AN AXENIC CULTURE OF *OPALINA OBTRIGONOIDEA* (METCALF).

by

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ABSTRACT

An axenic culture of Opalina obtrigonoidea (Metcalf), recovered from Rana pipiens, was developed by modifying Kidder and Dewey's basic medium for the culture of Tetrahymena geleii. A Millipore filter apparatus was employed and screw-top test tubes utilized in order to obtain anaerobic, bacteria-free cultures. The need for certain ions was demonstrated by omitting them from the medium. Magnesium, phosphorus, potassium, calcium, sodium and sulfur were found essential. Sodium acetate was needed as a carbon source. The following amino acids were found necessary for the maintenance of the organism: l-Arginine, l-Histidine, dl-Isoleucine, l-Lysine, l-Phenylalanine, l-Leucine, dl-Threonine, l-Tryptophan, dl-Valine, and l-Cysteine. Additional amino acids, minerals, and certain growth factors may be required but were not demonstrated due to the use of liver extract concentrate in the medium. The final solution contained the following: magnesium sulfate, potassium phosphate dibasic, calcium chloride, sodium acetate, l-Arginine HCl, l-Histidine HCl, dl-Isoleucine, l-Leucine, l-Lysine HCl, l-Phenylalanine, dl-Threonine, l-Tryptophan, dl-Valine, l-Cysteine, and liver extract concentrate.

INTRODUCTION

The opalinids are a group of multinucleate ciliates, lacking a cytostome, which are almost universally parasitic in the large intestine of amphibians, with but a few species parasitic in fish and snakes.

By far the most extensive work on this large group of organisms has been done by Maynard M. Metcalf of Johns Hopkins University. In his first paper, Metcalf (1909) gives a comprehensive review of the literature dealing with every phase of the study involving the Opalinidae. According to Metcalf in this review, Purkinje and Valentini were the first to use the generic name Opalina in 1835. He also found Zeller's report of 1877 as "the most accurate paper written on the family during the nineteenth century." Metcalf did extensive research on the life-cycle of these protozoans and delineated in detail the discoveries of previous workers in the field; i.e., Engelmann in 1875 and 1876, Bezzenberger in 1904, Léger and Duboscq in 1904, and Neresheimer in 1906 and 1907.

The galvanotaxis of these unicellular organisms was studied by Jennings (1899) and by Wallengren (1903). Various investigators have been interested in the taxonomy of the group,

among them Metcalf (1923), Gatenby and King (1925), Chatton and Brachon (1936), and in the last decade, Corliss (1961). The latter taxonomist has disagreed with Metcalf's classification of the Subclass Protociliata. Metcalf classified them on the basis of their nuclear dimorphism. Corliss (1963) has proposed placing the group as a new superclass in the Subphylum Sarcomastigophora.

Metcalf (1923) reviewed the morphology of the opalinids. Only a few papers dealing with this aspect of research have appeared since then; including those by Bhatia and Gulati (1927), Ivanić (1936), and Brookes and Mohr (1963). Metcalf (1940) has also reported on the geographical distribution of the group, attempting to correlate their distribution with the phylogeny of their Anuran hosts.

McConnachie (1960) has reported the latest work on the life-cycle of these multinucleated ciliates. This particular phase of investigation has been hampered by the lack of a good medium for in vitro studies of these osmotrophic protozoans. These microorganisms do not survive long outside their natural environment in the large intestine of Amphibia. In water they live a few hours, in frog Ringer's solution somewhat longer. Pütter (1905) developed a culture medium consisting of 0.8 % sodium chloride, 100 parts; 30 % potassium-sodium tartrate (Rochelle Salts), 5 parts; and distilled

water, 5 parts. In this fluid, and in the absence of free oxygen, opalinids, if provided with nutrients, would live three weeks.

Konsüloff (1922) used Pütter's fluid and added at each change of solution either a drop of fresh or boiled bouillon made of frog intestinal contents, or a drop of egg albumen dilution. He maintained cultures of Cepedea dimidiata and Opalina ranarum for as long as three months, changing them daily. Konsüloff also suggested keeping the cultures in the dark so as to approximate more nearly the normal surroundings.

Larson, van Epp, and Brooks (1925), Larson (1928), and Larson and Allen (1928), studied the reaction of Opalina obtrigonoidea to various laboratory media, including Pütter's, Locke's, and Ringer's solutions, to which were added egg albumen or blood serum for nutriment. They arrived at the following conclusions:

1. The addition of egg albumen or blood serum to any of the more generally used laboratory media considerably increases survival time in the media.
2. Pütter's solution with the addition of blood serum was found to be the most efficient in terms of survival time. The addition of egg albumen did not increase the survival time to as great an extent as the blood serum, but it did increase it beyond a non-enriched Pütter's solution.
3. The capacity of opalinids to survive in Locke's medium was increased several hours by the addition of blood serum or egg albumen.

4. The addition of either egg albumen or blood serum did away with the need to retain the rectal wall or part of its contents in the culture. This reduced the bacterial count - a very important factor in any kind of culture work.
5. Subculturing the opalinids every 24-48 hours made it possible to maintain a culture for about a month, depending upon the condition of the culture.

Lwoff and Valentini (1948) attempted to free Cepedea dimidiata from bacteria by repeated washings. They prepared cultures in a medium consisting of sodium chloride, 4 grams; magnesium sulfate, 0.1 gram; potassium sulfate monobasic, 1 gram; purified gum arabic, 10 grams; and distilled water, 1 liter. Extract of frog liver, yeast extract, beef extract, peptone, and either cysteine hydrochloride or Vitamin C were added to the medium. The organisms were cultured through 15 transfers, becoming as numerous as 120 organisms per cc.

Yang and Bamberger (1953) used a medium first developed by Boeck and Drbohlav (1925) for the culture of Entamoeba histolytica. The medium was composed of two portions; a solid egg slant, and a liquid overlay consisting of the following: sodium chloride, 4 grams; sodium phosphate dibasic, 2 grams; potassium phosphate monobasic, 0.3 grams; potassium chloride, 0.2 grams; calcium chloride, 0.02 grams; magnesium sulfate, trace; sodium carbonate, 0.4 grams; and enough distilled water to make 1 liter. Human serum and bacitracin, penicillin, or dihydrostreptomycin sulfate were added. Subculturing was done

at 48, 72, or 96 hour intervals depending on the abundance of the opalinids.

A more convenient method was developed by Yang (1960). It contained the same salts as were used in his previous work; however, liver concentrate (Wilson's) was also included. Before inoculation with the opalinids, 0.1 ml. inactivated human serum was added to each test tube. Subculturing was done weekly or biweekly.

McKinnon and Hawes (1961) maintained O. ranarum on Boeck and Drbohlav's medium for three months and observed plentiful division of the organism.

The purpose of the present study is to develop an axenic and anaerobic medium for the culture of opalinids which might facilitate life-cycle studies and the search for a completely chemically defined medium. The opalinid used throughout this experiment was O. obtrigonoidea.

MATERIALS AND METHODS

Opalinids from the rectum of Rana pipiens were used throughout the study. Hosts were obtained from three localities: 1. from E. G. Steinhilber and Co. Inc., 102 Josslyn, Oshkosh, Wisconsin; 2. from J. M. Hazen and Co., Alburg, Vermont, and 3. from the vicinity of Mesa, Arizona. Altogether 63 frogs were examined, 41 males and 22 females. Only 17 were found heavily infected with the opalinids, 11 males and 6 females. O. obtrigonoidea was found in the rectum of the anurans from Vermont and those from Mesa. Only one frog from Wisconsin was found infected but the species of opalinid was not determined. Opalinids from 14 Arizona frogs were the only ones used in these experiments. The frogs were pithed, the rectum quickly removed and placed in a sterile watch glass containing frog Ringer's solution, where it was teased open. If the animal was infected, the opalinids would swim out and they, with the aid of a dissecting microscope, could be easily identified by their characteristic spiral motion. The organisms were washed several times in frog Ringer's solution before serving as the inoculum. This procedure insured a minimum carry-over of the original rectal contents.

The amino acids and growth factors, including the liver

extract concentrate, were obtained from Nutritional Biochemicals of Cleveland, Ohio. The following chemicals were supplied by Mallinckrodt Chemical Works of New York: magnesium sulfate, potassium phosphate dibasic, cupric chloride, manganese chloride, zinc chloride, and sodium acetate. Calcium chloride, ferric chloride, ferrous ammonium sulfate, and dextrose were obtained from J. T. Baker Chemical Co. of New Jersey.

All glassware utilized was washed and rinsed three times with distilled water and all equipment was autoclaved before use. The media were prepared in 1000 ml. lots using a Beckman chainomatic analytical balance and distilled, de-ionized water. Each solution was then agitated and warmed for approximately fifteen minutes on a hot plate, then sterilized by filtering through a sterile Millipore filter of pore size 0.45 microns by vacuum. The filter flask was attached to a vacuum pump by means of a rubber hose. The filtered, bacteria-free solution was then poured into sterile 15 x 125 mm. Pyrex screw-top test tubes. Using these tubes made anaerobic conditions possible. Approximately 4 ml. of solution was placed in each tube before the tubes were inoculated by means of a sterile pipette. The tubes were kept at room temperature and were read by being emptied into sterile watch glasses and observed through the dissecting microscope.

EXPERIMENTS AND RESULTS

The basic culture medium used in this study was the original one utilized by Kidder and Dewey (1945) in the culture of Tetrahymena geleii with the following exceptions: instead of protogen, liver extract concentrate was used in the concentration of one unit per milliliter of solution, as suggested by Elliot (1949). The formulation of the modified medium is shown in Table 1.

Six tubes of the basic medium were prepared and each was inoculated with 12 opalinids. Relatively high concentrations of the microorganisms were used, since the results were more consistent and could be read much sooner. The first tube was inspected after 24 hours had elapsed, and it was re-inspected every 24 hours for the next five days. The second tube was inspected after 48 hours and again every 24 hours for the next four days. The third tube was inspected after 72 hours and again every 24 hours for the next three days. The fourth tube was inspected after 96 hours and again every 24 hours for the next two days. The fifth tube was inspected after 120 hours and again the next day. The sixth tube was inspected on the sixth day after inoculation of the tubes. This procedure

TABLE 1

COMPLETE MEDIUM*

Amino acids		Growth factors	
l-Arginine HCl	163	Ca. pantothenate	0.10
l-Histidine HCl	66	Nicotinamide	0.10
dl-Isoleucine	213	Pyridoxine HCl	2.00
l-Leucine	247	Riboflavin	0.10
l-Lysine HCl	196	Pteroylglutamic acid	0.10
dl-Methionine	214	Biotin	0.0005
l-Phenylalanine	110	Thiamine HCl	1.0
dl-Threonine	238	Choline Cl.	1.0
l-Tryptophan	60	Liver extract conc.	1.0
dl-Valine	96	Yeast nucleic acid	100
dl-Serine	317		
l-Glutamic acid	233	Salts	
l-Aspartic acid	61	$MgSO_4 \cdot 7H_2O$	100
Glycine	5	K_2HPO_4	100
dl-Alainine	55	$CaCl_2 \cdot 2H_2O$	50
l-Proline	175	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25
l-Hydroxyproline	75	$CuCl_2 \cdot 2H_2O$	5
l-Tyrosine	67	$FeCl_3 \cdot 6H_2O$	1.25
l-Cysteine	3.5	$MnCl_2 \cdot 4H_2O$	.05
		$ZnCl_2$	.05
Carbon source			
Dextrose	1,000		
Sodium acetate	1,000		

*All amounts are given in micrograms per ml. of final medium.

was carried out three times. Each time the opalinids were found to be more numerous than before. The results of this experiment are shown in Table 2.

The opalinids recovered from the preceding experiment were then subcultured following the same method. The results are shown in Table 3.

The components of the basic medium were then removed one by one in order to determine, with the use of the available equipment and materials, the requirements for maintaining life. Each new solution was poured into four test tubes and each tube was examined in the same manner as before. The first sign of death or inactivity on the part of the ciliates was a peculiar "balling up", that is, the organisms became almost perfect spheres. The results of this series of experiments are shown in Tables 4 and 5.

A final solution was prepared containing the salts, amino acids, and the liver extract concentrate found necessary for maintaining the organism alive. Four tubes were prepared, each containing 4 ml. of the solution. Opalinids were placed in the tubes and each tube was examined exactly as had been done earlier. At the end of the fourth day, the ciliates were still active in the medium. This experiment was carried out three times. The results are shown in Table 6. The components of this final, partially defined medium are shown in Table 7.

TABLE 2

GROWTH OF OPALINA OBTRIGONOIDEA IN BASIC MEDIUM

Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present					
		1	2	3	4	5	6
Tube 1	12	13	15	15	19	19	22
		14	17	18	23	23	26
		14	16	16	20	22	25
Tube 2	12		14	15	18	20	23
			15	18	21	21	24
			15	19	19	23	25
Tube 3	12			14	17	17	20
				17	19	20	23
				17	21	22	26
Tube 4	12				15	18	22
					23	23	25
					20	23	23
Tube 5	12					20	24
						20	24
						21	24
Tube 6	12						21
							24
							23
Total No. of Organisms							424
Average increase of individuals per tube							11.5

TABLE 3

SUBCULTURE OF *OPALINA OBTRIGONOIDEA* IN BASIC MEDIUM

Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present					
		1	2	3	4	5	6
	22	24	28	31	34	45	49
Tube 1	26	28	33	34	39	42	44
	25	27	29	32	32	39	42
	23		29	30	35	39	43
Tube 2	24		30	33	41	42	47
	25		28	34	35	41	44
	20			35	37	40	46
Tube 3	23			31	39	44	48
	26			32	37	38	41
	22				37	43	45
Tube 4	25				40	46	49
	23				31	35	42
	24					40	47
Tube 5	24					45	51
	24					40	43
	21						46
Tube 6	24						49
	23						47

Total No. of Organisms 823
Average increase of individuals per tube 22.2

TABLE 4

RESULTS OF EXPERIMENTS REMOVING ORGANIC COMPOUNDS FROM THE BASIC MEDIUM

Organic Compounds	Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present			
			1	2	3	4
l-Arginine HCl	Tube 1	12	12	9	6	3
	2	12		7	5	2
	3	12			none	none
	4	12				none
l-Histidine HCl	Tube 1	12	11	7	5	2
	2	12		10	4	none
	3	12			3	none
	4	12				none
dl-Isoleucine	Tube 1	12	12	10	7	4
	2	12		7	5	1
	3	12			2	none
	4	12				none
l-Leucine	Tube 1	12	10	7	5	2
	2	12		9	6	4
	3	12			3	none
	4	12				none
l-Lysine HCl	Tube 1	12	11	7	5	1
	2	12		11	5	2
	3	12			4	none
	4	12				none
dl-Methionine	Tube 1	12	12	12	13	14
	2	12		12	13	15
	3	12			13	14
	4	12				15
l-Phenylalanine	Tube 1	12	12	9	6	5
	2	12		8	5	1
	3	12			2	none
	4	12				none
dl-Threonine	Tube 1	12	11	9	6	4
	2	12		11	7	2
	3	12			5	1
	4	12				none

TABLE 4 (cont.)

Organic Compounds	Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present 1	2	3	4
l-Tryptophan	Tube 1	12	11	9	7	3
	2	12		6	4	2
	3	12			none	none
	4	12				none
dl-Valine	Tube 1	12	12	9	8	3
	2	12		7	5	1
	3	12			none	none
	4	12				none
dl-Serine	Tube 1	12	12	12	13	15
	2	12		12	14	16
	3	12			12	14
	4	12				14
l-Glutamic acid	Tube 1	12	13	15	16	19
	2	12		14	14	16
	3	12			17	18
	4	12				17
l-Aspartic acid	Tube 1	12	12	16	18	18
	2	12		15	16	19
	3	12			15	17
	4	12				20
Glycine	Tube 1	12	13	15	16	20
	2	12		14	15	17
	3	12			17	19
	4	12				21
dl-Alanine	Tube 1	12	12	12	17	19
	2	12		12	15	19
	3	12			13	16
	4	12				18
l-Proline	Tube 1	12	13	15	15	18
	2	12		15	17	20
	3	12			18	18
	4	12				19

TABLE 4 (cont.)

Organic Compounds	Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present 1	2	3	4
l-Hydroxy-proline	Tube 1	12	12	14	15	18
	2	12		13	16	17
	3	12			19	21
	4	12				20
l-Tyrosine	Tube 1	12	12	14	16	19
	2	12		12	12	15
	3	12			14	16
	4	12				16
l-Cysteine	Tube 1	12	11	9	8	5
	2	12		10	8	4
	3	12			4	1
	4	12				none
Dextrose	Tube 1	12	12	13	14	16
	2	12		12	12	14
	3	12			12	14
	4	12				15
Sodium acetate	Tube 1	12	11	9	4	3
	2	12		7	5	1
	3	12			2	none
	4	12				none
Calcium pantothenate	Tube 1	12	12	14	15	18
	2	12		12	16	17
	3	12			15	16
	4	12				20
Nicotinamide	Tube 1	12	12	14	14	18
	2	12		13	15	17
	3	12			13	13
	4	12				17
Pyridoxine HCl	Tube 1	12	13	14	17	21
	2	12		15	17	19
	3	12			19	22
	4	12				21

TABLE 4 (cont.)

Organic Compounds	Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present 1	2	3	4
Riboflavin	Tube 1	12	13	15	16	18
	2	12		17	20	22
	3	12			17	19
	4	12				20
Pteroylglutamic acid	Tube 1	12	12	13	16	20
	2	12		12	13	16
	3	12			15	18
	4	12				19
Biotin	Tube 1	12	12	14	14	17
	2	12		13	16	17
	3	12			14	15
	4	12				19
Thiamine HCl	Tube 1	12	12	14	17	18
	2	12		15	18	19
	3	12			15	17
	4	12				16
Choline Cl.	Tube 1	12	14	14	18	21
	2	12		17	18	22
	3	12			17	19
	4	12				20
Yeast nucleic acid	Tube 1	12	12	18	18	20
	2	12		12	14	16
	3	12			15	17
	4	12				21
Liver extract concentrate	Tube 1	12	8	5	1	none
	2	12		3	none	none
	3	12			none	none
	4	12				none

TABLE 5

RESULTS OF EXPERIMENTS REMOVING INORGANIC COMPOUNDS FROM THE BASIC MEDIUM

Inorganic Compounds	Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present 1	2	3	4
$MgSO_4 \cdot 7H_2O$	Tube 1	12	11	11	9	4
	2	12		7	5	2
	3	12			none	none
	4	12				none
K_2HPO_4	Tube 1	12	10	7	5	5
	2	12		7	7	3
	3	12			none	none
	4	12				none
$CaCl_2 \cdot 2H_2O$	Tube 1	12	6	4	4	1
	2	12		5	3	none
	3	12			none	none
	4	12				none
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Tube 1	12	12	15	15	19
	2	12		12	13	18
	3	12			15	17
						18
$CuCl_2 \cdot 2H_2O$	Tube 1	12	13	13	16	19
	2	12		14	14	16
	3	12			14	17
	4	12				15
$FeCl_3 \cdot 6H_2O$	Tube 1	12	12	13	15	17
	2	12		12	12	16
	3	12			14	16
	4	12				15
$MnCl_2 \cdot 4H_2O$	Tube 1	12	13	14	18	18
	2	12		13	14	17
	3	12			16	19
	4	12				17
$ZnCl_2$	Tube 1	12	12	12	16	18
	2	12		12	15	19
	3	12			14	17
	4	12				18

TABLE 6

GROWTH OF *OPALINA OBTRIGONOIDEA* IN PARTIALLY DEFINED MEDIUM

Culture No.	No. of Organisms Inoculated	Day Post Inoculation, No. of Organisms Present			
		1	2	3	4
Tube 1	12	12	12	13	14
		12	12	13	13
		12	13	13	13
Tube 2	12		13	14	15
			12	14	14
			13	14	14
Tube 3	12			13	13
				14	14
				14	14
Tube 4	12				14
					15
					14
Total No. of Organisms					167
Average increase of individuals per tube					2

TABLE 7

COMPONENTS OF FINAL MEDIUM*

$MgSO_4 \cdot 7H_2O$	100
K_2HPO_4	100
$CaCl_2 \cdot 2H_2O$	50
Sodium acetate	1,000
l-Arginine HCl	163
l-Histidine HCl	66
dl-Isoleucine	213
l-Leucine	247
l-Lysine HCl	196
l-Phenylalanine	110
dl-Threonine	238
l-Tryptophan	60
dl-Valine	96
l-Cysteine	3.5
Liver extract conc.	1.0

*All amounts are given in micrograms per ml. of final medium.

DISCUSSION OF RESULTS

Until recently, ciliates had been grown only in broths of unknown chemical composition. This cast much doubt upon the dependability of conclusions concerning basal food requirements. The development of almost completely defined media for a few of these protozoans has changed this situation. Since these microorganisms are similar to bacteria in the ease with which they can be handled and in their rate of growth, they offer material for studying animal metabolism under conditions which can be controlled to a degree not attainable in higher organisms. Furthermore, accurate control of the food supply favors their use in the search for new vitamins, as in the case of the discovery of protogen by Stokstad et. al. (1948) - a growth factor which may prove to be a fundamental requirement of all animals. To the parasitologist, parasitic protozoa in an axenic culture offer unprecedented opportunities for correlating metabolic activities and differences of parasites with the nature of the parasitic habit as well as parasitic susceptibility to chemotheraupetic agents (Hall, 1953).

The first ciliate to be successfully cultured in the absence of all other microorganisms was Tetrahymena geleii

(Kidder and Dewey, 1945). From this beginning, much information has been gained. Today it is the only animal which can be grown under rigidly controlled aseptic conditions in a completely chemically defined medium. Thus, a clear picture of its synthetic capacities is available. This is the reason why the basic medium for the culture of Tetrahymena was chosen for this study.

Not too much is known about the qualitative and quantitative mineral requirements of the protozoa. For this group in general, certain metal requirements are related to particular enzyme systems where they may be either integral parts of enzymes or "activators" whose function is not completely understood. Hence, it is possible that certain mineral requirements may vary quantitatively and perhaps qualitatively with the use of different substrates (Hall, 1953).

In the present study, the technique employed was adequate to indicate only the qualitative requirements of certain metals. The need for certain ions was demonstrated by simply omitting them from the medium. Magnesium, a component of carboxylase, was required, and may be a general requirement of all protozoa. Phosphorus in the form of the phosphate ion was needed and may be a general requirement for the phosphorylation of metabolites and vitamins. Potassium was also required and may be needed in certain phosphorylations.

Calcium is obviously essential for opalinids and may also be a general requirement for all protozoa. A need for sodium has apparently been shown. Sulfur, constituting part of several vitamins and amino acids is supposedly essential. The need for iron, copper, manganese, zinc, and chlorine could not be shown, but may not be significant, since these elements could be present in sufficient concentrations in other portions of the basal medium.

No vitamin requirements were observed, presumably due to the use of liver extract concentrate, which contains, among other things, the B complex vitamins. Therefore, this ciliate could possibly require one or all of the water soluble vitamins included in the liver extract portion of the medium, if such vitamins were present in adequate amounts. According to one source, all ciliates will eventually be shown to require thiamine in their diet under ordinary conditions (Bourne and Kidder, 1953). Kidder and Dewey (1948) used protogen in their experiments with Tetrahymena and eliminated the addition of liver extract concentrate to their cultures. It would be interesting to determine the vitamin requirements of O. obtrigonoidea using the same technique.

No nucleic acid requirements were observed in this study, perhaps also due to the use of liver extract concentrate.

Insofar as carbohydrate metabolism is concerned, O. obtrigonoidea does not require the addition of dextrose to the medium, but does need sodium acetate. Perhaps this organism is able to deaminate amino acids with the subsequent production of ammonia and a fatty acid. An amazing thing about previously studied ciliates is their inability to utilize sucrose; this most common of sugars seems to be inert nutritionally (Bourne and Kidder, 1953). In earlier studies fatty acids were routinely employed, but showed no evidence of being utilized (Kidder and Dewey, 1948). These were not included in this experiment due to their troublesome opalescence. Choline, a compound lipid, was included, but was not shown to be a requirement.

In the media used it was found that the following amino acids were necessary to maintain the organism: l-Arginine, l-Histidine, dl-Isoleucine, l-Leucine, l-Lysine, l-Phenylalanine, dl-Threonine, l-Tryptophan, dl-Valine, and Cysteine. Apparently Glycine, dl-Methionine, dl-Serine, l-Glutamic acid, l-Aspartic acid, dl-Alanine, l-Proline, l-Hydroxyproline and l-Tyrosine are not essential, but one or more of them could be present as constituents of the liver extract portion of the medium. It is doubtful if l-Glutamic acid, l-Aspartic acid, dl-Alanine, l-Hydroxyproline, or l-Tyrosine will eventually be found to be essential in a defined medium, since they have not been found either essential or stimulatory for any invertebrate

thus far investigated (Bourne and Kidder, 1953). This is not the case with Glycine, dl-Methionine, dl-Serine, or l-Proline. Glycine has been shown essential for Trichomonas foetus, while dl-Methionine has been found necessary for T. foetus, Tetrahymena geleii, Glaucoma scintillans, Tribolium confusum, and Attagenus sp. dl-Serine has been found essential for the growth of T. foetus and T. geleii, while l-Proline has been found necessary for the growth of T. foetus and G. scintillans (Bourne and Kidder, 1953).

In comparing the results of this study with the results of other investigators culturing opalinids, it is found that the mineral requirements agree with those of Pütter (1905) and Konsüloff (1922), except for the absence of magnesium in their cultures. These two workers added complex broths to their media for nutriment. The result was not defined and not axenic.

Larson (1928) introduced the addition of blood serum or egg albumen to her cultures with a resultant increase in the longevity of the organisms. If egg albumen is broken down into its constituents amino acids, it is found to be made up of the following: Alanine, Valine, Leucine, Isoleucine, Aspartic acid, Glutamic acid, Proline, Phenylalanine, Tyrosine, Cystine, Arginine, Histidine, Lysine, and Tryptophan (Best and Taylor, 1961). Human serum contains no Alanine, but contains all the aforementioned amino acids plus Amide N, Cysteine, and

Glycine (Giese, 1962). Since Larson (1928) found that her cultures lived longer with the addition of human serum than with the addition of egg albumen, perhaps the presence of one or both of the last two named amino acids is the cause for the greater longevity of the opalinids. However, these amino acids may not be absolutely essential for their maintenance.

Lwoff and Valentini (1948) were not sure of having bacteria-free cultures, and their cultures were complex brews. Yang and Bamberger (1953) added to their medium both human serum and whole eggs, but an antibiotic was needed to combat the invading bacteria. Yang (1960) removed the eggs and added liver extract concentrate. According to him, the liver concentrate medium was a poor breeding ground for bacteria and no antibiotics were necessary as long as subculturing was practiced weekly or biweekly. There was contamination with small flagellates and they eventually crowded out the opalinids (Yang, 1960).

McKinnon and Hawes (1961) made no attempt to obtain anaerobic conditions and the medium was susceptible to bacterial contamination.

The results of this investigation indicate there is still much work to be done in culturing this protozoan. As of today, no completely chemically defined medium has been found, since various workers have had to include yeast extract,

peptone, blood serum, egg albumen or liver extract concentrate in their cultures. This procedure brings in many unknown factors. As mentioned previously, the use of protogen may simplify the search for a completely defined medium by defining the vitamin requirements. The Millipore filter apparatus was used to obviate autoclaving certain heat-labile substances. This apparatus has been found to make the culture truly axenic and no further problems with bacterial contamination are foreseen in future studies.

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