growth curve in predicting the yield to be obtained and the different harvesting intervals. Practical considerations will determine the regime to be adopted. Thus, if a moderate concentration of cells is required, it may be wise to keep a culture at a high concentration and harvest a small fraction at short intervals, since it will be easier to separate a given quantity of cells from the solution by centrifugation if they are concentrated.

Growth rates on the order of a 1-2 day doubling time have frequently been observed, but an approximate

6-hour doubling time has been observed for A. cylindrica and Nostoc sp. and cell densities achieved range from about 0.2 to 5 g (dry-matter weight) per liter of medium (Kurz and Larne, 1971; Hoare et al., 1969; Hooganhout and Amesz, 1965; Allen and Arnon, 1955). The doubling times are changed depending upon the culture conditions such as nutrients, light intensity, temperature, aeration, and pH of the medium solution. Especially in autotrophic cultures of blue-green algae, availability of light limits growth in all but dilute solutions. To circumvent this problem, algae have generally been grown in flat or specially designed vessels for maximum illumination (Crespi et al., 1970). Either fluorescent or incandescent illumination may be used effectively to support high growth rates (Kratz and Myers, 1955). The relatively low yield of 650 mg dry matter per liter of medium in the present work was probably a result of insufficient light and carbon dioxide. The thickness of the layer of the medium and insufficient agitation might have reduced the penetration of light into the medium. Furthermore, as the cells grow, the penetration power of the light becomes weaker. More efficient agitation of the medium and increased illumination might have also improved the cell yield. Another reason might be related to the poor diffusion of carbon dioxide into the medium. Air bubbling through a glass tube (diameter 0.5 cm, 2.5 liter per min.) from the bottom of

the culture presumably did not diffuse enough air into a large volume of the medium. The amount of carbon dioxide in the atmosphere (about 0.03% carbon dioxide) may not have been sufficient for maximum growth.

(C) Composition of A. flos-aquae

A. flos-aquae cells contained 63.4% crude protein on a dry basis (Table 3) and about 87% of their total nitrogen was amino nitrogen (Table 4). The protein of algae falls as the culture ages, but this decline is very slight and the protein content associated to molybdenum content in medium (Colleyer and Fogg, 1955). It is known that the protein content is inversely related to mucilage content, and to the level of nitrogenous compounds in the medium (Wolk, 1973). The average protein content of blue-green algae such as Spirulina and Anabaena cylindrica has been found to be 50-65% of the dry weight basis (Clement et al., 1967, 1971; Cobb and Meyers, 1964) and that of Chlorella, a green algae, has been found to be 55-60% (Leveille et al., 1962a). Amino acid nitrogen of total nitrogen has been reported to be 88% in blue-green algae (Serenkov, 1957) whereas 82% in green algae (Leveille et al., 1962a).

(D) Decolorization of Algal Pigments

The importance of bleaching the bright greenish color of whole cells or isolated proteins has already been

Table 3.--General composition of freeze-dried algae (A. flos-aquae). Results expressed % dry weight.

Protein N x 6.25 %	Lipid %	Carbohydrate %	Ash %	Nucleic Acid %
63.4 ± 1.7	12.2 ± 0.1	14 ± 0.3	4.2 ± 0.009	4.0 ± 0.012

Means and standard errors are shown.

Table 4.--Nitrogen analysis of freeze-dried algae (% of dry matter).

Total-N %	Amide-N+NH_3 %	Humin-N %	Amino-N by diff. %	Amino-N % of total-N
10.1 ± 0.2	0.95 ± 0.003	0.32 ± 0.0007	8.83	87.4

Means and standard errors are shown.

mentioned in the Introduction. There are green, yellow, blue and red pigments in blue-green algae. Chlorophyll is the green pigment, and several carotenoids (carotene, echinenone, myxoxanthophyll, and zeaxanthin) are yellow. The major blue pigment is phycocyanin which is located near to chlorophyll and activates the fluorescence of chlorophyll, and the major red pigment is phycoerythrin on the photosynthetic lamellae (Wolk, 1973). It has been shown that elimination or destruction of these pigments is quite difficult in whole cells or protein isolates (Mitsuda et al., 1969).

Generally, three different approaches for decolorization have been reported. One is a biological induction of decolorization as the composition of the medium is changed, the second is a chemical extraction of the pigments with an organic solvent, and the third is a physical treatment such as the control of temperature and light intensities. Concerning biological decolorization, Shihira and Krauss (1963) reported that the algal cells altered their color under different culture conditions; supplying a high concentration of glucose to the basal medium (along with inorganic nutrients and thiamine) caused bleaching of the cells, but addition of casein hydrolysates induced greening of the cells in the light. The researchers suggest that the quantitative balance between carbon and nitrogen sources may control the amount of pigment in the

algal cell. In 1964 Shihira and Krauss found that the bleaching effect of glucose was primarily caused by its degradation effect on the chloroplast structure. In chemical extraction, the high bleaching was achieved with a mixture of methanol and hexane (4/3 ratio). Concerning physical bleaching, Brawerman and Charoff (1959, 1960) reported on the possibility of bleaching and greening Euglena by controlling temperature and light.

In our experiments, the absorption spectra of a cell suspension in the visible region (420-720 nm) were measured before and after exposure to light. Three main peaks at around 430 nm, 630 nm, 680 nm, were observed in the unbleached cell suspension, but the intensity of the peaks decreased with bleaching (Fig. 12). These findings suggest the possibility of bleaching the color of the algae by photolysis. The peaks were identified by comparison with previously published data. The absorption spectrum of a cell suspension of Spirulina maxia, one of the blue-green algae, has 5 peaks. These peaks were identified by Clement et al. (1967a) as follows: chlorophyll and a carotenoid at 440 nm, a carotenoid at 470-480 nm. Phycoerythrin at 560-570 nm, phycocyanin at 590-600 nm, and chlorophyll at 680 nm. Other investigators (Hattori and Myers, 1966; Haxo et al., 1955) have reported that, in vitro, the visible absorption peak of phycocyanin is at about 615-20 nm, of phycoerythrin at about 557-565 nm,

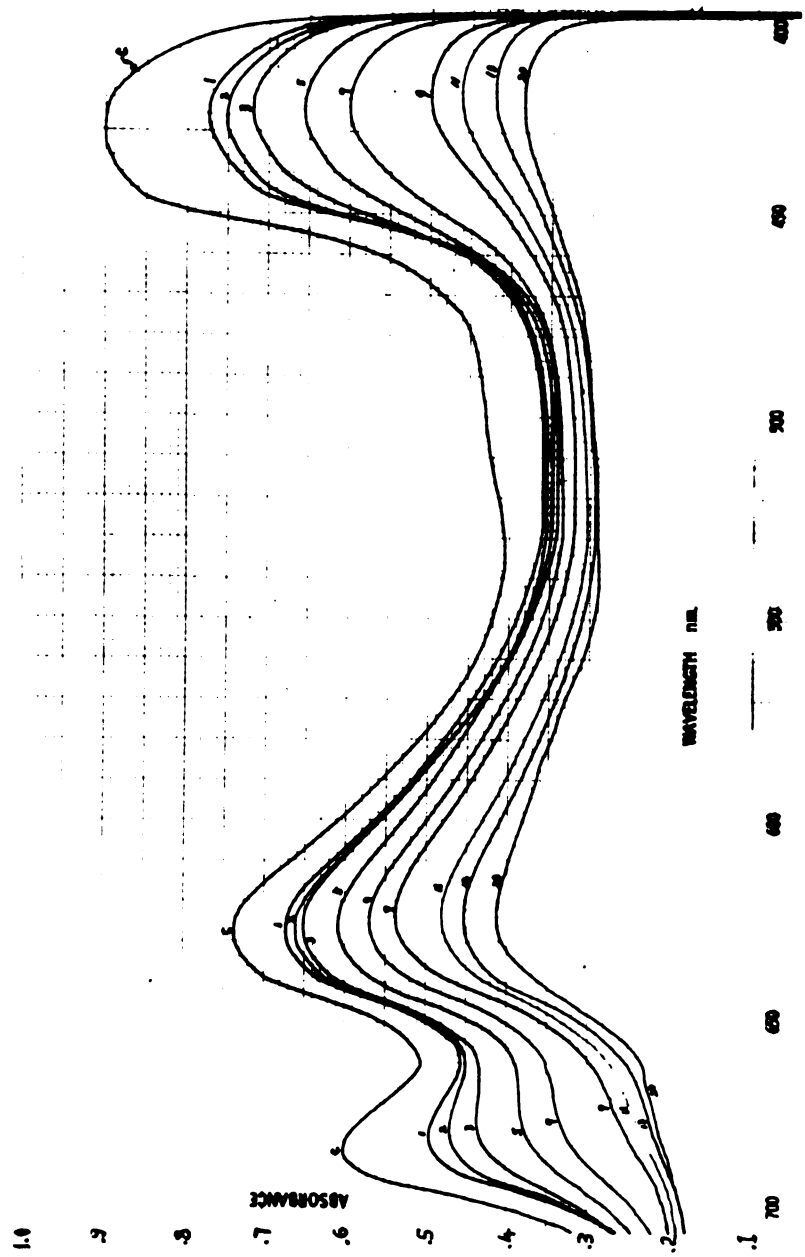

Fig. 12.--Changes in the absorption spectra of A. flos-aquae cell suspension during photolysis.
"C" represents the spectrum of the unphotolyzed cell suspension.
The numbers on the other curves indicate hours of exposure to light.
The cell concentration was 50 mg per 100 ml of water (pH 6.8). The light intensity and temperature during photolysis were 15,064 lux and 10 C.

and of allophycocyanin, a light blue biliprotein, at about 650 nm. In vivo, the absorption peaks are shifted to a wavelength about 13 nm higher than the absorption peak in vitro (Silberger and Haxo, 1950; Emerson and Lewis, 1942). On the basis of these reports the following deductions may be made; the absorption at 420-450 nm, region might be caused by chlorophyll and carotenoid. The peak at 620-640 nm might be caused by phycobiliprotein including phycocyanin, phycoerythrin, and the peak at 670-690 nm might be a result of chlorophyll.

All the pigments at the visible region completely disappeared after photolysis at room temperature for 30 hrs. under 15,064 lux fluorescence light. The bleached cells appeared light yellow, showing no peaks in the absorption spectrum.

Many factors may give rise to the broad flat absorbance peaks in Fig. 12 or 13. One of them might be related to the polarity or the polarizability of the solvent in which pigments are placed. Usually as the polarity of the solvent increases, absorbance bands become broader and flatter, or this leads to a shift in absorption maxima toward longer wavelength, and to a change in the shape of the absorption peaks (Rabinowitch, 1951). The other might be related to properties of the opalescent paper used in the measurement of spectrum. The paper should be uniform, moderate and constant opalescency over

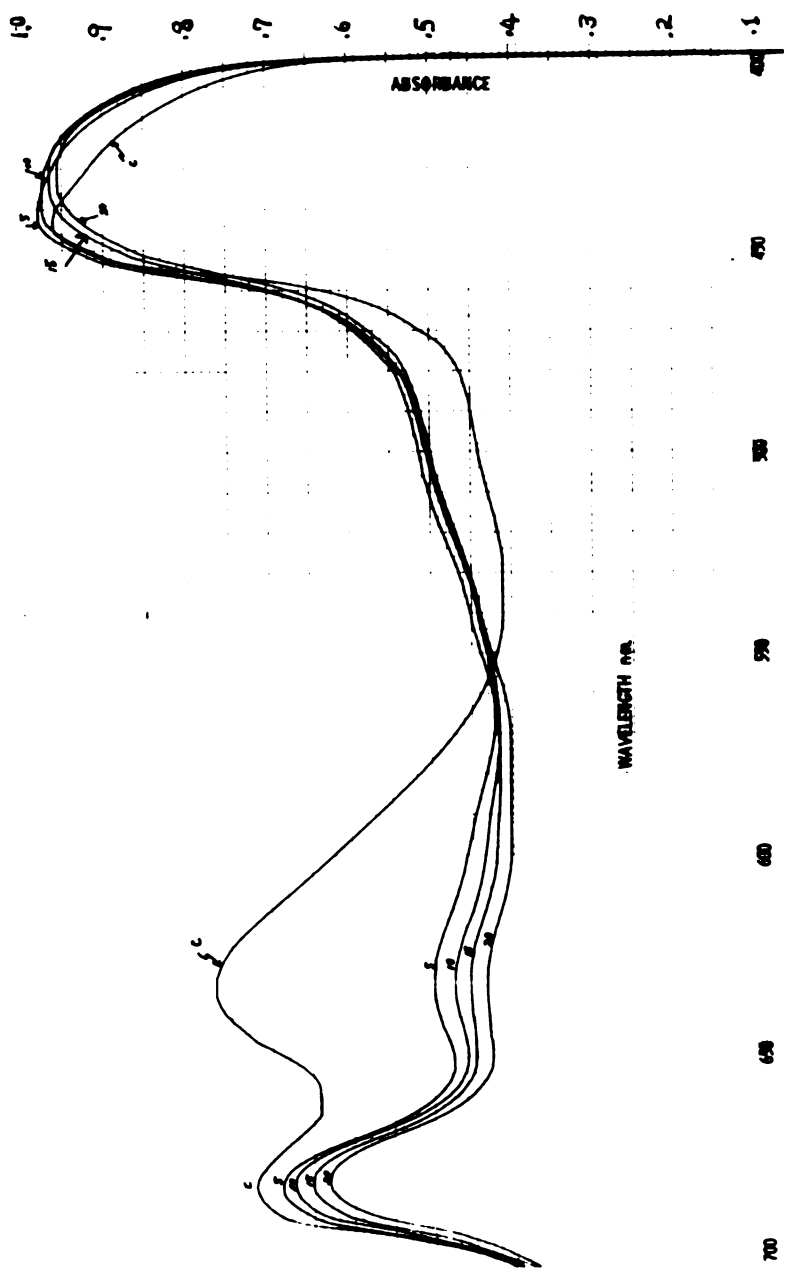

Fig. 13.-- Changes in the absorption spectra of an A. flos-aquae cell suspension during heating at 90 C. The letter "C" on the curves means control and the number on the other curves indicate minutes of exposure to heat.

a wide range of wavelengths. Too much opalescence made the wavelength range of observation narrower because of the low light intensity beyond the opalescent paper. It also broadened the absorption band excessively owing the wide slit requirement of the monochromator.

Fig. 12 shows that the pigments absorbing at around 430 nm and 680 nm were more sensitive to light exposure than were those absorbing at around 630 nm. In 9 hrs. of light exposure, the peak at around 680 nm completely disappeared and the peak at around 430 nm appreciably decreased. However, the cell suspension still had a blue-green color. By increasing the exposure time to 20 hours, the color disappeared and the suspension was very light green. Those observations suggest that phycobiliproteins may be appreciably more stable to light than are chlorophyll or carotenoids. Therefore, the color from chlorophyll or carotenoid was easily bleached by light exposure whereas light exposure alone could not completely remove the color from biliprotein.

The spectrum of the cell suspension after heating at 90 C for various periods of time was measured without photolysis (Fig. 13). In contrast to the changes of spectra by light, the pigments absorbing at around 630 nm were very sensitive to heat. This peak almost disappeared in 5 minutes heating whereas the peaks at 680 nm were only slightly reduced. The peak at 430 nm shifted to a shorter

wavelength (420 nm) without change in intensity. The rapid disappearance of the peak at 630 nm suggests instability of phycobiliprotein to heat. Fig. 12 and 13 indicate that the pigments stable to photolysis are unstable to heat, whereas the pigments stable to heat are unstable to photolysis. Thus the combination of heat and treatment with fluorescent light effectively bleached the cell.

Irradiation with ultraviolet light (253.7 nm) significantly decreased the absorption at 630 nm (phycobiliprotein) but slightly decreased the peaks at 430 nm and 680 nm (Fig. 14). Longer wavelength ultraviolet light (366.0 nm) did not affect the degradation of the pigments. The degradation of the chlorophyll of algae by ultraviolet light has also been reported by Lyman et al. (1961).

In order to determine the effect of pH of the cell suspension on bleaching of the cell, the pH of cell suspensions was varied from 1.0 to 12.0 and their absorption spectra were measured before or after bleaching (Fig. 15, 16, 17, 18). The spectra of the suspension at pH 3-9 showed similar, if not identical patterns. Differences were observed in the spectra at pH 1, 11 and 12. Exposure of the cell suspensions to 15,064 lux fluorescent light for 8 hours resulted in decrease of absorption at all pH levels, but the decolorization was more complete at higher pH levels 11, 12 (Fig. 18). The bleached cells were

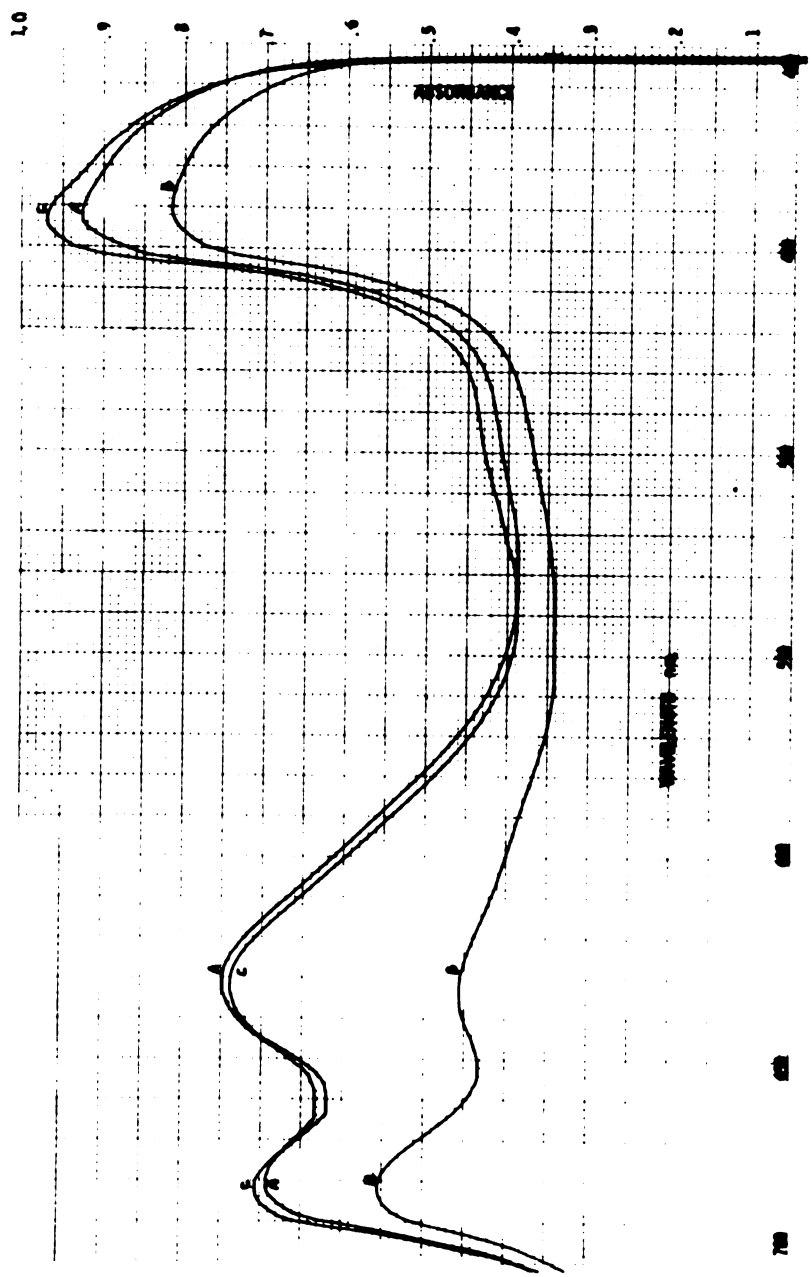

Fig. 14.--Changes in the absorption spectra of an A. flos-aquae cell suspension after exposure to ultraviolet light. "C" represents the control. "A" represents the spectrum of a cell suspension exposed to 366.0 nm u.v. light for 1 hour. "B" represents the spectrum of a cell suspension exposed to 253.7 nm wavelength u.v. for light for 1 hour.

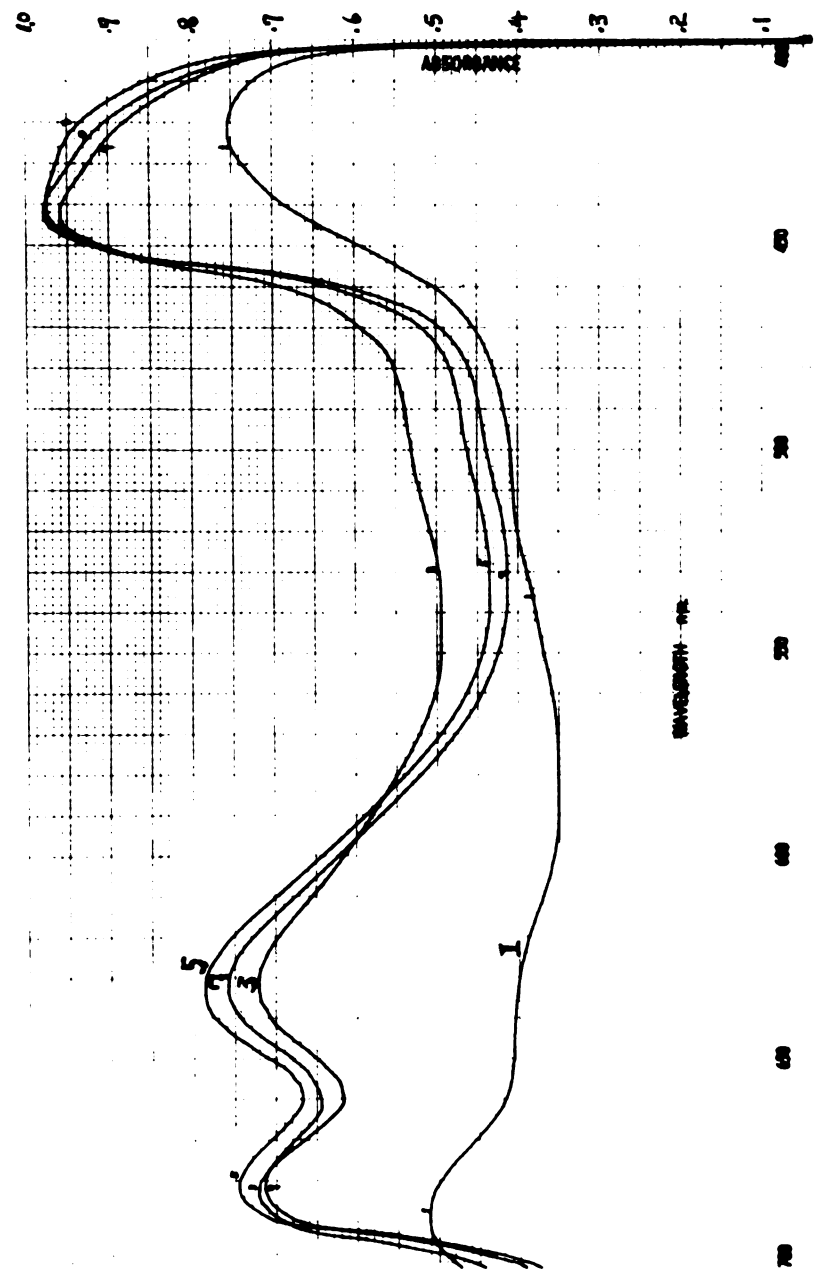

Fig. 15.--Absorption spectra of A. flos-aquae cells suspended in low pH solutions.
The number on the curves indicates pH of the solution.
Cell concentration was 50 mg per 100 ml.

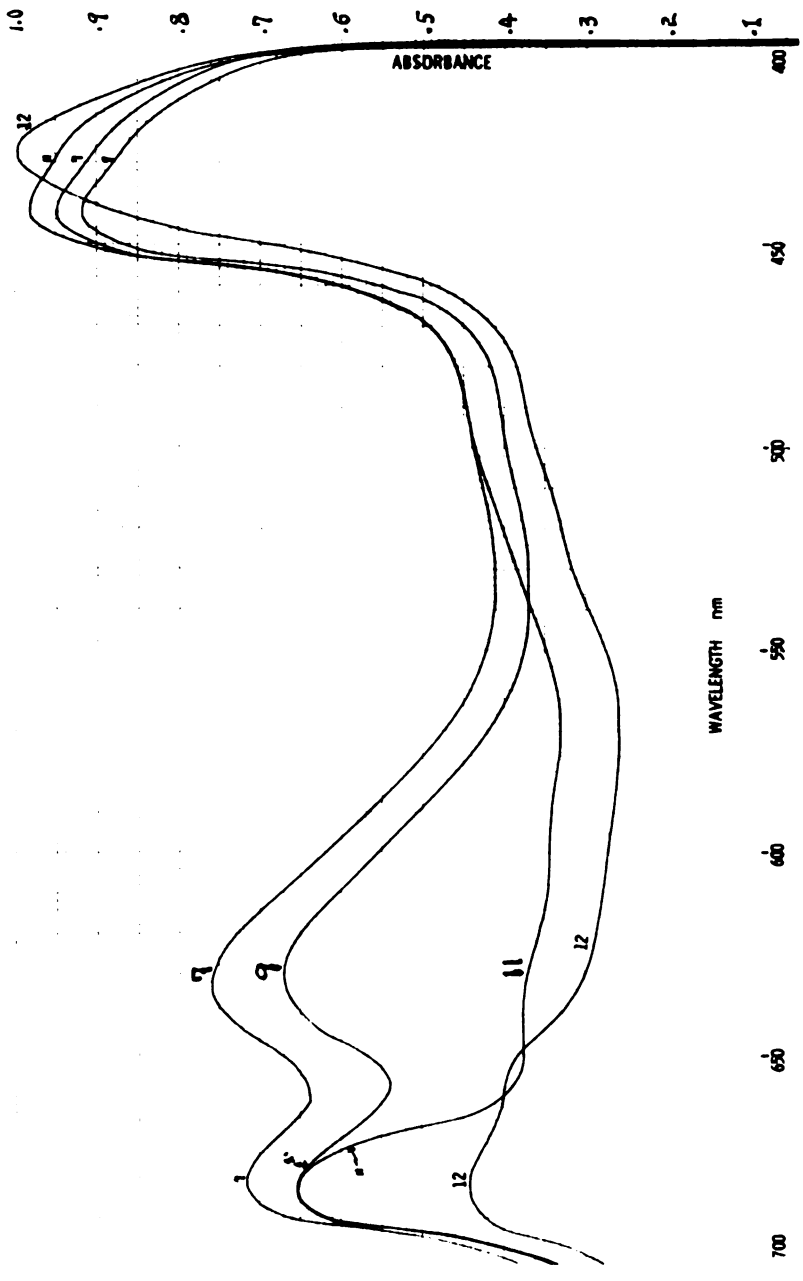

Fig. 16.--Absorption spectra of A. flos-aquae cells suspended in high pH solution.
The number on the curve indicates the pH of the cell suspension. The cell concentration was 50 mg per 100 ml.

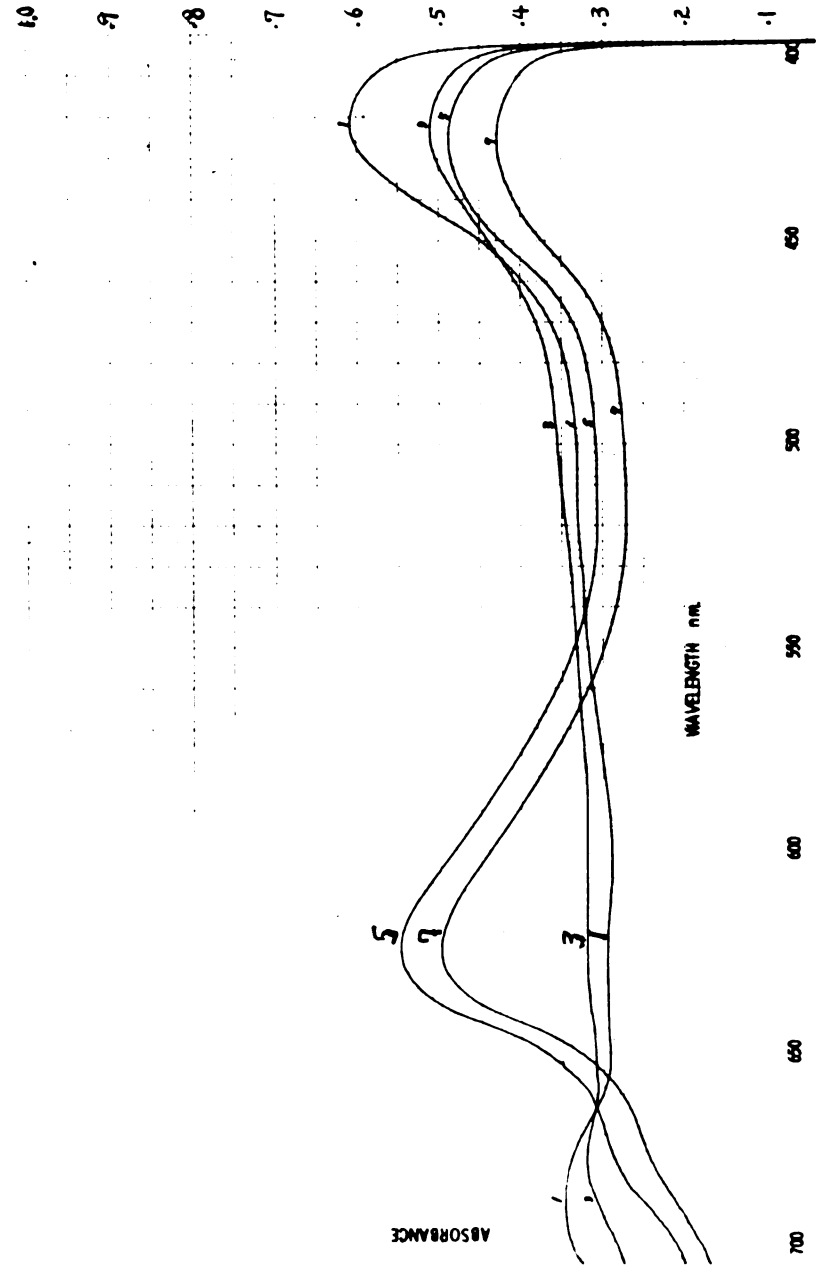

Fig. 17.--Absorption spectra of the cell suspension after bleaching at various pH.
The number on the curves indicates the pH of the cell suspension. The cell suspensions were photolyzed at 31 C for 8 hours under 15,064 lux light.

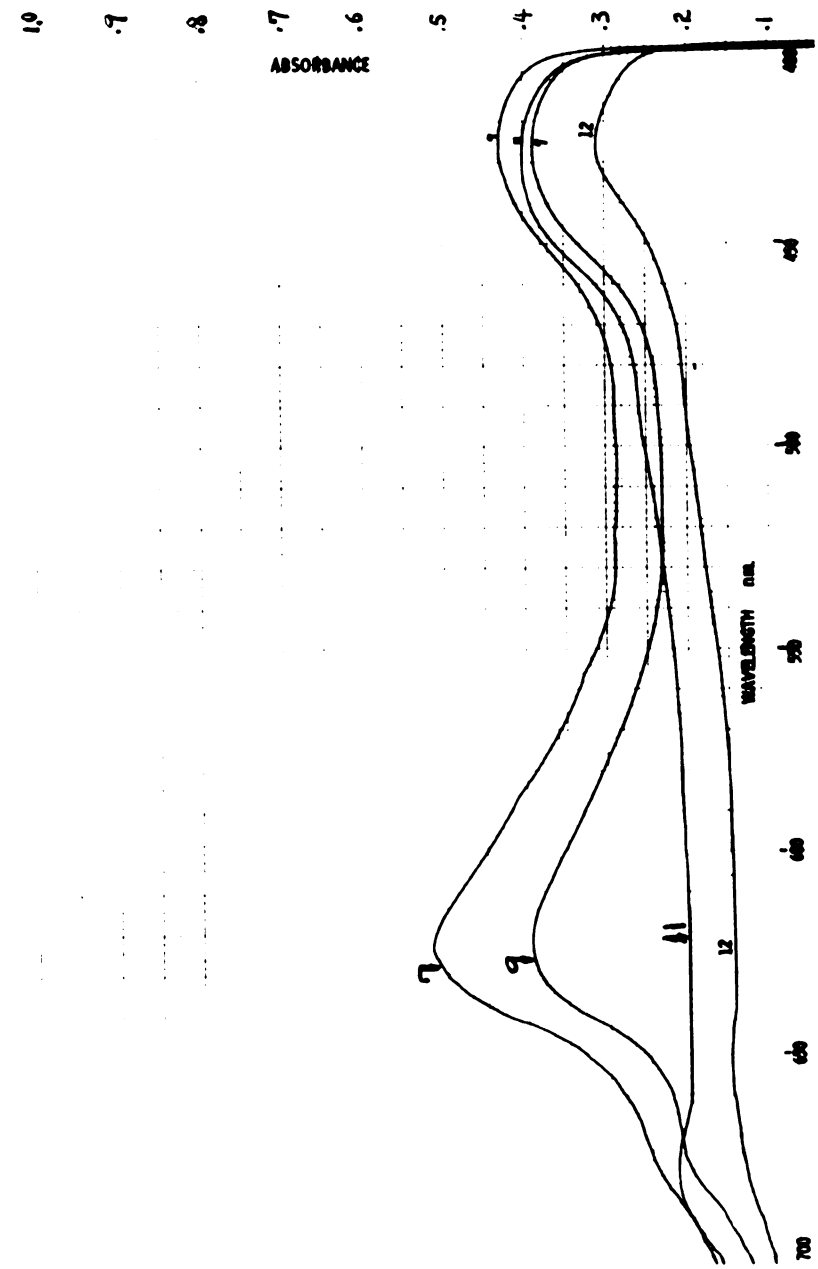

Fig. 18.--Changes of absorption spectra of the cell suspension after bleaching at various pH. The number on the curves indicates the pH of the cell suspension. The cell suspension was exposed to 15,064 lux fluorescent light for 8 hours, at 31 C.

yellowish white and possessed no grassy odor and no flavor whereas unbleached cell had a dark green color and strong grassy flavor (Fig. 19). Bleaching of the algal cells by photolysis was more simple and cheaper than solvent extraction or biological bleaching. However, denaturation of protein by high pH and photolysis was inevitable.

(E) Extraction of Protein

(E-1) Determination of Optimum Conditions for Protein Extraction

As the pH of the extraction solution affects the solubility of protein, the protein solubility or extractability in the aqueous solution was determined over a range of pH values. Fig. 20 summarizes the results obtained with 3 different methods under varying pH. The extraction medium remained in contact with the cells either (a) for 22 hours at 32 C in the dark or (b) for 20 minutes at 95 C in ordinary light or (c) for 22 hours at 32 C under 15,064 lux light. Under all three conditions the lowest extraction values were observed at around pH 4.0 approximating the isoelectric point of the algal protein and the highest at around pH 12.0. The higher extraction values at pH 11-13 in the dark compared with the values obtained under 15,064 lux suggests that light induces decomposition of the protein (Fig. 20). The yield of TCA-precipitable protein of the extract obtained by either HCl-pretreatment method or

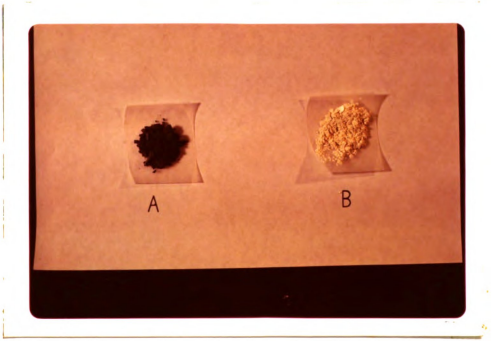

Fig. 19.--Photograph of bleached and unbleached A. flos-aquae cells. The cells were bleached at pH 12 under 15,064 lux fluorescence light for 8 hours (at room temperature). A letter "A" and "B" represent unbleached and bleached cell respectively.

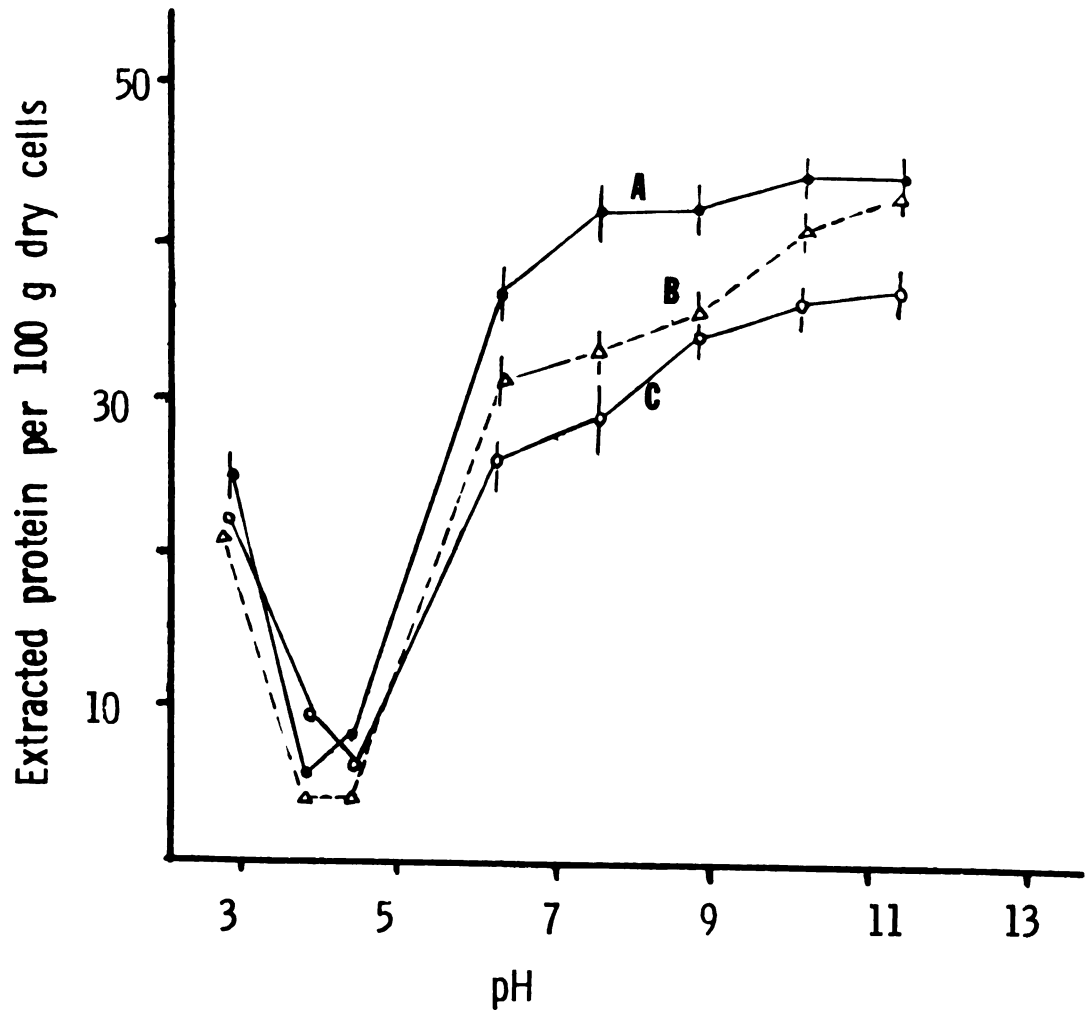

Fig. 20.--Protein extractability profiles for A. flos-aquae suspended in phosphate buffers of various pH under three different extraction conditions.
A: Extraction in dark, 32 C, for 22 hours.
B: Extraction for 20 minutes at 95 C
C: Extraction under 15,064 lux light (32 C) for 22 hours.
The cell concentration was 125 mg/100 ml. Means and standard errors are shown.

single alkali method was also decreased as much as 8% by exposure to light (Table 5).

As shown in Fig. 20 heating at 95 C for 20 minutes extracted as much protein as treatment at 32 C for 22 hours. A possible explanation for these observations is the destruction of hydrophic bonds between cell wall and protein by heat. The high extractability at high pH is consistent with the acidic nature of this protein (Table 12). The extraction of protein at pH 11-13 under light eliminated unpleasant odor and color.

The extraction of proteins from the cells increased with the temperature of the alkali solution (pH 11.0), but it was not significantly influenced by the cell concentration in the ranges from 44 mg/100 ml to 480 mg/100 ml (Fig. 21). High extractions of protein were obtained at around 95 C. Cell suspensions of higher concentration than 480 mg/100 ml were very gluey. Heating increased their stickness due to the swelling.

The maximum extraction of proteins was about 35% of the dry weight (50% of cell-nitrogen) during extraction at 95 C. Heating for more than 30 minutes was economically wasteful (Fig. 22). By pretreatment of the cell with dilute-HCl, the extraction of protein based on the extracted nitrogen of the cell nitrogen was raised to 84% (Table 9). Thus pretreatment is required to recover fully the protein.

Table 5.--Effect of photolysis of the protein extract on recovery of TCA-precipitable protein (TCA-P-N).

	Extracted N as % of cell N	TCA-P-N as % of cell-N	TCA-P-N as % cell N 20 hrs. after the ext.	
			in darkness	in light
Single alkali method	55.3 ± 2.0	43.3 ± 1.7	42.9 ± 1.7	40.2 ± 0.9
HCl-pretreatment method	81.4 ± 4.0	54.5 ± 1.4	53.2 ± 1.3	50.9 ± 0.8

The extraction followed (D-3-2) in Method section. Means and standard errors are shown.

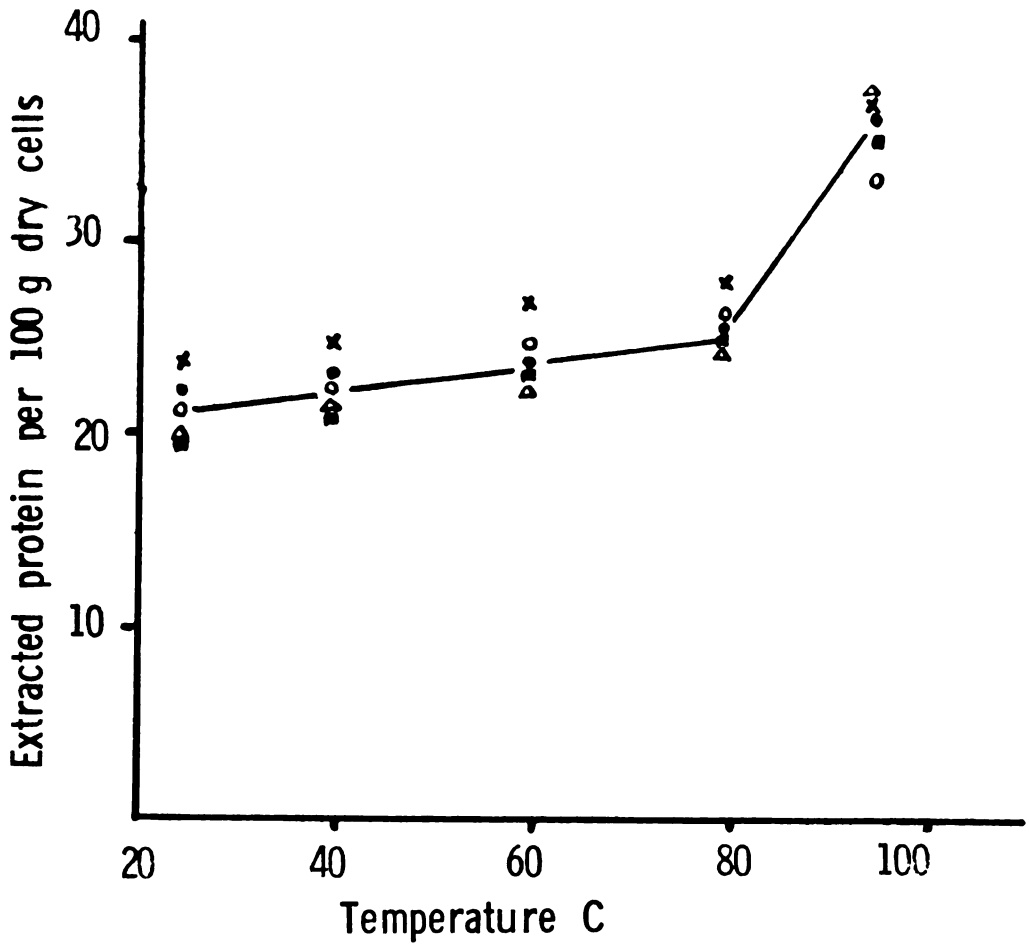

Fig. 21.--Effect of temperature and cell concentrations on the protein extraction from A. flos-aquae suspended in buffer solution (pH 11). The marks indicate cell concentration:

x: 44 mg/100 ml
•: 120 mg/100 ml
o: 240 mg/100 ml
Δ: 360 mg/100 ml
■: 480 mg/100 ml

The standard errors were less than 3%.

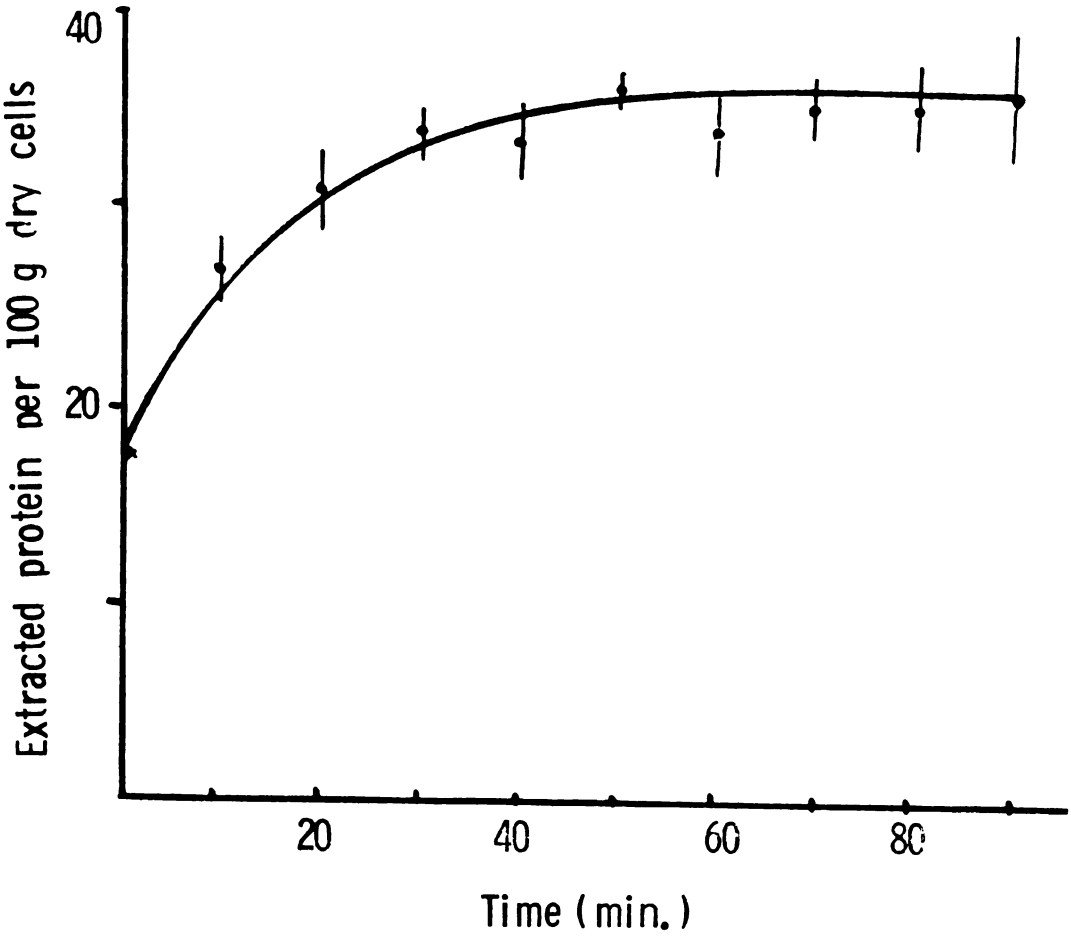

Fig. 22.--Effect of heating time at 95 C on the protein extraction from A. flos-aquae suspended in buffer pH 11.
The cell concentration was 400 mg/100 ml.
Means and standard errors are shown.

There is still a question on the optimum concentration of alkali solution to extract protein from microalga. Baba et al. (1960) recommended a high concentration of sodium hydroxide (10%), but some authors (Mitsuda et al., 1969) were able to obtain high extraction of protein with low concentration (0.2-1%). Because of this, various concentrations of sodium hydroxide were used under three different conditions: (a) extraction under light after preheating at 90 C for 30 minutes, (b) extraction in the darkness after preheating, (c) extraction under light without preheating (Fig. 23). The highest protein yield was obtained by using 0.5% sodium hydroxide at room temperature in all cases. The yield was decreased by high concentration of NaOH, photolysis and preheating. Concentrations above 0.5% NaOH not only decrease the amount of protein extracted but also resulted in black extracts. This observation was in agreement with the reports that recommended a low concentration of sodium hydroxide be used to extract the protein from microalgae (Klyushkina and Fofanov, 1969; Mitsuda et al., 1969).

(E-2) HCl-Pretreatment Extraction of Algal Protein

The purpose of this study was to develop a new extraction method to increase protein yield by facilitating the accessibility of the cell contents to alkali. Basically this entails partly or completely breaking the

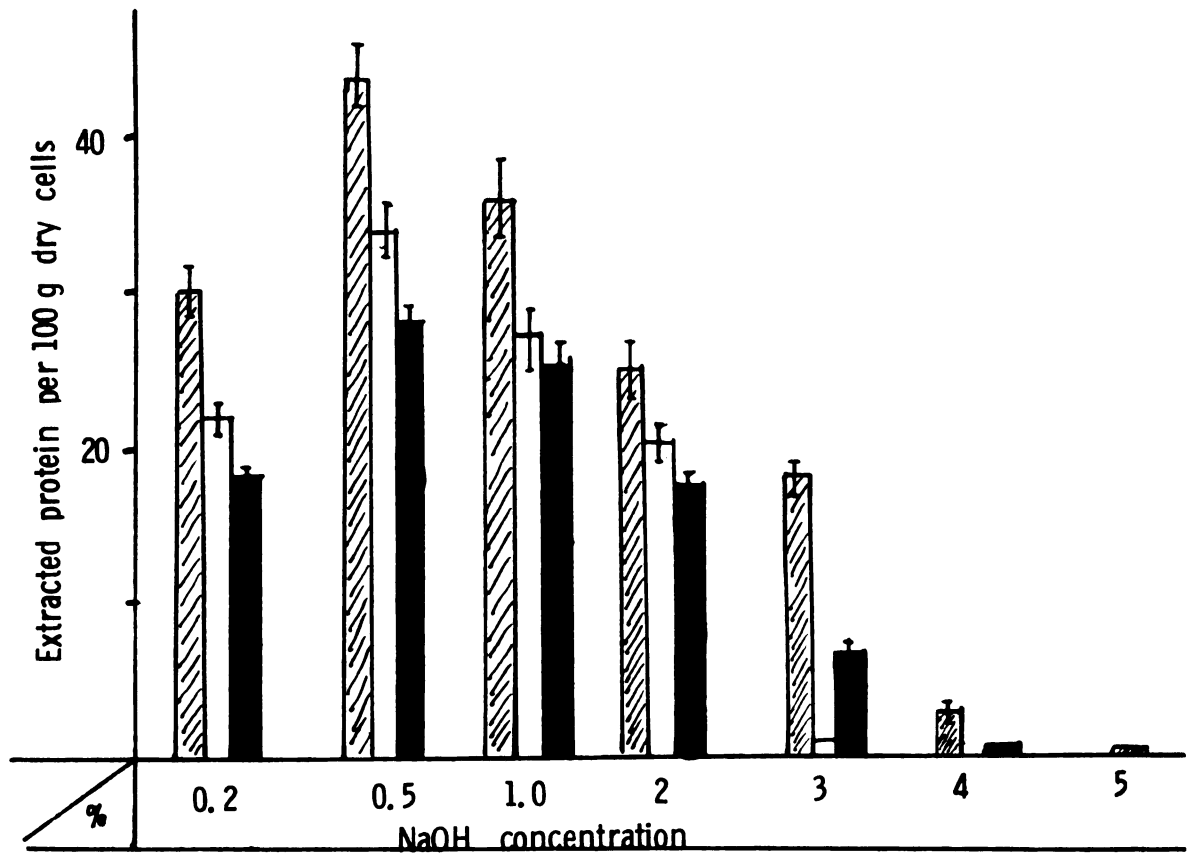

Fig. 23.--Effect of sodium hydroxide concentration on the extraction of protein from A. flos-aquae under various conditions
:extraction in dark, 32 C, for 12 hours after preheating at 95 C for 30 min.
:extraction at 32 C under 15,064 lux light for 12 hours after preheating at 95 C for 30 minutes.
:extraction at 32 C 15,064 lux light for 12 hours without preheating.

cell wall with HCl at high temperature for a short time and then extracting protein by increasing the pH to 11.0 at room temperature. The optimum conditions involving the concentration of HCl, heating temperature, extraction period at room temperature, and recovery of protein from the extract were determined (Table 6, 7, 8, Fig. 24).

The maximum yield was obtained by heating the cells with 3 N HCl at 95 C for 10 minutes and then extracting the cells with alkali solution (pH 11.0) within 1 hour at room temperature (Table 6, 7, Fig. 24). By HCl-pretreatment, about 84% of the total cell protein (Table 6, 7) was extracted compared with 56% obtained by alkali treatment alone which requires longer extraction period (Fig. 20). Therefore the single alkali method is rather unsatisfactory if mechanical disintegration is not involved. Mitsuda et al. (1969) achieved success breaking the cell wall and extracting the protein with a urea solution, but the protein recovery from Chlorella was only about 20% on a dry basis. One of the difficulties in extracting proteins from algae is that it has been impossible to extract more than 40% of the protein (on the basis of total cell nitrogen) from intact algal cells without using special mechanical or chemical methods. High levels of alkali (above 5% NaOH) have been used, but such a concentration induces substantial changes or hydrolysis in the protein. Attempts to destroy the cell wall by trituration with

Table 6.--The effect of HCl concentration for pretreatment of cells on extractability of protein from A. flos-aquae.

HCl-concentration for pretreatment	Protein extraction at room temperature for 2 hours without preheating % (on basis of dried weight)	Protein extraction at room temperature for 2 hours after preheating at 95 C for 5 minutes % (on basis of dried weight)
1.5 N	21.2 ± 0.9	40.4 ± 1.4
3.0 N	32.0 ± 1.1	54.0 ± 1.8
6.0 N	28.5 ± 1.3	53.7 ± 1.0

Protein content was determined by the Lowry method.

Means and standard errors are shown.

Table 7.--Effect of extraction period after HCl-pretreatment on the extraction of protein.

Extraction period	Protein yield as % of dry cell weight (N x 6.25)	N extracted as % of cell N
40 min.	52.7 ± 1.5	83.1 ± 0.9
72 hrs.	54.1 ± 0.5	84.4 ± 1.2
72 hrs. under 15,064 lux light	53.9 ± 0.8	85.0 ± 1.0

Cell protein was extracted at pH 11, and room temperature for various periods after pretreatment of HCl at 95 C for 10 minutes. Means and standard errors are shown.

Table 8.--Protein recovery in the extract by the HCl-pretreatment method after pH adjustment and zinc acetate addition.

pH to precipitate protein	Nitrogen precipitated by pH adjustment % of cell nitrogen	Nitrogen precipitated by zinc acetate from previous supernatant % of cell nitrogen	Sum of N of columns 2 and 3	Total precipitated N as % of extracted N
3.0	15.2 ± 0.5	26.0 ± 0.8	41.2	51.5
4.0	24.4 ± 0.7	15.3 ± 0.7	39.7	50
5.0	8.5 ± 0.1	30.7 ± 0.9	39.2	49
6.0	0	38.5 ± 1.0	38.5	48

The extraction followed (D-2-3) in Method section.

Means and standard errors are shown.

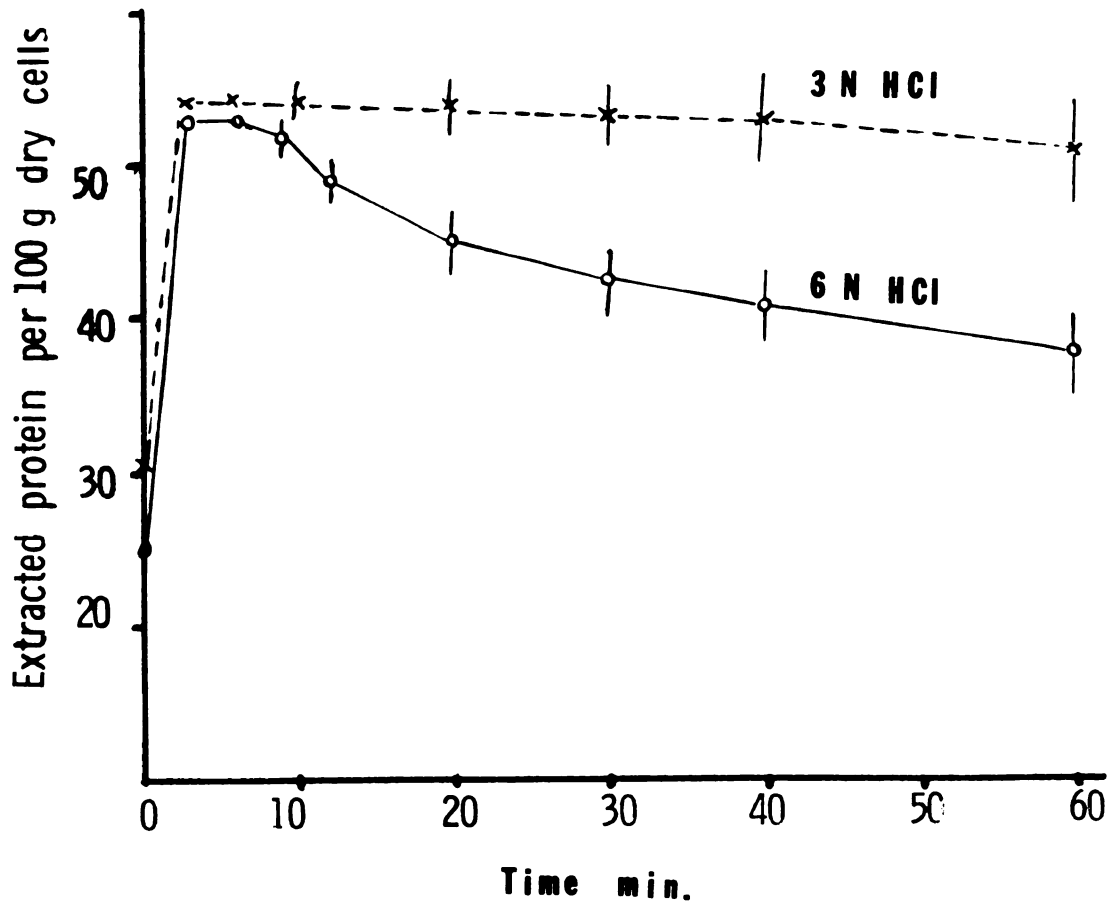

Fig. 24.--Influence of heating time of cells at 95 C on the protein extraction from A. flos-aquae suspended in 6 N and 3 N HCl. The extraction was performed at pH 11 for 2 hours at room temperature. Means and standard errors are shown.

marine sand with a mild concentration of alkali have been used. Grinding in a spherical mill was found by Hendenskog and Enebo (1969) to minimally increase the yield of extraction. Thus the method developed here offers not only better yield of protein but also removes the color from the product.

For protein intended for human consumption, precipitation by adjustment of the pH to the isoelectric point would be preferred and non-precipitable proteins by the pH adjustment were recovered again with zinc acetate or acetone. The maximum protein recovery from the HCl-pretreatment-extract was 24.4% of cell protein at pH 4.0. An additional 15.3% was recovered by zinc acetate precipitation (Table 8). Consequently, the total recovery of protein was 39.7% of cell protein (50% of extracted nitrogen). Using acetone dehydration instead of zinc acetate, about 30% of the extracted nitrogen could be additionally recovered (Table 9).

Of the three extraction methods, HCl-pretreatment, single alkali, and mechanical extraction, the highest extraction of nitrogen (81% of cell nitrogen) was obtained by the HCl-pretreatment method (Table 9). The low extractability by the mechanical method (41% of cell nitrogen) is probably due to incomplete disintegration of the cells. However, the extract obtained after mechanical treatment yield more pH 4.0-precipitated protein (26.2% of cell N)

Table 9.--Protein recovery by various extraction methods.

Extraction method	Treatment	Extracted-N as % of cell-N	Protein-N ppt at pH 4 as % of cell-N	Protein-N ppt with acetone from the supernatant of pH ppt as % of cell-N	Total recovered protein-N as % of cell-N	Recovery of protein-N as % of extracted-N
Single alkali method (Flow diagram in Fig. 4)	Preheated at 95 C for 10 min	70.1 ± 1.2	12.5 ± 0.3 (K-1-1)	43.1 ± 1.5 (K-1-2)	55.6	79.3
	Nonheated	59.7 ± 1.1	11.4 ± 0.4 (K-2-1)	37.1 ± 1.5 (K-2-2)	48.5	81.2
HCl-pretreatment method (Flow diagram in Fig. 3)	Preheated at 95 C for 10 min	80.7 ± 2.1	29.1 ± 1.9 (B-1-1)	35.1 ± 1.0 (B-1-2)	64.29	78.9
	Nonheated	41.5 ± 0.9	22.3 ± 1.0 (B-2-1)	10.0 ± 0.3 (B-2-2)	32.6	78.6
Mechanical method (diagram in Fig. 5)	Nonheated	41 ± 0.7	26.2 ± 0.6 (M-1)	10.3 ± 0.4 (M-2)	36.5	89.0

Means and standard errors are shown.

than the extracts by the single alkali method (11.4-12.5%). In terms of total protein-nitrogen recovered by pH adjustment and acetone precipitation, the method that resulted in the largest recovery of protein (64.2% of cell-N) was the HCl-pretreatment method, which entailed (i) the heating of the cells with 3 N HCl at 95 C for 10 minutes, (ii) extraction at pH 11.0 for 1 hour at room temperature, (iii) illumination of the supernatant solution from centrifugation with 15,064 lux fluorescent light. The product was the colorless and odorless protein preparation. Furthermore, the extract after HCl-pretreatment could be readily decolorized and deodorized by photolysis (Fig. 27), because the absorption spectrum of the extracted solution displayed two sharp peaks at 410 and 665 nm (Fig. 25) which were easily bleached by photolysis as shown in Fig. 12. The peak at around 630 nm (Fig. 13) which showed appreciable resistance against photolysis, disappeared. Thus extract protein solution was more easily and completely bleached than the whole algal cells. The mechanical extraction method appears to yield the less denatured form of protein. But specially designed equipment is required to increase the disintegration of the cells. According to Hedenskog and Enebo (1970), a 70% extraction of cell nitrogen was obtained using a high speed homogenizer with glass bead and Scenedesmus sp. for more than 30 minutes. They emphasized the requirement of more efficient

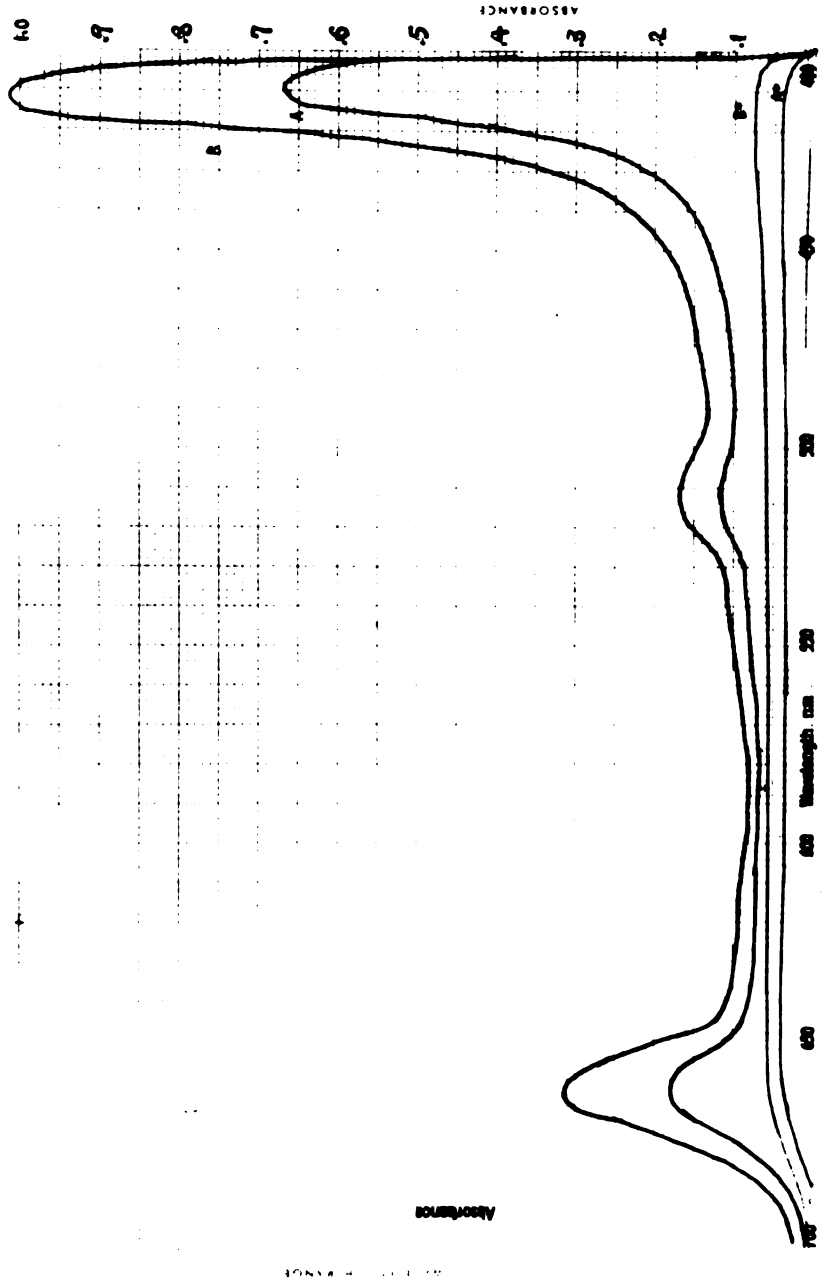

Fig. 25.--Changes in the absorption spectra of the extract obtained by the HCl-pretreatment method.
A: Spectrum of 40 mg cell dry matter per 100 ml suspension.
B: Spectrum of 80 mg cell dry matter per 100 ml suspension.
A* and B* are the corresponding spectra after photolysis (15,064 lux 8 hours, 30 C).

equipment for the cell disintegration. Many investigators have used chemical methods such as urea solution to increase the extractability of protein from algal cells (Mitsuda et al., 1969; Baba et al., 1960). According to the authors, the protein preparation was contaminated by cell fragments and was dark green in color. In addition, the unpleasant, grassy flavor of the preparation made it unacceptable for human consumption.

(F) Successive Fractionation of Algal Protein After Mechanical Disintegration of the Cells

The distribution of the components of the algal protein obtained after mechanical disintegration was determined by the procedure shown in Fig. 6 in the Methods section. The algal protein was separated into (1) an alcohol-extractable fraction, (2) a water-extractable, (3) a salt-extractable, and (4) alkali-extractable fraction (Table 10).

The 70% alcohol extractable fraction was 4.6% of the cell nitrogen, the water extractable fraction was 7.2% the salt (8% NaCl) extractable fraction was 5.7% and the alkali (0.2% NaOH) extractable fraction was 42.1%. There was in undissolved residue containing 26.8% of the cell nitrogen (Table 10). The result was quite different from that of general plant seed protein in which a large portion of protein is water extractable (56% of total N in Mung seed) (Loffe et al., 1968).

Table 10.--Distribution of nitrogen in fractions obtained from the mechanical disintegration extract of A. flos-aquae.

Fractions	Name of fraction	Nitrogen content (on dry basis) %	Nitrogen extractability (Extracted-N as % of cell N)
FC	Acetone extractable	1.1	0.9
FA	Alcohol extractable	3.6	4.61
			5.51
K_1	Water extractable	7.95	2.81
K_2	Water extractable	1.27	0.15
K_3	Water extractable	8.15	1.50
K_4	Salt extractable	13.5	3.53
K_5	Alkali extractable	15.0	37.26
Total N precipitated by pH adjustment			45.25
FW	Non precipitable-N by pH adjustment in water extract	6.15	2.73
FN	Non-precipitable-N in salt extract	3.72	2.20
FK	Non-precipitable-N in alkali extract	5.47	4.80
Total Non precipitable N by above treatment			9.73
Residue			26.80
Total accountable N			87.31
Unaccountable N			12.69

The algal protein was fractionated by the scheme described in Fig. 6.

It is evident that a suitable solvent for protein extraction from blue-green algae is a mild alkali solution. Remaining problems in these final purified protein were color and flavor as shown in Fig. 26. The fraction with lightest color was K_2 that is alkali-precipitated protein (pH 11.7) after water extraction.

(G) Composition of Protein Isolates Obtained by the Single Alkali and HCl-Pretreatment Methods

The freeze dried protein isolates obtained by single alkali and HCl-pretreatment contained 70 to 82% protein calculated as % N x 6.25 (Table 11). All but one isolate contained 0.1 and 0.2% nucleic acid. The remainder was carbohydrates (2-12%) and ash (8 to 16%). The determination of nucleic acid was based upon the extinction coefficient of 0.1% standard ribonucleic acid, which was a coefficient of 16 at 260 nm.

The nucleic acid content in the isolate was so low that it may not influence the uric acid metabolism in humans. High intake of nucleic acid has shown a possibility to lead to precipitation of uric acid crystals in joints and soft tissues or to lead to the formation of stones in the urinary tract (Endozien, 1970; Oser, 1965). This property is one of the reasons why single cells are hardly ever used for human consumption.

The protein isolated from the HCl-pretreatment extraction were devoid of their original characteristic

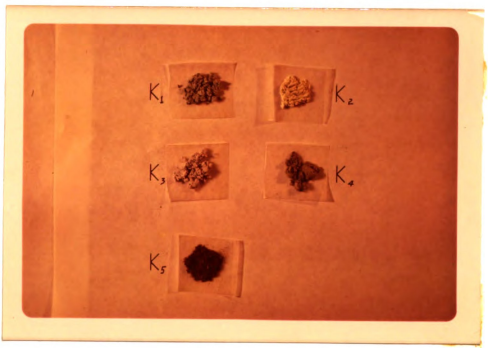

Fig. 26.--Photograph of protein fractions obtained by successive solvent extraction from A. flos-aquae; Fractions were obtained from scheme in Fig. 6 and lyophilized after dialysis.

K_1: Fraction precipitated at pH 4.0 after water extraction.

K_2: Fraction precipitated at pH 11.7 after water extraction.

K_3: Fraction precipitated at pH 5.5 after water extraction.

K_4: Fraction precipitated at pH 1.8 after 8% NaCl extraction.

K_5: Fraction precipitated at pH 4.0 after 0.2% NaOH extraction.

Table 11.--Composition of protein isolated from A. flos-aquae by various extraction methods.

	HCl-pretreatment extraction with photolysis		Single alkali extraction without photolysis		Single alkali extraction with photolysis	
	Protein isolate (B-1-1) from acid precipitation (pH 4.0) %	Protein (B-1-2) precipitated with acetone from previous supernatant %	Protein precipitated at pH 4.0 %	Protein precipitated with acetone from previous supernatant %	Protein isolate (K-1-1) from acid precipitation (pH 4.0) %	Protein isolate (K-1-2) precipitated with acetone from previous supernatant %
Protein (crude)	82.2 ± 2.1	81.8 ± 2.7	72.5 ± 2.5	71 ± 2.8	69.8 ± 1.6	70 ± 1.3
Carbohydrate	2 ± 0.04	4.7 ± 0.14	7.6 ± 0.26	3.7 ± 0.1	12.5 ± 0.5	3.7 ± 0.15
Nucleic acid	0.1 ± 0.005	0.1 ± 0.002	0.005 ± 0.001	0.2 ± 0.004	0.6 ± 0.02	0.2 ± 0.003
Ash	9.5 ± 0.21	10.3 ± 0.35	12.3 ± 0.4	13.4 ± 0.25	8.3 ± 0.2	15.8 ± 0.5

Protein isolates (B-1-1) (B-1-2) and (K-1-1) (K-1-2) were obtained by procedures described in Fig. 3 and 4. Means and standard errors are shown.

flavor, were light yellow, and were fairly water soluble. But the isolates from the single alkali extraction without photolysis were dark green in color and retained their characteristic grassy flavor (Fig. 27). HCl-pretreatment of the cells prior to the extraction of protein has a number of advantages such as increasing the protein yield and eliminating the unpleasant color and flavor. In this method dialysis is used only at the final stages of the work which is an advantage over other extraction methods. Dialysis may be easily applied as continuous dialysis or ultrafiltration.

(H) Digestibility of the Whole Cells and Protein Isolates of Blue-Green Algae

Fig. 28 summarizes the results of the pepsin digestibility tests of whole freeze-dried cells and protein isolates. It is shown that the protein isolates are much more digestible than the whole cells indicating that the process of isolation has increased the digestibility from 40% to 98% after incubation for 6 hours at 38 C. However, the digestibility of casein reached 100% under these conditions. The relatively slow rate of digestibility and the low ultimate value of the whole cells may be attributed to the protective action of the strong cell wall.

Fig. 27.--Comparison of color between protein isolates from various extraction methods.

A_1: Protein precipitated at pH 4 from single alkali extraction without photolysis.
A_2: Protein precipitated with acetone from supernatant of above precipitation.

L_1: Protein precipitated at pH 4 from single alkali extraction with photolysis.
L_2: Protein precipitated with acetone from supernatant of L_1 precipitation.

H_1: Protein precipitated at pH 4 by the HCl-pretreatment method with photolysis.
H_2: Protein precipitated with acetone from superantant of H_1 precipitation.

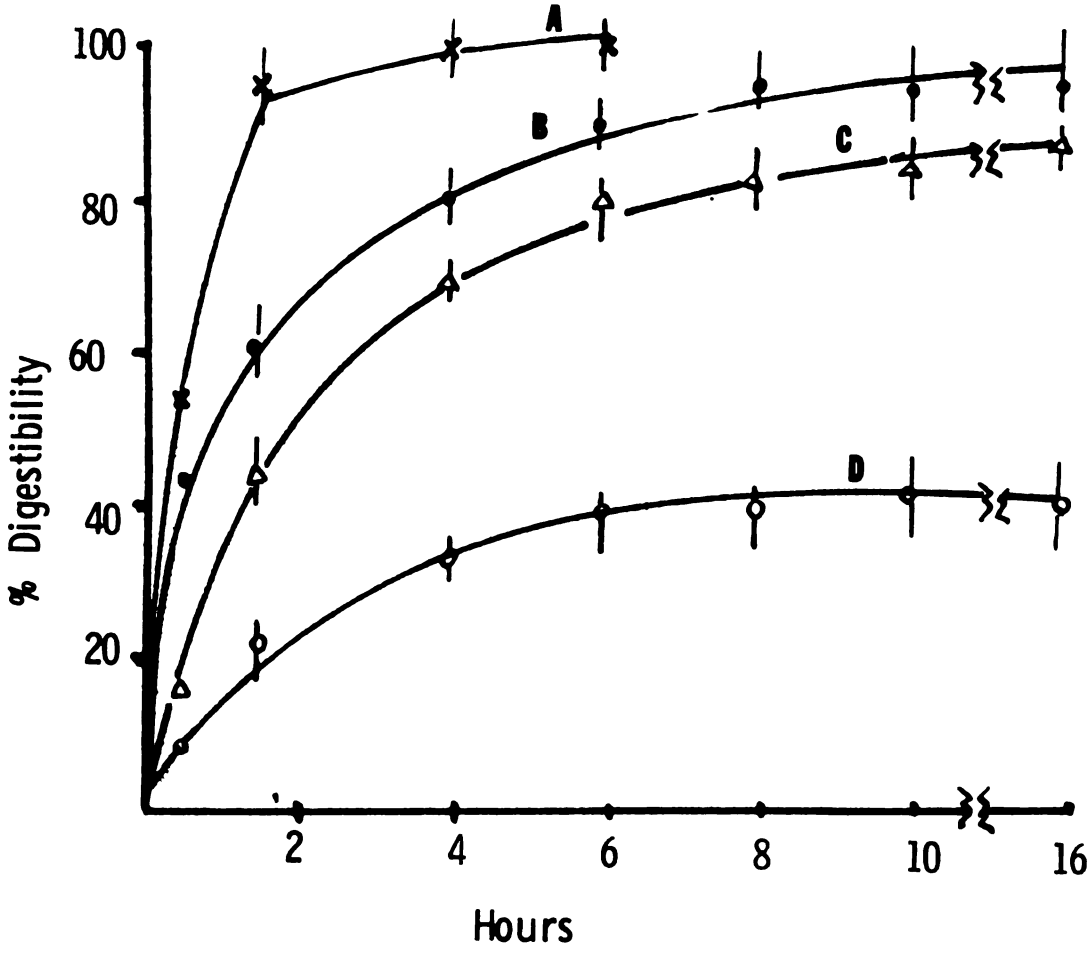

Fig. 28.--Digestibility of protein isolates and whole cells of A. flos-aquae by pepsin in vitro.

The protein isolates were obtained by HCl-pretreatment extraction. The whole cells were lyophilized cells.

A. Casein
B. Protein from acetone-precipitation
C. Protein from pH adjustment to 4.0
D. Whole cells

(I) Quality of Algal Protein Isolates as Judged by the Chemical Score

Table 12 shows the amino acid composition of freeze-dried algal cells, protein isolates (B-1-1 and B-1-2 shown in Fig. 3), hen's egg and the 1957 FAO provisional pattern (FAO, 1957). In the whole cells and protein isolates, methionine and cysteine are the limiting amino acids. Protein isolate (B-1-2) is strongly deficient in tryptophan in addition to being poor in the sulfur containing amino acids. The present work is in agreement with previous observations (Leveille et al., 1962, 1967; Miller et al., 1971) that methionine and histidine appear to be the limiting amino acids in micro algae. The amino acid composition of blue-green algae and their protein isolates compares favorably with that of other unconventional proteins--leaf protein, green algae and yeast (Mateles and Tannenbaum, 1968).

The chemical score (Cresta et al., 1969) of whole cells is 44 and that of protein isolate (B-1-1) is 46 (Table 13). The chemical score of B-1-2 is only 6 because of the extremely low concentration of tryptophan. Histidine and tyrosine were apparently destroyed by the HCl-pretreatment of the cells. The chemical score is an adequate first approximation for screening protein for nutritional quality. However the entire essential amino acid picture should be considered for more meaningful evaluation.

Table 12.--Amino acid composition of A. flos-aquae and its protein isolates (g Amino acid/100 g protein).

Amino Acid	Whole Cell	1957 FAO Provisional Pattern	Hen's Egg	Protein Isolate B-1-1	Protein Isolate B-1-2
Lysine	5.1	4.2	6.4	4.6	5.2
Histidine	1.8	---	---	0	0
Arginine	9.3	---	---	9.5	7.2
Aspartic acid	11.6	---	---	14.8	14.1
Threonine	5.8	2.8	5.1	5.6	6.6
Serine	4.1	---	---	4.8	5.6
Glutamic acid	11.6	---	---	13.2	14.1
Proline	3.4	---	---	3.7	4.3
Glycine	4.1	---	---	4.1	4.9
Alanine	7.6	---	---	7.6	9.2
Cysteine	0.6	2.0	2.4	0.9	0.8
Valine	8.2	4.2	7.3	7.3	7.1
Methionine	1.7	2.2	3.1	1.7	1.3
Isoleucine	5.5	4.2	6.6	6.7	6.2
Leucine	8.8	4.8	8.8	10.9	9.4
Tyrosine	5.5	2.8	4.2	0	0
Phenylalanine	4.8	2.8	5.8	4.8	4.2
Tryptophan	1.6	1.4	1.6	0.8	0.1
E/T ratio	3.0	2.0	3.2	2.7	2.5
E/N ratio	0.8			0.7	0.7

Protein isolates (B-1-1) and (B-1-2) represents the protein obtained by pH adjustment and acetone precipitation in Fig. 3. Data of the provisional pattern and Hen's egg were obtained from FAO/WHO Tech. Rept. Ser. 301, 1965. The E/T ratio is the proportion of essential amino acids of total nitrogen (g amino acids/g of total N). The E/N ratio is the ratio of essential amino acids to non essential amino acids.

Table 13.--The chemical score of blue-green algae and its protein isolates based on the essential amino acid pattern of whole egg.

Amino Acid	Whole Cell	Protein Isolate B-1-1	Protein Isolate B-1-2
S-cont. A.A.	44	46	38
Isoleucine	84	100	94
Leucine	100	100	100
Threonine	100	100	100
Valine	100	99	98
Total Aromatic A.A.	100	99	91
Lysine	80	72	82
Tryptophan	98	52	6

The content of each amino acid is expressed as a percentage of the same amino acid in the same quantity in the protein of a whole egg as a standard. The amino acid showing the lowest percentage is called the limiting amino acid; this percentage is the chemical score.

The whole cells and the isolates contain high levels of threonine, leucine and lysine which are often limiting in human foods. On the basis of this acids, blue-green algal protein is of low nutritional value for humans as the sole source of protein. It does, however, have supplemental value for other proteins.

Clements et al. (1967) reported that blue-green algae (Spirulina maxia) contained more than 60% protein in which all the essential amino acids except the sulfur-containing amino acid were present in relatively high concentrations and the chemical score was 43. This chemical score is similar to that of A. flos-aquae.

(J) Gel Electrophoresis of the Fractionated Protein or Protein Isolates

Gel electrophoresis is often used to test the homogeneity of protein preparations. Isolates B-1-1 (Fig. 3), K-1-1 (Fig. 4), and fractionated proteins (Fig. 6) were subjected to polyacrylamide gel electrophoresis at gel concentrations varying from 7 to 13%. The best resolution of the solvent-fractionated samples was obtained by using running gel pH 8.9, spacer gel pH 6.8, running buffer pH 8.3 and 13% gel (Fig. 29). However, a diffuse zone and a prominent, fast moving band were obtained in the isolates even though the gel concentration was increased to 13%; various electric currents in ranges from 2.5-3.5 mA per tube were employed, various pH's of

The electrophoresis was conducted in running gel (pH 8.9). space gel (pH 6.8) and running buffer (pH 8.3), 13% gel concentration. 3.2 mA of electric currents per tube.

Column	Protein
B-1-1:	pH-precipitate after HCl pretreatment (Fig. 3)
AW:	Unphotolyzed pH-precipitate after single alkali extraction
K-1-1:	Photolyzed pH-precipitate after single alkali extraction
K_1:	Water extractable and pH 4.0-precipitable protein in solvent fractionation (Fig. 6)
K_3:	Water extractable and pH 5.5-precipitable protein in solvent fractionation
K_4:	NaCl solution (8%) extractable and pH 1.8-precipitable protein in solvent fractionation
K_5:	Alkali extractable (0.2%) extractable and pH 4.0-precipitable protein in solvent fractionation

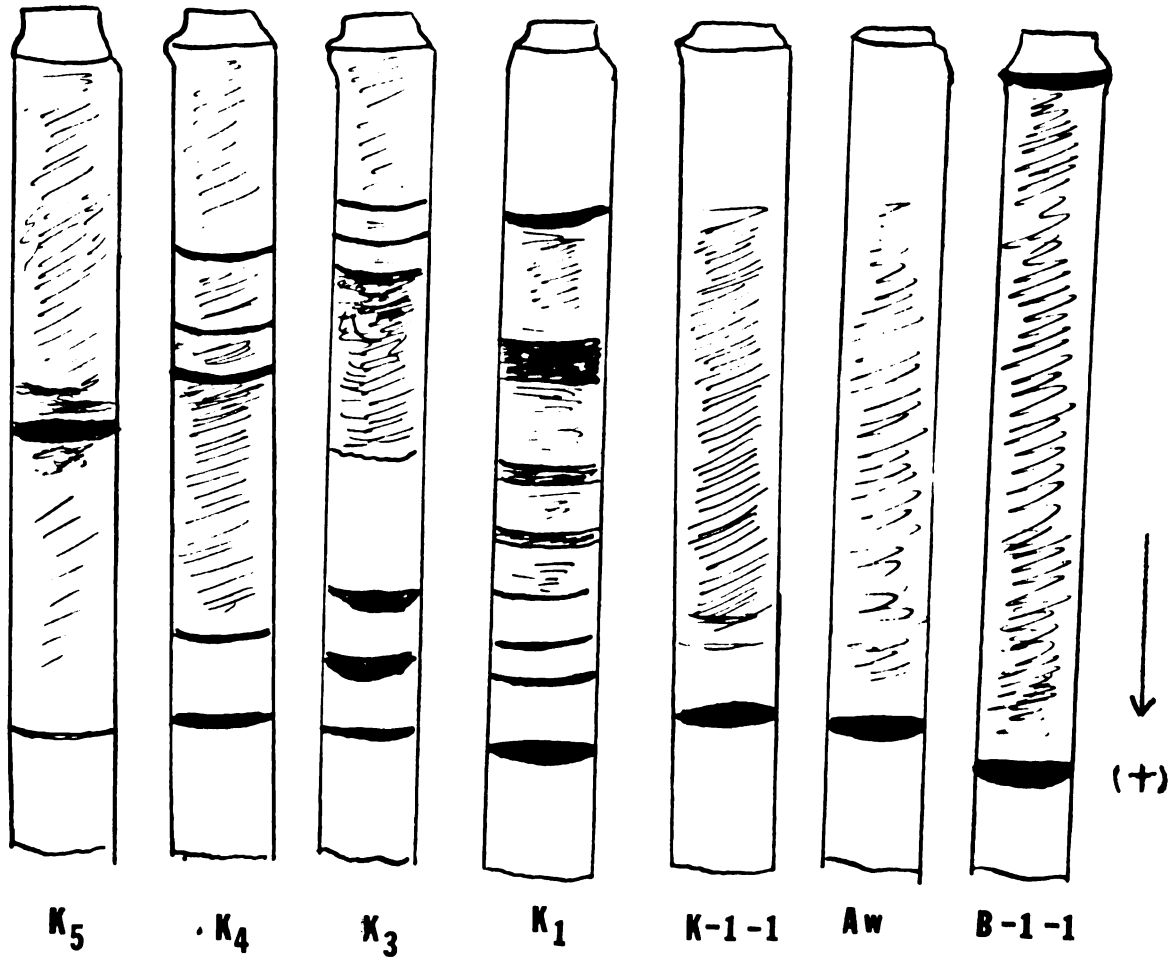

Fig. 29.--Polyacrylamide disc gel electrophoresis of seven isolated, fractionated proteins from A. flos-aquae.

space and running gel was used, and various amounts of samples (0.2-0.8 mg protein) were applied; whereas those of fractions K_1 and K_3 had many protein bands at the lower part of the gel; those of fractions K_4 or K_5 had few bands in the middle of gel (Fig. 29). This suggests that the size of the protein aggregates of the isolates may be smaller than that of the fractionates.

The SDS-electrophoretic pattern (Fig. 30) indicated that the water-extracted fractions (K_1, K_2, and K_3) contained protein subunits of wider size range than the salt or alkali-extracted fractions. The isolate K-1-1 is a photolyzed sample of AW. The disappearance of a number of bands from AW suggests an effect of photolysis on protein structure.

From a reference curve (Dunker and Ruecker, 1969) prepared with catalase, pepsin, trypsin and cytochrom C (Fig. 31) and the relative mobilities of 0.61-0.74 of the fractionated proteins, it appears that most of protein monomers of A. flos-aquae have a molecular size of 18,000 to 30,000 daltons. The relative mobilities of a few monomers obtained after alkali extraction and alkali precipitation were less than 0.17 indicating molecular weight higher than 60,000 daltons.

According to Myers and Kratz (1955), Kao and Berns (1968), Benett and Bogorad (1971) the biliprotein of blue-green algae can account for 60% of the total protein and

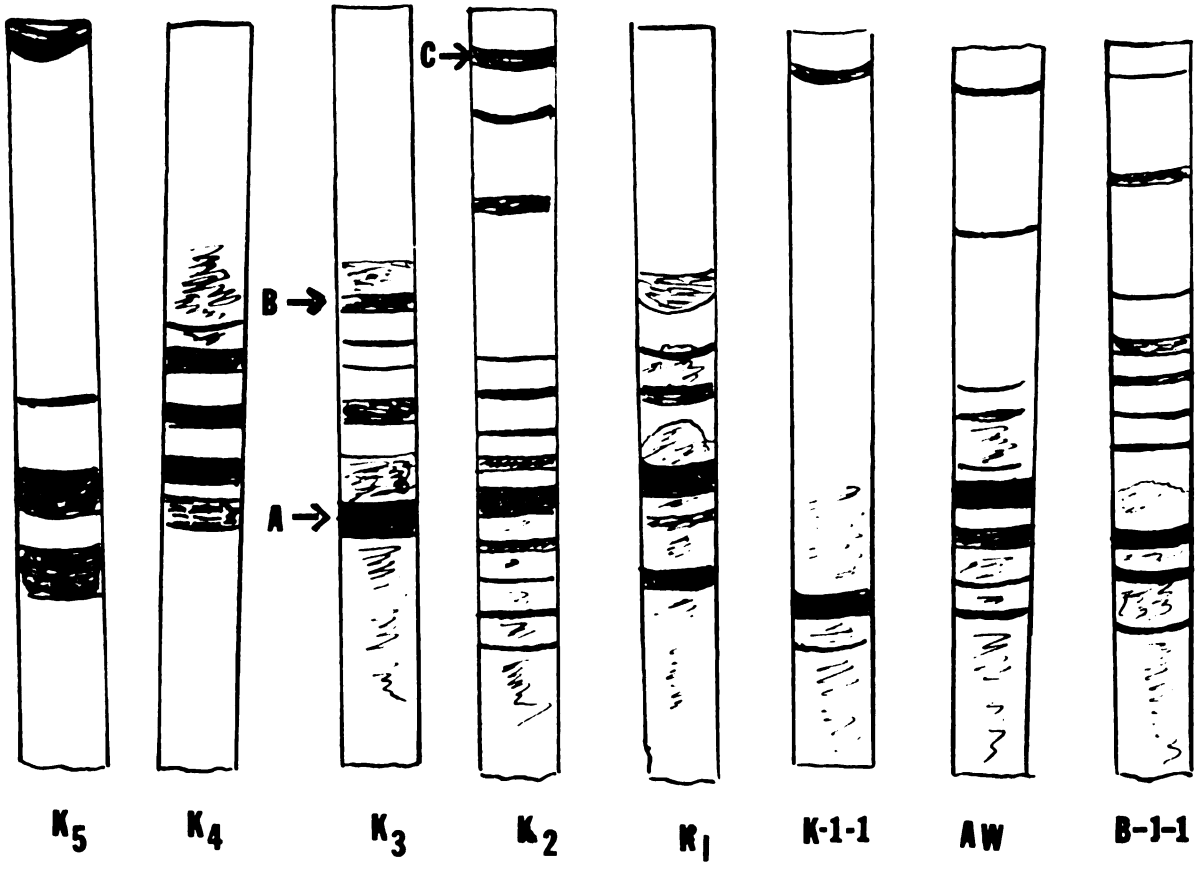

Fig. 30.--SDS-electrophoresis of proteins of isolated or fractionated proteins of A. flos-aquae.

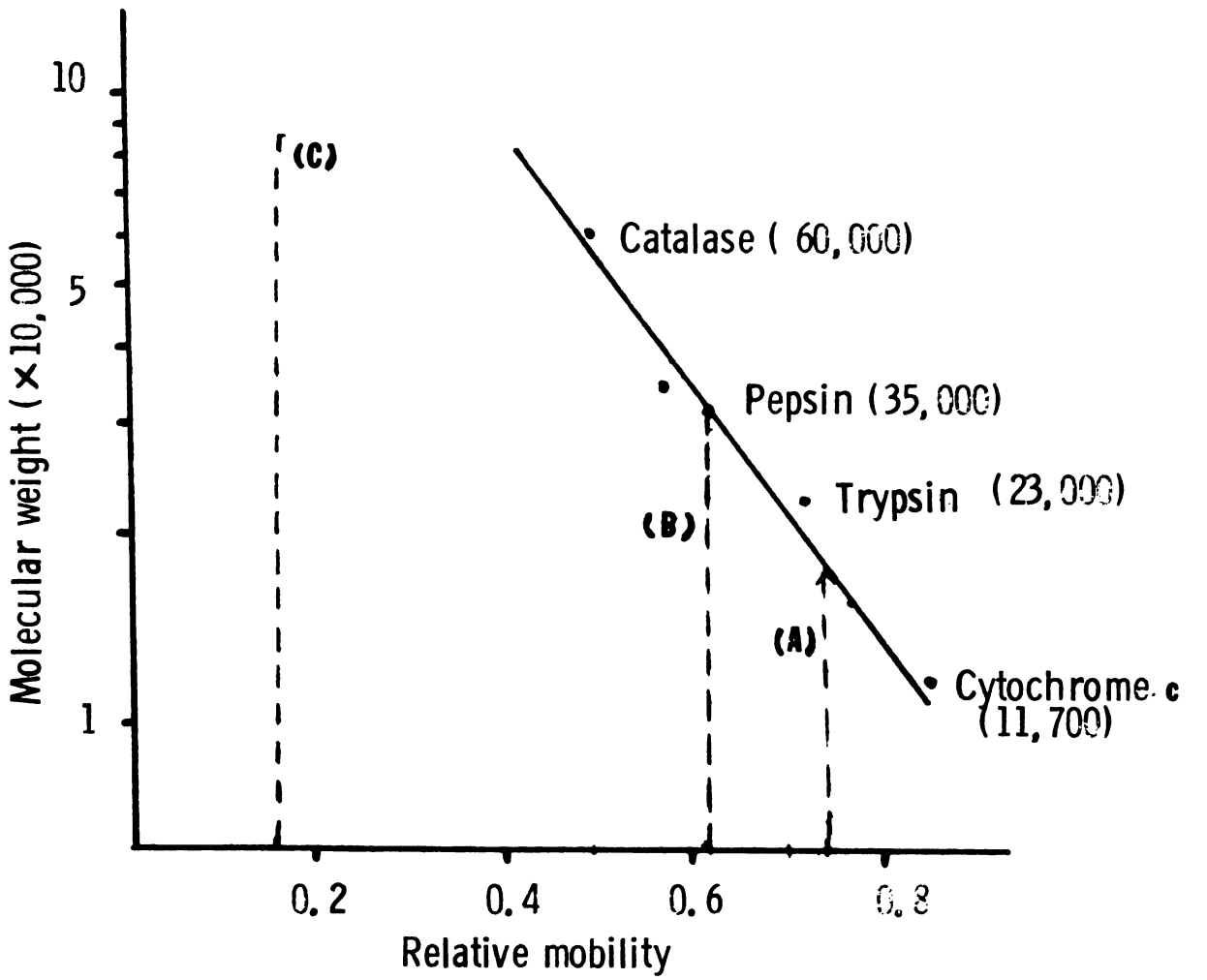

Fig. 31.--Semi-log plot of molecular weight against relative mobility in SDS-gel electrophoresis.

the molecular weight of a monomer of the biliprotein is reported to be 16,000-30,000 daltons.

SUMMARY

To determine the potential of the protein of blue-green algae (Anabaena flos-aquae) for human food, the growth conditions of the algae, the color and odor removal from protein extract, and the chemical and nutritional values of this protein were studied.

The blue-green algae was cultured in an autotrophic Kratz and Myers medium and in a heterotrophic medium containing urea (0.01% w/v), $NaNO_3$ (0.01%), $NaNO_2$ (0.01%), peptone (0.05%), glucose (1%), or glucose (0.02%) plus urea (0.02%). The addition of $NaNO_3$ and $NaNO_2$ to the autotrophic medium decreased the cell yields and protein contents of the cell. Neither urea nor peptone affected cell yields. However, the addition of glucose alone contributed to an increase in the cell yields. The maximum cell yield was obtained by including 1% glucose in the medium. The addition of more than 0.02% of urea retarded cell growth and this was only partially restored by the addition of glucose.

The cells were cultured in 16 liters of autotrophic medium, at 32 C under 15,064 lux light intensity and agitated at 2.5 liters of air per minute. Maximum

growth rate was 90 mg (dry cell) per liter per day and maximum yield (650 mg per liter) was obtained by culturing for 12 days. The blue-green algae cultured in the autotrophic medium contained 63.4% protein, 12.2% lipid, 14% carbohydrate, 4.2% ash, and 4% nucleic acid on a dry basis. The algal cells contained 0.95% amide and ammonia nitrogen, 0.32% humin nitrogen, and 8.83% amino nitrogen on a dry basis. Amino nitrogen comprised 87.4% of the total cell nitrogen.

The cell suspension displayed three light-absorption maxima, at 430 nm, 630 nm, and 680 nm. The pigments of the cell could be completely bleached by exposure to fluorescent light. Prolonged light exposure, heating, ultraviolet light, and high pH accelerated the decolorization. The pigments absorbing at 430 nm and 680 nm were markedly decreased by illumination. The pigments absorbing at 630 nm could be decreased by heating the cell, whereas the pigments absorbing at 430 nm and 680 nm were almost unchanged by heating alone. Ultraviolet light of short wavelength (233.7 nm) was more effective in bleaching the cells than fluorescent light or long wavelength (366.0 nm) u.v. light. The pigment absorbing at 630 nm was especially sensitive to ultraviolet light. The algal pigments were relatively stable at pH 3-7. The pigment absorbing at 630 nm was very unstable at low pH (1.0) and high pH (11), but the pigment absorbing at 680 nm was

stable at pH 1.0 and unstable at pH 11.0. The pigment at 430 nm was stable to the changes of pH of the cell suspension, but was very unstable to light exposure. It took 8 hours to bleach the cells completely at pH 11, 32 C, under 15,064 lux of fluorescent light. The bleached cells were light yellow.

It was found that the extraction of the cell protein was greatest at pH 11.0 and lowest in the range of pH 3.8-4.5, it was increased by increasing the temperature to 95 C. Optimum concentration of NaOH for algal protein extraction was 0.5%.

It was also found that efficient extraction of protein from blue-green algal cells and colorless, flavorless protein isolates could be obtained by the following procedures: (1) pretreatment of algal cells with 3 N HCl at 95 C for 10 minutes followed by increasing the pH to 11 at room temperature for 2 hours and centrifuging, (2) photolysis of the supernatant solution for 8 hours under fluorescent light of 15,064 lux, (3) precipitation of protein from the alkaline extract by lowering the pH to 4.0 followed by acetone precipitation. The total extracted nitrogen was 81% of the cell nitrogen and the isolated protein nitrogen was 64.2% of the cell N, which is considerably higher than that obtained by other workers. Treatment with 0.5% NaOH of cells previously ground resulted in extracts containing 41% of the N of the cells. From

this extract isolates were obtained by precipitation which contained 36% of the cell N. Single alkali extraction involving shaking the cells with 0.5% NaOH for 6 days at room temperature yield 70% extraction of cell nitrogen and 55.6% of cell nitrogen was recovered as protein nitrogen. The photolysis was able to remove unpleasant color and flavor but decreased the recovery of precipitable protein nitrogen. In successive solvent fractionation of algal protein a large portion of protein was extracted with a 0.2% sodium hydroxide solution (80% of total extracted protein nitrogen). The remainder was salt solution (8% NaCl) and water-extractable.

Protein isolates after HCl-pretreatment contained 82% protein on a dry basis and showed good digestibility (97%) by pepsin in vitro whereas the digestibility of freeze-dried cells was 40% while that of casein was 100% and was more quickly hydrolyzed.

Lysine, threonine, isoleucine, phenylalanine, leucine, and valine were found to be present in satisfactory quantities in whole cells and protein isolates, regarding human nutrition. The sulfur-containing amino acids, methionine and cystine, were the limiting amino acids. Tryptophan was lower, and histidine and tyrosine were absent in the isolates precipitated with acetone. The chemical scores of whole cells and acid-precipitated protein were 44 and 46, respectively.

Disc-electrophoresis (13% polyacrylamide) and SDS-electrophoresis on the proteins after HCl-pretreatment, from successive solvent fractionation, and from single alkali extraction showed that the water extractable fraction had many bands; there were 4 bands in the NaCl-extractable fraction and one band in the alkali extractable fraction. The isolates from the HCl-pretreatment or the single alkali extraction showed a fast moving band. The size of protein monomers from the fractionated protein were mostly in the ranges of 18,000 to 30,000 daltons. Photolysis reduced the number of protein bands on SDS electrophoresis.

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APPENDIX

APPENDIX

Table A_1.--Some important chemicals used in this study and their sources.

Chemicals	Sources
Acetone	Mallinckrodt Chemical Works
Trichloroacetic Acid	Mallinckrodt Chemical Works
Pepsin	Sigma Chemical Corp.
Mannose	Fisher
2-Mercaptoethanol	Fisher
Urea	Fisher
Sodium dodecyl sulfate	Fisher
Norleucine	Nutritional Biochemicals Corp.
Antifoam AF emulsion	Dow Corning Corp.
Standard amino acid calibration mixture	Bio-Rad Laboratories
Tryptophan	Calbio Chemical Calf.
Galactose	Phanstiehl
Ninhydrin	Bio-Rad Laboratories, Richmond, Calf.
Pronase	Calbiochem. Los Angeles, Calf.

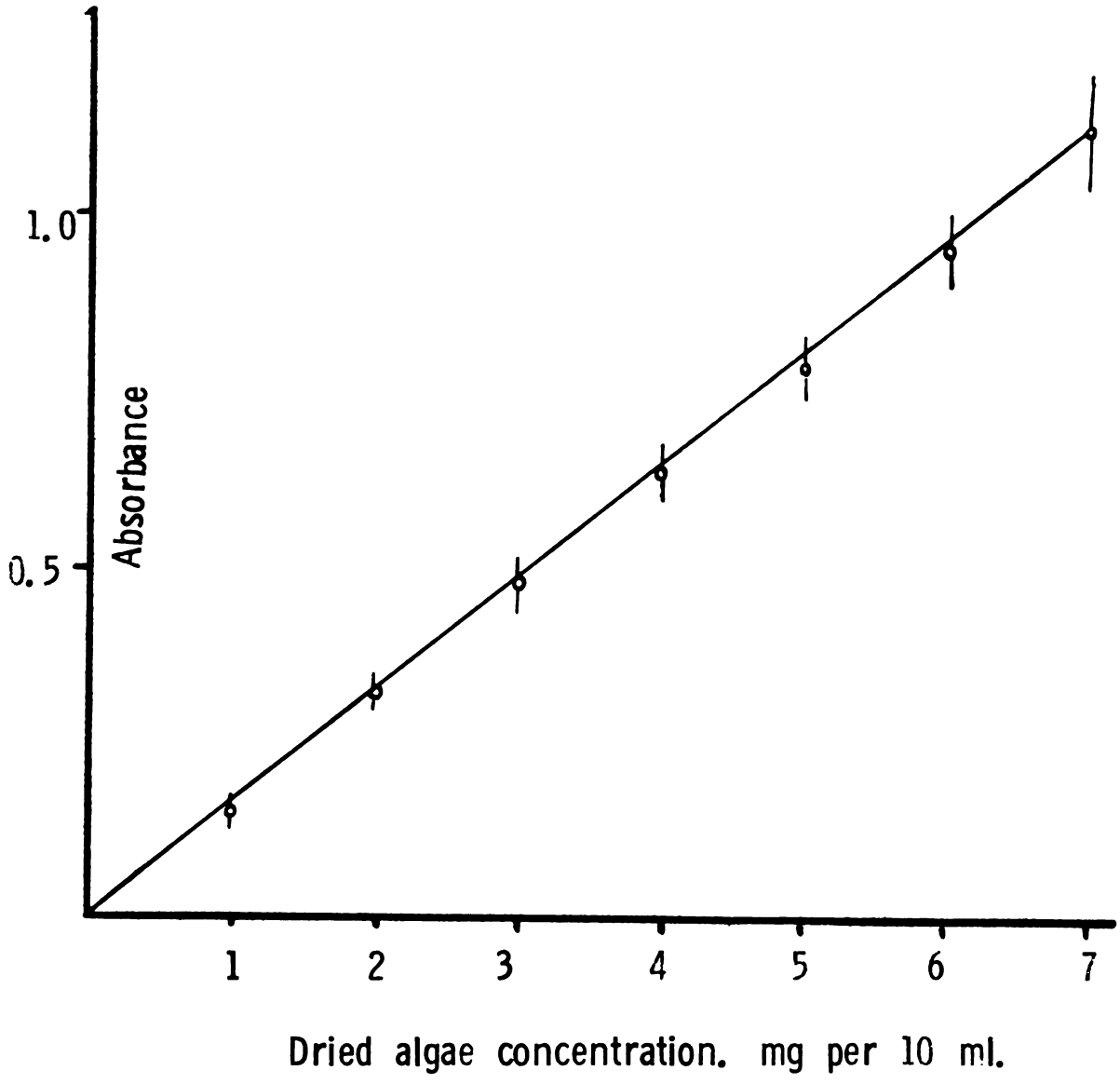

Fig. F_1.--Relationship between dried weight of algae and absorbance at 420 nm. The vertical line is a standard error. Absorbance was determined after suspending washed cells in distilled water.

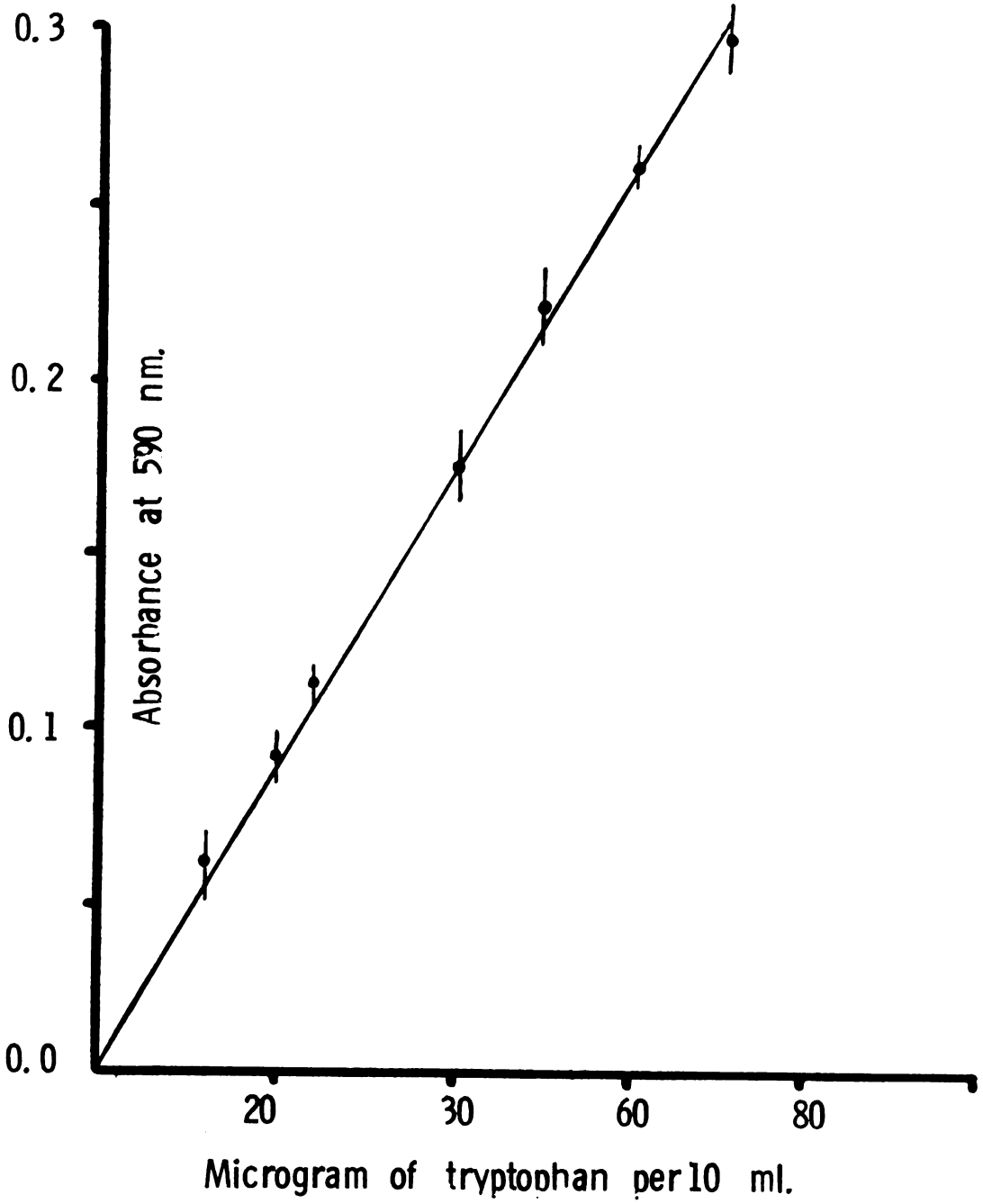

Fig. F_2.--Standard curve for tryptophan determination; vertical bars indicate standard errors.