I. IRRADIATION EXPERIMENTS WITH *NEUROSPORA CRASSA*

II. AN ELECTROPHORETIC COMPARISON OF THE SOLUBLE PROTEINS OF NORMAL AND VIRUS-INFECTED *ESCHERICHIA COLI*

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Part II

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ABSTRACT

Part I

1. A case is described and discussed in which a mutant strain of Neurospora which spontaneously reverts to wild type cannot be induced to revert by mutagenic agents.

2. The action of ultraviolet light on macroconidia of Neurospora is described.

3. The effect of heat and light post-treatment of ultraviolet irradiated macroconidia has been studied.

4. Data have been obtained which suggest that the killing effect of UV is more easily reversed by heat or light post-treatment than the mutagenic effect.

Part II

1. The soluble proteins of *Escherichia coli* were extracted by disrupting the bacteria in a colloid mill and centrifuging all particulate matter out of solution.

2. The soluble proteins so obtained, were resolved by electrophoresis.

3. A comparison was made of the electrophoretic scanning patterns of the soluble proteins of non-infected bacteria, bacteria infected with bacteriophage T2 during the "eclipse" stage of infection, and bacteria infected for longer than the "eclipse" with the following findings:

  a) The amount of free-moving desoxyribonucleic acid (DNA) present in the extracts of uninfected bacteria decreases during the "eclipse" stage of infection.

b) The amount of free-moving DNA increases strikingly after the end of the "eclipse."

4. Bacteriophage particles were disrupted in the colloid mill. The increase in free moving DNA in the extracts of bacteria infected for longer than the "eclipse" was found to be due to disruption of intracellular bacteriophage particles.

5. DNA was extracted both from bacteriophage particles and from <u>Escherichia coli</u>. A comparison of the two DNAs by electrophoretical and ultracentrifugal methods reveals a close similarity between them.

6. The experimental findings are discussed and several suggestions are made for further elucidating the course of virus multiplication.

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I. IRRADIATION EXPERIMENTS WITH *NEUROSPORA CRASSA*

I. INTRODUCTION

During the past 20 years the fungus *Neurospora crassa* has proved itself as a valuable experimental tool in the fields of biochemistry and genetics. Probably because Neurospora proved to be such an excellent organism for the study of the genetical control of biochemical reactions, the main emphasis of Neurospora research in the past ten years has centered about the problem of the interrelationship between genes and enzymes. However it has become increasingly clear that Neurospora is also well suited to the study of a host of other problems of interest to geneticists. For instance, there is the early work of the Lindegrens (1932-1939) and the more recent work of Whitehouse (1942) on the mechanisms of crossing over. McClintock (1945) and Singleton (1948) have uncovered many facts of interest relating to chromosomal mechanisms. Beadle and Coonradt (1944) and more recently Atwood (1950b) have studied problems of dominance and heterosis by the use of heterocaryons. Finally Hollaender et al. (1945), Emerson (1944), Giles et al. (1945), Dickey (1949), Atwood (1950), Kolmark and Westergaard (1949), and Goodgal (1950) have used Neurospora conidia in studies of the action of various mutagenic agents. In the present series of studies Neurospora has again been chosen to study non-biochemical genetics.

Two essentially unrelated problems have been considered which presented themselves in the course of developing techniques for the study of various irradiation effects on Neurospora conidia. They are:

1. A study of the differential effects of various mutagenic agents in inducing reversions in two phenotypically distinguishable mutant alleles of a gene.
2. The differential reversal of killing and mutagenesis by post-treatment of ultra-violet irradiated conidia.

Since unrelated problems are to be discussed, no general literature review has been included. Instead, pertinent literature is reviewed and discussed when it is warranted by the course of the discussion.

II. MATERIALS AND METHODS

A. Strains

Two strains of *Neurospora crassa*, each carrying a mutant allele of a gene for inositol independence, were used in these experiments. The original strains as selected from the Neurospora stocks of the California Institute of Technology were 37401a and 83201a. The mutant 37401 requires inositol for growth under all conditions so far investigated, and shows a colonial type of growth on low concentrations of inositol. It was originally derived by Beadle and Tatum (1945) from an

ascospore resulting from a cross between ultra-violet treated conidia of strain 25a and strain 1A. This strain has been used in investigations of the number of nuclei contributed by each parent to a cross in a single perithecium by Grant (1945). It has been proven suitable for microbiological assay of inositol by Beadle (1944), and it has been used by Giles and Lederberg (1948) in reversion experiments similar to those to be described here.

Strain 83201 requires inositol for growth above 28°. At 25° it will grow without inositol but added inositol will increase the amount of growth obtained. The strain was originally isolated by Beadle (1950) from an ascospore resulting from a cross between x-rayed (100,000r) conidia of strain Abbot 4A and strain 25a. Strain 83201 has been observed to revert spontaneously to wild type in the stocks. Beadle (1950) has noted that this strain will revert in the presence of a marker gene, thus ruling out contamination as an explanation for the observed reversion.

The strains actually used in the experiments were derived from the above described strains after a series of crosses designed to place both alleles into the same genetic background and to accumulate data bearing on the allelism of the two strains. The strains used were labeled 37401-212-1 and 83201-212-7. The pedigree of these strains is given in Figure 1. Strain 37401-212-1 is phenotypically

like the original 37401. It still requires inositol for growth at both high and low temperatures and it grows colonially at low concentrations of inositol. Strain 83201-212-7 is much like the original parent strain except that it has a semi-colonial growth habit. During the course of deriving the strains a colonial mutant appeared spontaneously in some of the segregants of one of the crosses. This colonial mutation was incorporated into 83201 for use in later work.

B. Media

Liquid and solid minimal and complete media used in these experiments are those described by Beadle and Tatum (1945). All crosses were performed on Westergaard's minimal medium (Westergaard and Mitchell, 1947) supplemented with small amounts (0.3 micrograms per ml.) of inositol. In some of the experiments a sorbose medium was used which is essentially the same as that of Tatum et al. (1949). This medium has the same salt composition as minimal medium but the carbon source, sucrose, is substituted by 0.5% sorbose and 0.1% sucrose. On this medium Neurospora adopts a colonial instead of the usual spreading growth habit.

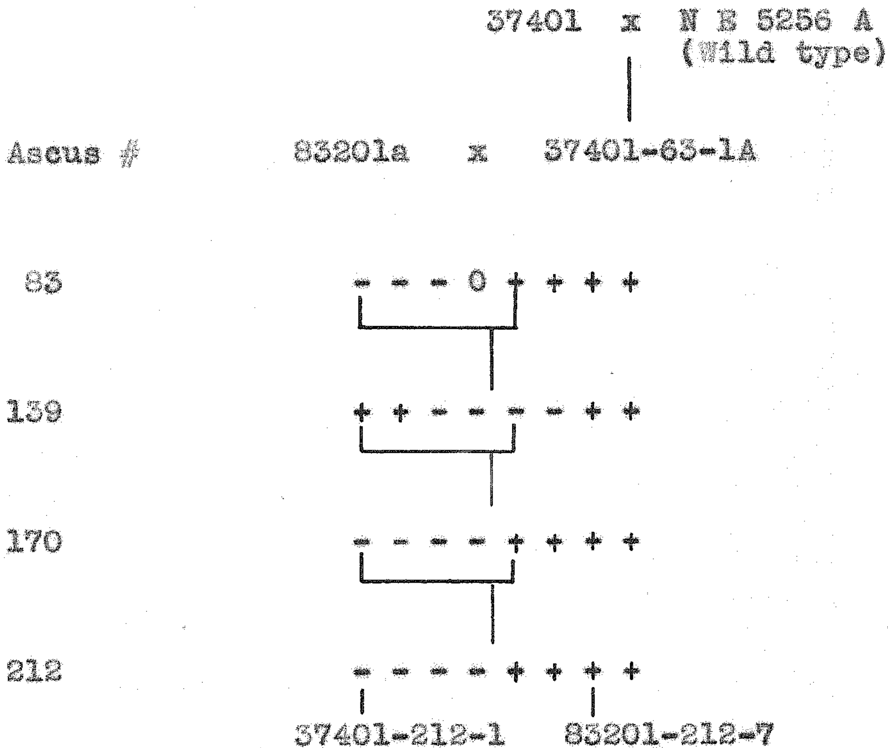
C. Method Used for Collecting Conidia for Experiments

Cultures were grown on complete agar medium either on slants in large test tubes or in Kolle flasks. Conidia, harvested from 5-7 day old cultures, were dispersed in

Figure 1.

Pedigree of Strains Used in the

Irradiation Experiments



+ = Growth at 25°C. on minimal medium

- = No growth at 25°C. on minimal medium

0 = Ascospore did not germinate

None of the cultures grew at 35°C. on minimal medium.

sterile distilled water and were filtered through absorbent cotton. Estimates of the conidial concentrations were made by direct count in a Spencer bright-line hemocytometer. Determinations of viable conidial concentrations were obtained by plating a proper dilution of the conidial suspension on minimal agar plates supplemented with 0.30 γ inositol per ml. (Giles and Lederberg, 1948). The values obtained by the dilution plating technique were consistently lower than those obtained by the hemocytometer count. The proportion of conidia counted in the hemocytometer which gave rise to colonies on plating varied from one batch of conidia to the next. A discussion of the possible causes for such a phenomenon is given by Ryan (1948). In some of the experiments the dilution platings were carried out on sorbose minimal medium in which case still a different value was obtained for viable conidia.

D. Irradiation Techniques

Conidia dispersed in distilled water were irradiated in 60 x 15 mm. petri dishes using an uncalibrated G.E. Sterile-lamp, Model UV -30, at a standard distance of 7.5 cm. A thin (approximately 1 mm.) layer of conidial suspension was agitated during the course of the irradiation to insure even distribution of the irradiation to all of the conidia in the suspension.

Visible light was supplied by a bank of four 40 watt flourescent tubes at a distance of 35 cm. Two of the

tubes were daylight and two were white.

E. Methods of Detecting Reversions

Two methods were used to determine the number of reversions present in the treated and untreated conidial suspensions. The first method consisted of diluting the treated suspension into an appropriate volume of liquid minimal medium and distributing aliquots of the diluted conidial suspension to a large number of small test tubes. A test tube with at least one reversion in it shows growth. The numbers of reversions in each tube follows the Poisson distribution. The average number of reversions in each tube is given by the following equation:

$m = -\log p$, where:

$m =$ average number of reversions per tube

$p =$ the proportion of tubes which do not show growth.

The standard error of m is given by Fisher (1937):

$$S.E._{(m)} = \sqrt{\frac{e^{m} - 1}{n}}$$

$n =$ total number of tubes to which aliquots were distributed.

The standard error is reduced to 10% when $p = .1$ to 0.33 and $n = 150$ to 200.

The second method used was that of plating large amounts of conidia on minimal sorbose plates. The reversions form easily countable colonies on this medium. Difficulty is encountered with this technique when very large numbers of conidia are plated due to a roughening of the plates

ascribed to a small amount of growth by the non-reversions under these conditions.

Details of variations in procedure will be given further in the text.

III. EXPERIMENTAL

A. Demonstration of Allelism of Strains 83201 and 37401.

During the course of studying various Neurospora mutants requiring the same substance for growth, many cases have been found in which such strains proved not to be allelic. In general such non-allelic strains are blocked at different points in the synthetic pathway of the substance required for growth. Other cases have been found, however, in which mutants requiring the same substance for growth are both allelic and blocked at the same point in the synthetic pathway of the substance. Two tests are available to demonstrate non-allelism of strains although neither test can prove critically that strains are allelic. However, when strains fail to show non-allelism in both tests a convincing argument can be set forth for allelism. The heterocaryotic test involves the assumption that two strains blocked at different points in the synthetic pathway of a substance essential for growth will show growth when inoculated simultaneously into minimal medium. Nuclei of each strain in this case are able to supply the intermediate that the other nuclei are unable to synthesize. However as Beadle and Coonradt (1944) have pointed out, one of the

wild type alleles may be present in too low a concentration to permit growth of the heterocaryon or other unforseen difficulties may occur which makes this test non-critical.

The other test involves the demonstration that no wild type segregants appear subsequent to a cross between the strains. It is difficult by this test, however, to prove non-allelism if the genes in question are very closely linked.

Strains 83201 and 37401 fail to show growth at 35° C. when inoculated together into minimal liquid medium and thus can be considered allelic barring the difficulties previously mentioned.

Ninety-five asci were dissected in order by the author from crosses of the two mutant strains. All of these asci had four ascospores requiring inositol for growth at both 25° and 35° and four ascospores requiring inositol only at 35°. Twenty-nine other such cases are given by Houlahan et al. (1949) making a total of 124 asci which failed to show any wild type spores. Thus, again the strains can be considered allelic barring a very close linkage.

The weight of the evidence favors the notion that the strains are allelic and they will be so considered during the course of this thesis.

B. Distance of the Gene from the Centromere.

New data bearing on the distance of the gene from the centromere are combined with those of other workers

into Table 1. According to Houlahan et al. (1949) the gene for inositol independence is located on linkage group E of Neurospora.

C. Survival of Conidia when Treated with UV.

Three main conclusions were reached early in the experimental work regarding the ultra-violet sensitivity of Neurospora macroconidia. In the first place it became quite evident that different harvests of conidia differed markedly in sensitivity to killing by UV. It was also noted that in general the more concentrated the irradiated suspension, the more resistant the conidia were to the killing action of the radiations. In all cases, however, a survival curve was obtained which could best be described as a multiple hit type of killing curve. The phenomenon of the concentration dependence of sensitivity to UV is demonstrated in Figure 2 which shows the results of an experiment in which several dilutions of one harvest of conidia were irradiated.

Since variable results were obtained from run to run as regards survival, each experiment was designed to be self contained. No attempt was made to reproduce exact results obtained in one experiment in other experiments; rather it was deemed more satisfactory to repeatedly demonstrate a phenomenon qualitatively.

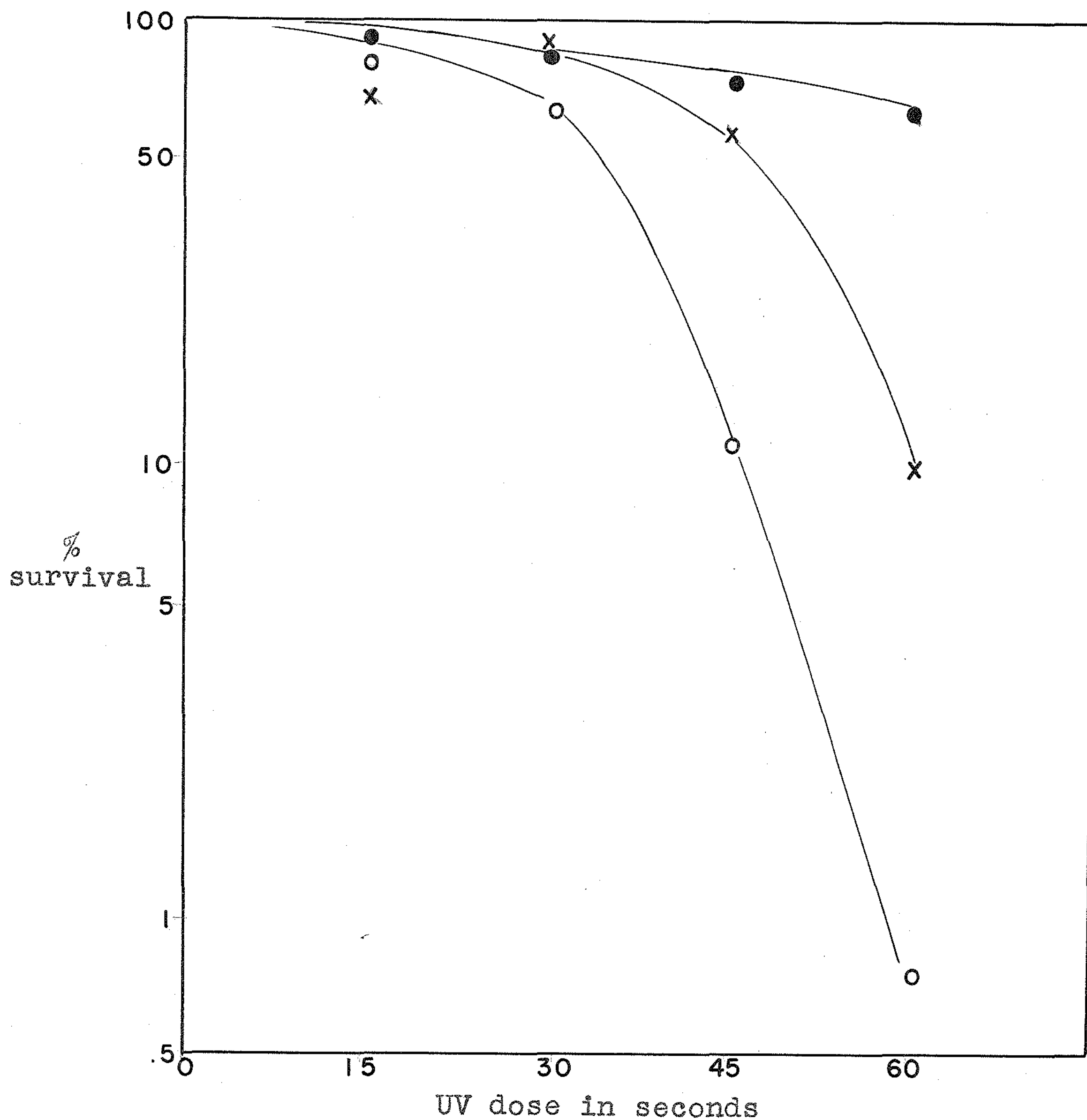
D. Spontaneous Reversion—Strain 37401-212-1

A total of over 100 million conidia were tested for spontaneous reversion. Out of this number only 16 conidia

Figure 2

Concentration Dependence of Sensitivity to UV

● initial concentration, 3 x 10^6 conidia per cc.
x initial concentration, 5 x 10^5 conidia per cc.
o initial concentration, 5.2 x 10^4 conidia per cc.



Survival curves of dilutions of one harvest of conidia.

Table I

Cross	First division segregation asci	Second division segregation asci	
37401 x 83201	33	62	
37401 x Abbot 4	22	21	Houlahan et al.(1949)
37401 x fluffy	8	12	" "
37401 x 83201	10	10	" "
37401 x peach	12	8	" "
	85	113	

% second division segregation = 57%

showed inositol independence. The distribution of reversions appearing among the various experiments is listed in Table II.

Whether the 16 reversions noted are actually reversions or merely contaminants awaits further verification. If the 16 cases of inositol independence are actually reversions, then the type of data obtained, that is a variation in the number of reversions noted from experiment to experiment, is compatible with the idea that spontaneous mutation is taking place, according to the analysis of Luria and Delbrück (1943).

Caution must be employed in analyzing results obtained in this system by the method of Luria and Delbrück for this method assumes that a reverted nucleus will propagate itself at the same rate as that of the inositol requiring nucleus. No data on this point are available, but the results of Ryan and Lederberg (1946) lead one to believe that it is unlikely that the two nuclei would reproduce at the same rate.

It seems quite plausible to assume that strain 37401-212-1 does have a low spontaneous reversion rate since Giles and Lederberg (1948) came to the same conclusion using a different method for detecting reversions.

2. Spontaneous Reversion—Strain 83201-212-7

The data relating to spontaneous reversion in strain 83201-212-7 are shown in Table III and are subject to the same limitations as apply to the data from strain 37401-212-1.

Table II

Spontaneous Reversion of Strain 37401-212-1

Exp. #	No. of Conidia Tested x 10^6	No. of Reversions
8	20	4
9	9	0
12	18	1
14	8	0
17	9	1
24	19	8
31	12.5	0
39	14	2
41	3	0
	112.5	16

Strain 83201 as previously discussed has been known to revert in the stocks of the Neurospora kept at the California Institute of Technology. Unpublished data of Beadle indicate that the apparent reversion is not due to contamination since an albino inositol requiring strain gives rise to albino inositol independent mutants. From the data obtained it seems likely that 83201-212-7 has a higher spontaneous reversion rate than does 37401-212-1.

F. Reversions Induced by Ultra-Violet Light—Strain 37401-212-1.

The data summarized in Table IV clearly demonstrate that reversions are induced in strain 37401-212-1 by UV. The data also demonstrate that the reversion frequency is increased with increasing dose of UV. Unfortunately the effect of higher doses of UV on reversion frequency was not studied. These results agree with those obtained by Giles and Lederberg (1948) using another technique for detecting reversions induced by UV in strain 37401.

G. Reversions induced by Ultra-Violet Light—Strain 83201-212-7.

Although conclusive evidence is available that strain 83201 will revert spontaneously, it does not appear to do so under the influence of UV. Table V summarizes the results bearing on this point obtained from four separate experiments. Experiments 11 and 13 were performed with conidia which had an easily measurable number of reversions

Table III

Spontaneous Reversion of Strain 83201-212-7

Exp. #	Conidia Tested x 10^6	No. of Reversions
8	12	1
9	19.5	1
11	23	39
13	12.5	64
16	34	1
18	19.5	0
24	7	1
38	2.6	0
	133.5	107

Table IV

Induction of Reversions in Strain 37401-212-7 by UV

Exp. #	Dose of Irradiation in seconds	Reversions per 10^6 surviving conidia
Pooled data	0	See Table II
9	10	2.6 ± .59
8	15	4.2 ± .75
12	15	7.0 ± 1.4
9	20	6.7 ± 1.2
12	30	14.2 ± 3.3
9	30	16.3 ± 3.5
14	30	11.0 ± 1.5
17	30	9.7 ± 1
12	45	13.9 ± 4.9

Table V

Demonstration of the lack of UV Induced Reversions in Strain 83201-212-7.

Exp. #	Dose of Irradiation in Seconds	Reversions per Number of Surviving Conidia
8	0	1 in 12 million
8	15	5 in 4 million
9	0	1 in 19.5 million
9	10	3 in 19 million
9	20	2 in 17 million
9	30	6 in 16.5 million
		Reversions per 10^6 Surviving Conidia
11	0	1.72 ± .36
11	20	1.90 ± .38
11	40	1.32 ± .36
13	0	4.46 ± .88
13	20	4.46 ± .88
13	40	4.08 ± .86
13	60	3.30 ± .65

before treatment. Ultra-violet light did not raise the number of reversions above that originally present.

H. Nature of the Reversions.

No experiments were performed to determine whether the reversions were back-mutants, suppressor type mutants, or due to some other phenomenon. A reversion, therefore, in these experiments refers to a change in a haploid macroconidium from inability to grow on a minimal medium to ability to grow on minimal medium. Giles and Lederberg (1948) tested two reversions of 37401 and found them to be mutations at the inositolless locus leading to the wild type condition. The reversions were indistinguishable from wild type in growth rate at 25° and in growth requirements. In these two cases the evidence seems fairly good that the reversions are actually back-mutations to the wild type condition.

I. Effect of other Mutagenic Agents on the Two Strains.

a) x-rays. No reversions were induced in strain 83201-212-7 in two experiments in which 20,000 r and 15,000 r were administered to 19 million and 12 million conidia, respectively. The doses administered caused 42% and 37% mortality. Doses of ultra-violet causing such mortalities in strain 37401-212-1 are sufficient to cause a drastic increase in reversion.

b) Nitrogen mustard. Conidia of both 37401-212-1 and 83201-212-7 were treated for 30 minutes with an 0.08%

Table VI

Reversions Induced by Nitrogen Mustard

In Strains 37401-212-1 and 83201-212-7

Strain	Treatment	Viable Conidia Tested x 10^6	No. of Reversions	Reversions per 10^6 Surviving Conidia
37401-212-1	Control	8.6	8	0.93
37401-212-1	N. Mustard	3.0	36	12.
83201-212-7	Control	7.2	1	-
83201-212-7	N. Mustard	2.8	1	-

solution of nitrogen mustard. The results are summarized in Table VI.

Although the data are meager it seems clear that as in the case with ultra-violet light, nitrogen mustard will induce reversions in strain 37401-212-1, but will not do so in strain 83201-212-7.

J. Discussion of Results.

Giles (1948) has studied a series of phenotypically indistinguishable allelic inositol requiring mutants and has found that some of them differ in their ability to revert under the influence of mutagenic agents. In particular he has compared strains 37401 and 46802. The results indicate that a situation exists similar to that reported here. Giles found that strain 46802 failed to revert with any treatment attempted. The same of course is true of strain 83201-212-7 as reported here. Strain 37401 and the derived strain 37401-212-1 yield the same type of results both in Giles' experiments and those presented in this thesis. Several differences exist between strains 46802 and 83201-212-7. Strain 46802 was derived from ultra-violet irradiated material while 83201-212-7 as previously mentioned was derived from x-rayed conidia. Strain 46802 is phenotypically indistinguishable from 37401 except for its reaction to mutagenic agents while 83201 is phenotypically distinguishable from 37401. Finally no data are available regarding the ability of strain 46802 to revert

spontaneously while strain 83201 has been shown to revert spontaneously.

The point of interest is that strain 83201-212-7 has been shown to revert spontaneously while it is incapable of being induced to revert by any of the common mutagenic agents. If the spontaneous reversions are in fact true mutations then we are presented with a case in which mutagenic agents are unable to induce the same type of mutation that occurs spontaneously. The experimental data are, however, subject to several other speculative interpretations.

It is possible that the UV induced reversions of strain 37401 do not actually represent back-mutations, but are suppressor type mutations at another locus. The results obtained could be explained by the assumption that UV could induce the suppressor mutation in both strains 37401 and 83201, but that the induced mutation was only capable of suppressing the allele of the inositol locus represented in strain 37401. That a suppressor mutation can affect alleles differentially has been demonstrated by Lewis (1950) in Drosophila. This interpretation, however, is contrary to the finding of Giles and Lederberg (1948) that in two cases studied, the UV induced reversions are in fact back-mutations. The suggestion is made that a wider study of the nature of the induced reversions is in order before the suppressor hypothesis can be ruled out.

Another interpretation which may be applied is that strain 37401 may represent a slight rearrangement of the

chromosomal material which is unstable towards mutagenic agents close to or involving the inositol locus. This notion implies that the biochemical function of the gene is not impaired because of actual damage to the gene, but rather by the change in the relative position of the gene. A reversion in this case would represent not the restoration of function to a "damaged" gene, but rather the reversal of a position effect.

K. Effect of Temperature on Ultra-Violet Light Action.

An attempt was made to see whether or not the complete inositol dependent allele would mutate to the temperature inositol-dependent allele. To this end, conidia of the strain 37401-212-1 were irradiated with ultra-violet light for 30 seconds and the reversion frequency was determined after incubation at both 25° and 35°. If an appreciable amount of temperature dependent mutants had been induced, they could now be detected by the fact that more reversions would be observed among the conidia incubated at 25°. However, when the experiment was performed and repeated, a significantly higher number of reversions was detected among the conidia incubated at 35°. Table VII summarizes the findings obtained in two such experiments.

The results suggest that perhaps temperature mutants were induced which required inositol for growth at 25° but which could grow on minimal at 35°, the reverse of the situation that exists in strain 83201.

Table VII

The Differential Appearance of Reversions after UV Treatment and Incubation at 25° and 35°

Exp.#	Treatment	Conidia per cc.	Vol dist to each tube	Temp of incubation	No. of tubes	No. of tubes Showing growth	Average no. of reversions per tube	Reversions per 10^6 Conidia	S.E.
14	Control	8.5×10^4	1cc	25°	50	0		0	
				35°	46	0		0	
	Irr. for 30 seconds	8.9×10^4	1cc	25°	99	44	.59	6.6 ±	1.0
				35°	93	57	.95	11.0 ±	1.5
						P of obtaining deviation by chance is 1/60			
17	Control	1.8×10^5	1cc	35°	50	1			
	Irr. for 30 seconds	1.8×10^5	1cc	25°	150	100	1.11	6.2 ±	.65
				35°	150	124	1.75	9.7 ±	1.0
						P of obtaining deviation by chance is 1/300			

The opportunity for testing this hypothesis was immediately at hand since merely by transferring cultures from all of the tubes which showed growth at 35° to fresh minimal medium and incubating at 25°, any of the cultures which contained only the reverse temperature mutants could be detected by lack of growth at 25°. The number of tubes which should show no growth can be predicted by the following considerations of the data obtained in experiment # 17:

The average number of reversions in each of 150 tubes incubated at 25° is 1.11. The average number of reversions in each of the tubes incubated at 35° is 1.75. We will assume that the tubes incubated at 35° have an average of 1.11 reversions per tube and an average of 1.75 - 1.11 or .64 reverse temperature mutants per tube. From the formula $p = e^{-m}$ we can calculate that 0.33 of the tubes should have no reversions and 0.47 of the tubes should have one or more reverse temperature mutants. The probability will, therefore, be that 0.33 x 0.47 or .16 of the tubes will contain only reverse temperature mutants. If all of the 124 tubes showing growth at 35° in experiment 17 were to be transferred to fresh minimal medium at 25° the probability would be greatest that 24 of the tubes should show no growth.

When the experiment is actually performed, however, all of the cultures obtained at 35° showed growth when transferred to fresh minimal medium and incubated at 25°. The discrepancy in the number of mutants obtained at 25° and at 35° must therefore be sought elsewhere. As a corollary to the above experiment and as a further check on whether the complete inositol dependent allele can be induced to revert

to the temperature inositol dependent allele, the 100 cultures obtained at 25° in experiment # 17 were also transferred to fresh minimal medium and incubated at 35°. Again all of the cultures showed growth at the alternate temperature.

The possibility that the discrepancy in the number of reversions observed at the two different temperatures might be due to the fact that more conidia germinate at 35° than do at 25° was examined. Accumulated data indicated that there was no such difference in untreated conidia and furthermore the 30 seconds of irradiation applied caused neither mortality of the conidia nor differential viability at the two temperatures.

Since it is believed that the observed phenomenon might be similar to that observed when irradiated conidia are treated with visible light further discussion of possible explanations for the temperature effect on reversion frequency will be deferred until the next section.

L. Effect of Light and Temperature on the Survival of Ultra-Violet Irradiated Conidia

For some time various investigators have noted that certain post-treatments of ultra-violet irradiated material would reverse to a lesser or greater extent some of the lethal effects of the radiation. Hollander and Emmons in 1940 noted that a variety of chemical reagents would reactivate ultra-violet killed conidia of the mold *Tricho-*

<u>phyton mentagraphytes.</u> More recently Kelner in 1949 discovered that visible light would reactivate conidia of an actinomycete. This discovery was quickly confirmed and extended by Dulbecco (1949,1950), working with bacteriophage and by Novick and Szilard (1949) working with bacteria. The phenomena associated with light post treatment have also been found to apply to the animal kingdom by Blum et al. (1950) and Marshak (1949) who studied light-induced recovery of the rate of cleavage of sea urchin eggs which had been irradiated with UV. Anderson (1949) subsequently discovered that heat would also reverse the effect of ultra-violet irradiation in bacteria. It is probable that still other post treatments will be found that can diminish the effectiveness of UV. In the course of study of visible light reactivation it was discovered that not all of the ultraviolet killing could be reversed. Dulbecco found that in each strain of bacteriophage investigated a certain definite amount of the ultra-violet damage could be reversed, while the rest of the killing apparently could not be reversed.

These recent findings arouse new interest in the mode of action of ultra-violet light. From the point of view of the geneticist, information of this nature is especially valuable since it is well known that ultra-violet light is a mutagenic agent. Several problems of immediate interest to the geneticist are already subject to attack. Atwood (1950) has recently demonstrated that much of the ultra-

violet killing of Neurospora conidia is probably not due to lethal mutation. The problem of whether all types of reactivation of ultra-violet damage are the same has not come under direct attack, but Anderson (1949) has noted that strain B/r of E. coli is subject to photoreactivation but not to heat reactivation, while strain B is sensitive to both post-treatments. Such information suggests that ultra-violet radiation may exert its total effect on living organisms through a variety of mechanisms. Different organisms may be differentially susceptible to the various mechanisms and different conditions of irradiation may favor one or the other of the various mechanisms. Extending the speculation further it is possible that several of the ultra-violet mechanisms may each be subject to reversal by proper post-treatment conditions.

Two major terminal effects of ultra-violet irradiation can be easily recognized and worked with experimentally; killing and mutation. As the data of Atwood (1950a) suggest it is probable that these effects of UV can be caused by different mechanisms in Neurospora conidia. It becomes of interest, therefore, to study the influence of post-treatment on both killing and mutation caused by UV. Novick and Szilard (1949), on the basis of preliminary data, conclude that both killing and mutation are equally reversed by visible light post-treatment in Escherichia coli strain B/r.

Newcombe (1950), in a later report confirms the results of Novick and Szilard (1949) for low doses of ultra-violet, but reports that at higher doses the mutagenic effect of ultra-violet is more stable to the action of visible light then is the killing effect. The mutagenic action of gamma irradiation is also apparently stable to the action of visible light according to Newcombe, although in this case no information is available as to the effect of visible light post-treatment on the killing effect of gamma radiation. It is reported by Goodgal (1950) that the frequency of morphological mutation induced by UV acting on Neurospora microconidia is reduced by the action of visible light to a lesser extent than is the killing effect. It is seen that by a proper study of the various aspects of the reversal of ultra-violet effect, much valuable information can be gained regarding the biological action of ultra-violet radiations.

Data collected in this laboratory confirm the reversal of ultra-violet killing by heat and light. Figures 3,4 and 5 represent the results obtained in three separate experiments in which conidial suspensions of strain 37401-212-1 were irradiated for varying periods of time and immediately diluted and plated. The conditions of incubation of the plates were in the dark at 27° C and 35° C and in the light at 27° C. The percentage survival is plotted against the ultra-violet dose for each condition of incubation. It can be clearly seen from the figures that at doses of UV

Figure 3

Light and Heat Reactivation of UV Treated Conidia—Exp. 38

○ conidia incubated in the light at 27°C.
● conidia incubated in the dark at 35°C.
x conidia incubated in the dark at 27°C.

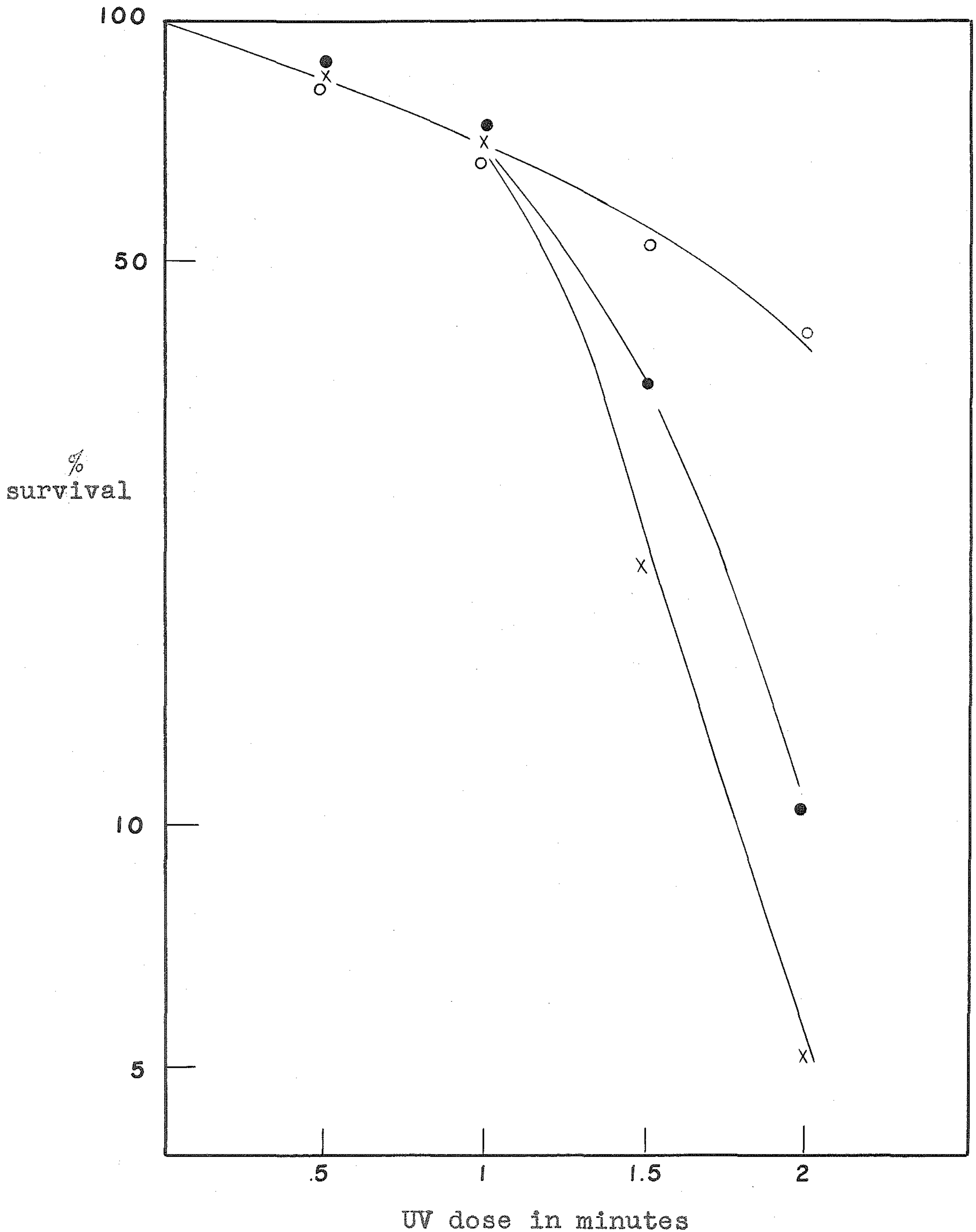
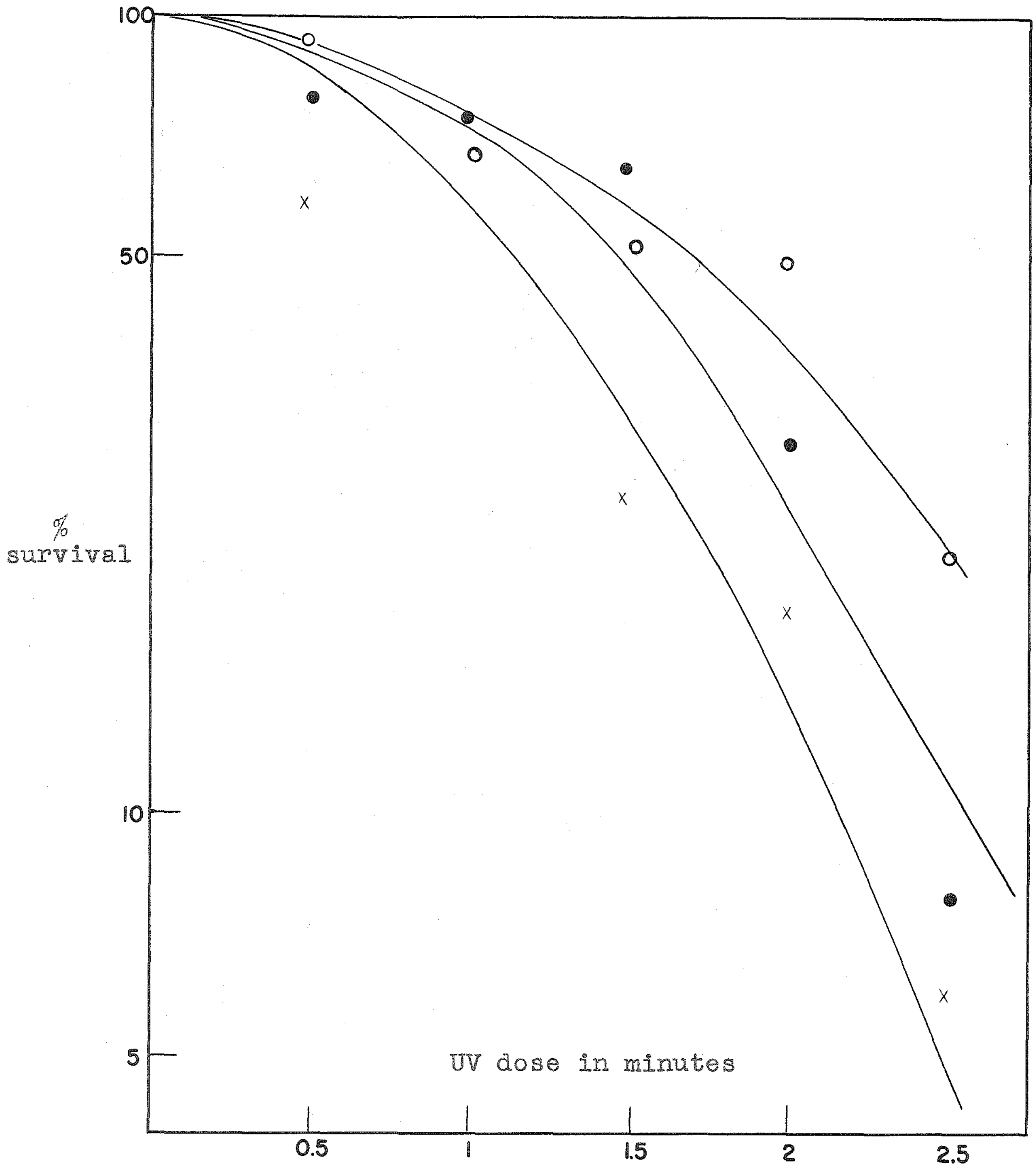


Figure 4

Light and Heat Reactivation of UV Treated Conidia—Exp. 39

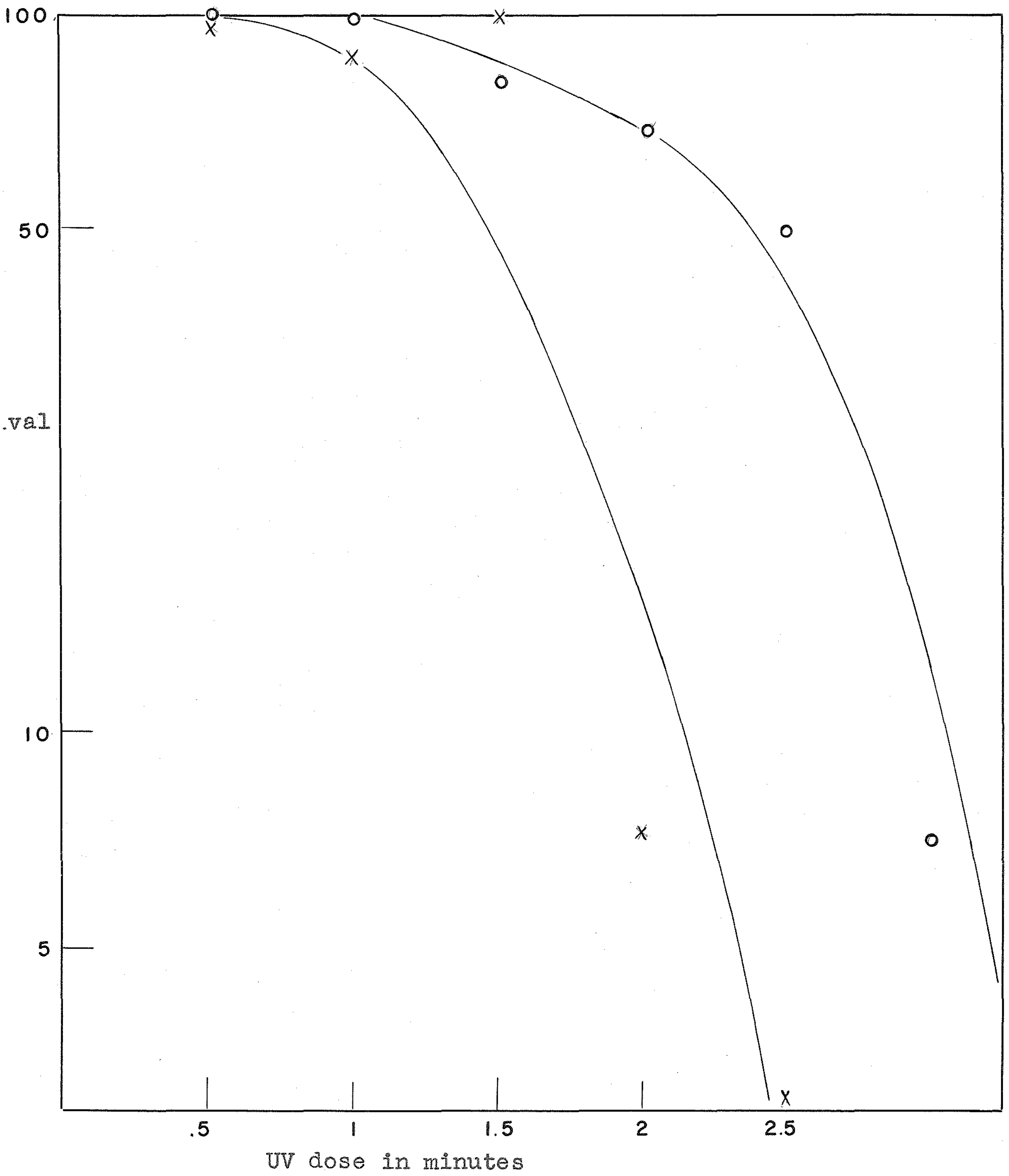


○ conidia incubated in the light at 27°C.
● conidia incubated in the dark at 35°C.
x conidia incubated in the dark at 27°C.

Figure 5

Light Reactivation of UV Treated Conidia—Exp. 41

o conidia incubated in the light at 27°C.
x conidia incubated in the dark at 27°C.



where appreciable killing takes place when the plates are incubated in the dark at 27°, incubating the plates in the dark at 35° causes a partial reversal of killing, while incubating in the light at 27° causes an even greater reactivation of the spores.

The results clearly demonstrate that the phenomena of light and heat reactivation of UV killed material observed by others also applies to Neurospora macroconidia.

Experiments were designed in an effort to determine whether the mutagenic action of ultra-violet was reversed to the same extent as its killing action. The macroconidia of Neurospora are multinucleate with a variable number of nuclei in each conidium. The survival curves of the conidia are clearly of the multiple hit type as demonstrated in an earlier section of this thesis.

If low doses of UV are administered to conidia so that there is little or no killing of conidia when they are germinated in the dark, then when such conidia are germinated in the light or heat there can be little evidence of reactivation. However, if we consider that the low doses of ultra-violet kill nuclei, then these nuclei should be subject to reactivation. On irradiation of a mutant strain such as 37401-212-1 with low doses of UV, the assumption is made that nuclei containing reversions to wild type will be distributed proportionately among the living as well as the killed nuclei. When a post-treatment is now given which

leads to reactivation of nuclei, three types of results can be expected as regards the frequency of reversion.

a) The frequency of reversion will decrease if reversal of mutagenic action is more pronounced than reversal of killing action.

b) The frequency will remain the same if the two actions are mediated by the same mechanism or if they are evenly balanced.

c) The frequency will increase if the reversal of the killing effect is more pronounced than reversal of the mutagenic effect.

Such experiments had already been performed in another context when it was realized that data bearing on the above analysis would be of value. Table VII gives the frequency of reversions found after 30 seconds of irradiation at 25° and at 35°. Clearly more reversions were found at the higher temperature indicating that the reversal of the killing effect is more pronounced than that of the mutagenic effect. Additional experiments were carried out with visible light as the reactivating agent using the sorbose plating technique to detect reversions. The results of three such experiments are listed in Table VIII. Here again the reversion frequency is increased in the presence of visible light indicating that the killing effect of ultraviolet can be reversed to a greater extent than the mutagenic effect.

If the conidia are given high doses of ultra-violet

Table VIII

Visible Light Post-Treatment Effect

on Killing and Reversion of UV Treated Conidia.

Exp. #	UV Dose in Seconds	% Survival		Reversions per 10^6 Surviving Conidia.	
		Dark	Light	Dark	Light
31	0	100	100	0	0
	120	83	100	11.3	41.5
39	0	100	100	0	0
	30	87	92	6	11
	60	58	76	15	15
	90	30	58	11	26
41	0	100	100	0	0
	30	100	100	3	10
	60	86	100	8	15
	90	45	85	22	29

such that the amount of killing both in the dark and in the light falls on the exponential part of the survival curve, then we may again expect three types of results.

a) The reversion frequency will be higher in the light than in the dark if visible light is inducing reversions or if killed reverted conidia are selectively reactivated.

b) The reversion frequency obtained before and after post-treatment will be the same if only the killing effect is reversed but the mutagenic effect remains unmodified by post-treatment.

c) The reversion rate will be reduced after post-treatment if the mutagenic effect is reversed. Careful quantitative experiments are required to determine the extent of mutagenic effect reversal.

This type of experiment is difficult to perform since at high doses the large numbers of non-viable conidia which must be plated with the relatively scarcer viable conidia may interfere with the determinations of the reversion frequencies. However, preliminary results of high dose experiments gave results of type c, from which it may be concluded that all or part of the mutagenic effect can be reversed. The results obtained in a high dose experiment are presented in Table IX. Only one experiment was performed, but the results agree quite well with results reported by Goodgal who performed essentially the same experiment with microconidia of Neurospora. Unfortunately this work was discontinued before the results could be confirmed by other experiments.

M. Discussion

Preliminary data have been accumulated which indicate that when mutant Neurospora conidia are subjected to low doses of UV such that the mortality is very low, the frequency of reversion is increased by subsequent visible light or heat post-treatment. However, at higher doses of UV, where the mortality of the conidia is high, the frequency of reversion is decreased by visible light post-treatment. During the course of presenting the data, the argument was put forth, in a qualitative manner, that these results indicate that post-treatment can differentially reverse the lethal and mutagenic effects of UV. The results, moreover, indicated that the lethal effect of UV is reversed more efficiently than the mutagenic effect.

The phenomenon, if confirmed, can be accounted for by the following theoretical scheme suggested by Dr. R. Dulbecco.

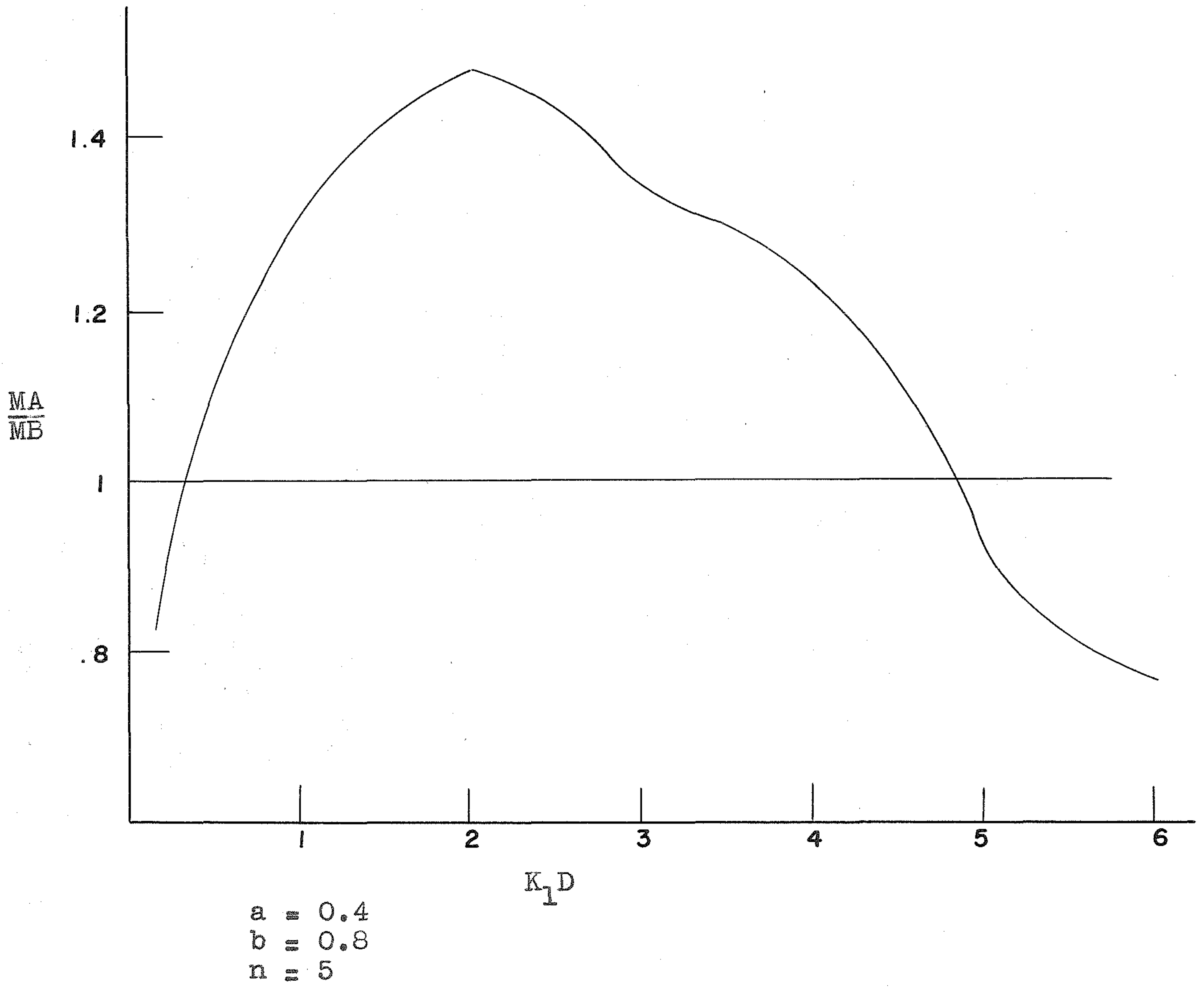
If we make the preliminary assumptions that nuclei are subject to one-hit killing by UV and that non-genetic killing is more easily induced than lethal mutation killing, then the probability that a given nucleus will survive after a given dose D is equal to $e^{-k_1 D}$.

The probability that a given nucleus will be inviable after a given dose D is equal to $1-e^{-k_1 D}$.

If we assume that a macroconidium has n nuclei, then the probability that a given conidium will be inviable after dose D is equal to $(1-e^{-k_1 D})^n$.

Figure 6

Plot of $\frac{MA}{MB}$ vs. K_1D



a = 0.4
b = 0.8
n = 5

Table IX

Visible Light Post-Treatment Effect on Killing and Reversion of Conidia Treated with High Doses of UV.

Exp. #	Dose of UV in Seconds	% Survival		Reversions per 10^6 Surviving Conidia	
		Dark	Light	Dark	Light
41	0	100	100	0	0
	120	14	68	31	14
	150	2.6	42	69	20

The probability that a given conidium will be viable after dose D is equal to $1-(1-e^{-k_1 D})^n$.

The probability that a mutation is induced in a given nucleus after dose D is equal to k_2D.

The probability that a nucleus is mutated and physiologically active is equal to $k_2De^{-k_1 D}$.

The probability that a conidium will contain at least one physiologically active and mutated nucleus is equal to $nk_2De^{-k_1 D}$.

So that without post-treatment:

$$\frac{\text{Detectable Mutations}}{\text{Surviving Conidia}} = \frac{nk_2De^{-k_1 D}}{1-(1-e^{-k_1 D})^n}$$

After post-treatment:

k_1D is reduced to ak_1D where a is less than or equal to 1. This represents the reactivation of the non-genetic killing.

k_2D is reduced to bk_2D where b is also less than or equal to 1. This represents the reversal of the mutagenic effect.

The mutation frequency among the surviving conidia after post-treatment then becomes:

$$\frac{\text{Detectable Mutations}}{\text{Surviving Conidia}} = \frac{bnk_2De^{-ak_1 D}}{1-(1-e^{-ak_1 D})^n}$$

If we now compare the mutation frequency before and after post-treatment:

$$\frac{MA}{MB} = \frac{\text{Mutation frequency after post-treatment}}{\text{Mutation frequency before post-treatment}}$$

$$= \frac{bnk_2De^{-ak_1D}}{nk_2De^{-k_1D}} \quad x \quad \frac{1-(1-e^{-k_1D})^n}{1-(1-e^{-ak_1D})^n}$$

The equation can be reduced as follows:

$$\frac{MA}{MB} = be^{(1-a)K_1D} \quad x \quad \frac{1-(1-e^{-k_1D})n}{1-(1-e^{-ak_1D})n}$$

On figure 6 $\frac{MA}{MB}$ is plotted against k_1D with an arbitrary value for n of 5; a is set equal to 0.4 and b is set equal to 0.8. It is seen that the type of results obtained in the preliminary experiments are predicted by the foregoing theoretical discussion. At low values of k_1D $\frac{MA}{MB}$ is greater than 1, while at higher values of k_1D $\frac{MA}{MB}$ becomes less than 1.

It will be of interest to confirm the preliminary experiments and to extend the work in a quantitative manner to see whether or not the theoretical scheme has any basis in fact. Such a system, if verified, should be useful for separating and studying the various effects of UV on living material.

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II. AN ELECTROPHORETIC COMPARISON OF THE SOLUBLE PROTEINS OF NORMAL AND VIRUS-INFECTED *ESCHERICHIA COLI*.

I. INTRODUCTION

A. General Statement of the Problem

When a bacterium is presented with a phage particle to which it is susceptible, the phage particle will adsorb onto the bacterium and disappear as a free infectious unit. After a specified length of time, depending both on the system and the environment of the system, the bacterium will burst and release many new infectious units. The virus-host relationship just described may be likened to other host-parasite systems in which the parasite attacks and completely destroys the host. Just as in other biological systems there are cases in which the virus may live in relative harmony with its host. The viruses, however, differ from other parasites in several notable respects. Parasites in general can complete at least a part of their life cycle in the absence of the host, either in nature or under special laboratory conditions. Viruses, on the other hand, are characterized by being completely inert in the absence of the living host. Viruses are further characteristically different from other parasites in that they are smaller and exhibit no metabolic activity outside of a living cell.

Parasites in general are thought to have evolved from free-living forms, the adaptation to parasitism being associated with loss of structure and function which would otherwise enable the parasite to live in the absence of its host.

It may be that viruses have arisen in a similar manner, in which case viruses may be thought of as the most parasitic of all parasites, having lost all function except that immediately related to self-replication. The possibility has also been suggested that viruses may be gene complexes which have escaped from the usual cellular environment and have become invasive for other cells. Still another theory is that viruses are very primitive organisms growing in an organic environment which once existed in an extracellular state, but which at the present time is found only in the protoplasm of living organisms.

Regardless of the origin of viruses, it seems clear that in many respects they resemble genes or gene complexes. The viruses do not merely use the host as a nutrient source as do other parasites, but they are actually capable of altering the host metabolism to serve the ends of their own replication. If we consider that an organism's metabolic processes are under the master control of its genetic apparatus, then it becomes reasonable to suppose that the virus is acting as genic material when it alters the host metabolism. Animal and bacterial viruses are composed primarily of desoxyribonucleoportein, the same basic material that is thought of as comprising genic material.

If it is true, then, that viruses resemble genes in so many respects, it becomes of interest to the geneticist to study the course of a virus infection in an effort to

gain insight into the primary action of genes. The opportunity is presented to determine how freshly introduced genic material affects a living cell.

Perhaps the easiest system in which to study the host-virus relationship is the bacterium-bacteriophage system. The equipment required for such a study is considerably less than that required for plant or animal virus investigations. Quantitative assay procedures have been developed and much information has been garnered to date as to the events that occur when a bacteriophage attacks a bacterium. Especially well studied is the system with which the experimental part of this thesis deals, viz., the bacterium *Escherichia coli* strain B and the *T* series of phages which attack it. The phages of the T series have been investigated with refined chemical and physical methods and many characteristics of these viruses have been established. The system of bacterium and phage together, has been under the concentrated attention of several groups of investigators. The pertinent information uncovered will be reviewed in the next section of this thesis.

The course of a phage infection can be divided into essentially three phases. The first phase deals with mechanisms of bacteriophage adsorption onto and entrance into the bacterial cell. During the second phase of the infection the virus loses its extracellular characteristics and cannot be recovered from the cell as an infectious unit. This phase of the infection is called the "eclipse" and

very little is known about it. During the third phase new infectious units appear within the bacterium in increasing numbers until the end of the latent period when the bacterium bursts and releases the new phage. The latent period refers to the period between the onset of the infection and the release of new phage. In the systems investigated, the latent period lasts from 13 minutes to a little over an hour depending upon the system and on the environmental conditions. The first phase of the infection lasts only a short period of time; the second phase lasts approximately for one half of the latent period, and the third phase occupies the second half of the latent period. In certain strains of phage, bacteria can be prevented from bursting for a period of several hours after the end of the normal latent period by a secondary phage infection.

The discussion so far has chiefly been concerned with a definition of the bacterium-bacteriophage system. Much work, however, in elucidating the general course of virus infections has been carried out with plant and animal viruses. Only work having a direct bearing on the problem to be considered in this thesis will be mentioned. Tobacco mosaic virus can easily be resolved from other proteins by electrophoresis and ultracentrifugation of leaf cytoplasm derived from infected tobacco plants. The results of a series of experiments suggest that tobacco mosaic virus is synthesized at the direct expense of a bulk cytoplasmic protein which

comprises approximately 85% of the total soluble cytoplasmic protein of uninfected tobacco leaves. This bulk protein has shown little or no enzymatic activity.

It was thought that the methods used so successfully in the plant virus research might yield further information about the course of virus infection by their application to the better defined bacterial virus system. The immediate points of attack were as follows:

1. To determine whether or not proteins and other high molecular weight substances derived from bacteria could be satisfactorily examined by electrophoresis and ultracentrifugation.

2. To investigate what, if any, high molecular weight components of the bacterium contribute directly to phage synthesis.

3. To determine the nature of the changes taking place in the bacterial cell during a phage infection.

4. To detect postulated viral precursors during the eclipse phase of the bacterial infection.

In this introductory statement no reference to authority was made to avoid disrupting the continuity of the presentation. An extensive coverage of the literature will be made in the following section.

B. Literature Review

The history of investigation of bacterial viruses is relatively young. Only thirty-five years have elapsed

since Twort in 1915 noted that a culture of staphylococci could be turned into a glassy mass by an agent which could be filtered through bacterial candles. The agent was active in extreme dilution, and could be transmitted in an endless series. Twort considered it most likely that the agent was secreted by the bacteria, but he did not rule out the possibility that the agent was an animal virus. In 1917, d'Herelle independently noted a similar phenomenon and contributed the words bacteriophage and bacteriophagy to the literature. The so-called Twort-d'Herelle phenomenon was studied for the next 25 years by a host of workers. The field of bacteriophage investigation from 1920-1930 is characterized by a great outpouring of published material. A review of the field by d'Herelle in 1926, only eleven years after the original observation by Twort, lists no less than 690 references. The investigations were further characterized by uncertainty as to the nature of the phenomenon. In hindsight the uncertainty may be attributed to the qualitative nature of the work performed, the eagerness of the earlier investigators to generalize the phenomenon on the basis of inconclusive data, and the inability to obtain reproducible results with the crude techniques employed.

Some of the early theories as to the nature of the bacteriophage were reviewed by Hadley in 1928. d' Herelle originally postulated in 1917 that bacteriophage is a filterable virus which parasitizes bacteria and causes their destruction by lysis. Kabashima (1920) postulated that the

bacteriophage is an enzyme which activated a pro-enzyme present in the bacterium. Bordet and Ciuca (1920) thought that the phenomenon could be explained by an autolytic product of the bacterium which under certain conditions could become pathogenic for other cells in a culture. Kuhn (1924) was under the impression that the lytic principle was a stage in the life cycle of the bacteria. In 1927 Béguet introduced the notion that bacteriophagy is due to an imbalance in the osmotic pressure between the bacteria and the colloids in the culture filtrates. The Wollmans (1926) were the first to suggest that the bacteriophage particles are hereditary units. Their ideas center around the postulate that the bacteriophages arise in some manner from the bacterial cell. Having so arisen, they acquire a certain degree of autonomy and can produce modifications in a normal bacterial cell. The bacteriophages carry the determinants of the modifications which they produce in the bacteria. Quite a different theory from those previously mentioned is that developed by Hadley in 1928. His hypothesis represents the bacteriophage as something akin to a gamete which is necessary for the reproductive processes of the bacteria.

The foregoing listing by no means exhausts the various notions which different workers held in the early days of bacteriophage research. No useful purpose would be served

in viewing in detail the early ideas which were developed before the advent of more refined quantitative experimental techniques.

Burnet, in a review published in 1930, did much to clarify the conflicting ideas as to the nature of bacteriophage. He states, "There is no serious conflict in the evidence as to the mode of action of strong phages which give rise to classical bacteriophage lysis. The differences of opinion arise fundamentally from the conflicting nature of the evidence as to whether or not bacteriophage can be derived from normal bacteria in the absence of pre-existing phage." Burnet is willing to leave aside for the time being questions as to the origin of bacteriophage in order to be able to concentrate more fully on the phenomenon of the already existing phage. On this account also he finds it more expedient to concentrate on those cases where phage must be added to a bacterial culture to make more phage and to lyse the bacteria. Thus, the problem of lysogenesis, that is, those cases in which no phage need be added to the bacterial culture in order to obtain phage production is pushed aside. The facts, accumulated at that date, considered by Burnet to be of most importance are that the phage is of a particulate nature and that the bacteriophage particles appearing after lysis of a bacterial culture are genetically derived from the particles used to initiate lysis.

By 1934, when Burnet again reviewed the subject, the

particulate nature of the phages was definitely established through the work of Elford and Andrews by ultra-filtration and by Schlesinger through the use of analytical centrifugation and light scattering. These workers also demonstrated that phage particles of any one strain are homogeneous in size, but they differ in size considerably from strain to strain. This was a notable advance since previously many theories had been built around d'Herelle's assumption that all of the phages belong to a single species. The final proof of the sizes of the bacteriophages awaited the development of the electron microscope. The observations with the use of this instrument showed not only that phages are particulate and that they vary in size, but also that many of them have a complex morphology.

d'Herelle (1926) had originally observed that when a small amount of phage was added to a growing bacterial culture, the phage concentration did not increase gradually but did so in steps 20-30 minutes apart. Burnet (1934) confirmed this observation and offered an explanation for the results of Krueger and Northrop (1931) who were led to believe that an equilibrium between intra-cellular and extra-cellular phage was set up during phage growth. Krueger and Northrop were not actually studying the effect of a single or a few phage particles on a single bacterium. On the contrary, they were making a study of the changes taking place in a mixed population of phage and bacteria. The

dynamics of such a situation are indeed of interest but are not to the immediate point in the study of the bacteriophage infection on a cellular level.

Bail in 1923 was the first to start differentiating phages according to plaque size and host range but the notion of the existence of only one species of bacteriophage prevailed until the early thirties. However, work by Morison (1932), Burnet and McKie (1933), Asheshov et al. (1933), and Burnet (1930), demonstrated clearly that the phages could be classified according to host range, particle size, plaque size, serological specificity, and the resistant bacteria which are found after lysis of a culture. This work tended to stress both the diversity which is found in the phage world and also the relationships which exist between different groups of phages.

Both Schlesinger (1934) and Northrop (1936) were able to demonstrate that the phage particles are primarily nucleo-protein in nature.

In 1937 Burnet et al. reviewed their work on the serological specificity of phage. The work deals mainly with distinguishing between the specific sites on the surface of the phage which act as antigens and those sites at which the phage becomes attached to the bacterium. Since this work deals mainly with the first phase of infection it shall not be reviewed here.

A notable advance in bacteriophage study was made in 1939 by Ellis and Delbrück who developed the one-step growth experiment which not only confirmed the previous findings of d'Herelle and Burnet that phage particles are produced by a bacterial culture in bursts, but clearly isolated and defined the latent period of phage multiplication in a single bacterium. This development opened for study the course of events during the now well-defined latent period. Until this time, work had been done with a large number of bacteriophages which were active against several genera of bacteria. Since 1945, however, the main stream of investigation has centered around seven specific phages, T 1-7 as defined by Demerec and Fano (1945), which attack strain B of Escherichia coli. The period of classification of the various phages had more or less come to a halt and attention was primarily directed to the study of the intracellular growth of bacteriophage in the E. coli-T system.

In 1942 Delbrück and Luria initiated the study of mixed infection of bacteria with two different phages. One of the first findings from this type of experiment was the discovery that only one of the two phages will multiply in such a mixedly infected bacterium.

In a series of two papers Luria and Delbruck (1943) and Luria (1945) demonstrated the occurrence of spontaneous mutations among both bacteria and bacteriophage. In the bacteria the mutations were of a type which imparted re-

sistance to the attack of some of the phages. The phage mutants had the ability to attack the resistant bacteria. These mutations both in the bacteria and in the phage had previously been observed but had been assumed to be either induced changes or adaptations. These experiments provided clear evidence that the phage was of such a nature as to be able to undergo spontaneous hereditable change thus pointing out its similarity to genic material. Mutations of phage affecting plaque characteristics were analyzed shortly thereafter by Hershey (1946a) who put forward a convincing argument that the variants observed were in fact mutations and that a bacteriophage had several loci at which it could mutate. The discovery of a variety of phenotypically recognizable characteristics for bacteriophage made possible the type of experiment which led to the finding by Delbrück and Bailey (1946) that the mutant phages were actually capable of exchanging genetic material during the course of an infection. This finding was subsequently expanded by the intensive work of Hershey (1946) and of Hershey and Rotman (1948, 1949) who found that there were what could be called by analogy with classical genetics three linkage groups in the bacteriophage studied. At the same time a possibly related phenomenon was being studied by Luria, who discovered in 1947 that ultra-violet inactivated phage were capable of infection and growth when two or more such inactivated

particles took part in the infection. This study was expanded by Luria and Dulbecco (1949), who on the basis of quantitative experiments suggested that the phenomenon might be the result of recombination of the undamaged parts of the irradiated bacteriophage. They postulated that upon entering the cell the phage particle broke down into component self-reproducing entities. Damage to any one of these entities could inactivate the bacteriophage in the extracellular state. However, if two phages, inactivated due to damage in different entities, took part in the infection of a single bacterial cell, then each phage could supply undamaged entities to take the place of the damaged ones of the other phage. Under such a scheme, the phage upon entering the cell would break up into its component self-reproducing entities which would individually undergo reproduction. At the end of the reproductive period, the individual entities would again be reassembled into complete phage particles. As a result of quantitative experiments Luria (1947) estimated that the number of self-reproducing entities which make up the extra-cellular phage particle would have to be of the order of 25-50, depending upon the phage.

Additional evidence that a drastic change takes place in the bacteriophage after it has entered the bacterial cell is supplied by the irradiation experiments of Luria and Latarjet (1947) and Latarjet (1948) who showed that the sensitivity of the phage to both x-ray and ultra-violet light

damage decreases markedly after the phage enters a bacterium. By the use of acriflavine, a poison which inhibits phage growth but which permits an infected bacterial cell to lyse at the end of the normal latent period, Foster (1948) demonstrated that complete inhibition of phage release can be obtained only if the acriflavine is added during the first half of the latent period, thus indicating that the critical period of phage synthesis occurs during this time. By a more direct attack Doermann was able to show that during the first half of the latent period no phage particles are recoverable from infected bacteria when they are disrupted, not even the original infecting particle. During the second half of the latent period, however, new infectious units appear within the bacterium in increasing numbers until the time of lysis.

Several workers have been concerned with the biochemical changes taking place in a bacterium during infection. In a series of papers Cohen and coworkers have described some of these changes. In 1945 Cohen and Anderson noted that upon infection bacterial multiplication is halted and that both the respiratory rate and the respiratory quotient remain at a level equal to that just prior to infection. Monod and Wollman (1947) were able to demonstrate that infected bacteria are no longer capable of producing adaptive enzymes. Further work by Cohen (1947, 1948a,b) demonstrated that protein synthesis continues in the bacteria

at the same rate after infection at which it took place at infection. Desoxyribonucleic acid (DNA)* is the only nucleic acid synthesized after infection, whereas prior to infection the rate of ribonucleic acid (RNA)* synthesis is considerably higher than that of DNA synthesis. Another point of interest is the fact that there is a lag in the onset of DNA synthesis after infection. DNA synthesis starts shortly before the appearance of intact phage in the bacterium and continues at a rate comparable to the rate of appearance of intracellular intact phage. By the use of radioactive phosphorus Cohen found that most of the DNA in newly synthesized virus is present in the medium as inorganic phosphorus at the time of infection and is not derived from constituents present in the bacteria at the time of infection.

In an expansion of this type of work, Kozloff, Putnam, and Evans (1950) and Barry, Gollub-Banks, and Koch (1950) using isotopes of phosphorus and nitrogen were led to the conclusion that the total amount of DNA present in the bacterium at the time of infection is incorporated into the DNA of the virus. Their idea is that the bacterial DNA is somewhat degraded and rebuilt into phage DNA. However, most of the phage DNA is synthesized from constituents still in the medium at the time of infection. Whether or not the constituents of the medium are first built into

*For convenience, desoxyribonucleic acid will be abbreviated to DNA throughout the rest of this thesis. Similarly, ribonucleic acid will be abbreviated to RNA.

bacterial DNA or directly into phage DNA was not determined. The workers cited also found that phage protein is derived in its entirety from constituents present in the medium at the time of infection. They came to the conclusion that the bacterial protein does not contribute to the bulk of the phage protein. In a very recent publication, Labaw, Mosley, and Wyckoff (1950) present evidence to the effect that all of the phosphorus taken up from the medium by infected bacteria is directly incorporated into the DNA of phage.

In summary, the early workers in bacteriophage research were interested mostly in the clinical applications of bacteriophage as a tool in combating bacterial infections. A great volume of work was done during the twenties revealing little of fundamental significance to the knowledge of virus multiplication. During the thirties there was a marked slackening of interest in the bacteriophage problem since it proved a failure in clinical application. This period did see, however, the intensive development of bacteriophage as a tool in immunological research by Burnet and coworkers in Australia. Interest in the problem has been revived in the past decade by the realization that bacteriophages offer an excellent tool for the study of the fundamental problem of virus multiplication.

Information which has been collected to date as to the events which take place during a bacteriophage infection may be summarized as follows. Upon entry into the bacterium

the bacteriophage loses some of its extracellular characteristics. The metabolic processes of the bacterium become reorganized in such a manner that only phage material is synthesized. The DNA of the phage is composed of DNA contributed by the bacterium as well as of newly synthesized DNA. The protein of the phage consists entirely of material synthesized during the infection. Certain critical events occur in the bacterium during the first half of the latent period about which little is known. During the second half of the latent period the end products of the machinations of infected cells, newly synthesized phage particles, appear in the bacterial cell in increasing numbers until after a well-defined time period the bacterium bursts and liberates its contents. The problem of what occurs during the "eclipse", the first half of the latent period, is becoming the subject of attack of present day bacteriophage research.

Turning now to some of the techniques used in plant virus investigations, which have been applied in this thesis to a study of bacteriophage infection, Wildman, Cheo, and Bonner (1949) have been able to follow the course of the changes taking place in the proteins of tobacco leaves during a tobacco mosaic virus infestation. Their method was to disrupt the leaf cells at different periods after infection enabling them to recover the soluble proteins of the leaf. The changes in protein composition during infection could be followed by an electrophoretic analysis of the protein so-

lutions. They were able to detect a protein with the physical characteristics of tobacco mosaic virus in the extracts of the plant leaves soon after infection and found that the synthesis of this material is accompanied by the decrease of a cytoplasmic nucleoprotein which comprises from 75-85% of the total soluble protein of the leaf. It was thought possible that the approach of following the changes taking place in the cytoplasm of infected bacteria would yield valuable information as to the nature of the changes taking place during the "eclipse".

Before concluding this literature review, I should like to mention a recent general review of the status of fundamental knowledge in plant, animal, and bacterial virus research. This review is called "Viruses 1950" and was published as a result of a conference held at this institute. I would also like to mention Burnet's book "Virus as Organism" and Beadle's review, "Genes and Biological Enigmas" which treat the more general aspects of the meaning of viruses as a biological phenomenon.

II. MATERIALS AND METHODS

A. Materials and Techniques.

1. Strains of Bacteriophage and Bacteria. Throughout the course of the experiments to be described attention has been confined to one bacterium and one virus. These are, respectively, *Escherichia coli* strain B which has been described many times in the literature, and bacteriophage T2 first designated as such by Demerec and Fano (1945). Both the bacterial and the phage stocks were originally obtained from Professor Max Delbrück of this institute.

2. Media used for Cultivation of Bacteria. The bacteria were cultivated in a nutrient broth medium which contained 8 gm. Difco nutrient broth, 5 gm. NaCl, and 1 gram glucose per liter of H_2O.

High titer phage lysates were prepared in M-9 medium, a synthetic medium buffered with phosphate and having glucose as a carbon source. The formula for this medium is as follows:

Na_2HPO_4, 7.0 gm.; KH_2PO_4, 3.0 gm.; $MgSO_4$, 0.2 gm.; NaCl, 0.5gm; NH_4Cl, 1.0gm; $CaCl_2$, 15 mg; H_2O, 1.0L. Nine parts of sterilized salt solution are mixed with one part of a sterile 4% glucose solution.

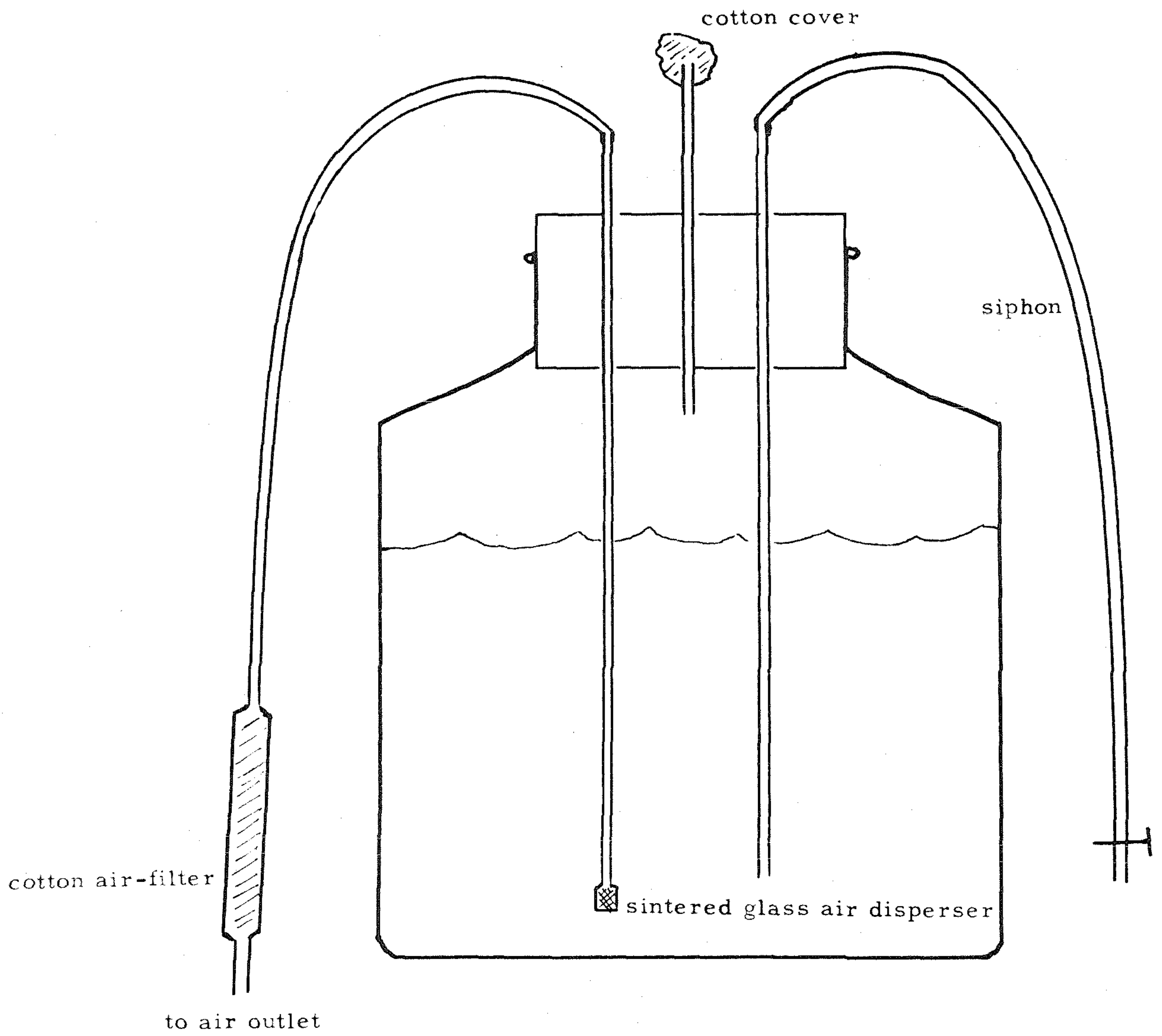
3. Culture Methods. The bacterial strain was maintained on an agar slant in a refrigerator. A fresh agar slant was prepared monthly. Before each experiment, a 24 hour broth culture was prepared to be used as inoculum.

Approximately 20 cc. of broth contained in a test tube was seeded by transfering some bacteria from the culture slant. The liquid culture was then incubated with aeration for 24 hours in a 37° water bath.

One day before the start of an experiment the appropriate amount of nutrient broth, usually 13 liters, was prepared in a 5 gallon jug. The jug was stoppered with a rubber stopper tied down with heavy twine. Three glass tubes entered the jug via holes in the stopper. At the end of one of the tubes inside the jug was attached a sintered glass air disperser connected on the outside of the jug with rubber tubing to a cotton air filter. The second glass tube was connected to rubber tubing on the outside of the jug and was used as a siphon to withdraw aliquots from the jug during the course of bacterial growth. The third glass tube entered the jug only a short distance and was used as an air outlet as well as an entry into the carboy through which the medium could be seeded with bacteria. This short tube was covered on the outside with a removeable cotton cover. The jug is pictured in Figure 1. To prevent foaming of the broth during aeration, 1 cc of DC-Antifoam A emulsion was added to each jug. The use of this antifoam agent was suggested by Fraser (1949) who found that the agent is innocuous towards the bacteria as far as could be determined. In the hands of the present author the agent also seemed to have no deleterious effects on the biological system employed. The jug was auto-

Figure 1

Experimental Set-Up For Growing Bacteria



claved for 45 minutes under 15 pounds of pressure and was allowed to cool overnight at room temperature. The jug was seeded with about 10 cc. of the 24 hour broth culture of bacteria prepared the previous day. Aeration was commenced and the temperature of the broth was maintained at 25° by controlling the room temperature. From time to time, the bacterial titer was measured by withdrawing an aliquot from the jug by means of the siphon arrangement and making a count of the bacteria in a Petroff-Hauser bacterial counting chamber. This method proved to be the most accurate and the most rapid of several methods tested.

4. Buffer. Unless otherwise mentioned the buffer employed for preparative as well as analytical work in all of the experiments was 0.1 ionic cacodylic acid buffer at pH 6.83. This buffer was used because it has been found (Longsworth, 1947) that a uni-univalent buffer gives the best results in electrophoretic analysis and because it was thought best to prepare and examine the protein solutions at a pH near neutrality. Cacodylic acid buffer is the only convenient uni-univalent buffer in the neutral pH range. The buffer consisted of 0.2 M cacodylic acid, 0.2M NaOH, and 0.8 M NaCl.

5. Preparation of Phage. Large amounts of high titer phage suspensions were required for the experiments since it was desired to infect 13 liters of growing bacteria with

5 to 10 times more phage than bacteria when the bacterial concentration reached 2-4 x 10^8 cells per cc. Thus, to infect the bacteria in one jug, approximately 2 x 10^{13} phage particles were required. The method adopted for preparation of the desired high titer phage suspensions was to seed four or five 250 cc. aliquots of M-9 medium contained in 300 or 500 ml. erlenmeyer flasks with small amounts of bacteria and allow the cultures to grow at 37° C either with aeration or with vigorous shaking on a mechanical shaker. When the cultures became quite turbid, indicating an approximate bacterial concentration of 5 x 10^8 cells per cc., they were infected with very small amounts of phage transferred from a previously prepared plaque with a sterile needle. The cultures were then allowed to incubate under the previous conditions for 15 to 24 hours.

During this period the amount of phage in the cultures increases at the expense of growing bacteria. After the end of the incubation period, the cultures are called lysates since they contain lysed bacteria. The lysates were filtered through Mandler candles to remove intact bacteria and stored at 20°C until use. The filtered lysates obtained in this manner usually contained from 2 to 6 x 10^{11} phage particles per cc.

When still larger quantities of phage were desired, the lysates were prepared in 18 liter lots in 5 gallon jugs. The crude lysates were passed once or twice through a steam-driven refrigerated Sharples supercentrifuge running at a

speed of approximately 30,000 r.p.m. with a flow rate of three liters per hour. The bowl was lined with cellophane prior to the run to facilitate the later removal of the sedimented phage. Three 250 ml. aliquots of buffer were passed through the Sharples after the lysate to remove the normal lysate holdup liquid. The cellophane sack containing the sedimented phage was then removed from the bowl to the buffer holdup liquid. The phage particles were easily resuspended in the buffer. The preparations were further purified by differential centrifugation, 2 or 3 cycles of slow and high speed centrifugation being employed. Slow speed centrifugation of resuspended phage was carried out at 5,000 x g. for 15 minutes to remove residual bacterial contaminant. High speed centrifugation was carried out at 20,000 x g. for one hour and served to sediment the phage. All operations were carried out in the cold (2°C). The centrifugation was performed in a Servall angle head centrifuge Model SS-1, using 50 cc. plastic centrifuge tubes. Difficulty was encountered in resuspending the large quantity of sedimented phage particles. This difficulty was partly overcome with the aid of a Potter-Elvehjem homogenizer. A plastic rotor, attached to a motor, which fitted loosely into the plastic centrifuge tubes was used to stir the sedimented phage vigorously into a small amount of added buffer.

Phage activity was determined by the plaque counting method first described by Gratia (1931).

6. <u>Method for Infecting Large Quantities of Bacteria.</u> Early in the experimental work it was discovered that it was not sufficient to add the required amount of phage to a jug of growing bacteria to obtain the desired degree of infection. If small amounts of phage suspension (100-250 cc.) are added to large amounts of bacterial suspension (13-18 liters), not all of the bacteria will become infected even though the numerical ratio of phage to bacteria may be 10 to 1 and the bacterial suspension is agitated vigorously during the addition of the phage. A standard procedure was therefore developed of diluting the necessary number of phage particles into five liters of broth, and using this diluted phage suspension to infect 13 liters of bacterial suspension. With this method more than 99% of the bacteria were infected. To determine the number of bacteria which had become infected, aliquots were withdrawn from the carboy just prior to infection and ten minutes after infection. These aliquots were immediately diluted and plated. Since only an uninfected bacterium will give rise to a colony upon plating, the proportion of bacteria left uninfected can be determined by comparing the number of colonies obtained from aliquots taken just prior to infection with the number obtained from aliquots taken ten minutes after phage had been added.

7. <u>Method of Arresting Phage Development.</u> To arrest phage development, sodium cyanide to a final concentration of 0.01 M was added to carboys of growing or infected bacteria.

For our purposes this method of poisoning is useful only if applied to uninfected bacteria or during the "eclipse" phase of the infection. Doermann (1948) has demonstrated that metabolic poisons added to infected bacteria during the last phase of infection, when newly synthesized intracellular phage particles are present, cause lysis of the bacteria. This induced premature lysis, although useful for obtaining certain types of information, makes it impossible to concentrate and wash the bacteria under controlled conditions before disruption.

8. <u>Collection and Washing of Bacteria.</u> Bacteria were collected from the medium in a Sharples supercentrifuge running at a speed of about 30,000 r.p.m. with a flow rate of 3/4 of a liter per minute. The bacterial mass was removed from the centrifuge bowl and the carboy lot of bacteria was resuspended in about 90 cc. of buffer. The bacteria were again sedimented at 10,000 x g. for 30 minutes in a Servall angle head centrifuge, resuspended in buffer and resedimented.

B. Analyses

1. <u>Electrophoretic and Ultracentrifugal Analysis.</u> Most of the electrophoretic determinations were carried out in a modified Tiselius electrophoresis apparatus designed by S. Swingle (1947). This instrument utilizes the Longsworth optical scanning method. In comparing infected and normal bacterial preparations, analysis was performed in paired

electrophoresis cells at identical total protein concentrations, usually 1 %. Most of the scanning patterns were taken after the boundaries had migrated for two hours. In some of the later experiments involving nucleic acid analysis the electrophoresis was performed in a Perkin-Elmer Tiselius electrophoresis apparatus Model 38.

The ultracentrifugal studies were carried out in an apparatus designed and built in the Chemistry Department of this institute. It is a synchronous motor, direct-drive instrument. The optical system is of the Philpot-Svensson type employing parabolic mirrors instead of lenses.

2. Elementary Analyses. Nitrogen determinations were performed by the Nessler colorimetric method. Half cc. aliquots were digested with 0.5 cc. concentrated H_2SO_4 for 45 minutes in a Folin-Wu digestion tube over a Fisher burner. One drop of 30% H_2O_2 was added to clear the digestion mixture. The tubes were then heated for an additional ten minutes. Color was developed with Nessler's solution. A Klett colorimeter with a blue filter was used to measure the optical densities of the solutions.

Phosphorus analyses were performed by the Allen colorimetric method (Allen 1940).

Desoxyribonucleic acid analysis was performed by the cysteine hydrochloride colorimetric method of Dische (1944).

Early in the experimental work it was determined that the dry weight, the weight of a trichloroacetic acid

precipitate, and the nitrogen content x 6.25 of the dialysed bacterial protein solutions were equivalent. Thereafter, the protein content of these dialysed solutions was determined by total nitrogen analysis.

C. General Experimental Method.

The technique consists of disrupting large numbers of bacterial cells which have been infected for varying lengths of time, removing the particulate debris, and analyzing the soluble protein components by electrophoresis. The number of bacteria which must be collected for one such experiment is determined by the fact that at least 150 mg. of protein in a 15 cc. volume are required for electrophoretic analysis in the apparatus used. It has been found in practice that approximately 3-4 x 10^{12} bacteria with a wet weight of 10-12 grams will yield 500 to 600 mg. of soluble protein by the techniques described.

The soluble proteins were examined in three stages of development of the bacteria and compared by the method of electrophoresis. The three stages studied are the uninfected state, the stage just prior to the end of the "eclipse", and the last stage of infection in which the bacteria contain newly synthesized intracellular phage but have not lysed. The method of growing and infecting all three types of bacteria are the same. Bacteria were grown in 13 liters of nutrient broth at 25°C until a concentration of 2-4 x 10^8

bacteria per cc. was attained. At this concentration the bacteria are in the logarithmic phase of growth. Infection was carried out by dumping into the bacterial culture 5 liters of broth, containing enough phage to give a five to tenfold excess of phage over bacteria. Five liters of sterile broth were dumped into control carboys containing bacteria to be examined in the non-infected stage.

It was determined by the one-step growth technique that the latent period under the conditions above described lasts for 35 to 40 minutes. If cyanide is added to a culture jug twenty-two minutes after infection, the bacteria lyse rapidly as indicated by a decrease in turbidity occurring a few minutes after addition of the poison. If cyanide is added twenty to twenty-one minutes after infection, no clearing is evident and it is possible to collect a sufficient amount of unlysed bacteria for further experimental work. This empirical finding indicates that the eclipse period of infection lasts approximately twenty minutes under the standard conditions set forth for these experiments.

The bacteria which are to be studied at the end of the eclipse are poisoned twenty to twenty-one minutes after the infecting phage is added. In the case of the carboy which served as a control to infected bacteria poisoned at twenty-one minutes, poison was also added twenty to twenty-one minutes after the addition of the five liters of sterile broth. The bacteria to be studied in the third phase of the infection were collected without being first poisoned at times

after infection which are to be indicated. The control bacteria for these experiments were also collected without poisoning.

The bacteria were collected in the Sharples centrifuge as previously described. The time required to collect the bacteria in this manner was 20 to 25 minutes. The bacteria which had been poisoned with cyanide represent a homogeneous population as far as the time of infection or growth is concerned. The bacteria which were not poisoned represent a population distribution of bacteria in different states due to the time period required to collect the bacteria. Since the centrifuge was refrigerated and the temperature maintained on the inside of the bowl was close to 0°C., it can be considered that changes in the bacteria can proceed only at a very slow rate as soon as they enter the centrifuge. Analysis of the bacteria in the third stage of infection gave similar results whether the collection had been started twenty-five minutes after infection or as late as 120 minutes after infection, thus indicating that the fact that a homogeneous population is not obtained is of little importance to the present study. It was mentioned previously that the latent period for the infection under the conditions of the experiment was 35-40 minutes. The phage strain used, however, is able to prevent lysis of the bacterium for several hours at the end of the latent period. This phe-

nomenon of lysis inhibition, which has been investigated by Doermann (1948), makes it possible to collect the unpoisoned infected bacteria before the onset of lysis.

After collection in the Sharples centrifuge, all bacterial preparations are treated in the same manner. They are washed twice in buffer and disrupted in the colloid mill.

III. EXPERIMENTAL RESULTS

A. Disruption of Bacteria and Resolution of Nitrogen Fractions.

The problem of how to disrupt bacteria has long troubled microbiologists (see, for instance, Chargaff, 1947). Methods which have been successfully used are grinding in a mortar and pestle with the aid of abrasives, the use of a Wood-Workman mill which involves forcing a mixture of bacteria and abrasive through two conical ground glass surfaces, and the use of a modified Potter-Elvehjem homogenizer. These three methods were recently compared and analyzed quantitatively for effectiveness of grinding under varying conditions by Dockstander and Halvorson (1950). Other methods which have been used successfully by various authors are intense sonic vibrations (Doermann, 1948) and the use of a specially designed mill by Booth and Green (1938). A new method for disrupting bacteria is presented in this thesis. The method involves the use of a colloid mill which has been used successfully for the disruption of thick-walled plant cells (Wildman, Cheo, and Bonner, 1949) and for disruption of animal nuclei (Mirsky, 1947). This method is especially suitable for disruption of large numbers of bacteria as used during the course of the present work. The details of the procedure giving the best results are as follows:

The bacteria after being washed are resuspended in a small amount of buffer to make a mixture comprising 1

part wet bacteria to 3-5 parts buffer to make a minimal volume of 30 cc. This slurry is then submitted for thirty minutes to the action of an ice-water-cooled Eppenbach Colloid Mill model QV-6, with the clearance between rotor and stator set at 25-50 microns. The bypass and outlet assembly of the mill was replaced by a piece of 1/4" x 3/16" rubber tubing to decrease the minimal capacity of the mill from 125 cc. to 25-30 cc. It was found in early experiments that the efficiency of disruption of the bacteria by the mill was greater the more viscous the circulating suspension. Since it was difficult to obtain sufficient quantities of bacteria from one experiment to make thick slurries in a large volume, the ability to decrease the minimal capacity of the colloid mill was of great advantage. During the course of milling the temperature of the circulating suspension rose to 15°-17° C.

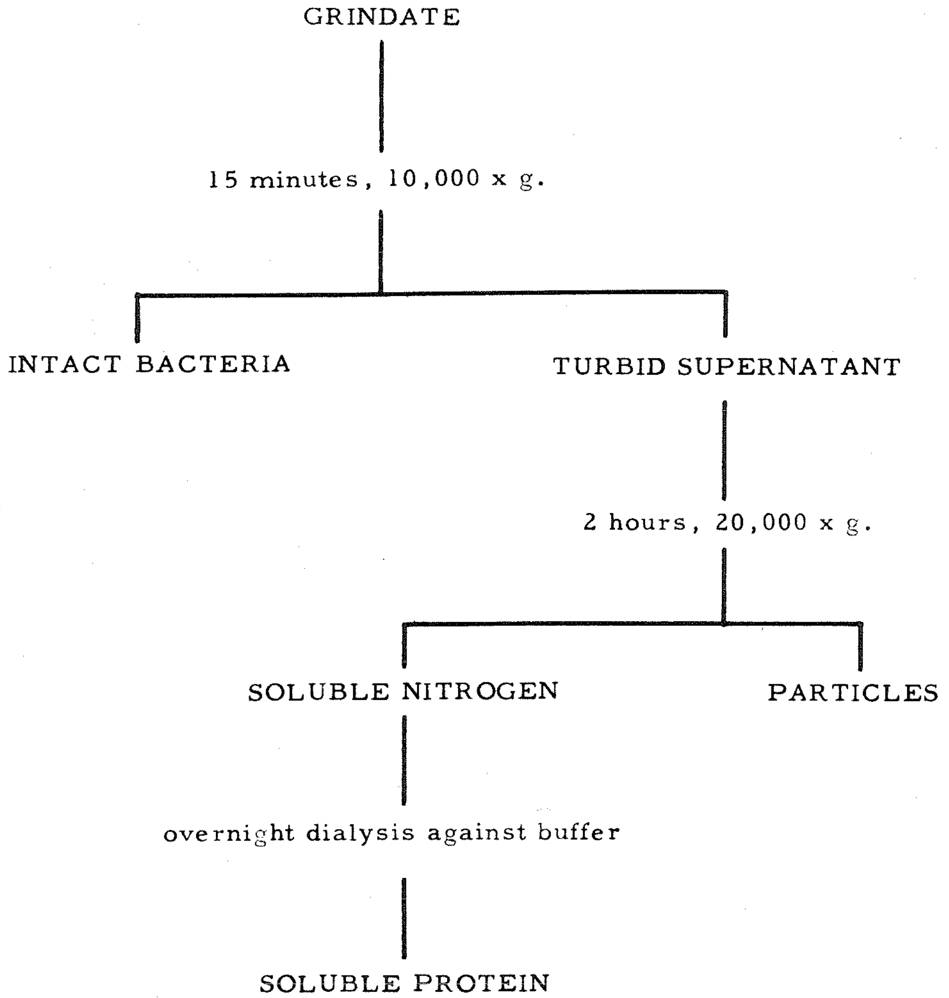
Before the colloid mill action, it is sufficient to centrifuge the bacterial suspension for 15 minutes at 10,000 x g to achieve complete sedimentation of the bacteria and a clear supernatant. After grinding, however, identical conditions of centrifugation leave the supernatant turbid, indicating that particles smaller than intact bacteria have been released from the bacteria by the colloid mill action. These particles represent either cell walls which have been torn apart or intracellular particles which are released from the cell upon disruption, or both. The turbid supernatant can be clarified by increasing the centrifugal field to 20,000 x g. for two hours. The sediment obtained under

these conditions will be referred to as "particles". The supernatant will be termed "soluble nitrogen". The soluble nitrogen fraction is dialyzed overnight against two to four liters of buffer to get rid of low molecular non-proteinaceous nitrogen, and to achieve the equilibration between the protein solution and buffer which is necessary for electrophoretic analysis. The soluble non-dialyzable nitrogen fraction is the experimental solution and will be called "soluble protein". The complete scheme for the preparation of the soluble protein preparations is diagrammed in Figure 2. The ground bacterial suspension as it comes from the colloid mill is termed the "grindate". The sediment obtained after low speed centrifugation is termed "intact bacteria," although it is not at all sure that this term represents the true situation since viability measurements were not made. The supernatant left after centrifugation at 10,000 x g. is termed "turbid supernatant".

Nitrogen analysis was performed on each of the fractions into which the grindate was separated in an attempt to detect differences in nitrogen distribution among bacteria in the different infected states studied. This procedure, however, proved to be too gross a tool for observing changes in the bacteria during the course of infection. The nitrogen distribution varied from one batch of bacteria to the next so as to obscure any consistent small change which might have taken place during infection. The reasons for the degree of variability of the results obtained is not known

Figure 2

Fractionation Scheme of the Bacterial Grindate



but several guesses as to the causes are ventured. The efficiency of disruption of cells in the colloid mill has not been well studied, and it is probably true that different proportions of bacteria were disrupted in different runs, thus contributing to fluctuations in the nitrogen fractionation. Another probable cause for the variable data obtained is that the conditions of centrifugation were not sufficiently studied. One object of this phase of the study was an attempt to isolate the intracellular particulate matter. This material may sediment very differently in suspensions of different densities and viscosities. The grindates obtained from the mill were always dense and viscous. The degrees of such density and viscosity depended on the original proportion of bacteria to liquid is difficult to control from run to run, again contributing variability to the data on nitrogen partition.

Results obtained in four separate runs as well as an average of 21 experiments are presented in Table I as an indication of the type of results obtained and of their reproducibility. Approximately 30% of the nitrogen in the grindate is recovered as soluble protein. Since the degree of breakage of cells is unknown and the proportion of nitrogen found in cell wall material and in the intracellular particles has not been well studied, the significance of this figure is questionable except to indicate that an appreciable amount of the protein of the bacteria is extracted as cell-free material by the analytical methods employed.

Distribution of Nitrogen Among the
Fractions of Colloid Milled Bacteria

Fractions	Avg. 21 expts. %*	Exp. 24 mg. N	Exp. 24 %	Exp. 33 mg. N	Exp. 33 %	Exp. 25 mg. N	Exp. 25 %	Exp. 13 mg. N	Exp. 13 %
Grindate	100	343	100	163	100	450	100	405	100
"intact" bacteria	55.1	147	43.1	100	61.4	267	59.3	169.5	42
turbid supernatant	42.6	186	54.6	59	36.2	172	38.2	214	53
particles	7.0	49	14.4	11.5	7.1	5	1.1	36.2	9
soluble nitrogen	34.2	136	40.1	49	30	136	30.2	157	39
soluble protein	27.3	120	35.5	48.3	29.8	123	27.4	132.5	33

*% of Nitrogen found in the grindate

Other facts that can be derived from the data are that there is relatively little low molecular weight nitrogenous material present in the bacteria and that there is a certain amount of insoluble nitrogenous material, probably proteinaceous in nature, represented by the fraction labeled "particles", which is being discarded in the present procedure. The suggestion is offered that perhaps some portion of these "particles" represent in the bacterium the analogue of the particulate enzyme complexes found in animal and plant tissues. A method is hereby available for recovery of significant amounts of this material for biochemical study.

To determine whether or not the method of preparation of the protein solutions grossly damaged the proteins, a series of rapid enzymatic assays were carried out. If at any stage in the course of preparation of the extracts nonspecific denaturation of the proteins had occurred, loss in the enzymatic activity of the preparations should result. The soluble nitrogen fraction was used for these tests rather than the soluble protein solution since coenzymes would have been lost from the latter upon dialysis.

Employing the Thunberg method in the manner of Bonner and Wildman (1946), the following dehydrogenases were found to be present and active; lactate, choline, malate, formate, and glutamate. Measuring liberated ammonia by the same method used generally in the nitrogen determinations, adenosine deaminase was also found to be present.

Since the study was undertaken merely to indicate that some of the enzymes present in the intact bacteria were not destroyed when the bacteria were disrupted and the proteins fractionated, further characterization of the bacterial enzymatic equipment was not undertaken. However, it is evident that many enzymes survive the colloid mill treatment and hence it can be concluded that the protein solutions used in this study were not seriously denatured during preparation.

B. Electrophoretic Study of the Soluble Proteins of Infected and of Non-Infected Bacteria.

In the first part of this study three bacterial states were examined:

1. Non-infected bacteria in the logarithmic phase of growth.

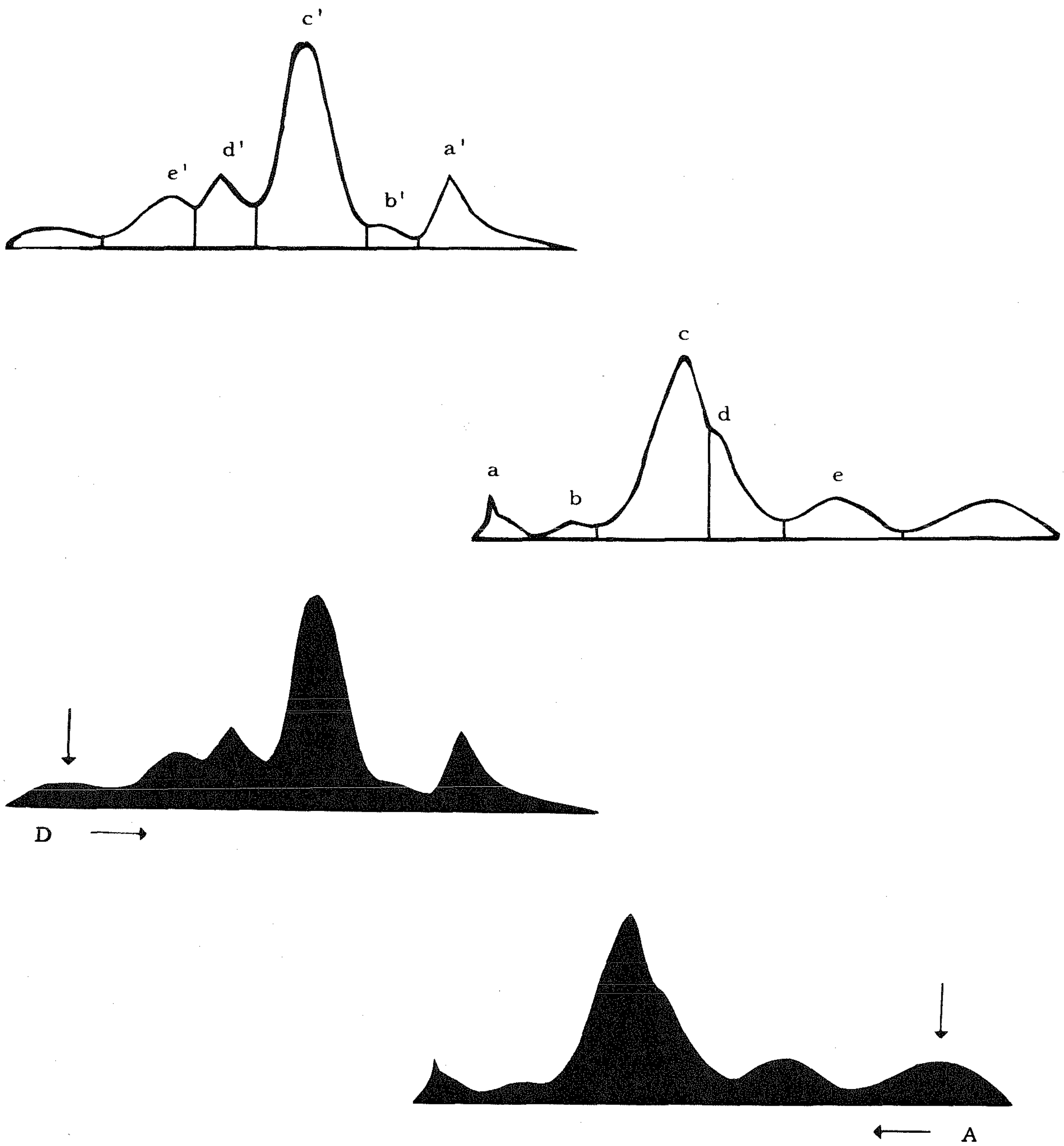
2. Infected bacteria poisoned to stop further phage development just prior to the end of the eclipse.

3. Bacteria in the third phase of infection, containing newly synthesized bacteriophage particles.

1) The resolution of the soluble protein components of non-infected bacteria.

Figure 3 represents 2 x enlarged tracings of the Longsworth scanning patterns of the soluble proteins of broth-grown bacteria, prepared by the grinding and differential centrifugal methods previously described. The patterns are reproducible from run to run. The individual peaks observed do not necessarily represent single protein species, but

Figure 3



The electrophoretic scanning pattern of the soluble proteins of uninfected bacteria. The lower two figures are 2x enlargements of the patterns obtained in the descending and ascending limbs of the electrophoresis cell. The upper two figures show the outline of these patterns and the arbitrary areas into which the patterns have been divided.

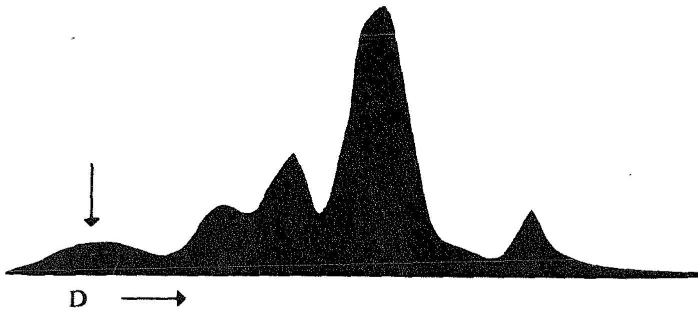
'he vertical arrows indicate the starting positions. A indicates the ascending pattern.
he horizontal arrows indicate the direction of migration. D indicates the descending pattern.

Migration time, 2 hours. Buffer, 0.1 ionic cacodylate. pH 6.83.
E', 3.47 volts/cm. Protein concentration 1%.

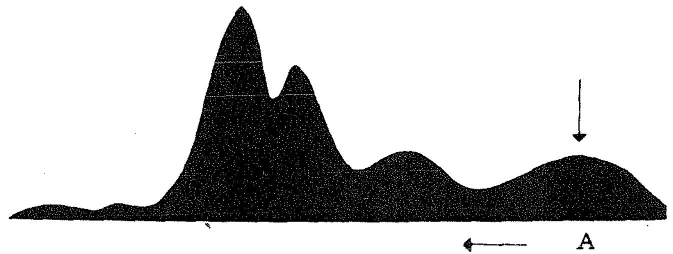
rather classes of proteins with similar mobilities which do not clearly separate under the conditions of electrophoresis used. As an aid in analyzing the patterns, arbitrary areas have been delineated as shown in Fig. 3, each characterized by a main peak in the scanning pattern. Because it is not certain that the areas delineated in the ascending and descending peaks represent identical material, the areas in the ascending pattern are represented by letters a, b, c, d, and e while the descending limb pattern areas are represented by letters a', b', c', d', and e'. The relative concentrations of the material represented by the different areas can be determined by relative spatial areas encompassed in the five defined areas (Bull 1943). These are listed in Table 2. Throughout the course of the experiments described in this thesis the soluble protein extracts of non-infected bacteria were prepared as controls and examined simultaneously with the soluble protein extracts of bacteria in the various infected states. These scanning patterns of the extracts of non-infected bacteria are presented in later figures as controls for further experiments.

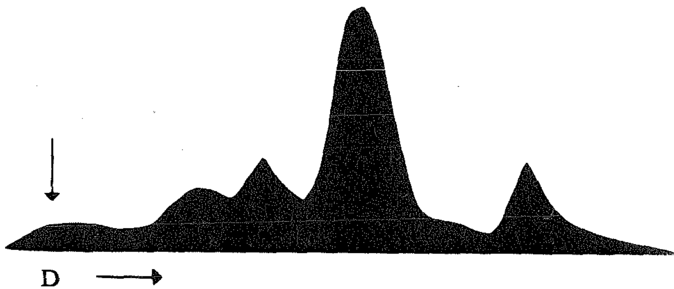
The discovery that it is possible to extract the soluble proteins of growing bacteria with methods which do not cause serious denaturation of enzymes and that it is possible to achieve a partial resolution of these proteins by electrophoretic means, laid the groundwork for the remainder of this thesis which deals with a study of the changes

Figure 4

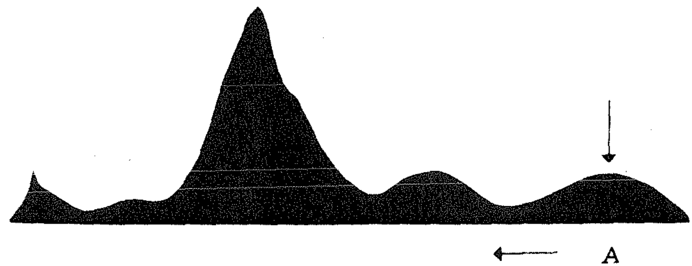


No. 42 "Eclipse" Bacteria





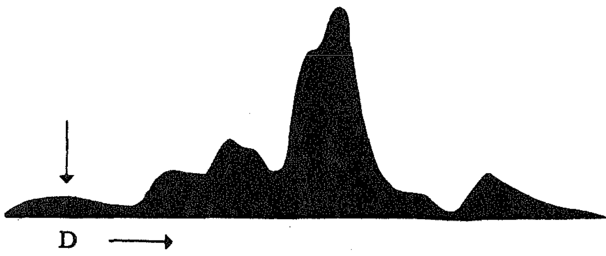
No. 41 Uninfected Bacteria



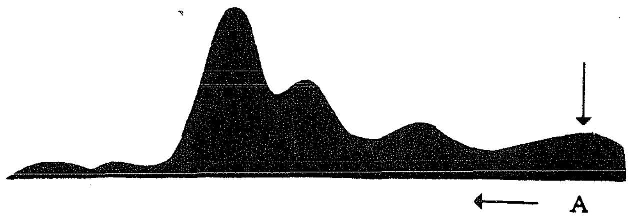
A comparison of the electrophoretic scanning patterns of the soluble proteins of "eclipse" and uninfected bacteria.

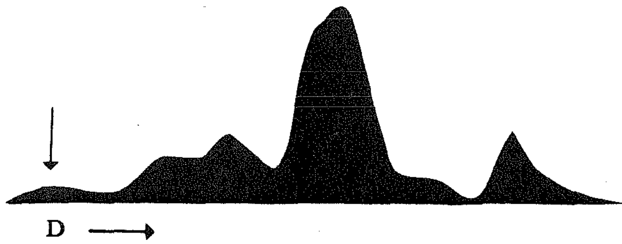
Note decrease in amount of the fast moving component in the patterns of "eclipse" bacteria.

Figure 5

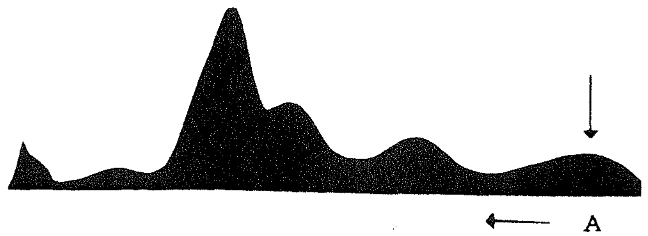


No. 46 "Eclipse" Bacteria





No. 45 Uninfected Bacteria



Same as Figure 4. A repeat experiment.

taking place in the bacterial proteins during the course of phage infection. It is hoped that this type of analysis will provide bacteriologists with an additional tool in the general study of the composition and metabolic processes of the bacterial cell.

2) Infected Bacteria Poisoned Prior to the End of the Eclipse.

Figures 4 and 5 present the electrophoretic patterns of the soluble proteins of bacteria which have been multiple-infected and poisoned just prior to the end of the eclipse together with the control pattern of uninfected bacteria. The patterns have been divided into the five areas discussed in the preceding section and the relative areas as determined by planimeter measurements are listed in Table 2. In general the patterns of uninfected and eclipse bacteria appear to be similar. However, there is a striking decrease in the relative area of the fastest moving peak in the patterns of the "eclipse" bacteria. This decrease is reproducible from experiment to experiment.

It will be shown in subsequent experiments that the component which is reduced in infected bacteria during the eclipse is desoxyribonucleic acid.

3) Infected Bacteria Collected After the End of the Eclipse Period.

The preparation of the solutions of the soluble proteins of bacteria collected in the third phase of infection was different in several respects from the preparation of

Table 11

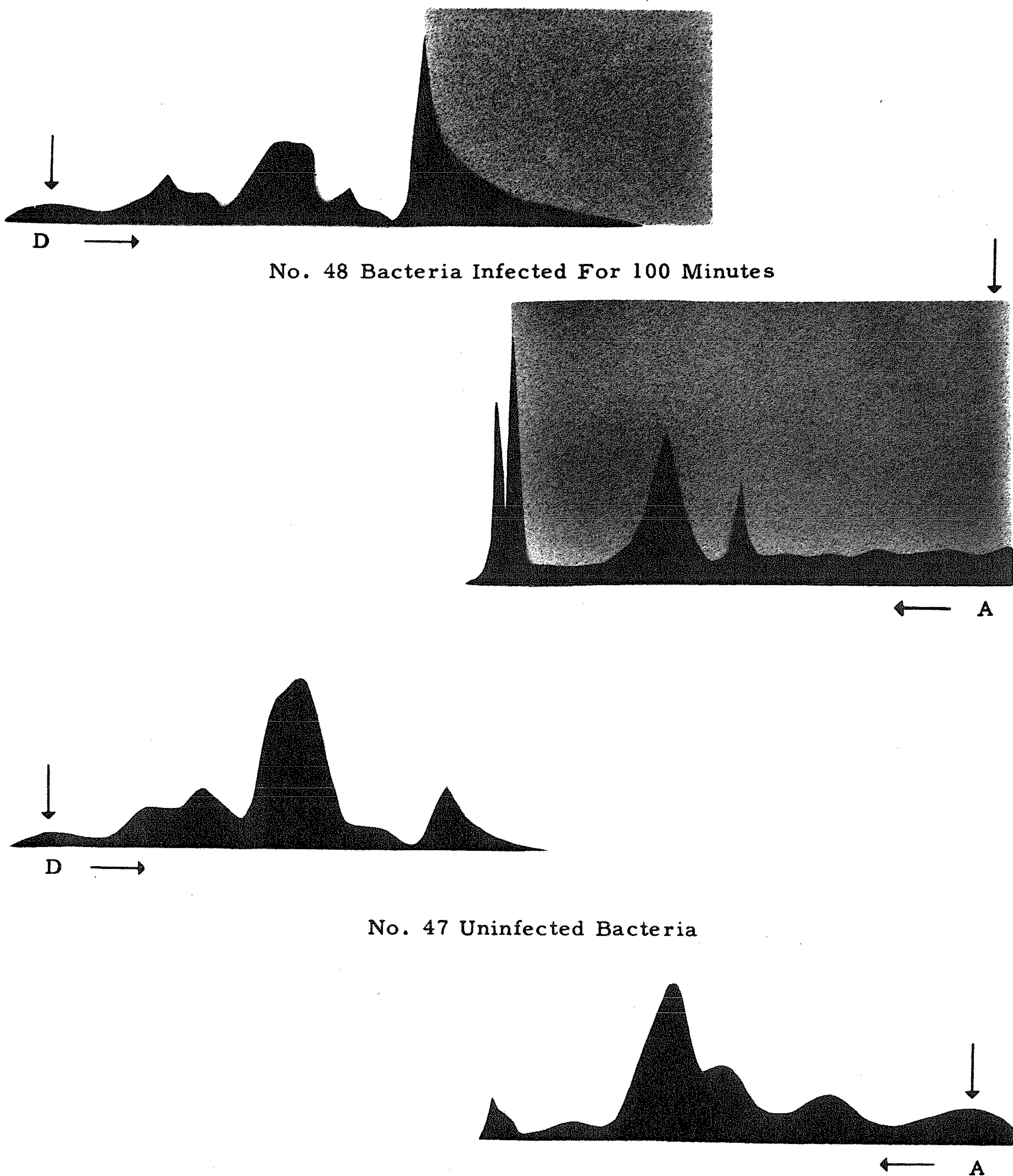
Relative Areas Under the Various Peaks in the Electrophoretic Scanning Patterns

Condition of Bacteria	Sample No.	Ascending Pattern % of Total Area in the Scanning Pattern					Descending Pattern % of Total Area in the Scanning Pattern				
		a	b	c	d	e	a'	b'	c'	d'	e'
Uninfected	#41 fig.4	6.4	4.1	49.7	22.2	17.6	16.8	4.2	51.7	12.9	14.5
	#45 fig.5	4.6	4.6	49.6	24.1	17.1	15.4	3.3	55.9	15.2	11.2
	#47a fig.7	5.3	5.3	55.0	10.7	23.7	12.4	2.5	60.1	14.1	12.9
"Eclipse"	#42 fig.4	2.2	2.3	46.5	29.7	19.3	8.3	3.4	59.1	16.2	13.1
	#46 fig.5	1.8	4.5	47.5	28.9	17.3	10.6	2.5	56.7	17.6	12.6
Infected for 100 minutes	#48a fig.7	17.7	5.4	41.2	15.6	20.0	32.0	2.7	53.0	6.9	14.8

either of the two previously discussed preparations. A deviation from the set procedure was found to be necessary on account of several observations which will now be recorded. In the preparation of sample 48, it was noted that a white, stringy substance appeared in the carboy which contained the bacteria after the infection had been allowed to proceed for longer than the end of the normal latent period. When the bacteria were collected, after the infection had proceeded for 100 minutes, the bacterial mass, when scraped from the bowl of the Sharples, appeared quite different in physical consistency from the previous preparations of bacteria. Whereas uninfected bacteria and bacteria poisoned prior to the end of the eclipse were paste-like and could be redispersed easily into a liquid suspension, the bacteria in this instance were gel-like in consistency, extremely viscous, and appeared to absorb liquid and swell when buffer was added. The bacteria apparently secrete some mucoid material during the prolonged infection period.

When the bacteria were subjected to treatment comparable to that given uninfected and eclipse bacteria, a viscous opalescent extract was obtained which upon electrophoresis yielded the scanning pattern pictured in Figure 6. Immediately obvious is the presence of two new peaks both with high mobility. It can be seen in Figure 6 that a significant optical density change occurs across the boundary of the slower-moving new peak. This shows that the opalescence in

Figure 6



A comparison of the electrophoretic scanning patterns of the soluble proteins of bacteria infected for 100 minutes and of uninfected bacteria. See text for further description. The shaded portions of the patterns indicate opalescence.

Note two new fast moving components in the ascending limb of the infected patterns and the general reduction in the quantity of the other components.

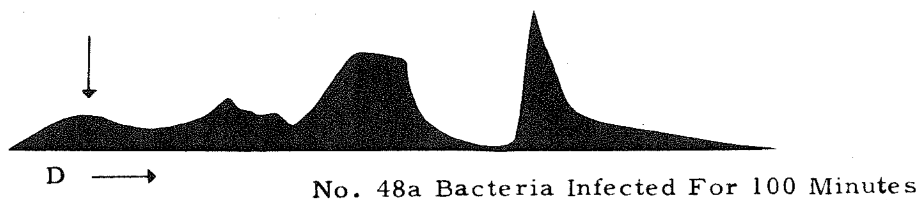
the solution is produced by the component represented by this new peak. In connection with another phase of the work, the extract whose electrophoretic scanning pattern is represented in Figure 6, was diluted with buffer to one-fifth of its concentration and was examined in the analytical ultracentrifuge, where it was noticed that the opalescence was removed from solution by a relatively low centrifugal force, leaving the rest of the components in a relatively clear residual solution.

During the course of preparation of the bacterial soluble protein solutions the extracts are routinely centrifuged at 20,000 x g for two hours. This procedure is usually sufficient to remove the last traces of turbidity from the solutions. In the case of sample 48, however, the supernatant remained opalescent and viscous. The ultracentrifuge data indicated that the material contributing the opalescence should have been sedimented at the centrifugal forces applied, and the question arose as to why it was still present in the solution. The preparative procedures did in fact differ in that a two-hour, 20,000 x g, centrifugal field had been applied to sample 48 when it was at a concentration of approximately 2%, whereas the subsequent electrophoretical and ultracentrifugal analysis had been performed after the solution had been diluted. This suggested that the sedimentation of the material causing opalescence in the solutions was being interfered with by the viscosity and density of the solution before dilution.

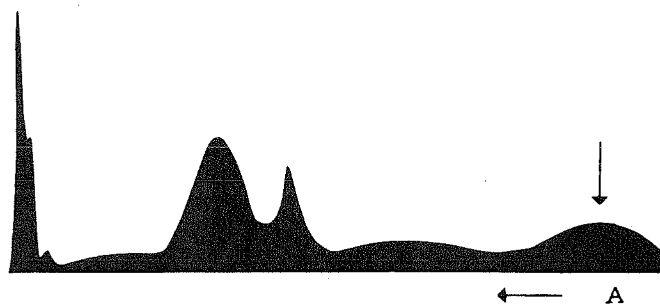
Sample 48 was therefore centrifuged at 28,000 x g for one hour after it had been diluted to 1%. The supernatant solution in this case appeared clear and had a much lower viscosity than the solution before centrifugation. The clear supernatant, 48a, was subjected to electrophoretic analysis. The scanning pattern obtained is shown in Figure 7. A comparison of Figures 6 and 7 demonstrates that one of the two new peaks present in Figure 6 is absent in Figure 7. Opalescence associated with this peak has also disappeared. The conclusion that can be drawn is that the material causing opalescence in the sample 48, represented in the electrophoretic pattern by one of the new fast-moving peaks, is in reality a rather large particle which can be centrifuged out of dilute solutions at 20,000 x g but whose sedimentation is interfered with in concentrated solutions by the fact that it imparts to the solution a high viscosity. It is suggested that this large particle material does not represent an intracellular bacterial component but is the same extracellular mucoid material previously encountered in this preparation.

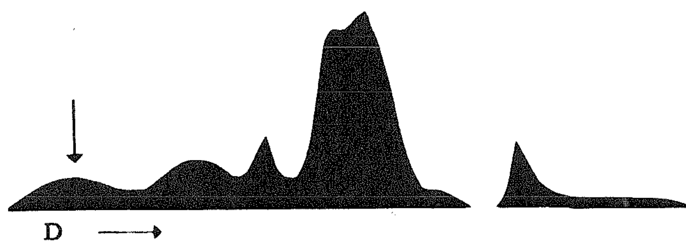
Sample 48a whose electrophoretic scanning pattern is shown in Figure 7 represents the intracellular protein components in the same manner that the previous preparations represent uninfected and eclipse bacteria soluble proteins. That the departure from the usual procedure, involving dilution and an additional centrifugation, is without effect on the intracellular soluble proteins is demonstrated by the electrophoretic patterns of the control uninfected bacterial solutions

Figure 7

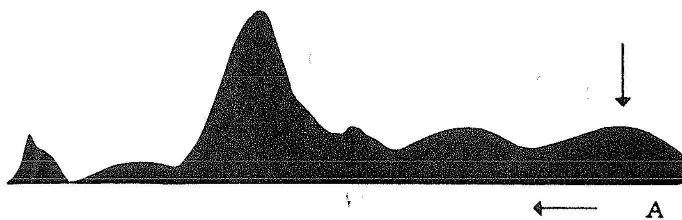


No. 48a Bacteria Infected For 100 Minutes





No. 47a Uninfected Bacteria

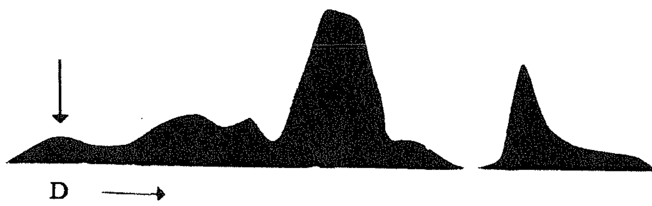


Same as figure 6 except that the preparations have been diluted and recentrifuged as described in the text. Note the absence of the slower moving new peak and the opalescence prominent in figure 6.

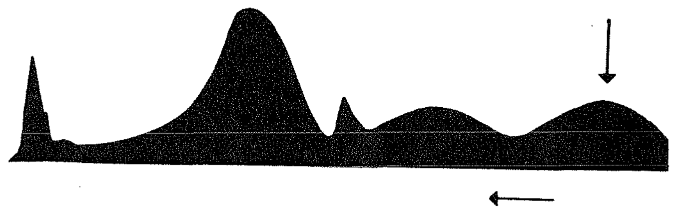
47 and 47a, prepared in parallel with solutions 48 and 48a and shown in Figures 6 and 7.

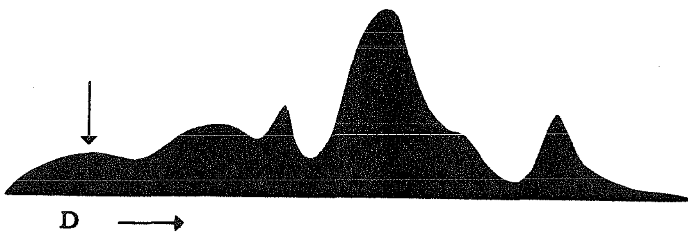
The experiment just discussed, involving a study of the soluble proteins of bacteria in the third stage of infection, was repeated in a somewhat more refined manner. In this case the infection was allowed to proceed for only 25 minutes before collection of the bacteria in the refrigerated Sharples super-centrifuge. The process of collection was completed 47 minutes after infection. Since under the conditions of the experiment, the latent period lasts approximately 40 minutes, most of the bacteria were collected before the onset of lysis inhibition. The consistency of the collected bacteria was intermediate between the paste-like qualities of uninfected and eclipse bacteria and the gel-like properties of the bacteria collected 100 minutes after infection in the experiment described last. The subsequent preparation of the experimental soluble protein solution was alike in all details to that used in previous preparations except that care was taken in this case to dilute the grindate before final centrifugation. The clear supernatant, sample #50, upon electrophoretical analysis yielded the scanning pattern shown in Figure 8. This scanning pattern again reveals the presence of a new peak with high mobility. A comparison of Figures 7 and 8 shows that the scanning patterns obtained after allowing the infection to proceed for 100 minutes in one case and 25 minutes in the other case are in

Figure 8

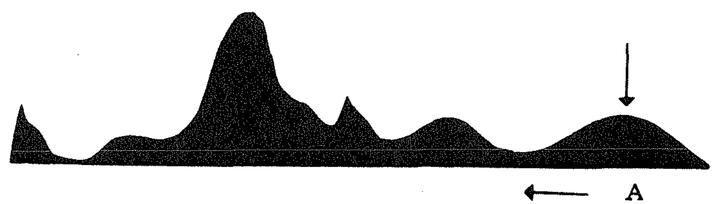


No. 50 Bacteria Infected For 25-47 Minutes





No. 49 Uninfected Bacteria



A comparison of the electrophoretic scanning patterns of the soluble proteins of bacteria infected for 25-47 minutes and uninfected bacteria. See text for further description.

general very similar, although the amount of the fast moving peak is less the shorter the infection. The conclusion is reached that the scanning patterns shown in Figures 7 and 8 are representative of the soluble proteins of bacteria in the third stage of infection regardless of whether they are or are not lysis-inhibited.

When these two experiments are considered and the electrophoretic behaviour of the proteins of the bacteria in the third phase of infection is compared with similar protein preparations obtained from the eclipse stage of infection and from non-infected bacteria, it is seen that radical differences are present. The most notable and marked difference is the appearance of a large amount of apparently homogeneous material with a high mobility in the solutions extracted from the bacteria in the third stage of infection. The sharpness of this component coupled with its very high mobility strongly suggests that this material is a nucleic acid, since most proteins give a more diffuse peak and have lower mobilities under the same conditions of electrophoresis (Abramson, Moyer, and Gorin, 1942). The nature and composition of the material represented by the spike-like fast moving peak was the subject for further experiments reported in this thesis. Therefore, further discussion of this matter will be deferred until other experimental data have been presented.

In previous experiments, it was shown that a fast moving component, present in the extracts of uninfected bacteria, disappeared from the scanning patterns obtained

from bacteria in the eclipse stage of infection. In this series of experiments it is shown that a component similar in mobility appears in high concentration later during the course of the infection.

C. The Disruption of Phage and the Resolution of Components Extracted from Phage.

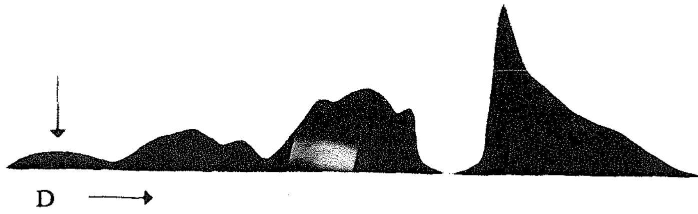
Since the principal difference in the patterns of the various bacterial soluble proteins is the appearance of a new, spike-like, fast-moving peak in the extracts of the bacteria in the third stage of infection, an effort was made to further elucidate the significance of this change in the electrophoretic patterns. Newly-synthesized, intact phage appear in infected bacteria after the end of the eclipse. The question arose, therefore, as to whether the new component appearing in the electrophoretic patterns is a bacterial component arising as the result of phage infection or whether it represents a component of the intracellular bacteriophage. The possibility that the new peak represents intact phage extracted from bacteria by colloid mill treatment is ruled out because previous centrifugal treatment of the preparation should have removed particles as large as phage, and because intact phage particles have a much lower mobility under similar conditions of electrophoresis than the new peak, as demonstrated by Putnam, Kozloff, and Neil (1949) and confirmed later on in this thesis.

The fact that the appearance of the new peak is

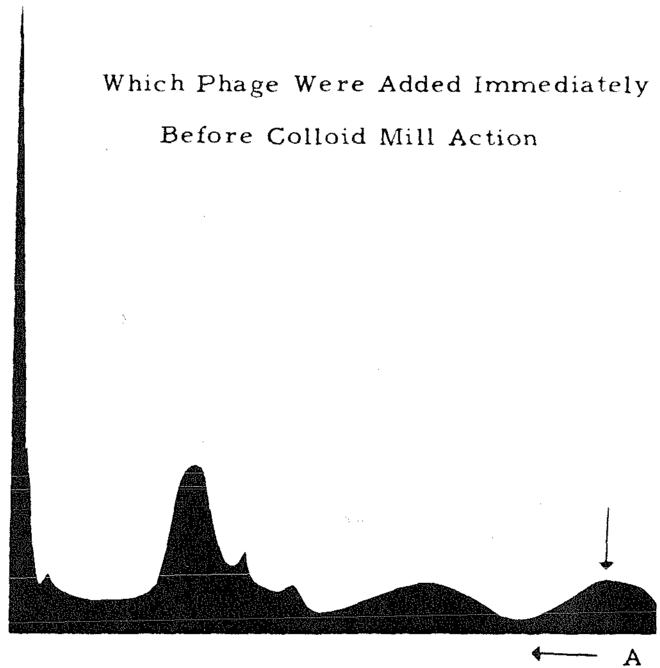
closely associated with intracellular phage particles is clearly demonstrated by the following experiment. To a paste of uninfected bacteria a concentrated phage suspension containing one hundred times as many phage particles as bacteria was added immediately before subjecting the mixture to colloid mill action. Such a ratio of phage to bacteria simulates the number of intracellular phage particles present in bacteria during the third phase of infection. The grindate, obtained after the colloid mill action, was subjected to the same centrifugal procedures applied to previous preparations yielding a clear supernatant solution. This solution, sample 54, upon electrophoresis gave the scanning pattern shown in Figure 9. Comparison of the pattern of sample 54 with those obtained from samples 50 and 48a, shown in Figures 8 and 7 respectively, shows that the new fast moving peak appears when a mixture of phage particles and bacteria are subjected to colloid mill action regardless of whether the phage particles are intracellular or extracellular. Also pictured in Figure 9 is the scanning pattern of sample 53, representing the soluble proteins obtained from a paste of bacteria to which no phage particles had been added prior to colloid mill action. This pattern is similar to other scanning patterns of uninfected bacteria. The conclusion drawn from this experiment is that the new peak is derived from intracellular phage particles which have been subjected to colloid mill treatment.

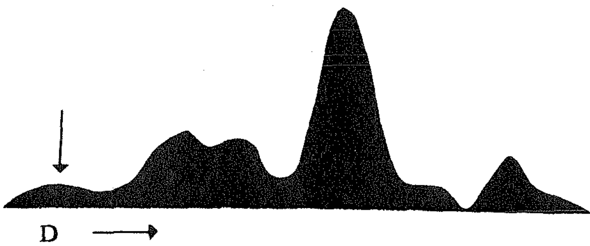
Figure 9

A comparison of the electrophoretic scanning patterns of the soluble proteins of uninfected bacteria and uninfected bacteria to which phage were added immediately before colloid mill action. See text for further description.

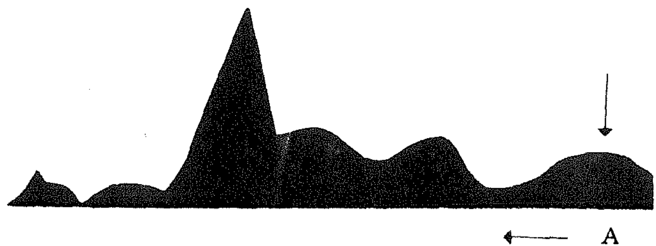


No. 54 Uninfected Bacteria To Which Phage Were Added Immediately Before Colloid Mill Action





No. 53 Uninfected Bacteria

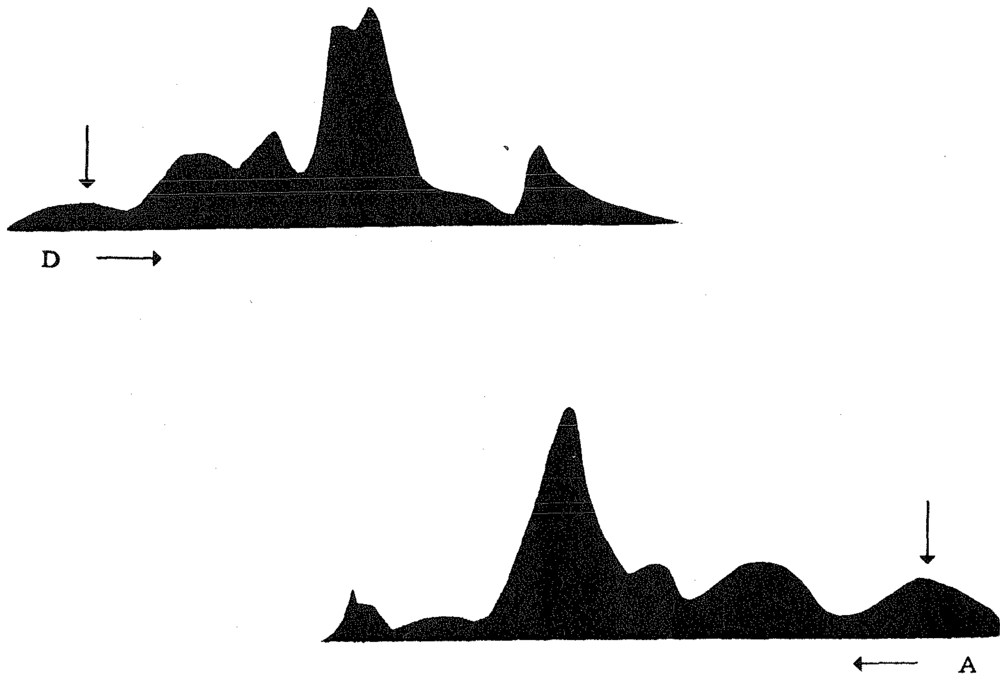


The next experiment was undertaken to prove conclusively that colloid mill action was necessary for the liberation of the material represented by the fast-moving peak. In the previous experiment, intact phage particles were added to intact bacteria just prior to colloid mill action. The possibility was present that the colloid mill disrupted only the bacteria and that some product of bacterial disruption was capable of disorganizing the bacteriophage in such a manner that material represented by the fast-moving peak was released into the solution. To test this possibility, intact phage particles were added to an uninfected bacterial suspension just after it had been subjected to colloid mill action. This mixture was allowed to warm up to room temperature and to remain thus for thirty minutes. The mixture was then cooled down to 2°, the working temperature for all of the experimental procedure in this thesis, and was subjected to the same centrifugal procedure used in previous experiments, 20,000 x g for two hours, which is sufficient to clarify the solution and also to sediment the intact phage particles. The resultant solution, sample 56, upon electrophoresis gave the scanning pattern shown in Figure 10. This scanning pattern is similar to those obtained from the soluble proteins of uninfected bacteria, and does not contain as much of the fast moving component as the scanning patterns of the experiments in which the phage and bacteria had been subjected to colloid mill action, thus demonstrating that colloid mill action is necessary for

release of the fast-moving component from the bacteriophage.

The question next arose as to whether phage particles alone in the absence of bacteria could be made to yield this new peak by colloid mill action. To settle this point, a purified phage concentrate was prepared, subjected to colloid mill action, and centrifuged at the usual gravitational field of 20,000 x g for 2 hours, which is sufficient to remove the intact phage particles from the suspension. The clear supernatant was analyzed by electrophoresis and the results obtained are shown in Figure 11. The scanning pattern demonstrates the presence of the material represented in electrophoresis by a fast-moving spike, showing clearly that colloid mill action on phage particles in the absence of bacteria is capable of liberating this material from the bacteriophage particles. In addition to the fast-moving spike, a slower more diffuse boundary is seen in the scanning pattern of colloid mill treated phage particles. That this component does not represent intact phage particles is quite certain since the preparation had previously been centrifuged at a force sufficient to sediment phage, and the mobility of this peak is different from intact phage particles as can be seen in Figure 11. This figure shows, in addition to the two peaks actually observed in the scanning pattern, the normal position intact phage particles would occupy under these electrophoretic conditions. The normal position of the phage particles was taken from the next experiment to be described. The foregoing experiment suggests strongly that the colloid

Figure 10



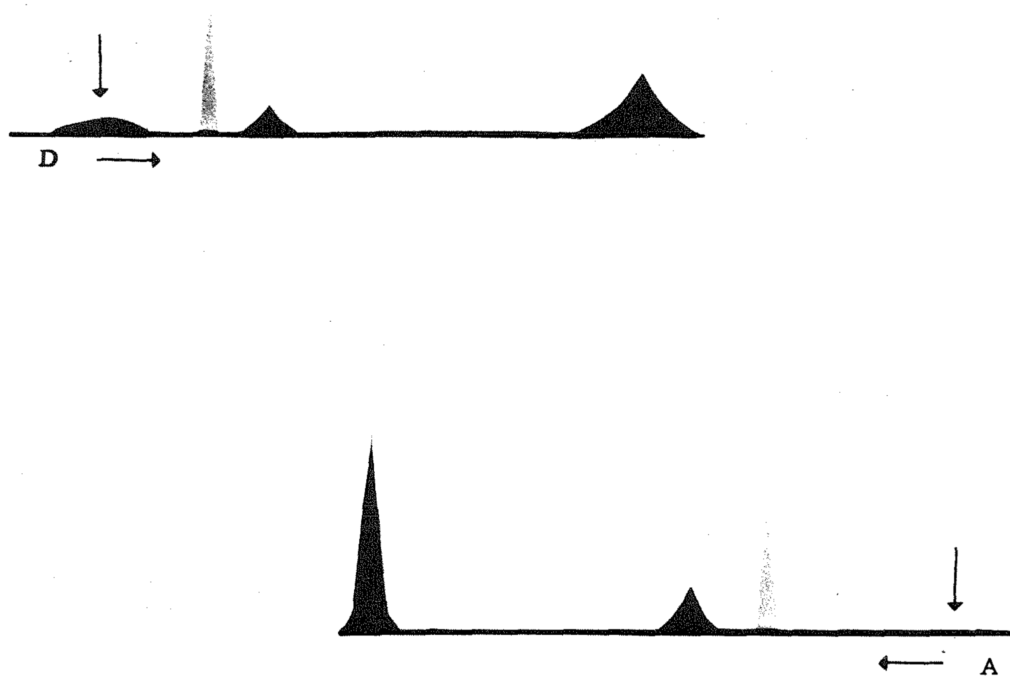
No. 56 Electrophoretic scanning pattern of the soluble proteins of a bacterial grindate to which phage had been added and then removed after 30 minutes at room temperature.

mill in addition to being capable of disrupting bacteria is also able to disrupt the much smaller bacteriophage particles.

Since the electrophoretic scanning patterns of disrupted, centrifuged phage particles revealed only two components, it was deemed necessary to confirm such simplicity of organization of the phage disruption products by some independent method. The analytical ultracentrifuge was chosen for such confirmatory analysis since the protein components in a solution are separated on the basis of mass and density by this experimental tool in contrast to separation on the basis of electric charge distribution achieved in electrophoresis. The results of the ultracentrifuge analysis of the phage disruption products, shown in Figure 12 again reveal the presence of only two components. One of the components is relatively large in mass and is estimated to be of the order of one-tenth to one-half the mass of intact bacteriophage. The other component, much smaller in mass, is represented in the patterns by a very sharp spike. This component has been the subject of further experiments to be discussed in a later section of this thesis. It appears, then, from both electrophoretic and ultracentrifugal analysis that only two kinds of high molecular weight components are present in the solutions obtained by disruption of bacteriophage particles.

The possibility still remained, however, that an equilibrium existed between the substance represented by the sharp, fast-moving peak and intact phage particles. Putnam, Kozloff, and Neil (1949) on examining purified phage sus-

Figure 11



No. 55 Electrophoretic scanning pattern of the soluble high molecular weight components obtained from disrupted phage. The peak with lighter shading indicates the position intact phage would occupy under these electrophoretic conditions.

Protein concentration 0.14%

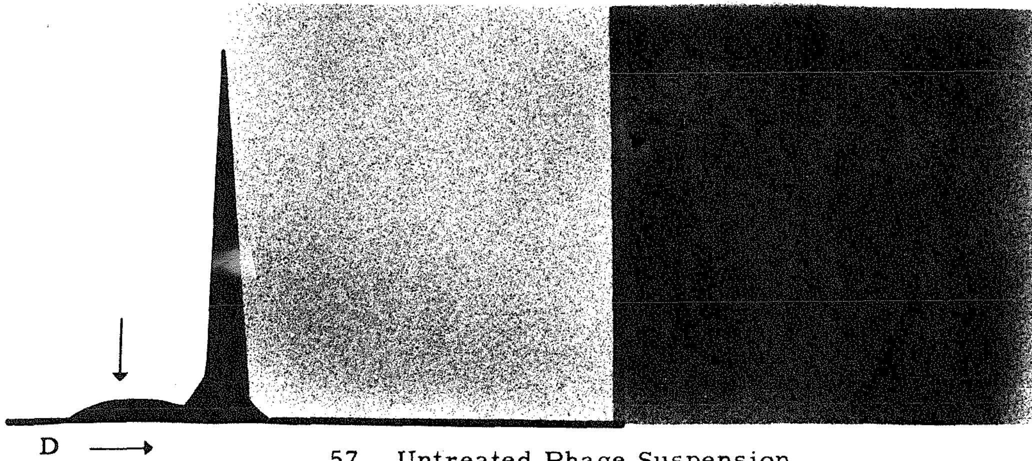
pensions of a similar phage, T6, noted that a small fast-moving peak was always present in electrophoretic scanning patterns of purified phage particles in addition to the main phage boundary. To eliminate the possibility that the appearance of the new peak was an equilibrium phenomenon, a purified phage concentrate was examined by electrophoresis before and after colloid mill action without introducing the complication of centrifugation designed to remove the intact phage particles. The scanning patterns of this experiment are shown in Figure 13. Since high titer, opalescent phage suspensions were used in this experiment the usually high optical resolution obtained in the electropheresis apparatus was interfered with. However, the point to be made from this experiment is evident in the scanning patterns shown. As a control, examination of the ascending pattern of unground phage reveals the presence of three areas of different intensities of shading. The first and darkest area represents the distance through which the front-moving boundary of intact phage has moved during the period of the application of the electric field to the solution. The second area of lighter shading combined with the first darker area represents the distance through which the front moving boundary of some unknown material, which perhaps is the same as that noted by Putnam, Kozloff and Neil (1949), has moved. However, when the phage suspension is first subjected to colloid mill treatment, the ascending scanning pattern of the phage preparation shows the absence of the first area of darker shading.

Beyond the area of lighter shading, the fast-moving spike previously noted is clearly visible. An examination of the descending patterns shows that the phage peak is reduced to approximately one-third its original size as the result of colloid mill treatment. The activity of the phage suspension was reduced correspondingly from 9×10^{12} infective particles per cc. to 3.2×10^{12} infective particles per cc. We conclude that the new fast moving spike noticed on the electrophoretic scanning patterns is indeed a component of bacteriophage particles which has been released as a result of phage particle disruption by colloid mill action.

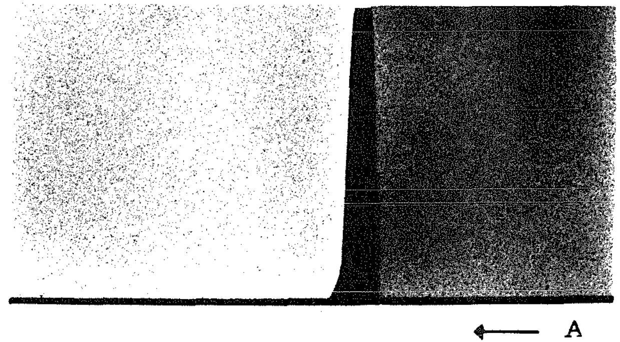
The remainder of the experimental work of this thesis is devoted to the isolation and characterization of one of the components released from phage by colloid mill action. It is perhaps appropriate at this point to review the work of other authors who in one way or another have disrupted bacteriophage organization. Anderson (1949) and Anderson et al. (1948) have demonstrated that certain strains of phages are inactivated when they are rapidly diluted into distilled water from a concentrated salt solution or when they are subjected to intense sonic vibrations. The suspensions, when viewed in the electron microscope, reveal the presence of phage "ghosts". These "ghosts" consist of a thin membrane with the tail still attached but the usual internal morphology associated with the head is absent. Dulbecco (1950) has recently observed that when certain phage suspensions are given very high doses of ultraviolet light, they

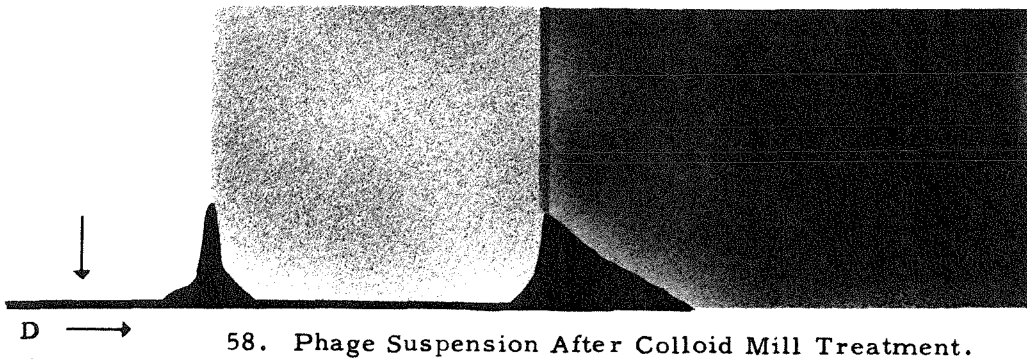
Figure 13

A comparison of the electrophoretic scanning patterns of untreated phage and phage after being colloid milled.

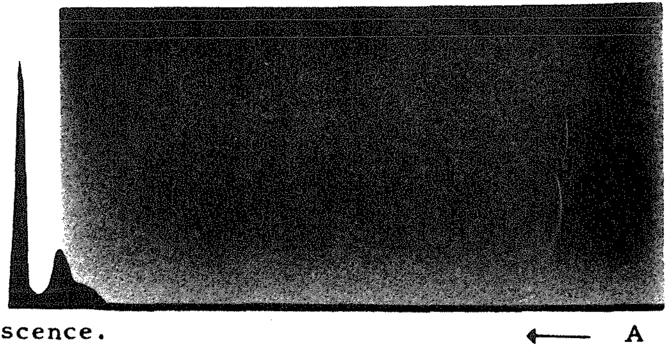


57. Untreated Phage Suspension.





58. Phage Suspension After Colloid Mill Treatment.



The shading indicates degrees of opalescence.
Protein concentration 0.41%.
The untreated phage suspension contained 9.0×10^{12} infective particles per cc.
The colloid milled phage suspension contained 3.2×10^{12} infective particles per cc.

liberate free nucleic acid into the medium. It is uncertain whether these disruptions involve something like the breaking of nucleic acid-protein bonds or whether they involve the disruption of a limiting membrane. Whether osmotic shock, sonic vibrations, ultra-violet light, and finally colloid milling all disrupt the phage organization in the same manner is another problem which awaits solution.

D. Comparison of Bacterial and Phage DNA.

Since the appearance of the sharp spike in the electrophoretic patterns of the bacteria in the third phase of infection could be attributed to disruption of intracellular phage and since a method for disruption of bacteriophage was available, an attempt to isolate and characterize the material represented by the fast moving electrophoretic spike was undertaken. The isolation of this material in a homogeneous state, as demonstrated by electrophoretic and ultracentrifugal analysis, proved to be a relatively simple task. Ultracentrifugal analysis of the phage grindate suggested that all of the material in the solution except that represented by the sharp spike in the ultracentrifuge pattern could be sedimented at a centrifugal force possible to attain in the Model L Spinco preparative centrifuge which was available.

A phage grindate prepared according to previously described methods was centrifuged for one hour at 108,000 x g. The clear supernatant remaining after centrifugation was analyzed in both the Tiselius apparatus and in the analytical

ultracentrifuge. The results of these analyses are shown in Figure 14. Only one sharp spike is present in each pattern. Thus, the correspondence between the sharp peaks observed in the patterns of the two analytical methods employed is established. The fact that only one peak is observed in each analytical pattern is taken as evidence that the material represented by this peak has been effectively isolated and purified.

Proof that the isolated material is DNA was obtained by performing the Dische cysteine hydrochloride test, determining the dry weight of the solution, analyzing the material for nitrogen and phosphorus content, and determining the ultra-violet absorption spectrum. The results of the first three analyses are listed in Table III while the ultra-violet absorption spectrum is shown in Figure 15.

It can be seen in Table III that DNA was present in the test solution by the Dische test. It will be noted further that the value obtained for the dry weight of material in the solution agrees with that obtained for the amount of DNA in the solution. This agreement attests the purity of the solution. The values obtained for Nitrogen, Phosphorus, and the Nitrogen/Phosphorus ratio agree well with other such determinations made on DNAs from other sources (Chargoff and Zamenhoff, 1948). The ultra-violet absorption spectrum obtained is that typical for a nucleic acid.

Table III

Analysis of a Solution of Phage DNA

Determination	Amount of Material in Solution	% of Dry Weight
Dry weight	3.6 mg/ml	
Dische cysteine hydrochloride	3.6 mg DNA/ml	100
Nitrogen	500 mg N/ml	13.9
Phosphorus	260 mg P/ml	7.2

N/P Ratio = 1.9

Figure 14

Electrophoretic and ultracentrifugal scanning patterns of phage DNA showing the presence of a single component by both methods of analysis.

Migration time, 200 minutes. The diffuse material moving slower than the spike is believed to be due to convective disturbance.

A. 900 r.p.s., 0 minutes after speed was reached.
B. 900 r.p.s., 20 minutes after speed was reached.
C. 900 r.p.s., 40 minutes after speed was reached.

DNA concentration 0.36%.

We conclude that a single substance is present in the purified solution and that this substance is DNA. It should be pointed out here that an easy method has been developed for the isolation and purification of a presumably native desoxyribonucleic acid. This method allows for the isolation of appreciable quantities of this material without the necessity of dehydration in the course of its preparation.

Other workers (Kahler, 1948; Tennent and Vilbrandt, 1943; Cecil and Ogston, 1948) working with thymonucleic acid noted that the sedimentation constant of this material upon analysis in the ultracentrifuge is extremely dependent upon the concentration of the material. A study was undertaken to see whether phage DNA also exhibited this behavior and to determine to what extent its sedimentation characteristics compare with that of thymonucleic acid as determined by the above mentioned workers. The sedimentation constants obtained at a variety of concentrations of phage DNA are listed in Table IV. The data show that the sedimentation constant of phage DNA is concentration dependent. In Figure 16 is plotted the reciprocal of the sedimentation constants obtained during the course of the experimental work as a function of concentration. On the same figure are plotted the curves obtained by other workers for thymonucleic acid. It will be noted that whereas the various preparations give similar zero extrapolated sedimentation constants, the slopes of the lines from which these constants were extrapolated are markedly different.

Table IV

Sedimentation Constants of Phage DNA
at Various Concentrations

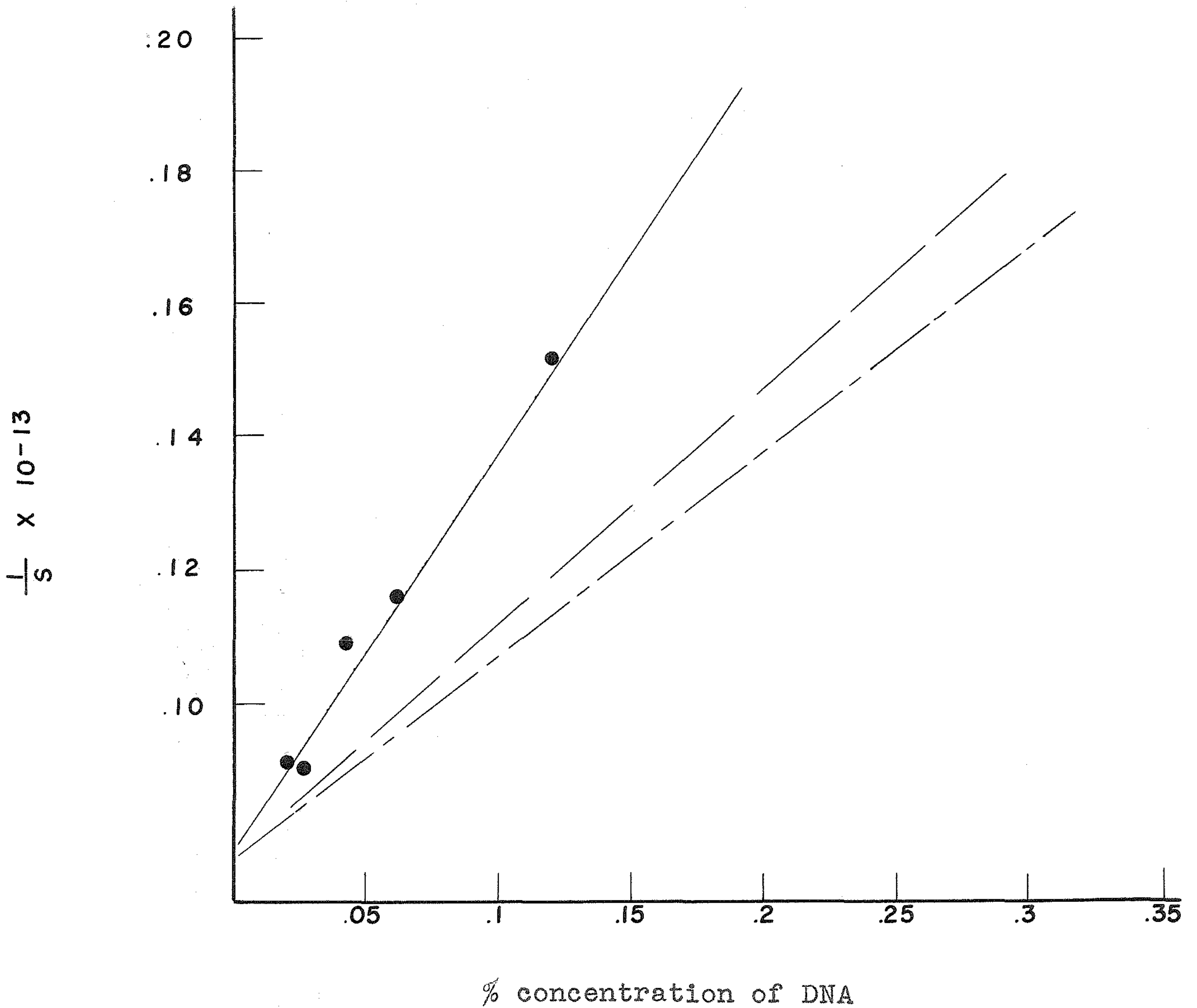
Concentration of DNA %	S_{20}^{B} * 10^{-13} Svedberg Units	1/S
.36	4.49	.222
.12	6.61	.151
.06	8.73	.115
.04	9.31	.108
.024	11.27	.089
.02	11.11	.090
.00 #	13.00	.077

* S_{20}^{B} designates the sedimentation constant at 20°C. in 0.2 ionic cacodylic acid buffer, pH 6.73.

Extrapolated from the curve shown in Figure 16.

Figure 16

The Concentration Dependence of the Sedimentation Constant of DNA



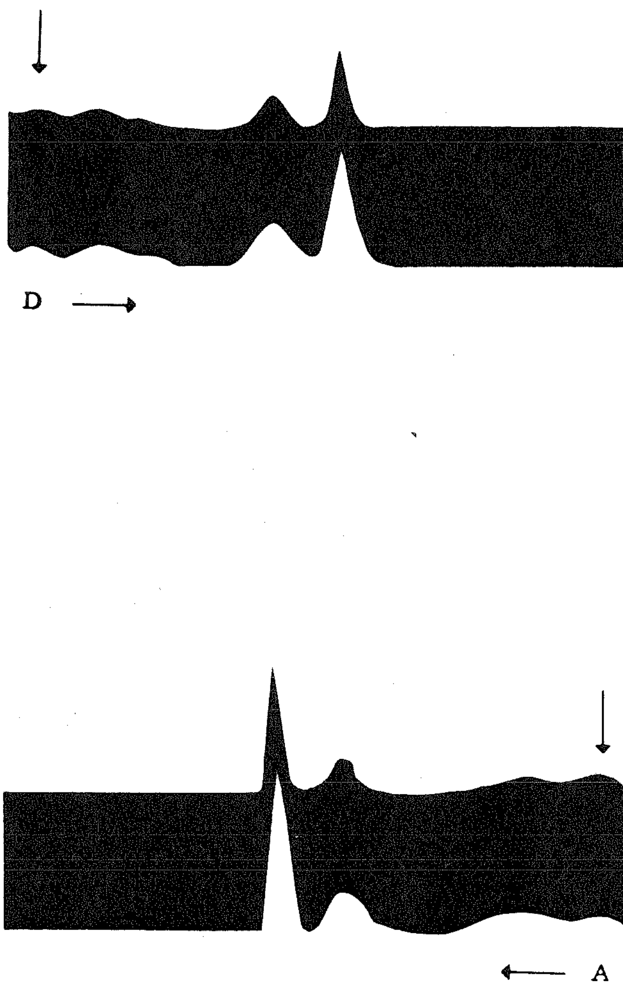
The evidence of Kozloff, Putnam, and Evans (1950) that phage DNA is derived in some manner from bacterial DNA has already been presented and discussed in the literature review section of this thesis. Since the evidence indicates that some sort of transfer takes place, it was thought of interest to compare DNA isolated from bacteria with that isolated from phage. An attempt was made to isolate bacterial DNA by the method used to obtain phage DNA. A grindate of uninfected bacteria, prepared in the usual manner, was centrifuged at 108,000 x g for five hours at which time no further sediment appeared on continued centrifugation. When the clear supernatant was examined both electrophoretically and ultracentrifugally, a considerable amount of residual protein was still present.

The method found to be successful for the isolation of bacterial DNA was that of Sevag, Lackman, and Smollens (1938). The bacterial grindate, after having been centrifuged at 108,000 x g for five hours, was shaken vigorously with an equal volume of a solution consisting of 35 parts of chloroform and 10 parts of amyl alcohol. Under these conditions, the protein in the experimental solution forms a chloroform-protein gel complex while the DNA remains in solution in the aqueous phase of the mixture. Upon centrifugation of the mixture, three layers are formed in the centrifuge tube. The bottom layer consists of the chloroform-amyl alcohol mixture, the middle layer comprises the chloroform-protein gel complex, while the aqueous phase

containing the DNA forms the uppermost layer. The aqueous phase is decanted or pipetted off and is again mixed with chloroform-amyl alcohol solution and the shaking and centrifuging process is repeated until no more chloroform-protein gel complex is formed. This process was repeated sixteen times, at the end of which there was still a minute amount of complex being formed. The aqueous phase was then subjected to negative pressure to remove the last traces of chloroform and amyl alcohol. After this, the solution was dialyzed against buffer and was analyzed by electrophoresis and in the ultracentrifuge. The results are shown in Figures 17 and 18 where it can be seen that most of the protein had been removed from solution. The scanning patterns of both the electrophoretic and ultracentrifuge analyses reveal the presence of two components. The faster moving component in the electrophoretic pattern is probably the bacterial DNA since it has the same mobility and spike-like character as phage DNA. The faster sedimenting material in the ultracentrifuge pattern is very likely the bacterial DNA because of the sharpness of this peak. The slower sedimenting material is thus taken to be the lower mobility component noticed in the electrophoretic scanning pattern.

A positive Dische test was observed for the bacterial preparation indicating that the solution did in fact contain DNA. The quantitative results of the Dische test compare well with the amount of DNA estimated to be present in the solution by a measurement of the area of the fast moving peak in the electrophoretic pattern of the bacterial

Figure 17



The electrophoretic scanning pattern of the bacterial DNA solution prepared as described in the text.

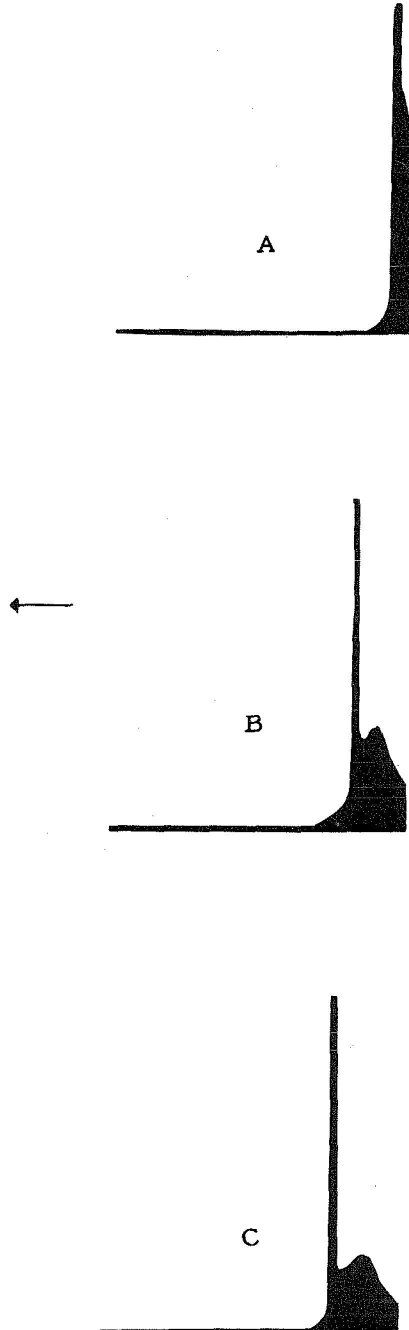
The fastest moving peak represents bacterial DNA. Several impurities are present. Analysis performed in the Perkin-Elmer electrophoresis apparatus.

Buffer, 0.2 ionic cacodylic buffer. pH 6.73 Time of migration, 67 minutes.

Concentration, 0.17% DNA. E, 8.17 volts/cm.

Figure 18

Ultracentrifugal scanning patterns of the bacterial DNA solution prepared as described in the text. The spike represents bacterial DNA. A slower sedimenting impurity is present.



A. 900 r.p.s., 2 minutes after speed was reached.

B. 900 r.p.s., 25 minutes after speed was reached.

C. 900 r.p.s., 37 minutes after speed was reached.

DNA preparation as seen in Table V. It is considered that bacterial DNA has been partially isolated by the methods described and that this DNA is identified in the scanning patterns of electrophoretic and ultracentrifugal analyses.

It will be recalled that a fast-moving peak observed in the electrophoretic scanning pattern of the soluble proteins of uninfected bacteria was diminished in the eclipse stage of infection. The material isolated as bacterial DNA has the same mobility as the component which disappears from the eclipse bacteria. It is therefore considered as highly probable that DNA disappears as a freely moving entity in the soluble proteins extracted from bacteria during the eclipse.

Since the bacterial DNA and phage DNA exhibited several common properties, i.e., sharpness of peak in both electrophoresis and ultracentrifuge, similar mobility, and similar sedimentation constant, an attempt was made to determine whether differences in the two materials could be detected by the most sensitive physical methods immediately available. Two methods were used both of which involve attempts to resolve the two substances in a mixture; one by electrophoresis, the other by analytical ultracentrifugation.

The dependence of the electrophoretic mobility on pH has been determined for thymonucleic acid by Creeth, Jordan, and Gulland (1949). Figure 19, taken from their paper, summarizes their results. This graph demonstrates that the mobility of thymonucleic acid changes with the pH

The Identification of the Faster Moving Peak in the Electrophoretic Scanning patterns of the Bacterial DNA Solution with DNA

Exp.#	DNA Concentration as determined by Dische test: %		Area[1] in electrophoretic scanning pattern under:		Area under fastest peak / Total concentration of DNA
	Phage	Bacterial	Slower Peak	Faster Peak	
PEIII	.072			174	2410
PE IV		.17	268	410	2410
PE V	.09	.085	134	422	2405

1 Area in arbitrary planimeter units.

The fact that the ratios in the last column are constant shows that the faster moving peak in the electrophoretic scanning patterns of the bacterial DNA solutions represents bacterial DNA.

throughout most of the pH range. It was deemed necessary, therefore, that attempts to resolve the phage DNA from bacterial DNA by electrophoresis should be performed at at least two different pHs, since it is possible that at any one pH the mobilities would be the same while the pH-mobility curves of the two nucleic acids might be somewhat different. The experiments were performed at pH 6.9 and 4.2. The results are pictured in Figures 20 and 21. These experiments were carried out in the Perkin-Elmer electrophoresis apparatus. Due to the rapid migration of the boundaries and the smaller size of the electrophoresis cells employed, the buffers used were made up to 0.2 ionic strength with NaCl instead of the 0.1 ionic strength buffers previously employed, in an attempt to eliminate convective disturbances and to allow the migration to continue for a longer period of time than otherwise possible. The results show that the DNA from phage and the DNA from uninfected bacteria migrate as a single peak at both pHs.

The mixture experiments performed in the analytical ultracentrifuge were carried out at a number of different total concentrations. In Figure 16 the reciprocal of the sedimentation constant is plotted as a function of the concentration, as obtained by various workers with different preparations of thymonucleic acid. It will be recalled that although the extrapolated sedimentation constants at zero concentration are all rather similar, the slopes of the

Figure 19

Dependence of the Electrophoretic Mobility of Thymonucleic Acid on pH

(Redrawn from Creeth, Jordan, and Gulland: 1949)

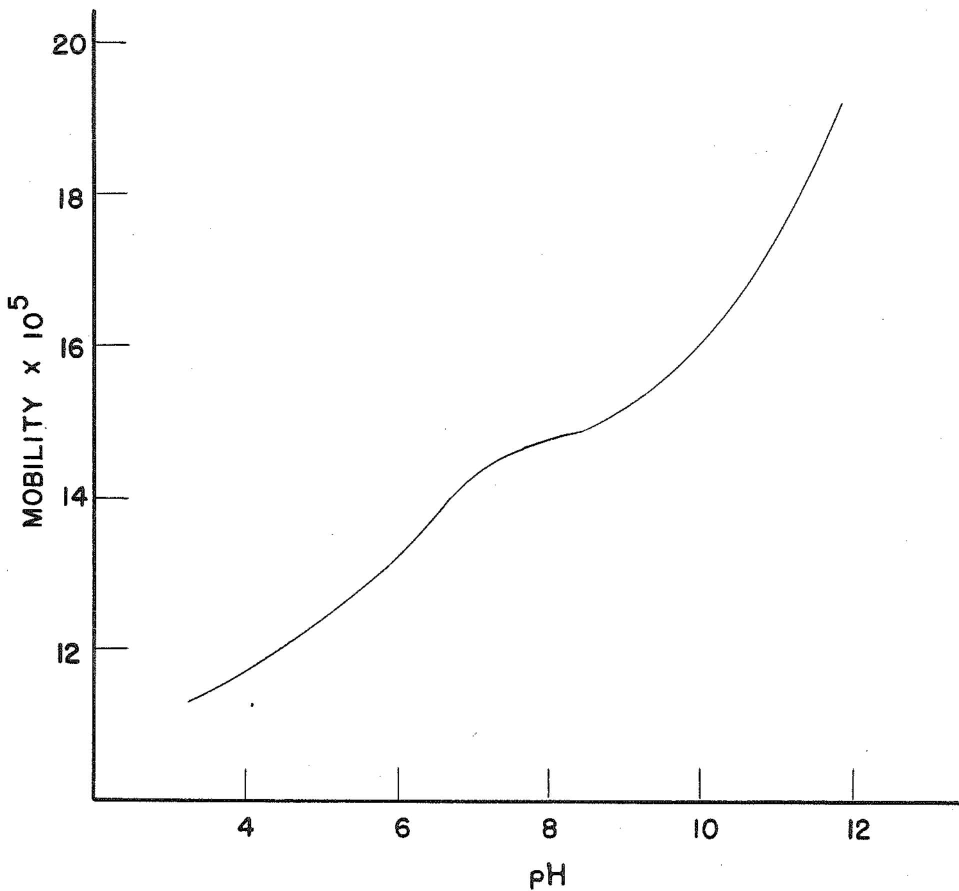
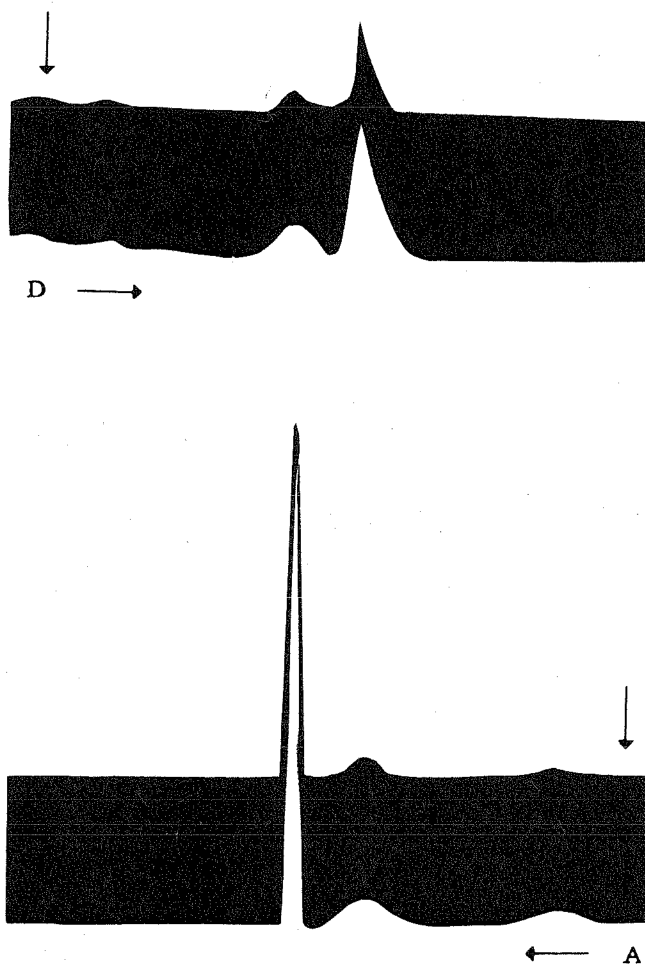


Figure 20

Electrophoretic scanning pattern of a mixture of bacterial and phage DNAs.

pH 6.73.



The sharp peak represents the mixture of DNAs. The other peaks represent the impurities in the bacterial DNA solution.

concentration, .085% bacterial DNA E, 8.17 volts/cm.
.09% phage DNA.

buffer, 0.2 ionic cacodylate

Migration time, 67 minutes.

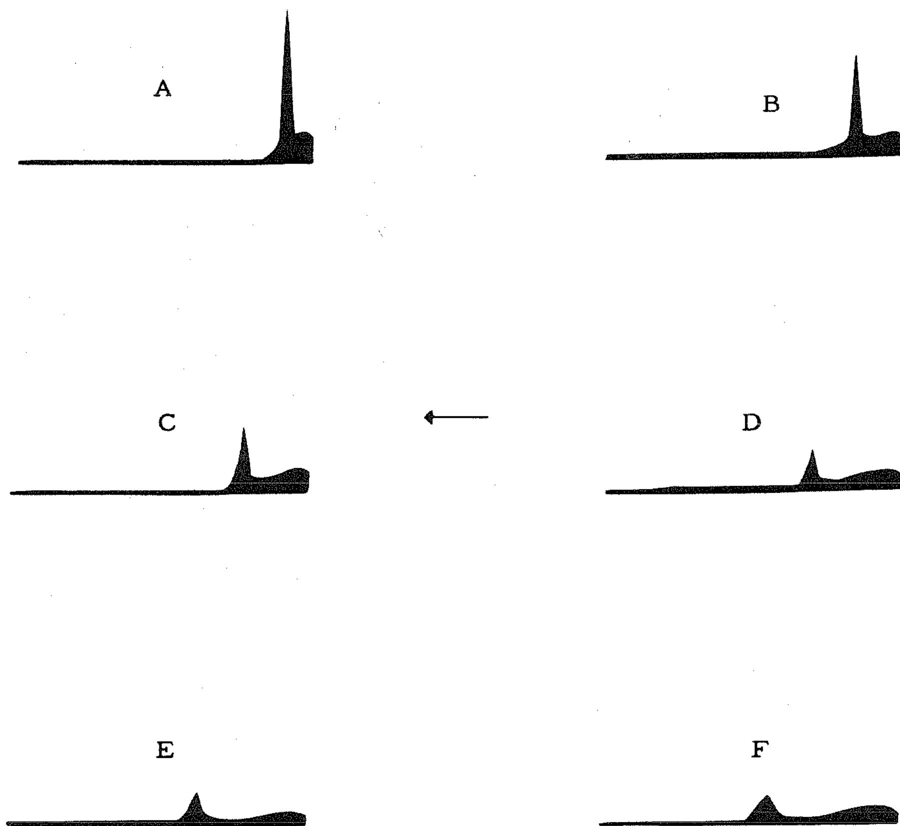
straight lines obtained in such plots differ significantly from one preparation to another. Therefore, the most sensitive method to use in attempting to resolve the two DNAs by the analytical ultracentrifuge is to do a series of experiments with mixtures of the two DNAs in roughly equal amounts at different total concentrations in order to account for the possibility that although at one concentration the sedimentation constants might be the same at other concentrations they might be different. Figures 22 and 23 show the results obtained in two mixture experiments at two different total DNA concentrations. In neither case is there evidence of a resolution of the spike into two components.

The results of both the electrophoresis and ultracentrifuge mixture experiments indicate that the bacterial and phage DNAs are very similar insofar as their physical properties are concerned. The possible significance of this finding will be discussed in the next section of this thesis.

Figure 22

Ultracentrifugal scanning patterns of a mixture of bacterial and phage DNAs.

Concentration .03% bacterial DNA
.03% phage DNA



900 r.p.s.

Times in minutes after speed was reached

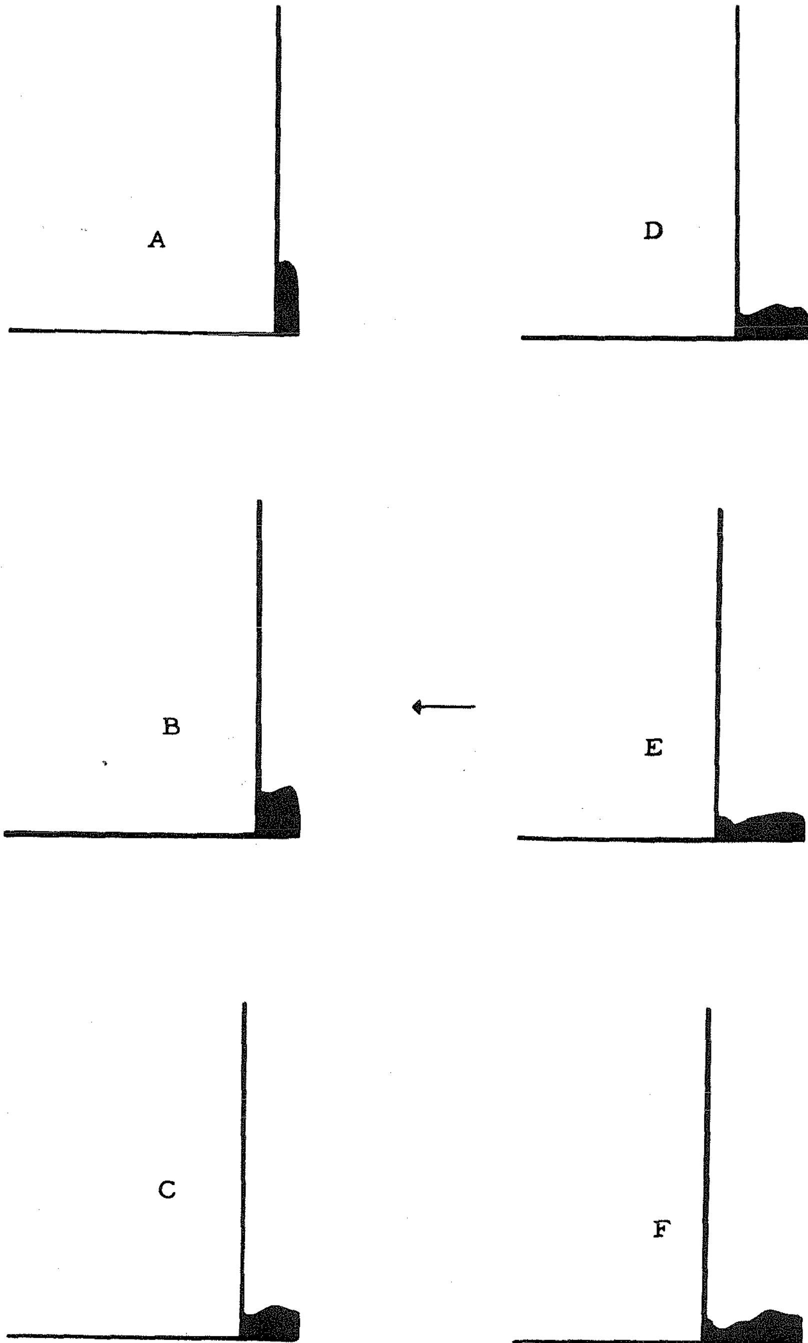
A-4, B-13, C-22, D-32, E-41.5, F-49

Buffer, 0.2 ionic cacodylate pH, 6.73

Figure 23

Ultracentrifugal scanning patterns of a mixture of bacterial and phage DNAs.

Concentration .09% bacterial DNA
.09% phage DNA



900 r.p.s.

Time in minutes after speed was reached.

A-4.5, B-14.5, C-27, D-35, E-43, F-52.5.

Buffer, 0.2 ionic cacodylate pH, 6.73

IV. DISCUSSION

The comparatively well studied host-virus system consisting of the bacterium Escherichia coli strain B and one of the phages which attack it, T2, has been utilized in this thesis for an experimental approach relatively new to biology. This approach involves the detection of changes induced by the virus in the soluble proteins extracted from bacterial protoplasm by using a combination of physical and chemical methods. This experimental approach has yielded valuable information relating to such diverse biological problems as plant virus synthesis (Wildman, Cheo, and Bonner 1949) and muscle contraction (Dubisson, 1950). In the present problem four distinct findings have been obtained:

1. It is possible to disrupt bacteria and to extract and resolve their high molecular weight substances.

2. At least some of the DNA in the soluble protein extracts obtained from uninfected bacteria migrates as a freely moving entity in an electric field, while extracts prepared in a similar manner but obtained from infected bacteria poisoned prior to the end of the eclipse contain very little, if any, free-moving DNA.

3. It is possible to disrupt phage particles and to recover the products of such disruption. Disrupted phage suspensions yield two components, which can be distinguished both in the electrophoresis apparatus and in the ultracentrifuge. One of these is pure DNA, as shown by chemical tests.

4. DNA isolated from phage particles and from uninfected host bacteria have very similar physical properties.

At the present time any scheme of phage replication is of necessity speculative in nature. An admittedly speculative description of the course of a phage infection has recently been presented by Luria (1950) in an attempt to combine all of the current knowledge of the coli-phage system into a consistent scheme and to point out the gaps in our knowledge of reproduction of the phage. The findings of this thesis will be discussed insofar as they can be applied to the general course of virus infection as it has been elucidated to date by a mass of experimental work and also in the light of the current theories of phage replication.

Electrophoretic analysis obtained from both uninfected and infected bacteria reveal the complexity of the bacterial soluble protein organization compared with that found in a plant leaf. In a system of such complexity only extremely gross changes might possibly be detected and changes of vital interest might be obscured in the multicomponent system. At the outset of the work it had been hoped that the system might be of a comparatively less complex nature, such as that found in the plant leaf, and that such a system would permit the identification and characterization of phage intermediates present in the bacteria during the eclipse. Although the hopes did not materialize, several observations were made during the course of the work which provide circumstantial evidence about the

nature of the postulated phage intermediates.

The finding that no gross changes take place in the soluble protein composition of bacteria during the eclipse should not be too surprising since the indications from several lines of attack are that phages are synthesized by the enzymatic equipment of the bacteria. It should be possible, however, by a refinement of technique, to identify phage intermediates. The soluble protein extracts of the bacteria could be further defined by additional electrophoretic and ultracentrifugal analysis at a series of concentrations and pHs. Further information might be gained by a study of fractions of the soluble protein mixture instead of, as has been done in this thesis, examining the mixture in its entirety. Finally Luria (1950) has suggested the use of specific immunological reactions for the isolation of phage intermediates. These suggested refinements in technique were not resorted to in the present work since enough material was made available for immediate experimental attack through the observation of the changes taking place in the desoxyribonucleic acid organization of the bacteria during infection. These observations could be made despite the complexity of the analytical patterns because of the peculiar physical properties of DNA which consist of a very high mobility in electrophoresis at pHs above 4 and the formation of a very sharp boundary under conditions of analytical centrifugation. By studying the internal changes of a bacterium during infection valuable information has been

accumulated as to the course of virus synthesis. Further work in this direction employing a combination of physical and chemical tools may yield more insight into the biology of viruses.

The similarity of the DNA in uninfected bacteria and free phage, respectively, is subject to several interpretations. The interpretation applied depends on the nature and extent of variability found among DNAs in general. Unfortunately the state of DNA study has not reached the stage where DNAs obtained from different sources have been classified as to physical and chemical properties. If DNAs obtained from a variety of sources do indeed all appear to behave in the same manner in the analytical procedures of electrophoresis and ultracentrifugation used in this thesis, then the observation of the similarity of the phage and bacterial DNAs is of little interest. That DNAs may exhibit extreme biological specificity, however, has been adequately demonstrated by the work of Avery et al. (1944) and Boivin (1947) in the type-transforming principles of Pneumococcus and E. coli. The question resolves itself into the problem of whether such biological specificity associated with DNA is also reflected in changes in the DNA molecule which can be detected by physical and chemical means. This problem may be subject to elucidation by the adaptation of the coliphage system used in this thesis to the transforming and transformable strains of E. coli used by Boivin. Boivin has extended the basic work of the Avery school on Pneumococcus

to E. coli by the discovery that the DNA of a certain smooth strain can transform a rough strain derived from a different smooth strain into the smooth strain from which the DNA was derived. The reaction is extremely specific as regards the strains employed. If the adaptation of the biological system used in this thesis to the strains used by Boivin is possible, then it would be possible to isolate the DNAs from a variety of bacterial as well as phage strains and compare them by electrophoresis, analytical centrifugation, and for transforming ability. It should then be possible to determine whether biological differences of DNAs are reflected in physical and chemical differences. It would also be of interest to compare the nucleic acids derived from a series of phage strains which attack the same host in order to determine whether the host is responsible for the specificity of phage nucleic acid or whether the DNA is phage specific. The data accumulated by such a proposed study would probably lead to a greater insight into the biological function of DNA through a better understanding of the nature of the specificities involved.

I wish to turn now to a discussion of the finding that freely-migrating DNA largely disappears during the eclipse of infection as it may be applied to conceptions of both phage growth and DNA function in general. The Swedish school headed by Caspersson (see reviews by Caspersson, 1947, Thorell, 1947, Hyden, 1947) by entirely different

methods than those used by the author have come to certain general conclusions regarding the biological function of DNA.

Through a study of the changes in the concentration and distribution of DNA during cell division, the view has been evolved that the reproduction of the gene elements is conditioned by the presence of DNA. The observations which led to this view involve the attachment to the chromosomes of DNA at the onset of cell division and the subsequent detachment of DNA from the chromosomes at the conclusion of active cell division. RNA, in contrast to DNA, in the opinion of this group of workers is tied up in some manner with the synthesis of the cytoplasmic proteins.

Investigation of a series of animal viruses, graded in organizational complexity, has led to the view that the viruses are self-reproducing units which act as parasites upon the nucleoprotein forming parts of the host cells and that different viruses attack the nucleoprotein synthesizing mechanism of the cell at different points in the mechanism.

The notion which the Swedish school has developed as to the function of viral nucleic acid is that the more complex viruses require DNA since they contain several protein entities. The DNA is present in its capacity to organize postulated chromosome like elements of the more complex viruses and to supervise the equal distribution of the newly synthesized material to daughter viruses.

It is possible to correlate the findings of the Swedish school with those of other workers. I wish to recall the conclusion mentioned previously that when a bac-

teriophage attacks a bacterium, the bacterium ceases to synthesize its own components and proceeds to synthesize viral components. This finding fits in very well with the idea that the viruses are indeed parasites on the nucleoprotein forming parts of the host cells. The notion that a primitive chromosomal mechanism may be operating in the proliferation of the DNA-containing animal viruses finds support in the Hershey linkage groups of the bacterial viruses. Another point of interest that is pertinent to the present discussion is the recent finding by Luria (1950) that in the early stages of bacterial virus infection, there is an autocatalytic replication of genetic material followed by a phase during which recombination of the genetic units takes place. Probably only after these processes are completed do fully formed phaged particles appear which are now capable of infecting other bacteria. These findings are very suggestive of something like an intense mitotic activity of the phage parts followed by something comparable to a meiosis during which crossing over takes place.

An attempt will now be made to outline a speculative scheme of the events taking place during the infection of a bacterium by a bacterial virus. Biological significance is attached to the author's finding that DNA disappears as a freely moving entity during the eclipse. This scheme is meant to apply chiefly to the infection of Escherichia coli strain B by one of the T even phages but perhaps it is of more general significance.

Upon entering the cell, the virus breaks up into its component parts and in some manner causes the nuclear apparatus of the bacterium to disintegrate (Luria and Human, 1950). Probably by disruption of the cellular nuclear apparatus the bacterium is prevented from synthesizing further bacterial material. The component parts of phage, which are probably chromosomal in nature, start undergoing intense mitotic activity. The cellular enzymatic apparatus is immediately directed by the phage genetic complex to synthesize the building blocks which supply the necessary proteinaceous material for the intense mitotic activity. Since the component chromosomal material is rapidly increasing, a large amount of DNA is required to organize the chromosomal duplicating activity. Thus, the DNA of the bacterium becomes attached to the primitive chromosomes of the phage during the eclipse and disappears as a freely moving entity. After an initial lag, the bacterium starts synthesizing DNA, perhaps because the bacterial reservoir of DNA has been exhausted. After a time of mitotic activity, some sort of pairing and exchange of parts of homologous chromosomal material takes place and complete phage units are formed. The chromosomal material, probably after the primitive meiotic activity and before infectivity is gained by the new unit, undergoes the same sort of change that leads to interphase chromosomes, that is the DNA is released from its tight bonding with the chromosome, and thus is again found as a free-moving entity when the phage particles are disrupted.

The above exposition is subject to experimental attack in several manners. Of extreme interest would be an exhaustive study of phage constituents by a variety of methods. A method has been described in this thesis for the disruption of intact phage. Two kinds of components appear as a result of such disruption. One of these has been isolated and has been characterized as DNA. The other component has not yet been examined. It is suggested that examination of this material by chemical and physical methods may reveal relationships with classical chromosomal material of higher organisms. Electron microscopic observation might immediately reveal whether the foregoing postulates have any basis in fact by possibly revealing a chromosome-like structure for this material. Further chemical and physical characterization may possibly reveal relationships with the chromosomin of the Stedmans (1947) or the residual chromosomes of Mirsky (1947). It is realized that any material extracted from the intact phage may bear little relationship to the phage components once they have started on their reproductive cycle within the bacterial cell. However, valuable hints as to the nature of viral replication may be gained from such a study. For instance, comparisons could be made between material extracted from intact phage with material extracted from resting nuclei, actively dividing nuclei, and sperm to see what if any relationships between such materials are present, since they all represent concen-

trations of genetic material in different states.

Of equal importance is the isolation and examination of phage intermediates which appear during the course of active phage multiplication. Suggested approaches towards the isolation of such material have already been mentioned in an earlier section of this discussion. When the technical difficulties involved in such an isolation have been overcome, it will be possible to examine such material by the same methods as those suggested for the characterization of the unstudied component observed in extracts of disrupted phage.

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