A Further Study of the

ANTIGENIC PROPERTIES OF EUGLENA GRACILIS KLEBS

with related phenomena

by

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CONTENTS

page

GENERAL INTRODUCTION 1

GENERAL REVIEW OF LITERATURE 3

MATERIALS AND METHODS 3

EXPERIMENTAL WORK

I. The Cytotoxic Principle in Anti-Euglena Rabbit Serum 15

Introduction 15

Literature references to specific cytotoxic sera acting without serum complement 17

Additional technic 20

Experimental work on nature of the cytotoxic principle

1. Thermostability 21

2. Specificity 21

3. Adsorption 26

4. Role of electrolytes 27

5. Passive transfer 27

6. Dialysis 29

7. Concentration 30

8. Relationship to normal antibody 43

Discussion 47

II. The Production of Anaphylaxis with <u>Euglena gracilis</u> Klebs and certain other Unicellular Chlorophyll-bearing Organisms

Page

Introduction 49

Materials and methods 49

Experimental work 51

Discussion 58

III. A Study of the Chlorolytic Effect of Serum

Introduction 61

Experimental work 61

1. The chloroplast factor 61

2. The serum factor 63

3. Analysis of resulting supernatant fluid 66

4. Rate of chlorolysis 66

Discussion 68

GENERAL DISCUSSION 73

SUMMARY AND CONCLUSIONS 75

ACCESSORY FINDINGS 77

BIBLIOGRAPHY 78

APPENDIX 86

INDEX TO FIGURES

page

1. *Euglena gracilis* Klebs 9

2. Apparatus used for single cell isolation 12

3. Unicellular algae 14

4. *Euglena polymorpha* 23

5. Diagrammatic representation of serum proteins . . 35

6. Right uterine horn of virgin guinea pig no. 36 56

7. Right uterine horn of virgin guinea pig no. 39 57

8. Rate of chlorolysis with different sera 69

INDEX TO TABLES

page

1. Simple data on organisms used 16

2. Cytotoxin reaction with various strains and forms of *Euglena gracilis* Klebs 25

3. Absorption of the cytotoxic principle from immune rabbit serum with the homologous organism . . 28

4. Cytotoxic action of various serum fractions 39-41

5. Comparison of cytotoxic effect of anti-euglena rabbit serum with and without complement 46

6. Results of Dale tests 52-55

7. Comparative chlorolytic power of sera 65

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with related phenomena

Euglenas, aside from their occasional production of fishy and violet odors in water supplies, are of little direct economic importance. Nevertheless, they occupy a position of peculiar interest among the forms of microscopic life. The order Euglenida (Stein) is cosmopolitan, and approximately three hundred species have been described (Walton, 1915). Some of these at times occur in great abundance. Ponds of considerable size may become completely covered with a characteristic yellow-green tissue paper scum, consisting almost entirely of euglenas. Other species are responsible for "pools of blood". A few are parasitic.

It is this group which forms the classical example of unicellular organisms intermediate between plants and animals. Steinecke (1925) gives them a unique position in nature, stating that it is with the descendants of the euglenas that we are to look for the foundation of the animal kingdom. As this is the only work of its kind with a serological basis, it is of interest in this

connection. He concluded as follows: Autotrophic bacteria were the first living things. Bacteria show protein relationship with the blue-green algae, but none at all with the fungi. The lower green algae developed from the blue-green algae. Two great groups of green flagellates must be considered. One (Volvocales) has developed directly from the green algae. The other represents reproductive cells of the higher green algae which have developed into independent organisms. This latter group of flagellates, among which are the euglenas, forms the basis of the animal, but not of the plant, kingdom. The animal kingdom, then, does not go back to the amebas, but to the higher algae.

It is thus apparent that the euglenas, in addition to their wide distribution and abundance in nature, have considerable theoretical implication. Inasmuch as antigen-antibody reactions are generally considered to indicate biological relationships, it is of particular interest to inquire into the antigenic constitution of this group. Surprising as it may seem, they have been little studied from this standpoint.

The present work includes a consideration of the nature of the cytotoxic principle in anti-euglena rabbit serum, the production of anaphylaxis with the organisms used, and a study of the chlorolytic effect of serum.

REVIEW OF LITERATURE

Previous to 1925, as far as the writer is aware, no immunological study of any species of the order Euglenida (Stein) had been made, even under the handicap of impure material. In that year, Steinecke, whose work was mentioned above, published results of an extensive study of the antigenic relationships among the algae. His antigens, which were prepared from dried material collected from ponds, included one strain of *Euglena viridis* Ehrenberg. In this instance he also demonstrated that the immune serum gave identical results with a pure culture of *Euglena viridis*.

Elmore (1928), working with pure cultures of *Euglena gracilis* Klebs, described a specific immune thermostable cytotoxin for this organism.

MATERIALS AND METHODS[1]

Euglena gracilis Klebs[2] was selected for the most extensive investigation because it is a typical member of the genus and grows more abundantly in artificial culture media than any other euglena. Four distinct

[1] Consult the appendix for a general description of the group.

[2] See figure 1.

forms of this species have been described. These are as follows, the arrows indicating the described origins of each form:

1. Normal green form grown in the light.
2. Hyaline form grown in the dark.
3. Intermediate form.
4. Hyaline form grown in the light.

1. Normal green form - This is the only form encountered in nature. Later investigators have added little to the original description of the species by Klebs (1883). The average cell dimensions are 63 by 10 micra. The body in the swimming condition is the shape of a long cylinder or a slender egg. The delicacy and transparency of the cell are characteristic. There are delicate spiral striations on the pellicle. The flagellum is little sensitive and approximately the length of the body. "Metabolie" is very active. The chloroplasts are disc-shaped. They vary greatly in number, with an everage of perhaps ten. They appear circular or polygonal in surface view, and each contains a pyrenoid center surrounded by paramylon. The chloroplasts often lie so close together that the body appears homogeneously green. The very delicate color of green is noticeable. The nucleus is usually central. Division may occur both in the motile con-

dition after loss of flagellum, and in the encysted stage. The maximum number per day is one and one-half.

2. Hyaline dark form - This is the form of *Euglena gracilis* in organic media in the dark or in very rich organic media in the light. Zumstein (1900) gave the first description. It is only an environmental modification of the normal green form; the two can be changed into one another at will. It corresponds in all respects to the normal green form except that it is colorless on account of the absence of chlorophyll. The chloroplasts develop as leukoplasts, and since light is even more of a deterrent to chloroplast division than to cell division, there are many small leukoplasts. It is well known, for example, that white leaves grow from vegetables, such as potatoes and turnips, stored in the dark, and this form of *Euglena gracilis* is considered analogous to such etiolated higher plants. But *Euglena gracilis* reduces its chlorophyll content in the dark more than any other known organism. Some algae are even as green in the dark as in the light. The maximum multiplication rate for this form is two divisions per day. Paramylon is produced as in the light.

3. Intermediate form - This is of infrequent occurrence, and is defined by Ternetz (1912), who first described it, as any euglena which in later generations

splits into the normal green form and the hyaline form occurring in the light. Thus, it is very unstable, and "pure cultures" could not exist. It results from a discontinuance of chlorophyll formation, and may occur as completely colorless except for the poorly developed stigma, as highly vacuolated with red or yellow spots, or as a homogeneous light yellow. Reproduction is as rapid as with the normal green form. Motility is marked. Ternetz states that the intermediate form represents a variation analogous to the colored leaf varieties of higher plants.

4. Hyaline light form - Ternetz (1912) discovered colorless individuals arising spontaneously in the light in organic media. This is the only one of the modifications of *Euglena gracilis* which is constant. The cells measure 48 to 68 micra in length by 4 to 10 micra in width. The flagellum is two-thirds to more than the length of the body, and stronger than in the other forms. The maximum rate of division is 0.7 per day. Heliotaxis is absent. Resting stages are limited to poor media, so that in favorable media many dead organisms occur early in the culture. The individuals are unable to maintain themselves in competition with the green form, explaining why only green forms are the end result of

a culture containing "intermediate forms". This permanently colorless form bears no trace of a stigma, and has neither chloroplasts nor leukoplasts, which explains its inability ever to become green. Except for a slight difference in its manner of division, it is indistinguishable from Astasia. There are two ways in which this form may originate. Chloroplasts arise only by division of preexisting chloroplasts, which takes place entirely independently of cell division. Thus a form without chloroplasts could result from an unequal division of a chloroplast-poor individual. Origin is also from the intermediate form by destruction and resorption of the chloroplasts.

Pure cultures of six strains of Euglena gracilis Klebs of widely different origin were used in the present work. All of these were submitted to Dr. L.B. Walton for identification. Data concerning each follow:

1. "Noland" strain - From an impure culture obtained in the summer of 1926 at the University of Wisconsin through the courtesy of Dr. Lowell E. Noland. He had identified the euglena as gracilis, and had maintained it for some time because of its luxuriant growth under cultivation. Single cell isolation was carried out on Oct. 5, 1926.

2. "Turtox" strain - From an impure euglena culture purchased from the Chicago Biological Supply House in 1927. Single cell isolation was carried out on March 29, 1927. The growth was luxuriant.

3. "Jewell" strain - Appeared in laboratory culture at William Jewell College, Liberty, Missouri, in the fall of 1927. Single cell isolation was carried out on April 5, 1928. The growth was luxuriant.

4. "Prague" strain - Pure culture of *Euglena gracilis* obtained as such in August 1928 from the Pflanzenphysiologisches Institut of the German University at Prague, Czechoslovakia, through the courtesy of Dr. Felix Mainx. Single cell isolation was carried out on May 19, 1932. The growth was scanty.

5. "Rochester" strain - From an impure euglena culture purchased from the Biological Supply Company, Rochester, New York, in 1932. Single cell isolation was carried out on May 18, 1932. The growth was scanty.

6. "Michigan" strain - From an impure euglena culture purchased from the Michigan Biological Supply Company, Ann Arbor, Michigan, in 1932. Single cell isolation was carried out on July 18, 1932. The growth was scanty.

The six strains of *Euglena gracilis* described above were maintained as the normal green form. The hyaline

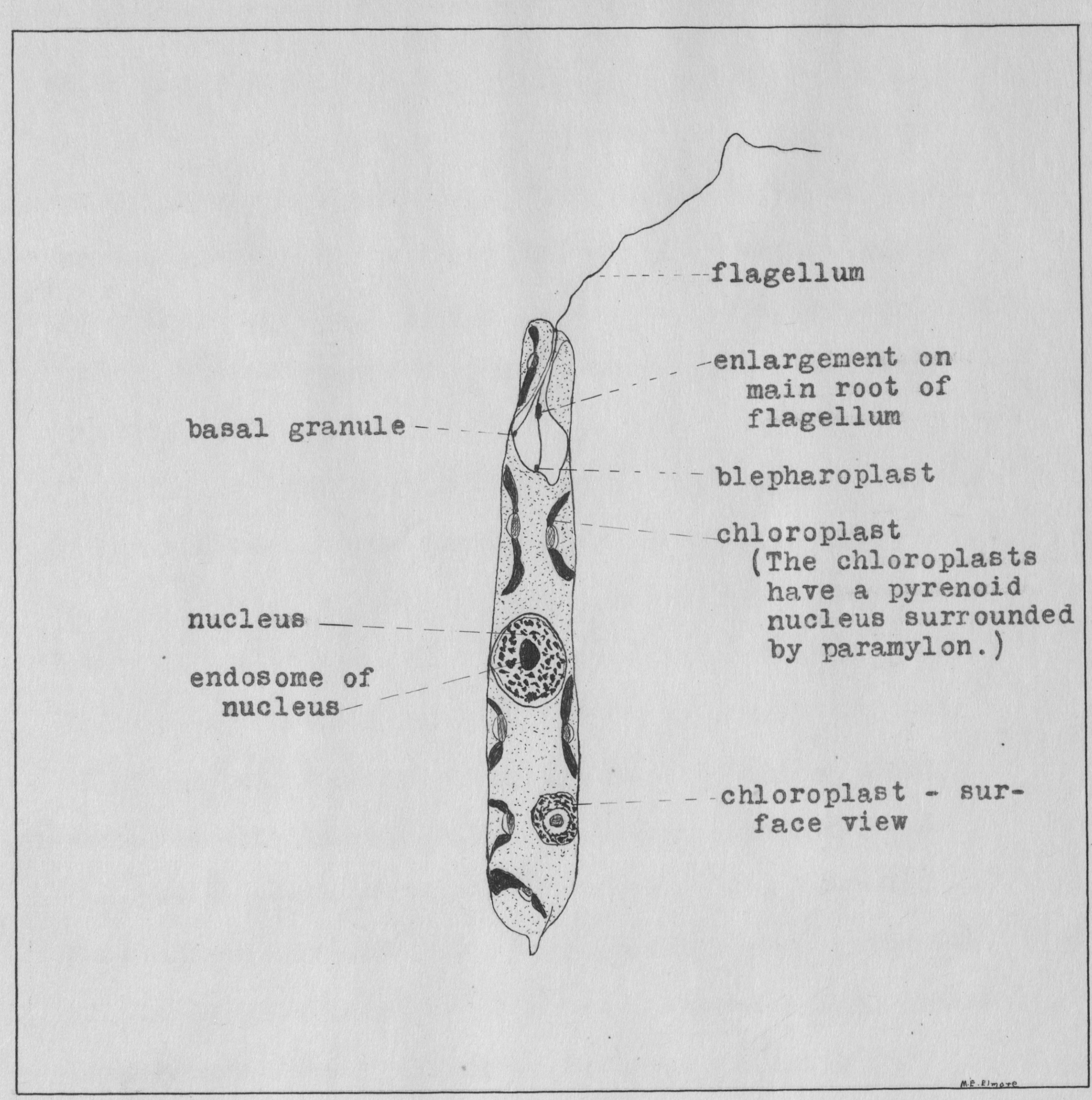

Figure 1

Euglena gracilis Klebs X 1500

Two-week growth in peptone broth of normal green form of "Noland" strain, after more than six years continuous (transfers at one to two month intervals) growth in pure culture. Fixation in hot Schaudinn's fluid; staining with iron hematoxylin, long method; counterstain Bordeaux red. Stigma is present in living organisms, but does not show with this stain. Length excluding flagellum is 60 micra.

dark modification of each could be produced at will by growth in the dark. The "violet" odor typical of euglenas was noticeable in the pure cultures in peptone broth, and a fishy odor, ascribed by Bütschli (quoted from Walton, 1915, p. 349) to the oil vacuoles contained within the cell, was present whenever the cells were disintegrated.

In addition to the cultures in the light, the "Noland" strain was also kept in the dark from the time of its first isolation in 1926. After more than five years of continuous cultivation in the dark, it was found upon bringing transfers into the light that one of the tubes remained colorless in the light. This, then, was the hyaline light form which had arisen from the hyaline dark form. It is conceivable, however, that origin may have been through the normal green form or the intermediate form present in the original inoculum, as observed by Ternetz, darkness offering more favorable conditions for the survival of the hyaline light form. Single cell isolation was carried out on May 19, 1932.

Pure cultures free from bacteria were insured in every case by single cell isolation with the Barber (1914) technic. The majority of the isolations was carried out without the aid of a special pipet holder.

The centering of the tip of the pipet in the field of the microscope is not particularly difficult with any apparatus. After this is accomplished there is need for movement in three planes. Two of these are obtained by the mechanical stage of the microscope. The third (up-and-down) must be supplied in connection with the pipet holder. Barber (1914) described how an ordinary dissecting microscope could be employed quite satisfactorily for this. Malone (1918) used a second microscope stand.

In the present work a moist chamber approximately 43 by 70 by 20 mm. was made from glass cemented together with hard DeKhotinsky cement. The cover glasses were 43 by 70 mm., and of number one thickness to permit use of the oil immersion objective. A Spencer microscope stand number 7 was used for the observation microscope. The large circular mechanical stage permits the use of the entire area of the large cover glass used. The regular substage condenser was removed, and replaced by a single lens in a substage mounting with an iris diaphragm and of such a focus (approximately 1 inch) that the light could be accurately focused upon the under surface of the cover glass. A discarded microscope stand served as the pipet holder. A Luer syringe, also attached to this stand, was found pre-

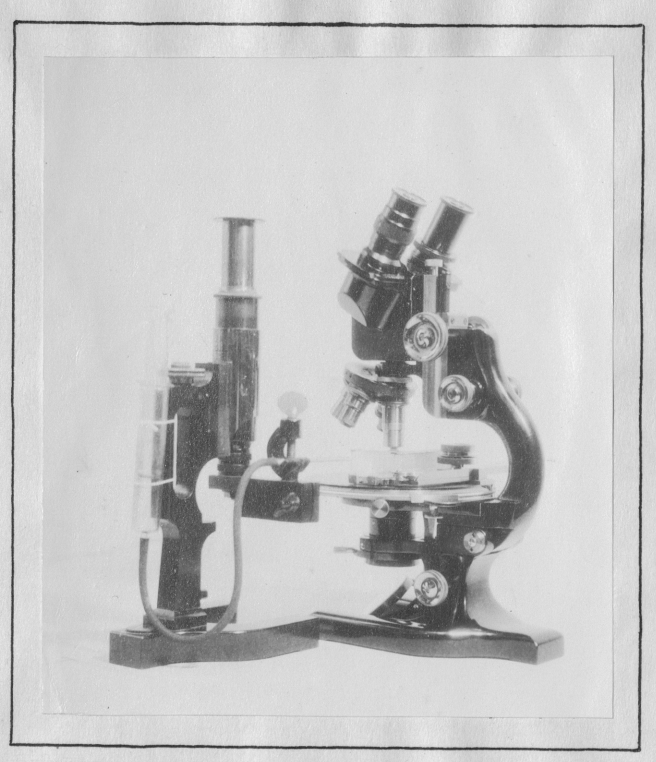

Figure 2

Apparatus used for single cell isolation

ferable to mouth control for pressure and suction. The apparatus is shown in figure 2.

The medium used in the cultivation of Euglena gracilis was a 0.25 % peptone broth to which inorganic salts had been added (Elmore 1928). Bacto-peptone has recently been substituted for the Merck peptone used in the earlier work, as being a more completely digested and more uniform product.

In addition to Euglena gracilis, use was made in the present work of pure cultures of the following unicellular algae. These were obtained in October 1927 through the courtesy of Dr. Frank B. Wann of the Utah Agricultural College, and were cultivated upon the medium used by him (1.5% agar containing inorganic salts and 1 % dextrose). Some of these forms are illustrated in figure 3.

1. Stichococcus sp.

2. Chlorella sp. (# 5) - Shape of cells usually oval. Size variable, average 6 by 9 micra. Many oil droplets in cytoplasm. Chloroplast cup-shaped. Luxuriant growth. This species was submitted by Dr. Wann (personal communication) to various authorities without definite identification. Wille in 1919 had considered it as probably a Coccomyxa.

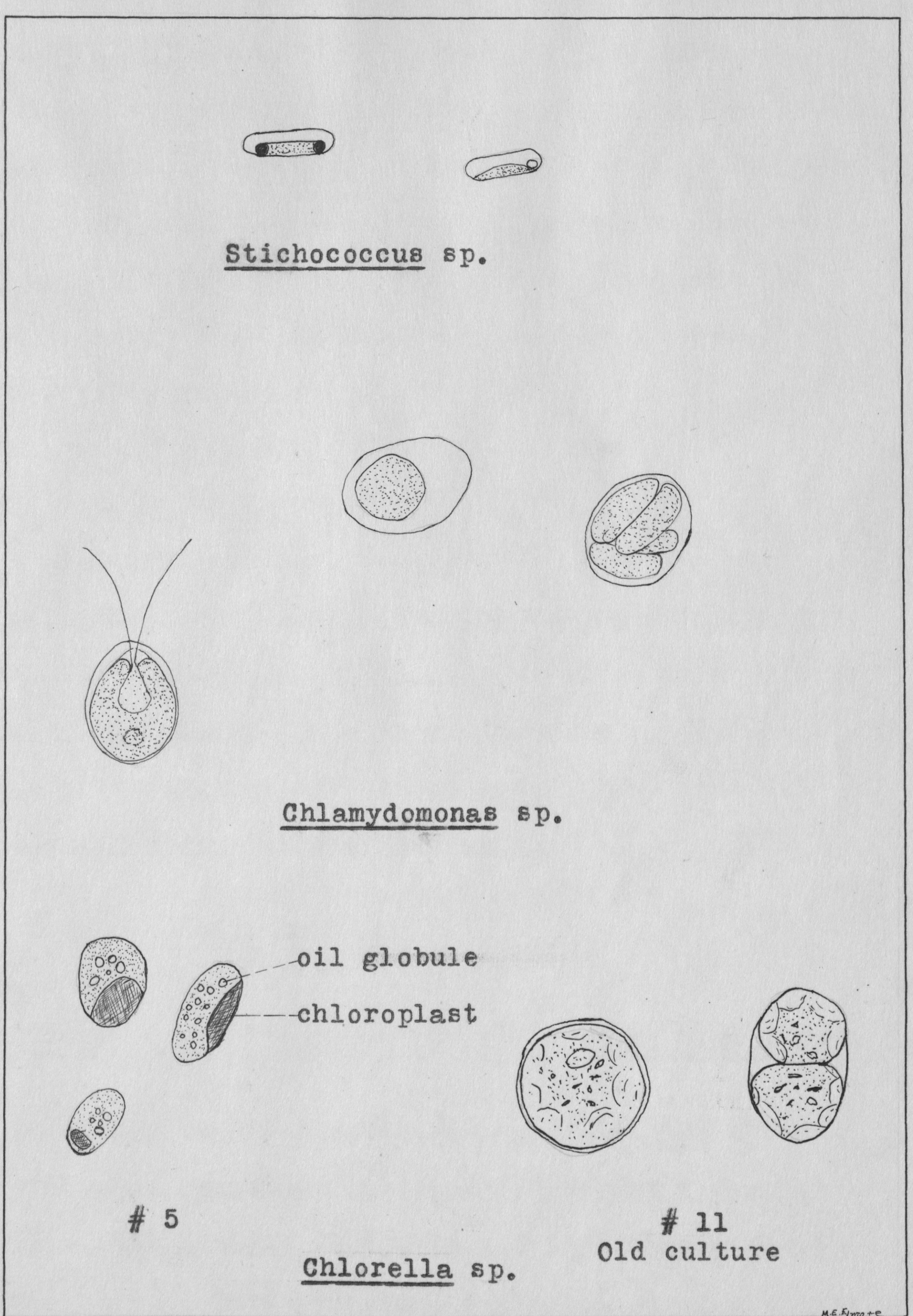

Figure 3

Unicellular Algae X 1500

Drawn from unstained preparations.

3. Chlorella sp. (# 11) - Large cells, average 15 micra in diameter, within which many non-motile daughter cells form. Clathrate chloroplast. Luxuriant growth. This species was likewise submitted by Dr. Wann to various authorities for identification without definite determination. Wille in 1919 reported it as probably an undescribed species.

4. Protosiphon botryoides (Kg.) Klebs

5. Chlamydomonas sp.

6. Unidentified species (# 9)

As a first step in a better understanding of the nature of the materials used, some simple data were obtained. The results are shown in table 1. The percentage error in the analyses is necessarily high due to the small amount of material available.

EXPERIMENTAL WORK

I. THE CYTOTOXIC PRINCIPLE IN ANTI-EUGLENA RABBIT SERUM

According to the present conception of the mechanism of immune reactions, there first occurs a specific coating of the antigen particles with antibody-globulin from the serum. This gives the antigen particles the characteristics of denaturized globulin particles, with which the surface charge (repellent force) is depressed

TABLE 1

Simple data on organisms used

Organism	% moisture			% lipoids[2] based on dry weight			Thermal death point
	Results	Mean	% error	Results	Mean	% error	
E. gracilis (green)	69.9 73.1	72[1]	4	9.4 11.4	10.4[3]	19.	45° C.
Chlorella sp. (# 5)				8.0 11.7	9.9	37	48° C.
Chlorella sp. (# 11)				2.2 3.2	2.7	37	52° C.
Protosiphon botryoides							50° C.

[1] Noland strain.

[2] Extraction with redistilled petroleum ether in cold.

[3] Mixture of Noland and Turtox strains.

by electrolytes much more than is the cohesive force; when this decrease is sufficient, the particles clump together. The alteration in the surface properties of the antigen by a sensitizing substance from the serum also is such as to prepare the antigen particles for phagocytosis (Mudd, et al., 1929).

In the case of the great majority of the cellular antigens, the adsorption of antibody does not seem to be harmful to the cell. It is well known, for example, that agglutinated bacteria grow readily. However, if "complement", an accessory thermolabile enzyme-like constituent of serum which is adsorbed non-specifically by the globulin-coated antigen, is present, the cell may be sensitive to this union. Although some bacteria are unaffected, others are killed, a few even disintegrated. Erythrocytes are laked, and specific toxic effects have been described for a great variety of cells.

Review of Literature

As will be noted later, serum complement is not essential for the demonstration of the immune cytotoxin reaction dealt with here. Although in general a cell is not appreciably injured by its antiserum in the absence of serum complement, such cases are not unknown. Rössle (1905) states that heating an immune serum, produced

by injection of living *Paramecium caudatum* into rabbits, at 53° C. for thirty minutes did not alter its specific paralyzing action. He implies that exposure to a higher temperature did do so, for he says that another serum produced with dried heated paramecia differed from the first in that it could not be inactivated, even by heating at 70° C. for thirty minutes. However, he does not mention controlling this with similarly treated normal serum.

Hamilton (1908) reported for a diphtheroid termed the "Ruediger" bacillus an immune bactericidal substance which seemed to act without complement. Bactericidal substances for the same organism in normal serum also withstood a temperature which would be considered to destroy all complement, but were not as thermostable as the immune bodies.

The work of Sellards (1911) with a specific cytotoxic serum for amebas is inconclusive. While no inactivation resulted after exposure to a temperature of 60° C. for periods varying from thirty minutes to three hours, or of 70° C. for thirty minutes, he found that normal serum heated for one hour at 60° C. had acquired an equal toxicity.

A similar work by Heathman (1932), however, was apparently controlled by normal serum. She produced

an antiserum with a precipitin titer of 1:100,000 by injection of a free-living ameba. A 1:10 dilution of this antiserum killed the amebas within one hour, and a 1:1000 dilution killed them within eight hours. The immune serum did not lose its toxicity upon heating at 56° C. for one-half hour, and normal serum apparently was not toxic in 1:10 dilution either heated or unheated.

Elmore (1928), after giving rabbits intraperitoneal injections of the whole cells from pure cultures of *Euglena gracilis* Klebs, found that the serum of the treated rabbits caused complete sedimentation of the euglenas up to 1:1000 original serum dilution (1:2000 ultimate dilution). Above 1:50 dilution complete recovery often took place. This immune serum property was in sharp contrast to the toxic effect of normal rabbit serum in that (1) normal serum had no effect above 1:50 dilution; (2) the immune property was thermostable and did not require serum complement for its demonstration, while the toxicity of normal serum was completely lost upon inactivation. Sedimentation was accompanied by discarding of the flagellum, a common injury reaction; recovery by its regeneration. Other antigen-antibody reactions were absent or indistinct.

Mention should also be made here of the bacteriophage-inhibiting antibody. The majority of workers

agree that complement is not necessary for the action of anti-bacteriophage serum; however, it has been suggested that the mechanism is that of a toxin-antitoxin neutralization (Bronfenbrenner 1928).

Additional Technic

For more exact work it was found necessary to carefully standardize the euglena suspension used for serum titration. Freshly opened cultures of three to four weeks growth were used. Younger cultures were more sensitive to the serum, while in older cultures there was usually some sedimentation in the salt control. Cultures which had remained in the refrigerator for a day or longer seemed more resistant to the serum.

The euglena suspension used was adjusted so that it contained 500 organisms per cu. mm.. This was done as follows: One cubic centimeter of a culture, concentrated somewhat by light centrifugation, was mixed with 1.0 cc. of a 1:1000 mercuric chloride solution. One drop of this mixture was put into the hemacytometer counting chamber, and an average count obtained. The culture was then adjusted by the addition of 0.9 % NaCl solution so that it contained 500 euglenas per cubic millimeter (25 per square millimeter of counting chamber).

Williams and Jacobs (1931) have recently called

attention to marked physiological differences between different preparations of so-called "chemically pure" sodium chloride. While no extensive study of the comparative toxicity of different preparations to euglenas has been made, in a concentration of 0.9 % one sample of Mallinckrodt's chemically pure granulated sodium chloride was found to be toxic, while one sample of Baker's analyzed sodium chloride was entirely non-toxic.

Nature of the Cytotoxic Principle

1. Thermostability of the cytotoxic principle - The thermostability of the antiserum has been mentioned previously (Elmore 1928). Heating for thirty minutes at 60° C. causes no decrease in titer, while the corresponding dilutions of normal serum are entirely inactive. This temperature would be certain to destroy all serum complement, hence any role of it is eliminated. The control with normal serum shows that the heating does not introduce an additional toxic element.

2. Specificity of the cytotoxic principle - It was to be expected that no reaction would occur between an anti-euglena serum and other unicellular organisms. Treatment of the immune serum with *Chlorella* sp. (# 11) caused no adsorption of the cytotoxic principle, while similar treatment with *Euglena gracilis* did do so (El-

more (1928). Identical results were obtained when the unidentified alga species # 9 was used in place of *Chlorella* sp., as reference to table 3 will show.

The effect of the anti-*gracilis* serum on other species of euglenas was studied directly. A form identified according to the classification of Walton (1915) and of Lemmermann (1913) as *Euglena polymorpha* Dangeard (see figure 4) was found in large numbers in a fair state of purity in a pond scum. Another euglena occurring similarly was submitted to Dr. L.B. Walton for identification. He reported that it was *Euglena proxima* Dangeard or perhaps a variety of this species. The anti-*gracilis* serum had no effect on either of these species. A 1:50 dilution of the serum in centrifuged pond water was mixed with an equal volume of euglena suspension, and observed microscopically. Similar treatment of *Euglena gracilis* caused all of the organisms to round up and settle to the bottom within a few minutes.

The strains of *Euglena gracilis* cultivated fell into two distinct groups as to their growth in the liquid medium used. Jahn's (1931) partially hydrolyzed casein medium for *Euglena gracilis* was tried, with the same results:

Luxuriant growth	Delicate growth
Noland	Prague
Turtox	Michigan
Jewell	Rochester

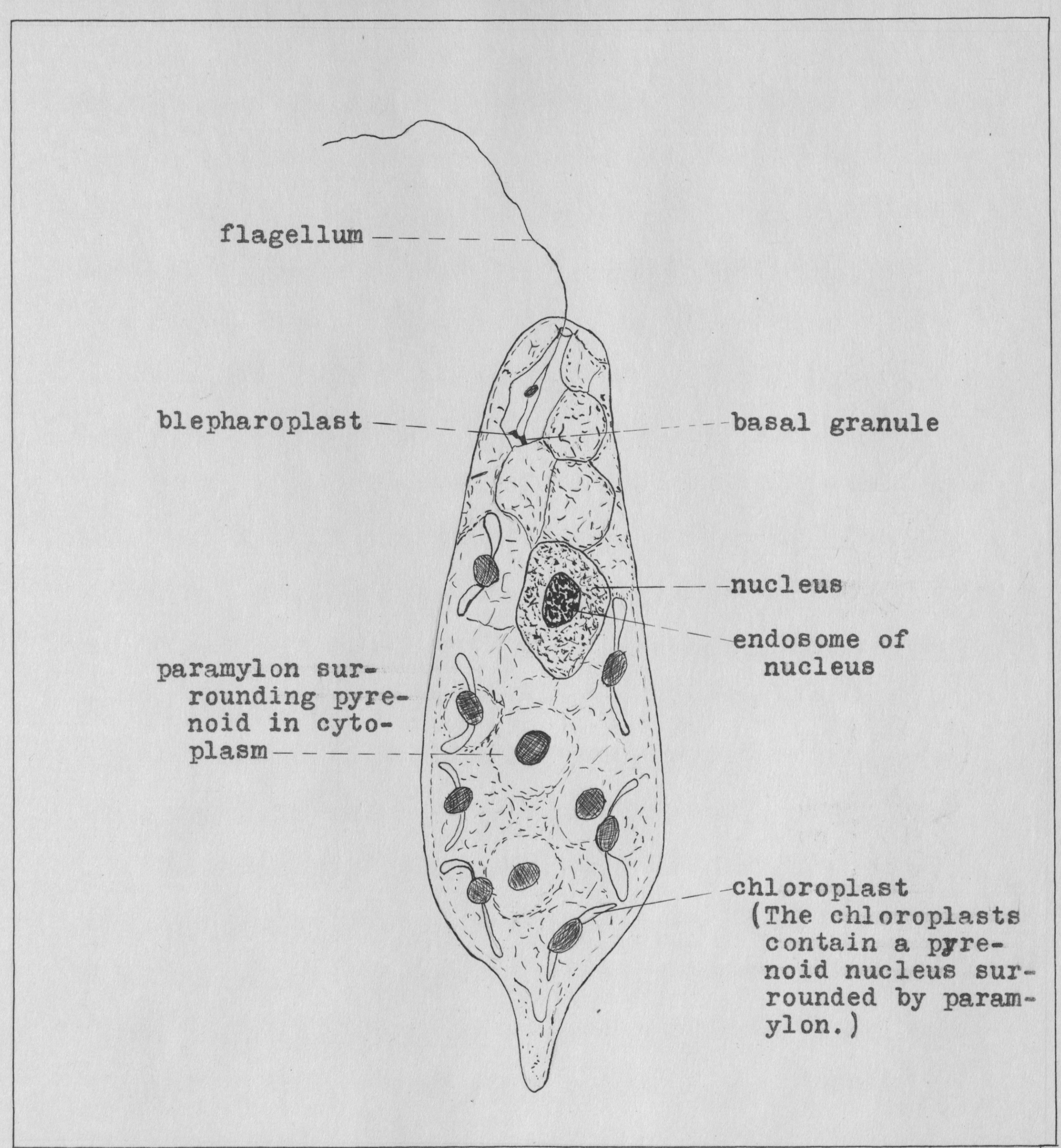

Figure 4

<u>Euglena</u> <u>polymorpha</u> Dangeard X 1500

Fixation in hot Schaudinn's fluid; staining with iron hematoxylin, long method; counterstain Bordeaux red. Length excluding flagellum is 80 micra. Stigma does not show with this stain.

Mainx stated (personal communication) concerning the "Noland" strain, which was sent to him in exchange for the "Prague", that it was entirely identical physiologically with his own cultures of this species. He uses a different medium, and did not give any basis for this opinion. It is hardly thought that this difference in manner of growth is an adaptive change on the part of the organism to prolonged cultivation, for this difference has been noted since their first isolation in pure culture, and with continued cultivation the delicate forms have shown no change. It is thought rather that this is one indication of at least two distinct gracilis types. In case this is true, immunological tests should bring out this difference even more clearly.

Accordingly, suspensions containing approximately the same number of organisms from cultures of the same age were prepared of each of the various strains and forms of Euglena gracilis available. Dilutions of anti-"Noland" (normal green) serum were prepared. Equal volumes of serum dilution and euglena suspension were mixed in small glass cells, and observed under low magnification for cessation of movement and sedimentation. The results of this experiment are shown in table 2. It is seen that the strains fall into two distinct groups, and that these are exactly correlated with the growth

TABLE 2

Cytotoxin reaction with various strains and forms of Euglena gracilis Klebs, using anti-"Noland" (normal green) serum, titer 1:1000

Organism	Serum dilution					
	1:50	1:250	1:500	1:1000	1:5000	saline
Noland						
green	++++[1]	++++	++++	++++	-	-
hyaline light	++++	++++	++++	++++	-	-
hyaline dark	++++	++++	++++	++++	-	-
Turtox						
green	++++	++++	++++	++++	-	-
hyaline dark	++++	++++	++++	++++	-	-
Jewell						
green	++++	++++	++++	++++	-	-
hyaline dark	++++	++++	++++	++++	-	-
Prague						
green	+	+-	-	-	-	-
hyaline dark	-	-	-	-	-	-
Michigan						
green	+[2]	+-	-	-	-	-
hyaline dark	-	-	-	-	-	-
Rochester						
green	-	-	-	-	-	-
hyaline dark	-	-	-	-	-	-

[1]++++ indicates no motile forms, complete sedimentation.

[2]Fewer organisms in suspension used.

characteristics as mentioned above. The "Turtox" and "Jewell" strains react to the titer of the serum, while the serum is practically without effect upon the "Prague", "Michigan", and "Rochester" strains. The hyaline light form of the "Noland" strain and the hyaline dark form of each strain react just like the corresponding green form. That at least the dark form is not identical antigenically with the green form will be shown in a later section; but that the various forms (normal green, hyaline light, hyaline dark) of one strain are more closely related serologically than corresponding forms of different strains is indicated by the above results.

3. Adsorption of the cytotoxic principle - Specific adsorption by its antigen is recognized as one of the essential characteristics of an antibody. Taliaferro (1932) alone, in his study of rats infected with *Trypanosoma lewisi*, has described an antibody which entirely lacks *in vitro* affinity for its antigen. For this reason he considers it a new type of antibody not reconcilable with the unitarian conception, and proposes the term "ablastin" since it is manifested by its inhibition of reproduction.

Some degree of adsorption by the homologous antigen was readily demonstrated in the present work, and it is believed that complete adsorption could be obtained

by proper adjustment of all of the factors involved. An experiment similar to that previously reported (Elmore 1928) is given in table 3. The titer of the serum was 1:500 (original dilution). Well washed cells were used for the adsorbing material in the proportion of one part cells to nine parts of 1:10 antiserum. The tubes were shaken thoroughly and incubated at 37° C. for three hours, followed by twenty hours in the refrigerator. The clear serum dilution remaining after centrifugalization was used for the test.

4. Dependence of the cytotoxin reaction upon electrolytes - Dilutions of dialyzed serum in distilled water were set up with a washed euglena suspension in distilled water. The results were identical with those of similar dilutions of the dialyzed serum in physiological saline solution, using a euglena suspension in saline. This may indicate that the mechanism of the reaction is not, like the agglutinin and precipitin reactions, a sensitization to electrolytes. However, this is in no sense proved. At least traces of electrolytes were present, as the distilled water used was not conductivity water; and in no case could the electrolytes within the cell be excluded.

5. Passive transfer with serum containing the cytotoxic principle - Reference to table 6 shows that

TABLE 3

Absorption of the cytotoxic principle from immune rabbit serum with the homologous antigen*

Previous adsorption with	Serum dilution				
	1:100	1:250	1:500	1:1000	saline
Euglena gracilis	////	//	-	-	-
Unidentified alga number 9	////	////	////	//	-

*Results refer to the degree of sedimentation observed macroscopically after one hour of incubation at 37° C. . For the test 0.5 cc. of the homologous euglena suspension was added to 0.5 cc. of the serum dilution indicated.

the immune rabbit serum, with which the cytotoxic principle is demonstrable, passively sensitizes guinea pigs to a protein extract of the homologous organism (guinea pig number 68).

6. Dialysis of the cytotoxic principle - The antibody is non-dialyzable, as reference to the various fractional precipitation procedures carried out (see table 4) will show.

The method of dialysis evolved in the present work will be described in some detail, hoping that it may be of use to others working with small amounts of material. Collars about 3 cm. in length were cut from tubing of 17½ mm. internal diameter. Both ends were fire-polished, and one end was flared. Test tubes of 14½ to 15 mm. external diameter were next selected. A sheet 30 cm. square of ordinary non-moisture proof cellophane* paper (purchasable from any bakery supply house) was moistened with water and draped over the test tube. The glass collar was then slipped over the tube outside of the cellophane, flared end foremost. The tube was then withdrawn, leaving the cellophane sac undisturbed. The material to be dia-

*Cellophane paper is coming widely into use as a semi-permeable membrane. The writer is indebted to Dr. C.M. Downs for first calling her attention to this.

lyzed was then put into the sac, and the neck of the tube was reinserted in the inverted position where it served as a tight stopper. A large glass jar of distilled water, covered with a heavy cardboard containing a number of holes about two centimeters in diameter, served as the dialyzing bath. The sacs were held suspended in this by means of the lip on the collar. The whole was kept at about 5° C. in a refrigerator, and the water was replaced daily or oftener, depending upon the number of such dialyzing units being accomodated, for five days. After dialysis, the sac was inverted and the contents allowed to drain into the tube. Besides the convenience, rapidity, and large surface area, with this arrangement the dialyzing material is visible.

<u>7. Concentration of the cytotoxic principle</u> - It has long been thought by immunologists that if the antibodies could be separated from their inactive serum accompaniments, a better understanding of their nature could be had, as well as the more effective therapeutic use of some. The majority of attempts in this direction have been toward finding with which of the serum proteins they are associated. A survey of the literature shows that there is general agreement that the albumin fraction is inactive; but as to the distribution between euglobulin and pseudoglobulin, there is entire lack of agreement.

Antibodies are still reported to be entirely in the euglobulin fraction (Ahuja 1931); entirely in the pseudoglobulin (Mallick and Maitra 1932); distributed between the euglobulin and the pseudoglobulin (Kendrick and Kahn 1926); or even sometimes in euglobulin alone and sometimes in both euglobulin and pseudoglobulin (Taliaferro 1932); or, finally, in neither euglobulin nor pseudoglobulin (Felton 1928). The following paragraphs are designed to show in part why this confusion of thought exists.

Serum proteins can be quite satisfactorily separated into an albumin fraction and a globulin fraction. The method commonly used to bring this about is one-half saturation with ammonium sulfate. Upon adding an equal volume of a saturated solution of ammonium sulfate to the serum or serum solution and allowing the mixture to stand for several hours, an abundant precipitate settles out. This precipitate is the globulin fraction.

Klobusitzky (1931) has presented some important considerations regarding the theory of "salting out", which is the term commonly applied to the precipitation of proteins and other colloids by salts. The ability of a colloid to be salted out depends first of all upon the degree of dispersion. The more finely distributed a lyophile colloid is, the higher the salt concentration

necessary to separate it out of solution. The action of the salt depends at least partly upon dehydration, i.e., upon the withdrawal of the means of solution, which is the water. The more finely distributed a protein is, the greater is its surface and, other things being equal, its surface energy with which it binds water to itself; therefore, the more difficult it is to withdraw water from it. It is inherent in the colloidal solution that the size of the dispersed particles, even with no external cause whatsoever, within certain limits is constantly changing. It is therefore very probable to assume that in a solution consisting of a mixture of globulin and albumin, at the moment of addition of the salt there are present globulin particles more finely dispersed than the average and also albumin particles with a lesser degree of dispersion than their avarage. The only legitimate conclusion to be drawn from the salt concentration necessary to effect precipitation is one concerning the degree of dispersion.

Thus, it is clear that the albumin and globulin fractions obtained by one-half saturation with ammonium sulfate are never to be considered completely free each from the other. However, the separation here is sufficiently distinct for practical purposes.

Kauder's publication in 1886 of the ammonium sul-

fate fractionation method worked out by Hofmeister forms the point of departure for attempts at subdivisions within the serum globulin fraction. It was found upon dialysis of the fraction precipitated at one-half saturation with ammonium sulfate that only a small part of the globulin was insoluble in distilled water. Hofmeister applied the term euglobulin to the portion which remained insoluble upon dialysis, and to the water-soluble portion he applied the term pseudoglobulin. This concept has been modified by Pauli (1924). He has shown by a careful electrodialysis of serum, the progress of which was controlled by electrical conductivity determinations, that even the pseudoglobulin previously considered water-soluble actually represents a water-insoluble globulin held in solution by a residual salt that has not been removed. If all salt is removed electrically so that the serum approaches the conductivity of the purest water, a completely globulin-free albumin solution results.

Approximately one-third saturation with ammonium sulfate has been considered to precipitate the euglobulin, and this assumption has been employed in much of the work upon serum fractionation. Klobusitzky (1931), however, has shown that it is entirely incorrect to carry out the separation of the globulins into euglobulin

and pseudoglobulin on the basis of ammonium sulfate concentration. The source of error mentioned above in regard to the separation of globulin from albumin is present to a far greater degree in the fractionation of the globulins, for globulin is a far more labile protein than albumin, and the size of the dispersed particles is much more variable. Figure 5 is an attempt to illustrate Klobusitzky's (1931) views diagrammatically. Probably the best evidence we have for the existence of two distinct proteins in the globulin fraction is that of solubility, since solubility is a very characteristic property of chemical compounds. Further subdivisions of the globulins are probably not justified with present knowledge.

Klobusitzky's (1931) objection does not apply to the fractionation of the globulin of immune serum by salting out when this is found a useful means of concentration of antibody, but merely to the consideration of the fractions obtained as independent globulins. It merely happens that the antibodies may be associated with globulin particles of a definite size. He insists that a transition of globulin into albumin or vice versa does not occur. In immunization and other conditions where an increase in globulin takes place at the expense of the albumin, there has been no chemical transforma-

% saturation with ammonium sulfate	Serum fraction precipitated	Particle size
0		
33 1/3	euglobulin	
50	pseudoglobulin	
100	albumin	

Figure 5

Diagrammatic representation of serum proteins according to Klobusitzky's views, showing that the size of the particles rather than their chemical nature determines their precipitation by salts

tion, but merely a change in the degree of dispersion. This question has recently been raised anew. Fischer (1932) suggests that the globulins of serum are merely albumin-heparin compounds, basing his opinion upon the fact that the addition of heparin to crude serum albumin caused the precipitation of a substance which possessed physical characteristics (solubility, precipitation by salts, etc.) of globulin, therefore must be globulin. Although Fischer's (1932) observations have been verified by others (Hooker and Boyd 1932; Earle and Howe 1933), these present additional data which invalidate his conclusions. Hooker and Boyd (1932) found that Fischer's results could not be obtained with purified crystalline serum albumin. Moreover, testing Fischer's hypothesis with the more delicate and specific methods of immunology, these authors found that the albumin-heparin compound of Fischer still reacted to the same degree as albumin with an anti-albumin serum, and did not react with anti-globulin serum.

Other considerations which are of interest in connection with serum fractionation will be mentioned briefly. Uniformity in results could hardly be expected with such variations in methods as have been employed. Klobusitzky (1931) points out that results with ammonium sulfate precipitation can not be considered comparable

unless consideration has been given to the method of preparation of the ammonium sulfate solution. The saturation must be done at room temperature, for an acid solution results from disintegration of the ammonium sulfate when this is heated.

Heating of serum is well known to augment the globulin fraction at the expense of the albumin fraction, and to augment the euglobulin at the expense of the pseudoglobulin.

There is some evidence that the animal producing the antiserum is a factor.

The degree of dilution employed is responsible to no small extent in the composition of the resulting precipitate. Banzhaf and Gibson (1908-'09) state that at dilutions of horse plasma in the precipitated mixtures of 1:1.5, 1:5, and 1:10 the ammonium sulfate "euglobulin" fraction amounted to about 67 to 70, 20 to 24, and 10 to 15 percent., respectively, of the total serum globulin in both normal and diphtheria antitoxin plasma. This behavior of euglobulin results in an increasingly diminishing amount of precipitate when the euglobulin is washed or reprecipitated (see numbers 3, 5, 7, and 10 of table 4).

In summary of the above discussion, it may be said that there is evidence for the existence of three dis-

tinct serum proteins; namely, albumin, pseudoglobulin, and euglobulin. Antibodies are not found with the albumin fraction. Attempts to identify them with one or the other globulin fraction have been legion, and the results conflicting. These are chiefly of interest to the extent that they have resulted in a means of concentration of antibody.

The results of an attempt to concentrate the cytotoxic principle in anti-euglena rabbit serum are shown in table 4. In practically every case three serum fractions were obtained. The designation of these as euglobulin, pseudoglobulin, and albumin is merely for convenience, no implication being intended as to their nature. Each serum fraction after dialysis was made up to the original volume of the serum with 0.9 % sodium chloride solution. Dilute sodium carbonate solution was added to the euglobulin dialysate until the reaction was alkaline to litmus in order to effect solution. Dialysis as a means of separation of the globulins, although theoretically correct, practically yields only a minute amount of precipitate (Banzhaf and Gibson 1908-'09; see numbers 1 and 23 of table 4). The method devised through the New York State Board of Health for the concentration of diphtheria antitoxin (Wadsworth 1927) left a portion of antibody in each globulin frac-

TABLE 4

Cytotoxic action of various serum fractions

No.	Method of serum fractionation	Serum	Results			Remarks
			Euglobulin	Pseudo-globulin	Albumin	
1	50% sat. with $(NH_4)_2SO_4$ + dialysis	Immune Titer, 1:1000	-	1:1000 = ++++	-	
2	Like 1	Normal	-	-	-	
3	Wadsworth (1927)	Immune Titer, 1:1000	1:1000 = ++++	1:1000 = ++++	-	Eu. ppt. not washed.
4	Like 3	Normal	-	-	-	
5	Like 3	Immune Titer, 1:1000	1:50 = ++++ 1:250 = +++	1:1000 = ++++	-	Eu. ppt. washed once.
6	Like 3	Normal	-	-	-	
7	Like 3	Immune Titer, 1:1000	1:10 = ++++	1:1000 = ++++ 1:2000 = +++	Not run	Eu. ppt. washed thoroughly, thereby losing much through solution.
8	Like 3	Normal	-	-	Not run	
9	Felton (1928)	Immune Titer, 1:1000	No separation resulted		-	

TABLE 4 - continued

No.	Method of serum fractionation	Serum	Results			Remarks
			Euglobulin	Pseudo-globulin	Albumin	
10	Ppt. eu. by 34% sat. with $(NH_4)_2SO_4$, reppt. with 36%. Saturate filtrate to 48% to ppt. pseudo. Dialyze.	Immune Titer 1:50	-	1:50 = ++++	-	
11	Like 10	Normal	-	-	-	
12	50% sat. with $(NH_4)_2SO_4$ to ppt. both globulins. Redissolve and sat. with NaCl to ppt. eu. Ppt. pseudo with acetic acid. Dialyze.	Immune Titer 1:2000	1:50 = ++++ 1:250 = ++	1:50 = ++++ 1:250 = −	-	Shows that exposure to acid entails loss in antibody. Cf. with 14 below. Serum diluted 1:1.
13	Like 12	Normal	-	-	-	
14	Like 12, except concentration of pseudo with acid omitted.	Immune Titer, 1:1000	1:100 = ++++ 1:500 = +++ 1:1000 = −	1:250 = ++++ 1:500 = ++ 1:1000 = +	-	Eu. reppt. once Serum diluted 1:5.
15	Sat. with NaCl to ppt. eu. Filtrate to 48% sat. with $(NH_4)_2SO_4$ Dialyze.	Immune Titer, 1:250	1:50 = ++++	1:250 = ++++	-	Eu. reppt. once Serum diluted 1:1
16	Like 15	Normal	-	-	-	
17	Like 15	Immune Titer, 1:1000	-	1:1000 = ++++	-	Serum diluted 1:5
18	Like 15	Immune Titer, 1:1000	±	1:500 = ++++ 1:1000 = −	Not run	

TABLE 4 - concluded

No.	Method of serum fractionation	Serum	Results			Remarks
			Euglobulin	Pseudo-globulin	Albumin	
19	34% sat. with $(NH_4)_2SO_4$. Reppt. with NaCl. Increase filtrate to 48% to ppt. Pseudo. Dialyze	Immune Titer, 1:1000	1:100 = ++++	1:500 = ++++ 1:1000 = ++	-	
20	38% sat. with $(NH_4)_2SO_4$. Reppt. with NaCl. Increase filtrate to 48%. Dialyze.	Immune Titer, 1:1000	1:100 = +++±	1:1000 = ++++	-	
21	42% sat. with $(NH_4)_2SO_4$. Reppt. with NaCl. Increase filtrate to 48%. Dialyze.	Immune Titer, 1:1000	1:100 = ++++ 1:500 = +++ 1:1000 = —	-	-	Shows that 42% precipitates all antibody.
22	Like 15, except that $(NH_4)_2SO_4$ sat. to only 42%.	Immune Titer, 1:1000	-	1:250 = ++++	-	Used only 2 cc. of Serum. Apparent loss in antibody may be due to inaccuracy in dilutions.
23	42% sat. with $(NH_4)_2SO_4$. Dialyze	Immune Titer, 1:1000	Not run	1:500 = ++++	—	Ppt. in globulin dialyzing bag negligible

tion (see numbers 2 - 8, table 4). Felton's (1928) method for pneumococcus antibody was not applicable. Sodium chloride precipitation in some cases seemed to give all antibody in one fraction (see numbers 17, 18, 21, and 22 of table 4), though frequently with some loss in titer (see numbers 18, 21, and 22 of table 4). These results were not uniformly consistent however, even with the same serum (compare numbers 17, 18, 21, and 22 of table 4 with numbers 12, 14, and 15). The use of acid as a precipitant was avoided since it was found that this entailed considerable loss in antibody (compare number 12 of table 4 with number 14).

Preliminary experiments had shown that the cytotoxic principle in anti-euglena rabbit serum is associated with the globulins, and not with the albumin (see table 4). The filtrate obtained after 48 to 50 per cent. saturation with ammonium sulfate was always inactive (see numbers 1 - 8 and 10 - 21 of table 4). Moreover, it was later found that the antibody was entirely removed by 42 per cent. saturation with ammonium sulfate of 1:5 serum dilution (see numbers 21 - 23 of table 4). Since a considerable amount of extraneous substances are thus removed in one step, 42 per cent. saturation with ammonium sulfate could logically be taken as the starting point for further concentration.

<u>8. Relation of the cytotoxic principle in immune serum to that present in normal serum</u> - It has already been mentioned that the toxic effect upon euglenas of normal serum readily disappears upon heating the serum at 56° C. for one-half hour, while the serum immune to euglenas is unaffected by such treatment. This indicates that complement is a factor in the normal serum reaction but that it is not necessary in the case of the immune serum. This difference between normal and immune serum finds its only important analogy in immunology in the opsonins and tropins of normal and immune sera respectively. In "the case of all other antibodies (agglutinins, lysins, etc.) it has been shown that in structure and mode of action the antibodies of immune serum are in every way qualitatively similar to the corresponding ones of normal serum, representing merely a specific quantitative increase of substances originally present in small amount" (quoted from Zinsser 1931, p. 333). However, it is "now the generally accepted view that both normal opsonins and immune opsonins, or bacteriotropins, have a dual structure, in the sense that they involve, or may involve, the combined action of a specific sensitizing antibody and complement; but that the very low concentration of the specific antibodies in normal serum necessitates the adjuvant action of

a considerable amount of complement before its presence can be detected, so that the complementary action appears to dominate the picture, while the high concentration of the specific antibody in an immune serum reduces the adjuvant action of the complement to a mere enhancement of an effect which takes place in its absence" (Topley and Wilson 1931, p. 176).

Zinsser (1931) suggests that the leukocytes themselves furnish sufficient complement for the action of tropins. If such an assumption seems necessary, it could be made as well in the case of antibodies producing an injurious effect upon their living cellular antigens apparently without the aid of complement; for the cells involved thus far - paramecia, amebas, certain diphtheroid bacilli, and euglenas - for the most part are large cells which could be assumed to furnish enzymes with function analogous to complement as well as the leukocytes can. Hamilton's (1908) work with the Ruediger bacillus need not be considered further here, for there is little doubt that she was dealing with a simple thermostable antibody in both normal and immune sera. Unfortunately, with the amebas and paramecia no study has been made of the normal antibody.

Experiments were carried out to determine whether the toxic effect of an immune serum upon euglenas is

enhanced by the addition of complement. There is considerable evidence that this does occur in the case of tropins. It has already been shown that the toxic titers of fresh unheated and of heated anti-euglena rabbit sera run parallel. Although this indicates that an antibody is present which does not require free complement for its demonstration, it does not show the comparative effect of the antibody with and without free complement, for the amount of complement present in 0.0005 cc. of rabbit serum (0.5 cc. of 1:1000 dilution) would be negligible. Accordingly, the effect of the addition of serum containing complement was studied. The results are shown in table 5. The high toxicity of the guinea pig serum was a disadvantage, but the amount of complement used was shown to be fully adequate in the hemolytic system with excess of hemolysin. The results clearly indicate that the toxic effect of the immune serum is increased by the presence of free complement.

The question arises as to the evidence for the existence of an anti-euglena antibody in normal serum. The work of Mackie and Finkelstein (1931) makes it seem improbable that the bactericidal effect of fresh normal serum is due to complement alone. However, this question is still controversial. Gordon and Carter (1932) hold

TABLE 5

Comparison of cytotoxic effect of anti-euglena rabbit serum with and without free complement[1]

Serum	Serum dilution							
	1:500	1:750	1:1000	1:1250	1:1500	1:2000	1:2250	1:2500
Without complement[2]	++++	+++	++	+	—	—	—	—
With guinea pig serum[3]	++++	++++	++++	+++	+++	+++	++	+
With rabbit serum[4]	++++	++++	++++	++++	++++	+++	++	++

[1]0.25 cc. of immune serum dilution and 0.5 cc. of standard euglena suspension in each tube. Total volume 1.0 cc.

[2]Saline control negative

[3]0.25 cc. of 1:20 pooled guinea pig serum added to each tube. This amount of unheated guinea pig serum shows one-plus toxicity per se.

[4]0.25 cc. of 1:10 fresh rabbit serum added to each tube. This amount of unheated rabbit serum showed two-plus toxicity per se.

that variations in the bactericidal power of normal serum against different organisms depend merely upon the sensitiveness of these organisms to non-specific factors in the serum. Heated normal serum retains a very slight opsonic action. Heated serum in low dilution likewise is toxic to euglenas, but it must be admitted that other substances than an antibody could contribute to this.

Specific absorption of the toxic substance from normal serum could not be demonstrated. Since the antibody would be present in small amount only, it might not be possible to distinguish it from other toxic serum constituents.

It is well known that addition of complement to heated normal serum restores its full opsonic action. However, no evidence could be obtained that this also restored the toxic action toward euglenas. Remembering the quantitative relationships which must exist between antibody and complement, this is not surprising. With the small amount of antibody that can be present in normal serum, there may well be required a larger amount of complement than can be used without exceeding the toxic dose of fresh serum.

Discussion

Earlier immunologists assumed that the different

effects that could be produced by an immune serum were each due to a different constituent of the serum. According to the more recent "unitarian" theory, to each chemically distinct antigenic substance in a complex antigen the animal body responds with the production of a single immune substance. Considered from this viewpoint, one would look to an unusual sensitiveness of the cell to alterations in its environment rather than to any peculiar characteristics of the antibody for an explanation of the specific toxic action of an immune serum unaided by free complement. The fact that the presence of serum complement definitely enhances the reaction also tends to align this antibody with the other known antibodies, and gives basis for the assumption that the injury to the cell may after all be the result of an antibody sensitization to complement. The following statement by Mudd et al. (1929) concerning certain acid-fast bacteria which do not agglutinate although the surface potential difference is reduced well below the level necessary for related forms, and concerning others which are phagocytized to a high degree even without serum sensitization, is pertinent here also. They state that phagocytosis and agglutination can not be formulated in terms of the sensitizing substance alone, but depend both on the properties of the sensitizing substance and on the in-

trinsic properties of the bacteria undergoing sensitization. This of course assumes that the sensitizing film never becomes complete, for in that case the properties of the antigen would be masked.

II. THE PRODUCTION OF ANAPHYLAXIS WITH EUGLENA GRACILIS KLEBS and certain other Unicellular Chlorophyll-Bearing Organisms

A study of anaphylaxis with euglenas and with the unicellular algae available in pure culture was undertaken first to determine the applicability to the problem at hand of methods depending upon the production of hypersensitiveness, and second to investigate antigenic relationships among these organisms by this means if found applicable. The smooth muscle reaction of Dale, clinical anaphylaxis, and Arthus' phenomenon were studied.

Materials and Methods

The organisms used in addition to the various forms and strains of *Euglena gracilis* were *Stichococcus* sp., *Chlamydomonas* sp., and the two species of *Chlorella* previously designated as number 5 and number 11.

A total of 68 guinea pigs was used in this study. The majority of these were virgins injected intraperi-

toneally with a protein extract and tested for active sensitization by the Dale (1913) technic.

A cellular extract modified from the technic of Besredka (1906) was used for the antigen both for injection and for the tests. The volume used (see table 6) is that of this undiluted extract. Its preparation consisted in mixing the dried cells with c.p. sodium chloride crystals, in the proportion of 0.45 gram sodium chloride per gram of dried cells, and grinding in a mortar to a fine powder. Distilled water was added drop by drop with continuous grinding until a liquid resulted. This was then shaken vigorously for an hour or two, after which sufficient distilled water was added to bring the concentration to that of a physiologically normal solution. Preliminary results on virgin uterine horns seemed to indicate that 1.0 cc. of euglena extract and 5.0 cc. of extracts of the other forms were non-toxic doses. However, extracts made from time to time varied, so that occasionally toxic reactions were encountered with much smaller doses. No toxic reactions were encountered when 0.1 cc. quantities were used in the test. As a protein extract of the forms used, the Besredka modification was considered inferior to the Hopkins et al. (1930) extract, but its use was continued for the sake of uniformity in results.

Tyrode's solution (Sollmann 19) was used as the bath in the Dale method with one-half the usual amount of calcium chloride, as recommended by Dale (1913).

Experimental Work

In spite of the low agglutinin and precipitin titers obtained, experiments were carried out to determine whether it might be possible to sensitize guinea pigs to the various organisms, either actively with a protein extract or passively with the serum of the treated rabbits.

With the virgin guinea pigs, clearly positive reactions with resulting desensitization were obtained with the majority of living uterine strips. The most significant results are shown in table 6 and in figures 6 and 7. Both active and passive sensitization to *Euglena gracilis* were demonstrated. This was also true of the other organisms in as far as experimented with.

With the small series of guinea pigs in which an attempt to demonstrate clinical anaphylaxis was made, only doubtful and negative reactions were obtained.

Arthus phenomenon was clearly demonstrated in the case of three rabbits injected subcutaneously for this purpose, one with *Stichococcus* sp., one with *Chlorella* sp., and the third with *Euglena gracilis*. The serum

TABLE 6

Results of Dale tests

Guinea Pig Number	Weight at date of testing	Record of injections	Days between last injection and test	Uterine horn	Antigens in order tested*	Reaction to each antigen
	grams					
4	155	E. gracilis (Noland green) 1 injection of 3.0 cc.	22	Right	0.5 cc. Chlorella sp. (#1)	−
					0.5 cc. " " (#5)	−
					0.5 cc. E. gracilis (Noland green)	+
					0.5 cc. E. " (" ")	−
					5.0 cc. 1% $BaCl_2$	+
36	215±	E. gracilis (Noland green) 1 injection of 4 cc.	29	Right	0.1 cc. Chlamydomonas sp.	−
					0.1 cc. E. gracilis (Noland dark)	−
					0.1 cc. " " (" green)	+
					0.1 cc. " " (" ")	−
					5.0 cc. 1% $BaCl_2$	+
37	214±	Like 36	28	Left	0.1 cc. E. gracilis (Turtox green)	+
					0.1 cc. " " (" ")	−
					0.1 cc. " " (Noland ")	−
					5.0 cc. 1% $BaCl_2$	+
39	239±	Like 36	20	Right	0.3 cc. E. gracilis (Turtox green)	+
					0.3 cc. " " (" ")	−
					0.3 cc. " " (Noland ")	−
					5.0 cc. 1% $BaCl_2$	+
40	223±	Like 36	29	Right	0.2 cc. E. gracilis (Noland dark)	−
					0.1 cc. " " (" green)	+
					0.1 cc. " " (" ")	−
					5.0 cc. 1% $BaCl_2$	+

TABLE 6 - continued

Guinea pig number	Weight at date of testing	Record of injections	Days between last injection and test	Uterine horn	Antigens in order tested	Reaction to each antigen
	grams					
42	242±	Chlorella sp. (#5) 12 daily injections of 1 cc. each.	22	Left	0.2 cc. Chlorella sp. (#5)	+
					0.2 cc. " " (#5)	±
					5.0 cc. 1% $BaCl_2$	+
				Right	0.2 cc. Stichococcus sp.	−
					0.2 cc. Chlorella sp. (#11)	−
					0.2 cc. " " (#5)	−
					5.0 cc. 1% $BaCl_2$	−
						+
43	248±	Chlorella sp. (#5) 10 daily injections of 1-2 cc. each.	24	Left	0.2 cc. Chlorella sp. (#5)	+
					0.2 cc. " " (#5)	−
					5.0 cc. 1% $BaCl_2$	+
				Right	0.2 cc. Stichococcus sp.	−
					0.2 cc. Chlorella sp. (#11)	−
					5.0 cc. 1% $BaCl_2$	+
50	261	3.0 cc anti-Stichococcus rabbit serum, precipitin titer 1:50.	2	Right	0.2 cc. Chlorella sp. (#5)	−
					0.2 cc. Stichococcus sp.	+
					0.5 cc. " ".	−
					2.5 cc. 1% $BaCl_2$	+
				Left	0.5 cc. Chlorella sp. (#11)	−
					0.5 cc. Stichococcus sp.	+
					0.5 cc " "	−
					2.0 cc 1% $BaCl_2$	+
55	265	Chlorella sp. (#11) 9 daily injections of 1.0 cc. each.	23	Right	0.5 cc. Chlorella sp. (#11)	+
					0.5 cc. " " (#11)	−
					5.0 cc. 1% $BaCl_2$	+
				Left	0.5 cc. Chlorella sp. (#5)	−
					0.5 cc. " " (#11)	±
					0.5 cc. " " (#11)	−
					5.0 cc. 1% $BaCl_2$	+

TABLE 6 - continued

Guinea pig number	Weight at date of testing	Record of injections	Days between last injection and test	Uterine horn	Antigens in order tested	Reaction to each antigen
	grams					
58	335	E. gracilis (Noland green) 4 daily injections of 1.0 cc. each.	21	Left	0.4 cc. E. gracilis (Noland hyaline light)	+
					0.4 cc. " " " " "	±
					0.4 cc. " " (" green)	—
					5.0 cc. 1% $BaCl_2$	+
60	358	E. gracilis (Turtox green) 2 daily injections of 2 cc. each.	21	Right	0.5 cc. E. gracilis (Turtox green)	+
					0.5 cc. " " (" ")	—
					5.0 cc. 1% $BaCl_2$	+
				Left	0.5 cc. E. gracilis (Rochester green)	—
					0.5 cc. " " (Michigan ")	±
					0.5 cc. " " (Prague ")	—
					0.5 cc. " " (Turtox ")	±
					0.5 cc. " " (" ")	—
					5.0 cc. 1% $BaCl_2$	+
61	300	E. gracilis (Jewell green) 2 daily injections of 2.0 cc. each.	22	Right	0.5 cc. E. gracilis (Jewell green)	+
					0.5 cc. " " (" ")	—
					5.0 cc. 1% $BaCl_2$	+
				Left	0.5 cc. E. gracilis (Turtox green)	—
					0.5 cc. " " (Jewell ")	—
					5.0 cc. 1% $BaCl_2$	+
62	312	E. gracilis (Jewell green) 3 daily injections of 1.0 cc. each.	21	Right	0.6 cc. E. gracilis (Jewell green)	+
					0.6 cc. " " (" ")	—
					5.0 cc. 1% $BaCl_2$	+
				Left	0.4 cc. E. gracilis (Turtox green)	—
					0.4 cc. " " (Jewell ")	+
					0.4 cc. " " (" ")	±
					5.0 cc. 1% $BaCl_2$	+

TABLE 6 - concluded

Guinea pig number	Weight at date of testing	Record of injections	Days between last injection and test	Uterine horn	Antigens in order tested	Reaction to each antigen
	grams					
68	210	4½ cc. immune (E. gracilis-Noland green) rabbit serum, sedimentation titer 1:1000.	2	Right	0.67 cc. E. gracilis (Noland green)	+
					0.67 cc. " " (" ")	—
					5.0 cc. 1% $BaCl_2$	+
				Left	0.5 cc. E. gracilis (Noland dark)	+
					0.5 cc. " " (" ")	±
					0.5 cc. " " (" green)	—
					5.0 cc. 1% $BaCl_2$	+

*Bath volume 25 cc. Bath changed after each addition. Volume added is of undiluted Besredka extract. ±indicates a doubtful reaction.

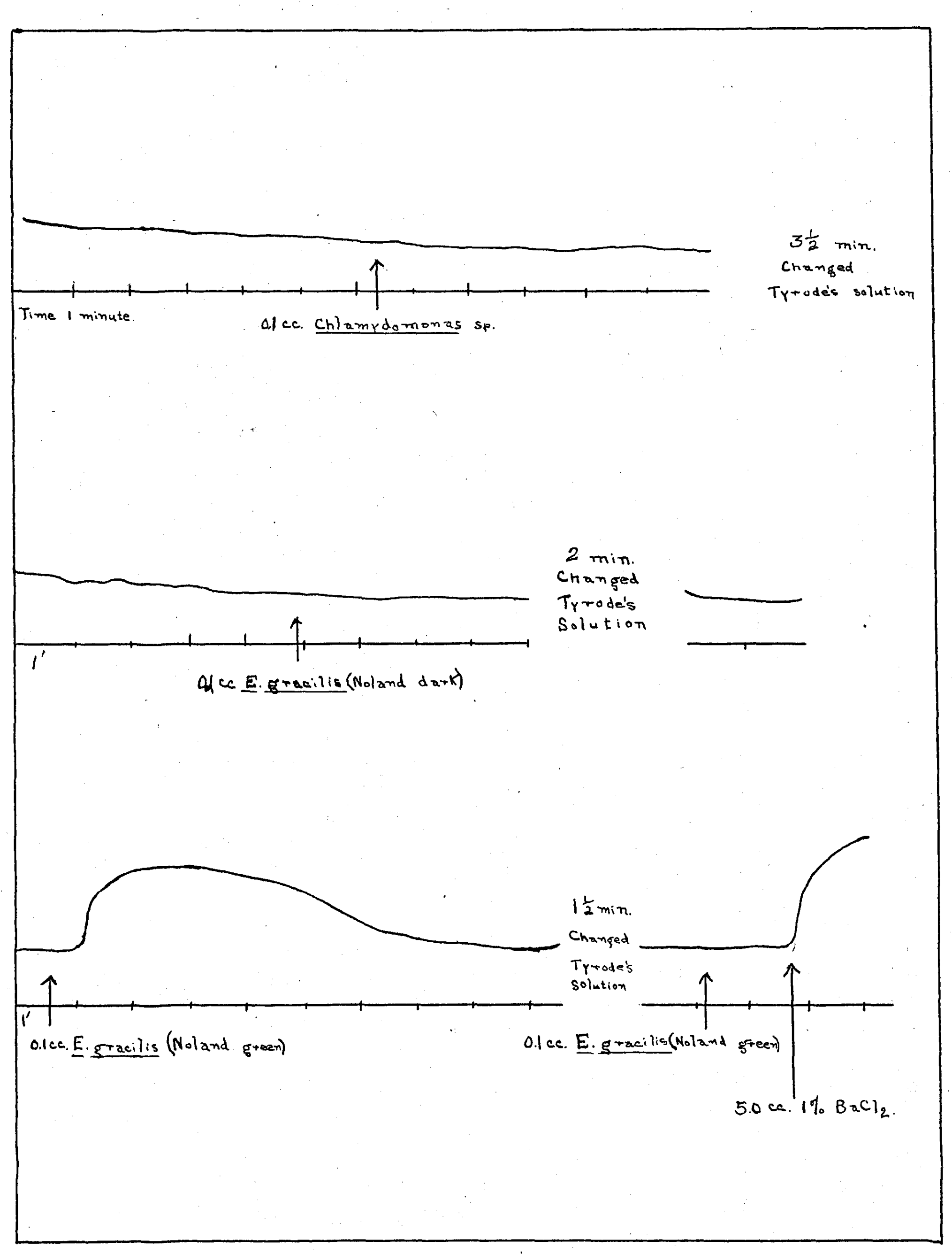

Figure 6

Right uterine horn of virgin guinea pig no. 36

(Illustration is tracing from a photographic reproduction of original chart.)

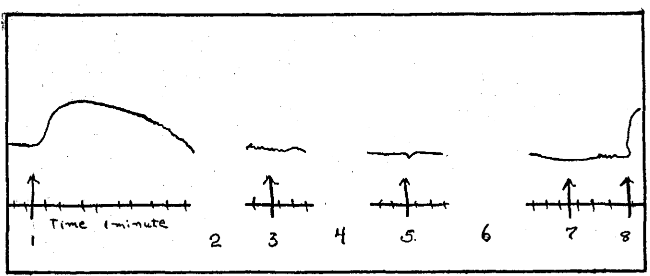

Figure 7

Right uterine horn of virgin guinea pig number 39

(Illustration is tracing from a photographic reproduction of original chart)

1. 0.3 cc. E. gracilis (Turtox, green)
2. Changed Tyrode's solution, 1½ minutes.
3. 0.3 cc. E. gracilis (Turtox, green)
4. Changed Tyrode's solution, 1 minute.
5. 0.3 cc. E. gracilis (Noland, green)
6. Changed Tyrode's solution, 1½ minutes.
7. 0.3 cc. E. gracilis (Noland, green)
8. 5.0 cc. of 1.0% barium chloride.

of the *Stichococcus*-immune rabbit contained precipitins (1:50 Besredka extract).

Discussion

No indication of an antigenic relationship between the euglenas and the lower green algae was found (see right horns of numbers 4 and 36 of table 6, and figure 5).

By means of the smooth muscle anaphylactic reaction, it has been possible to distinguish between the normal green and the hyaline dark forms within one strain of *Euglena gracilis* (see right horns of numbers 36 and 40 of table 6, and figure 5). It will be recalled that it was not possible to do this by means of the cytotoxin reaction. The result obtained is in harmony with the work of Rosenblat (1913) and of Lieske (1916). Rosenblat (1913) demonstrated distinct agglutinins for green and colorless forms of *Chlorella protothecoides*. Lieske (1916) could detect no serological difference between a green culture grown in the dark and a colorless form of the same organism, while differences could be detected between these and green cultures grown in the light. He concluded that it was not the presence or absence of chlorophyll which was responsible for the serological difference between green and colorless cultures of the same species, but the autotrophic or heterotrophic mode of nutrition conditioned thereby. This ascribes

the difference between green and colorless cultures to chemical changes in the protoplasm more fundamental than the mere absence of chlorophyll, which Sherwood (unpublished work) has shown is not antigenic. Reference to number 58 of table 6 shows that no difference could be found in this one instance between the hyaline light form and the normal green form. Comparison with number 68 shows that this does not necessarily mean that the hyaline light form bears a closer antigenic relationship to the normal green form than does the hyaline dark form, but rather indicates that the method gives such variable results that great caution must be exercised in drawing conclusions from a small series.

The cytotoxin reaction has shown the probable identity of the normal green forms of the Noland, Turtox, and Jewell strains. The left horn of number 37 (table 6) and the right horn of number 39 confirm this as to the Turtox and Noland strains, but the left horn of number 62 suggests the possibility of subgroups within the two main groups that have been established in this work for _Euglena gracilis_. However, the results of Dale (1913) with multiple sensitization should make us cautious about drawing conclusions as to identities based upon a supposed specific desensitization. He found that a non-specific desensitization readily occurred.

One example will suffice. A virgin guinea pig was sensitized to a mixture of horse serum, sheep serum, and egg white. If the antigens were tested in the order listed, a reaction could be obtained to each; however, if egg white were given first no response could later be obtained to either of the sera. In numbers 37 and 39 of table 6 a common antigen is indicated, but whether there is identity or non-specific desensitization can not be determined by this means. The above statement also holds true for the normal green and hyaline light forms as reacting in the left horn of number 58.

The significance of results such as those obtained with numbers 42, 43, 55, 61, and 68 of table 6 is not understood. This has occurred too frequently to be an accidental variation. Specific sensitization is proved with one horn. The other horn, tested first with another antigen, does not react to this antigen nor later to the homologous one.

The great variation of a method based upon an experimental animal has been emphasized above. Comparison of numbers 42, 43, and 55 with number 50 furnishes another example.

III. A STUDY OF THE CHLOROLYTIC EFFECT OF SERUM

Sherwood (1925) has described a liberation of greenish-yellow pigment from the chloroplasts of certain higher plants which was brought about by the serum of various animals in the presence of plant extracts. This effect, which he called "chlorolysis", was noticeable after one hour at 37° C., and did not occur without the plant extract. Although the word "hemolysis", from which the term "chlorolysis" was coined, concerns only one pigment, the latter term was intended to cover any pigments involved.

Elmore (1928) adopted this term for a result obtained by the action of serum alone. The present section is a further study of this phenomenon, which it will be shown is not identical with that described by Sherwood.

Experimental Work

1. The chloroplast factor - It had previously (Elmore 1928) been found that euglenas killed in various ways underwent chlorolysis when in contact with serum. Such chlorolysis had not been obtained with unicellular algae heated to the temperature that killed the euglenas. Hence, a study of the effect of heat on these organisms

was undertaken. Table 1 gives the thermal death points of three unicellular algae and one species of euglena, determined in the usual way.

Heavy suspension of these organisms were prepared. Each was heated to its thermal death point and incubated with an equal quantity of serum, but again chlorolysis occurred only in the case of the euglenas. However, when Chlorella sp. (# 5) was heated for thirty minutes at 56° to 60° C., chlorolysis, though less in quantity, was obtained just as with the euglenas heated only to their thermal death point. Evidently, with this species of Chlorella, it is necessary to heat to a temperature somewhat above the thermal death point in order to obtain chlorolysis. However, with the other two algae, Chlorella sp. (# 11) and Protosiphon botryoides, chlorolysis could not be obtained even after heating at 75° to 80° C. for one-half hour. With the euglenas the amount of chlorolysis was only slightly greater with organisms heated for ten minutes at 50° C. than with those heated at 45° C.

In addition to the unicellular organisms mentioned above, similar tests were made with the chloroplasts contained in finely ground suspensions of leaves of alfalfa (Medicago sativa), red clover (Trifolium pratense), and the common broad-leaved plantain (Plantago major).

Heavy washed suspensions of ground leaves (10 to 20 %) were used. Chlorolysis occurred with serum, but the color of the supernatant fluid was slight as compared to the very intense blue-green color resulting when a heat-killed ten per cent. euglena suspension was incubated with an equal or smaller quantity of serum. Serum was necessary for chlorolysis, as shown by the fact that the control tube containing 0.9 per cent. sodium chloride solution in place of serum in every instance remained perfectly colorless.

2. The serum factor -- Chlorolysis occurred from every serum experimented with, whether fresh or inactivated, recently drawn or preserved for many weeks. Dog, guinea pig, and numerous samples of human and rabbit serum were used. No attempt was made to collect the blood in the fasting state. The amount of chlorolysis varied with different serums, as will be discussed below.

It is stated elsewhere (Elmore 1928) that the amount of chlorolysis obtained with rabbit serum is not increased by immunization. The serum used was an anti-euglena rabbit serum with which a cytotoxin was readily demonstrated in a dilution of 1:2000, although the customary antigen-antibody reactions were negligible. In agreement with Sherwood (1925), this chlorolytic property of serum was not found in common plant extracts. Inasmuch

as chlorophyll is lipoid soluble, it was thought probable that the lipoid content of the serum was responsible for the chlorolysis. The following findings tend to support this view.

Bloor (1923) states that the values for total lipoid show wide variations in different species and to a less extent in individuals of the same species. Since human serum seemed consistently to have a distinctly greater chlorolytic effect than rabbit serum, a series was set up to better determine quantitative variations. Table 7 shows that the chlorolytic power of the sera used decreases in the following order: dog, human, rabbit, guinea pig. Bloor (1923) gives 0.490 as the average total lipoid by weight per 100 cc. plasma for the dog without food for twenty-four hours. Mathews (1930) gives 0.1 to 0.3 per cent. as the normal figure for fat in human serum. Büssen's (1927) range for normal rabbits on normal feedings taken at different times of the day, and determined by Bang's micromethod is 0.057 to 0.063 per cent. total fat. Figures for guinea pig were not available.

The effect of increased fat in the serum following excessive fat ingestion was studied. A dog was bled nine hours after a carbohydrate and protein meal and again three hours after a meal rich in fat. The

TABLE 7

Comparative chlorolytic power of sera*

Species	Serum dilution				
	Undi-luted	1:5	1:10	1:25	1:50
Dog	4	4	4	3	1
Human	4	4	4	2	1
Rabbit	4	4	3	1	0
Guinea pig		3	2	1	0

*0.5 cc. of 12 % suspension of *Euglena gracilis* in 0.9 % sodium chloride solution was added to 0.5 cc. of the serum dilution indicated. Suspension had been heated in a water bath at 50° C. for ten minutes. Incubation period 1½ hours at 37° C., followed by thorough shaking, then 24 hours in refrigerator. Figures represent arbitrary degrees of chlorolysis.

chlorolysis resulting from the latter serum was much greater. The tests with the two sera were carried out at the same time and the tubes read in a comparator. Also, fatty human sera had a greater chlorolytic effect than other normal samples.

Considerable chlorolysis resulted from an emulsion containing the lipoids extracted from serum by alcohol and ether, following the method of Bloor, Pelkan, and Allen (1922). The filtrate was evaporated, and sufficient physiological saline solution added to make the volume equal to that of the original serum. From these experiments, it appears that the amount of the chlorolysis varies with the lipoid content of the serum used.

3. Analysis of resulting supernatant fluid - The absorption spectrum of the supernatant fluid from euglena "chlorolysis" was compared with that of an alcoholic extract of fresh euglenas, using a spectroscope provided with a comparison prism. The two spectra were identical. This indicates that the chlorolyzed pigments are in true solution, for in colloidal solutions of chlorophyll the absorption bands are shifted to the red end of the spectrum as compared to those of a true solution (Spoehr 1926).

4. Rate of chlorolysis - The above work was concerned merely with the total amount of chlorophyll that

diffuses out within a definite period of time. While it has been shown (Elmore 1928) that the amount of chlorolysis is not greater for immune than for normal serum, the possibility remained that immune serum might bring about more rapid chlorolysis than normal serum. Preliminary experiments showed that some chlorophyll was transferred to the serum within a few seconds. In order to study the rate at which diffusion goes on a method was worked out by means of which the process is slowed down sufficiently to record the early changes.

The method consists of repeated filtrations of the serum through a pad of asbestos fibers on which euglenas or other cells are held, in a Gooch crucible. With the suction applied, 2 cc. of a 1:10 dilution of serum in physiological saline solution were added. The filtrate was collected in a test tube, and the time recorded in seconds from the moment of adding the serum to the moment of release of suction when filtration was complete. After each filtration the filtrate was quickly matched in a comparator with a standard tube, and the filtration was then repeated.

The amount of chlorolysis after successive filtrations was determined by comparison with a series of standard solutions. Standards prepared from an alcoholic extract of euglenas were too yellow, while extracts

of chlorophyll alone were too blue for satisfactory comparison. By the method of Thatcher (1921) the chlorophylls and yellow pigments were separated, and half of the yellow pigments were put back with the chlorophyll. This gave color standards which closely matched in color the solution obtained by chlorolysis. The standard solution was brought by evaporation to a volume representing a 3 per cent. suspension of the original cells from which it was prepared. Using 50 per cent. methyl alcohol as a diluent, a series of concentrations was made, based upon this standard pigment solution, each successive tube decreasing by approximately 20 per cent. .

The results of this study are shown in the accompanying diagram (figure 8). The rate of chlorolysis is seen to be no greater with immune than with normal rabbit serum. With human serum the diffusion is more rapid than with rabbit serum.

Discussion

The chlorolysis described above results from the action of serum alone. The fact that it is not increased by the addition of plant extract distinguishes it from that described by Sherwood (1925). Sherwood did not observe chlorolysis with serum alone, probably due to

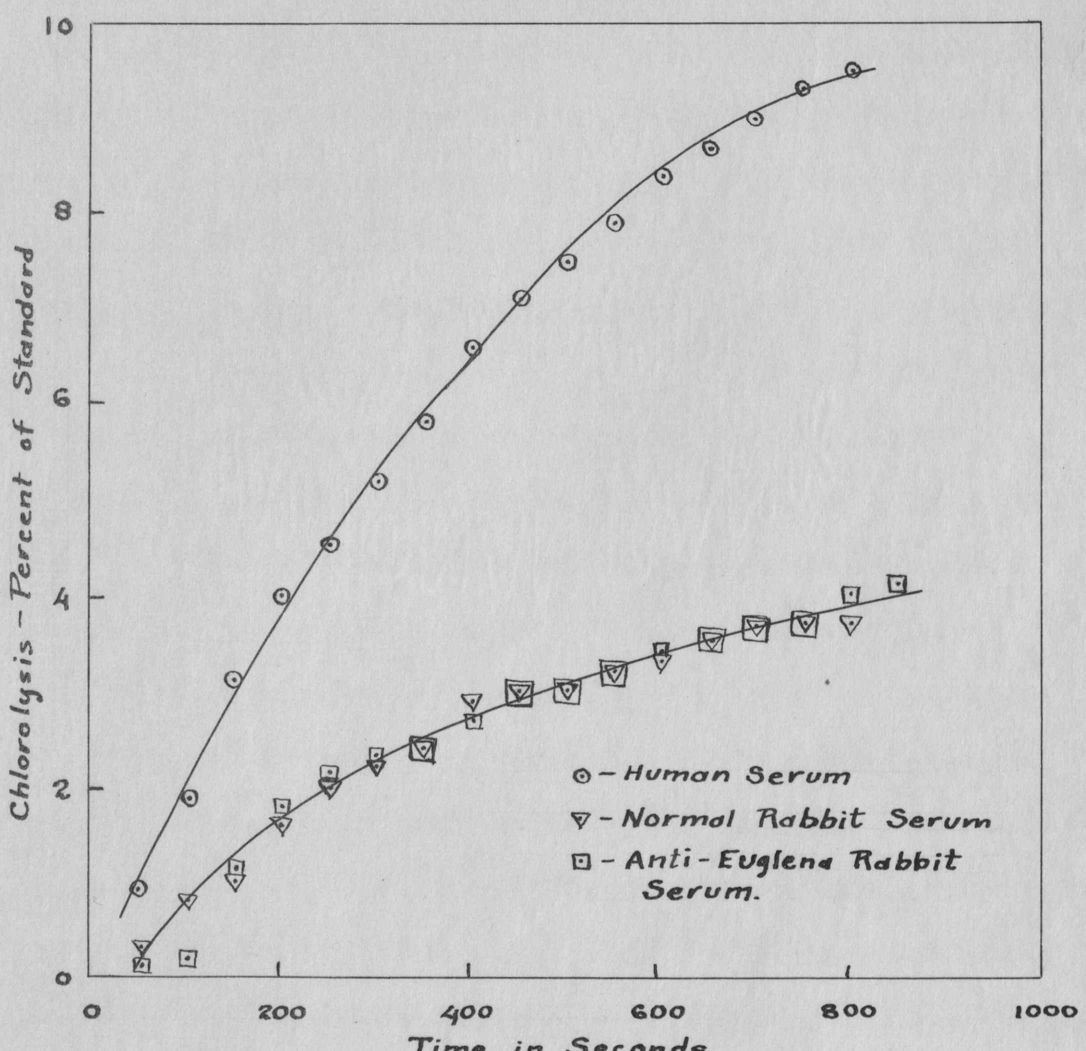

Figure 8

Rate of chlorolysis with different sera

the more dilute chloroplast suspension with which he worked. In repeated attempts to duplicate his results, the phenomenon he described has been observed only after the tubes have stood at low temperature for 24 hours or more. An incubation period up to four hours produced no change, but after standing in the refrigerator for 72 hours the tube containing plantain extract in addition to serum and chloroplasts was distinctly more yellowish brown with a tinge of green than the color control. However, even after one hour of incubation a blue-green color in the control tube with serum and chloroplasts alone was always detectable if a sufficiently heavy chloroplast suspension was used.

The total amount of chlorophyll that diffuses out during chlorolysis must depend primarily upon the comparative solubility of the chlorophyll in the medium within and without the cell. When, with similar cell suspensions, one serum produces considerable chlorolysis while another serum produces only slight chlorolysis, it is probable, as has been discussed above, that this is due to the former serum being richer in lipoids than the latter.

Why the same serum releases different amounts of chlorophyll from different types of cells is less clear. The condition of the chlorophyll within the living chlor-

oplast is debated. Zirkle (1926) gives evidence that it is not in a lipoid solution, as has been held. Whatever the condition of the chlorophyll within the dead and altered cells with which we are dealing, chlorolysis appears to be a process by which the chlorophyll concentration reaches an equilibrium between the cell and the surrounding serum. The total amount released varies with conditions under which equilibrium is reached. Inasmuch as lipoids are present in all cells, it was thought of interest to determine whether the amount is greater in the cells releasing more chlorophyll. Reference to table 1 shows that this is actually the case. _Chlorella_ sp. # 11 contains only a negligible amount of lipoid as compared to _Euglena gracilis_ and to _Chlorella_ sp. # 5. It will be recalled that chlorolysis resulted from the latter organisms but that it could not be obtained with the former. Jacobs (1924) warns against drawing conclusions from work with plant cells where the cellulose cell wall may be a complicating factor. This may account for the greater ease of chlorolysis in euglenas than in unicellular algae with similar lipoid content.

If chlorolysis were more rapid with immune than with normal serum, it would indicate that the immune serum had increased the permeability of the cell to its chlorophyll. That this is not the case is shown

by figure 8. The rate of chlorolysis, as well as the amount of chlorolysis, varies with the lipoid content of the serum, but no effect of immunization upon chlorolysis has been found.

The theory is offered that the cell death or further necessary preliminary treatment enables some chlorophyll to diffuse from the chloroplasts into the lipoids of the cell. Upon contact with the serum, chlorophyll passes to the serum lipoids up to the point of equilibrium between cell contents and serum. Hyaline euglenas left in contact over night with the supernatant fluid from euglena "chlorolysis" gave suggestive evidence. Although no change in the euglenas could be detected microscopically, and there had been no appreciable loss in chlorophyll from the liquid, the cell sediment was distincly pale green.

Although the effect of temperature upon chlorolysis was not studied carefully, it is assumed that it would be slight. The temperature coefficients for the entrance of at least some of the more readily penetrating organic compounds are low, i.e., of the order of magnitude characteristic of diffusion processes rather than of chemical reactions (Jacobs 1924).

The occurrence of chlorolysis with heated serum and with heated cells eliminates the possibility of enzymic factors.

That mere cell death is a sufficient change to enable chlorolysis to occur with euglenas, that heating somewhat above the thermal death point is necessary for the same result with certain algae, and that others are entirely resistant to the serum, indicate some fundamental differences in the microchemical structure of these cells. The role of lipoids and of a cellulose cell wall have been considered.

GENERAL DISCUSSION

The foregoing study, with the exception of the work of Steinecke reviewed above, is the first contribution to our knowledge of the immunological behavior of the euglenas. It is to be considered as such, for it is incomplete in many places and is largely confined to the most readily cultivated member of the group.

The ultimate goal in taxonomy is a classification showing the true evolutionary relationships. In determining these, immunological reactions must be given consideration. The tendency has been in recent years to place euglenas with the animal rather than with the plant kingdom. In agreement with Steinecke (1925), no relationship to the algae was found. However, the Dale test might be too specific to show this.

The division of Euglena gracilis into two groups by means of the cytotoxin antibody and the hitherto unnoticed physiological difference in manner of growth is so clear that it would seem to be of fundamental significance. Other antigen-antibody reactions failed to show this as nicely, due to their greater specificity.

Judging from the reactions obtained with bacteria in various stages of dissociation, it is to be expected that serological differences would occur for each of the distinct morphological types of Euglena gracilis. To the extent that morphology and physiology are expressions of chemical constitution, they are best distinguished by immunological means. An alteration of the protein dependent upon the mode of nutrition has been shown by others working with green and colorless algae. This has been corroborated in the present work with the euglenas.

The cytotoxic principle previously found (Elmore 1928) needed further study, because of its apparently unique behavior. The data secured, however, have shown that it is a true antibody, which may involve the action of serum complement.

Except for the well known polysaccharide "paramylon", the chemical structure of the euglenas is an unexplored field. Their high lipoid content has led to the discovery of a phenomenon which must be considered in any serum study of chlorophyll bearing organisms.

SUMMARY AND CONCLUSIONS

1. The presence of a specific cytotoxic principle in anti-euglena rabbit serum which does not require serum complement for its action has been confirmed.

2. The cytotoxic principle in anti-euglena rabbit serum behaves like a true antibody in its specificity and adsorption reactions, and in its association with the non-dialyzable globulin constituents of serum. It is completely removed from serum by 42 per cent. saturation with ammonium sulfate.

3. The cytotoxic effect of an immune serum is enhanced by the presence of complement. Electrolytes may not be necessary for the reaction.

4. *Euglena gracilis* Klebs is not a homogeneous group, but consists of at least two distinct types. This is clearly demonstrated by the cytotoxic effect of an immune serum, and is correlated with the growth characteristics. The various forms (normal green, hyaline light, hyaline dark) within one type show a closer antigenic relationship to each other than they show to corresponding forms of the other type.

5. With the smooth muscle anaphylactic reaction

active sensitization of virgin guinea pigs was demonstrated to *Euglena gracilis* and to *Chlorella* sp.

6. With this reaction passive sensitization to *Euglena gracilis* and to *Stichococcus* sp. was demonstrated.

7. With this reaction it was possible to distinguish between the normal green and the hyaline dark forms within one strain of *Euglena gracilis*.

8. Arthus' phenomenon was clearly demonstrated with *Euglena gracilis*, *Stichococcus* sp., and *Chlorella* sp.

9. No indication of antigenic relationship between euglenas and the lower green algae was found.

10. Serum will cause a liberation of chlorophyll from many cells or chloroplasts, if these have been sufficiently altered by heat or contact with strong serum.

11. This effect is associated with the lipoid content of the serum, and appears to require the presence of lipoids in the cell.

12. Immune serum does not increase the permeability of the cell to chlorophyll.

ACCESSORY FINDINGS

1. The hyaline light form of *Euglena gracilis* Klebs may originate from the hyaline dark form.

2. The cells of *Euglena gracilis* as cultivated in this study consist of approximately 70 % water. Approximately 10 % of the dry weight is lipoid.

3. A method is described (appendix) for observation of the motor apparatus of the euglenas in the living unstained condition.

4. A simple arrangement for the dialysis of small amounts of materials is described.

5. A simple method for determining the rate of chlorolysis is described.

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APPENDIX

GENERAL CHARACTERISTICS OF THE EUGLENAS

(See figures 1 and 2)

The euglenas constitute one group of unicellular microorganisms which possess characteristics of both plants and animals. They are generally included among the Protozoa as follows (Calkins 1926):

Class - Mastigophora
  Subclass - Phytomastigoda
    Order - Euglenida
      Family - Euglenidae

The family Euglenaceae likewise is considered among the flagellates of the plant kingdom.

The shape of the body is that of a long spindle. Under favorable conditions the organisms move forward freely, always with rotation of the body, and the direction of their motion is influenced by light. This swimming motion is due to the action of one flagellum, located anteriorly. The euglenas can throw off the flagellum; they can then no longer swim, but can change the body form through a peculiar squirming type of movement, early designated by Perty as "Metabolie". The loss of the flagellum seems to be a general injury reaction, probably analogous to the well known autotomy of the crab, cray-

Appendix, continued

fish, and lizard. A disc-like swelling first appears at the tip of the flagellum, interpreted as beginning degeneration. Under unfavorable conditions the euglenas can enclose themselves within a mucus cyst.

All euglenas possess a differentiated layer of the outer surface of the protoplasm, the pellicle. This differs from the cell wall of plants in being protein containing and non-cellulose, and also does not show plasmolysis with concentrated salt solutions. Only mechanical force can separate it from the cytoplasm. The pellicle is covered with spirally arranged stripes consisting of rows of little knobs concerned with the secretion of mucus. This is always secreted in individual threads which flow together into a homogeneous mass.

Within the reticular cytoplasm is the large round or oval nucleus, frequently in the middle of the body. The nucleus contains a larger darker staining central body, the endosome. This is surrounded by a chromatin network.

At the anterior end of the euglena is a narrow funnel, covered with the pellicle. This enlarges at its base into a naked reservoir. A series of pulsating vacuoles arise beside the reservoir, enlarge, and flow

Appendix, continued

together to form one large vacuole, which finally empties into the reservoir. The flagellum passes through the funnel and reservoir, and is attached to the base of the latter by means of two roots, each with a granule at the base. The granule at the base of the main root is the blepharoplast, the other is the basal granule. The flagellum and basal granule have their origin from the blepharoplast. The blepharoplast itself arises from the endosome of the nucleus, and occasionally in stained preparations rhizoplasts can be seen connecting blepharoplast and nucleus. On the main flagellar root above the blepharoplast is a swelling of unknown function.* A stigma containing red pigment lies adjacent to the reservoir.

The euglenas typically are green in color, due to the presence of chlorophyll. The chloroplasts are band- or disc-shaped, variously arranged, usually in the peri-

*I have found the following method unusually successful in demonstration of the motor apparatus in living Euglena gracilis in the unstained condition. Upon mixing the euglenas with a ten per cent. gelatin solution, the flagellum is discarded, and the cytoplasm is largely drawn into the posterior portion of the cell, leaving the reservoir in the anterior portion. Due to the unusual transparency of this species, the two roots of the flagellum with blepharoplast, basal granule, and swelling above blepharoplast can be clearly seen through the pellicle and reservoir. Observation was with apochromatic oil immersion objective, N.A. 1.4, with exacting lighting conditions.

Appendix, continued

phery of the cell. The organic food supply is furnished both by photosynthesis and by absorption from the surrounding medium. The starch-like body, paramylon, is a characteristic product of metabolism. It occurs as colorless highly refractive granules which vary in size, shape, amount, and arrangement. Pyrenoids, which have their origin in mitochondria, are considered to be the centers of paramylon formation.

Division can take place either in the motile condition, after discarding the flagellum, or within the cyst. The flagellum does not divide, but is thrown off, and an entire new motor apparatus is constructed for each daughter cell. Mitosis is accomplished in a manner quite similar to that described for higher plants and animals. Definite chromosomes are formed, divide longitudinally, and are distributed equally to the daughter cells. The chromosomes are formed from chromatin of the outer nucleus and not from the endosome. The endosome gives rise to the kinetic elements. Division of the nucleus is preceded by its migration anteriorly in the body until it comes into close contact with the lower border of the reservoir. Division of the cell takes place by a longitudinal splitting, beginning at the anterior end and continuing downward. A process known as endomixis, a sort of internal conjugation, has been described