FOREWORD

This program was conducted at Armour Research Foundation of Illinois Institute of Technology, Technology Center, Chicago 16, Illinois, under the sponsorship of the 6570th Aerospace Medical Research Laboratories. The program was initiated and monitored by 1/Lt. Eugene G. Sander and Dr. Alton E. Prince of the Biospecialties Section, Physiology Branch of the Biomedical Laboratory. The work was conducted under Contract No. AF 33(616)-7964, Project No. 7164, "Space Biology Research," Task No. 716403, "Environmental Biology." At Armour Research Foundation the research program was designated ARF Project C 193. Dr. M. E. King, Mr. A. M. Shefner, Dr. B. Kohn, and Mr. J. Kyle planned and conducted this program.

This has been designated Report No. ARF 3193-5. The authors are M. E. King and A. M. Shefner. The studies performed and compiled into this report were conducted during the period of 1 March 1961 to 31 April 1962.

ABSTRACT

Untreated algal cells are incompletely digested in man's alimentary canal. Therefore, various enzymes were investigated in an effort to develop an enzyme supplement that would increase the nutritive value of an algal ration. Such an enzyme additive would function by directly degrading the algal cells or by making the cells more susceptible to the action of the normal digestive enzymes.

Enzymes were evaluated by in vitro digestion for 2 hours in artificial gastric juice followed by digestion for 4 hours in artificial intestinal juice. The commercial cellulases were not effective. Favorable results were obtained with enzyme systems derived from the snail Helix pomatia and the mold Myrothecium verrucaria. Pectinase was also effective, both alone and in combination with the snail and the mold enzymes. These results are consistent with the studies of Northcote and others on the composition of the algal cell wall.

PUBLICATION REVIEW

This technical documentary report has been reviewed and is approved.

Jos M Quashnock

JOS. M. QUASHNOCK
Colonel, USAF, MC
Chief, Biomedical Laboratory

ENZYMATIC DIGESTION OF ALGAL CELLS

INTRODUCTION

One of the major problems of extended space flights is that of ensuring an adequate diet for the crew. Since algae are being contemplated for oxygen-carbon dioxide gas exchange in the life support system, the possibility of using the algae as a source of nutrients for the astronauts is of considerable interest. The human digestive system, however, does not possess enzymes capable of degrading the algal cell wall. In the present study various enzymes were added to a simulated human digestive enzyme system in order to derive the maximum quantity of nutrients from the intact cells of alga Chlorella pyrenoidosa 71105.

BACKGROUND INFORMATION

Algae of the genus Chlorella consist of unicellular spheroid organisms in which the cell contents are surrounded by a cell wall composed of protein, carbohydrate, and lipid material. We studied a variety of enzymes in an effort to increase the degradation of the algal cell wall. This study was based on two assumptions, which are closely interrelated.

1. The algal cell wall is composed of material not readily digested under the conditions prevalent in the human digestive tract. In order to utilize these substances for nutrition under conditions which preclude chemical or physical processing of the algae, an enzyme mixture that could degrade the cell wall and could act within the human digestive tract would be highly desirable. To prove of value, the enzyme supplement would have to increase the utilization of the algae to an extent sufficient to compensate for the weight of enzyme being added to the diet.

2. The intact cell wall acts as a barrier to the digestion of the contents of algal cells by the normal human digestive enzymes. The cell wall should be ruptured in order to make the contents readily accessible. In addition, certain of the polysaccharides of the cell contents may not be digestible by human digestive enzymes, and the enzyme supplement would be necessary to increase their utilization.

Our interests thus encompassed the structure and composition of the algal cell wall, enzymes which degrade cell walls and enzyme activities over the pH ranges of the gastrointestinal tract. Enzymes that are judged to be active by in vitro methods of analysis must be shown to be effective in whole-animal feeding studies. The algae-enzyme foodstuffs must not only be shown to be adequate nutritionally but must also be shown to be free from toxic manifestations.

Two excellent studies of the structure and chemical composition of algal cell walls have been conducted by Northcote et al. (ref. 12) and by Cronshaw et al. (ref. 2). Northcote utilized differential centrifugation of mechanically disintegrated Chlorella pyrenoidosa cells to obtain his cell wall fraction. Cronshaw studied a variety of green, red, and brown algal species and investigated the fraction of ground frozen cells that was insoluble in boiling ethanol. Both groups of workers utilized electron microscopy to supplement chemical and chromatographic analyses for specific components. Although there are, naturally, some differences in interpretation of results, both groups present a similar picture of the algal cell wall.

The wall appears to be composed of two distinct phases: an organized microfibrillar structure and a surrounding continuous matrix. In the Chlorella cell wall Northcote found the microfibrils to be irregularly interwoven in a continuous network over the wall. The microfibrils (30 to 50 A in diameter) lie in two main directions, at right angles to one another. No definite lamellae were found. In Cladophora and Chaetomorpha Cronshaw found many lamellae consisting of straight microfibrils arranged parallel to each other and surrounded by amorphous material. The direction of orientation of the microfibrils in adjacent lamellae was approximately at right angles. When the matrix was removed, the microfibrils became easily separated. The cell walls of the other algal species investigated by Cronshaw showed a basic structure of randomly arranged microfibrils embedded in an amorphous matrix.

In Chlorella the cell wall is approximately 210 A thick. After digestion by an enzyme preparation from the snail Helix pomatia (ref. 9), the remaining material shows no microfibrillar structure but resembles the granular matrix. Sections of these digested walls have a laminated appearance. Two distinct layers approximately 50 A thick can be seen, one near the outer edge and one near the inner edge, separated by a space of approximately 100 A. This seems to indicate local concentrations of some of the materials of the matrix in these outer and inner lamellae. Northcote et al. identify the microfibrils with the α-cellulose fractions of the cell walls. They are composed of polymers of glucose, galactose, mannose, arabinose, and rhamnose. The protein, which may exist in part as a glycoprotein, is associated with the hemicellulose and with these polysaccharides makes up the greater part of the continuous matrix. The hemicellulose was isolated as an electrophoretically pure particle and upon acid hydrolysis yielded galactose, mannose, arabinose, xylose, and rhamnose.

Hemicellulose refers to those cell-wall polysaccharides which can be extracted from plant tissues by treatment with either hot or cold dilute alkalies but not with water, and which can be hydrolyzed to constituent sugar and sugar-acid units by boiling with hot dilute mineral acids (ref. 11). α-Cellulose refers to the cell-wall polysaccharides that are insoluble in cold alkalies (ref. 14).

From the yield of α-cellulose in the whole cell and in the cell walls Northcote calculated that the wall represents 13.6% of the dry weight of the cell. The composition of the cell wall and the degree to which each of these components is digested by the snail enzyme preparation are listed in table 1.

Table 1

CONSTITUENTS OF CHLORELLA CELL WALLS*

Constituent	% in Intact Cell Walls	% Liberated by Snail Enzyme
Protein	27.0	--
Lipid	9.2	40
α-Cellulose	15.4	70
Hemicellulose	31.0	13
Glucosamine	3.3	
Ash	5.2	
Total Recovered	91.0	

*Data from Northcote et al. (ref. 12).

The data of Cronshaw et al. agree in the main with the information cited for Chlorella. They, too, consider the microfibrils to be cellulosic in nature. However, in the case of Cladophora and Chaetomorpha they consider the microfibrils to be pure Cellulose I, containing only glucose residues. In the other algal species they studied, glucose is still the major structural unit but it is invariably associated with other sugars, frequently xylose. Other hexoses, pentoses, and uronic acids may also be associated in the microfibrillar fraction and in the amorphous matrix. In Porphyra mannose appears to replace glucose as the basic building unit of the microfibrils.

The general conclusion that the cell walls contain material other than a classical glucosidic α-cellulose has been reinforced by investigations on other algal systems. Myers and Preston (ref. 10) report that the α-cellulose fraction of the cell wall of Rhodymenia palmata, a red alga, is composed of approximately equal portions of glucose and xylose residues. In other red algae galactose is also present, and in Porphyra they found that glucose is replaced by mannose. Iriki and Miwa (ref. 7) isolated crude fiber from a green alga and found it to be composed of 91 to 93% mannose. They believe this fiber from Codium to be a β-1,4-mannan, but other Codiaceae yielded β-1,3-xylans. Peat (ref. 13) has recently published an extensive analysis of the carbohydrate of the red alga Porphyra umbilicalis and reports a variety of hexoses, pentoses, methyl- and anhydro- derivatives, and sulfate esters. Hirst (ref. 6), in a general review on polysaccharides of marine algae, lists the following components as being present in one or another species:

D-glucose
D-galactose
L-galactose
3,6-anhydro-D-galactose
3,6-anhydro-L-galactose
6-o-methyl-D-galactose
D-mannose
L-fucose
L-rhamnose
D-xylose
L-arabinose
D-mannuronic acid
L-guluronic acid
D-mannitol
glycerol
pyruvic acid

This list of components is an indication of the complexity of the structural elements of the algal cell wall and the cell contents. Obviously a mixture of enzyme activities should prove more useful in degrading the cell wall than a purified enzyme capable of acting on only a single class of substrates.

PRELIMINARY STUDIES

Our initial work was concerned with screening commercial cellulase preparations for their ability to hydrolyze Solka-floc, a highly purified wood cellulose. This work was predicated on the assumption that the algal cell wall is composed principally of cellulose containing β-glucosidic linkages; thus, hydrolysis by β-glucosidases would expose the cell contents to the normal digestive secretions. The following enzyme preparations were used: Mylase SA, from Wallerstein Company, Incorporated; Cellulase 35 and 36, from Rohm and Haas Company; Takamine cellulase 4000, from Miles Chemical Company; cellulase 4000, from Nutritional Biochemicals Corporation; and a crude cellulase, from Sigma Chemical Company.

For these experiments 200 mg of Solka-floc and 1 mg of enzyme were incubated, with shaking, at 37 °C for 4 hours in 10 ml of buffer at pH values ranging from 1.2 to 9.9. The buffer systems were as follows: pH 1.2 and 2.1, hydrochloric acid-potassium chloride buffer; pH 3.2, 4.2, and 5.2, citrate-phosphate buffer; pH 5.8, 6.8, and 7.8, phosphate buffer; and pH 8.9 and 9.9, carbonate-bicarbonate buffer (ref. 4). At the end of the incubation period the mixtures were heated in boiling water for 5 minutes to inactivate the enzyme and were filtered through sintered-glass filters of medium porosity. The amount of glucose in the filtrate was determined by the glucose oxidase method (ref. 16).

The results are listed in table 2. Except for cellulase 35, maximum activity was obtained at pH 5.2. None of the enzymes exhibited much activity at pH 2 and 8, the pH values of interest in digestion. The crude cellulase produced the largest quantity of glucose and was active over the widest pH range.

Table 2

LIBERATION OF GLUCOSE FROM 200 MG OF SOLKA-FLOC AFTER INCUBATION AT 37 °C FOR 4 HOURS

	Glucose Liberated, μg/ml					
pH	Cellulase 35	Cellulase 36	Cellulase 4000	Mylase SA	Takamine Cellulase 4000	Crude Cellulase
1.2	1.1	1.47	4.4	0.0	0	3.03
2.1	1.1	2.94	4.4	0.0	3.3	25.0
3.2	0	2.94	2.94	0.0	3.3	37.8
4.2	2.27	10.3	17.6	4.4	19.7	60.5
5.2	7.95	11.8	25.0	13.2	25.0	66.5
5.8	12.5	8.82	19.1	10.3	16.7	56.0
6.8	2.27	1.47	2.94	4.3	4.54	34.8
7.8	0	1.47	1.47	1.47	1.51	
8.9	2.27	1.47	2.94	1.47	1.51	6.05
9.9	1.1	1.47	1.47	1.47	1.51	6.05

A similar type of screening using Chlorella 71105 as the substrate was attempted. Samples containing 200 mg of the algae and 2 mg of cellulase 35 were suspended in 10 ml of buffer at pH values of 2, 5, and 8. The mixtures were incubated, with shaking, at 37 °C for 4 hours. At the end of the incubation period the mixtures were heated in boiling water for 5 minutes to inactivate the cellulase. The mixtures were cooled to room temperature and centrifuged, the residue washed, and the washings added to the supernatant liquid. The resulting samples were of varying intensities of green: light transparent green at pH 2, medium transparent green at pH 5, and opaque dark green at pH 8. Control samples that did not contain enzyme were similarly pigmented.

The presence of chlorophyll precluded the use of colorimetric methods for estimating the amount of glucose. Therefore a qualitative determination was attempted by paper chromatography. The samples were evaporated to dryness and extracted with methanol; the residue was dissolved in water. Spot chromatograms of the aqueous and the methanol extracts were run on Whatman No. 1 filter paper by descending technique using a 5:1:2 solvent system of n-butanol, acetic acid, and water. The reducing carbohydrates were detected by using aniline oxalate spray.

No differences were observed between the chromatograms of controls and of samples with the enzyme. However, there was considerable difference in reducing sugars present at the different pH levels. Three reducing substances were present at pH 8. The methanol extract contained galactose and an unidentified compound, probably a disaccharide, but not cellobiose, lactose, or maltose. The aqueous extract contained primarily a third compound, possibly a trisaccharide. At pH 5 only a trace of this compound was present, and none was detected at pH 2. The compound referred to as a disaccharide was present in considerable quantity at both pH 5 and pH 2, and galactose was present at pH 5 but not at pH 2. Glucose was absent from all samples.

Since it had become obvious that substances other than glucose residues were being produced, we next chromatographed a 5-hour acid hydrolysate of the algae (figure 1). The hydrolysate of Chlorella 71105 (column 1) contained an unidentified disaccharide or trisaccharide (D), galactose (GA), glucose (GL), two other compounds that are probably hexoses or hexose derivatives such as deoxy-sugars (H), and three compounds that are pentoses or pentose derivatives (P). Chromatography of the enzymatic and acid hydrolysates demonstrated that the cell wall polysaccharide of Chlorella is not a typical cellulose composed of β-1,4-linked glucose residues as was originally assumed. Therefore, further screening with Solka-floc would be of no value.

EXPERIMENTAL WORK

Mixtures of enzymes in an artificial gastric and intestinal enzyme system were screened by using Chlorella 71105 as the substrate.

Preparation of Enzymes

Crude cellulase was isolated from the snail Helix pomatia by a method described by Karrer (ref. 8) and purified by a modification of the method of Grassman (ref. 5). The snails were removed from their shells and the gastrointestinal tract with its contents separated. This material was ground with fine sand and toluene in a mortar, extracted with water, and filtered through asbestos. The resulting suspension was dialyzed against distilled water, lyophilized, and stored in the freezer until used. About 1 gram of the crude preparation was obtained from 20 snails. For purification, the crude enzyme was dissolved in distilled water and adjusted to pH 3.5 with acetate buffer. This mixture was treated with activated alumina and centrifuged, and the precipitate was discarded. The supernatant liquid was dialyzed three times against distilled water and lyophilized after each dialysis. The purified enzyme contained 66.4% protein, whereas the crude starting material contained 56.9% protein.

Cellulase was also prepared from two cultures of the mold Myrothecium verrucaria, grown on a mineral medium. In one culture 30 grams of Solka-floc was added to 1 liter of the medium. In the other culture 30 grams of lyophilized pasteurized Chlorella ellipsoidea was added to 1 liter of the medium. Flasks containing 150 ml of the culture medium were inoculated with M. verrucaria spores and incubated, with shaking, at 28 °C for 2 weeks. At the end of the incubation period the flasks were centrifuged, and the residual solutions were purified by a modification of the method of Whitaker (ref. 15). The filtrates were adjusted to pH 6.5 with phosphate buffer and brought to 30% saturation with ammonium sulfate. This mixture was refrigerated overnight and centrifuged, and the precipitate was discarded. The supernatant was dialyzed at 4 °C against distilled water to remove the ammonium sulfate, lyophilized, and the residue stored in the freezer. The preparation from the material grown on the cellulose contained 71.9% protein and that from the material grown on the algae contained 47.2% protein.

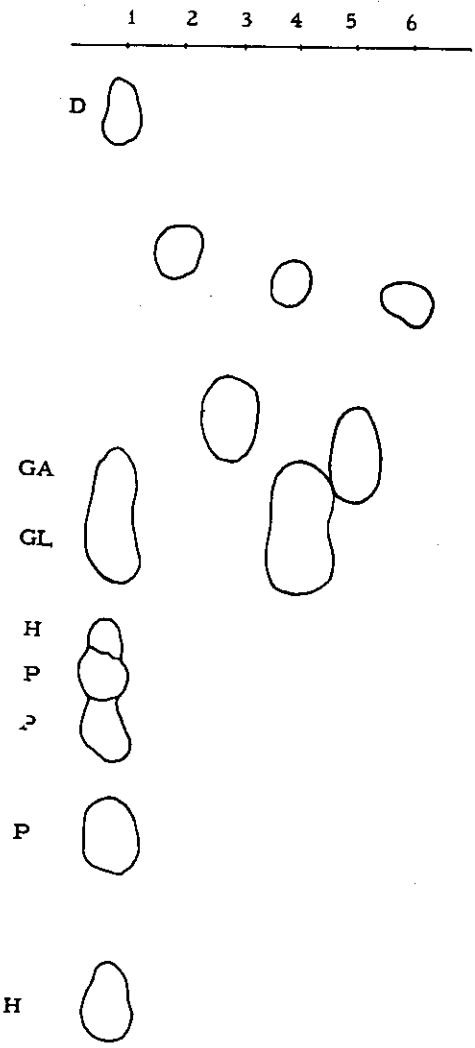

Figure 1

DRAWING OF CHROMATOGRAM OF ACID HYDROLYSATE OF CHLORELLA 71105

Column 1 Chlorella hydrolysate
Column 2 Lactose
Column 3 Glucosamine-hydrochloric acid
Column 4 Cellobiose, galactose, glucose, from top to bottom
Column 5 Sodium glucuronate
Column 6 Maltose

The commercial enzymes used were obtained from the following sources: pepsin 1-15,000 and pancreatin USP, from General Biochemicals; Takamine cellulase 4000, from Miles Chemical Company; pectinase and hemicellulase, from Nutritional Biochemical Corporation; and hemicellulase, from Wallerstein Company.

Preparation of Digestive System

The degradation of Chlorella 71105 to hexose and nitrogen was tested in an artificial gastric and intestinal system to which test cellulases were added. Samples containing 200 mg of algae and 5 mg of pepsin in 10 ml of pH 2 buffer were incubated, with shaking, at 37 °C for 2 hours. When pectinase was used, it was added to the algae-pepsin mixture before incubation. Next 10 ml of phosphate buffer containing 10 mg of pancreatin, 0.03 mg of trypsin, and 5 mg of the test cellulase were added. This mixture, now at pH 8, was incubated at 37 °C for 4 hours. The samples were heated for 5 minutes at 100 °C to inactivate the enzymes and were then centrifuged. The supernatant liquid was refrigerated.

Determination of Degradation Products

Hexose was determined by the quantitative Kiliani method (ref. 3). Aliquots were passed through a mixed-bed ion-exchange column and concentrated to dryness. The residue was dissolved in water and treated with 5 ml of 0.4 N acetic acid and 5 ml of 0.8 N potassium cyanide. The mixture was heated at 39 °C in a stoppered flask for 3 hours. The contents of the flask were acidified, and the excess hydrogen cyanide was driven off by passing air through the solution. The resulting nitrile was hydrolyzed to ammonia by addition of 20% sodium hydroxide, and the ammonia was steam-distilled into boric acid and determined titrimetrically.

Nitrogen was determined by the micro-Kjeldahl method (ref. 4). Aliquots were treated with an equal volume of 10% trichloroacetic acid and centrifuged. The supernatant was digested for 4 hours with 1.5 ml of sulfuric acid, 50 mg of sucrose, 40 mg of mercuric oxide, and 0.5 g of potassium sulfate. The resulting ammonia was liberated by addition of 50% sodium hydroxide, steam-distilled into boric acid, and determined titrimetrically.

Results

The results, listed in table 3, are expressed as the difference in recovery between the enzyme system under test and the digestive control for that run. Calculations in this fashion were found necessary since the material recovered from the digestive enzyme controls showed considerable variation from one experiment to the next. The results with commercial cellulase preparations either alone or in combination with other enzymes differed only to a minor degree from the digestive enzyme controls.

Cellulase from M. verrucaria grown on cellulose, alone and in combination with other enzymes, produced the highest recovery. Its combination with pectinase and pancreatin was the most effective in terms of overall recovery. But in terms of total weight of enzymes added compared with the total weight of protein and carbohydrate recovered, this combination was not as effective as other combinations. Purified Helix cellulase and pectinase were individually effective in increasing the amount of nutrient recovered in comparison with the weight of enzyme added.

DISCUSSION

The assumption that hydrolysis by β-glucosidases would be sufficient to degrade the algal cell wall was negated by the results from chromatography of acid hydrolysates of Chlorella 71105 and a report by Northcote (ref. 12) on the cell wall composition. Northcote found the wall to be composed of two distinct phases: (1) an organized microfibrillar structure made up of α-cellulose, and (2) a surrounding continuous matrix made up of protein, perhaps as a glycoprotein, and hemicellulose. Acid hydrolysis of the α-cellulose yielded glucose, galactose, mannose, arabinose, and rhamnose; while hydrolysis of the hemicellulose yielded the same products except that xylose rather than glucose was found. The analytical methods used for evaluating enzyme activity were such that they could detect the liberation of nutritionally available substances. Thus the methods measured (1) amino acids and peptides too small to be precipitated by trichloroacetic acid and (2) the increase in reducing sugars in soluble carbohydrate substances, i.e., utilizable material. Because of the complex nature of the cell wall, a mixture of enzymes was expected to be more effective for degradation than a purified enzyme capable of acting on only a single class of substrates. In addition, the complex polysaccharides of the cell interior may also be refractory to digestion by normal human digestive enzymes and may require supplementation for complete utilization.

Table 3

RECOVERY OF UTILIZABLE PROTEIN AND CARBOHYDRATE FROM 200 MG OF CHLORELLA 71105 UPON DIGESTION WITH VARIOUS ENZYMES FOR 4 HOURS AT 35 °C

Cellulase	mg	Enzyme Added	mg	Material Recovered* Protein, mg	Carbohydrate, mg
From snail, crude	5	None		4.4	1.8
	5	Lysozyme	5	5.6	2.2
	5	Pepsin	5	10.6	10.2
		Pectinase	5		
	5	Pancreatin	5	11.0	6.4
		Trypsin	5		
		Pectinase	5		
From snail, purified	5	None		5.6	5.0
	5	Lysozyme	5	7.8	4.2
	5	Pectinase	5	7.6	8.8
	5	Pectinase	3	-9.2	4.8
	5	Pectinase	1	-2.8	4.2
	3	Pectinase	5	-3.2	7.0
	1	Pectinase	5	-6.2	5.6
	3	Pectinase	3	-5.8	3.6
	1	Pectinase	1	-3.6	7.2
From mold grown on algae	5	None		-3.6	0.0
	5	Pectinase	5	-1.4	5.0
	5	Pectinase	3	-8.0	4.2
	5	Purified snail	3	-5.4	2.2
	5	Lysozyme	3	4.2	2.0
From mold grown on cellulose	5	None		8.2	8.8
	5	Lysozyme	5	4.6	8.4
	5	Pepsin	5	6.2	14.4
		Pectinase	5		
	5	Pancreatin	5	11.8	17.8
		Trypsin	5		
		Pectinase	5		
	5	Purified snail	3	1.6	10.4
	5	Pectinase	5	7.8	13.4
	5	Pectinase	3	5.0	10.4
	5	Pectinase	1	0.0	8.8
	3	Pectinase	5	0.0	12.0
	1	Pectinase	5	-2.2	8.4
	3	Pectinase	3	-4.4	6.4
	3	Pectinase	1	-3.0	5.8
	1	Pectinase	3	3.2	0.0
	1	Pectinase	1	2.0	2.6
None		Pectinase	5	8.4	6.0
Takamine 4000	5	None		-0.2	1.2
Hemicellulase (Wallerstein)	5	None		1.0	-0.2
Hemicellulase (Nutritional Biochemical)	5	None		5.2	0.2

*Expressed as the difference between the test system and the control system for that run.

The over-all results of this study support the assumption that enzymes that show activity toward complex cell wall polysaccharides are most likely to be effective in our Chlorella test system. No increase in utilizable material was produced by the commercial cellulase preparations. On the other hand, the snail cellulase, the mold cellulase, and pectinase were effective individually and in various combinations. The yield of additional utilizable substances surpassed the weight of added enzyme, thereby compensating for the additional input required.

In summary, the use of enzyme additives in an algal diet for human nutrition appears to offer a means of obtaining maximal nutritional benefit. However, the usefulness of these additives must be demonstrated in whole-animal feeding studies and the additives must be nontoxic.

REFERENCES

1. Ballentine, R., Methods in Enzymology, Vol III, p 984, Academic Press, New York, 1957.

2. Cronshaw, J., A. Myers, and R. D. Preston, "A Chemical and Physical Investigation of the Cell Walls of Some Marine Algae," Biochim. et Biophys. Acta, Vol 27, pp 89-103, 1958.

3. Frampton, V., et al., "Quantitative Application of the Kiliani Reaction," Anal. Chem., Vol 23, p 1244, 1951.

4. Gomori, G., Methods in Enzymology, Vol I, p 138, Academic Press, New York, 1955.

5. Grassman, L., et al., "Über den Enzymatischen abbau der Cellulose und ihrer Spaltprodukte," Ann., Vol 503, p 167, 1933.

6. Hirst, E. L., "Polysaccharides of the Marine Algae," Proc. Chem. Soc., pp 177-187, 1958.

7. Iriki, Y., and T. Miwa, "Chemical Nature of the Cell Wall of the Green Algae, Codium, Acetabularia, and Halicoryne," Nature, Vol 185, pp 178-179, 1960.

8. Karrer, P., "Der Enzymatische abbau von Native und umgefallter Zellulose von Kunstseiden und von Chitin," Kolloid Z., Vol 52, p 304, 1930.

9. Keilin, J., "Helicorubin and Cytochrome h," Biochem. J., Vol 64, p 663, 1956.

10. Myers, A., and R. D. Preston, "Fine Structure in the Red Algae. II. Structure of the Cell Wall of Rhodymenia palmata," Proc. Roy. Soc. London, Vol B-150, pp 447-455, 1959.

11. Norman, A. G., "Chemistry of the Carbohydrates and Glycosides," Ann. Rev. Biochem., Vol 10, pp 65-90, 1941.

12. Northcote, D. H., K. J. Goulding, and R. W. Horne, "Properties of Cell Wall of Chlorella pyrenoidosa," Biochem. J., Vol 70, p 391, 1958.

13. Peat, S., "Carbohydrates of the Red Alga, Porphyra umbilicalis," J. Chem. Soc., pp 1590-1595, 1961.

14. Pigman, W. W., and R. M. Goepp, Jr., Chemistry of the Carbohydrates, p 529, Academic Press, New York, 1948.

15. Whitaker, D. R., "Purification of Myrothecium verrucaria Cellulase," Arch. Biochem. Biophys., Vol 43, p 253, 1953.

16. Worthington Biochemical Corp., "Glucostat," Freehold, N.J.