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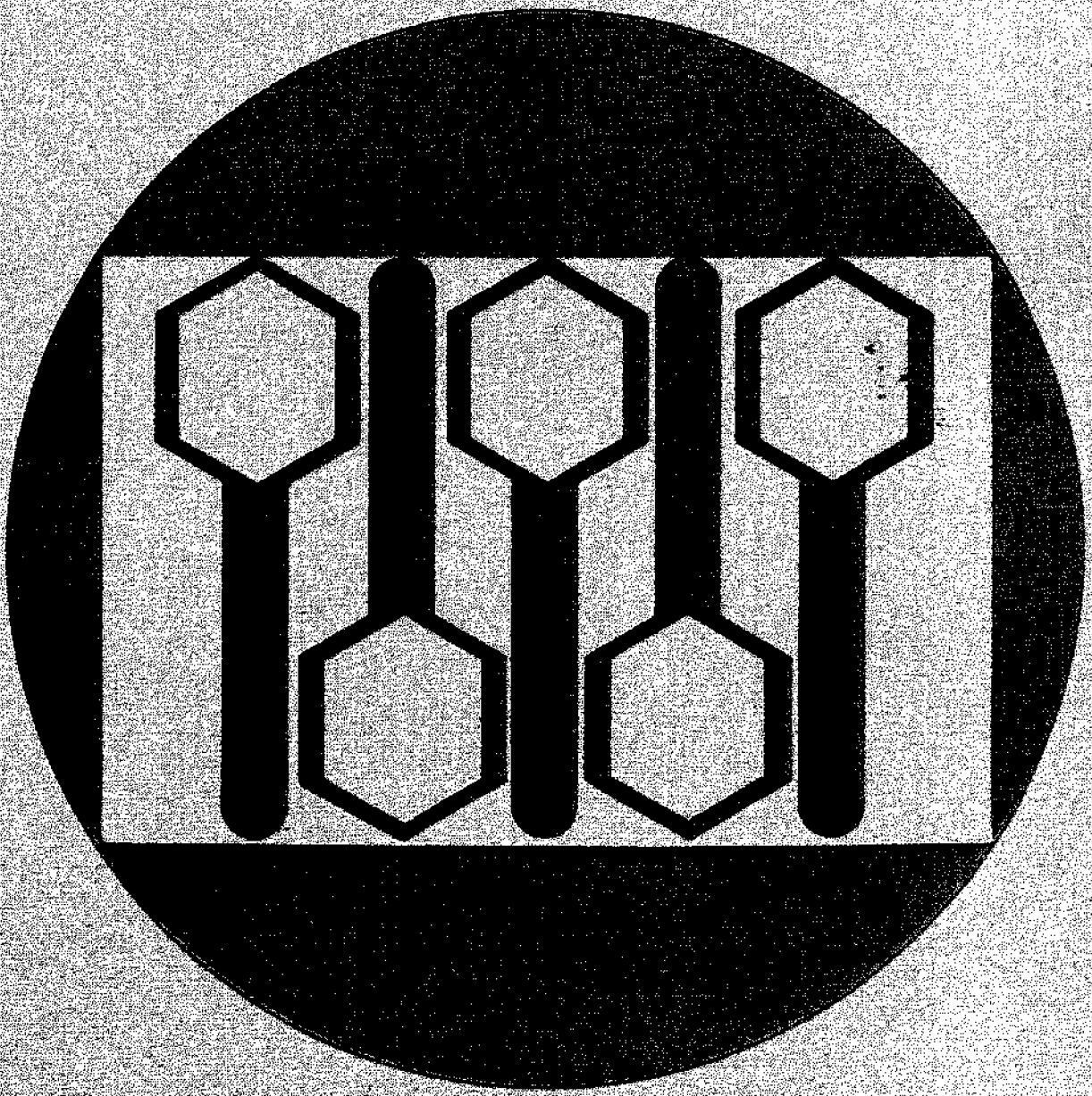

HOST RANGE STUDY OF BLUE-GREEN ALGAL VIRUSES

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INTRODUCTION

Foreword

Much of the data contained in the host-range tables which comprise this study is not readily interpretable at the present time, but may be useful in further investigations into the molecular genetics of the blue-green algae. The present paper is a progress report summarizing the information gained on the molecular ecology of a host/virus system covering a rather wide range of blue-green algal viruses and their hosts. Knowledge gained to date from the host-range data has been applied in: interpreting survival curves of blue-green algal viruses as a function of radiation dose (10); formulating a methodology for the examination of fish kills; characterizing new and old viruses; and establishing a blue-green algal host/virus system as a model of principles and mechanisms in the examination of viruses in polluted or eutrophic water.

The Molecular Environment in the Estuary

During several summers, high school students in various programs (supported by NSF, AEC, ACS and local workshops as well as by NOAA) examined waters of all kinds for the presence of blue-green algal viruses. From these assays we concluded that areas of streams enriched by domestic sewage carry increased counts of blue-green algal viruses; these viruses were also abundant in wastes from feed lots, duck ponds, pig pens, wild bird reservations, sludge, composting debris and swamplands (8,9). Runoffs from these places enter the estuary and since many types of viruses are stable in eutrophic water, it appears that the blue-green algal host/virus system is an efficient monitor of the viral content of water and an indicator of

eutrophic conditions.

Since the concentration of viruses in water is often less than one detectable count/ml, the water sample being examined must usually be incubated on an algal host in order to develop a readable count. Since we have, as yet, no information on the genetic make-up of our stock hosts, we are forced to inquire whether there is a remote possibility that we may be developing a count from latent viruses carried in our own stock. If we were to use our blue-green algal host/virus system only as a monitor of sewage pollution, it might matter little whether we were measuring a quality of water able to induce a virus count or whether we were measuring the viruses themselves. But if our host/virus model is to monitor viruses and to give information on principles and mechanisms of virus transfer in water, we must know more about the virology, molecular ecology and biochemical genetics of the blue-green algal system. Then we can advance aqua culture, improve marine industry, control pollution, enjoy conservation and provide safe recreation.

High algal counts were found not only in sewage-receiving waters where numbers of enteric and pathogenic viruses are high, but also in regions where limnological studies (15) showed low "diversity" indexes and the stream was judged "unhealthy" or "semi-polluted." The limnological station immediately below the sewage plant on the Red Clay Creek gave evidence of such stream degradation; at this same station, we repeatedly found high virus counts of about 50 P.F.U. (plaque-forming units)/ml (9). Normally, the diversity of planktonic blue-green algae in these regions of streams is also low (14). However, we find high virus counts in oxida-

tion ponds and other eutrophic streams and bodies of water where unialgal blooms exist. A blue-green algal bloom, then, can originate in the midst of a high count of blue-green algal viruses! The bloom species must therefore be immune or resistant to the viruses present. In this situation, too, explanations require increased information on viral ecology. It is apparent that, in order to understand and control productivity in potentially polluted water, much more must be learned at the molecular level about interactions among viruses and microorganisms.

Molecular Genetics of the Blue-Green Algae: State-of-the-Art

In the light of the wealth of information available on the Escherichia coli host/virus system, it is apparent that the ecology of the blue-green algal viruses, as we have viewed it from thousands of field observations and laboratory cultures, presents a parallel array of phenomena. The pioneer in blue-green algal genetics is faced at the outset with evidence for host cell modification of virus, virus conversion of host, lysogeny, multilysogeny, defective lysogeny, cryptic virus, transduction, interspecies transfection, drug transfer factors, extrachromosomal DNA, intracellular symbiosis and parasitism and possible sexually-mediated genetic transfer which has not been formally recognized as an attribute of the blue-green algae. In the midst of all these phenomena (5,6), the initiator must obtain some base line from which he can begin to build. The host-range, plaque-morphology charts have provided this base.

The first requisite of any genetic study is the preparation of reliably pure stock. With the exception of a Nostoc donated by the Radiation Laboratories of the Smithsonian Institute, none of the algae obtained from outside collections was sufficiently clean for immediate use. However,

most of the cultures obtained from established collections were unialgal and apparently well cloned, for different batches and subcultures gave consistent results on our host-range studies. We found axenic culture of oscillatoreacean, nostocacean and other filamentous blue-greens unusually difficult because of intracellular parasites capable of passing a 0.45 micron filter (cf.4) and contamination by fungus-like organisms producing extensive, very fine mycelia. No secure, uniform methodology of isolation can yet be advanced; for each separate culture, patience, persistance and ingenuity must combine standard methods for the isolation of microorganisms with whatever tricks of the trade can attain success. The preparation of suitable strains has required several years.

The use of highly purified algal strains greatly simplifies the development of pure virus strains. The chief diffuculty is the repeated cultivation of the virus in a stable, reproducible manner. In order to choose a suitable host on which to cultivate the viruses, it was necessary to make a detailed study of the differences in host ranges as the viruses are cultivated on various hosts. Lacking other markers, these host-range, plaque-morphology descriptions provided our first genetic identification. Though cumbersome, perhaps, to use as markers, they have proved useful in bringing about an organization of the various viruses infecting blue-green algae, so that differences among them can de defined and interrelationships can be demonstrated.

METHODS AND MATERIALS

Algal Strains

Over 30 strains of Schizothrix calcicola showing host-range differences for at least one virus have been isolated. The various strains and their sources are listed in Table 1. Isolates procured from special niches in nature probably represent various adaptations to polluting effluents. Many other strains have been derived from algal growths resistant to various infections (often fungal) in established cultures and may represent drug-resistant mutants. Virus resistant (receptor site) mutants and host-strain-modifying or restricting mutants are also present. All strains have a similar morphology consisting of long trichomes of uniform cells.

To recognize interrelationships among the various viruses for blue-green algae of species other than S. calcicola we added cultures of other blue-greens to the host range. These are also listed in Table 1.

Algae are cultivated under cool fluorescent lighting at room temperature in an entirely inorganic medium (Table 2) based on Hughes medium (1), omitting EDTA and citrate and reducing nitrate to 2/3. We also allow the precipitate in the prepared medium to settle, and siphon off the upper layers. This procedure lowers the pH to about 8.2 but prevents occurrence of an objectionable precipitate after autoclaving. At each transfer all cultures are inspected microscopically and plated out on Trypticase-Soy agar to detect chance contamination.

Elimination of fungal contamination was a long and tedious process. The strains used have undergone extensive cloning and repurification. Ionizing radiation has been used freely in preparing bacteria-free cultures (7

TABLE 1

BLUE-GREEN ALGAL HOST STRAINS USED IN HOST-RANGE, PLAQUE-MORPHOLOGY STUDY

ALGA		SOURCE, ISOLATION AND REMARKS
STRAINS	Schizothrix calcicola:	
	CAT	isolated as thin filaments from S. calcicola BB (7)
	CRY	from warm tributary of Christina R. (M.P.K.)
	CUF	(M.P.K.) from White Clay Creek, Pa.; irradiated 0.6 Mrads
	FAV	Indiana University (I.U.) (19) #427, repurified
	FCY	(M.P.K.) from White Clay Creek, Pa.
	HOS, TAF, BOR	I.U. #594. repurified; TAF from R. S. Safferman
	HRC	(M.P.K.) from Red Clay Creek, Hercules Sta., Del.
	LUR	I.U. 426, repurified.
	NEM	(M.P.K.) from salt marshes, Lewes, Del.
	TWS	(M.P.K.) White Clay Creek, Lovers' Lane, Pa.
	SCH	(M.P.K.) from filter beds, Springfield, Mass. water treatment
	VAL	recovered from so-called "lysogenic plectonema, (LPP-1D)"
	SAF, TFS	"resistant" strain from R. S. Safferman
	MAN	mandelamine-resistant mutant from S. calcicola CRY
Lysogenized strains:		
	CAT(716)	immune to superinfection by LPP-2-related strains
	CRY(716)	" " " " " "
	NEM(716)	" " " " " "
	TWS(716)	" " " " " "
	FAV(1818)	" " " " S3-related "
	HRC(1818)	" " " " " "
	TWS(1818)	" " " " " "

TABLE 1 (continued)

RCAT	immune to superinfection by uncharacterized virus
RCRY	" " " " " "
RCUF	" " " " " "
RTWS	" " " " " "
NEM(15F)	" " " " " "
FCY(15F)	" " " " " "

OTHER BLUE-GREEN ALGAL SPECIES:

Oscillatoriacea:

PRO	Oscillatoria prolifera; I.U. 1270, bacteria-free, (M.P.K
FRM	Fremyella diplosiphon; I.U. #481
TOL	Tolypothrix tenuis from L. Gantt
VAC	Microceleus vaginatus M.P.K.
JAM	S. calcicola (Anacystis nidulans); short trichomes; from Jamaica, L.I. sewage treatment plant.

Chroococcaceae:

UNI	Anacystis montana; (M.P.K.) from Indian Run, White Clay Creek, West Grove, Pa.
CRO	Anacystis montana; (M.P.K.) from Puerto Rican stream

Nostocaceae:

BRY	Nostoc
CAR	Nostoc carneum; (M.P.K.)
ANA	Anabaeniopsis; I.U. #137
ELL	Nostoc ellipsosporum; Gift from T. Kantz
FLO	Anabaena flos aquae; I.U. #1440
LIN	Nostoc linkia; (M.P.K.)
VAR	Anabaena variabilis; (M.P.K.) isolation from soil

Its use resulted in no essential alteration of the fundamental characteristics by which a strain was customarily recognized. It may be that our practice of preparing pure algal strains through the use of high doses of ionizing radiation has inadvertently provided us with a group of organisms uniquely suitable for studying the genetics of autotrophic metabolism.

Blue-Green Algal Viruses

Considerable information is available on the properties of the blue-green algal viruses [see reviews (2, 14, 17)].

Recently we have introduced the blue-green algal virus, S3, whose serology, plaque morphology, and host range differs from that of the others. The parent S3 virus used in these studies was isolated from the Connecticut River by incubation on S. calcicola NEM and plaque-picked from the sensitive host S. calcicola TWS, which produced uniform plaques distinguished by concentric pigmented rings. The geometry of the ring formation varies according to the host on which the virus is plaqued. The S3 virus is characterized by formation of turbid plaques on S. calcicola FAV.

Other viruses were isolated from water samples encountered in the course of monitoring streams. If the virus concentration is less than 1 PFU/ml, the water sample must be incubated to multiply the virus count. For this, 25-35 ml of water sample are added to 25-15 ml of freshly transferred algal culture. The mixture is allowed to incubate in the light 1-10 days. Lysis often occurs in 1-2 days. To remove bacteria, 10 ml of the lysate is thoroughly mixed with 0.2 ml of chloroform, allowed to settle, and the supernate removed for use. Alternatively, the lysate may be passed through a 0.45 micron millipore filter.

TABLE 2

GROWTH MEDIUM

	Stock, gms/2L	ml. stock/5 gal.
$NaNO_3$	200.0	200
$K2HPO_4$	7.8	200
$MgSO_4$	15.0	200
$NaCO_3$	4.4	200
$NaSiO_3$	11.6	200
$CaCl_2$	5.6	200
$FeCl_3$	1.5 gm/500ml.	10
Trace elements:/L		
H_3BO_3	2.86	20
$MnCl_2.4H_2O$	1.81	
$ZnSO_4.7H_2O$	0.222	
MnO_3(85%)	0.178	
$CuSO_4.5H_2O$	0.079	
$Co(NO_3)_2.6H_2O$	0.049	

Host-Range Determinations

The properties of the various derivative viruses which we have encountered are recorded in terms of host-range and plaque-type as a function of host history. By means of these descriptions we have been able to organize the various characteristics of host/virus relations and to provide guidelines for further experimentation.

To construct host-range, plaque-type charts, algal-host lawns are prepared as soft agar overlays (16) using about 20 algal hosts representing a range of responses. The host strains had previously grouped themselves into categories according to their most generally encountered reactions to virus and the hosts are listed in the charts in this order:

(a) sensitive (clear plaques)

(b) variable (turbid or haloed plaques, small, minute, mottled or late-appearing plaques)

(c) resistant (no plaques).

For base layers, growth medium containing 1 1/2% agar is sterilized and poured into petri dishes. Also small test tubes containing 3 ml agar are prepared and kept in readiness. In making soft agar overlays, the agar in tubes is melted in a boiling water bath and transferred to a 55°C holding bath. Two and a half to 3 ml of late log phase algal culture is added to the melted agar and poured over the hardened base layer. These plates are allowed to develop in the light for a day or two. They are then marked in sections. Viruses, serially numbered, are spotted onto corresponding sections of each algal host plate. On the host-range chart, the algal hosts are listed, and the viruses, with their most recent host history, head the columns. Plaque morphology is recorded on the 1st, 2nd, 3rd, 5th and 10th

TABLE 3

HOST-RANGE, PLAQUE-MORPHOLOGY SYMBOLS

+	lysis
-	no lysis
0	turbid plaques
h	clear halo
H	turbid halo
m	mottled plaques
p	picked plaque, clone
R	resistant growth after lysis
y	yellow halo or lysis
*	stimulation of algal growth
rl	rapid lysis, within two hrs.
r	rings, concentric and deeply pigmented, green or brown
L	lacunae: irregular, non-transferable plaques
s	clear, single plaques within the spot test, i.e., single centers of infection.
S	turbid, as above
B	"blue"cells in plaque
BL	spontaneous or virus-induced lysing of culture with release of phycobilin pigment
d	small or irregular diffuse plaques; defective plaques produced by chloroform treatment of culture; cannot be transferred
dep	depression in agar, with or without bacterial growth or lysis

days according to the symbols shown in Table 3.

As the plates are read, lytic spots, or those characterized by special morphology as significant for further study, are picked and suspended in 5 ml of growth medium and re-run on another host range. The aim is to define a plaque morphology characteristic of a certain virus and to discover which hosts are suitable for repeated cultivation of a virus with constant characteristics. As the viruses become more clearly defined and patterns of host/virus behavior begin to be discovered, we extend the host range to other genera and even to organisms outside the algal classification, e.g., bacteria. It is possible then to discover defectiveness in one alga by its ability to form plaques on another, and to ascertain whether bacteria participate in the acquisition of virally transferred characteristics. The extended host range enables us to follow the loss and acquisition of phenotypic markers occurring in the passage of a virus through a series of hosts, some of which are not necessarily algae.

Serology

Three viruses, exhibiting the greatest differences in plaque morphology shown by the various viruses available, were used in preparing antisera:

1. Virus #716, obtained from the headwaters of the Elk River, was serologically typed as an LPP-2 virus by Safferman and Morris (personal communication).
2. Virus #15 is a derivative of #1504, obtained from a stabilization pond for dairy wastes. Virus #1504 was typed by Safferman and Morris also as an LPP-2. Virus 15 produced uniform small plaques when picked from host S. calcicola FAV and grown on host S. calcicola TWS.
3. Virus #1818 is an S3 virus, characterized by ringed plaques. These viruses have been repeatedly found in sewage effluent.

In preparing antiserum for each virus, two rabbits were injected interdigitally. Pre-immune serum was obtained one and two weeks prior to innoculation. An emulsion of 2 ml 1:1 high titer (1×10^9) virus and Freund's adjuvant was injected into the foot pad. A 1 ml booster-shot into the ear vein was given six weeks later. Serum was collected from a series of bleedings and stored frozen.

In running neutralization tests, virus was diluted in growth medium to give a count of 1×10^5 P.F.U./ml. Serum was similarly diluted so as to give final dilutions of 1/5, 1/10, 1/20, 1/100, etc. when mixed with the virus. Virus and serum were well mixed and allowed to stand 1/2 hour or more before plating. Observations of neutralization of virus in serum preparations were compared with a virus control by dilution plating on a suitable host alga.

Alternatively a more rapid spot-test assay was used. In this method, overlay plates containing viruses were prepared to give a just confluent lysis on a host plate. The three antisera were prepared in a series of dilutions and dropped onto sections of the hardened agar. The plates were allowed to incubate in the light several days. Algae then grow where the serum has neutralized the virus. Once the highest dilution at which the serum is effective has been determined for a given tube of serum, serum can be conserved in further testing. By this method, much information on a series of viruses and hosts can be quickly obtained.

RESULTS

While the establishment of definite genetic or even phenotypic markers describing algal and viral characteristics is not yet possible, many interrelations among the various viruses and hosts have been discovered. Host-

range, plaque-morphology descriptions can differentiate various properties of virus behavior. In summing up the data in the numerous charts, host-range, plaque-morphology descriptions furnish distinctions between S3 and LPP-2 viruses and give indications of other viruses. The structure of ringed plaques has been analyzed and evidence for bacteriocinogeny in the blue-greens has been found. By analogy with the Escherichia coli system, Cyanocin is the term coined for the protein analog of Colicin, and Cyan factor as the term for the DNA coding for the cyanocin. Much more detail still remains uncovered in the charts. Therefore, the first volume of charts, A and B, has been deposited in the Marine College Library; the rest are available from the author. Altogether, about 500,000 observations on 4,000 viruses have been made.

The S3 Virus

The most important aid in bringing about organization was the discovery of the S3 virus. The S3 virus was repeatedly found in sewage effluent and we believe it enters the algal host/virus system via a bacterium (12). The S3 virus was first characterized by turbid-plaquing on Schizothrix calcicola FAV. When these plaques were picked and cultured on other hosts, ringed plaquing developed. When grown repeatedly on the same host, resistant strains were segregated. The transfer of ringed-plaque formation to a series of hosts hints that S3 is capable of transduction.

Serology

Our antiserum prepared against the original S3 virus, #1818, appears to be a reliable check for the presence of S3 character in a virus suspension. Table 4 (b) shows complete neutralization of S3 virus by S3 antiserum and Table 4 (a) indicates zero neutralization by the LPP-1 and LPP-2 antisera of

TABLE 4

NEUTRALIZATION OF VIRUS BY VARIOUS ANTISERA

(a) LPP-1 and LPP-2 ANTISERA OF SAFFERMAN AND MORRIS (18)

NEUTRALIZATION TEST DATA 8/21/72

Virus	Control	LPP-1 Antiserum* 1:10	LPP-1 Antiserum* 1:100	LPP-2 Antiserum* 1:10	LPP-2 Antiserum* 1:100
LPP-1	2.2×10^9		1.2×10^5		
LPP-2	3.6×10^7				1×10^3
1818	2.1×10^6	2.6×10^6	2.0×10^6	2.2×10^6	2.6×10^6

* Note that only 1:100 antisera dilutions were used against LPP-1 and LPP-2 viruses while an additional 1:10 dilution was used against 1818 with no apparent neutralization.

(b) ANTISERUM PREPARED AGAINST 1818 VIRUS ANTIGEN

Virus	Control	1:5	1:10	1:20	1:100	1:1000
1818	4.3×10^5	100%	100%	100%	100%	95.5%

(c) SPOT TESTS USING ANTISERA FOR VIRUSES, 716 (LPP-2) and 15

Virus	1:20 antiserum dilution		
	15	716	1818
275 (S3)	-	-	+
J32 (S3)	-	-	+
17J	+	-	-

Key: + neutralization of virus by antiserum; - no neutralization

Safferman and Morris (18). As yet, little work has been done on the serology of the various viruses. In those tests that have been run, the neutralization data check well with the immunity shown to S3 viruses in hosts lysogenized by 1818 virus, as indicated by host-range, plaque-morphology assay. Two such cases for virus 1818 are shown in Table 4 (c). Also shown in Table 4 (c) is 17J, neutralized by antiserum for virus 15. Virus 17J has a host range (cf. Table 6, column 1, 20V) which gives lytic plaques on nearly all S. calcicola strains used. Viruses such as 21A (Table 5, column 6) do show partial neutralization by 716 and 15 antisera. However, not enough work has been done to make definite assignments about 716 and 15 antisera.

Lysogeny

The apparent ability of the S3 virus to be easily lysogenized in a number of hosts (FAV, HRC, TWS), which were then immune to superinfection by the same or related viruses, not only provided a means for identification of related viruses but also contributed a feeling of security in the interpretation of "negative count" as immunity in hosts lysogenized with the LPP-2 virus, 716. It also indicated that other different viruses may be lysogenized in other R-designated hosts. This appears to be borne out in Table 5, where viruses in columns 3, 4, and 5 give uniformly different host ranges on S3- and 716-lysogenized hosts.

Virus Differentiation by Host Range

In column 1 of Table 5 is shown the host-range, plaque-morphology data for virus 20V which lyses all hosts tested. This virus came originally from the St. Jones River, Dover, Del., (Station 22, which is just below Silver Lake). It was repeatedly passed through VAL and SAF hosts which are "normally resistant hosts," being resistant to the viruses LPP-1 and LPP-2.

TABLE 5

DIFFERENTIATION OF VIRUSES BY HOST-RANGE AND PLAQUE TYPE

	VIRUS SERIAL NUMBER									
ALGAL LAWN	20V	11K	14A	J32	140	21A	Z53	15E	K34	K98
HOS	0ss+ s		-s++		000+	0+++		--00	----	++++
CAT	++++	++++	++++	++++		0+++	++++		---h	
TAF	++++		++++ HH		0+++	0+++	++++	--00		
TWS	++++	++++	0+++	++++	0+++	0+++	0+++	--00	---h	0+++ H
HRC	++++		0+++ HH	++++	0+++	0+++	0+++	--00		
FAV	++++	++++	0+++	0000 K34	0+++	0+++	++++	--00	----	--00
CRY	++++	++++	0000	0+++	----	0+++	----	--00	----	++++
RCAT	++++	++++	0+++	++++	----	----	----	--00		
RCRY	++++	++++	0+++	0+++	----	----	---+		----	
RTWS	++++	++++	0+++	0+++	----	----	0+++	---0		
(15)									++++ K98	
CAT(716)	+++	+++	+++	+++	----	----	----	--00	----	++++
CRY(716)	++++	++++	++++	++++	----	----	----	--00 h		
TWS(716)	+++	++++	0+++	++++	----	----	----		--0+	++++
FAV(1818)	0+++	sss+	----	----	-0++	----		-0++	----	
HRC(1818)	++++	s+++	----	----	-0++	----	-00+ H			----
TWS(1818)	+++	s+++	----	----	-0++	----	-00R r	-+++		
MAN	++++	0+++	-000	----	----	----	0sSS h	-000	-000 h	----
SCH	++++	0+++	-+++	----	----	----	---+	-0++		
VAL	++++	++++	0+++	++++		----				-00+
SAF	++++	++++	0+++	++++	++++	----	----	-+++	----	----

Key: +, lysis; -, no lysis; 0, turbid plaque; s, single plaque; r, rings; R, resistant algal growth; h, clear halo; H, turbid halo; the four symbols correspond to readings on the 1st, 3rd, 5th and 10th days.

The original virus from the St. Jones formed ringed plaques and the virus 20V was obtained from the outermost lytic ring. The host range data suggests that this virus should have a serology different from the others. If virus 15 is identical with this virus, as suggested above, then LPP-2 is not a pure virus, for virus 15 was a small plaquing variety obtained from the 1504 virus which was designated as LPP-2 (18).

In Table 5, column 2, 11K virus was obtained, originally, from the raw sewage at the Kennett Square, Pa. sewage treatment plant. It passed through Anacystis montana CRO and Coccochloris peniocystis hosts. We find a host range similar to that in column 1 but with a restricted (s) count on 1818-lysogenized hosts. Such counts are generally about 10^{-2} lower than that of the initially virulent (+++) hosts.

In column 3, 14A is from a lytic plaque produced on Tolypothrix tenuis by a bacterially contaminated sewage sample from the Kennett plant. As seen, this virus shows the immunity on 1818-lysogenized hosts accorded an S3 virus.

J32, in column 4, has the host range characteristics originally ascribed to an S3 virus, particularly the turbid-plaquing on FAV. It is a derivative of 1818 with a long and varied host history. As the virus is recultured on FAV, it develops a lysis on FAV which comes from a lytic s plaque developed within the turbid plaque. The lytic s plaque is shown in 140 (column 5), while the turbid plaque is shown in K34 (column 9). In K34, we pick up a count only on NEM(15), and the host range of this virus is shown in the last column, as K98. Here results may indicate recombination of superinfecting virus with a carried virus (if virus 15 has become lysogenized in host NEM). Uniform small-plaque-morphology was found on many of the hosts and small

plaques are a characteristic of virus 15. The host range is more similar to J32 than to virus 15 which, according to the serology, should be similar to 20V (column 1).

Z53 also came from raw sewage (from a trailer camp). As previously, virulence in the algae appeared to be established through a single plaque on a bacterially-infected spot. The s-plaque was carried through hosts CAT and TAV. The host range shows ringed plaques established in the 1818-lysogenized hosts. S3 virus and ringed plaques are repeatedly obtained in sewage effluent.

15E, column 8, also arose from a bacterially contaminated spot on Tolypothrix tenuis. The lysis on SAF and 1818-lysogenized hosts is also typical of a bacterially-assisted lysis. The bacterium, when plated out on agar, gives rise to lytic areas. From these areas, resistant bacteria arise. We have reason to believe (8,9) that Tolypothrix is defectively lysogenized (3) and suspect that considerable transduction of lysogeny can occur, even among non-related species.

Ringed-Plaque Formation

Since ringed plaques vary from one host to another, they should be useful analytically. The ringed plaques appear to develop day by day; the demarcation between pigmented and lytic rings becomes sharper in bright light. Once a plaque has formed a series of rings, it can be transferred intact from the start. A host-range, plaque-morphology assay was attempted on several large plaques and, although lytic rings appear to be made up of different viruses, no definite sequence of viruses seemed to be uniformly followed. In the turbid center of a ringed plaque no virus count was obtained on host-range, plaque-morphology. The sequential rings appear to have a

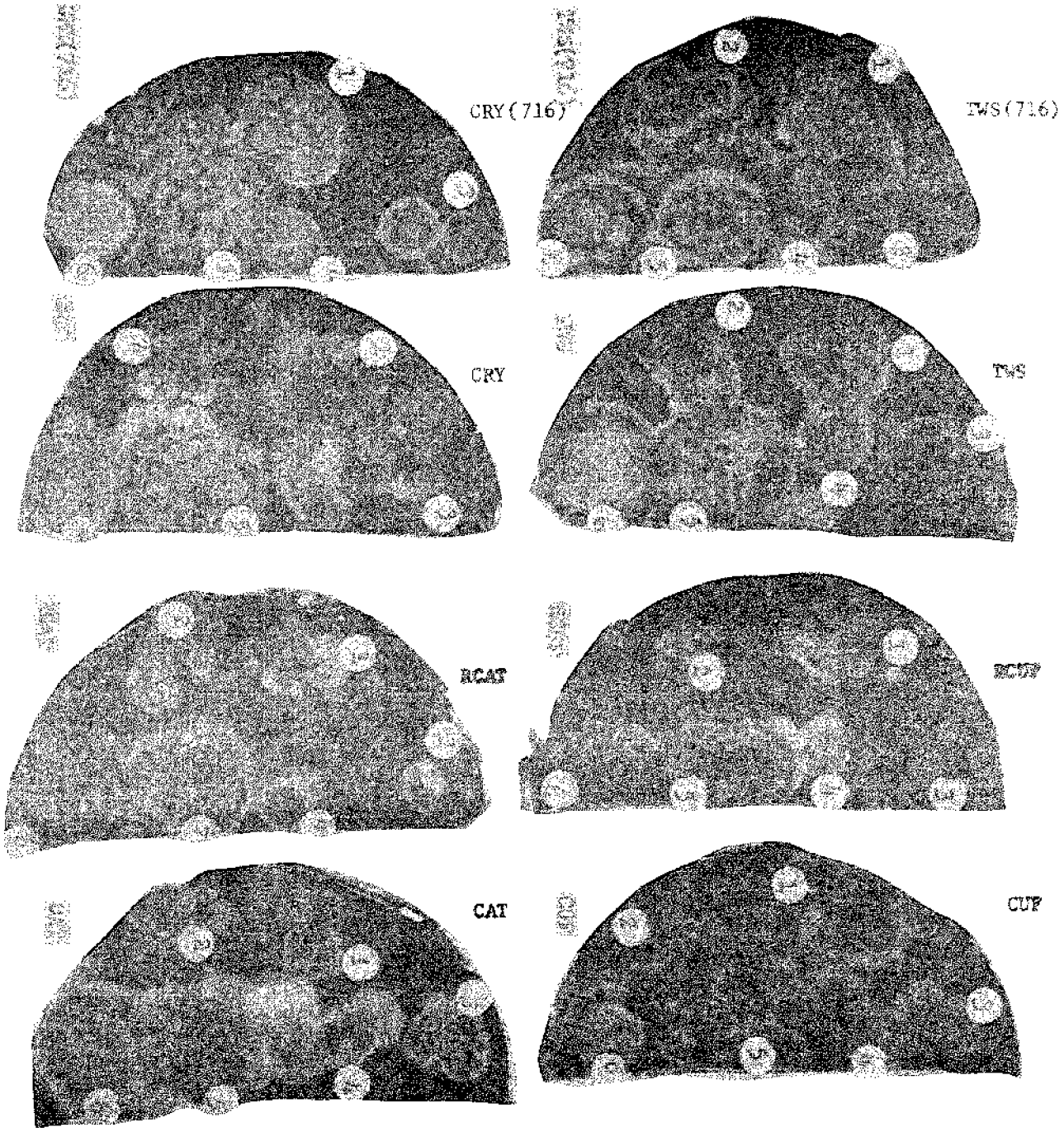
TABLE 6

HOST-RANGE AND PLAQUE-MORPHOLOGY OF RINGS OF A RINGED PLAQUE

	8G center 1st ring	8H 2nd R ring	8I 3rd R ring	8J 4th lytic ring	8K 5th R ring	8L outer lytic ring
RWS						
TWS(716)						
CRY						
CRY(716)						
CAT						
RCAT						
CUF						
RCUF						
HRC						
NEM						
FAV						
HRC(1818)						
TWS(1818)						
MAN						
SCH						
SAF						

PLATE I

PLAQUE MORPHOLOGY DIFFERENCES OF THE S3 VIRUS (5A)
AS A FUNCTION OF ALGAL HOST



Six rings of a ringed plaque of S3 virus, 5A, were dissected off, ring by ring, and suspended in 5 ml of growth medium. The host-range, plaque-morphology descriptions of these rings are given in Table 6. In the above photograph, (taken from the dried agar of the petri plates), the differences in the plaque morphology developed by the virus composition of the successive rings is shown on four different pairs of hosts.

different virus composition depending on the hosts, as shown in Table 6. The morphology of the plaques bears this out, as shown in Plate I, where different morphologies are observed between closely-related strains. It is evident that the plaques are uniform for a single host, but that there are small differences between hosts that need explaining. Plate I shows successive rings of virus 5A, a derivative of a virus obtained from the "polishing pond" at the Kennett Square Sewage Treatment Plant (12). This virus was incubated on an S. calcicola host and passed also through Anacystis montana CHR and Anabaena flos aquae FLO. The host range study is given in Table 6. The virus is clearly an S3 from the immunity shown by 1818-lysogenized hosts; the negative counts on other hosts probably represent absence of virus. Essentially all the plaques on any host are turbid or ringed plaques. Even in the outer lytic ring the original lytic count is developing resistance; perhaps this illustrates the formation of the next pigmented ring. There are really no symbols capable of describing the developing plaques shown on Plate I. The specificities of virus variation as a function of host can be more clearly demonstrated by following the host history of an outer lytic ring of a ringed plaque obtained from sample 102 at Station 16 of the St. Jones River. The original water sample after incubation on an S. calcicola host was run through a 0.45 millipore filter and several drops of the filtrate were placed in the center of a CAT and a TWS lawn. After the plaques developed, the outer lytic rings of each plaque were carefully picked up with a pasteur pipette and well distributed by squishing back and forth in 5 ml of growth medium in a 1 oz. prescription bottle. A host range was then run as shown in columns 1 and 2, Table 7. Certain of these plaques were picked and re-run: the encircled notations indicate the nature of the

TABLE 7

HOST HISTORY AND PLAQUE MORPHOLOGY OF A RINGED PLAQUE

ALGAL LAWN	VIRUS SERIAL NUMBER							
	SJ 102 CAT	SJ 102 TWS	Y84	Y86	Y63	Y64	Z34	Z35
HOS	000+	0000_{h}	++++	0+++	0+++	0+++		
CAT	000+ 716 Y84	00++ Y86	++++	++++ S2, 716	++++	++++	----	++++
TWS			0+++	$0+++_{h}$	-+++	-+++	----	++++
HRC	0+++ Y63	00++	-+++	--SS	0+++	0+++	----	0+++
NEM			++++	0+++	++++	++++	----	++++
FAV	0+++ Y64	00++	++++	++++	++++	++++	$---_{r}^{s}$	++++
CRY	----	----	++++	++++	---0	---0	----	---0
RCAT	----	----	$---+_{Y}$	-000 S2	----	----	----	----
RCRY	----	----					----	
RTWS			++++	0+++	----	----	----	
NEM(15)			++++	0+++				
CRY(716)					----	----	----	----
NEM(716)			----	-000_{h}	----	----	----	----
TWS(716)	$---_{ss}^{s}$	-sss	----	000_{h}^{R}	----		----	----
FAV(1818)	--++	--s+			-000 Z35	--SS Z34		++++
HRC(1818)				--ss 716			----	
TWS(1818)	$---_{ss}^{s}$	--S+		$--_{s}^{++}$ S5				
MAN	$---_{S}^{s}$	$--_{ss}^{00}$	$--_{s}^{00}$	$---_{s}^{0}$	$---_{S}$	----	----	----
SCH	---m	$\frac{R}{m}$	----	---0 S3, s 716	----	----	----	----
SAF	----	----	----	--00	++++	++++	----	

Key: see Table 5

virus present. (For convenience, we will let S2 represent any virus appearing to be immune on the hosts named in rows between FAV and RTWS; and we will let S3 signify a virus, as in column 1, Table 5, which lyses all hosts). From columns 1 and 2, the plaques on CAT were picked and re-run on a host range. A noticeable difference is shown on HRC and RCAT hosts: the V86 virus is no longer resistant on 716-lysogenized hosts. If we pick and compare the plaques of HRC and FAV from column 1, 63 and Y64, we find only a slight difference in host range occurring on FAV(1818). These plaques are picked and re-run as Z34 and Z35. The host-range, plaque-morphology picture between these two is very different, and illustrates the specificities which are occurring. The fact that Z34 is a turbid s-plaque is the significant distinction, representing the ability of Z34 to become lysogenized.

Cyanocins and Cyanocinogeny

We have long been suspicious of the presence of bacteriocins for the blue-green algae (8,9). Bacteriocins can be placed in the category of defective viruses (3,13). Lysogeny and defective lysogeny are probably as common in the blue-green algae as has been claimed for the bacterial systems (5). By analogy with the coliform system, we will term a cyanocin as the product of a gene and a cyan factor as the DNA which codes for the product. There are many different types of bacteriocins, but the present experiment shall be concerned with cyanocins which sit at a receptor site and effect a killing of the cell. This cyanocin might properly be regarded as the part of a virus which recognizes a receptor site and possesses the ability to "take command" over host synthesis. Since the cyanocin is a protein, its effectiveness can be destroyed by the proteolytic enzyme, trypsin (13). Trypsin-treatment of a cyanocin-infected cell, within a limited time after its attachment to the

receptor site, can reverse the killing effect.

Cyanocins first appeared on plates as an indistinct plaque often produced within two hours after spotting algal lawns with chloroformed algal suspensions. Since the usual time for viral plaques to appear is 18 hours or more, this was not a normal virus action; no multiplication of virus occurred and the lytic activity could not be transferred. However, since superinfection by other chloroform-treated cells often produced single plaques, it appeared to be a virus-related event.

Since the descriptions of the phenomena corresponded to bacteriocin-like activity, a series of chloroform-treated cultures were examined. A series of concentrated cultures (prepared by pouring off most of the supernatant medium from a mature culture) of about 10 ml were thoroughly mixed with 1 ml of chloroform. After standing two hours or more, or overnight, the supernatant was removed and part of it was spotted on a range of algal lawns. Generally the upper twelve sections of a 24-sectioned lawn were spotted with the untreated supernatant while a mirror image on the lower half was spotted with the same material treated with trypsin at a concentration of from 300-500 microg./ml. The results are shown in Table 8.

It is seen that the chloroform-treated samples have various kinds of lytic activity, some of which corresponds to that defined for bacteriocins.

It is also apparent from these results that it is probably very important to investigate further the virus characteristics of the supernatant medium of the cultures and their chloroformed extracts.

Host Modification and Restriction

A "restricted" count appears often on hosts which have been lysogenized or are suspected of a lysogeny. While this restricted count is noted by

Table 8

"CYANOCINS": BLUE-GREEN ALGAL BACTERIOCINS

CHLOROFORM-TREATED CELLS	ALGAL LAWN											
	TWS(1818)	HOS	CAT	VAL	NEM(716)	FAV	RCAT	FAV(1818)	CUF	RMAN	CRRY	CRY
TWS s1	-	-	-	-	o	d	-	d	o	o	-	cy
TWS h	-	-	-	-	-	-	-	d	+	cy	cy	d
FCY(15F)	-	-	-	-	-	cy	d	d	+	cy	cy	d
NEM(S3) b	-	-	-	-	-	d	-	d	o	cy	d	d
NEM(S3)	-	-	-	-	-	-	-	d	+	cy	d	d
RAM	-	-	-	-	o	d	d	d	+	d	cy	d
FAV	-	-	-	-	-	cy	cy	-	-	d	d	cy
RMAN	-	-	-	-	o	cy	cy	d	-	cy	cy	cy
TAF	-	-	-	-	-	cy	cy	-	-	cy	cy	cy
VAR	-	-	-	o	o	-	cy	-	o	cy	cy	cy
BRY	-	-	-	o	o	-	-	cy	-	-	-	-
TOL	-	-	-	-	o	-	dy	d	-	d	d	cy
ASP	-	-	-	-	o	cy	cy	-	o	-	cy	cy
UNI	-	-	-	-	o	cy	cy	cy	o	-	cy	cy
FLO	-	-	-	o	o	-	cy	cy	-	cy	cy	-
CAR	-	-	-	o	o	-	cy	d	-	cy	cy	-
JAM	-	-	-	o	o	-	d	d	o	-	d	cy
ELL	-	-	-	-	+	-	d	d	o	d	cy	d

Trypsin sensitive:

cy: cyanocin; "killing" plaque destroyed by trypsin
\+ : defective to lytic plaque
o : defective to turbid plaque

Trypsin insensitive:

\- : lytic or resistant plaque
d : defective: "killing" plaque

s-plaquing and can be backed up by actual plate counts, we have not studied it in any further detail.

CONCLUSIONS

The host-range, plaque-morphology studies have brought about a practical organization of interrelationships among blue-green algal viruses and their hosts.

Definition of Viruses

In addition to the S3 virus, which is already well defined, we can identify another virus, which we term S5, as having a host range which lyses all commonly-used hosts, including those resistant to the LPP-1 and LPP-2 viruses. We find by serology that such a virus may be similar to virus 15, which is possibly lysogenized in two hosts, NEM(15) and FCY(15). If the S3 and S5 viruses are suitable pure, it will then be possible to superinfect single hosts with two viruses and study recombinations leading to genetic mapping of the viral chromosome.

Host-Range, Plaque-Type Markers

The long range of hosts which have been assembled for these studies now can be used for coordinating interrelationships of viruses for species other than S. calcicola. We have demonstrated that viruses from S. calcicola and other species are capable of cross infecting. (Though not mentioned in these results, virus SM-1 can be picked up on the host range, being able to multiply in a single S. calcicola strain).

Ringed Plaque Morphology, Lysogeny and Transduction

It has been demonstrated that ringed plaques have stable, definite characteristics and that establishment of rings is somehow connected with

the ability of the S3 virus to lysogenize and to transduce this ability into many hosts.

Bacterial-Algal Virus Transfer

The repeated occurrence of a bacterial-associated virulence in raw sewage samples raises the important question as to whether enteric and pathogenic viruses can be adapted to multiply in other hosts and thus be transferred to other future hosts of a species different from the original host species. The high increase of algal virus in sewage effluent and the uncertainty which exists concerning viral transmission in renovated water makes this problem one which should have further detailed attention.

Utility of the Host-Range Data

The host-range data have been used as a genetic marker to define differences in virus properties as altered by radiation dose. The host-range data are useful in setting up future experiments to determine which hosts and viruses are suitable for measuring certain effects.

REFERENCES

1. Allen, M. M. (1968). Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4: 1-4.

2. Brown, R. J., Jr. (1972). Algal viruses. Advan. Virus Res. 17: 243-277.

3. Campbell, A. M. (1968). Techniques for studying defective bacteriophages. In Maramorosch, K. and Koprowski, H., (eds.) Methods in Virology V, Academic Press, N. Y.

4. Gold, K., and U. Pollingher (1971). Occurrence of endosymbiotic bacteria in marine dinoflagellates. J. Phycol. 7: 264-265.

5. Hayes, Wm. (1968). The Genetics of Bacteria and Their Viruses. John Wiley and Sons, N. Y.

6. Hershey, A. D., (ed.) (1971). The Bacteriophage Lambda. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

7. Kraus, M. P. (1966). Preparation of pure blue-green algae. Nature 211; 310.

8. _____ (1970). The distribution and molecular ecology of the blue-green algal viruses. J. Physol. 6 (suppl):5.

9. _____ (1971). The role of molecular ecology in the management of waste stabilization lagoons. Proc. 4th Mid-Atlantic Ind. Waste Conf. Dept. Civ. Eng., Univ. of Del., Newark, Del. Nov. 18-20, 1970.

10. _____ (1972). Interaction of a blue-green algal host/virus system with ionizing radiation. Document COO-3007-23, U. S. Atomic Energy Commission.

11. _____ (1972). Blue-green algal genetics: a new methodology. Document COO-3007-24, U. S. Atomic Energy Commission.

12. _____ (1974). Host Range Studies of Blue-Green Algal Viruses in a Polishing Pond. (submitted).

13. Ozeki, H. (1968). Methods for the study of colicins and colicinogeny. In Maramorosch, K. and Koprowski, H., (eds.) Methods in Virology IV, Academic Press. N. Y.

14. Padan, E., and M. Shilo (1973). Cyanophages: viruses attacking blue-green algae. Bacteriol. Reviews 37 (in press).

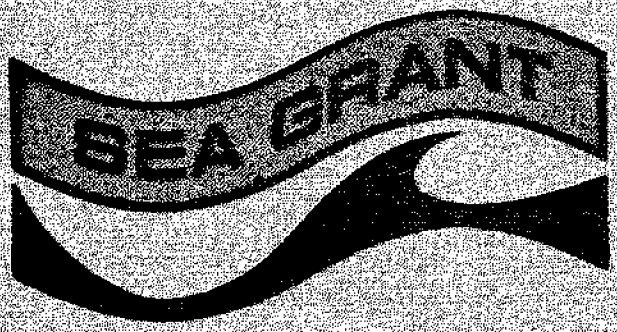
15. Patrick, R. (1967). Red Clay Survey. Limnology Dept., Academy of Natural Sciences, Philadelphia, Pa. 19103.

16. Safferman, R. S. and M. E. Morris (1964). Growth characteristics of the blue-green algal virus, LPP-1. J. Bact. 88: 771-775.

17. Safferman, R. S. (1973). Phycoviruses. In The Biology of the Blue-Green Algae, N. G. Carr and B. A. Whitton, (eds.) Blackwell Sci. Pub., Oxford, England.

18. Safferman, R. S., and Morris, M. E. (1972). Personal communication.

19. Starr, R. C. (1971). The culture collection of algae at Indiana University -- additions to the collection July 1966 -- July 1971. J. Phycol. 7: 350-362.



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