




1972

## The chemical analysis of *Velella Lata* float

Ralph Lee Gainey  
*University of the Pacific*

Follow this and additional works at: [https://scholarlycommons.pacific.edu/uop\\_etds](https://scholarlycommons.pacific.edu/uop_etds)

 Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

---

### Recommended Citation

Gainey, Ralph Lee. (1972). *The chemical analysis of Velella Lata float*. University of the Pacific, Thesis. [https://scholarlycommons.pacific.edu/uop\\_etds/1772](https://scholarlycommons.pacific.edu/uop_etds/1772)

This Thesis is brought to you for free and open access by the Graduate School at Scholarly Commons. It has been accepted for inclusion in University of the Pacific Theses and Dissertations by an authorized administrator of Scholarly Commons. For more information, please contact [mgibney@pacific.edu](mailto:mgibney@pacific.edu).

THE CHEMICAL ANALYSIS OF  
VELELLA LATA FLOAT

A Thesis

Presented to

the Faculty of the Graduate School

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Ralph Lee Gainey

November 1972

## ACKNOWLEDGEMENT

The author wishes to acknowledge:  
the courage of Dr. Otis Shao, who opened the door; the  
faith of Dr. Emerson Cobb, who kept it open; and  
the dedication of Dr. Paul Gross, who gen-  
erously provided space in his labor-  
atory and patiently demonstr-  
ated its effective use  
in the direction  
of this work.

## DEDICATION

This thesis is dedicated to:

Dr. Manfred Rimpler

of Hannover, Germany, whose encouragement  
and technical guidance are appreciated

more than he knows;

and to:

Carol

my wife, who knows.

## TABLE OF CONTENTS

CHAPTER	PAGE
1. Introduction . . . . .	1
A. General Considerations of Molecular Architecture . . . . .	1
B. Components of Connective Tissue . . . . .	3
C. Investigation of Chitin . . . . .	5
D. <u>Verella lata</u> . . . . .	7
2. Experimental Procedure . . . . .	11
A. Determination of Moisture Content . . . . .	13
Summary . . . . .	15
B. Determination of Inorganic Content . . . . .	16
Summary . . . . .	20
C. Determination of Ether Soluble Material . . . . .	21
Summary . . . . .	22
D. Determination of Carbohydrate Content . . . . .	23
Summary . . . . .	34
E. Determination of Protein Content . . . . .	36
Summary . . . . .	45
3. Brief Summary of Results . . . . .	46
4. Discussion of Results . . . . .	50
A. Moisture Content . . . . .	50
B. Inorganic Content . . . . .	53
C. Lipid Content . . . . .	58
D. Carbohydrate Content . . . . .	60
E. Protein Content . . . . .	64
5. Conclusion . . . . .	71
References . . . . .	76
Appendix . . . . .	82

## LIST OF FIGURES

FIGURE	PAGE
1. <u>Veilella lata</u> . . . . .	8a
2. Relationship of Cell Layers to Float . . . . .	8a
3. Orthogonal Projection of <u>Veilella</u> Float . . . . .	8b
4. Detail of Horizontal and Vertical Margins . . . . .	8b
5. The Time Dependence of the Water Uptake of Dried KOH-Treated Float Material . . . . .	15a
6. Determination of Optimum Hydrolysis Conditions . . . . .	28a
7. Colorimetric Determination of Carbohydrate . . . . .	29a
8. Thin Layer Chromatography . . . . .	32a
9. Hypothetical Macromolecule Containing Six Lysine- Linked Octasaccharides . . . . .	99
10. Hypothetical Construction of <u>Veilella lata</u> Float Based on a Topologically Continuous Concentric Membrane Model . . . .	100

LIST OF TABLES

TABLE	PAGE
1. Amino Acid Results . . . . .	39
2. Protein Found in Amino Acid Analysis . . . . .	40
3. Calculation of Protein and Carbohydrate Ratio . . . . .	40
4. Lysine Residue in Standards and KOH-Treated Material . . . . .	42
5. Ratios of Lysine to Other Amino Acids . . . . .	43
6. Mole Ratio of Lysine to Carbohydrate . . . . .	43
7. Mole Ratio of Protein to Carbohydrate . . . . .	44
8. Systematic Identification of <u>Verella lata</u> . . . . .	97
9. Phyla of the Animal Kingdom . . . . .	98

LIST OF INFRARED SPECTRA

SPECTRUM	PAGE
1. N-acetylglucosamine	(a)* . . . . . 83
	(b) . . . . . 84
2. Glucosamine hydrochloride	(a) . . . . . 85
	(b) . . . . . 86
3. Shrimp Shell	(a) . . . . . 87
	(b) . . . . . 88
4. <u>Verella</u> Float Material (6N KOH, 8 days at 25°C)	(a) . . . . . 89
	(b) . . . . . 90
5. <u>Verella</u> Float Material (1N KOH, 24 hr at 100°C)	(a) . . . . . 91
	(b) . . . . . 92
6. Crystalline Material from Acid Hydrolysate of <u>Verella</u> Float Material	(a) . . . . . 93
	(b) . . . . . 94
7. <u>Verella</u> Float Material (washed and dried)	(a) . . . . . 95
	(b) . . . . . 96

\*(a) 500-1200  $\text{cm}^{-1}$

(b) 1200-3500  $\text{cm}^{-1}$

"Thus, the cellular function of the multi-cellular organism cannot be understood fully without complete knowledge of the structure and function of the (extracellular) matrix."

Endre A. Balazs<sup>1</sup>

## INTRODUCTION

### A. General Considerations of Molecular Architecture

Our universe exhibits a unity which extends to all observable phenomena, and allows us to confidently propose the structure of the hydrogen molecule as the basis for a code to communicate with intelligent beings anywhere within it. This unity is expressed nowhere more dramatically than in the world of living things, where all of the physical laws are obeyed and a continuous spectrum of molecular evolution exists. A comparison of proteins from different animals shows amazing similarities in the amino acid sequences of molecules engaged in similar functions (i.e. the oxygen carrying molecules, pituitary hormones, and various insulins).<sup>11</sup> The more closely related the two animals, the more similar the proteins are. The distinction between two species is primarily based on macroscopically observable differences in shape and pigmentation, which we expect to be caused by molecular variation at the site. Differences in behavior are usually considered an adaptation to the environment and in lieu of shape and pigment differences are seldom sufficient for the discrimination into a new species or subspecies. Examination of biological structure reveals a hierarchy of levels of organization from the small to the large and the simple to the complex. In cotton, for example, the



organization is pentafid: glucose molecule, fibril, microfibril, macrofibril, and cotton hair.<sup>32</sup>

Everything should be ultimately explainable in terms of the components of which it is composed. In the animal kingdom well over ninety-five percent of all recognized species are invertebrates, a distinction based on connective tissue, though most of our efforts have been directed towards vertebrates for reasons of convenience and closeness to man. Investigation of the various chemicals which make up plants and animals has been diverse, allowing us to get a general picture of what molecules to expect at each stage of the evolutionary progression, but for no animal is the information complete, and for invertebrates the information is particularly sketchy. It would be convenient to have certain animals and plants completely known chemically, so that biological molecules subsequently discovered could be compared to these standards. Velella lata may be a candidate for such a standard, having a number of unique advantages. It occurs in the evolutionary chain at that point where a true multicellular animal begins, and it is the simplest animal from which genetically determined internal structural material is conveniently isolated and purified.

In plants the connective tissue is considered to be almost entirely carbohydrate polymer and in vertebrates the connective tissue is considered to be almost entirely protein (amino acid polymer), while many invertebrates have almost an even mixture of protein-carbohydrate in an unknown relationship. Velella presents an oppor-

tunity to examine closely the nature of the protein and its relationship to the carbohydrate.

The carbohydrate of various invertebrate connective tissue matrices has been extensively studied and the structure and linkages reasonably well defined. Some questions remain regarding the length of the smallest oligosaccharide and the manner in which these are built up into higher levels of organization. Inorganic material (primarily in the form of calcium), lipid in small amounts, and moisture are also present in the invertebrate matrix. While calcium is generally considered to be in the form of the carbonate, the possibility that it is not all in this form awaits investigation, and the recent report that silicon is essential in the formation of chick connective tissue is interesting.<sup>5</sup> Lipids are not a major component in connective tissues studied to date, and this is true of Velella also. This does not preclude, however, the possibility that lipids may play some role in the formation of membrane-like sheets which are observed in the laminations of various mollusk-arthropod matrices. Velella offers an opportunity to examine invertebrate connective tissue in an intense way to gain an understanding of the morphology and developmental dynamics which hopefully would be extensible to the vertebrate matrix.

#### B. Components of Connective Tissue

The present picture of biological activity indicates that all events originate in the genetic code of phosphate-sugar polymers in the cell nucleus. The code is apparently only for proteins, to the exclusion of lipids, carbohydrates and other non-protein molecules. The most obvious visible expression of this genetic code is shape

and pigmentation, and the presence of non-protein molecules in the matrix leaves a gap in our understanding. The mediation of proteins (especially as enzymes) in species-specific events is observed in the biosynthesis of matrix precursors and is expected to determine in some fashion the final products. Self-assembly (aggregation, polymerization, crystallization) is a common phenomenon of cellulose,<sup>32</sup> chitin,<sup>17,18</sup> and collagen,<sup>15,44</sup> the matrix materials of widest distribution. The self-assembly of both intracellular and extracellular "organelles" is receiving increasing attention. Only two or three "forms" of chitin are organized into a great variety of shapes, and one is tempted to account for this anomaly in terms of an attached protein moiety. Can the extracellular self-assembly of specific macromolecules account entirely for the specificity of the matrix? To what extent is the adjacent cell layer a factor? Cell surface proteins are involved in the aggregation of dissociated cells<sup>29,47</sup> and specific carbohydrates are identified with the irregularities exhibited by neoplastic cells.<sup>29</sup> Glycoproteins are commonly isolated from matrix materials.<sup>27,36</sup> Surely these facts are related in some way. The golgi apparatus apparently functions to synthesize or organize these protein-carbohydrate complexes<sup>38,56</sup> and this suggests a pathway between DNA and final shape. If this sequence is generally accurate it should be possible to define in detail the reactions involved and demonstrate the presence of the necessary intermediates and enzymes. Velella appears to be an animal sufficiently simple to facilitate this, yet complete enough to illustrate the generality of the scheme; a solid matrix is present without problems of isolation and purification. The related "protein"

may be polymerized and branched in an intricate way (e.g. by disulfide and  $\epsilon$ -lysine crosslinks) so that what we conceive as an individual amino acid polymer becomes a monomer at a higher level of organization. It is thus of interest to examine in detail the invertebrate matrix proteins, of which resilin, arthropodin and sclerotin have so far been isolated and studied. The protein from Velella lata float, which may be called "velatin", can now be added for comparison. The sequencing of these structural proteins may reveal the phylogenetic progression we have learned to expect.

### C. The Investigation of Chitin

The history and occurrence of chitin have been recently reviewed.<sup>19</sup> The identification of the carbohydrate moiety as the  $\beta$ , 1 $\rightarrow$ 4 linked polymer of N-acetylglucosamine seems well established.<sup>25,40</sup> Not all of the peaks in the ir spectrum have been identified,<sup>35</sup> but the absorption at  $891\frac{1}{2}$   $7\text{ cm}^{-1}$  is characteristic of the beta linkage.<sup>35</sup> The purest form was found in the internal skeleton of the squid.<sup>19</sup> Chitin was found to give three distinct x-ray diffraction patterns which were designated alpha, beta and gamma. Beta and gamma were convertible into alpha.<sup>18,19</sup> The beta form was interpreted to indicate the monohydrate, with the water molecule bound between the C-3 of one N-acetylglucosaminyl unit and an hydroxymethyl group in an adjacent chain.<sup>10</sup>

Elemental analyses have not settled the degree of acetylation. However, Hunt found no free amino groups using dinitrophenylation.<sup>19</sup>

The aggregation of chitin with various peptides and amino acids in

solution was studied and the complexes formed were found to be sensitive to ionic strength and pH, completely dissociating above pH 9.<sup>17</sup>

The general occurrence of a protein moiety tightly associated with chitin has been found and studied in attempts to identify the nature of the linkage and the amino acid(s) involved.<sup>19</sup> Asparagine and histidine were found still associated after 60 hr in 1N NaOH at 100°C,<sup>18</sup> and chitin dissolved in saturated lithium thiocyanate contained protein when reprecipitated by the addition of acetone.<sup>13</sup> Several studies indicated that the carbohydrate-protein linkages identified so far invariably involve the anomeric carbon of the carbohydrate.<sup>19</sup>

The oligosaccharide length has been studied in depth.<sup>2,4,40</sup> Column chromatography of acid hydrolysates separated the first seven homologous oligosaccharides.<sup>2</sup> Polarized ir evidence supports x-ray powder diagrams which gave a repeat distance of 41 Å for the axial direction, corresponding to eight N-acetylglucosaminyl units.<sup>42</sup>

X-ray powder diagrams were interpreted to indicate that the chains are oriented so that adjacent chains are in the same direction, but that alternate pairs of chains are oriented in the opposite direction.<sup>6</sup> This interpretation was supported by polarized ir work which showed a lack of free OH and NH groups and no C=O...HO bonding.<sup>7</sup> A structure occurred in the powder diagrams from a variety of alpha and beta chitins at repeat distances of 31 Å, corresponding to six monomers.<sup>42</sup> This repeat survived boiling in benzene for 40 hrs. Electron micrographs seemed to show a regular hexagonal array of carbohydrate surrounded by protein.<sup>42</sup> Gamma chitin showed the repeat of 41 Å and was

interpreted to indicate a slightly different spatial array, and removal of the protein gave the usual alpha chitin pattern after spontaneous aggregation.<sup>42</sup>

There are similarities between collagen and chitin. Invertebrate collagens high (10-15%) in carbohydrates<sup>16</sup> and hydroxylysine linkages<sup>50</sup> have been found. A glycoprotein is reported to be always linked to collagen<sup>21,27</sup> and protects it from collagenase in the same way that the protein of chitin protects it from chitinase and chitobiase.<sup>22,36,52</sup> Lysine has been implicated in many of these papers, especially in its ability to combine with itself to form a crosslink. A deaminated crosslink has been proposed,<sup>14</sup> and the precursor of such a crosslink (alpha-amino-adipic acid-delta-semialdehyde; an aldol condensation product) has been found in tropocollagen.<sup>28</sup> A lysine tie using lysinonorleucine (two lysines condensed) has been suggested,<sup>14</sup> and four lysines were found to combine into desmosine and isodesmosine, which were isolated from an acid hydrolysate of elastin.<sup>14,34</sup> Elastin contains a large amount of lysine.<sup>43,49</sup> A macromolecule identified as glucosylgalactosylhydroxylysine is reported "widely distributed throughout the animal kingdom as the carbohydrate moiety of collagen."<sup>24</sup> The present study supplements many of these findings in an interesting way.

#### D. Velella lata

The full systematic identification of Velella lata is given in the Appendix (Tables 8,9). It is a coelenterate (Order hydroida)

which occurs in the Pacific Ocean as the pelagic, free-floating medusa (figure 1).<sup>23</sup> It is generally considered to be a colony of polyps (cup-like forms).<sup>20</sup> It appears as a small (3-5 cm) pigmented jellyfish and has the common names "purple sailor" and "by-the-wind sailor".

Velella is rarely found in the intertidal region but onshore storms often sweep the surface-dwelling animals onto the beaches of N. California and Oregon, and the dried floats are sometimes found in rows several inches thick along the beach for miles. Masses of Velella have been reported at sea to extend for 260 km.<sup>23</sup> References for the complete distribution were not found, but world-wide ten species are known.<sup>23</sup> Porpita is a closely related animal also having a disc-shaped float, but without the vertical "sail" component. The orientation of the sail is 40° to the wind (identical to Physalia...the Portugese man-of-war) and is the sole means of transportation. The survival value of this structure may be inferred from the discovery of Velella floats in rocks dated 450 million years old.<sup>8,12</sup> The occurrence of solid structural material in the lower animals is rare and the presence of the invertebrate analogue of bone in a "jellyfish" is strange indeed. Invertebrates commonly exhibit what is generally termed an "exoskeleton", while Velella lata has a "float" (figure 2,3) which is completely internal. The formation has been viewed as an exoskeleton formed internally by a folding over and inward of the external cell layer. The float material as found on the beach is a light tan or dull white, turning yellow in the sun. The yellowing is associated with the protein moiety and when it is removed the carbohydrate is a

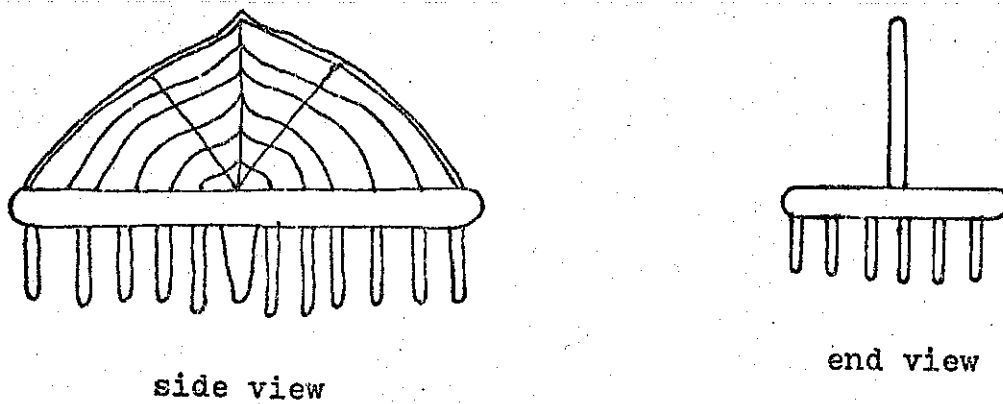


Figure 1. Velella lata

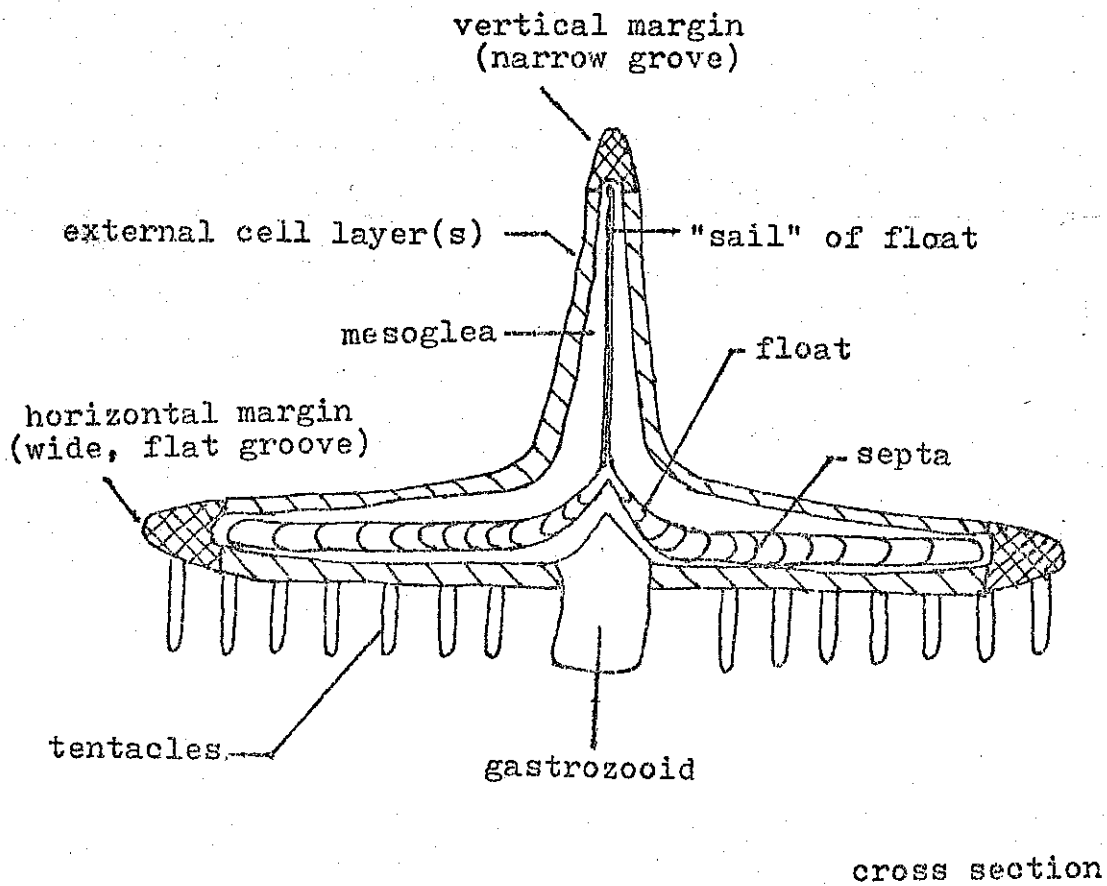


Figure 2. Relationship of Cell Layers to Float



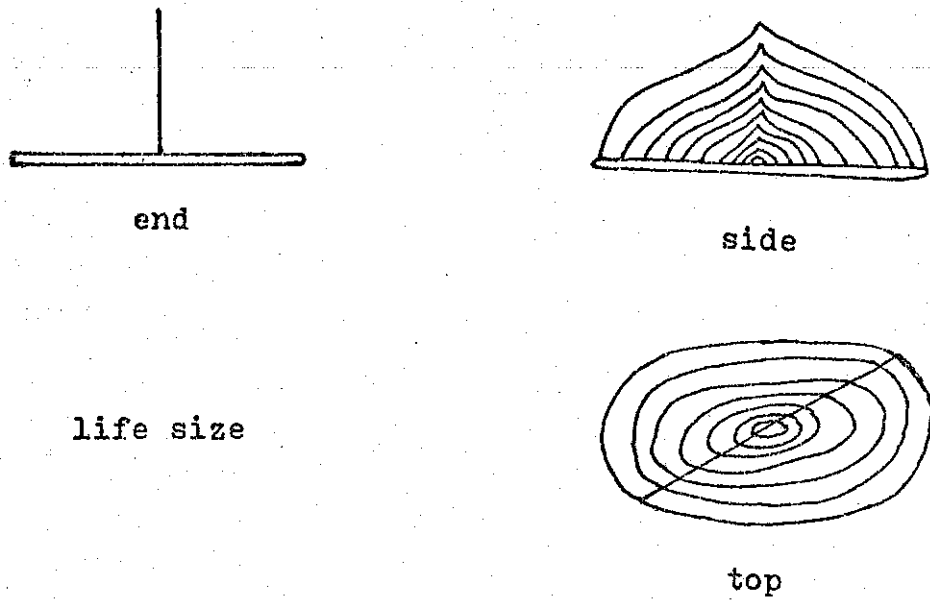


Figure 3. Orthogonal Projection of Velella Float

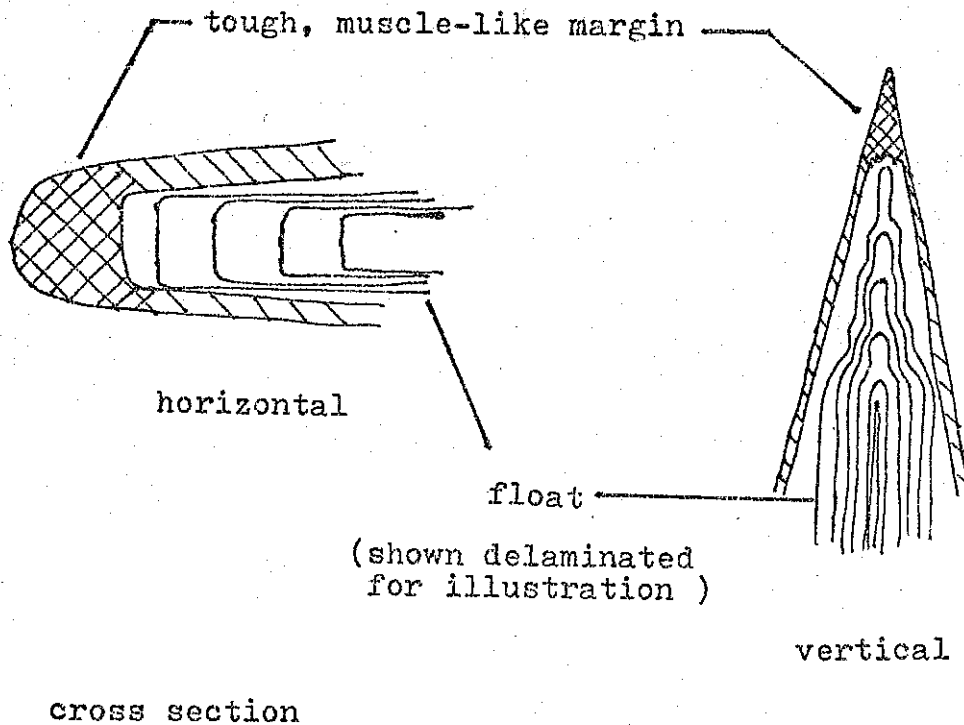


Figure 4. Detail of Horizontal and Vertical Margins

clear dull white. The untreated float gives a negative Molisch test for carbohydrate. The float from formalin-preserved specimens separates easily from the fleshy tissue much like a foot from a shoe, with no apparent connection to the adjacent cell layers. Careful examination did not reveal any connection to the fleshy tissue, nor any associated membrane systems. The float material seems to be laid down in layers in a cyclical manner. The edges of the float are extended by an increase in the diameter of the muscle-like peripheral tissue, which Hyman states is contractile.<sup>20</sup> Examination of floats separated from formalin-treated specimens revealed that the float is constructed by the laying down of flat sheets of material, each slightly larger than the last, resulting in a concentrically symmetrical structure with concentric lines where the overlap between layers occurs (figure 4). The formalin-treated float material readily "peels" apart like an onion, revealing the identical, though smaller, float beneath it. The layers of the vertical component are continuous with those of the horizontal component so that the synthetic activity of the entire cell layer seems coordinated. The horizontal component differs from the vertical in that it contains monolayered septa which result from the flat surface of the horizontal marginal groove (figure 4). The vertical edge is sharp, and apparently the float is bilaminar along the vertical ridge, although these layers could not be separated (figure 4). It appears that the entire adjacent cell layer is engaged in synthesizing float material subunits and these then spontaneously orient (self-assemble?) themselves into the flat sheets which adhere smoothly

to the previous layer (figure 10). Growth apparently involves a cycle of mitosis-synthesis, which may be circadian. Such light-related phenomena have been reported in the biosynthesis of chitin in an arthropod skeleton.<sup>30</sup> The ubiquitous distribution of a purple pigment in invertebrates suggests the possibility of an energy-trapping system such as occurs associated with the green chlorophyll pigment of plants. Photosynthesis is sometimes defined as the direct utilization of light energy for the biosynthesis of carbohydrate polymers.<sup>51</sup> Since chitin is a carbohydrate, might not such a system be suspected? Enzymes in a suggested carbohydrate biosynthetic pathway<sup>19</sup> are indirectly light sensitive (by their ATP requirements) and aequorin, a calcium-specific protein which occurs in other jellyfish, bioluminesces in the presence of free calcium<sup>48</sup> - also a component of the matrix. The float is central to all of these questions and its chemical analysis forms the basis of the present study.

## EXPERIMENTAL

### Preface

Weights were taken on a Mettler analytical balance (type H15) provided with silica gel desiccant in the weighing chamber. Crucibles and glassware used in weight determinations were pre-rinsed in a  $H_2SO_4$  mixture. All glassware was cleaned by washing with detergent, rinsing with distilled water and with an acetone/methanol mixture and dried at  $110^\circ C$  in the oven for at least ten minutes. Weights were taken after the crucibles and glassware had cooled to constant weight in the weighing chamber. Infrared spectra were taken on a Perkin-Elmer 337 spectrophotometer, on KBr pellets at a concentration of 1-2mg/100 mg KBr. Amino acid analyses were carried out on a Bio-cal 201 Amino Acid Analyzer, using the single column technique. Tlc was carried out on glass plates coated with silica gel G and GF (2:1) by developing in one of two solvent systems:

System #1;  $CHCl_3$ , MeOH, EtOH (90/5/5)

System #2; n-propanol, ethyl acetate,  $NH_4OH$  (25%),  $H_2O$  (80/10/10/30)

Spots were visualized by spraying with  $H_2SO_4$  (10% in MeOH) and charring in an oven at  $125-150^\circ C$ . Elemental analyses were done by Microanalytisches Laboratorium Beller, Göttingen, Germany. Melting points were determined on a Thomas-Hoover "Unimelt" apparatus and are uncorrected. In vacuo means less than 0.1 mm Hg unless otherwise specified. "Wig-L-Bug" is made by Crescent Dental Manufacturing Co., Chicago, Illinois. The words "float" and "float material" are used interchangeably and refer to the entire float without distinction between the vertical and

horizontal components. The material described is that which is easily separable from the intact animal and survives washing with water.

## CHAPTER 2

### EXPERIMENTAL PROCEDURE

#### PART A

#### Determination of Moisture Content

##### Whole Float

Over CaCl<sub>2</sub>. Approximately 2 g of diced float material were carefully washed in tap water and dilute HCl (1N) to free it of sand and extraneous material. After being rinsed in distilled water until neutral, the float material was allowed to dry in air until the weight was constant. In a crucible the float material (1.8696 g) was then placed in a constant temperature desiccator over CaCl<sub>2</sub> at 60°C at less than 0.1 mm Hg. Two days later the material was allowed to cool to room temperature and was quickly weighed. The loss of weight (0.1927 g) was 10.30% of the weight of the float material before drying.

Over P<sub>2</sub>O<sub>5</sub>. Whole float material (0.9184 g) which had been diced, washed and dried in air as above, was placed in a test tube (constant weight), and dried over P<sub>2</sub>O<sub>5</sub> at 65°C and less than 0.1 mm Hg. After 67 hrs the float material was weighed at room temperature (0.7851 g). The loss of weight (0.1333 g) was 14.5% of the weight of the float material before drying.

KOH Treated Float Material

Over P<sub>2</sub>O<sub>5</sub>. Approximately 0.1 g of float material which had been freed of protein by the Rudall method (1N KOH at 100°C for 24 hr), washed in distilled water until neutral, and dried in the lab atmosphere to constant weight, was added to one vial. After weighing, three vials with float material were dried over P<sub>2</sub>O<sub>5</sub> at 65°C and less than 0.1 mm Hg. After 75 hr heating, the float material was allowed to cool to room temperature in the drying pistol. After the material was removed, it was quickly weighed. The weight loss of each vial, calculated as a percentage of the weight of the float material was: #1 - 14.14%; #2 - 14.10%; #3 - 15.3%.

Resorption of Moisture by KOH Treated Float Material. Vials #2 and #3 were covered with aluminum foil with small holes and kept in the lab atmosphere. The vials were weighed periodically:

<u>Time in air</u>	<u>Resorbed water (% of original loss)</u>	
	<u>Vial #2</u>	<u>Vial #3</u>
4 hr	40.5%	38.8%
10 hr	70.5%	74.1%
79 hr	78.5%	81.5%

The two vials were then redried over P<sub>2</sub>O<sub>5</sub> in vacuo at 65°C as before for 46 hr. After cooling to room temperature, the weights were the same as on the previous drying. Again the two vials were allowed to stand in the lab atmosphere as before and the weights periodically taken:

<u>Time in air</u>	<u>Resorbed water (% of original loss)</u>	
	<u>Vial #2</u>	<u>Vial #3</u>
24 hr	75.8%	77.0%
49 hr	83.3%	85.0%
82 hr	82.0%	83.8%
32 days	106.0%	104.0%

A graph of the resorption data was prepared (figure 5), which indicated non-linear resorption.

### Summary

#### Loss on drying of whole float material

Over  $\text{CaCl}_2$ , 48 hr, 60°C in vacuo 10.3%

Over  $\text{P}_2\text{O}_5$ , 67 hr, 65°C in vacuo 14.5%

#### Loss on drying of KOH treated float material

Over  $\text{P}_2\text{O}_5$ , 75 hr, 65°C, in vacuo 14.1%

14.1%

15.3%



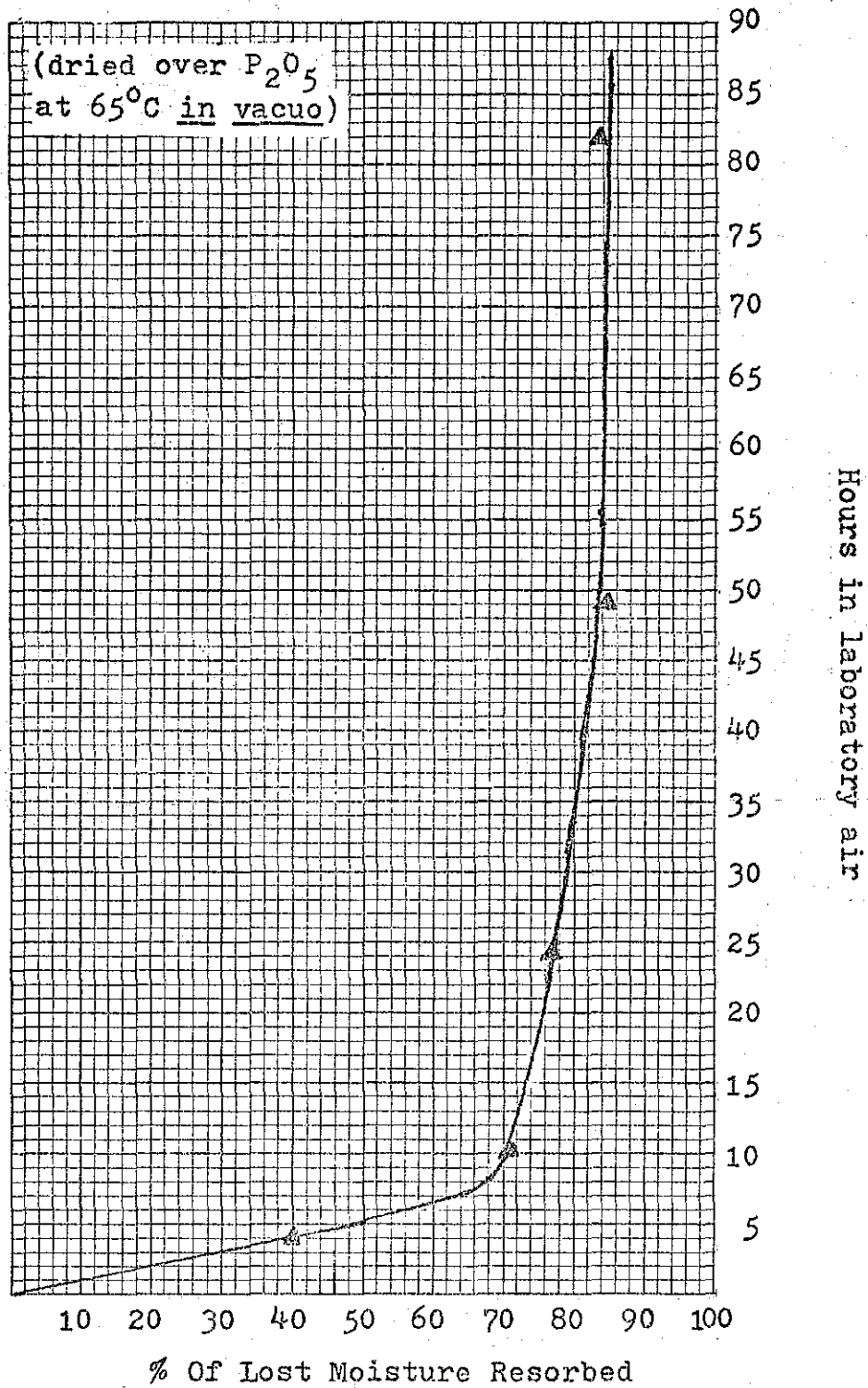


Figure 5

THE TIME DEPENDENCY OF THE WATER UPTAKE  
OF DRIED KOH-TREATED FLOAT MATERIAL.

## EXPERIMENTAL PROCEDURE

### PART B

#### Determination of Inorganic Content

Material Extracted From Whole Float Material With Water. Whole float material which was collected from the beach was washed with vigorous shaking in distilled water only. The water extract was suction filtered and evaporated in a Nickel crucible (heating gently over a bunsen burner) to give 0.2146 g of residue. After ashing over a Meker burner the residue was weighed again (0.1483 g; 69.0% of the dry residue).

Clean crab shell which had been ground to a powder and treated in a manner similar to the float material gave 72.4%.

#### Whole Float Material

Sand Content. Float material was washed in distilled water, followed by decantation of the suspended float material to separate out sand. This process was repeated for a prolonged period, interspersed with manual separation of any wood or other extraneous material. Very thorough washing did not remove sand which was found to be trapped within the float structure. The float material was air dried on aluminum foil. The weighed dry float material was placed in a constant weight crucible, ashed to whiteness and the ash residue was determined. The results of several determinations, expressed as a percentage of the weight of the washed float material, were: 2.8%, 3.0%, 3.7%. If sand was trapped higher percentages up to 14% could be found.

Float Material Extensively Washed in Distilled Water. Float material, extensively washed in distilled water, was cleaned and selected to be free of visible sand grains. It was shaken in a separatory funnel with distilled water, and sand was removed through the stopcock. After blending with distilled water, in a Waring blender, the procedure was repeated. Finally, the material was air dried and placed in a platinum crucible (0.8532 g). The crucible with float material was put into a muffle furnace at 110°C for 15 minutes, after which the temperature was increased to 450°C for 2 hr. After allowing to cool to room temperature, the ash weighed 0.0071 g (0.83% of the weight of the washed and dried float material).

The crucible and ash were returned to the muffle furnace for 1 hr at 550°C and cooled to room temperature. The residue (0.0045 g), expressed as a percentage of the weight of the washed and dried float material, was 0.528%.

The residue was transferred with conc  $\text{HNO}_3$  (20 ml), to a platinum crucible. The solution was heated in the crucible to dryness, allowed to cool to constant weight, (0.0196 g). Conc HF (10 ml) was added and the residue was heated to dryness on an asbestos pad over a bunsen burner flame (natural gas), allowed to cool to constant weight and weighed. The procedure was repeated twice with 5 ml HF until the weight remained constant (0.0096 g). The weight of the residue, expressed as a percentage of the original starting material, was 1.13%.

### Wet Ashing

Acid Digestion. (1.8195 g) of the float material (free of visible sand and other extraneous matter, dried in air) was treated with conc  $\text{HClO}_4$  (10 ml) and conc  $\text{HNO}_3$  (10 ml) and heated until the solution was clear. A small amount of gray material remained suspended. After removal of the acid by heating, the weight of the residue was determined. The residue, expressed as a percentage of the washed and dried float material, was 2.7%, (2.8% after 12 hr in air).

Ashing to Whiteness. The residue from (a) was quantitatively transferred to a platinum crucible, with conc  $\text{HNO}_3$ , and heated to dryness with conc  $\text{HNO}_3$  and conc  $\text{HClO}_4$  (2-3 ml) several times, until the residue was white. The residue, expressed as a percentage of the weight of the washed and dried float material, was 2.9%.

Conc HF Treatment. The white residue from (b) was further treated by adding conc HF (2-3 ml), heating to dryness and cooling to constant weight. This treatment was repeated until the weights became constant (3X). The residue was 2.64% of the weight of the washed and dried float material. Recalculation considering 14% moisture gave 3.1%. The platinum crucible did not change weight during the analysis.

### KOH Treated Float Material

Mild KOH Treatment. Float material (washed, selected to be free of visible sand material) was treated with 1N KOH (100°C for 24 hr), rinsed in distilled water to neutrality, rinsed in acetone (3X), and

allowed to dry in air on aluminum foil. This float material (approximately 0.2 g each) was weighed into three crucibles which were placed in an electric furnace (about 400°C) until the ash was white and the weight constant. The weight of the ash was determined by difference. The results, expressed as a percentage of the weight of the pre-ashed float material, were: 1.84%, 3.60% and 3.21%. The residues were dissolved in hot conc HCl and transferred with washing (3X) to flasks to which ammonium oxalate was added. After several hours, flask A had a barely perceptible precipitate, flask B a copious precipitate and flask C, a moderate precipitate.

Intensive KOH Treatment. Float material which had been kept in 6N KOH for 3 days at 100°C, washed with distilled water until neutral, rinsed with acetone (3X), and allowed to dry in air on aluminum foil, was placed equally in three crucibles and ashed over a Meker burner to constant weight. The weight of the residue was determined by difference and found to be: 3.43%, 3.34%, and 7.67% (red ash).

Desiccated KOH Treated Material. A porcelain crucible was brought to constant weight. Float material, approximately 0.1 g, which had been carefully washed, was treated with 1N KOH (100°C for 24 hr), rinsed with distilled water until neutral, rinsed in acetone (3X), and dried over  $P_2O_5$  at 65°C and less than 0.1 mm Hg, for 24 hr, was placed in the crucible and was quickly weighed (0.1031 g). Now it was ashed to constant weight in an electric furnace between 350-450°C (several hr). The weight of the residue at room temperature was 4.35%.

Summary of Inorganic Determination

(Unless otherwise indicated, all percentages are  
of the air-dry float material)

Aqueous Extract 69.0%

Whole Float Material (Dry Ash)

Washed; visible sand present 2.8%, 3.0%, 3.7%

Washed, blended and sorted; free of  
visible sand

450°C ash 0.8%

550°C ash 0.5%

After HF treatment 1.1%

Whole Float Material (Wet Ash)

Conc  $\text{HNO}_3/\text{HClO}_4$  2.7%

Reweigh after 12 hr 2.8%

After treatment with conc  $\text{HNO}_3$  2.9%

After treatment with conc HF 2.6%

Subtracting 14% moisture factor 3.1%

KOH Treated Float Material

1N KOH (24 hr at 100°C)  
(Oxalate positive for calcium) 1.8%, 3.6%, 3.2%

6N KOH (3 days at 100°C) 3.3%

3.4%

(red ash) 7.7%

Desiccated KOH treated float material 4.4%

EXPERIMENTAL PROCEDURE  
PART C

Determination of Ether Soluble Material

First Determination. Whole float material to be extracted was cleaned as before and dried in air to constant weight. Approximately 2 g was weighed and was carefully transferred to the ether pre-washed cellulose cup of a soxhlet into the extraction apparatus with pure diethyl ether (approximately 75 ml). After 24 hr, the extracted float material was allowed to dry in air on aluminum foil. The extracted float material was weighed and the loss of weight was 0.14% of the pre-extraction weight of the float material. Recalculation after subtracting a factor of 14.5% for moisture content gave a value of 0.17%.

A second determination gave a weight loss of 0.15% of the pre-extraction weight of the float material. After the extraction, the cup and float material were placed in a desiccator over  $P_2O_5$  at  $65^\circ C$  and less than 0.1 mm Hg. After three hr the apparatus was allowed to cool to room temperature and the float material was removed and was quickly weighed. The loss of weight expressed as a percentage of the pre-extraction weight was 13.7%. The loss of weight due to ether extraction expressed as a percentage of the desiccated float material was 0.17%. The ether solution was left for evaporation to constant weight and the remaining light brown syrup was weighed. The weight of the extracted material was determined by difference. The loss of

weight expressed as a percentage of the pre-extraction weight of the float material was 0.23%. The loss of weight expressed as a percentage of the weight of the desiccated float material was 0.27%.

### Summary

#### 1st Determination

Loss in weight of air-dried float material during ether extraction	<u>0.14%</u>
Recalculated after subtracting a 14.5% moisture content factor from the weight of the air-dried sample	<u>0.17%</u>

#### 2nd Determination

Loss in weight of air-dried float material during ether extraction	<u>0.15%</u>
Loss in weight expressed as a percentage of desiccated float material	<u>0.17%</u>
Weight of ether extracted material (direct weight from flask) expressed as a percentage of the air-dried float material	<u>0.23%</u>
Weight of ether extracted material (direct weight from flask) expressed as a percentage of the desiccated float material	<u>0.27%</u>



## EXPERIMENTAL PROCEDURE

## PART D

## Determination of the Carbohydrate Content

Treatment with Base

6N NaOH at Room Temperature. Whole float material which had been washed in tap water, dilute HCl (1N), and rinsed in distilled water until neutral was dried in air. The dry float material (0.5485 g) was agitated with 6N NaOH (10 ml). After 7 hr, a few drops of the clear supernatant were spotted on a silica gel tlc plate. No charring was observed for the spot indicating that no carbohydrate had been solubilized. After 9 days at room temperature, the float material was quantitatively transferred onto a sintered filter, washed with water until neutral (3X), and rinsed with acetone (2X). The sintered filter and contents were dried to constant weight under an infrared lamp. The weight of the NaOH treated float material was determined by difference, and expressed as a percentage of the weight of the air dried starting material (53%; uncorrected for moisture content or inorganic residue).

A sample (approximately 30 mg) of the NaOH treated float material was dissolved in conc HCl (a few ml). Small white needle-shaped particles remained in suspension and were visible only in bright sunlight. The solution was spotted on a tlc plate. The float material extinguished fluorescence, did not move in solvent system #1,

and charred with  $H_2SO_4$ .

The yellowish colored filtrates and water washings of the NaOH treatment were combined and concentrated on a rotovac apparatus. A few drops of the concentrate were spotted on a tlc plate. The spot did not move in solvent system #1 (uv light), and charring with  $H_2SO_4$  gave a light brown spot, which faded with heating time. A small black spot appeared at the solvent front line.

From the original alkaline filtrate a precipitate resulted after 4-5 hr at room temperature.

The acetone washings were concentrated in a rotovac apparatus and subjected to tlc. No fluorescence was observed. A small spot appeared at the front on charring. On standing, a gray precipitate appeared as before.

#### The Effect of Base Treatment on Shape, Refractive Index and Texture

Shape. The shape as found for the float in the live animal, is maintained during treatment with base, whether 6N KOH for a prolonged period at room temperature or 1N KOH for 24 hr is used. On drying there was a slight shrinkage and resultant wrinkling, with minor distortion of the overall shape.

Refractive Index. The whole float material, separated from the fleshy part of the animal, was observed to be almost invisible in sea water. This was also observed when the float was immersed in tap water and base solutions. No alteration is apparent when the float material is kept in strong base for prolonged periods.

Texture. Whole floats, which have been washed and dried, were smooth, flexible and tough, being somewhat difficult to tear with the hands. After treatment with base, followed by rinsing and drying, the texture became less smooth and the material was more brittle and less pliable.

#### Purity of the Base Treated Float Material

Millon Test. Whole float material (washed and dried) was placed in a few ml of conc  $\text{HNO}_3$ . The float material became light red in color. Subsequent addition of conc  $\text{NH}_4\text{OH}$  produced a bright orange color. However, the KOH treated float material (6N KOH, 8 days at  $25^\circ\text{C}$ ) did not change in color from the original dull white when subjected to the same test.

Ninhydrin. Whole float material (washed and dried) was sprayed with fresh ninhydrin solution. The color of the heated float changed to light purple. The KOH (6N, 8 days at  $25^\circ\text{C}$ ) treated float material also produced a color reaction, but the color was only light pink. Glucosamine hydrochloride and N-acetylglucosamine were sprayed with ninhydrin solution on a tlc plate. The glucosamine hydrochloride gave a light pink color, but the N-acetylglucosamine gave no color reaction, as expected.

Amino Acid Analysis. Whole float material (washed and dried) was hydrolyzed in 6N HCl and placed on the column of an amino acid analyzer. The analysis indicated a high protein content (see section on protein determination). Material which had been treated with 6N KOH for 8 days was analyzed in the same manner, and was found to be free of amino acids (except for a small lysine peak).

Determination of Carbohydrate/Protein Ratio in Whole Float Material

Starting Material. Whole float material, approximately 0.5 g each, washed and dried in air, was put into two test tubes, and dried over  $P_2O_5$  ( $65^\circ C$ , in vacuo, 24 hr). The drying apparatus was allowed to cool to room temperature and the weight of the starting float material was quickly determined by difference (0.5195 g and 0.4847 g). The float material was quantitatively transferred to two round-bottom flasks and was heated (28 hr) in refluxing 1N KOH (17 ml) with occasional shaking. The contents were quantitatively washed into two tared Gooch crucibles, washed with 1N KOH (2X), distilled water until neutral (3X), acetone (3X), and placed in a warm oven until the odor of acetone disappeared. After drying over  $P_2O_5$  at room temperature in vacuo (40 hr, 20mm Hg) the weight of the KOH treated float material was determined by difference. The loss of weight during KOH treatment, expressed as a percentage of the weight of the washed and dried whole float material, was 40.9% and 39.8%. Conc HCl was added to each crucible (mounted on a vacuum filter flask) to dissolve the float material. After 2 1/2-3 hr, the dissolved float material was suction filtered through the bottom of the crucible. After washing with conc HCl, the crucibles and remaining sandy residue were heated at  $110^\circ C$  for 30 min, cooled to constant weight and the weight recorded. The weight of the sand and residue, expressed as a percentage of the weight of the original whole float material, was 10.7% and 10.0%.

Summary of Results

	Actual Weights Recorded		Expressed as a percentage of the Starting Mtl.			
	(in grams)		including residue		excluding residue	
	#1	#2	#1	#2	#1	#2
Sand and residue	0.0557	0.0489	10.7%	10.0%		
Loss on KOH digest	0.2123	0.1941	40.9%	39.8%	<u>45.8%</u>	<u>44.3%</u>
Carbohydrate	0.2512	0.2442	48.4%	50.2%	<u>54.2%</u>	<u>55.7%</u>

(all values uncorrected for soluble  
inorganic material)

Acid Hydrolysis of KOH Treated Float Material

Method of Hydrolysis. Pyrex tubes (inner diameter 5-6 mm) approximately 18 cm long were cleaned, dried, sealed at one end, and provided with a narrow neck on the other end. These ampoules were cleaned again and dried at 110°C. Float material (approximately 2 mg), treated with KOH, and dried over  $P_2O_5$  at 65°C in vacuo for 24 hr, was placed in the tube and the accurate weight of the float material was determined by difference. 6N HCl was added through a finely drawn glass funnel until the height in the tube was 5-6 cm. The contents were frozen in shaved dry ice and the narrow neck of the ampoule was sealed after evacuation of the tube (20 mm Hg). The ampoules were stored frozen until hydrolyzed. Hydrolysis was carried out by immersing the ampoules in a vigorously boiling water bath. Three ampoules were hydrolyzed 1, 2 and 3 hr. The contents were diluted in volumetric flasks to 50 ml and colorimetrically determined (described below) for maximum recovery of hexosamine. For storage the solutions were frozen. A second set of samples was

hydrolyzed and colorimetrically determined in the same way. When obvious errors were discovered during the analysis, the ampoules were discarded. A graph was prepared (figure 6) indicating the optimum hydrolysis time of 3 hr.

#### Colorimetric Determination of D-glucosamine

Equipment. The colorimetric determinations were made according to the method of Rondle and Morgan<sup>39</sup>, using a B&L Spectronic 20. The test tubes used were chosen for optical match by sequentially transferring a dilute  $\text{KMnO}_4$  solution to each test tube and observing the transmittance at the test frequency, 530 mu. Test tubes selected had a similar % Transmittance within 0.5%. The readings were taken from a digital readout apparatus furnished by B&L. The Spectronic 20 and the digital readout were supplied by a regulated 110V power supply. Readings were not made prior to at least 30 min warmup.

Reagents. The acetylacetone was freshly distilled before the start of the determinations. All of the determinations were made within two months of the distillation and the acetylacetone was stored in a brown bottle at room temperature during this time. The ethanol was taken directly from the container of the supplier and was not denatured. The p-Dimethylaminobenzaldehyde had a higher m.p. than recommended by Rondle and Morgan and was not recrystallized. The DMAB reagent solutions were stored at  $-10^\circ\text{C}$  in 50 ml portions.

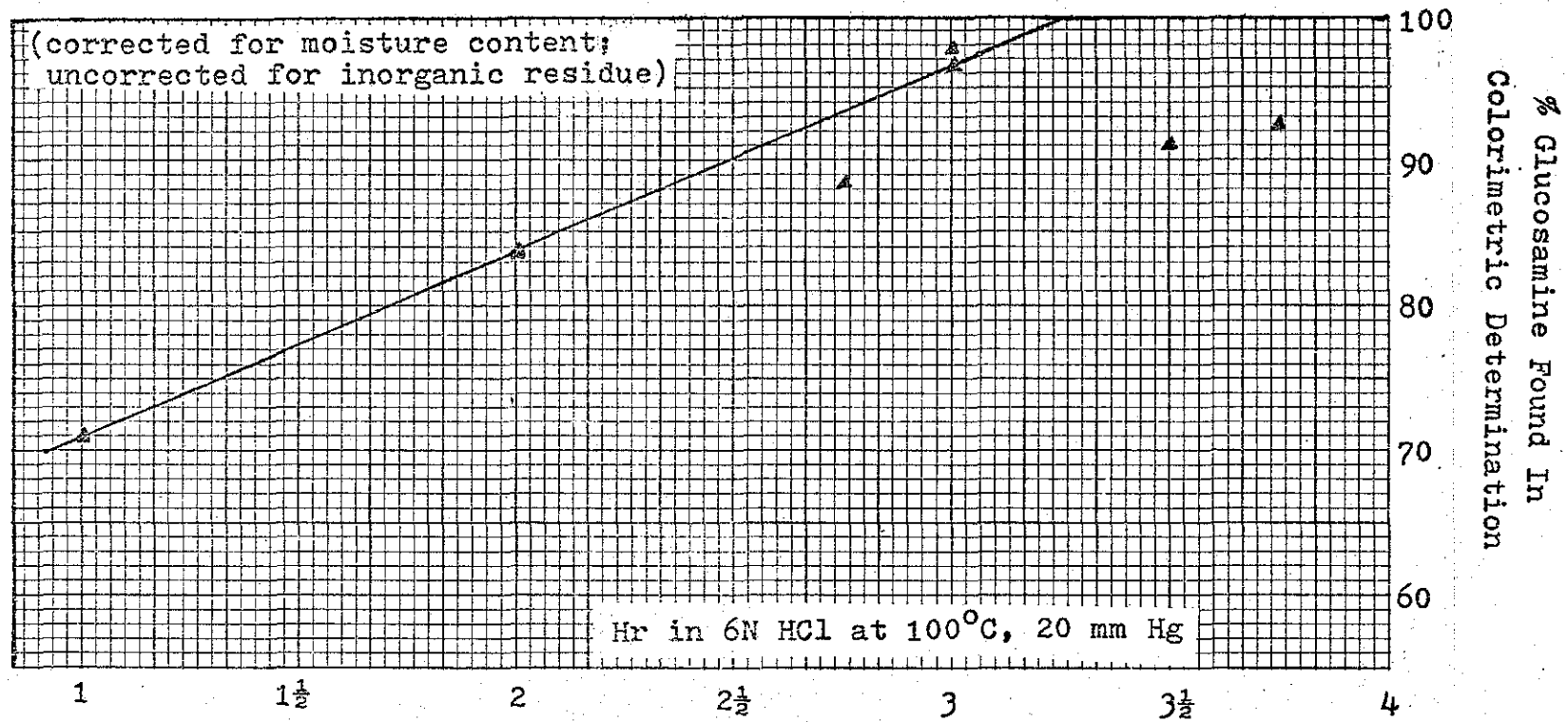


Figure 6

DETERMINATION OF OPTIMUM HYDROLYSIS CONDITIONS

Standards and Blanks. The standards were made up fresh from the crystalline glucosamine hydrochloride supplied by Pfanstiehl Labs, Waukegan, Illinois (lot #8684). The standard was found to be free of moisture when dried over  $P_2O_5$  at  $65^\circ C$ , in vacuo for 67 hr. There was no weight loss for any of three samples (approximately 1 g each) which were dried simultaneously. The standard was submitted for amino acid analysis and found to be free of detectable amino acids (except for a small lysine peak). The standard solutions were made up by dissolving 0.2150 g glucosamine HCl in 50 ml distilled water in a 100 ml volumetric flask to give a 0.01M solution of sugar. The standard solutions were further diluted by transferring 1 ml portions to six volumetric flasks (20-, 25-, 35-, 50-, 100-, and 250 ml). The colorimetric results obtained for these known concentrations were used to prepare a graph as a basis for the determination of the experimental samples, which were always determined simultaneously with the prepared standards and blanks. The blanks were made by substituting distilled water for the sugar solution, keeping all other variables constant. Erroneous results were obtained when standards and analyses were not carried out simultaneously. Figure 7 shows a typical graph.

Pipets. The pipets used were calibrated by filling to the mark with distilled water at room temperature and weighing.

Glucosamine from KOH treated float material. A sample (2.5 mg) of KOH treated float material which had been dried over  $P_2O_5$  ( $65^\circ C$ , in vacuo 24 hr) was exposed to room humidity (4 hr) and hydrolyzed for 3 hr



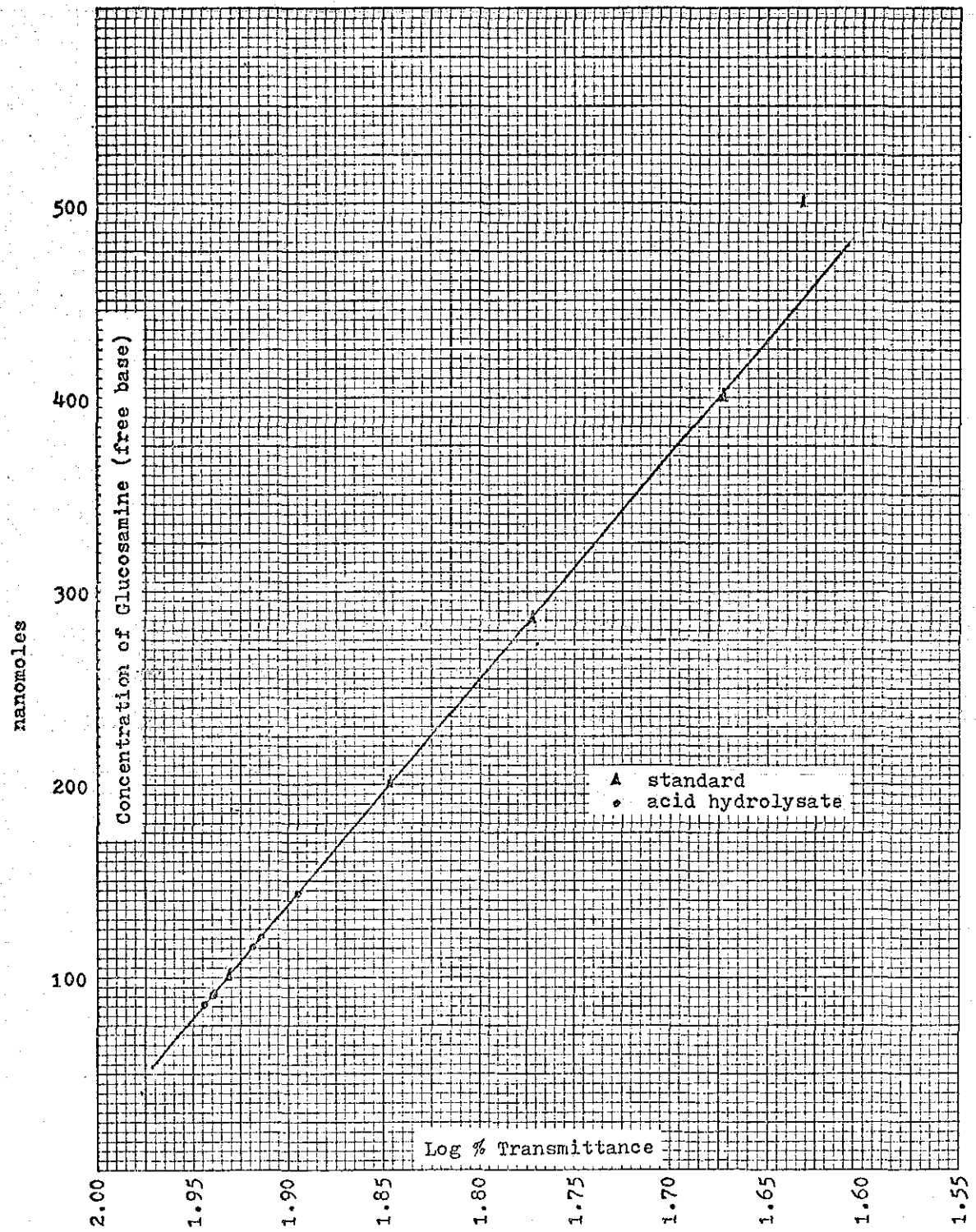


Figure 7

Colorimetric Determination of Carbohydrate

and colorimetrically determined as above. Extrapolation gave 93.5%. When a moisture factor of 5.6% (from graph) was subtracted from the weight of the starting material the results were 99.1%. A second sample (2.8 mg) was determined in the same way and gave 94.0% and 97.7% (after a 3.6% moisture factor for 3 hr at room humidity before hydrolysis). Both samples are uncorrected for any inorganic or amino acid impurity.

#### IR Comparison of Carbohydrate Material

Shrimp Shells. Fresh shrimp shells (species unknown) from the Pacific Ocean were obtained from a local restaurant within 12 hr of removal from the frozen animal. The shells were kept chilled until cleaned several days later. After being washed thoroughly in water to remove all fleshy material (including tails and legs), the shells were kept under 6N KOH at room temperature for 15 days. After being rinsed with water the shells were light pink in color with white patches. The shells were then stirred in 200 ml HCl (2N) for 5 hr at room temperature, filtered by suction, and washed with distilled water (3X), and acetone (2X). The shell material (dried over  $P_2O_5$ , in vacuo at 65°C for 24 hr) was chalky white in color.

Whole Float Material. Whole float material (washed, dried over  $P_2O_5$  at 65°C, in vacuo for 24 hr) was powdered in a stainless steel capsule with a stainless steel insert on a "Wig-L-Bug" and a KBr pellet was made. The spectrum (7a,b) was compared with Spectrum 3a,b obtained in the same way from shrimp shell, and known N-acetylglucosamine

(Spectrum 1a,b). Spectrum 3a,b was virtually identical to Spectrum 7a,b while the N-acetylglucosamine (Spectrum 1a,b) bears a close general relationship.

KOH Treated Float Material. The ir spectrum of whole float material which had been treated with 6N KOH at room temperature for 8 days (Spectrum 4a,b) or 1N KOH at 100°C for 24 hr (Spectrum 5a,b) were compared. There is no detectable difference, although the pellet made from the material treated for a longer time has slightly better resolution. The KOH treated float material does not show a strong absorption at  $865\text{ cm}^{-1}$  which is prominent in the N-acetylglucosamine spectrum. A comparison with glucosamine hydrochloride (Spectrum 2a,b) shows a general overlap of absorption peaks, but the glucosamine hydrochloride has more and sharper peaks.

$\alpha$ -D-Glucosamine hydrochloride from KOH treated Float Material.

IR. Float material (approx. 0.4 g) (treated with 6N KOH for 8 days at room temperature) was hydrolyzed in conc HCl at 65°C for 3-4 hr to give a clear solution, evaporated to dryness on a rotovac with methanol several times, and recrystallized from methanol, to give 0.25 g (63%). A KBr pellet was made from material ground in an agate mortar. The spectrum obtained (Spectrum 6a,b) was identical to that obtained from the known glucosamine (Spectrum 2a,b). All peaks are present (with no extraneous ones) in the same proportional absorption.

TLC. A few drops of the  $\alpha$ -D-glucosamine hydrochloride prepared as above, were chromatographed on a tlc plate with known glucosamine

hydrochloride and N-acetylglucosamine, with solvent system #2 and charred. The  $R_f$  of N-acetylglucosamine was 0.41, while that of the glucosamine hydrochloride and the experimental sample were identical at 0.32. This solvent resolves the experimental sample into one major and three very minor fractions. The minor fractions may be a product of the hydrolysis conditions or may indicate the presence of other similar sugars in minor amounts (figure 8).

#### Elemental Analysis of Carbohydrate

For elemental analysis, a sample of float material (treated: 1N KOH, 100°C, 24 hr) was carefully selected to be free of visible sand, suspended in 6N HCl and deaerated in vacuo (20 min), filtered, washed with distilled water to neutrality, and with acetone (3X). It was dried at 65°C in high vacuum to constant weight. The calculated values are based on an expected N-acetylglucosamine polymer of infinite length (m.w. 203).

$C_8H_{13}NO_5$ (203)	Calc. C, 47.29; H, 6.40; N, 6.90; O, 39.41;
	CH <sub>3</sub> CO, 21.18.
	Found C, 47.28; H, 6.43; N, 6.95; O, 39.48;
	CH <sub>3</sub> CO, 21.53.

#### Amino Acid Analyzer Results

Whole float material which had been washed and dried, float

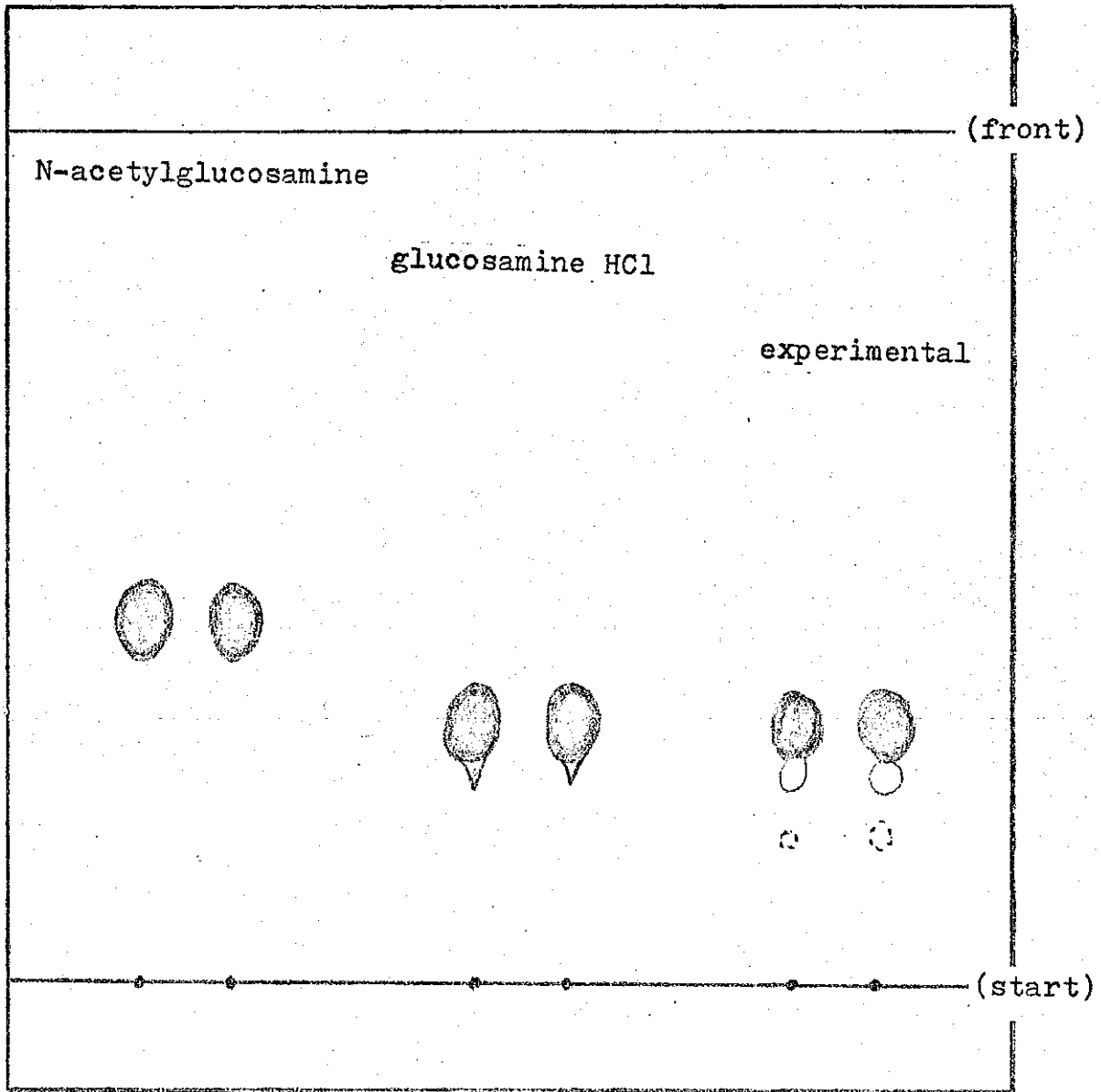


Figure 8

Thin Layer Chromatographic Comparison of the Acid Hydrolysate of KOH Treated Float Material with N-acetylglucosamine and glucosamine hydrochloride

material which had been treated with 6N KOH for 8 days, and known samples of glucosamine hydrochloride and N-acetylglucosamine were analyzed on a Bio-cal 201 Amino Acid Analyzer. The peaks indicated a large concentration of glucosamine in the whole float material and in the hydrolysate of the KOH treated float material. When quantitative calculations of glucosamine content were made from the chromatograms using the known N-acetylglucosamine as the standard, the KOH treated material gave a 96.5% recovery. Whole float material gave 33.8% N-acetylglucosamine content. A correction factor (15%) for moisture increased this to 38.5%. A second sample gave 40.6% and 49.6% respectively. When the found carbohydrate content was calculated as a percentage of the total protein and carbohydrate found, values of 60.4% and 59.4% were obtained.

Summary of Carbohydrate Determination

By Difference

1st KOH treatment	53%
2nd KOH treatment	54.2%
	55.7%

Direct Determination (Amino Acid Analyzer)

Whole float material

<u>1st determination</u> (inaccurate standard)	33.8%
Subtracting 15% moisture factor	38.5%
<u>2nd determination</u>	40.6%
Subtracting 15% moisture factor	49.6%

KOH Treated Float Material

(Recovery as a percentage of known standard hydrolyzed simultaneously)	96.5%
---	-------

Carbohydrate Percentage

(Carbohydrate as a percentage of total protein and carbohydrate found)	60.4%
	59.4%

Colorimetric Determination of Hexosamine Content

1st determination

KOH treated material (uncorrected for any inorganic residue)	93.5%
corrected for 5.6% moisture content	99.1%

2nd determination

corrected for 3.6% moisture content	97.7%
-------------------------------------	-------

Elemental Analysis

Analysis matches for  $C_8H_{13}NO_5$  (203).

Acetyl group analysis indicates complete acetylation.

IR

Whole float material matches known chitin.

Acid hydrolysis product of KOH treated material matches glucosamine hydrochloride.

TLC

Acid hydrolysate of KOH treated material and glucosamine hydrochloride have identical  $R_f$  values.



## EXPERIMENTAL PROCEDURE

### PART E

#### Determination of Protein Content

##### Millon Test

Whole Float. Approximately 100 mg float material (washed and dried) was treated with 1 ml conc  $\text{HNO}_3$ . The float material became red in color and changed to bright orange when 3 ml  $\text{NH}_4\text{OH}$  was added.

KOH Treated Float. Float material (washed and dried) treated with KOH (1N,  $100^\circ\text{C}$  for 24 hr; 6N, room temperature for 7 days) was washed to neutrality in water, and acetone and dried in air. Treatment with  $\text{HNO}_3$  and  $\text{NH}_4\text{OH}$  as before did not change the color.

##### Ninhydrin

Whole Float. Whole float material (washed and dried) was sprayed with ninhydrin solution. The material was placed in an oven ( $105^\circ\text{C}$ ) for 10 min during which a purple-pink color developed.

KOH Treated Material. Float material washed and dried and treated with KOH (1N,  $100^\circ\text{C}$  for 24 hr) was sprayed with ninhydrin. A light pink color developed.

##### Ashing

Whole float material (washed and dried) was ashed in a crucible to whiteness. During the ashing a distinct odor similar to that of burning hair was detected.

Elemental Analysis for Phosphorus

Whole float material (washed and dried) was analyzed for C, H, O, N, and P. Found: C = 41.3%; H = 6.56%; O = 43.15%; N = 8.99%; P = 0.0% (less than 0.2%).

Sulfur

Sodium Fusion. Whole float material (100 mg; washed and dried) was heated to redness with metallic sodium. The hot test tube was shattered in 20 ml distilled water. The filtrate (2 ml) was treated with 10 drops of 5% lead acetate solution. A very faint black precipitate was observed. Float material (washed and dried; treated with KOH as before) gave in comparison no black precipitate.

Formalin Preserved Float

A velëlla specimen which had been kept in a conc formaldehyde solution for a number of years was examined periodically during treatment with 6N KOH (25°C for 25 days). In bright sunlight flat portions of the float were observed separating from the vertical component and smaller portions were observed separating from the horizontal component. The materials were not analyzed. The formalin treated floats showed pronounced darkening and shrinkage when washed and dried after KOH treatment.

### Percentage of Protein by Difference

KOH Treatment. The loss of weight which occurred when whole float material was treated with KOH was determined as described in the carbohydrate determination. This loss (from two determinations) was 45.8% and 44.3%. The filtrate did not contain any material that could be charred on a tlc plate. The KOH treated float material was examined on a Bio-cal 201 Amino Acid Analyzer and found to be virtually free of amino acids, with the exception of a small amount of lysine, which still persists in the KOH treated material. The glucosamine hydrochloride and N-acetylglucosamine standards were tested in the same way and were found to contain also detectable amounts of lysine. A small peak tentatively identified as galactosamine also occurs in all of the chromatograms.

### Percentage Protein By Direct Method (Amino Acid Analyzer)

Hydrolysis. Float material (washed and dried in air) and redistilled 6N HCl (1 ml/1 mg) were frozen in a glass tube at  $-70^{\circ}\text{C}$ . The tube was evacuated (less than 0.1 mm Hg) and sealed. The contents were hydrolyzed at  $105^{\circ}$  (16 hr). The clear solution was transferred to a crucible, dried over  $\text{P}_2\text{O}_5$  and KOH (less than 0.1 mm Hg), redissolved in water, transferred to a 10 ml volumetric flask and brought with buffer solution to pH 2.2.

Analysis. For the analysis an Amino Acid Analyzer, BC 201 - Biocal Instrument, München, West Germany, was used. The one-column technique with suitable arranged buffer changes gave chromatograms which were compared with known mixtures of amino acids.

Evaluation of the Chromatograms. For the calculation of the amino acid content of the hydrolysis, the method of CREMER<sup>9</sup> was used which is supposed to be the best choice for manual evaluation<sup>46</sup>. The peak-height (h) and the peak-diameter at the half height (1/2 b) were measured with a calibrated magnifying lens. Then the F-values were calculated by the formula  $F = h \cdot 1/2b$ . These values were compared with the known areas of standard chromatograms and the amount of amino acids calculated. The results for the mole ratios (Leu = 1) are given in Table 1. N-acetylglucosamine was hydrolyzed and determined in the same manner and used as a standard for the glucosamine calculations. The carbohydrate results are given in Table la.

Table 1  
Amino Acid Analyzer Results

Amino Acid	1 2	Nanomoles Found	Nanograms (thousands)	Ratio of Nmoles /Leu	No Residue	M.W.	Total M.W.
Aspartic Acid	1 2	312.2 314	32.0 36.1	3.85 3.95	4 4	115	460 460
Threonine	1 2	260 228	25.6 23.0	3.2 2.87	3 3	101	303 303
Serine	1 2	314 311	27.4 27.0	3.9 3.7	4 4	87	348 348
Glutamic Acid	1 2	585 644	75.6 83.0	7.2 8.1	7 8	129	903 1032
Proline	1 2	396 491	77.9 96.5	4.9 6.2	5 6	97	485 582
Glycine	1 2	90.8 126	5.16 7.19	1.1 1.57	1 1	57	57 57
Alanine	1 2	125.2 118	8.95 8.46	1.55 1.47	1 1	71	71 71
Valine	1 2	362 412	35.8 40.7	4.5 5.2	5 5	99	495 495
Isoleucine	1 2	105 92	11.8 10.39	1.3 1.16	1 1	113	113 113
Leucine	1 2	81 79.5	9.15 8.98	1 1	1 1	113	113 113
Tyrosine	1 2	112 88.1	18.3 14.36	1.4 1.11	1 1	163	163 163
Phenylalanine	1 2	124.9 161	18.3 23.6	1.54 2.0	2 2	147	294 294
Lysine	1 2	471 523	60.4 66.9	5.8 6.6	6 6	128	768 768
Arginine	1 2	166.5 172	26.0 26.8	2.0 2.16	2 2	156	312 312
Half Cystine	1 2	102 264	10.4 26.7	1.25 3.3	1 3	102	306 918
Totals		3733 4088	442.8 526.7		44 48	5191	6029

Calculated  
Minimum M.W.

Table 1a  
Carbohydrate Found

N-acetyl- glucosamine	1	3320	3800	675
	2			771

Any tryptophan present was degraded in the acidic hydrolysis. Histidine and methionine were not present in the sample.

Sample #1 = 2.0 mg for hydrolysis

#2 = 1.9 mg for hydrolysis

A moisture factor of 15% was subtracted from the starting weights and the results calculated for both the dried and undried material. The results of two determinations, expressed as a percentage of the dried and undried starting material were:

Table 2  
Protein Found in Amino Acid Analysis

Sample	#1	#2
Room Humidity	22.1%	26.3%
15% Moisture Factor Subtracted	25.0%	31.0%

The total protein and carbohydrate found were summed, and their relative percentages calculated. The results were:

Table 3  
Calculation of Protein and Carbohydrate Ratio

		mg Found		% Of Total Found	
		1	0.443		39.6%
Total Protein	2		0.527		40.6%
Total Carbohydrate	1	0.675		60.4%	
	2		0.771		59.4%
Total Found	1	1.118		100.0%	
	2		1.298		100.0%

The recovery of the starting material after hydrolysis was calculated for both dry and room humidity conditions. The results were:

Table 3a

Total Protein and Carbohydrate Found as % of Starting Weight

Sample	Weight (air dry)	Total Found	% Of Starting Weight	
			Air Dry	15% Moisture Factor Applied
1	2.0 mg	1.118 mg	55.9%	65.7%
2	1.9 mg	1.298 mg	68.2%	80.4%

Mole ratios for the total amino acids found and the total N-acetylglucosamine found were calculated for both samples:

Table 3b

Mole Ratio of Total Amino Acids to Carbohydrate

sample	total amino acids found (nanomoles)	N-acetyl- glucosamine (nanomoles)	mole ratio
1	3733	3320	1.12 :1
2	4088	3800	1.08 :1

Chromatograms of KOH treated float material were examined for amino acid content. Three peaks were present in the glucosamine-ammonia region and labeled #1, #2, and #3 in order of elution pending identification. The peaks were also present in the commercially prepared N-acetylglucosamine standard and glucosamine hydrochloride. Muramic acid prepared in this laboratory was free of peaks #1, #2, and #3, but contained a new peak in the same region. Peak #3 was tentatively identified as lysine; and though peaks #2 and #3 were unidentified, galactosamine and hydroxylysine were considered possibilities. The peak areas were determined as before. Mole ratios for lysine : N-acetylglucosamine gave an average of one lysine for 8,000 carbohydrate monomers. The complete results were tabulated (Table 4).

sample	unknown peaks			lysine		N-acetyl-glucosamine		mole ratio
	#1 mm <sup>2</sup>	#2 mm <sup>2</sup>	#3 mm <sup>2</sup>	test mm <sup>2</sup>	nano- moles	micro- grams	micro- moles	
vertical component (KOH treated)	9.0	4	8.8	$\frac{8.8}{436} \times 50 =$	1.02	1,850	9.1	1: 8900
horizontal component (KOH treated)	11.7	4	7.9	$\frac{7.9}{436} \times 50 =$	0.91	1,360	6.7	1: 7400
whole float (KOH treated)	20.6	4	11.5	$\frac{11.5}{436} \times 50 =$	1.32	1,930	9.42	1: 7140
N-acetylglucosamine (standard)	15.4	4	10.2	$\frac{10.2}{436} \times 50 =$	1.17	2,000	9.85	1: 8400
glucosamine hydrochloride	9.3	3.5	9.9	$\frac{9.9}{436} \times 50 =$	1.13	1,880	9.25	1: 8170
					avg. 1.37			avg. 1: 8000

Table 4

Lysine Residue in Standards and KOH Treated Material

The mole ratios of lysine to other amino acids present were calculated from Table 1. Samples #1 and #2 averaged 5.5 and 6.1 lysine residues respectively, and 5.8 when combined. The complete results were:

Table 5  
Ratios of Lysine to Other Amino Acids

Amino Acid	sample	Moles Lysine/ Mole(s) Other Amino Acid	
Leucine	1	5.8	
	2		6.6
Isoleucine	1	4.5	
	2		5.7
Tyrosine	1	4.2	
	2		5.9
Glycine	1	5.2	
	2		4.2
Phenylalanine = 2	1	7.5	
	2		6.5
Arginine = 2	1	5.7	
	2		6.1
Threonine = 3	1	5.4	
	2		6.9
Asparagine = 4	1	6.0	
	2		6.7
Average		5.5	6.1
Grand Average (of all 16)		5.8	

The lysine : carbohydrate mole ratios for whole float material were 1 : 7.1 and 1 : 7.3 for samples #1 and #2 respectively (Table 6).

Table 6  
Mole Ratio of Lysine to Carbohydrate

sample	lysine found (nanomoles)	N-acetyl- glucosamine found (nanomoles)	mole ratio
1	471	3,320	1 : 7.1
2	523	3,800	1 : 7.3



Lysine : carbohydrate mole ratios expected for the minimum molecular protein weights determined in the amino acid analysis (Table 1) and protein : carbohydrate weight distributions suggested by the quantitative results were calculated. The molecular weight of an hypothetical protein with lysine links to six octasaccharides (figure 9) was included for comparison. Values of 1 : 5.2 to 1 : 8.6 were obtained.

The complete results were:

Table 6a

Calculated Mole Ratio of Lysine to Carbohydrate

protein to carbohydrate weight ratios	lysine to carbohydrate mole ratios		
	m.w. of protein		
	#1 5200	#2 6000	cal. 6923
45 / 55	1: 5.2	1: 6.0	1: 7.0
41.54 / 58.46	1: 6.0	1: 7.0	1: 8.0
40 / 60	1: 6.9	1: 7.4	1: 8.6

A similar calculation for the protein : carbohydrate mole ratios gave values of 31.4 - 51.6 N-acetylglucosaminyl units per protein.

The complete results were:

Table 7

Mole Ratio of Protein to Carbohydrate

protein to carbohydrate weight ratios	protein to carbohydrate mole ratios		
	m.w. of protein		
	#1 5200	#2 6000	cal. 6923
45 / 55	1: 31.4	1: 36.1	1: 41.6
41.54 / 58.46	1: 36.0	1: 41.7	1: 48.0
40 / 60	1: 38.4	1: 44.3	1: 51.6

Summary of Protein DeterminationNinhydrin Test

Whole Float	positive
KOH treated float material (diminished; with color change)	positive

Millon Test

Whole Float	positive
KOH treated float material	negative

Ashing

Definite odor of burning protein

% Protein by Difference

Loss on KOH treatment	47.0%
" " " " (quantitative)	45.8%
	44.3%

% Protein by Direct Determination (Amino Acid Analysis)

Found in air dried sample	22.1%
	26.3%
15% moisture factor applied	25.0%
(uncorrected for loss in hydrolysis)	31.0%
As a percentage of the total protein and carbohydrate found	39.6%
	40.6%
(uncorrected for loss of amino acids in hydrolysis)	

## CHAPTER 3

## Brief Summary of Results

Moisture ContentWhole Float

Over $\text{CaCl}_2$ (48 hr, 60°C <u>in vacuo</u> )	10.3%
Over $\text{P}_2\text{O}_5$ (67 hr, 65°C <u>in vacuo</u> )	14.5%

KOH Treated Float Material

Over $\text{P}_2\text{O}_5$ (75 hr, 65°C <u>in vacuo</u> )	14.1%
	14.1%
	15.3%

Inorganic Content

Aqueous Extract 69.0%

Whole Float Material (Dry Ash)

Washed; visible sand present	2.8%, 3.0%, 3.7%
Washed, <u>blended</u> and sorted; free of visible sand	
450°C ash	0.8%
550°C ash	0.5%
After HF treatment	1.1%

Whole Float Material (Wet Ash)

Conc $\text{HNO}_3/\text{HClO}_4$	2.7%
Reweigh after 12 hr	2.8%
After treatment with conc $\text{HNO}_3$	2.9%
After treatment with conc HF	2.6%
Subtracting 14% moisture factor	3.1%

KOH Treated Float Material

1N KOH (24 hr at 100°C)	1.8%, 3.6%, 3.2%
(Oxalate positive for calcium)	
6N KOH (3 days at 100°C)	3.3%
	3.4%
(red ash)	7.7%
Desiccated KOH treated float material	4.4%

Lipid Content1st Determination

By Difference	0.14%
Applying 14.5% moisture factor	0.17%

2nd DeterminationBy Difference

Air Dry	0.15%
Desiccated (over P <sub>2</sub> O <sub>5</sub> , 60°C 3 hr, 20 mm Hg)	0.17%

Direct Weight

Air dry	0.23%
Desiccated (as above)	0.27%

Carbohydrate ContentWhole Float1st Determination

room humidity/6N NaOH 9 days, 25°C (dried under infrared lamp to constant weight) 53.0%

2nd Determination

over P<sub>2</sub>O<sub>5</sub> at 65°C for 24 hr in vacuo/1N KOH 24 hr at 100°C, washed and dried as before. 54.2%

55.7%

Amino Acid Analyzer Determination

Found in air dried sample 33.8%

38.5%

15% moisture factor applied 40.6%

49.6%

As a percentage of total material found 60.4%

59.4%

Protein ContentPercentage Protein by Difference (KOH treatment)

nonquantitative 47.0%

quantitative 45.8%

44.3%

Direct Measurement (Amino Acid Analysis)

Found in air dried sample 22.1%

26.3%

15% moisture factor applied 25.0%

31.0%

As a percentage of total material found 39.6%

40.6%

Composition of Velella lata Float Collected From The Beach  
As Suggested By The Analysis

Moisture content (at room humidity) 14-15 wt %

---

As % of Dry Weight

Inorganic Content	3-4 wt %
Lipid "	0.2-0.3 wt %
Carbohydrate "	55-58 wt %
Protein "	42-45 wt %
Protein/Carbohydrate	45/55 wt %

## CHAPTER 4

### DISCUSSION OF RESULTS

#### PART A

##### Moisture Content

The float material is saturated with water in the intact animal. The removal of water represents a departure from the physiological condition. When the moisture content at room humidity was determined the amount of water in a saturated float was not investigated since it did not seem to be germane to the analysis. For further work, it would be interesting to examine the rate and amount of water exchange out to the saturation point.

The determination of moisture content at room humidity allowed the application of a moisture factor to material which had never been dried. The average humidity in the laboratory was not determined and no graph was prepared showing the moisture factor at different levels of humidity, pressure and temperature. To this extent the moisture factor is unreliable. When whole float material was dried over  $\text{CaCl}_2$  and  $\text{P}_2\text{O}_5$  a difference of 5% was noticed and thereafter all drying was done over  $\text{P}_2\text{O}_5$ . The loss of weight on drying of whole float material was compared with that of the KOH treated material and found to be the same within 1%. This seems to indicate that the removed protein absorbs water to approximately the same extent as the carbohydrate.

The resorption of water was studied to give some indication of how fast the dry material regained the lost water. A rapid water uptake was not found and no special weighing or handling was required. The resorption of approximately 10% of the lost water in one hr indicated that appreciable errors could be avoided by quickly weighing the material. The delay in resorption indicated that, once dried, the float material requires several days before it can be treated as equilibrated with the room humidity.

The increased drying over  $P_2O_5$  relative to  $CaCl_2$  raises the question as to whether all moisture was completely removed by the  $P_2O_5$  either. The recovery of carbohydrate was over 96% in the colorimetric determinations under optimum conditions. In the amino acid analysis there was a small amino acid impurity (lysine), an undetermined peak (possibly galactosamine or hydroxylysine), plus an undetermined inorganic residue. These factors, plus any loss of the carbohydrate in the hydrolysis, suggest that the residual water is less than 1-2% when the material is dried over  $P_2O_5$  in vacuo at  $65^\circ C$  for 24 hr.

Float material which has been washed equilibrates on drying in air overnight. This time is shortened by washing in acetone. The weight attained remains reasonably constant over a period of time and suggests a special relationship between the water and the polymeric matrix. When the resorption of water is calculated for the KOH-treated material as a ratio of moles of water resorbed to moles of polymeric matrix (using the weight of the N-acetylglucosaminyl residue-203- for the calculation) it is found that the ratio is between 1-2 moles water to 1 mole of monomer. Where the ratio goes from 0 : 1 to 1 : 1, the water uptake appears linear, but thereafter the rate of resorption



decreases sharply. This may indicate that  $\beta$  chitin, described as the monohydrate by Dweltz<sup>10</sup>, may give the  $\alpha$  diffraction when completely dry. Very accurate determinations of moisture content require that the inorganic content be determined and corrected for by ashing the dried sample. Unfortunately there are difficulties in the drying form of calcium, and coupled with the unknown silicon relationship, the question of free calcium versus the carbonate, the presence of other unknown inorganic material, and the difficulty of handling the large sample sizes required for accuracy, it is doubtful that a significant improvement in the moisture values would result. The acceptance of a 14-15% moisture content in both the untreated and KOH-treated float material under ordinary conditions of temperature and humidity seems to be justified by the data.

A more detailed investigation of the drying and resorption rates might give some indication of how the water is attached to the polymer.

## DISCUSSION OF RESULTS

## PART B

Inorganic Content

The Velella float material for this study was collected on the beaches of Northern California and Oregon over the past two years. The removal of sand, which adheres tenaciously to the dried float, represents a challenge which was not completely met. Sand grains come in all sizes, and the assumption that the sand could be removed manually by visual inspection proved fallacious. The fact that the horizontal portion of the float is a "double" membrane with enclosed air spaces compounds the problem. When sandy float material is washed carefully by hand, one float at a time under a running tap, the remaining sand content can easily amount to 10-15% of the total weight. With careful selection of sand-free material this can be reduced to 2-3%. The best sample, which was blended in a Waring blender, was reduced to 0.8%, but the blending may have produced unknown fractionations and it was not used as a general cleaning technique.

The residue after ashing was treated with conc HF to determine the silicon content of one sample by converting the silicon to gaseous silicon tetrafluoride. The sample increased in weight almost 100% and leaves the question undecided. Such an increase can be partially accounted for if the anion is converted from oxide to fluoride. The small sample size (4.5 mg) of the ash makes possible an appreciable error in the weighings.

Does Velella use silicon as a component of the float structure?

The present collection method renders this question unanswerable, as a silicon compound (e.g.  $\text{SiO}_2$ ) may be removed as extraneous material in the cleaning method that was used. In this connection one may recall that silicon is used for structural purposes in silicoflagellidae, sponges, and radiolaria, and is essential for the synthesis of connective tissue of the chick<sup>5</sup>. This means that a preferred method of collection would be to scoop the live Velella animals from the open sea and freeze them until a careful separation of the float could be made in the laboratory.

Sea water contains a high concentration of dissolved salts, and to some degree the Velella exodermis serves as a semipermeable barrier behind which inorganic material may accumulate. The float, once freed from the protection of the intact animal, would be expected to equilibrate with the salt concentration. This expectation is tempered by the observation that the external coverings of many marine animals do not appear to be protected from the sea and do not appear to lose their inorganic content. These materials contain a high calcium content in a "chitinous" matrix similar to that found for Velella, and the extrapolation might be made that the inorganic content is not diluted appreciably by exposure to the sea and to rain. A value of 69% for the inorganic content of filtered wash water suggests an appreciable inorganic content in vivo. A better answer awaits improved collection technique.

Several attempts were made to separate the water and acid soluble

components from the ash. The small quantities used and the minor differences noted did not allow any conclusions other than that produced by other methods, i.e. that the inorganic content is a very small percentage of the total weight.

It was of interest to determine if the residual inorganic material survived the KOH treatment or was removed with the protein. Three simultaneous determinations were made in the same way on KOH treated samples and calcium was precipitated with ammonium oxalate by the method described by Kolthoff and Sandell<sup>26</sup>, indicating that KOH treatment does not remove all of the inorganic material.

Treatment with acid would be expected to dissolve any calcium that was originally present as the carbonate. Hydrolysis of the float material with strong acids did not result in a completely clear solution however, and this acid insoluble residue remains a mystery. The residue constitutes an insignificant amount of the total weight and is only mentioned as a suggestion for further work. White needle-shaped particles remain suspended when the KOH treated material is dissolved in concentrated HCl, gray suspension remains after hot concentrated  $\text{HNO}_3$ - $\text{HClO}_4$  oxidation, and red particles appear on prolonged treatment with concentrated HCl.

Values of about 4% inorganic residue for the whole float and 1% for the KOH-treated material were found by the Beller Microanalytical Laboratory.

The primary interest in the inorganic material was to try to eliminate it entirely so that it did not obscure the protein and

carbohydrate determination, but it is interesting to consider the possible function of the minute amounts which are present. The finding of 0.8% inorganic material seems woefully insignificant, but a mole of calcium (m.w. 40) represents only 0.67% of the weight of a protein with a molecular weight of 6,000 when the mole ratios are 1 : 1. A carbohydrate polymer of eight N-acetylglucosaminyl residues (m.w. 203) has a molecular weight of about 1640 and could coordinate with a divalent cation (four ligands) in a mole ratio of 4 : 1 and the cation could still be well under 1% of the total weight.

The presence of aequorin in other jellyfish raises interesting questions regarding its possible biological function. Aequorin is a protein which specifically bioluminesces in the presence of free calcium ion<sup>48</sup>. The energy is released at contact, so that the aequorin-calcium complex may be assumed to be a lower energy and thermodynamically more stable conformation<sup>53</sup>. Phosphorescence is a common observation of disturbed marine animals. Does physical disturbance of the animal (and the matrix) dislodge free calcium, which then triggers the stored energy of aequorin-like proteins? If so, how is the calcium subsequently freed? Is light required to free the calcium, which might then be available for incorporation into the matrix? It seems clear that there are other alternatives to accepting the view that the calcium is present in the matrix only as the carbonate.

What can be said with confidence regarding the inorganic material? The difficulty of removing the sand can be overcome with improved collection techniques, and the loss of soluble material minimized,

so that reliable information regarding the in vivo condition can be accumulated. Calcium is certainly present, and so tenaciously as to arouse suspicions of coordination in the matrix. Material collected from the beach has a residual inorganic content which can be lowered to 3-4% with careful washing. Blending and meticulous sorting will give material with less than 1% residue. The possibility of determining the specificity of calcium for the extracellular matrix is suggested.

## DISCUSSION OF RESULTS

### PART C

#### Lipid Content

The first attempts to determine if a significant ether soluble fraction existed in the float material gave inconclusive results. The possibility that the washing procedure was extracting the lipid fraction was considered. A large portion of unwashed sail material was cleaned of debris and free sand and washed extensively in a minimum amount of diethyl ether several times. The ether was subsequently evaporated to dryness at room temperature. A thin film of oily residue remained with the slight odor of cod liver oil, indicating either a negligible lipid fraction, or a tightly bound one.

Carefully conducted quantitative extraction in a soxhlet apparatus gave results under 0.2% in two determinations.

When a portion of an animal is removed for investigation, there is always the question of surface contamination. The float material is non-cellular, but occurs in the animal immediately adjacent to actively synthesizing cell membranes. Is it possible that the float material absorbs a portion of the lipid material found in membranes when the animal dries on the beach? The exposure to the sea and rain might not remove lipid material which is only slightly soluble in water. Is the lipid an integral part of the float in the live animal, associated with the protein or carbohydrate, or both? These questions remain unanswered for the moment. When the protein is removed in the KOH treatment an oily film is observed on the wash water, indicating

the necessity for washing with acetone. This makes it probable that the small lipid content is removed with the protein.

The distinction between "ether soluble" and "lipid" is significant. The presence of a large lipid of 16-18 carbons represents a mole ratio of about 1 : 1 to the protein. A small ether soluble molecule, with 2-3 carbons, would have a mole ratio of about 1 : 6 to the carbohydrate. In both cases a significant role can be seen for such a molecule in the matrix, and it should not be altogether forgotten. The identification of the lipid would be helpful in resolving its role relative to the matrix.

The float material was dried after extraction to prevent possible loss during the drying process. It would be interesting to determine the volatility of the ether soluble component, since it might be included in, or be related to, the moisture loss.



## DISCUSSION OF RESULTS

### PART D

#### Carbohydrate Content

When Rudall examined a wide variety of invertebrate connective tissues using x-ray techniques, he obtained a characteristic diffraction pattern by which he defined "chitinous" material<sup>40</sup>. Although he treated the float material with 5% KOH at 100°C for 24 hr prior to his analysis to free it of protein he failed to get such a "chitinous" pattern from Verella spirans float material and suggested that additional purification would yield the characteristic diagram. Float material which was so treated in this work was submitted to amino acid analysis and found to be at least 96.5% pure N-acetylglucosamine. The same material when hydrolyzed and determined colorimetrically again gave 96.5% recovery of N-acetylglucosamine. The determinations were made for glucosamine after hydrolysis but the elemental analysis of this material indicates that it is completely N-acetylated. The major impurity appears to be lysine, which remains after base treatment and survives subsequent acid hydrolysis, and possibly a minor amount of galactosamine and hydroxylysine which occurs on the amino acid analyzer chromatograms. Rather than purity, heat is more likely to be responsible for the x-ray results. The longer treatment (6N KOH, 6-8 days at 25°C) would probably give the usual diagrams. Thin layer chromatography in a variety of solvent systems indicated that the carbohydrate was pure, but when solvent #2 was tried, late in the analysis, it was discovered that a separation

into one major and three minor components occurred. These findings do not necessarily contradict high recovery of N-acetylglucosamine and the question of other sugars being present is still open. No muramic acid was found when amino acid analyzer chromatograms of known muramic acid were compared with chromatograms of Velella float material.

The identification of the monomer rests upon: The ir comparisons with known chitin, N-acetylglucosamine and glucosamine hydrochloride, the colorimetric tests which are specific for hexosamines, the amino acid analyzer results, the elemental analysis, and tlc comparisons with known glucosamine hydrochloride and N-acetylglucosamine. Melting points matched for chitin and glucosamine hydrochloride, but were not considered significant for identification.

The use of enzymes to degrade the polymer (chitinase and chito-  
biase) would give a direct recovery of the monomer without the deacetylation which occurs with acid hydrolysis. These enzymes were not available for the present study.

The high recovery of the N-acetyl group in the elemental analysis indicates that the 6N KOH treatment (7-9 days at room temperature) does not appreciably deacetylate the polymer and that the carbohydrate is probably completely N-acetylated in vivo.

The elemental analysis on the whole float indicates that phosphorus is not present. The metabolic pathways found for carbohydrate biosynthesis<sup>19</sup> include phosphorylated intermediates. By the time the carbohydrates are incorporated into the polymeric framework of the Velella float these intermediates are probably absent, unless lost in

the collection and washing methods used.

The polymeric matrix is organized in an unknown way. The possibility that the polymer is one long infinite chain is unappealing, and biologically illogical, since the float appears to be synthesized and laid down as a topologically continuous membrane on three surfaces simultaneously. The association of the monomer into subunits is a more attractive concept, and is supported by suggestions that a polymer 8 monomers long is in evidence<sup>2,42</sup>.

Some evidence on the levels of organization was offered by observing the float material during the acid hydrolysis. When KOH treated material was hydrolyzed in 6N HCl in a large test tube at 100°C the initial condition after shaking was the sedimentation of the entire mass. After several hours and intermittent shaking the mass distributed itself evenly throughout the test tube. Sand grains which were free in the solution did not fall to the bottom of the tube. After another hour of heating at 100° the sand grains slowly tumbled down through the matrix to the bottom of the tube. This change was reasonably abrupt, suggesting a change in the structure. After more heating and several hours later, sand grains brought to the top fell without interruption to the bottom of the tube. Placing the tube in an oven at 125°C for ten minutes completely cleared the solution. Browning occurred during the hydrolysis and later the solution assumed a distinct dark brown color. After hydrolysis, when the tube was held against bright sunlight, small needle-shaped particles were visible which appeared crystalline. These five abrupt

changes may correspond to the five levels of cellulose organization<sup>32</sup>.

The amino acid analyzer results suggest a protein of 44-60 amino acids with a m.w. of 5,000-7,000. The mole ratios for the N-acetylglucosaminyl monomers give values suggesting 31-52 monomers per protein. If the carbohydrate is linked to every protein by six linkages, as the x-ray and electron micrographs<sup>41,42</sup> seem to show, then an oligosaccharide length of 5-9 monomers is indicated. The acid hydrolysates produce a seven monomer unit<sup>2</sup> (which is better evidence for eight since the end sugar would be expected to degrade in the hydrolysis) and the  $\gamma$ -chitin studies<sup>41,42</sup> indicate that eight is the proper number. If the eight monomer unit is used with the weight distributions and the m.w. findings, a protein with a m.w. of 6,800-7,000 is required (if there are six octasaccharides per protein). It is interesting that the ideal weight distribution calculated for the hypothetical macromolecule (figure 9) gives values of six, seven or eight depending on the m.w. of the protein. Considering the variety of animals and shapes containing chitin, one may suspect some universality of these molecular weights and their associated oligosaccharide lengths.

## DISCUSSION OF RESULTS

### PART E

#### Protein Content

The determination of the protein content by difference raises a question of accuracy. When the lipids, moisture and inorganic material have been removed, the remaining weight should be completely accounted for by the protein and carbohydrate contents. The amino acid analysis results indicate that almost all of the protein is removed by KOH treatment with only minor persisting amounts of amino acids, principally lysine. But is the carbohydrate degraded at the same time? Testing the supernatant in the KOH treatment periodically, does not reveal any charable material. Unfortunately this does not resolve the question since the carbohydrate might be degraded in base to compounds which may not be detected by charring. The stability of the carbohydrate polymer to base (6N KOH at 100°C) was tested, and showed no appreciable loss in three days. But the issue is not settled. The surviving carbohydrate does set an upper limit of 45% on the protein and the surviving protein in the amino acid analysis sets a lower limit of 31%.

Since there was appreciable loss during the acid hydrolysis, especially of tryptophan and methionine, neither of which were found at the end of a 16 hr hydrolysis, the higher value is more attractive. The direct results might be even higher if any appreciable inorganic residue remained in the test sample or if the humidity in the testing

laboratory required a higher moisture factor than was applied. The results could be improved by determining the tryptophan content in a separate determination by basic hydrolysis. Methionine, if present, can be determined by using a shorter acid hydrolysis time.

When the protein and carbohydrate found after hydrolysis were compared, the results agreed within 1% for the two samples even though a subtle error was possible. The carbohydrate content (and hence the distribution) of sample #1 was evaluated with a standard which was made simultaneously with sample #2 but with a different ninhydrin solution. The agreement suggests a negligible error in this case, but a second consideration is more significant. The protein content was determined with the aid of amino acid standards which were not hydrolyzed for 16 hr. The carbohydrate standard received the same treatment as the samples, so that the hydrolysis loss was compensated for in the calculation. This means that the protein found is low and that the 31/69% distribution should be corrected toward the 45/55% value. The chromatograms can be re-evaluated when graphs of amino acid hydrolysis are available. Applying a reasonable value of 10% hydrolysis loss to the protein gives a 43/57% distribution.

Thus a large amount of protein is found in the Velella float and is not all removed by extensive washing. Is this protein a component of an adjacent membrane? Is it perhaps some component of the mesoglea which becomes enmeshed in the carbohydrate matrix? Or is it a true component of a glycoprotein macromolecule of which the float is composed?

The Millon and ninhydrin treatments indicate that the protein is evenly distributed in the lateral dimensions of the float. It was possible to delaminate the formalin preserved floats, and this suggests that the initial synthetic process is the development of a membrane-sheet which is of uniform thickness and topologically continuous over the entire outer surface (figure 10). The detail of the horizontal component (figure 4) indicates that this membrane sheet is completed before it contacts the prior layer. The picture emerges as an alternating mitosis-synthesis cycle, possibly circadian. The growth process is reminiscent of a man constructing a ladder as he climbs. This model is supported somewhat by the observation that continuous synthesis is illogical in view of the structure and that mitosis and synthesis generally are considered mutually exclusive cellular events. The failure to find a membrane between the float and the adjacent cell layer of the preserved specimen seems to eliminate this as a possible site for the protein. The protein is removed by treatment with KOH without disturbing the shape. The filtrate does not contain charable material. Examination of the formalin treated floats during KOH treatment reveals large patches of material sloughing off the float. These events seem contradictory. If the "patch" material observed sloughing off is pure protein, how is the internal protein removed? The patches were not analyzed and perhaps the observed darkening and shrinking of the formalin treated floats result from incomplete removal of protein. If the "patch" material is a portion of the outermost layer, which has been degraded by formaldehyde, it

should contain protein and carbohydrate, an hypothesis not investigated. Formic acid is reported to "dissolve" chitinous material<sup>40,45</sup> and perhaps this is a related phenomenon. Formic acid and formaldehyde both react with proteins. A study which combined these reagents with the demonstrated acid and base effects might prove effective in determining the exact organization of the layers.

The amino acid analyzer results do not reveal the number of proteins which are present, whether there are disulfide links (the presence of half-cystine indicates the possibility), or other protein-protein crosslinks, or whether the true molecular weight is a multiple of the minimum calculated. The similarity between the samples of whole float material gives confidence in the results. When the vertical and horizontal components were analyzed separately a difference was obtained which has not been studied further. Initially this seems to indicate at least two different proteins in the float, one for the vertical layers and another for the horizontal layers. The layers are observed to be smoothly continuous at the junction and such a conclusion seems untenable. Sequencing the protein(s) may resolve the question.

The presence of six lysines in the minimum m.w. protein and its persistence in the KOH treated float material suggested that a special relationship may exist between lysine and the carbohydrate. Lysine was then given special attention. X-ray diagrams of "chitin" from other animals<sup>19</sup> indicate a recurring structure of six and eight monomer distances. Electron micrographs of "chitin" from hymenopteran



ovipositor<sup>41</sup> indicate a six-sided carbohydrate structure surrounded by protein. While not confirmed, the conversion of the six monomer pattern into the eight monomer pattern when the protein is removed suggests that the protein has six linkages per unit. The amounts of glutamic acid (7-8 residues), proline (5-6) and valine (5) make them candidates for such linkages, and the linkages may involve more than one amino acid. If a one protein model with six links to an octasaccharide is proposed so that all of the evidence from the literature and this analysis is satisfied, a protein with 44-60 amino acids (m.w. 7,000) results (figure 9). The name "velatin" is suggested for this protein(s) in order to emphasize species specificity.

What can be said about the relationship of the protein to the moisture, the lipid and inorganic contents? The relative moisture content of the KOH treated material (all carbohydrate) is the same as in the whole float so that the protein seems to be as hygroscopic as the carbohydrate. The presence of one water molecule per amino acid seems to be indicated for the protein in the intact layer at room humidity.

The lipid (or ether soluble component) defies evaluation other than its possible participation in the organization of the float at some level. It might participate in a hydrophobic-hydrophilic transition associated with the aggregation phenomena.

The reports of microtubule reaggregation<sup>3,54</sup> describe a calcium dependence. The presence of calcium in the whole float and its presence after KOH treatment make possible its participation in the aggregation

events. The presence of a calcium-specific protein in other jellyfish implies that such a protein is to be expected in Velella, but there is no indication that aequorin is identical to "velatin" or that velatin is calcium specific.

What significance can be placed on the amino acid/carbohydrate monomer correspondence? The results indicate that spatially the carbohydrate and protein are equivalent. How can we remove such a volume of protein without destroying the structure? Do the carbohydrates rearrange to fill the void? The lateral shrinkage is minor, so any significant rearrangement must result only in a thinner layer and would not be observable. One gets the impression that the protein is not very important in the maintenance of the shape, although chitinase and chitobiase do not degrade the structure while the protein is present<sup>11,22</sup>. (Collagen is protected from collagenase by a glycoprotein in a similar way<sup>36,52</sup>). Does the protein exercise its biological function and specificity in the initial construction of the layer? The overall shape of the float seems to be determined by differential mitosis in the adjacent cell layer, the layer itself being sufficiently flexible (at least initially!) to match the contours of the previous layers. The protein function seems limited then to the organization of the layer and the layer-layer interactions. It is spatially oriented relative to the carbohydrate chains so that the glycosidic linkages are shielded from marauding enzymes.

The reaggregation of the centrifuged washings might tell more and the significance of such a study is indicated by the protein-

carbohydrate participation in specific cell-cell aggregation and the failure to reaggregate after treatment with trypsin<sup>29,31,47,55</sup>.

To what extent does velatin participate in the extracellular building of the layer? Since cellulose (a carbohydrate) and collagens (proteins) both aggregate spontaneously no preference is apparent. There is probably a concerted effect.

## CHAPTER 5

### CONCLUSION

How does one evaluate scientific work? For analytical results and personal observations the author's statement of reproducibility invites ready confirmation if the reports are sufficiently detailed. Another evaluation requires an objective appraisal of how the investigator's time and sacrifice have been rewarded. What really has been accomplished? What value is there to the average person of a study of "jellyfish bones"? The words themselves invariably provoke humor and ridicule. The objectivity of the author's evaluation is thus confronted by his desire to find significance in the results and the discomfiting suspicion that nothing which could not have been predicted was found. A reasonable middle path is perhaps a statement of what has been confirmed, what is felt to be new, and what possibilities are suggested for further work.

The presence of chitin and protein in Velella float was confirmed and the common value of 45/55% for the weight distribution was found. Calcium was again found associated with connective tissue. The carbohydrate was shown to be a  $\beta$ -linked polymer of N-acetylglucosamine, and the spontaneous aggregation of chitinous material was again observed.

The definition by Hunt<sup>19</sup> of chitin as a protein-carbohydrate complex conveys accurately the general picture found in Velella, although some indication of the N-acetylglucosaminyl oligosaccharides

and the associated species-specific(?) protein as a glycoprotein subunit in sequentially synthesized layers will probably prove to be a more descriptive identification. When all of the levels of organization and their linkages have been determined the definition will cause less difficulty.

Some hesitation attaches to the designation of a finding as "new" and perhaps a more tentative phraseology would be more appropriate. However, it is cautiously put forward that the statement by Brimacombe and Webber that "Precise methods for the quantitative determination of chitin do not exist because the isolation and purification of the polysaccharide normally result in its modification. Moreover, it is difficult to assess the purity of chitin<sup>4</sup>" should be modified for Veilella float material. The basic treatment of chitin at room temperature was shown to give a product of defined purity, and the elemental analysis seems to resolve the question of the degree of N-acetylation. The optimum hydrolysis conditions for chitin were found and the application of Rondle and Morgan's colorimetric determination methods<sup>39</sup> were shown to be useful for chitin studies. The absence of phosphorus was noted. The utility of the amino acid analyzer was demonstrated for chitin investigations and as an analytical tool in assaying purification and separation techniques. An amino acid analysis was obtained which was reproducible and quantitative determinations were found feasible for both the protein and carbohydrate. The calculations of the amino acid results provided the basis for the construction of an hypothetical glycoprotein which seems to correlate

much of the evidence in the chitin literature. The author hopes that a good case has been made for reporting amino acid analysis results in mole ratios rather than the more common "residues per 1,000 residues". The observation that the layers can be delaminated with formalin treatment should prove useful in the future and suggests a sequence of events in the morphological development of the float. The observation of a topologically continuous concentric membrane-like (TCCM) structure provided an unexpected dividend for the author as it provides support for a general hypothesis of biological activity<sup>58</sup> which is based on the TCCM concept.

The Velella investigation seems to offer new insight into a number of biological events. The gap between DNA and final shape seems somewhat narrower in the case of Velella and hopefully the intermediates can be isolated and identified. The relationship between specific cell-cell attachment, differential mitosis, and the extracellular matrix can probably be determined more readily in Velella than in a more complex animal. The expected phylogenetic progression of structural proteins can be studied using velatin as a basis. A comparison of the sequence of velatin with other structural proteins is to be eagerly anticipated. New insight is provided into the general synthesis of membranes, in which glycoproteins and glycolipids unite into a continuous sheet, and into mucopolysaccharide "mucous" layers, in which they do not. The peculiar correspondence in the amino acid and carbohydrate mole ratios suggests an undiscovered relationship between proteins and hexose-hexosamine, and an explanation for the use of these particular molecules for morphological events.

The work which is left to do is humiliating on the one hand and inspirational on the other in the offering of a lifetime of challenging pursuits. As a reminder for further work one might mention the various linkages awaiting study, the amino acid sequence of velatin, the oligosaccharide length, the isolation of the glycoprotein from the mesoglea, the position of the oligosaccharide linkages (presumably 1,4), x-ray diffraction comparisons with other chitinous materials, detergent separation methods, a study of the interrelationship of light-sensitive calcium-specific proteins and of purple pigments in circadian rhythms, the relationship of calcium, silicon and water to the matrix, the relationship between calcium and the contractility of the muscular margin, and the medical uses of a chemically defined chitin (intra-articular injection of chitin induces an arthritic condition<sup>33</sup>). This list is incomplete but it is clear that Velella represents a scientific feast for many disciplines.

An extension of the introductory quotation might be appropriate for a final statement. The extracellular matrix has its beginnings within the cell, and there is a continuum which includes the golgi apparatus and the cell membrane surface<sup>37</sup>. The function of the matrix is concerned with all substances, structures and events which occur in the extracellular space: e.g. membranes, tissue fluid, blood, ion concentrations, phagocytosis, immune response, hormones, toxicity, synaptic phenomena, cell-cell interactions, nutrients, and gas exchange. The "complete knowledge of the structure and function of the matrix" is thus an ambitious goal. Hopefully the present work is

a contribution.

One is naturally reluctant to conclude an obviously incomplete work; the author therefore announces ....

-a beginning-



## REFERENCES

## REFERENCES

1. Balaz, Endre A. (1970) in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 1, p. xxiv.
2. Barker, S.A., A.B. Foster, M. Stacey and J.M. Webber (1957) *Chem. Ind. (London)* 208; and (1958) *J. Chem. Soc.* (1958) 2218.
3. Borisy, G. and J. Olmstead (1972) "Nucleated Assembly of Microtubules in Porcine Brain Extracts" *Science* 177, 1196.
4. Brimacombe, J.S. and J.M. Webber (1964) Mucopolysaccharides, Elsevier, Amsterdam, chap. 2.
5. Carlisle, E.M. (1972) "Silicon: An Essential Element for the Chick" *Science* 178, 619.
6. Carlstrom, D. (1957) *J. Biophys. Biochem. Cytol.* 3, 669.
7. \_\_\_\_\_ (1962) *Biochim. Biophys. Acta* 59, 361.
8. Caster, K.E. (1942) *Pal. Americana* 3, No. 14, pp. 1-34.
9. Cremer, E. and L. Roselius (1958) *Angew. Chem.* 70, 42
10. Dweltz, N.E. (1961) *Biochim. Biophys. Acta* 51, 283.
11. Florkin, M. (1966) A Molecular Approach to Phylogeny, Elsevier, Amsterdam.
12. Flower, R.H. and R. Wayland-Smith (1947) *Bull. Geol. Soc. Amer.* 58, 1180.
13. Foster, A.B. and R.H. Hackman (1957) *Nature (London)* 180, 40.
14. Franzblau, C., B. Faris, R.W. Lent, L.L. Salcedo, B. Smith, R. Jaffe, and G. Crombie (1970) "Chemistry and Biosynthesis of Crosslinks in Elastin" in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 1, pp. 617-639.

See also Yarnas, I.V. (1972) *Rev. Macromol. Chem.* 8, 49; and W. Traub and K.A. Piez (1971) *Adv. Protein Chem.* 26, 243 which gives a comprehensive survey of the various proposals for crosslinking in collagen.

- Also valuable is J.G. Nold, A.H. Kang and J. Gross (1970) *Science* 170, 1096 which presents "Conclusive evidence that each TC (tropocollagen) molecule in mammalian skin is made up of two alpha 1 and one alpha 2 chains....(there is) agreement that the alpha chains are crosslinked intramolecularly by bonds located in their respective non-helical N-terminal ends and formed by aldol condensation of aldehydes derived from lysine...the nature of the....crosslinks has remained open." I.V. Yannas (1972).
15. Green, H. and B. Goldberg (1963) *Nature* 200, 1097; and (1964) *J. Cell Biol.* 22, 227.  
See also H. Green, B. Goldberg and G.J. Todaro (1963) *Exptl. Cell Res.* 31, 441.
  16. Gross, J., B. Dumsha and N. Glazer (1958) *Biochim. Biophys. Acta* 30, 293.
  17. Hackman, R.H. (1955a) *Aust. J. Biol. Sci.* 8, 83; (1955b) *Aust. J. Biol. Sci.* 8, 530.  
See also J. Gross and D. Kirk (1958) *J. Biol. Chem.* 233, 355; J. Gross (1964) *Science* 143, 960; and J.M. Cassel (1970) National Bureau of Standards Report 10271, U.S. Department of Commerce.
  18. \_\_\_\_\_ (1960) *Aust. J. Biol. Sci.* 13, 568; (1962) *Aust. J. Biol. Sci.* 15, 526.
  19. Hunt, S. (1970) Polysaccharide-Protein Complexes in Invertebrates, Academic Press, New York, chap. 8.
  20. Hyman, Libbie H. (1940) The Invertebrates, McGraw-Hill, New York, vol. 1, pp. 90, 300-301, 475-485.
  21. Jackson, D.S. and J.P. Bentley (1968) "Biology of Collagen", part A, in Treatise on Collagen, B. Gould ed., Academic Press, New York, vol. 2, p. 189.
  22. Jeaniaux, C. (1963) Chitine et Chitinolyse, Masson, Paris.
  23. Kaestner, A. (1964) Invertebrate Zoology, trans. from the 2nd German edition by H. and L. Levi, Interscience Publishers, New York, vol. 1, pp. 43-88.
  24. Katzman, R.L. and R.W. Jeanloz (1970) "The Carbohydrate Chemistry of Invertebrate Connective Tissue" in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 1, p. 217.

The authors state that "Glucosylgalactosylhydroxylysine is apparently widely distributed throughout the animal kingdom as the carbohydrate moiety of collagen. Thus far, no other carbohydrate moiety has been found."

25. Kent, P.W. (1964) in Comparative Biochemistry, M. Florkin and H.S. Mason eds., Academic Press, New York, vol. 7, p. 93.  
See also P.W. Kent and M.W. Whitehorse (1955) Biochemistry of the Aminosugars, Academic Press, New York.
26. Kolthoff, I.M. and E.B. Sandell (1952) Textbook of Quantitative Analysis, 3rd ed., Macmillan, New York, p. 339.
27. Meyer, K. (1970) in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol.1, pp. 5-24.
28. Miller, E.J., G.R. Martin and K.A. Piez (1964) Biochem. Biophys. Res. Comm. 17, 248; and E.J. Miller (1971) Biochem. Biophys. Res. Comm. 45, 444.
29. Moscona, A.A. (1971) "Embryonic and Neoplastic Cell Surfaces: Availability of Receptors for Concanavalin A and Wheat Germ Agglutinin" Science 171, 905; and (1968) in In Vitro, M.M. Sigel ed., Williams and Wilkins, Baltimore, vol. 3, pp. 13-21.
30. Neville, A.C. (1965) Q.J. Micros. Sci. 106, 315.
31. Nicolson, G.L. and R. Yanagimachi (1972) "Terminal Saccharides on Sperm Plasma Membranes: Identification by Specific Agglutinins" Science 177, 276.
32. Nissan, A.H., G.K. Hunger and S.S. Sternstein (1965) "Cellulose" in Ency. of Polymer Sci. and Tech., Wiley, New York, vol. 3, p. 168.
33. Page Thomas, D.P. (1970) "Experimental Arthritis and Acid Hydro-lases" in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 3, p. 1711.
34. Partridge, S.M., D.F. Elsdon, J. Thomas, A. Dorfman, A. Telser, and P-L. Ho (1966) Nature 209, 399.
35. Pearson, F.G., R.H. Marchessault and C.Y. Liang (1960) J. Polymer Sci. 43, 101; and (1960) Biochim. Biophys. Acta 45, 499.
36. Quintarelli, G. and M.C. Dellovo (1970) in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz, ed., Academic Press, New York, vol. 3, p. 1403.

The authors state that "The results reported here sustain

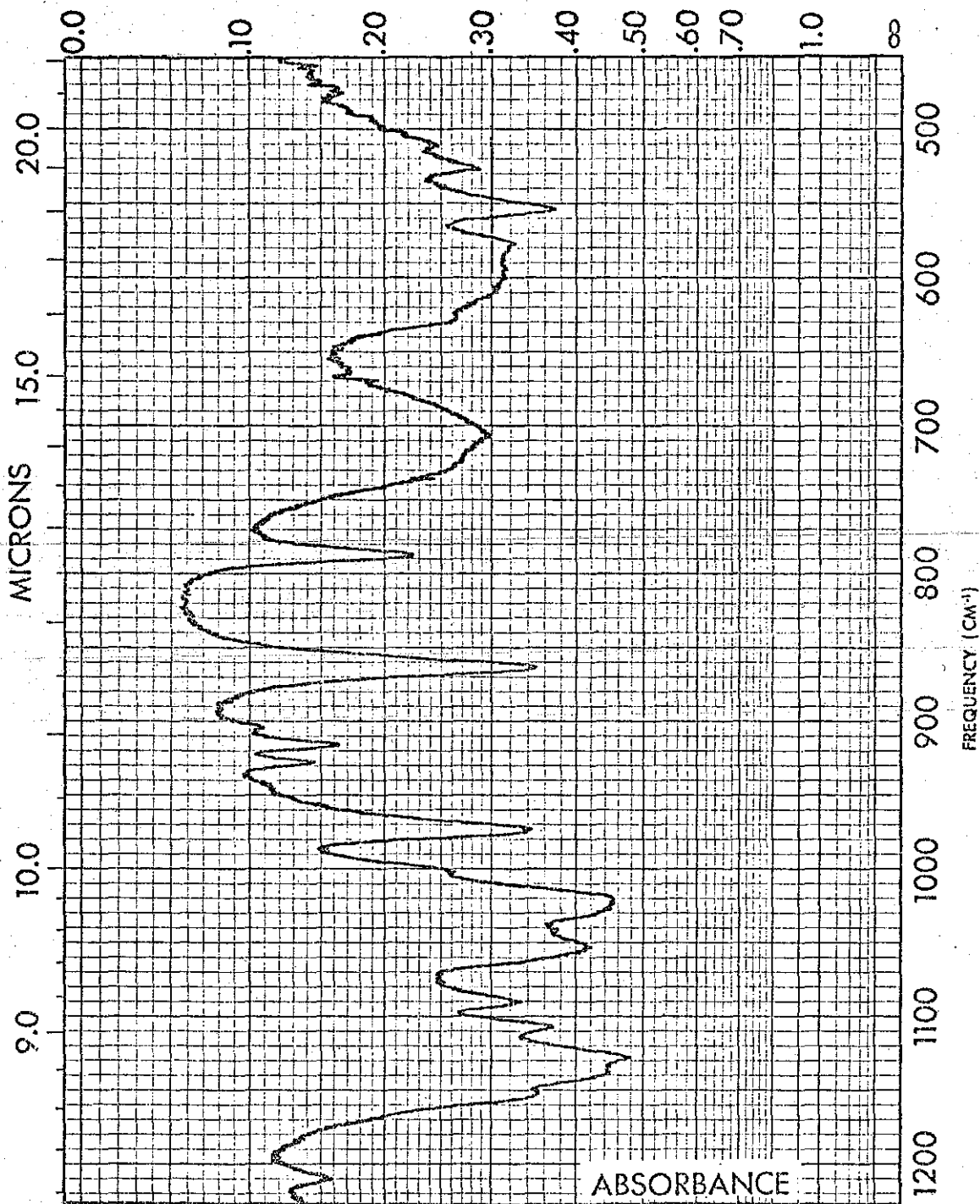
- (our earlier opinion; 1966, 1967) and are in agreement with the findings of Steven et al (1968), that a glycoprotein linked with collagen prevents in some as yet unknown manner, the hydrolysis of the collagen fibers by collagenase." See also G. Quintarelli and M.C. Dellovo (1966) *Histochemie* 7, 141; and (1967) *Histochemie* 8, 216.
37. Revel, J.P. and S. Ito (1967) in The Specificity of the Cell Surface, Warren and David eds., Prentice-Hall, Englewood Cliffs, New Jersey, p. 211.
  38. \_\_\_\_\_ (1970) "Role of the Golgi Apparatus in the Elaboration of Matrix Glycosaminoglycans" in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 3, p. 1485.
  39. Rondle, C.J.M. and W.T.J. Morgan (1954) *Biochem. J.* 61, 586.
  40. Rudall, K.M. (1955) "The Distribution of Collagen and Chitin" in *Symposia Soc. Exptl. Biol* 9, 49.
  41. \_\_\_\_\_ (1963) *Adv. Insect Physiol.* 1, 257.
  42. \_\_\_\_\_ (1965a) *Biochem. J.* 95, 38P; and (1965b) in Structure and Function of Connective Tissue, Butterworth, London, p. 191.
  43. Sandberg, L.B., T.N. Hackett and W.H. Carver (1969) *Biochim. Biophys. Acta* 181, 201.
  44. Schmitt, F.O., J. Gross and J.H. Highberger (1953) *Proc. Natl. Acad. Sci.(US)* 39, 459.
  45. Schulze, P. and G. Kunike (1923) *Biol. Zentr.* 43, 556.
  46. Scott, R.P.W. and D.W. Grant (1964) *Analyst* 89, 179.
  47. Sharon, H. and H. Lis (1972) "Lectins: Cell-agglutinating and Sugar-specific Proteins" *Science* 177, 949.
  48. Shimomura, O., F. Johnson and Y. Saiga (1962) "Extraction, Purification and Properties of Aequorin from Aequorea" *J. Cell Comp. Physiol.* 59, 223-239.  
 See also J.R. Binks, P.H. Mattingly, B.R. Jewell, and M. Van Leeuwen (1969) *Fed. Proc.* 28, 781; B. Kaminer and J. Kimura (1972) *Science* 176, 406; and C.C. Ashley and J. Ridgway (1970) *J. Physiol. London* 209, 105.
  49. Smith, D.W., N. Weissman and W.H. Carver (1968) *Biochem. Biophys. Res. Comm.* 31, 309.

50. Spiro, R.G. (1970) "Carbohydrates in Collagen" in Chem. and Mol. Biol. of the Intercellular Matrix, E. Balaz ed., Academic Press, New York, vol. 1, pp. 195-215.

The author states: "The density of the (hydroxylysine-linked) carbohydrate units varies from 1 per 1000 amino acid residues in scleral collagen to 27 per 1000 amino acid residues in lens capsule." It is further noted that in tendon collagen many single galactose residues are present with the glucosylgalactosylhydroxylysine and galactosylhydroxylysine, while in the basement membranes almost all units are disaccharides and only glucosylgalactosylhydroxylysine is found.

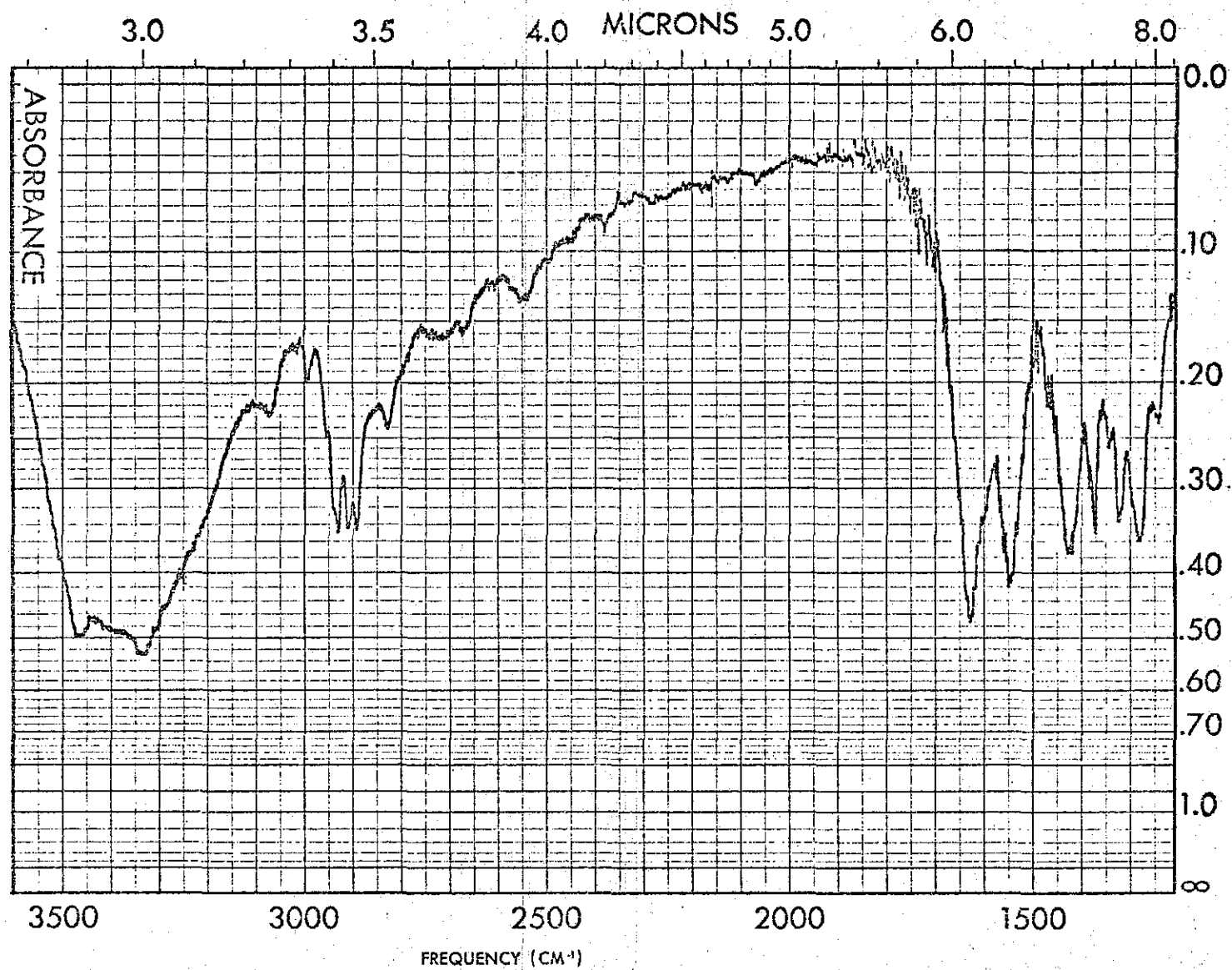
51. Steiner, R.F. (1965) The Chemical Foundations of Molecular Biology, Van Nostrand, Princeton, New Jersey, p. 396.
52. Steven, F.S., D.S. Jackson and K. Broady (1968) Biochim. Biophys. Acta 160, 435.
53. von Hippel, P. and T. Schleigh (1969) "Ion Effects on the Solution Structure of Biological Macromolecules" Accts. of Chem. Res. 2, 257.
54. Weisenberg, R.C. (1972) "Microtubule Formation in vitro in Solutions Containing Low Calcium Concentrations" Science 177, 1104.
55. Weiser, M.M. (1972) "Concanavalin A Agglutination of Intestinal Cells from the Human Fetus" Science 177, 525.
56. Whaley, G.W., M. Dauwalder and J. Kephart (1972) "Golgi Apparatus: Influence on Cell Surfaces" Science 175, 596.
57. Yannas, I.V. (1972) "Collagen and Gelatin in the Solid State" Rev. Macromol. Chem. 8, 49.
58. Presented in a seminar at U. of the Pacific, Stockton, California, 12 July, 1972. In preparation.

A P P E N D I X

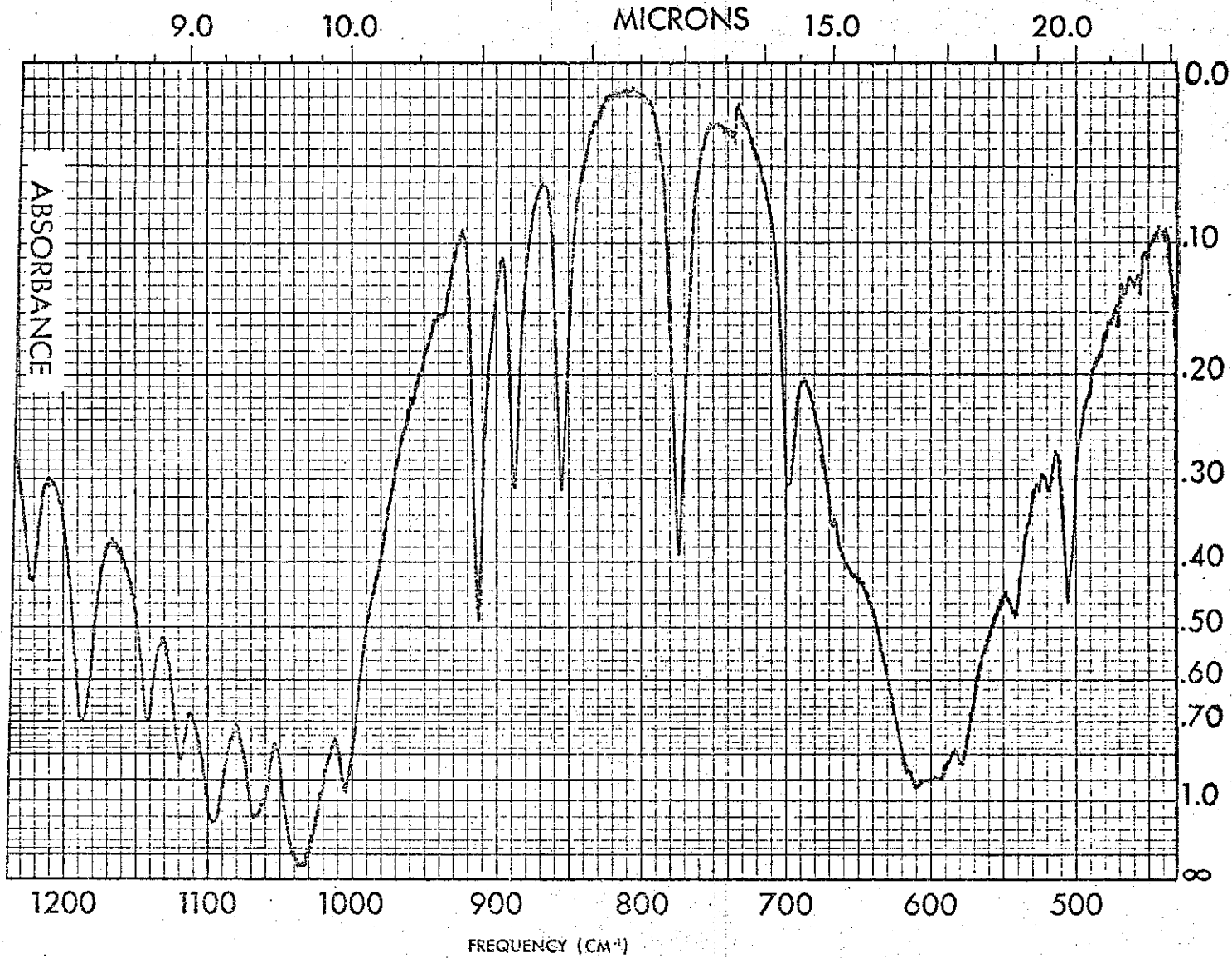


Infrared Spectrum 1(a). N-acetylglucosamine

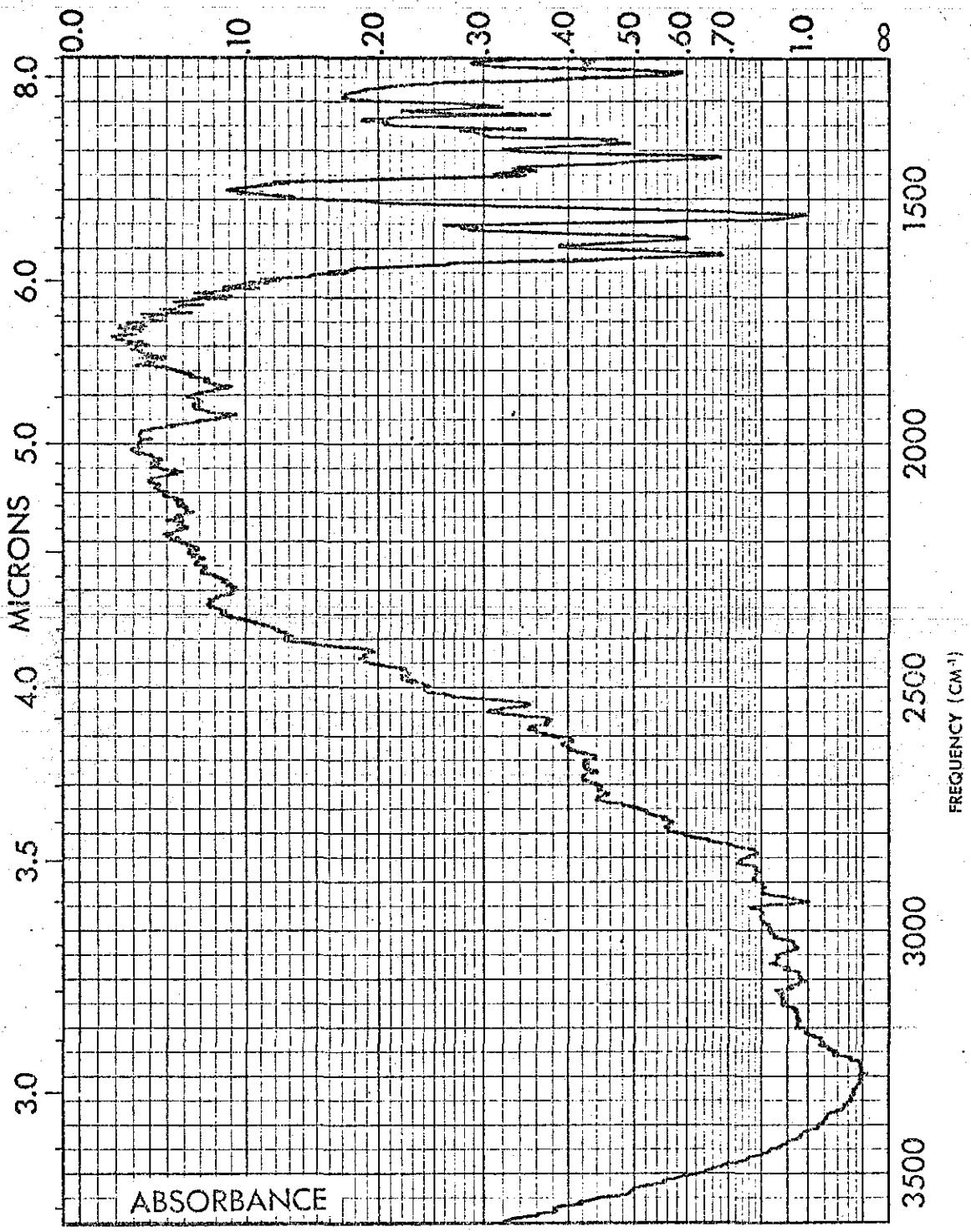




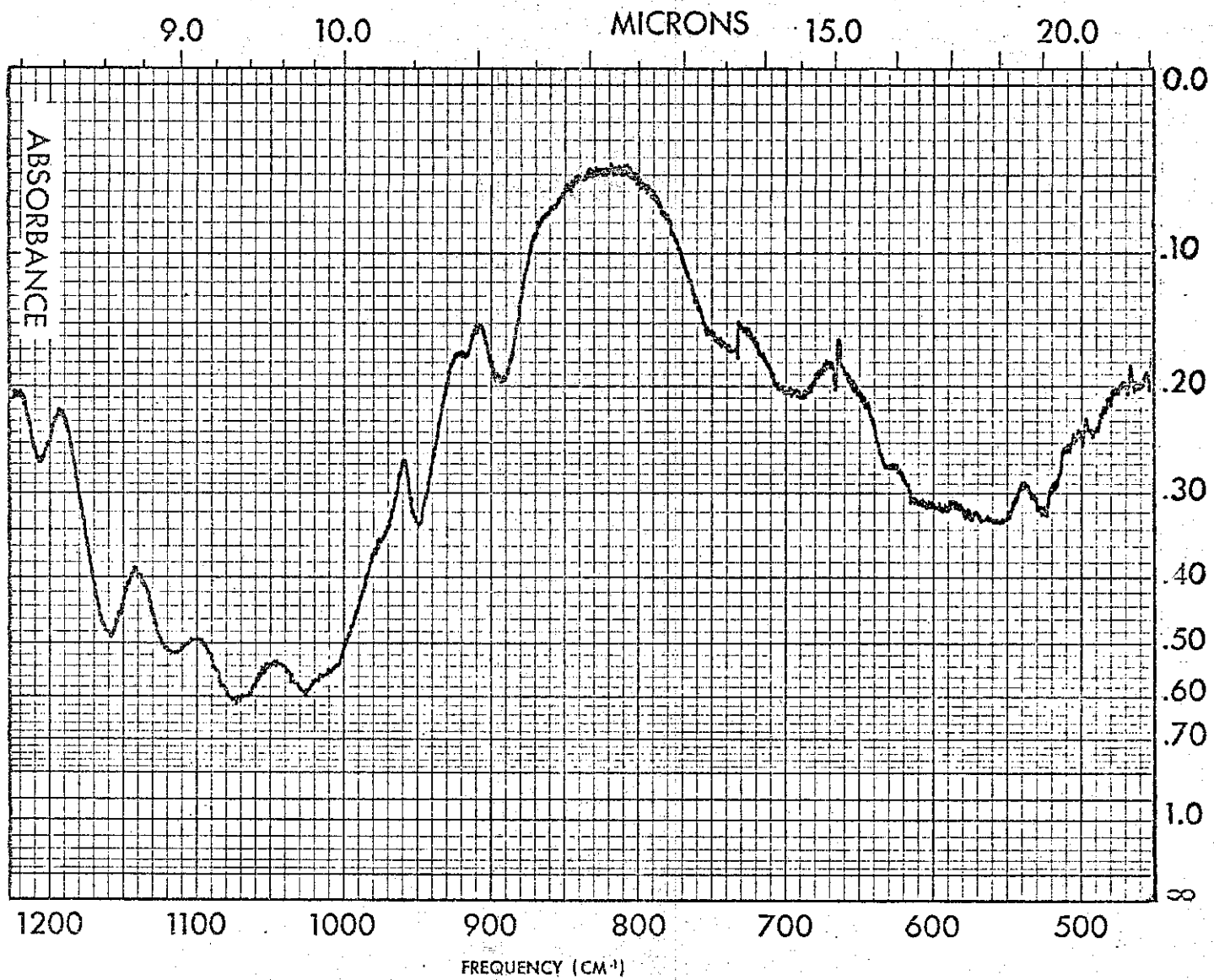
Infrared Spectrum 1(b). N-acetylglucosamine



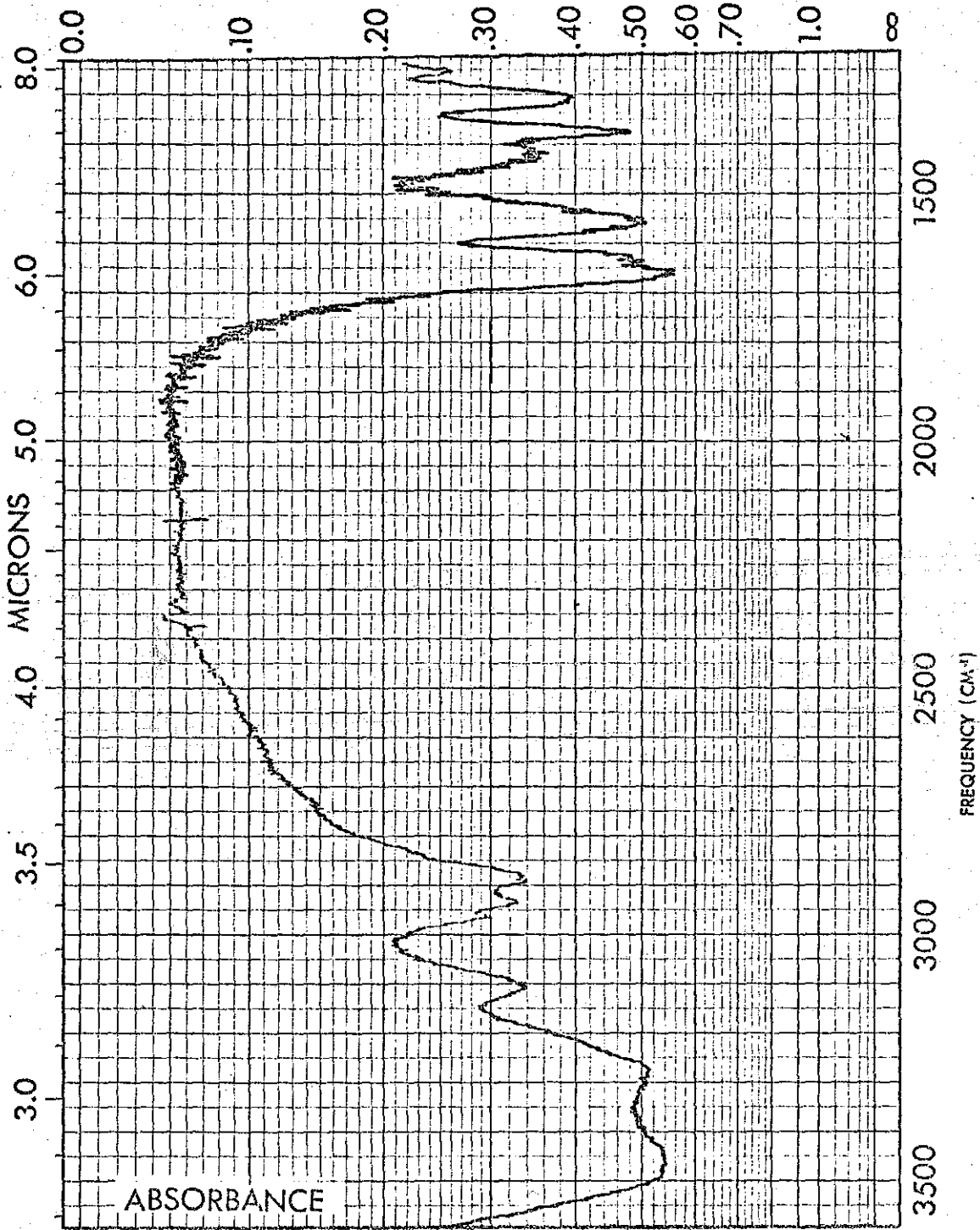
Infrared Spectrum 2(a). Glucosamine hydrochloride



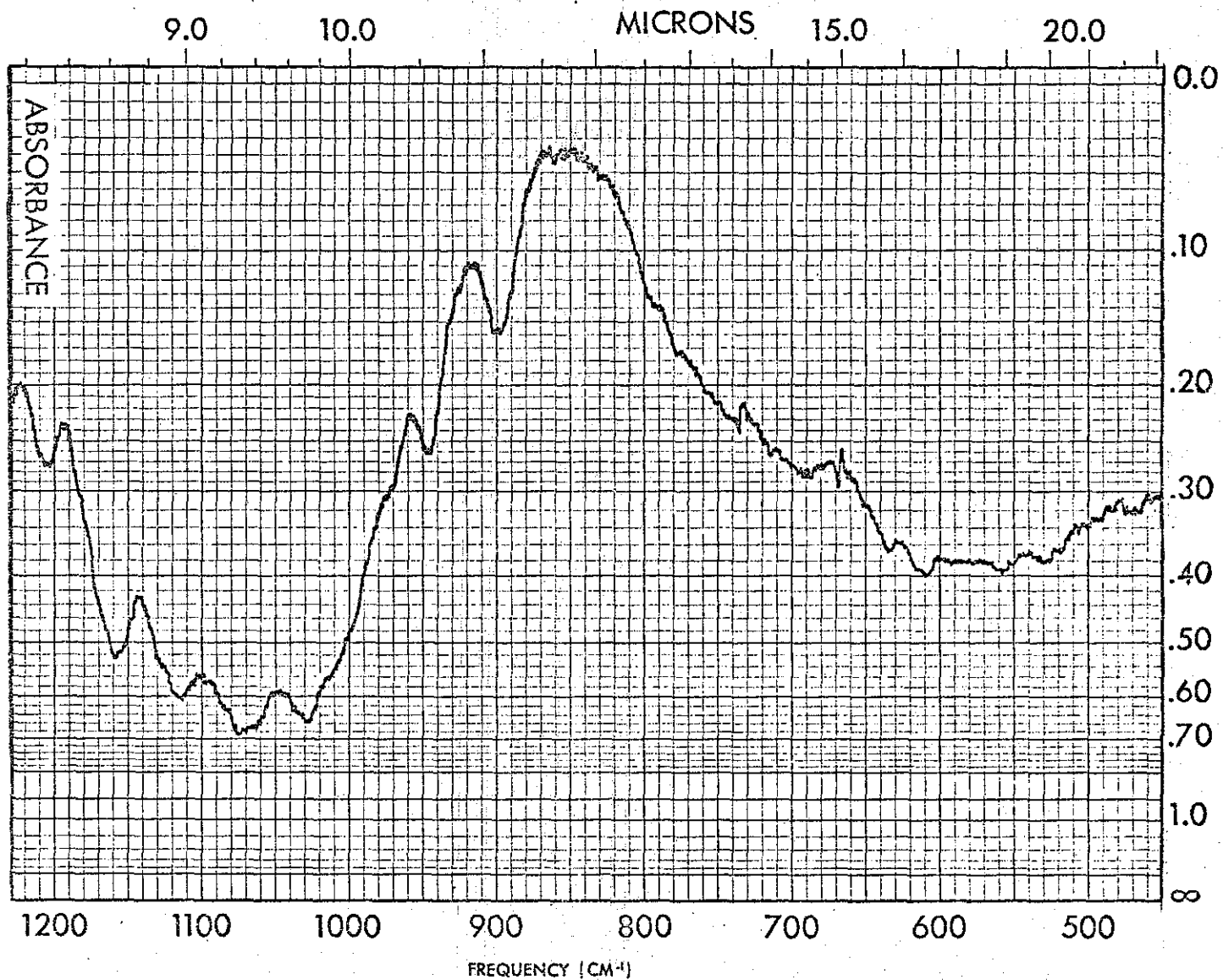
Infrared Spectrum 2(b). Glucosamine hydrochloride



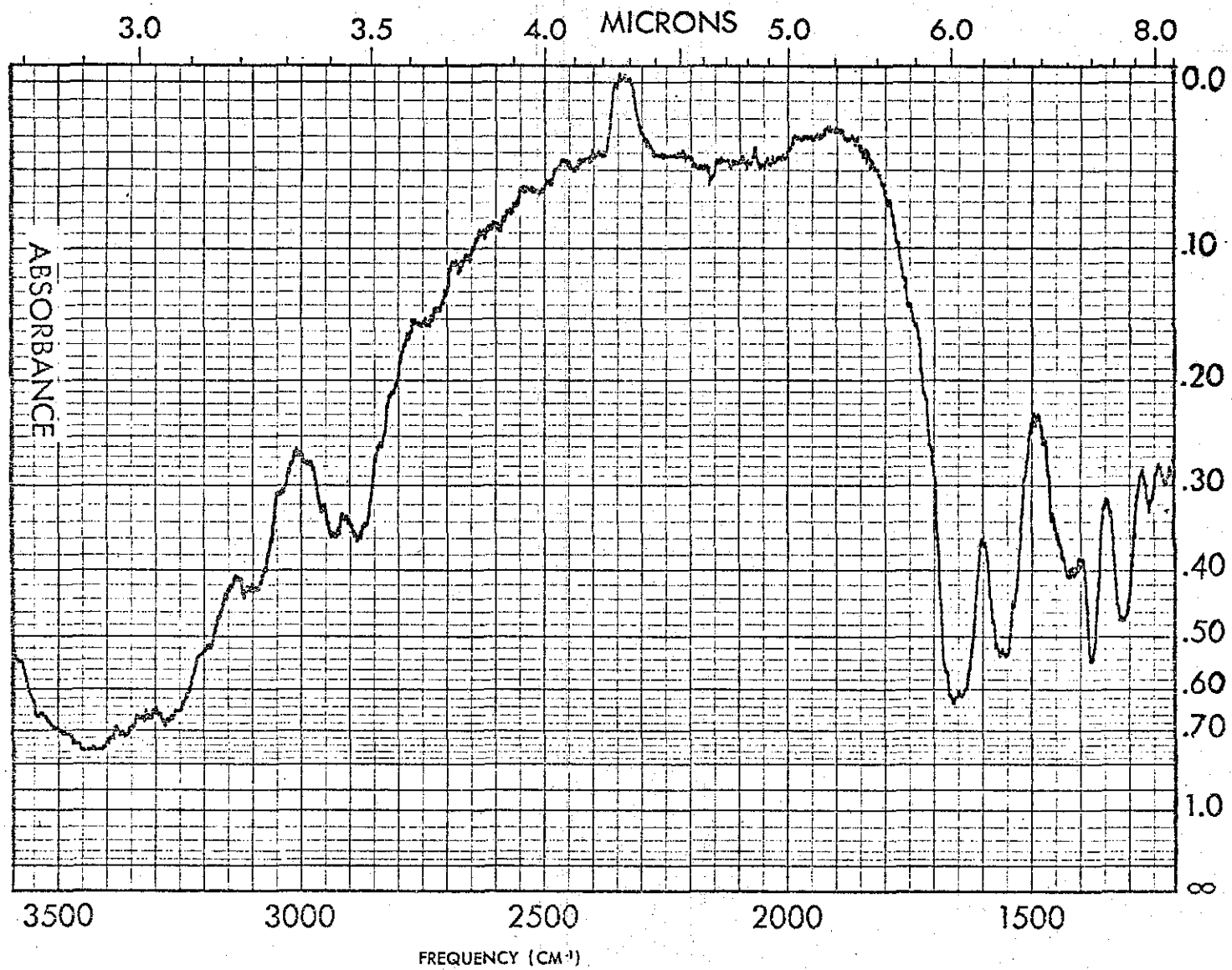
Infrared Spectrum 3(a). Shrimp shell



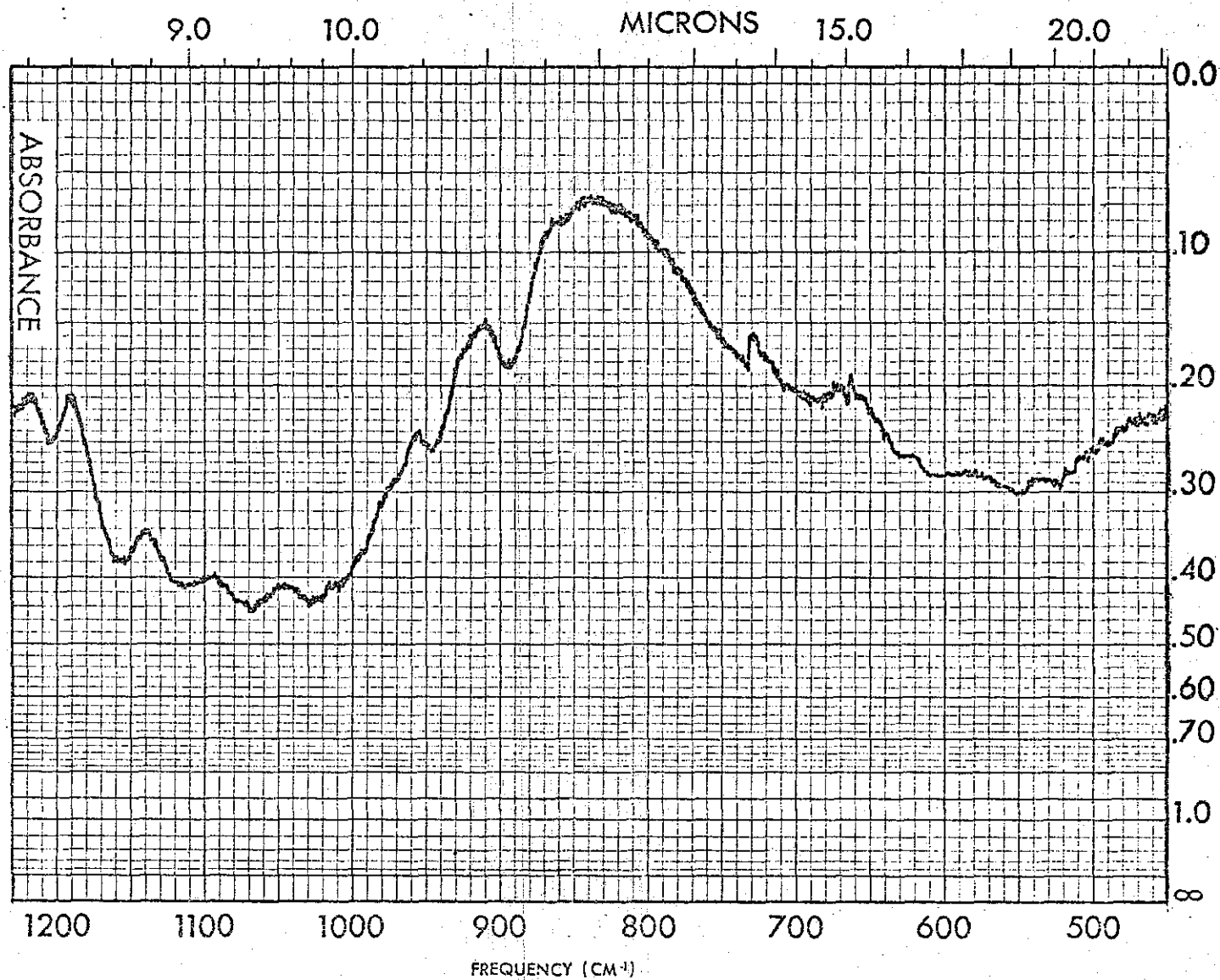
Infrared Spectrum 3(b). Shrimp Shell



Infrared Spectrum 4(a). Velella Float Material: (6N KOH, 8 days at 25<sup>0</sup>C)

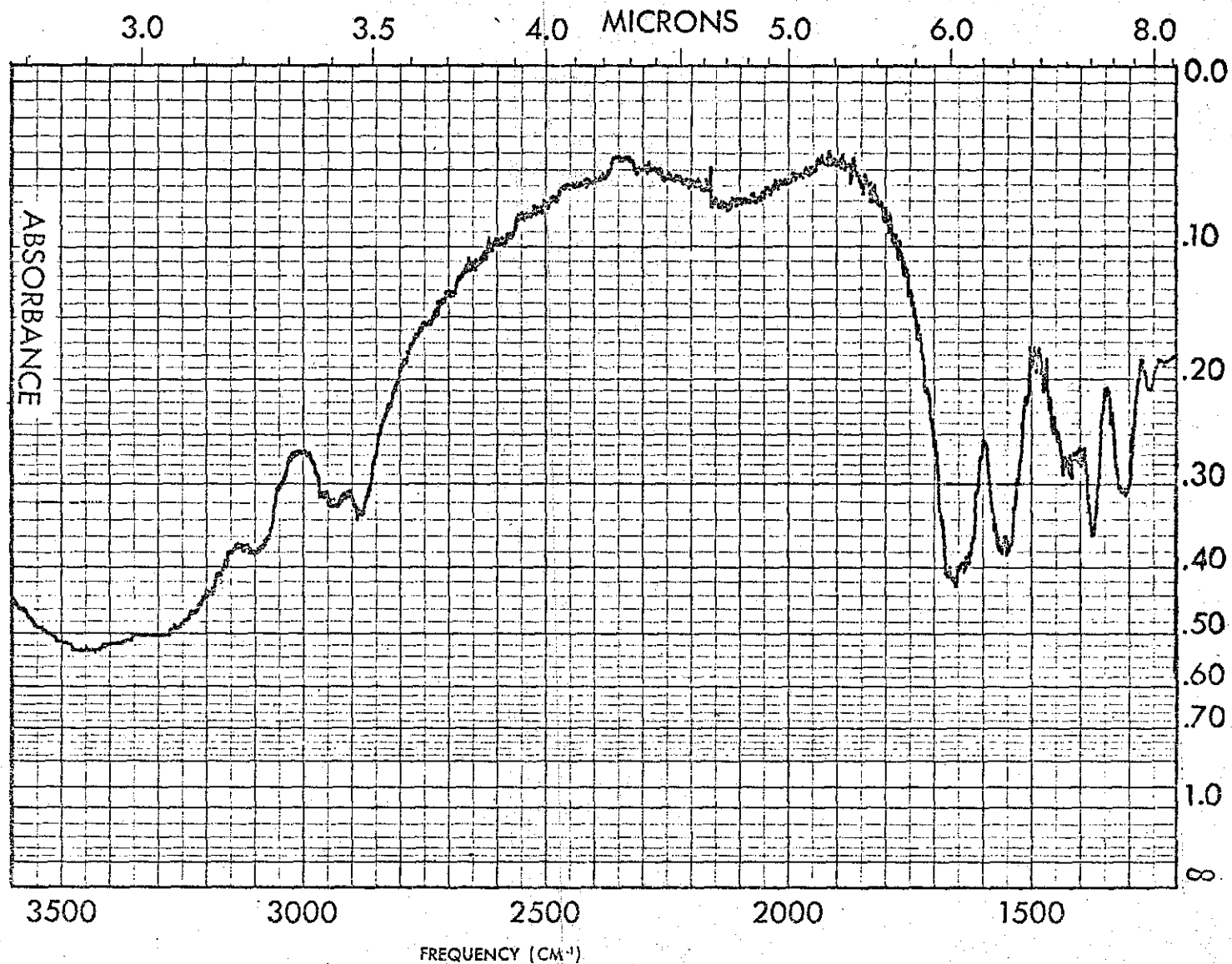


Infrared Spectrum 4(b). Veilella Float Material (6N KOH, 8 days at 25°C)

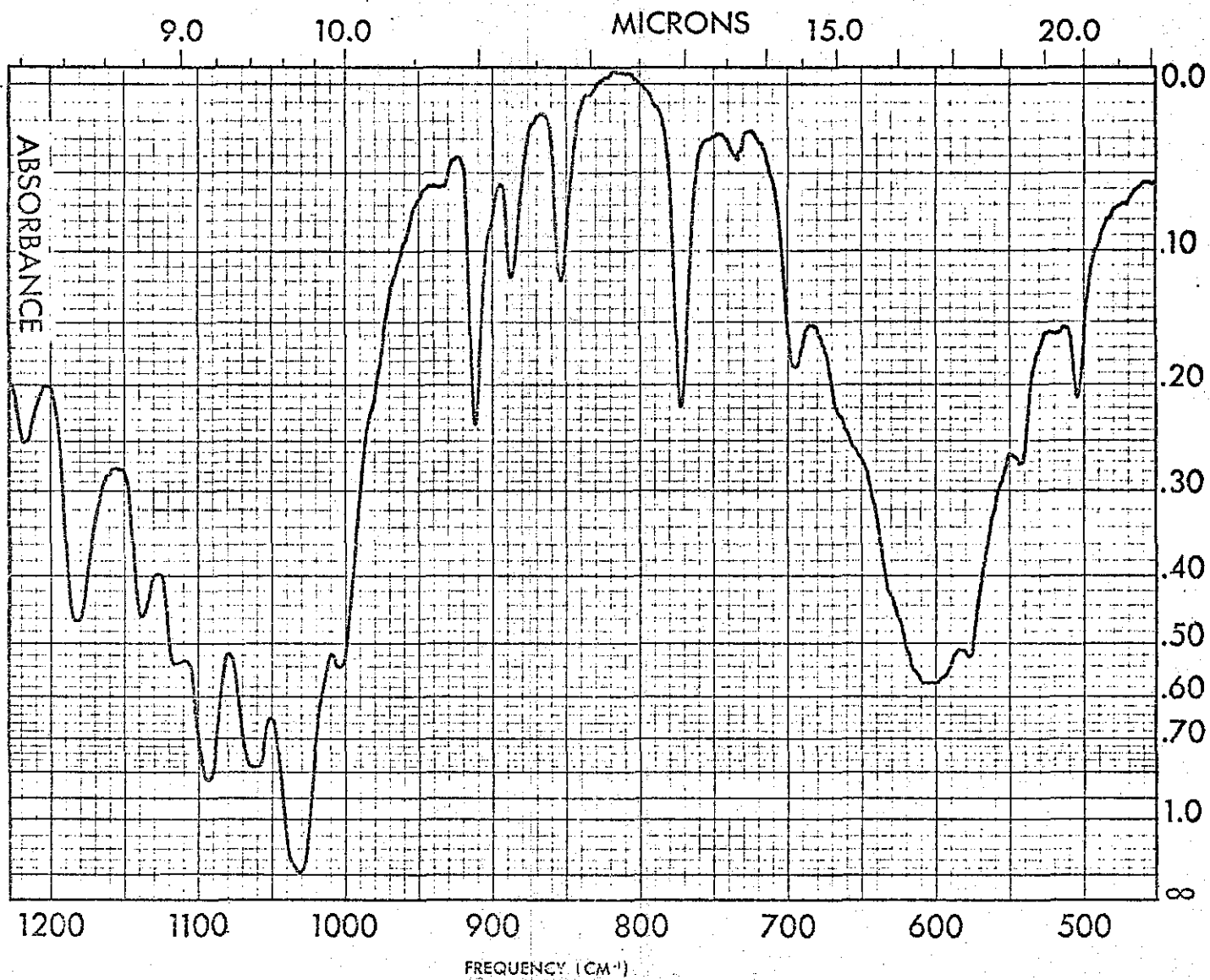


Infrared Spectrum 5(a). Veilella Float Material (1N KOH, 24 hr at 100°C)

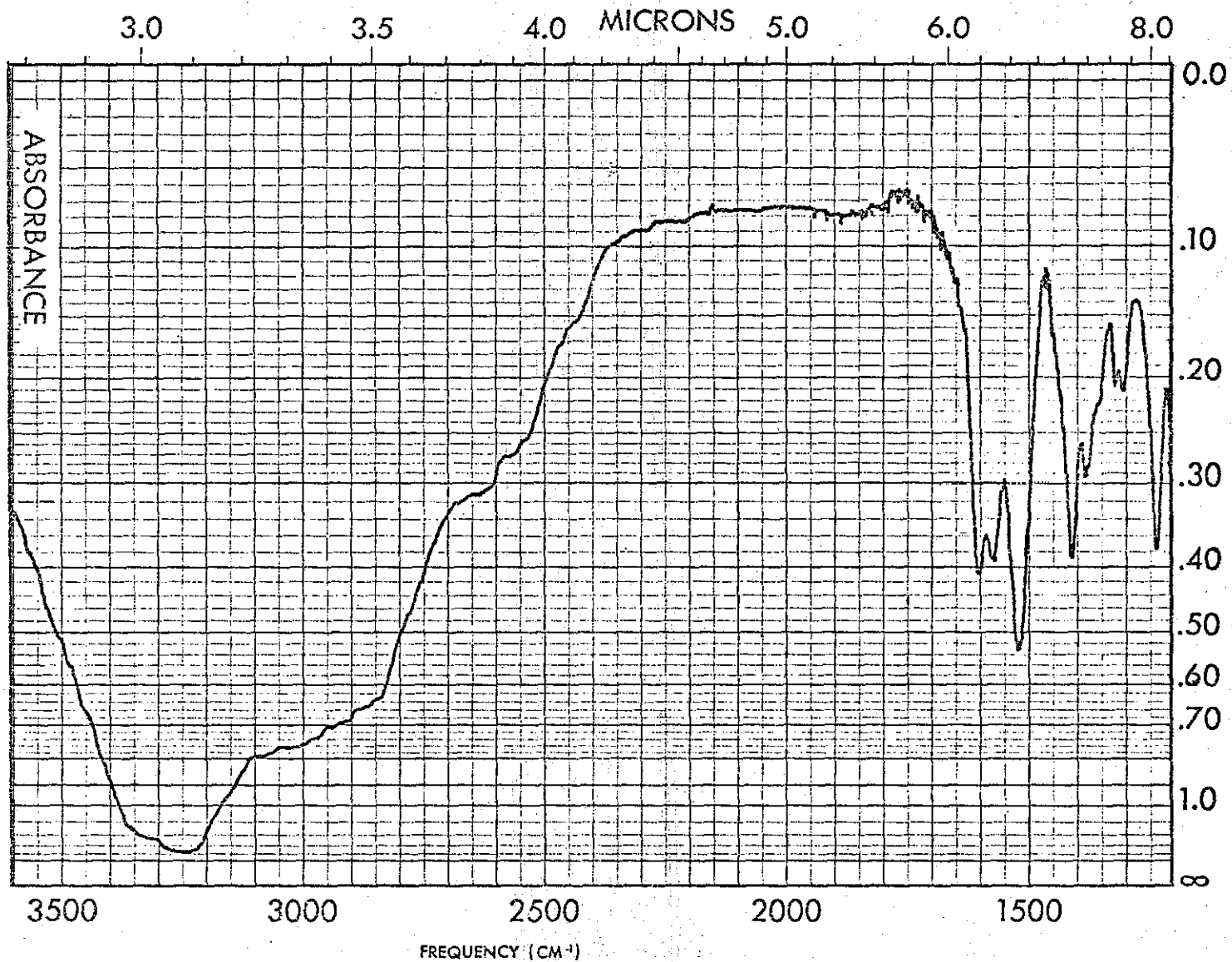




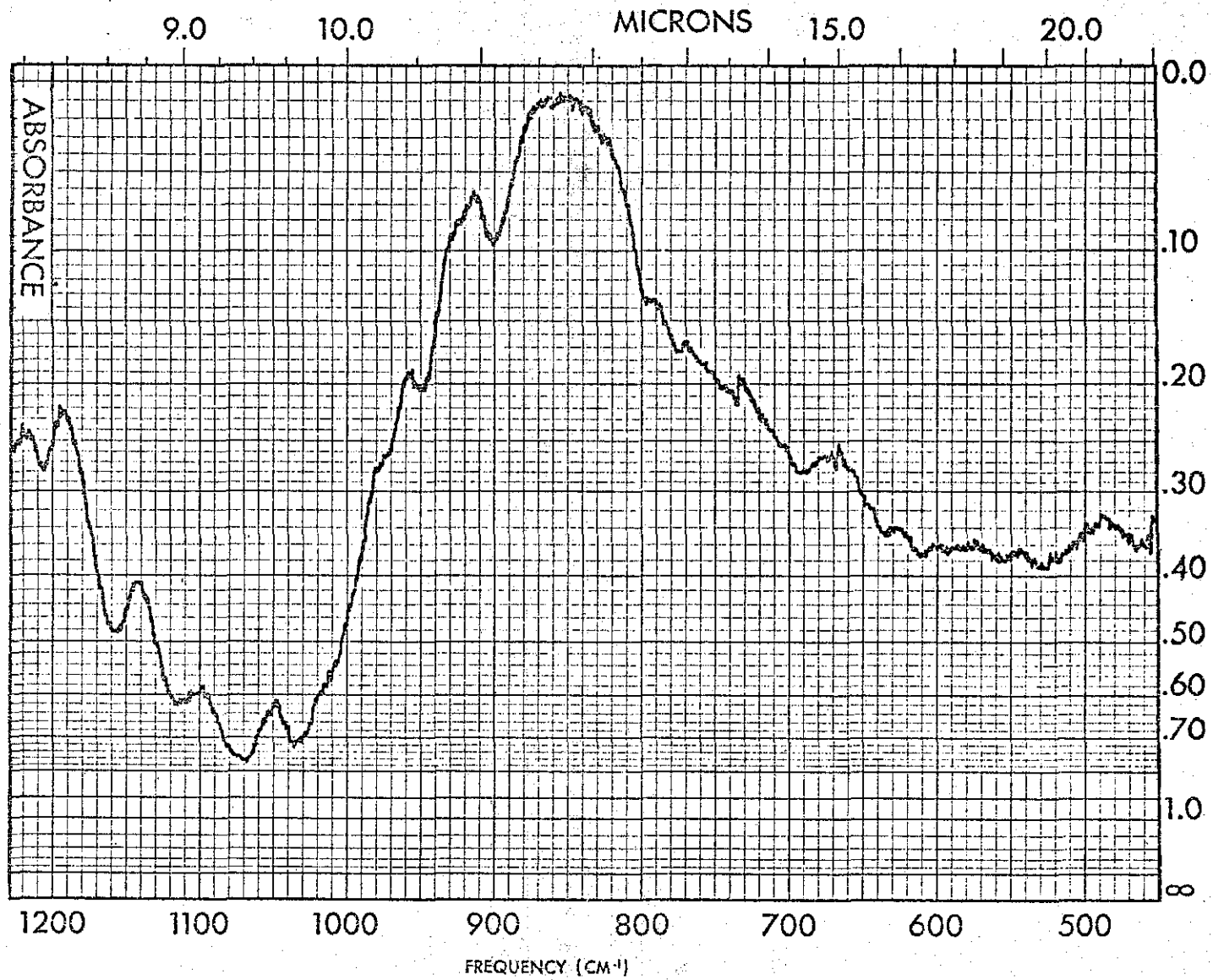
Infrared Spectrum 5(b). Velella Float Material (1N KOH, 24 hr at 100°C)



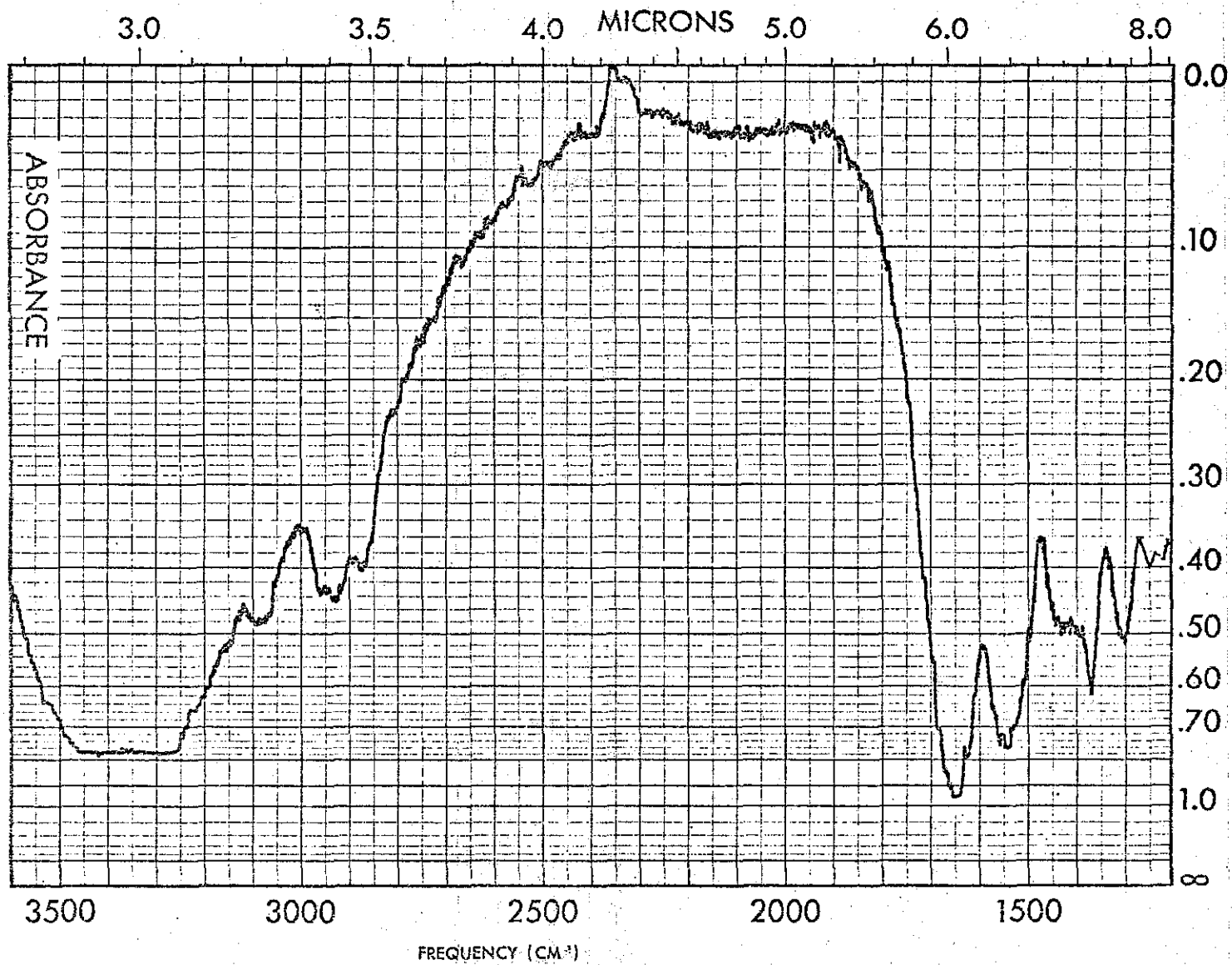
Infrared Spectrum 6(a). Crystalline Material From Acid  
Hydrolysate of Veilella Float Material



Infrared Spectrum 6(b). Crystalline Material From Acid  
Hydrolysate of Veilella Float Material



Infrared Spectrum 7(a). Veilella Float Material (washed and dried)



Infrared Spectrum 7(b). Velella Float Material (washed and dried)

Table 8

Systematic Identification Of Velella lata

Phylum Coelenterata (Cnidaria). Coelenterates. Symmetry radial or biradial; the individual either a cylindrical sessile polyp, often in colonies, or a free-floating bell-like medusa with much mesoglea; with stinging nematocysts; digestive cavity (enteron) sac-like, sometimes branched; soft tentacles about mouth; no anus, no head, no other organ systems; nervous system diffuse; some with eyespots, or statocysts; reproduction usually asexual in polyps and sexual in medusae; dioecious or monoecious; no sex ducts; all aquatic, chiefly marine, attached or floating; Lower Cambrian to Recent, 10,000 species.

Class Hydrozoa. Hydroids (and some medusae). No stomodeum; enteron lacks partitions and nematocysts; mesoglea noncellular; medusae usually small and with velum (craspedote); chiefly in shallow salt waters; colonial or solitary; 3,700 species.

Order Hydroida. Polyp generation well developed, solitary or colonial, usually budding off small free medusae that bear ocelli and ectodermal statocysts.

Suborder Chondrophora (Physophorida). Polymorphic, colonial, free-floating; gonophores feed as medusae; upper end of colony a float or pneumatophore. Velella, float thin, with erect sail; Porpita, float disc-shaped.

(from Storer, Tracy I. and Usinger, Robert L., "General Zoology," 4th ed., McGraw-Hill, New York, 1957)

## Table 9

## Phyla Of The Animal Kingdom

Protozoa

Mesozoa

Porifera

Coelenterata

Ctenophora

Platyhelminthes

Nemertinea

Entoprocta

Aschelminthes

Acanthocephala

Ectoprocta

Phoronidea

Brachiopoda

Mollusca

Annelida

Sipunculoidea

Echiuroidea

Arthropoda

Chaetognatha

Echinodermata

Pogonophora

Hemichordata

Chordata

schematic representation of protein (m.w. 6800-7000) 44-60 amino acids

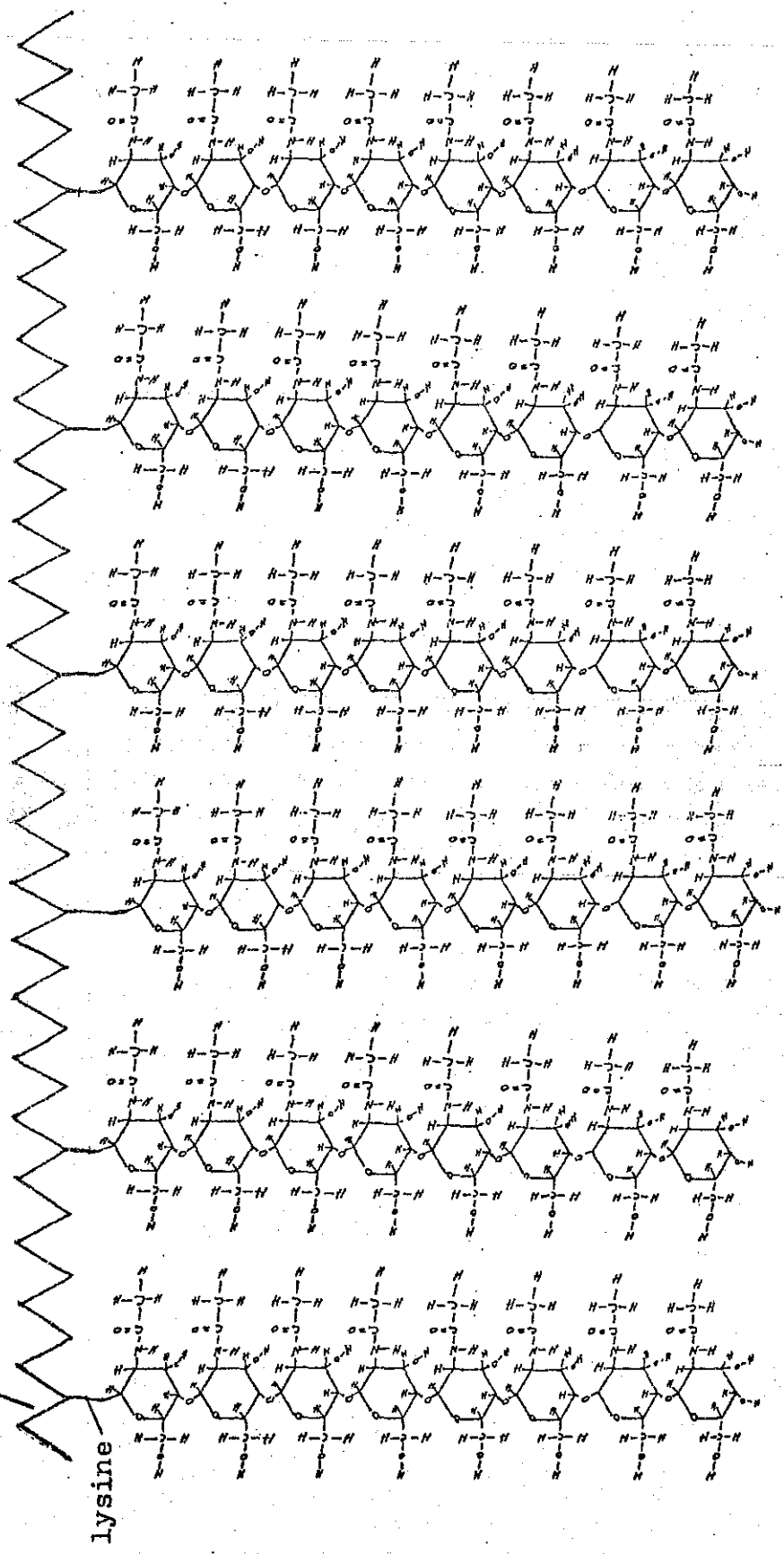
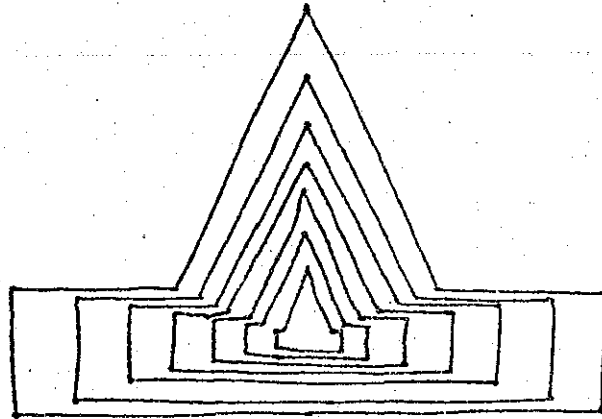


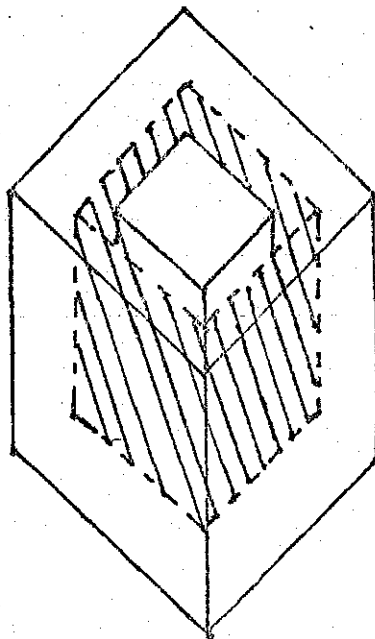
Figure 9

Hypothetical Macromolecule Containing Six Lysine-Linked Octasaccharides





(a) two dimensional representation (end view)



(b) three dimensional schematic representation illustrating TCCM model

Figure 10. Hypothetical Construction of Velella lata Float  
Based on a Topologically Continuous Concentric  
Membrane (TCCM) Model