COEVOLUTION

IN MODEL ECOSYSTEMS

WITH *Escherichia coli* **AND COLIPHAGE** *Lambda·virulent*

by

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ABSTRACT

The evolution of chemostat $E.\ coli$-λvir communities was studied with
the aim of clarifying the mechanism of their persistence. Under the
physical and nutrient conditions of the experiments these organisms
may coexist indefinitely but the pattern of population dynamics
varies considerably from culture to culture. Generally, the
evolution of phage-resistant bacteria stabilises the total bacterial
numbers and reduces the density of the phage population. The
latter persists, however, at very high densities and fluctuates
irregularly. Two obvious evolutionary events, the evolution of
resistance in bacteria and of a delayed-plaque-growth phenotype in
phage, were associated with major changes in population densities.
Through extensive phenotypic characterisation of the "selected" strains,
however, many more evolutionary events were traced which did not
correspond to density fluctuations in any obvious manner.

The "selected" bacterial population consisted of several distinct $mal^{\pm}$
(ability to utilise maltose) phenotypic subpopulations some of which
were totally resistant to both the "selected" and the original phages;
some were sensitive to the selected phage and to host-range mutants of,
but resistant to, the original phage (partially resistant); some
appeared resistant but acquired sensitivity in the presence of
maltose (semiresistant); some were fully sensitive to all phages.
Other characteristics of the "selected" bacteria included mucoidity,
larger cell size and longer intrinsic generation time, high spontaneous
mutability, inhibition of phage intracellular development in the
presence of mitomycin C and low frequency of lysogenisation by temperate
strains, all suggestive of evolution of their SOS-repair, recombination,

DNA metabolism and cell division gene-system.

The altered antigenic specificity, the increased sensitivity to chloroform and the increasedadsorbing ability of the "selected" phage are interpreted as pleiotropic effects of the evolution of its adsorption site. Its ability to infect partially resistant bacteria was, however, due to a second evolutionary event since the partially resistant isolates were found to block the infective process at a stage subsequent to the irreversible adsorption reaction. The evolution of the phage's adsorption site is explained as a response to the evolution of "semiresistance". A third independent response of the phage was an extraordinary increase in its burst size which was expressed specifically on the genetic background of the coexisting selected hosts. An alternative to the existing model of the genetic control of λ-development is proposed, to explain the usual variation in burst size and in frequency of lysogenization as well as the evolution of these parameters; according to this model no "decision" is taken as to which developmental pathway will be followed. The delay of the selected phage in forming plaques on all hosts is suggested to be a side effect of the evolution of phage genes which control its development.

The proposed model of λ-development predicts that, although not isolated, lysogens and plasmid-carriers do exist in such "selected" communities and may, under certain circumstances, play important evolutionary roles. However, for as long as the extracellular conditions do not inactivate the virulent variants, the latter may persist by evolving their virulence (host range and productivity of infection) in response to any evolution of resistance, or immunity, in the coexisting bacteria.

The extent and, mainly, the nature of evolution in these cultures, the conclusions of an extensive discussion of the $E.\ coli$-λvir system's

biology, as well as the conclusions of a theoretical and empirical study of "fitness" and its determinants, severely contradict fundamental assumptions and specific (for this sytem) predictions of previous equilibrium and non-equilibrium theories of ecological stability. It is proposed that coexistence of *E. coli* and λvir is achieved through continuous, random, indefinite coevolution of resistance and virulence, respectively, and that the apparently erratic deviations of densities from constancy are the ecological manifestations of the successive extinction and replacement of genotypic subpopulations.

« τά πάντα ῥεῖ τά πάντα χωρεῖ καί οὐδέν μένει »

Heraclitus

"Look abroad through nature's range

nature's mighty law is change!"

Robert   Burns

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1.1 *I N T R O D U C T I O N*

1.1.1 *Coevolution*

The idea that genetic changes in a population cause density fluctuations is an old one (Ford, 1931) but it is only since Pimentel's paper on "Animal Population Regulation by the Genetic Feedback Mechanism" in 1961 that progress has been made towards a rigorous evolutionary theory of population dynamics.   In that, and in subsequent papers (Pimentel, 1963;   1964;   1968;   Pimentel and Al-Hafidh, 1965;   Pimentel and Soan, 1970), Pimentel and his colleagues developed a theory that populations which interact through competition, predation or parasitism coevolve so as to make their coexistence more stable. The little supporting evidence of evolution in experimental host-parasite systems presented by the above authors was challenged however by Lomnicki (1971) who suggested that the observed population fluctuations are attributable, not to evolutionary changes, but rather to "strictly ecological phenomena".

Since then, the study of interactions between ecological and genetic parameters of populations has been intensified.   In addition to the genetic feedback models, which are concerned with the genetic consequences in one population of density changes in a second population coexisting with it (those of Pimentel and their mathematical elaboration by Levin, 1972), there exist models of density-dependent selection (Anderson, 1971;   Roughgarden, 1971;   Charlesworth, 1971; Clarke, 1972), which focus on the interactions between density and gene frequencies of the same population, and of frequency-dependent selection (Ayala, 1971;   Ayala and Cambell, 1974;   Kojima 1971a; 1971b;

Cockerham *et al* 1972; Jayakar, 1970; Yu, 1972) which concentrate on the interactions between genotypes in the same population or between the genotypic distribution of two populations.

Coevolutionary models, such as those of Levin and Udovic (1977), Roughgarden (1976), Slatkin and Wilson (1979) and Wilcox and Walters MacCluer (1979), are more complex in the sense that they consider at least two genetically heterogeneous populations, and they take into account all the possible types of population interactions; these are (i) intraspecific and interspecific density-dependent regulation, (ii) intraspecific and interspecific frequency-dependent selection, (iii) density-dependent selection, as well as (iv) interspecific genetic feedback loops between the densities and the gene frequencies of the coexisting populations.

Much of this complexity is explicit in Roughgarden's (1976) crude definition of coevolution as "... evolution where the fitness of each genotype in a species depends on the population size of that species and both the population sizes and gene frequencies of other species". Janzen (1980) defines coevolution more precisely as an evolutionary change in a trait of individuals in one population in response to a trait of individuals of a second population followed by an evolutionary response by the second population to the change of the first; *diffuse* coevolution occurs, according to the same author, when either, or both populations in the above definition are represented by an array of populations that generate a selective pressure as a group.

The need that Janzen (1980) points out of a precise definition of coevolution which would remove it from synonymy with the terms

"interaction", "symbiosis", "mutualism" and "animal-plant interaction" indicates the narrowness of meaning with which the term has been used. The restrictions imposed by the word "population" and by the distinction between "diffuse" and simple coevolution in Janzen's definitions seem, however, unnecessary. For two traits of individuals to coevolve, two conditions are necessary: first, that the traits are genetically heterogeneous; second, that the traits coexist in the same habitat and interact. The traits may, therefore, represent subpopulations in the same local population or Linnaean species in a community. Because, on the other hand, no two traits are isolated in nature and each trait always interacts directly or indirectly with more than one biotic component of its environment, coevolution occurs only in its diffuse mode.

The importance of coevolution in population dynamics increases with the accumulating evidence of genetic heterogeneity of natural populations. Based on the results of their theoretical study, Levin and Udovic (1977) suggested that the rates of evolution in natural ecosystems are relatively high and that selection pressures and rates of population growth are sensitive to changes in gene frequencies. Similarly, Wilcox and Walters MacCleur (1979) suggested that seemingly erratic population fluctuations may simply be the result of evolutionary change, whereas substantial evolutionary change may have relatively little effect on population size.

Despite the number of theoretical models of coevolution there remains very little experimental evidence against which these hypotheses can be tested. Of the systems in which experimentalists have tried to study coevolution the continuous cultures of bacteria and bacterio-phages have given few, but most encouraging results.

1.1.2 *Phage-Host Model Systems*

A phage-host system is an example of a community where interpetation of
the dynamics on the basis of purely ecological criteria would be
inadequate.   This was realised by Campbell (1961) who first studied
the persistence of such systems theoretically.   After examining a
simple predator-prey ecological model, Campbell noted that although
"... the simplicity of the phage-host system, together with the ease
of making measurements, suggests an ideal situation for studying
predator-prey equilibria... things are not so simple because bacteria
can mutate to phage-resistant types... Before an equilibrium can be
established the culture becomes overgrown with such mutants."
Continuing, he considered further complications such as: (i) the
rise of phage host-range mutants, which are able to infect first order
resistant hosts, and a possible continuation of bacterial mutation to
phage-resistance and phage, host-range mutation cycles; (ii) the
possibility that either the host or its phage-resistant competitor can
undergo adaptive mutations unrelated to the existence of phage;
(iii) that a temperate phage may mutate to a virulent one or *vice-versa*;
(iv) a number of other possibilities concerning the selective value of
resistance, of the immunity of lysogens to superinfecting temperate
phages, of carrying a matter and energy consuming prophage, and of
transduction mediated by a temperate, but also to a lesser extent (?)
by a virulent (Starlinger, 1958) phage.   Finally, he considered (v)
the case of reproductive (recombinational) isolation and speciation of
phages having different host-range.

A phage-host system therefore is not a simple predator-prey system
but a complex community of predators, competitors, parasites, symbiotes,
mates, extrachromosomal genetic elements, and so on, each of which

is genetically variable and potentially evolving. The simplicity and convenience of such systems, on the other hand, rests on the following: (i) both organisms have short generation times and give rise to large populations, both necessary conditions for rapid evolution; (ii) both organisms are haploid and, therefore, any genetic/evolutionary changes can be traced in the phenotype; (iii) they show no "behaviour" (such as searching for, or fighting over, food, nesting, hunting and so on) and, therefore, in a homogeneous environment, their interactions are governed by the mass-action laws; (iv) they feed upon single molecules, or cells, which eliminates the problem of resource independence; (v) the large number of biochemical and genetic techniques, developed specifically for these organisms, allows relatively easy detection, mapping and analysis of evolutionary changes while the heteroduplex-mapping technique enables visualisation of large scale evolutionary events (Szybalski and Szybalski, 1974).

The continuous culture technology (Monod, 1950; Novick and Szilard, 1950a, b; Herbert *et al*, 1956; Herbert, 1958; Bungay and Bungay, 1968; Veldkamp and Jannasch, 1972; Meers, 1973) allows for a theoretically indefinite length of life for a microbial community in a well defined area. Chemostats eliminate, or control to a minimum, complications arising from immigration, differential emmigration and spatial and temporal variation of the physical, chemical and nutrient environment, other than that caused by the community itself. Thus, chemostats allow for specific assumptions to be made about the environmental factors involved in the community's dynamics and evolution.

Chemostat populations may be expected to undergo two types of evolution; firstly, *adaptive* evolution, which is caused by stable, abiotic factors, and secondly, *coevolution*, caused by the biotic environment, presented by other, coexisting populations. Coevolution which results in a maintainable, equilibrated, autoregulated community is referred to as *coadaptation*. In chemostats one can show correlations, or absence of correlations, between ecological and evolutionary events and between evolutionary events and specific abiotic or biotic environmental factors.

How sure can one be that natural selection operates in the chemostat in the same manner as it does in the wild? Natural selection is nothing but the differential effect of the environment on the reproductive and survival rates of coexisting clones and chemostats present relatively controllable and repeatable environments where the magnitude of many of the selective variables can be monitored or controlled. Generalisations concerning coevolution and its effects on community dynamics are particularly legitimate since coevolution does not depend on the imposed selective factors but on the biology of the inter-clone interactions.

The experimental community chosen for this study of evolution and persistence is one which allows for specific and testable assumptions about the genetic variation of its members to be made. It consists of strain of the bacterium *Escherichia coli* and a virulent strain of the *coliphage Lambda* (*λvir*). In terms of Linnaean systematics, therefore, this community is a simple one; it consists of only two interacting species. However, the wide range of different genetic types which occur within these species actually results in a very complex population as will become evident throughout this thesis.

The phage-host relationship has been seen as a predator-prey one, in the case of virulence, or as a symbiosis, in the case of lysogeny (Lwoff, 1953; Bertani, 1955).

1.1.3 *Phage-Host Coexistence and Evolution in Continuous Culture*

Paynter and Bungay (1968) appear to be the first to have made detailed studies of phage-host population interactions in continuous culture. Working with phage T2 and its host *E. coli* B they showed that the two populations can coexist, developing a relationship in which bacterial peaks and declines were accompanied by fluctuations in phage titre. During the course of this coexistence the bacteria became resistant to phage but sensitive cells persisted as significant proportions of the population. The resistance was associated by the authors with capsulation and mucoidity. They also observed several distinct phage mutants based on plaque morphology (Paynter and Bungay, 1969). In contrast with the virulent bacteriophages, a λ-lysogenic culture of *E. coli* acts, according to the same authors, in a self-regulatory manner and returns to a steady state condition after undergoing induction and lysis (Paynter and Bungay, 1970). Such a relation was predicted by a mathematical model developed by Noak (1968).

Using the virulent phages T3 and T4 grown in chemostats with their host *E. coli* B for very long periods of time, Horne (1970a; 1970b) showed that these too establish a stable state of coexistence. The stability of these systems increased with time as a result of selective adaptation. Similar dynamics were obtained at different dilution rates which seemed to influence the equilibrium titres but not the establishment of the equilibrium itself. The evolutionary

changes observed with T3 and T4 systems are apparently very similar to those observed with phage T_2; Horne attributed the coexistence of his organisms to successive cycles of bacterial resistance and phage host-range mutations and to the existence of sensitive, partial back mutants which arise from the evolved resistant population and on which phage can multiply. He interpreted the reduction of the plaque size as a result of accumulated mutations which effect the reproductive potential of the phage, in contrast with the interpretation of Paynter and Bungay (1971) as the appearance of distinct plaque-size mutants. Indeed, the latent period of both his selected phages was considerably extended and their burst sizes decreased when these were measured on control, non-selected hosts. This "weakening" of the phage-host interactions in continuous culture led to the suggestion that lysogeny, being less harmful for the host and most safe for the phage relationship, may be the ultimate outcome of their coevolution (Horne 1970a).

Cowlishaw and Mrsa (1975) studied the interactions of the filamentous blue-green alga *Plectonema boryanum* and its cyanophage LPP-1 in quasi-continuous culture. After some inversely correlated fluctuations in virus and cell densities the organisms established stable equilibrium with the host population at preinfection levels. There was some evidence of physiological resistance of the host during the initial period of fluctuations but genetically resistant strains eventually dominated the culture and no lysogeny was found. The derived phage was found to be antigenically similar to the parental and to have similar adsorption ability, burst size and latent period on parental cells although its plaque morphology has somewhat altered. These authors estimated that a conversion of resistant cells to sensitive ones at a rate of 0.004 per generation would be necessary to

maintain the phage and host populations at the steady state concentrations observed; but, as they stated, they found evidence of a more frequent but "less infective" pairing of selected organisms.

Coexistence of the phages with their hosts was established in all of these cases. In no case was there rigid evidence, however, of genetic stability or at least of a circularity in population fluctuations which would enable the authors to predict future events of their populations with reasonable accuracy.

The existing ecological models of predator-prey interactions which account for stable coexistence in homogeneous habitats (May, 1972; 1973) describe stability in mathematical rather than biological terms and are, thus, of limited utility for describing mechanisms by which specific phage and host populations coexist. Campbell (1961) and Horne (1970a; b), on the other hand, based their theories solely on the biology of phage-host interactions without making assumptions about the nature of the habitat. They both made coevolutionary rather than ecological suggestions. An alternative theory was recently formulated by Levin and his colleagues who claimed to have taken into account the nature of both, the habitat and the phage-host interactions. This theory is a very clear example of the current ecological theories of density and frequency-dependence, and it is worth looking at in detail.

Levin *et al* examined the cases of one-resource, one-prey, one-predator and one-resource, two-prey, one-predator and they showed that "... all populations can coexist in structurally stable equilibria if the number of distinct predator populations is not more than the number of distinct prey populations and the number of the latter is not more than the sum of the number of resources and the number of predator

populations". This principle describes no theoretical limit to the number of distinct prey and predator populations that can coexist in a one-resource habitat. An important question arises: can, and will, the number of such populations increase indefinitely? If it does, could such situations be described as "stable equilibria" to which the principle refers?

When applied to the case of phage-host systems, this principle would allow two host and one phage, three host and two phage populations and so on, to develop stable states of coexistence in homogeneous, single-resource limited environments. The requirement for successful coexistence in a system with one resource, one host and one totally resistant competitor, and one phage population is that the resistant population should be less fit in competition with the sensitive population, by having a lower intrinsic rate of increase; but, if the resistant population became slightly sensitive to phage predation the resulting increase in phage numbers could be sufficient to eliminate the original host.

To test their model, the above authors experimented with the virulent phages T_1 and T_2 in continuous cultures with *E. coli* B. They observed stable coexistence, but the experimentally estimated values of phage titres, resource uptake rates, adsorption rates, latent periods and burst sizes were outside the range where stable coexistence had been predicted by the model, suggesting that the real systems were more complex than the model imagined.

In a subsequent paper (Chao *et al*, 1977) the same workers reported more detailed studies of phage-host communities employing phage T_7 and *E. coli* B. They were able to show coexistence of two phage strains – the original and a host-range mutant – with three host

populations of which one was sensitive to both phages, one was
sensitive to the host-range mutant and the third was resistant to
both phages. In some of their cultures they observed development
of a small-plaque forming phage population but they did not consider
this fact as important for the establishment of equilibrium (see
below). They also found that the original host and phage strains
were fitter in competition with the corresponding selected strains,
partially and totally resistant hosts and host-range phage, in mixed
cultures.

These results confirmed the prediction of the authors that maintenance
of a relatively complex community of phages and hosts is possible in
a simple, one-resource habitat. They also appear to confirm that
differential susceptibility to a co-occurring parasite or predator is
a sufficient condition for stable coexistence of competitors
(Barbehenn, 1969) and that spatial or temporal "patchiness" of the
environment is not necessary as originally thought (Slobodkin, 1961;
MacFadyen, 1963).

The *development* of the phage-host community was, clearly in this case,
the result of coevolution of bacterial resistance and phage host-range.
The mechanism that Chao and his colleagues proposed for the *maintenance*
of such a community is, however, a purely ecological one. Their basic
assumptions are: (i) that there is a "numerical refuge" for the
sensitive bacteria, i.e. a density below which the number of cells
killed by the phage (dependent on the densities of both, phage and
host) equals the number of cells produced by cell-division, and
(ii) that the growth rate of the "wild-type", sensitive bacteria is
higher than that of the resistant bacteria, i.e. the generation time
of the sensitive bacteria is shorter due to more efficient

utilisation of the limiting resource.

They proposed, therefore, that as the phage density increases, the density of sensitive bacteria decreases but not below the numerical refuge; as a result resources become available for the resistant bacteria of which the density increases; the rise of the resistant bacterial population causes a fall of the phage population; this fall of the phage population allows an increase in density of the sensitive bacteria due to their growth-advantage against their resistant competitors.   An analogous mechanism was proposed for the coexistence of "wild-type" and host-range phage.   When the relative frequency of "wild-type" bacteria is sufficiently high, the disadvantage associated with the narrower host range of the "wild-type" phage would be offset by their relatively higher growth rate on the sensitive hosts.

The authors found the term "genetic feedback" inappropriate for the description of the underlying processes of the community's persistence. If, however, coevolution had led the community to a situation where "... the relative fitness of different bacterial genotypes depends on the densities of the different phage types and the relative fitness of the different phage clones depends on the densities of the various orders of bacteria" (Chao *et al*, 1977) in a way that the coexisting clones regulate one another, this would be the essence of "genetic feedback".

The above mechanism is, according to its proponents, one by which the polymorphism in the bacteria is made possible by the presence of phage, and the host range polymorphism in the phage develops in response to the polymorphism in the bacteria.   This again raises the

problem: if the introduction of one phage allows coexistence to two bacteria and the presence of two bacteria allows coexistence of two phages, there is nothing in the biology of bacteria to stop them increasing to $n+1$ in presence of n phages and nothing in the biology of the phage to stop it increasing to $n+1$ variants in presence of $n+1$ bacteria. Continuous extinction of original variants could be expected in finite populations.

There are, nevertheless, other complications which render this mechanism too simple to be acceptable. In an earlier paper Levin (1972) reported coexistence of two *E. coli* strains grown in single-resource chemostats (without phage) which he attributed to some environmental heterogeneity due to metabolic byproducts which could be used as secondary resources. Van den Ende (1973) suggested that wall-growth may also account for coexistence of competitors in chemostats. Nevertheless, the most important heterogeneity in resources results from phage predation: when cells lyse they liberate a substantial amount of amino-acids, sugars and nucleotides. Thus, it is not clear that polymorphism is maintained by a density- or frequency-dependent regulatory mechanism and not by the environmental heterogeniety created, for example, by the phage; in other words, it is not clear whether the phage acts as a *controller* of the bacterial populations and not as "*re-elaborator*" of the niche-hyperspace of the chemostat (Whittaker, 1977).

The complications noted by Campbell (page 4) still stand. It became evident from the pioneer studies of phage-host systems that evolution occurs not only towards resistance and host-range enhancement but also in other directions. The clearest and commonest manifestation of this is the evolution of small-plaque forming phage populations

of which Chao *et al* (1977) took no theoretical account. "These

small-plaque phage, however, had the same host range as $T7_1$"

(selected host-range mutant). "Consequently, we cannot attribute

the persistence of the viral population to their evolution". In

view of such complications, the assumption that the fitness of the

wild-type, or any bacterial or phage clone, in competition with

newly evolved clones is constantly higher or density- or frequency-

dependent, may be a serious oversimplification.

The pilot experiments that follow were to determine whether the

behaviour of the *E.coli-λvir* system was consistent with that of

typically virulent phage-host systems reported previously, and whether

lysogeny might evolve as predicted by Horne (1970a). Emphasis has

been placed on the measurement of phage growth parameters on its

co-occurring hosts(s) which, although most important for assessing

possible mechanisms of coexistence, has not previously been accomplished.

1.2   G E N E R A L   M A T E R I A L S   A N D   T E C H N I Q U E S

1.2.1   *Chemostats*

The apparatus, similar to that used by Chao *et al* (1977), was designed to minimize the likelihood of contamination and simplify sampling (the design is shown in *figure 1.1*).   500ml glass vessels with a constant culture volume of 300ml were used.   Aeration and stirring were achieved with humidified sterile air which maintained a small positive pressure within the vessel.   The units were connected, autoclaved together at 121 C^o for 30 min and left to cool overnight in the autoclave; it was observed that cooling in open air was almost certain to result in contamination.   The chemostats were operated for two days prior to inoculation to ensure that sterile conditions had been achieved.   Sterile medium was added by peristalsis at a dilution rate (D) of 0.04 hr^{-1}.   Inoculation was by injection through a rubber seal, while samples were taken from a special port at the outflow tube.   All experiments were at 37°C and the same dilution rate of 0.04 hr^{-1} at which a bacterial population doubles every 17.3 hr; very similar dilution rates were used in most of the reported studies and this was chosen here for comparative purposes.   The pH was not controlled but was measured at most samples and found to be consistently 8.0$\pm$0.2 in all cultures.

1.2.2   *Strains*

Escherichia coli C600 (Appleyard, 1954), a F$^-$, lambda-sensitive derivative of strain K12, carrying the markers *thr*$^-$, *leu*$^-$, *thi*$^-$, *lac*$^{-}$[1]

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[1] Unable to synthesize *thr*eonine, *leu*cine and *thi*amine and utilise *lac*tose.

FIGURE 1.1:   *The Chemostat Apparatus.*

air pump

rubber seal
rubber tubing
glass tubing

air
pressure
control

humidifier

cotton wool
air filter

air flow
control ▶

peristaltic
pump

inoculation

sampling
port ▶

≈ 30 cm

sampling

medium
reservoir

37 °C
water-bath

culture
300 ml

waste
collection
vessel
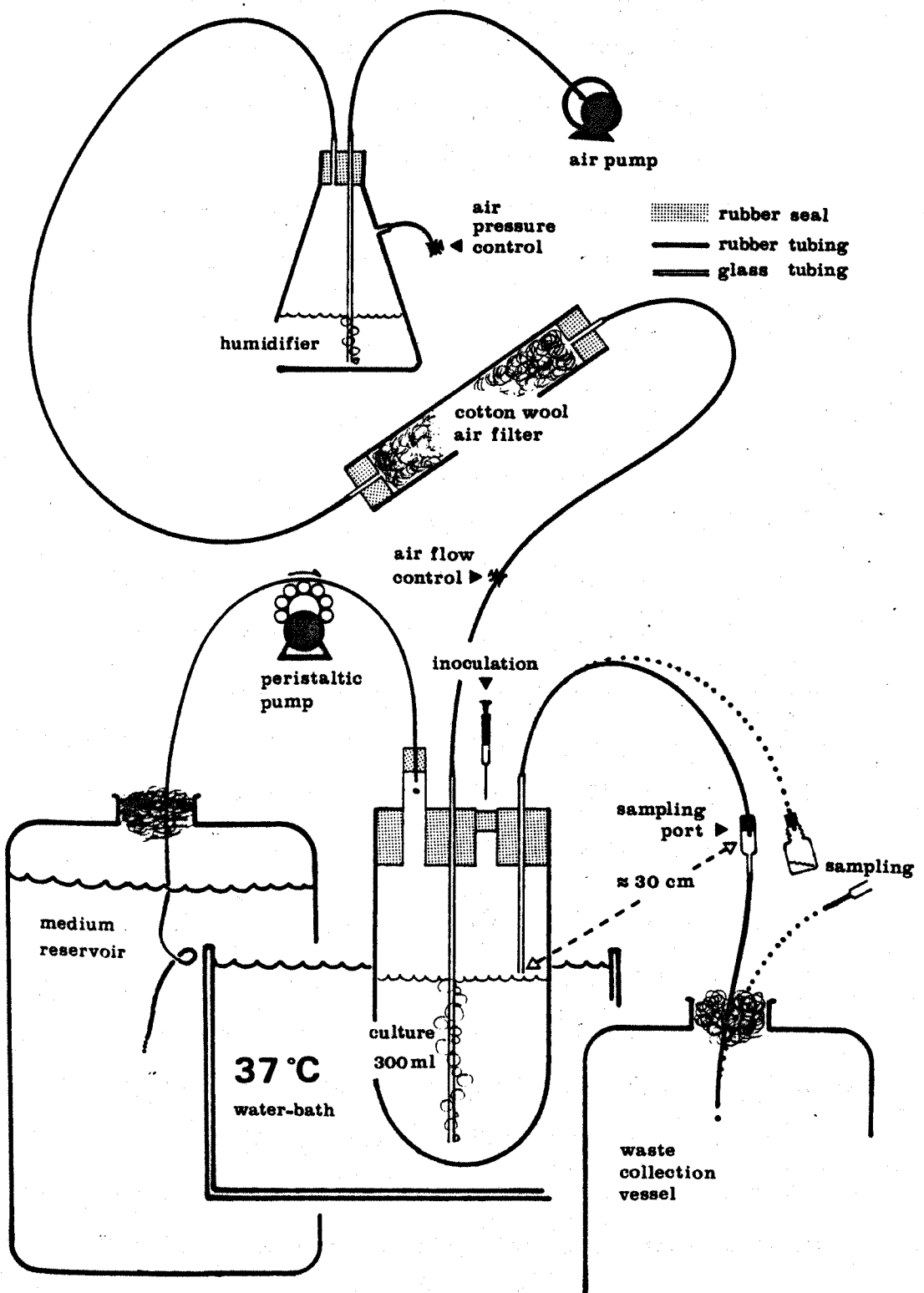
and resistant to coliphage Tl was used as host in the chemostats and for the preparation of lysates. Strains C600/λ*vir*, C600/T4*r* and C600*str* (resistant to streptomycin) were isolated from this strain. *E. coli* K12 (λ) and phage λ*vir* (Jacob and Wollman, 1954) were obtained from Prof. D. Ritchie, Liverpool University, U.K. and phage T4*r* from Dr. M.T. Horne, Stirling University, U.K. The strains isolated from the chemostat are described, and appropriate abbreviations are given, in the text.

1.2.3 *Media and Identification Tests*

The growth medium used in all experiments was "Lambda Broth" (Davison and Freifelder, 1966) at half strength and consisted of *Tryptone (OXOID L42)* 5g, sodium chloride 5g and magnesium chloride 2g per 1000ml distilled water (final pH = 7.5). This medium (TB1), for which the growth limiting factor was not determined, supported phage growth satisfactorily, unlike several minimal salts media tested, and did not foam in continuous culture conditions. The same medium was used for dilution, analysis and storage purposes.

Solid media, TA1 and TA2, were prepared by adding 0.7% *Agar No 1 (OXOID L11)* to TB1 and normal strength "Lambda Broth" (TB2) respectively and were used for colony and plaque counts as indicated in the text. Only a single soft-agar layer was used as the addition of a bottom-agar layer did not effect colony or plaque numbers; freshly made soft agar was kept molten at 46°C. For free phage counts sterile solution of streptomycin sulphate was added to a final concentration of 300μg/ml.

A minimal salts medium with potassium *di*-hydrogen orthophosphate 3g, *di*-potassium hydrogen orthophosphate 7g, sodium citrate 5g, magnesium

sulphate 1g, ammonium sulphate 0.5g, glycerol 5g, *Purified Agar*

(OXOID L28) 10g per 1000ml water with appropriate amino-acid (10µg/ml)

and vitamin (1µg/ml) supplements was used to confirm the presence

of the auxotrophic markers in culture isolates.   The *lac*-character

was tested on *Eosin-Methylene Blue Agar (LEVINE) (OXOID CM69).*

Further identification was made with standard biochemical tests using

API-20E Identification System and by testing for sensitivity to T4*r*

coliphage.

The phage was identified by its plaque-forming ability on the strains

C600 (original and *str*), C600/T4*r*, C600/λ*vir* and K12 (λ); wild-type

λ phage fails to grow on C600/λ*vir* and K12 (λ) strains while λ*vir*

grows on all but the C600/λ*vir* strain.

1.2.4   *Stocks and Samples*

The original bacterial strains were obtained by subculturing single

colonies in TA1.   The resulting cultures were identified and maintained

by subculturing 1ml aliquots in TB1.   Only fresh, overnight cultures

were used to minimise error due to uncommon genotypes overgrowing at

the stationary phase.

The stock lysate of the original phage (to be used in the chemostats)

was prepared from a single λ*vir* plaque.   To obtain high-titre lysates

phage was added to a small volume of dense host culture and allowed to

adsorb at 37°C for 5 min after which time the mixture was transferred

into a growing host culture.   Lysates prepared in this way contained

approximately 5×10^9 pfu/ml.   To eliminate viable cells from phage

preparations the lysates were treated with chloroform and/or

centrifuged and the supernatant was filtered through *Millipore* type

GSWP (0.22μm) filters.

The chemostat populations were sampled, titred, separated and
stored as following.   10ml of the culture were collected from the
special port at the outflow tube.   These mixed samples were titred
for total viable cells and plaque-forming units (pfu) and for free
phage particles, using C600*str* as indicator bacteria and streptomycin.
The dense mixed samples were then centrifuged at low speed; the
supernatant was filtered whereas the bacterial pellet was re-
suspended in TB1 containing λ*vir*-antiserum, incubated for 5min at
37°C and washed again with fresh TB1 to remove the antiserum and more
phage.   At first, the phage-containing supernatant was treated
with chloroform to ensure that no bacteria remain in the phage stock
but this procedure was omitted later when it was realised that
chloroform affected significantly the titre of phage coming from the
chemostat.   Filters effectively removed bacteria but no phage.   In
no case did the total phage titre differ from the free-phage titre
obtained on streptomycin containing lawns implying that the chemostat
phage was principally in the free-particle form.   At times, three
independent 10ml samples were taken in order to estimate the sampling
error in the estimation of titres, but only from one of them were the
populations separated and stored.   For further examination the
separated phage and bacteria were stored at 4°C.

The "selected" bacterial and phage strains (see section 1.3.1) were
similarly separated from a 10ml mixed chemostat sample.   The bacteria
were cured of phage by subculturing 1ml of their suspension in TB1
containing λ*vir*-antiserum and were maintained in TB1 using 1ml, or even
heavier, inocula to avoid loss of rare subpopulations.   All tests
were performed with the largest possible proportions of the populations

so that the results represented their average behaviour.   Unless

indicated otherwise, all tests and measurements concerning

chemostat phage were performed with the original, or diluted, cell-free

preparation from the sample,   All preparations of phage and bacteria

were titred before use in quantitative experiments.

1.2.5   *Immunology*

λ*vir* antiserum was prepared in rabbits using high titre lysate injected

introperitoneally with adjuvant.   The serum was incubated with heat-

ruptured *E. coli* C600 at room temperature for 3hr, centrifuged at low

speed and stored at 4°C.

To follow the kinetics of phage inactivation antiserum was diluted 1:100

into phage suspension of known titre;   at time intervals the mixture

was diluted appropriately to stop the reaction and assayed for residual

plaque-forming particles.   The reaction constant K (Burnet *et al*,

1937) was estimated from the slope of the regression line

1.1: $\log N_t = \log No - \dfrac{K}{2.3D} t$

where No is the phage titre before the addition of antiserum, N_t is

the titre at time t and D is the final dilution of the antiserum

(D=100 when the dilution is 1:100).

1.2.6   *Lysis Induction by   Mitomycin C*

Cultures to be induced were halved;   one half was incubated at 37°C

for 2hr with 1μg/ml mitomycin C and assayed for phage;   the other half,

incubated without the drug, was used as a control (Otsui *et al*, 1959).

A similar procedure was employed for the estimation of the number of

lysogenic cells in bacterial populations; samples were diluted appropriately and plated with 1μg/ml mitomycin C and also without the drug, using λ-sensitive C600 bacteria as indicator. Induced, lysogenic cells produce clear plaques on the sensitive indicator. This technique gives a minimum estimate of the titre of lysogenic cells (since some lysogens may fail induction) but tested with K12 (λ) strain its accuracy was very nearly 100%. Such estimates can be obtained, however, only when the titre of free phage in a mixture is significantly lower than the titre of lysogenic bacteria. This condition is tested in control plates without mitomycin C.

1.2.7 *Estimation of Adsorption Rates*

Phage and overnight cultures of streptomycin-sensitive bacteria were mixed at a multiplicity of infection of 0.01-0.04 in growth medium at 37°C. The titres were chosen to reflect those of equilibrated populations in the chemostats. At subsequent time intervals samples were diluted to prevent further adsorption and plated on *E. coli* C600*str* lawns with streptomycin. This drug interferes with the protein synthesis preventing intracellular phage growing in sensitive bacteria from the adsorption mixture so that adsorbed phage does not form plaques. The method, therefore, assays residual free phage the titre of which subtracted from the total phage titre gives an estimate of the number of adsorbed phage at time t (Symonds, 1957). The adsorption constant K can be calculated from the slope of the line:

1.2: $\log Nt = \log No - \dfrac{K\,B}{2.3}\,t$

where No and Nt is the phage titre at times zero and t, and B is the bacterial titre in the adsorption mixture (Schlesinger, 1932).

This, in common with all the traditional methods of measuring adsorption, however, is useful only when the titre of free phage is significantly lower than the total phage titre measured at the same time. In the case of selected bacteria this technique was coupled with a procedure for reducing the residual free phage to levels well below those of infected bacteria.

20μg/ml chloramphenicol was added to the bacterial culture immediately before the addition of phage; this antibiotic prevents phage multiplication without inhibiting adsorption and therefore synchronizes phage production (Hershey and Melechen, 1957; Melechen, 1955). The mixture was incubated for 50 min at 37°C to increase the number of infected bacteria (it was found that longer incubation with the drug may result in a permanent inhibition of phage growth); then, the drug and a large proportion of free phage were washed out by centrifugation with a *SM-24* rotor for 10 min using a *SORVALL RC2-B* centrifuge of which the rotor and the chamber were pre-warmed to approximately 37°C. The pellet was re-suspended in fresh medium containing λ*vir*-antiserum at a final dilution 1:100. Centrifugation was repeated to remove more free phage and the antiserum. With the first re-suspension chloramphenicol is diluted and intracellular growth of phage begins; therefore, the total experimental procedure, following removal of the chloramphenicol should not last longer than the minimum latent period of the particular phage-host system. The final suspension was assayed for total and free phage using streptomycin as above.

This method is not suitable for following the kinetics of phage adsorption but can give a fairly accurate estimation of the number of infected bacteria and the adsorption rate at a desired time interval

(in this case 50 min).

1.2.8 *One-Step Growth Experiments*

Phage growth parameters were obtained by the one step growth method (Ellis and Delbruck, 1939). Phage was added to approximately 5×10^8 cells/ml at multiplicities of around 0.01 and allowed to adsorb for 15 min, or 50 min with chloramphenicol. Time was counted from mixing or from the removal of chloramphenicol. Free phage was removed with antiserum and dilution. The sampling times immediately before and immediately after the first burst occurred were used as minimum and maximum estimates of the minimum latent period. The average burst size was calculated as the ratio of the mean phage titre during the final plateau (corrected for free phage present during the latent period if significant) over the mean titre of infected bacteria estimated during the latent period using the streptomycin and chloramphenicol techniques described above.

The distributions of phage release were studied, and the mean latent periods with their confidence limits were obtained, from the probit-transformed rise periods (Adams and Wassermann, 1956). This analysis was based on the "Maximum Likelihood" method (Finney, 1971) and was made on a *TI programmable 59* calculator with a specially devised program, described in the appendix.

As phage growth parameters are greatly dependent not only on the genetic composition, but also on the physiological condition of the host population and the physical and nutrient environment, for comparisons between phages these parameters were measured on halved bacterial cultures.

1.3 *P E R S I S T E N C E   O F   T H E* E.coli-λvir *S Y S T E M*

1.3.1 *Population Dynamics*

Culture 1. Phage λ*vir* was added to an equilibrated chemostat

culture of *E. coli* C600 at a final titre of approximately 2×10^1 pfu/ml.

The dynamics of the total host and phage populations in this culture

(culture 1) are shown in *figure 1.2.* The phage population, growing

at its maximum rate, achieved its highest density within three days

from inoculation. This peak was followed by a steady decline which

continued until days 40–45 to a density of just above 10^3 pfu/ml.

The rate of this decline was at first equal or similar to the

dilution rate of the culture but some considerable phage reproduction

was evident when the population had reached 10^5 pfu/ml or lower

densities. The period of decline was followed by a period of

continuous increase and the titre stabilised at around day 110 at a

level as high as 10^8 pfu/ml.

At day 3 the total bacterial population fell from its equilibrium

titre of about 5×10^8 cells/ml to 8×10^7 cells/ml, due to excessive

phage activity, but recovered to pre-phage levels by day 4. This,

relatively small drop in bacterial titre was perhaps due to some

physiological (phenotypic) resistance, analogous to that causing the

characteristic rings at the periphery of λ*vir* plaques on C600 lawns;

a number of other possibilities will, however, be examined later.

The difference in phage titre of 1.5×10^3 pfu/ml at day 42 and 3.5×10^8

pfu/ml at day 113 produced no significant change in bacterial numbers.

Bacteria and phage sampled from culture 1 at day 135 were studied in

detail and are referred to in the data below as "selected strains"

FIGURE 1.2:   *Population Dynamics in Culture 1.*

□ Bacteria   D : Dilution Rate
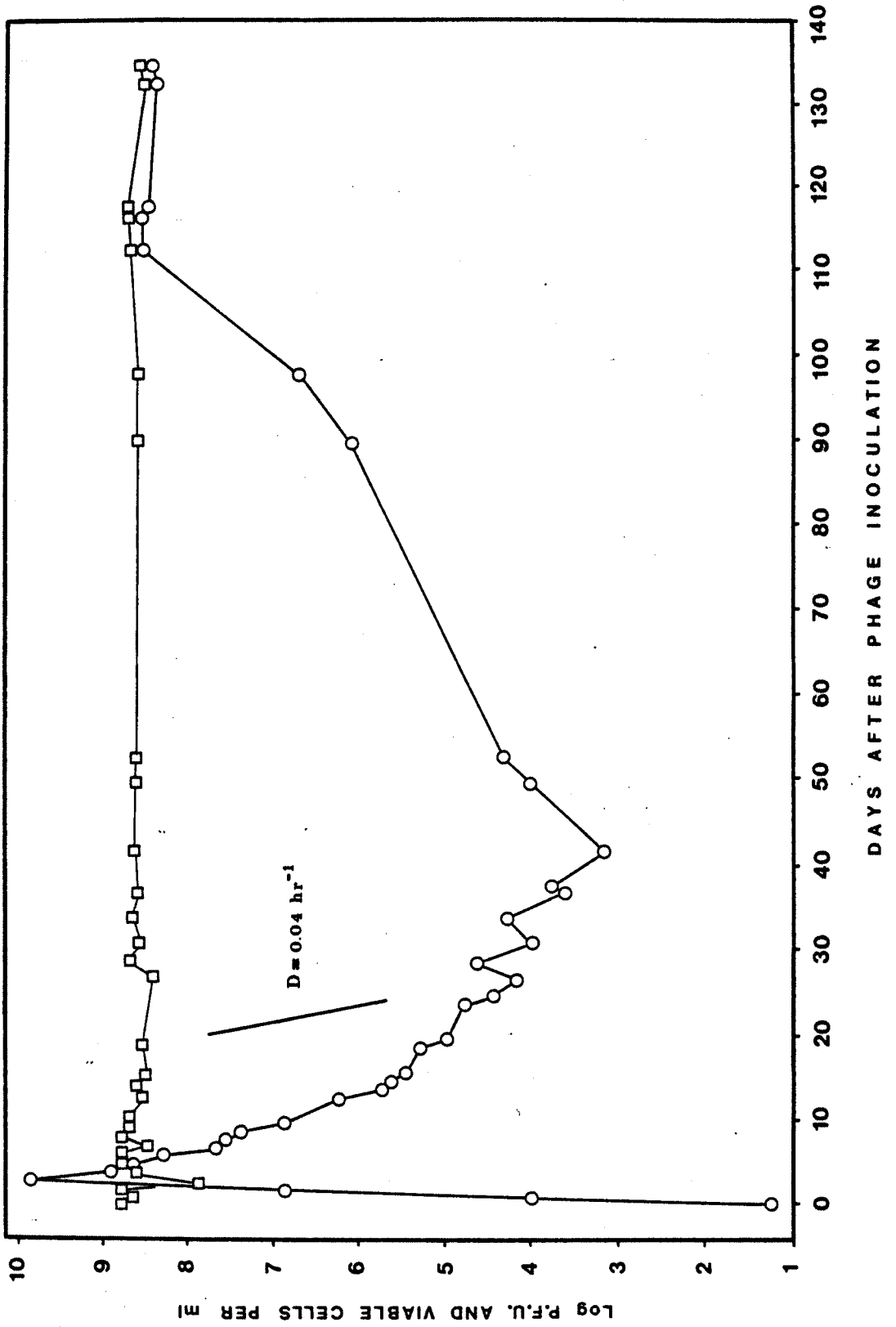
O Phage

(S will be used to designate selected bacteria and λs for selected phage) and the original strains as "controls" (C will be used to designate control *E. coli* C600; λ will be for the temperate, wild-type, phage and λv for the original λ*vir* phage).

Culture 2. Phage-cured selected bacteria were allowed to grow and equilibrate in a reconstituted culture (culture 2) to which selected phage was introduced to a final concentration of approximately 50 pfu/ml after 10 days. No free phage could be detected at a dilution of 10^{-1} of this chemostat culture prior to, and at the day of, phage inoculation confirming that curing had been achieved.

The population dynamics of this culture (*figure 1.3*) were considerably different from that of culture 1. The phage titre increased at a relatively slow rate and was soon stabilized without undergoing the peak and decline stages characteristic of culture 1. Similarly, the bacterial population seemed not to be affected by the early phage growth in culture 2. The sampling error was consistently less than 50% allowing for the observed fluctuations to be considered as genuine.

1.3.2 *Preliminary Characterisation of Selected Bacteria*

Resistance. Routine biochemical tests as well as confirmation of the specific markers *lac, thr, leu* and *thi* showed that the selected strain was identical to the control. On plates it supported growth of T4r-phage but appeared resistant to high titre suspensions of λ, λv and λs which formed no plaques. This resistance developed immediately after the phage peak in culture 1 and it was undoubtedly associated with the recovery of the bacterial population and with the rapid decline of the phage; the bacteria remained resistant

FIGURE 1.3:   *Population Dynamics in Culture 2.*

          □ Bacteria   D : Dilution Rate

          O Phage

D = 0.04 hr⁻¹

DAYS AFTER PHAGE INOCULATION

Log P.F.U. AND VIABLE CELLS PER ml
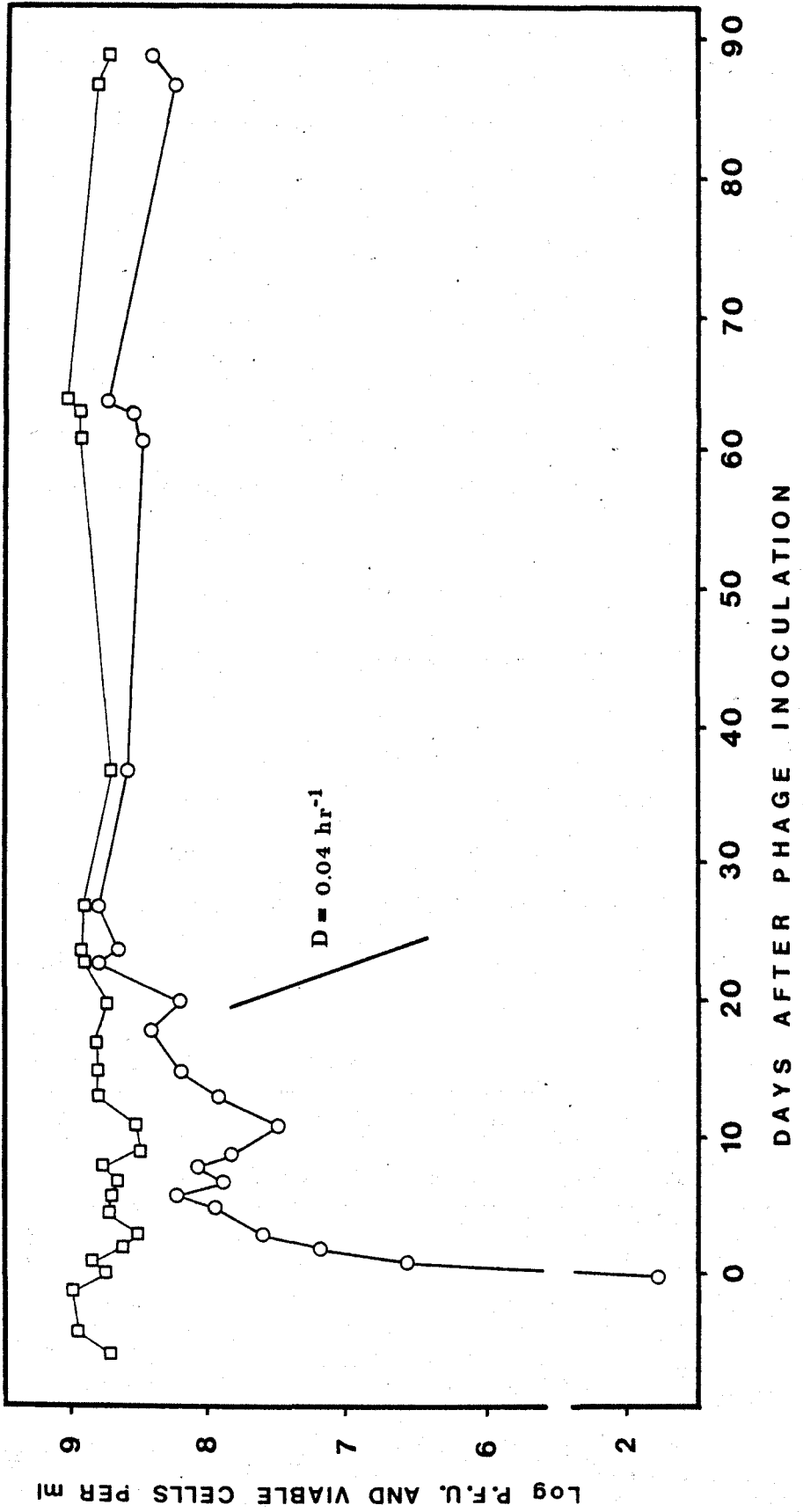
during the 10 days of growth prior to phage inoculation in culture 2.

There was no direct evidence of partial resistance and host-range mutation; bacterial populations from any stage of the cultures were highly resistant not only to their coexisting phage but also to all subsequently sampled phages.

Phage Carrying. Lysis inhibition was shown to be absent by treatment with chloroform which reduced the titre of viable cells by more than 4 log units but caused no increase in phage titre in the selected mixture. Any other kind of infective-phage carrying seemed to be unlikely since even large proportions of the selected bacterial population (of the order of 10^7-10^8 cells/ml) could be cured of phage by a single subculture in the presence of λv-specific anti-serum.

When the cured bacteria were treated with mitomycin C no evidence of phage induction, and therefore of lysogeny, was found while induction was effective with the lysogenic *E.coli* K12(λ) strain which was used as control *(table 1.1)*.

TABLE 1.1 : Lysis Induction with Mitomycin C.

Bacterial Strain	Phage Titre (pfu/ml)[a]	
	Before Treatment	After Treatment
K12 (λ)	7.5×10^4	2.2×10^9
Selected from culture 1	0	0
Selected from culture 2	0	0

[a]Values are the means of three replicate induction experiments.

1.3.3 *Preliminary Characterisation of Selected Phage*

Virulence and Morphology.   The selected phage formed clear plaques on all control C600 strains except C600/λ*vir*.   It also formed clear plaques on the lysogenic K12 (λ) and C600 (λ) strains confirming its virulent character.   Electron microscopy revealed no differences in size and general morphology between original and selected phage.

Antigenic Properties.   The serotype of λs was tested by spotting undiluted rabbit antiserum prepared against λv on plates which contained a total of 10^4 pfu (so many particles are needed for confluent lysis of the bacterial lawn).   The λv-antiserum prevented growth of λs completely, confirming that the latter was of the same serotype as λv.   T4*r* phage, used as a control was not affected by this antiserum at all.

The kinetics of the phage-serum reaction (*equation 1.1*) did, however, differ between λv and λs (*figure 1.4*) suggesting that some change had occurred in the antigenic properties of the phage during its growth in culture 1.   Namely, the reaction constant, K, was only 295.65 min^{-1} for λs compared with 527.36 min^{-1} for λv;   the expected rate of inactivation for t = 30min was, at 5% confidence level, between 88.3 and 94.1% for λv, but only between 71.8 and 80.5% for λs.

The Small-Plaque Phenotype.   It was observed that phage sampled from culture 1 after day 50 showed a considerable delay in forming plaques on control bacterial lawns in TA1;   a great proportion of plaques appeared after the second day of incubation at 37°C while, under the same conditions, almost all the plaques of control phage appear within two days.   The delay was less obvious when normal strength, TA2, medium was used on which only some difference in plaque size was

FIGURE 1.4:   *Kinetics of Inactivation of λv and λs by λv-Specific Antiserum.*

t : time from mixing phage with antiserum; N_t : residual pfu/ml at time t; N_o : pfu/ml at time zero.   Values are the means of three replicate measurements.   Standard errors and confidence zones are at $P = 0.05$.

Regression Analysis

phage	λv (O)	λs (●)
correlation coefficient	−0.982	−0.954
slope	−0.0382±0.010	−0.021±0.006

$F = 1.66$ $(P > 0.05)$
$t = 3.40$ $(P < 0.001)$
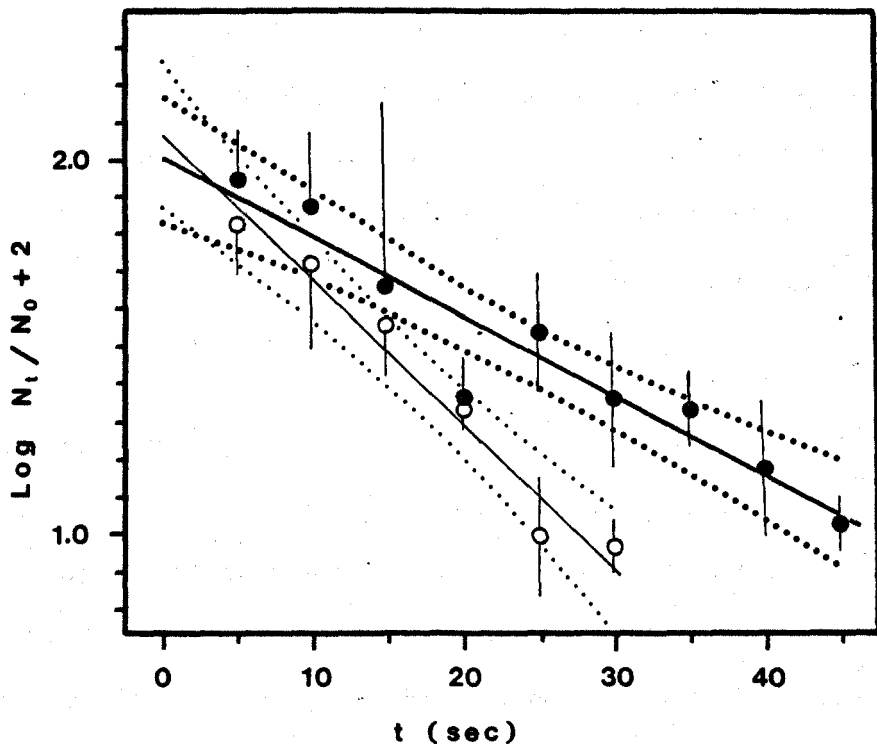
evident.   Attempts to isolate early , or late, plaque formers from

the selected population were unsuccessful;   lysates prepared from

either small latterly formed, or larger early formed, plaques

presented the same distribution of plaque-sizes and plaque-formation

rates as the parental population.

Large numbers (of the order of 10^4) of plaque-forming λv and λs

particles were used on several identically prepared plates with

control bacterial lawns to study the distribution of individual

plaque-forming times in each population.   Plaque counts were taken

at subsequent time-intervals of incubation (at 37^oC) to see what

proportion of the particles had formed plaques.   The incubation

continued until plaques ceased to appear.   The experiment was

performed with both, TA1 and TA2, media.

The percentage of plaque yield increased with incubation time in a

sigmoid manner.   The time of incubation needed for 50% of the plaques

to appear (the distribution's mean) and the variation in time of

appearance of individual plaques of a particular type (the

distribution's variance) can be estimated from such data by trans-

forming the "raw" sigmoid curves into probit-regression lines (see

appendix).   The results of this analysis are shown in *figures 1.5* and

1.6.

As the χ^2-values show none of the relationships departed systematically

from linearity;   the distributions of plaque-formation times could,

therefore, be considered as normal and the phage populations homo-

geneous in respect to plaque-formation rates.   The composition of

the medium did not affect the variance of the distributions but the

use of TA1 amplified the difference between the variances of the two

FIGURE 1.5: *Distribution of Plaque Formation Times* FIGURE 1.6: *Distribution of Plaque Formation Times*
  in λv Populations *in λs Populations*

N.E.D. : Normal Equivalent Deviate corresponding to the percentage of plaques on control bacterial lawns which become
    visible by the end of a particular period of incubation; confidence zones are at P = 0.05.

Probit Analysis

Medium	TA1 (▲)	TA2 (●)	TA1 (▲)	TA2 (●)
ML-Rounds	2	2	3	1
Degrees of Freedom	3	2	2	2
χ^2	6.437	0.687	0.319	0.865
P	0.092	0.709	0.822	0.649
α	-3.555811397	-3.999060257	-8.343072605	-7.093755502
β (P=0.05)	2.838013587 $\pm$0.3518265135	3.754684285 $\pm$0.8017880369	4.908854589 $\pm$0.272270395	4.937630490 0.5621509342

Log hr OF INCUBATION

N. E. D.
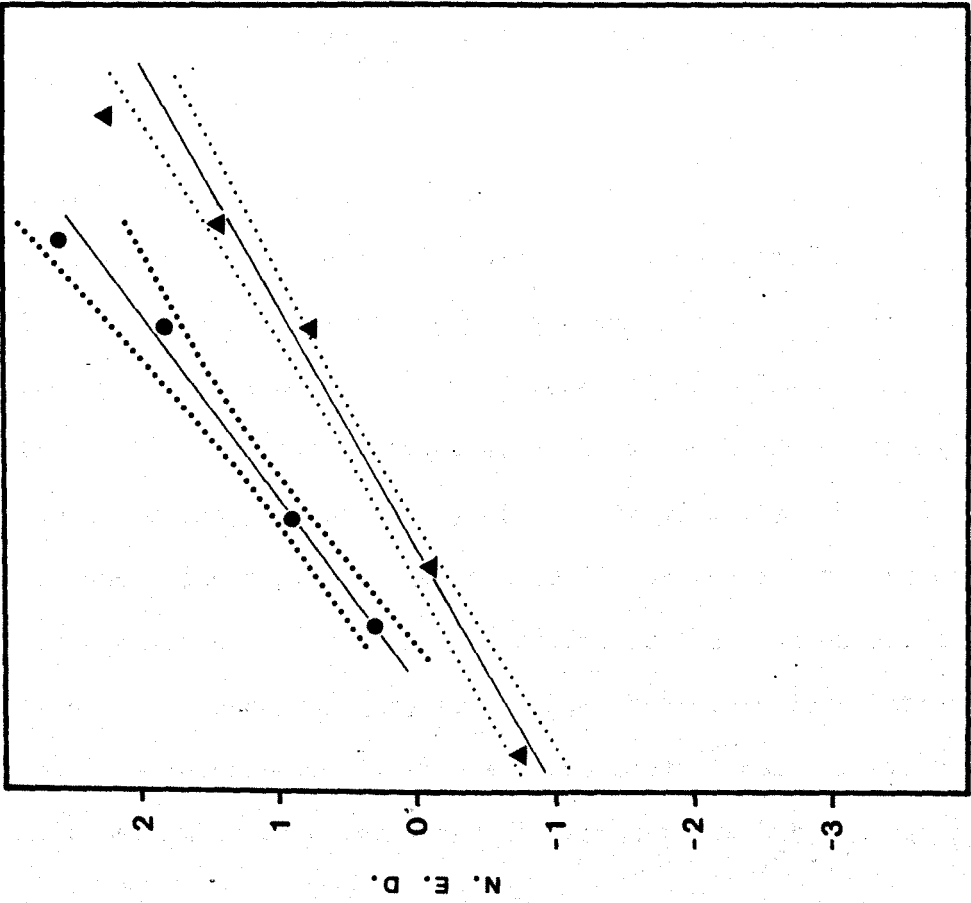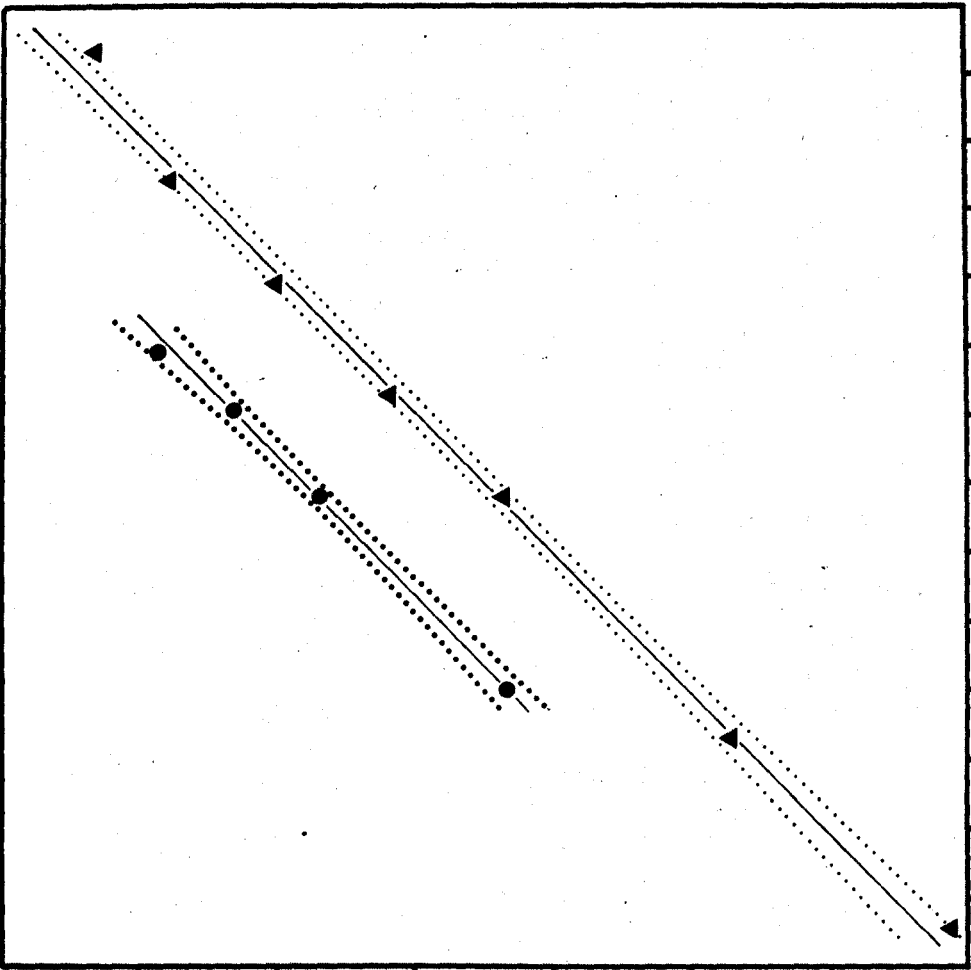
populations. This is seen when the slopes (*b*) are compared; the distribution of plaque-formation times of the λs population has a larger standard deviation compared with the control. The expected 50% plaque-formation times are approximately 18 hr for λv and 50 hr for λs in TA1, and 11 hr for λv and 20 hr for λs in TA2; the differences between these values are highly significant (P<0.001).

The results of this analysis, therefore, suggest (i) that the variation of plaque sizes in the λs population may be considered as phenotypic rather than due to the existence of several distinct mutant subpopulations; (ii) that the smaller size of λs plaques compared to the control (see *figures 1.7* and *1.8*) is the result of delayed growth, rather than of an early inhibition of growth, on plates and (iii) that the availability of nutrients (in TA2 medium) has an accelerating effect on plaque-formation, so that differences of this kind between the original and the selected phage would be likely to escape attention in very rich media if appropriate measurements were not made.

Phage Growth in Batch Cultures. The resistance of the selected bacteria could only be superficial since phage persisted at high densities. Their ability to support growth of the co-selected and control phages was tested in batch cultures (*figure 1.9*). λs multiplied well on selected bacteria, although much slower than it did on its original host; only after approximately 5 hr did growth become evident by which time some 60% of the input particles appeared to have been inactivated. A similar loss was observed in the case of the control phages, λv and λ. Four more interesting phenomena were revealed: (i) from the two control phages, λv showed some net growth whereas λ recovered its input titre but did not grow

FIGURE 1.7: *Plaques of* λv

FIGURE 1.8: *Plaques of* λs
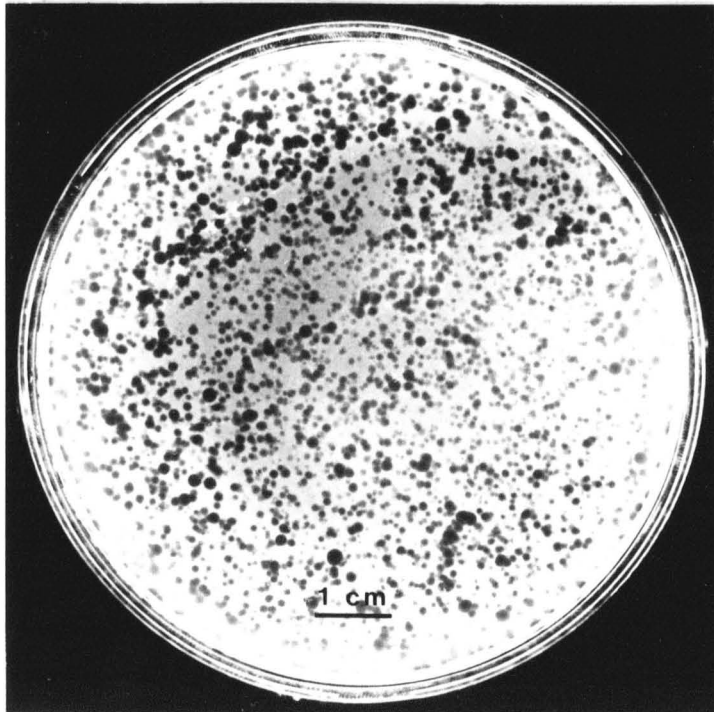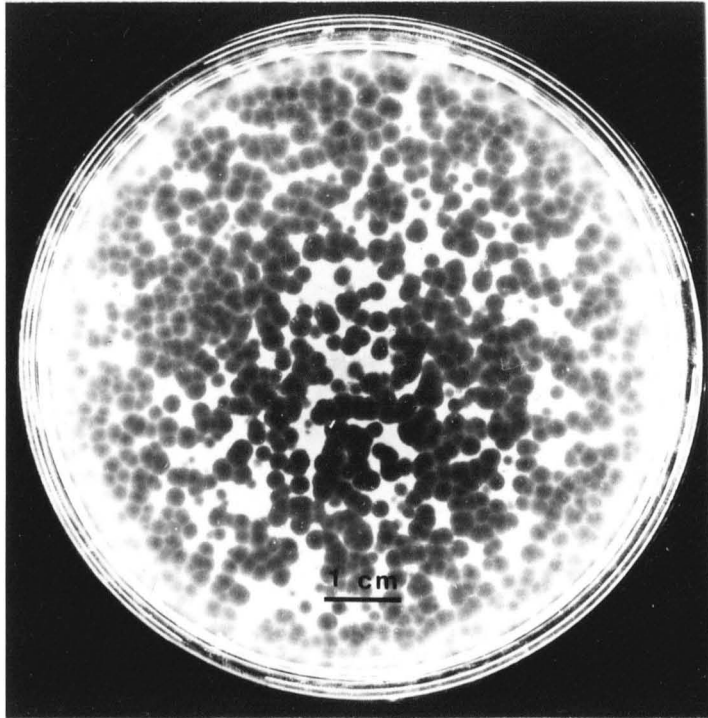
FIGURE 1.9:   *Phage Growth in Batch Cultures*

The experiment was performed at room temperature; N_O: pfu/ml at time zero; N_t pfu/ml at time t;   circles: λv;   squares: λs;   triangles: λ(wild type); blank symbols indicate growth on control bacteria and solid symbols indicate growth or selected bacteria.
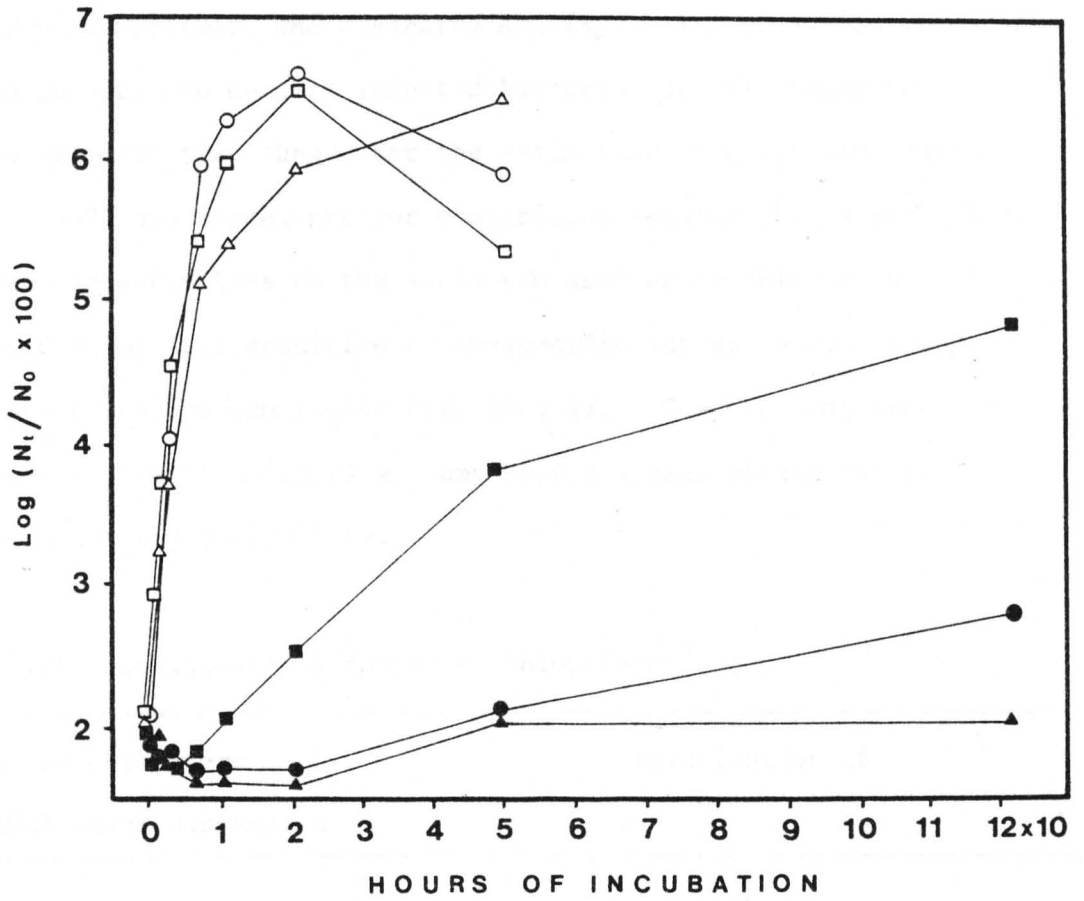
further; (ii) λv grew to a considerably lower titre than λs; (iii) in control bacterial cultures all phages suffered some considerable inactivation after reaching their maximum densities but not upon inoculation; (iv) λs suffered the highest rate of such inactivation in control cultures.

Chloroform Sensitivity and Adsorbing Ability. The protocols which use anti-phage serum to measure infected bacteria, or chloroform to measure residual free phage, for the estimation of adsorption rates (Adams, 1959) are inadequate for comparisons between phages which have different sensitivities to the antiserum used or to chloroform. λs was found to be less sensitive to λv-specific antiserum and more sensitive to chloroform (*table 1.2*) than λv. The protocol with streptomycin (section 1.2.7) was employed instead, chosen for its high accuracy and reliability.

TABLE 1.2: Sensitivity of Phages to Chloroform [a]

Drops of Chloroform per 10ml Phage Suspension	% Inactivation of λv	λs
5	0	17
10	25	33

[a] Chloroform was added to phage suspensions in TB1 and the mixture was shaken vigorously. Counts were taken before, and 30 min after, the treatment.

Two control experiments were performed to ensure that streptomycin would not introduce bias of that kind. First, the effect of 300µg/ml streptomycin on the infectivity of λv and λs particles was tested by

comparing the titres of cell-free λv and λs suspensions measured on

C600str lawns with and without the antibiotic; ten replicate plates

were used for each measurement and no significant difference was

found. Second, to test that neither phage could grow in

the presence of the above amount of the drug, each phage was allowed

to adsorb onto streptomycin-sensitive control bacteria at 37°C for

15 min at which time total and free phage counts were taken and

300μg/ml of streptomycin were added to the adsorption mixture;

incubation continued for 2 hr from infection and bacterial and phage

counts were taken again. The phages were found at numbers lower

than the free-phage counts taken at 15 min while there were no

surviving cells; the drug did inhibit, therefore, intracellular

growth of both phages as well as cell-division.

Simultaneous measurements of total plaque forming units, taken on

plates without streptomycin, give information on whether non-productive

adsorption, or any kind of physical inactivation of phage, takes

place after mixing with bacteria. When no significant inactivation

occurred, such total-pfu counts were considered as replicate

measurements of the input titre.

The kinetics of adsorption of selected and control phages on control

bacteria are compared in *figure 1.10*. The highly significant

difference in slopes indicates that λs adsorbed better than λv. The

adsorption constants (K; see *equation 1.2*) calculated from the slopes

and the bacterial concentration used (5.0×10^{8} cells/ml) were, for λv,

$(1.916 \pm 0.875) \times 10^{-10}$ mlcell^{-1}min^{-1} and, for λs, $(5.25 \pm 1.138) \times 10^{-10}$

ml cell^{-1}min^{-1}. The 15 min adsorption rates expected at 0.1%

confidence level were 64.5 to 85.9% for λv and 95.8 to 99.0% for λs.

The rise in free phage counts observed at 30 min from mixing, and

FIGURE 1.10:   *Kinetics of Adsorption of λv (a) and λs (b) on Control Bacteria*

t: time from mixing; N_o: pfu/ml at time zero; N_t: pfu/ml at time t.   Triangles indicate residual free phage particle counts taken on plates with streptomycin and C600*str* lawns;   circles indicate total pfu counts taken on the same indicator bacteria but without streptomycin.   Each value is the mean of five replicate measurements.   Standard errors are at P = 0.05 and confidence zones at P = 0.001.   The thick dotted lines are explained in the text.

Regression Analysis

Phage	λv	λs
correlation	−0.976	−0.993
slope (P = 0.001)	−0.042 $\pm$ 0.019	−0.114 $\pm$ 0.025

F = 2.158 (P > 0.05)

t = −11.568 (P << 0.001)

Log $N_t/N_0 + 2$

t (min)

a

b
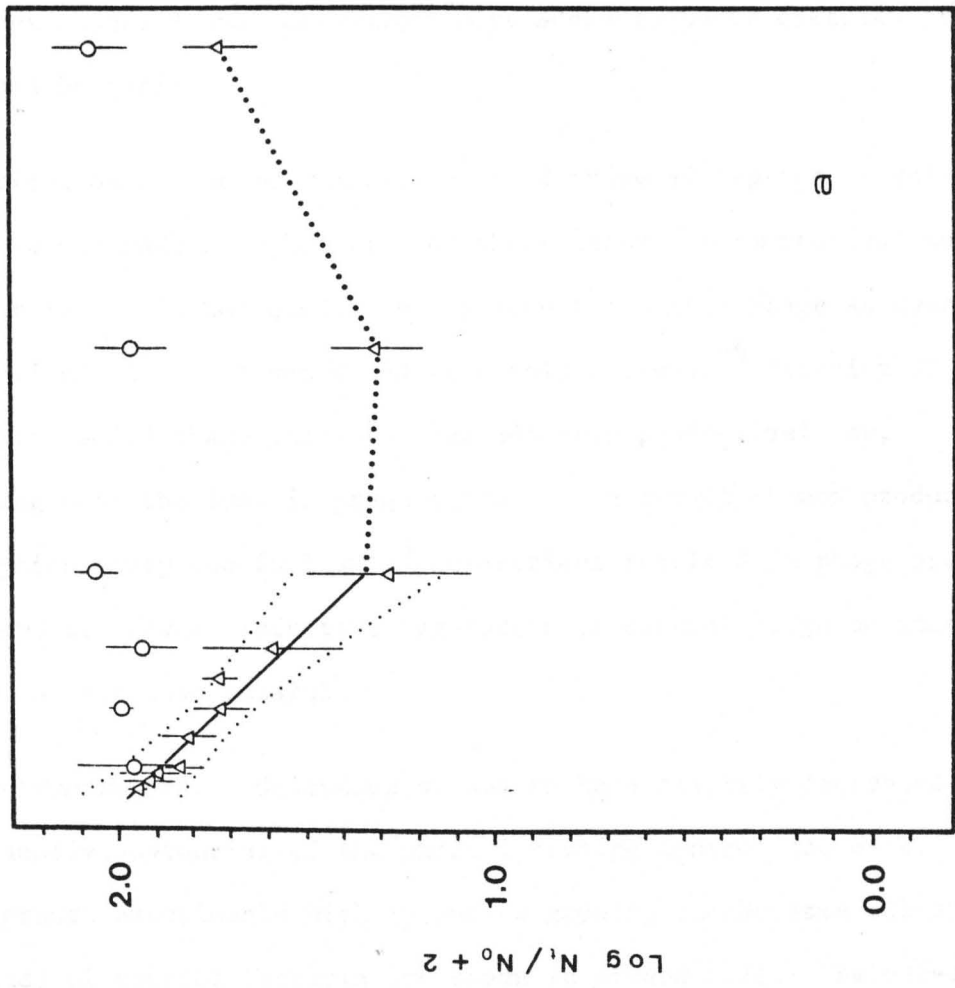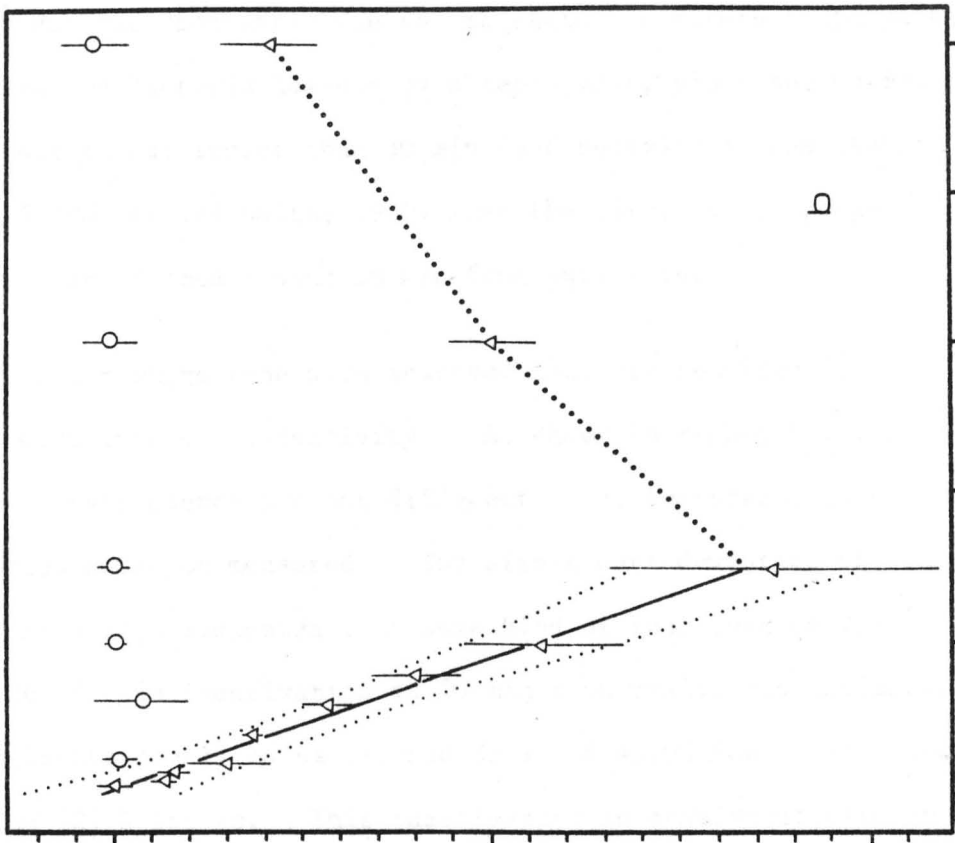
afterwards, was apparently due to liberation of mature phage particles from infected bacteria damaged by streptomycin, since the normal latent period was longer than 50 min (see below); it has been reported (Carter and Smith, 1970) that the first mature phage particles are formed around 30 min from infection.

Mixing either phage type with selected bacteria resulted in a significant loss of infectivity. As shown in *figure 1.11*, total and free phage counts are not different and, therefore, no productive adsorption could be measured. The significant deviation of both slopes from zero indicates that some kind of inactivation did occur. The rate of this inactivation at 50 min from mixing was estimated (at 5% confidence level) to be between 33.9 and 45.0% for λv and between 36.9 and 52.1% for λs. This inactivation in consistent with the loss in titre observed when the phages were added to batch cultures of selected bacteria.

An estimation of the 50 min rate of productive adsorption on selected bacteria was made for the selected phage using chloramphenicol to prevent intracellular growth and to reduce the free phage as described in section 3.2.7; it was found that only a 5.8×10^{-6} fraction of the initially added phage particles had adsorbed productively or, assuming that the loss in phage titre is the result of non-productive adsorption, only one in 1.3×10^{5} adsorptions resulted in phage production. Attempts to measure infective adsorption of control phage on selected bacteria were unsuccessful.

Growth Parameters. Selection seemed to have slightly decreased the reproductive potential of the phage infecting control bacteria. One-step growth experiments with λv and λs growing on the same culture (halved) of control bacteria are shown in *figure 1.12*. Selected

FIGURE 1.11: *Kinetics of Inactivation of λv (a) and λs (b) Upon Mixing with Selected Bacteria.*

Symbols are as in the legend of figure 3.10.

Regression Analysis

Phage	λv	λs
correlation	-0.867	-0.840
slope (P = 0.05)	-0.006 ± 0.001	-0.005 ± 0.002

F = 2.033 (P > 0.05)

t = -0.845 (P >> 0.05)
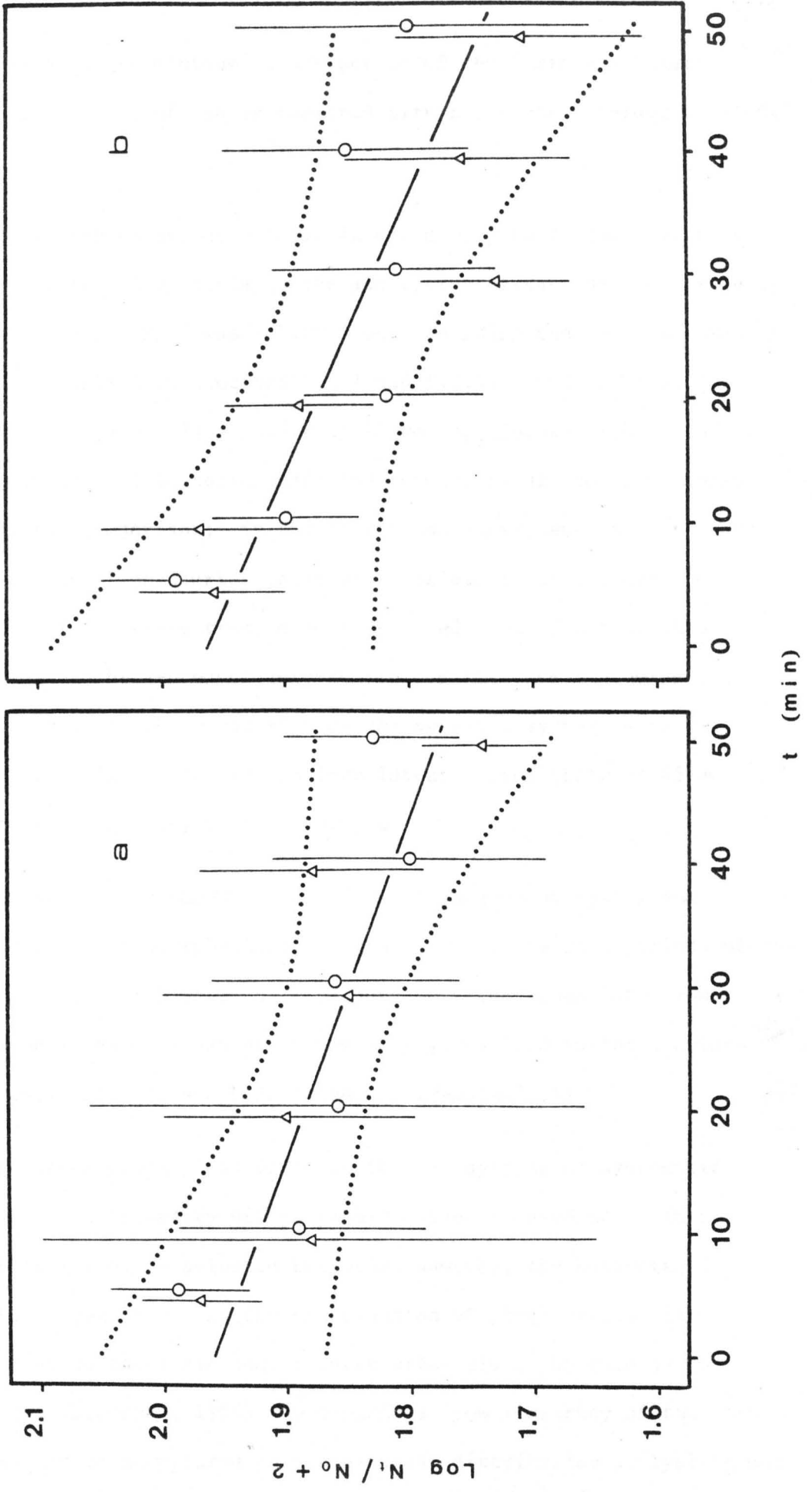
phage had a longer minimum latent period of 66-72 min and lower
average burst size of 154 as compared with the control values of 61-64
min and 181.

One-step growth on selected bacteria could only be followed when the
number of infected bacteria in the adsorption mixture had successfully
been estimated.   This was achieved with λs using the chloramphenicol-
protocol described in sections 1.2.7 and 1.2.8.   Such an experiment
is shown in *figure 1.14* together with two experiments with λv and λs
growing on control bacteria under the conditions of the chloramphenicol-
protocol for comparison;   in the latter two experiments the procedure
for reduction of unadsorbed phage was considered unnecessary and
omitted.   It appeared that, despite the reducing effect of chlor-
amphenicol on the reproductive potential of the phages growing on
control bacteria, the burst size of the selected system (406) was
considerably higher, and its minimum latent period (between 45 and 55
min) shorter, than any of the controls.

The distribution of phage release in each phage-host system and
the effect of   chloramphenicol was studied from the rise periods of the
growth curves, which were for this purpose transformed into probit
regression lines.   These are shown in *figures 1.13* (without chlor-
amphenicol) and *1.15* and *1.16* (with   chloramphenicol).

Although heterogeneity was found in the λ-C systems no systematic
departure from linearity of the probit curves is evident.   In the
case of λs infecting selected bacteria, however, the heterogeneity
seems to be genuine.   As the distribution of phage release is
insensitive to the variation of burst sizes along the rise period
(Adams and Wasserman, 1956) the departure from linearity of the λs-S
curve should be attributed to a non-normal distribution of lysis-times

FIGURE 1.12:   *One-step Growth of λv and λs on Control Bacteria*
                (Standard Protocol).

N_o: number of productively infected bacteria (per ml) in the growth
tube, measured immediately after the dilution of the adsorption mixture,
at 15 min from infection; N_t: total pfu/ml at time t.   Circles
indicate λv, and triangles λs, counts.

FIGURE 1.13:   *Distribution of Latent Periods of λv- and λs-*
                Infected Control Bacteria

N.E.D.: Normal Equivalent Deviate corresponding to the percentage
of phage progeny released by the time of sampling.   Confidence zones
are at P = 0.05.

Probit Analysis

Phage	λv (O)	λs (△)
M L Rounds	1	2
Degrees of Freedom	11	7
χ^2	29.37	14.02
P	0.002	0.051
h	6.363	
α	−5.336034741	−6.835409017
β (P=0.05)	0.0636242421 ±0.0153993903	0.0693237285 ±0.006154405
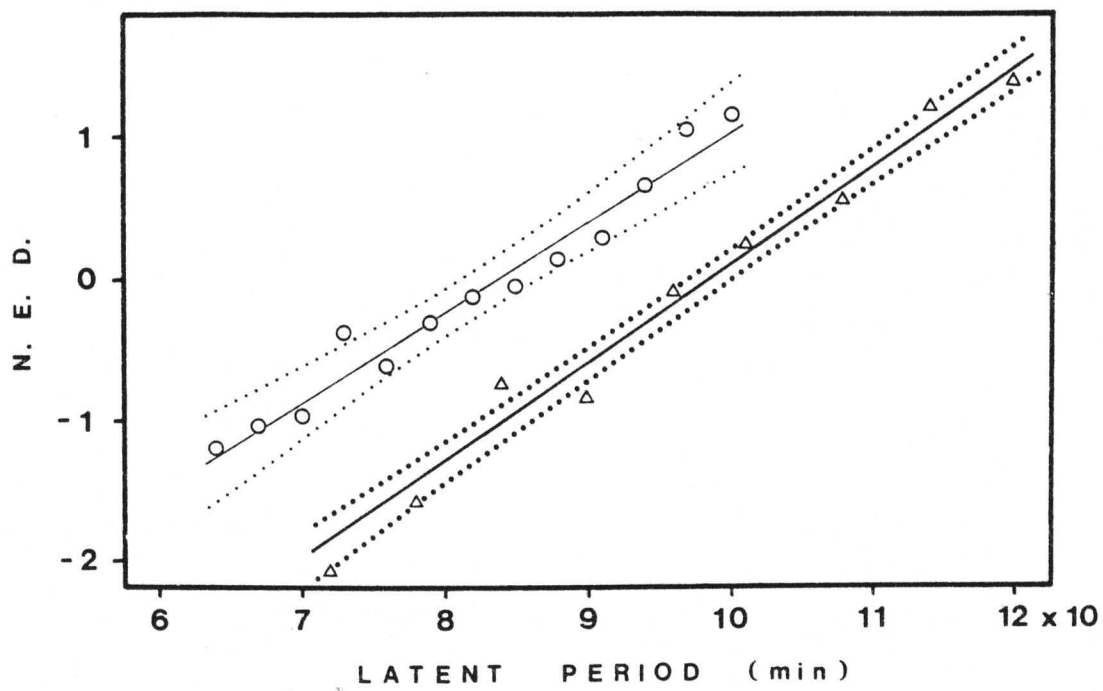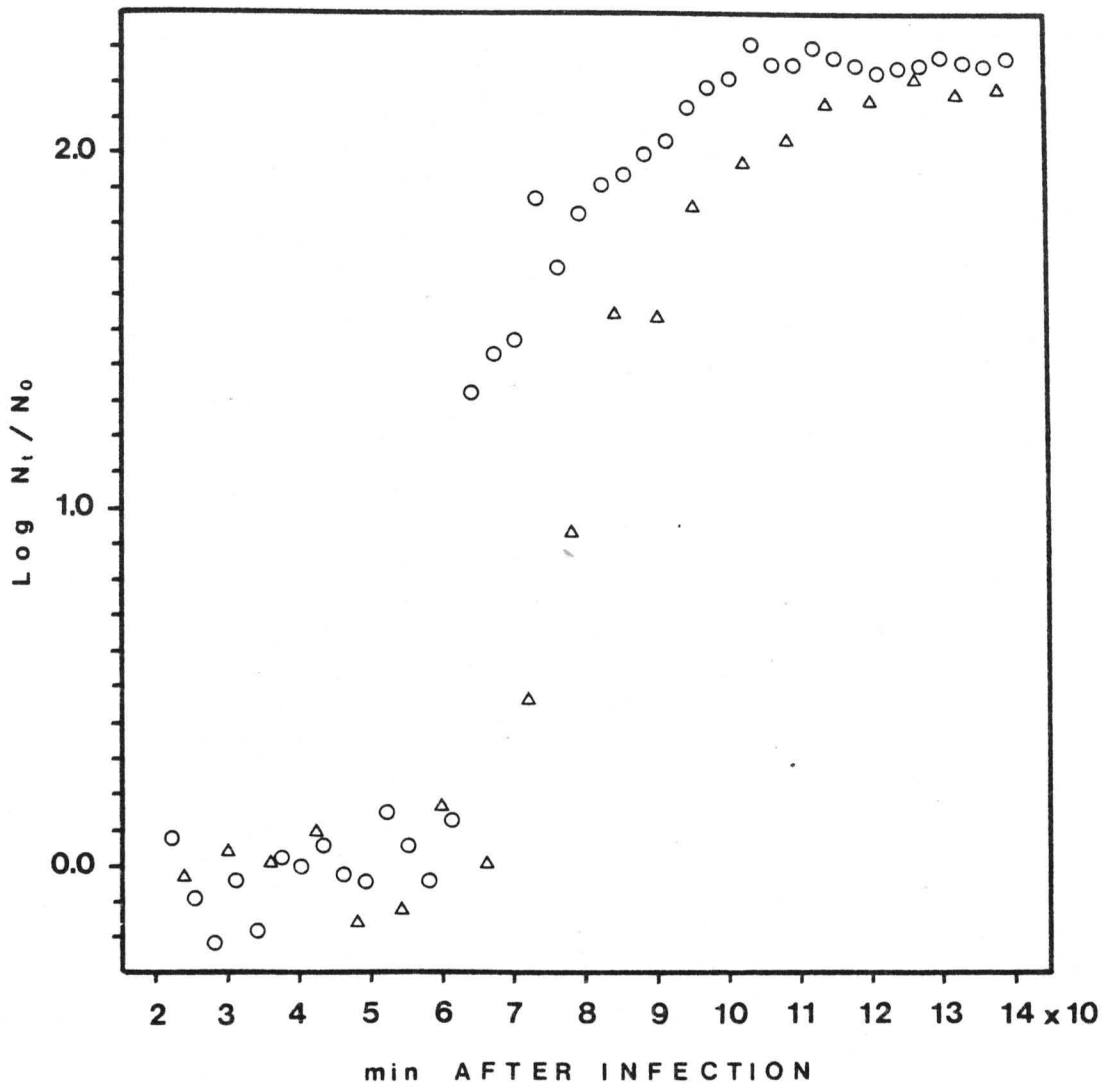
FIGURE 1.14:   *One-step Growth of λv and λs on Control Bacteria,*
                and of λs on Selected Bacteria (Chloramphenicol Protocol).

t: time from removal of chloramphenicol; N_o: titre of productively
infected bacteria measured immediately before the removal of
chloramphenicol; N_t: total pfu/ml at time t.   Blank circles: λv
on control bacteria;   triangles: λs on control bacteria;   solid circles:
λs on selected bacteria.
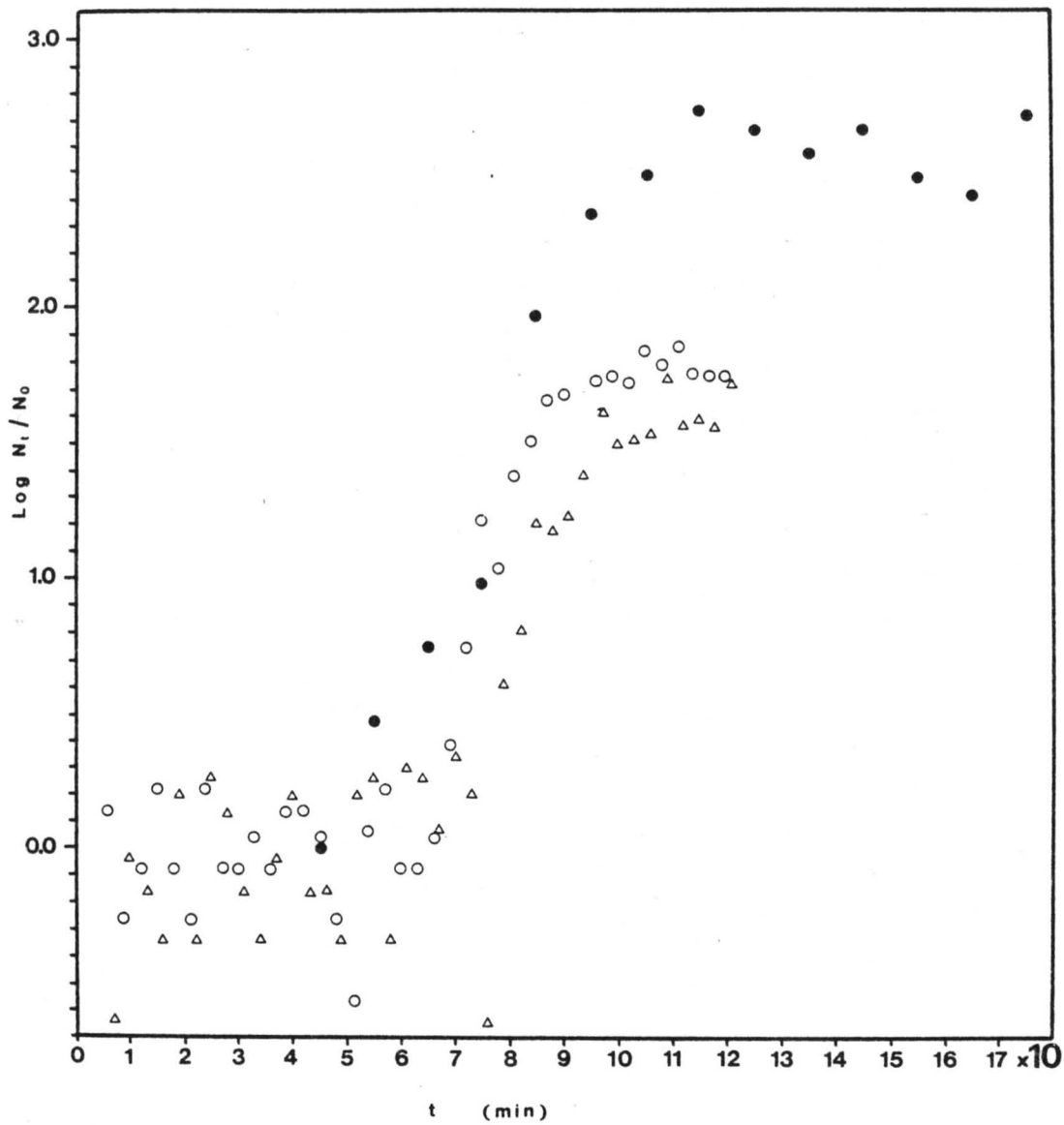
FIGURE 1.15: *Distributions of Latent Periods of λv- and λs Infected Control Bacteria (Chloramphenicol Protocol).*

The latent periods begin with the removal of chloroamphenicol; N.E.D.: as in the legend of figure 1.13.   Confidence zones are at P = 0.05.

Probit Analysis

Phage	λv (O)	λs (Δ)
M.L-Rounds	2	1
Degrees of freedom	7	4
χ^2	16.71	3.90
P	0.019	0.420
h	8.333	
α	−7.587337524	−8.175657255
β (P=0.05)	0.0901089727 +−0.0402633717	0.0892762191 ·+−0.0347267585

FIGURE 1.16: *Distribution of Latent Periods of λs-Infected Selected Bacteria (Chloramphenicol Protocol).*

The latent periods begin with the removal of chloroamphenicol; N.E.D.: as in legend of figure 1.13.   Confidence zone at P = 0.05.

Probit Analysis

M.L-Rounds	2
Degrees of freedom	1
χ^2	5.51
P	0.019
h	18.75
α	−7.216795659
β (P=0.05)	0.0756302066 +−0.0365197516
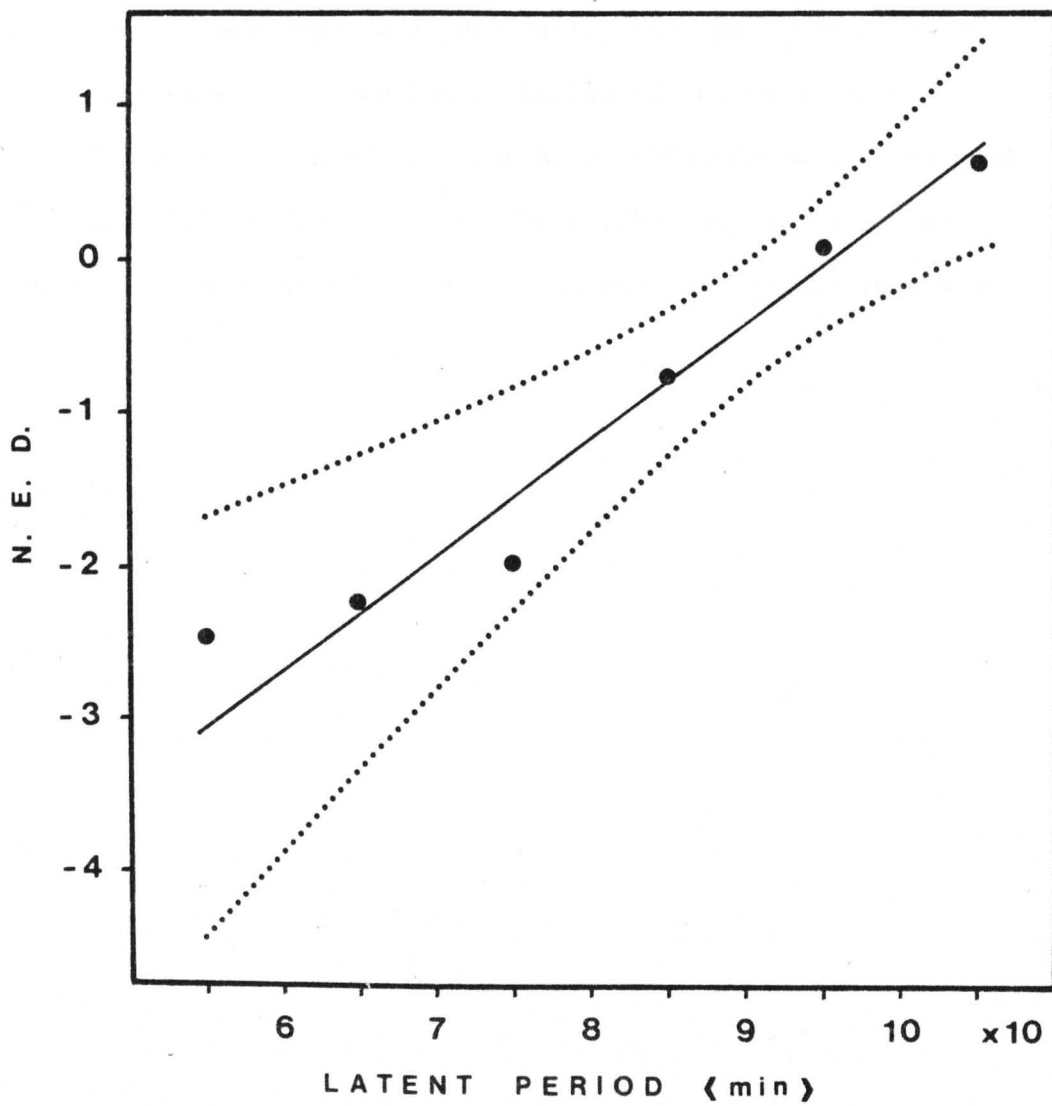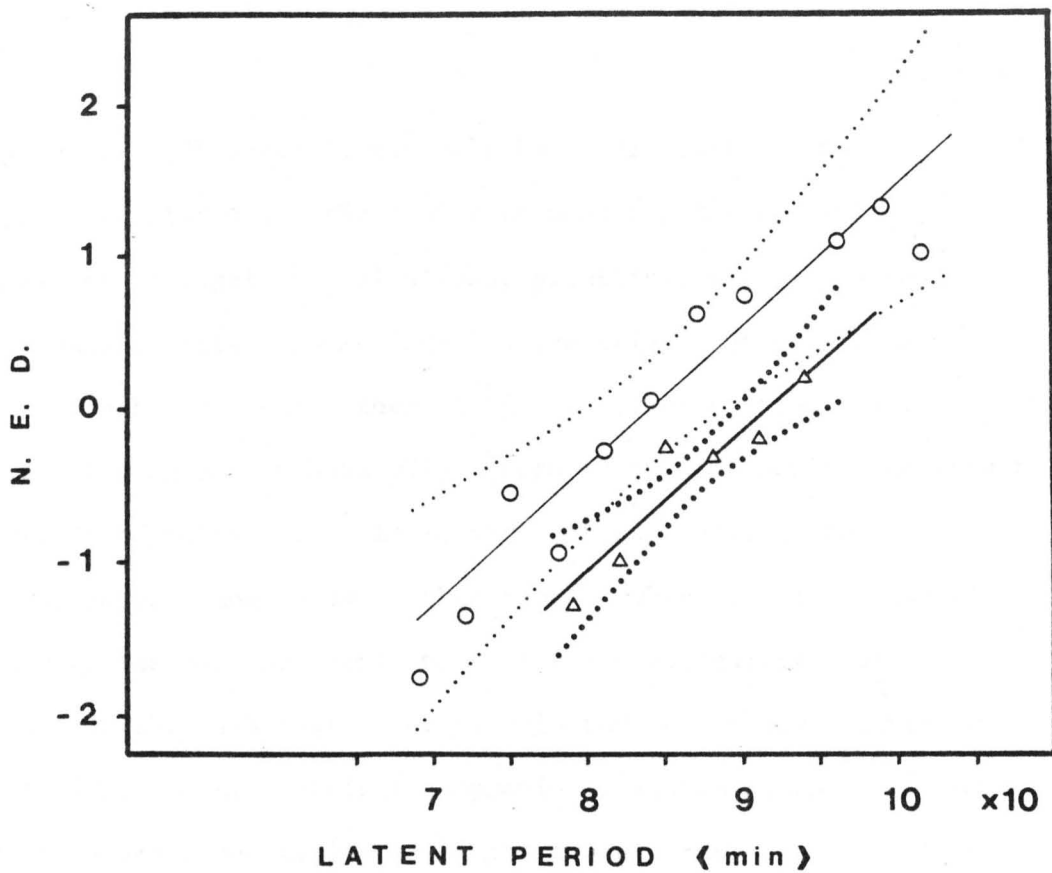
(see appendix).   This result may well be an artifact of the
chloramphenicol protocol: the hard treatment for the removal of
free phage (centrifugations, dilutions, pipetting, etc) could have
caused premature lysis of some infected bacteria.   On the other
hand, the possibility that either of the two selected populations
(bacterial or phage) is genetically heterogeneous, concerning the latent
period, cannot be ruled out.   It is worth noting, nevertheless,
that λs was found homogeneous in this respect when tested on control
bacteria with the same protocol and that the great majority of the
burst-times in the λs-S system are distributed in the same manner as
the burst-times in the λs-C (chloramphenicol) system (compare the mean
latent period and slope estimates of the two systems).

Chloramphenicol did not improve significantly the synchrony of phage
release, but decreased the mean latent period of λs, on control
bacteria.   The experiments without the drug demonstrated a difference
between the mean latent periods of λs (98.6 min) and λv (83.9 min)
but the variation in burst times of the two populations was very much
the same.

1.4 *THE FIRST SIGNS OF THE SYSTEM'S EVOLUTION AND ARISING QUESTIONS*

1.4.1 *The General Behaviour of the Model*

The *E. coli* − λvir system shows many similarities with the previously reported, typically virulent phage-host systems. First of all, the phage remained virulent during the course of its coexistence with its host. The inability of temperate λ phage to grow in batch cultures of selected bacteria may be interpreted as that the bacterial clones on which λv and λs multiplied were immune to λ by carrying, for example, a λ-prophage. The mitomycin C induction tests, however, demonstrated that typical λ-prophages, which would result from λvir by simple back mutations, did not exist at a detectable frequency. If λ-prophages did exist these should be non-inducible or defective, ie unable to multiply, lyse their host-cell and/or infect the control non-lysogenic cells. Such possibilities are examined in detail in chapter 3. Nevertheless, evolution of lysogeny does not seem necessary for the phage to persist.

In addition to modelling a virulent phage-host population interaction the *E. coli* − λvir system appears to be a fairly good model in which to study coevolution and its relation to community stability. The recovery of each population is associated with evolutionary changes (development of resistant bacteria and small-plaque forming phage) which increase the ability of the species to persist. The latter is suggested by the absence of explosion-crash episodes with the reconstitution of the selected community in culture 2. However, the evidence presented here is in contrast to previous expectations. In

an environment of resistant bacteria the phage appears to have

increased, rather than decreased, its growth potential, firstly, by

modifying its adsorption site so as to increase its infecting

ability and, secondly, by adapting its developmental functions to

the intracellular conditions of its coexisting host(s) so as to

increase its intracellular reproductive rate.

1.4.2   *Evolution of the Phage's Adsorption Site*

The altered antigenic properties, the increased sensitivity to chloro-

form and the improved adsorbing ability of the selected phage are

interpreted as pleiotropic effects of stereo-morphic changes of the

phage's adsorption site.   It is an old discovery (Lanni and Lanni,

1953) that, although an antiserum may contain more than one antibody

specific against distinct constituent antigens of the phage particle,

inactivation occurs only due to irreversible antigenic blockage of

the structures responsible for the infective attachment of the phage

onto the host-cell's surface;   such structures are, consequently,

known as the *serum-blocking* antigens of the phage.   Thus, the

reduction in the antiserum inactivation constant (*K* from *equation 1.1*)

indicates changes in the stereochemical specificity of the adsorbing

machinery of the phage in culture 1.

Such changes are usually associated with host range enhancement.

Streisinger (1956) showed that the host range and the serological

specificities of the phages T2 and T4 are controlled by the same locus.

In fact, the serological difference between T2 and T4, as reported by

this author, is not greater than the difference between λv and λs

found here.   The sensitivity to chloroform is also associated with

host-range mutations.   Newcombe and Rhynas (1958), for example,

observed that a host-range mutant of a clear-plaque forming $\lambda v2$ phage

was sensitive to chloroform and that survivors of chloroform treatment had lost their host-range character. Similar observations were made by Thirion and Hofnung (1972) on a host-range mutant of λ*vir* and by Horne (personal communication) on phages of the T-series selected from chemostats. Despite the consistency of these observations the way chloroform inactivates phage and the linkage of this inactivation with host-range mutations are not precisely known; some suggestions are given in chapter 2 where the subject is discussed in detail.

A change in the stereochemical conformation of the adsorption site does not necessarily imply enhancement of the phage's host range; it may also result in shortening of the host range. In addition, one may imagine two phages with stereochemically different adsorption sites having identical host range but different ionic, or co-factor, requirements for adsorption, or different preference for individual host clones within this range ("resource partitioning"). What the adsorption site of λs was selected for is not clear; whereas the comparatively good adsorption and growth of this phage on selected bacteria suggests that it has a wider host range than its ancestor (λv), its faster adsorption on control bacteria is better explained as an adaptation of its adsorption site to the chemical environment of the chemostat.

1.4.3 *Evolution of the Phage's Intracellular Interactions with its Hosts*

Of particular interest is the development of the small-plaque phenotype by the phage,which, in contrast to the opinion of Chao *et al* (1977; see page **54**), seems to be most responsible for the persistence of the phage. This phenotype has been attributed to distinct plaque-mutant

subpopulations constituting the phage population (Paynter and Bungay, 1969; 1971), or to the accumulation of mutations affecting the reproductive efficiency of the phage on its original host (Horne, 1970a; 1971). The first notion is obviously a notion of polymorphism, created by disruptive selection and maintained by some frequency-dependent mechanism, or which may be attributed to the neutrality of such plaque-mutants. Horne's notion is one of directional selection favouring genotypes which form smaller plaques, a property which he associated with decreased virulence. The comparison of the distributions of plaque formation rates revealed evidence in support of both views. The difference in variance (or in standard deviation) of the distributions indicates that disruptive selection or accumulation of selectively neutral mutant clones might have occurred but in addition to the occurrence of directional selection which is clearly seen as a shift of the mean plaque-formation time towards greater values. Therefore, two problems arise: (i) what is the selective advantage of small-plaque forming clones and (ii) how many such clones may coexist?

A key for a solution to the first problem is the observed change in the phage's reproductive potential. The small-plaque phenotype may well be a side-effect of an evolutionary adaptation of the phage to a chemostat condition which is absent in plate lawns of control bacteria. This is reasonable since the actual host of the phage in the chemostat may well be other than the original bacteria. Such adaptation would confer a density-independent advantage to λs against λv when the two phages competed for the same host; this would violate the assumption of Chao *et al* (1977) that the original phage has higher reproductive rate and that host-range mutations reduce the reproductive ability of phages. It would also violate Horne's prediction that the phage will evolve towards decreased virulence and, perhaps, lysogeny.

Unfortunately, the technical complications involved in the performance
and analysis of one-step growth experiments with such highly resistant
bacteria as the selected ones leave some doubts as to whether the
observed differences in growth parameters between the phages are
genuine.   In other words, it is not possible at the moment to say
whether the evolution suggested by these results was of bacterial or
phage genes involved in the intracellular development of the phage or,
simply concerned the organisms' response to chloramphenicol treatment.
These complications were resolved and answers to these problems are
given in chapter 3 where the phage's intracellular development is
studied in more theoretical and empirical detail.

The second problem, concerning the number of distinct plaque-size and
reproductive-potential mutants existing in the phage population, is
more difficult to resolve.   The statistical analysis of the times at
which individual λs plaques appear showed that these were normally
distributed around their mean, confirming the hypothesis that the λs
population was homogeneous in this respect.   The fact that the above
distribution did not differ in populations produced from small-plaque
and large-plaque isolates reinforces the hypothesis that the variation
of plaque size was not genotypic but phenotypic.   On the other hand,
if we accept that polymorphism may exist in other characters, such as
the host-range, there is no reason to reject that polymorphism may
also exist in genes determining the reproductive potential and/or
affecting the plaque-size.

1.4.4   *Phage Mortality*

The observed inactivation of all phages in mixture with selected bacteria
has an important ecological implication.   It demonstrates the existence

of factors which affect phage survival and which may act as selective

forces.   Such factors were not taken into account previously.

Instead, it was assumed that the only cause of "death" for the phage

was its random removal from the outflow of the chemostat (Chao *et al*

1977).   The most straightforward hypothesis for the nature of this

mortality is that there exist bacterial clones on which phage can

irreversibly adsorb but not multiply (non-productive adsorption).

Other explanations are given, and the evolutionary importance of such

mortality is discussed, in chapter 2 and again in subsequent chapters.

EVOLUTION OF RESISTENCE

2.1 *M O L E C U L A R   A N D   P O P U L A T I O N   B I O L O G Y*
O F   λ - R E S I S T A N C E

2.1.1   *The Two Meanings of Resistance*

Phages grow inside cells and are heavily dependent on the cell's
enzymatic and genetic controls.   For this, the process of their
reproduction may be impaired by host mutations at any stage where a
cell function is involved.   Such mutations are not necessarily lethal
for the cell.   Several aspects of the host's involvement in phage
reproduction are reviewed by Lewin (1977).   For example, in *E. coli*
mutations have been reported which interfere with successful injection
of the λ-DNA into the cell (Scandella and Arber, 1974;   Emmons *et al*,
1975) with transcription of early genes (Georgopoulos, 1971;   Ghysen
and Pironio, 1972;   Friedman and Baron, 1974), with DNA replication
(Georgopoulos and Herskowitz, 1971;   Gross, 1972) and with virion
morphogenesis (Georgopoulos *et al*, 1973;   Sternberg, 1973).   These
mutations, the number of which increases continuously, render the
bacteria resistant to the phage.   Phage mutants which are able to
bypass the developmental block imposed by the host have, however,
been isolated in most cases.   Genetic changes of these kinds should
certainly be considered in population studies under the general terms
of resistance and host-range and are particularly important as will be
discussed.

The main concern of this chapter is, however, the evolution of genes
and functions involved in the first of the phage-host molecular inter-
actions, the adsorption of the phage onto the cell's surface.   When
referring to bacterial resistance and phage host-range most previous

workers implied structural changes in the cell surface and the tip

of the phage tail which, both, affect the adsorbing ability of the phage.

The higher adsorbing ability and lower sensitivity to antibodies of

λvir after selection were interpreted here in the same manner (section

1.4.2).   Both these phenotypic changes manifest a structural change in

the phage's tail which presumably compensates adsorption difficulties caused

by host mutations towards resistance.   But was the selected bacterial

population homogeneous or heterogeneous?   Is λs's host susceptible to

the original phage?   The results of the preceeding preliminary

experiments are contradictory, suggesting that all imaginable types of

cells, this is λs- and/or λv-sensitive and λs- and/or λv- resistant

cells, might well have been present in the selected population.   To

obtain direct evidence of this, and to determine the nature of resistance

and host-range more precisely, the selected populations were analysed

on the basis of what is now known about adsorption and particularly

about λ-resistance due to changes in the bacterial cell surface.

2.1.2   *The λ-Receptor*

Adsorption is the allosteric reaction of the adsorption-site of the

phage with a molecule, or other structure, of the cell surface which

is generally called a *receptor* (Weidel, 1953;   Lindberg, 1973).   The

receptor of phage λ is a protein situated on the outer membrane of

the *E. coli* cell (Randall-Hazelbauer and Schwartz, 1973).   As it is

gradually being discovered for all phage- and colicin-receptors

(Braun and Hantke, 1977) λ-receptor's main functions are other than

reacting with the phage.   This protein is part of the maltose and

maltodextrin active transport (Szmelcman and Hofnung, 1975;

Szmelcman *et al*, 1976), and chemotaxis (Hazelbauer, 1975) systems.   It

forms large pores on the outer membrane (Boehler-Kohler *et al*, 1979)

that facilitate the diffusion of maltose and maltodextrins, and
functions as a molecular sieve for other saccharides (Nakae, 1979;
Nakae and Ishii, 1980). It is of primary importance for the transport
of large maltodextrin molecules (maltotetraose or larger molecules)
which, unlike maltose and maltotriose, cannot diffuse passively into
the cell (Wandersman *et al*, 1979). It is also necessary for growth
on maltose when this is provided at very low concentrations. Thus it
is not as puzzling as it was originally that the λ-receptor is coded
for by a structural gene (*lamB*) of a maltose operon and that its
expression is subject to the regulatory mechanisms operating in the
maltose regulon (Hofnung, 1974; Hofnung *et al*, 1974).

2.1.3 *Genetics and Phenetics of λ-Resistance*

The structural and functional analysis of the maltose regulon (summarised
in its latest form in *figure 2.1*) has provided a great deal of
information on λ-resistance at the level of adsorption. The *malA*
region, located at 74 min of the genetic map of *E. coli* K12, contains
the *malPQ* operon, of which the structural genes code for enzymes of
the maltose catabolism, and the regulatory gene *malT* (Raibaud and
Schwartz, 1980). The *malB* region, located at 90 min, contains two
divergent operons, *malK-lamB* and *malEFG* (Raibaud *et al*, 1979; Silhavy
et al, 1979), which are specialised in maltose and maltodextrin uptake
with the exception of the *malF* gene which codes for a cytoplasmic enzyme
of maltose catabolism.

All three operons are subject to positive regulation by the *malT*-protein
(*malT*p), a maltose-activated activator (Debarbouille and Schwartz,
1979; Debarbouille *et al*, 1979; Hofnung and Schwartz, 1971) and to
catabolite repression, and pleiotropic positive regulation, by the
cyclic adenosine monophosphate-receptor protein (CRP; Schwartz and

FIGURE 2.1:   *Genetic and Physiological Control of Maltose Utilization and λ-Receptor Production*

Dashed lines: transcription/translation;   dotted lines: regulation/catalysis;   solid lines: biochemical reactions; ADP: adenosine di-phosphate;   ATP: adenosine tri-phosphate;   CPS: capsular polysaccharide;   E: maltose-binding protein;   G: glucose;   G-1-P: glucose-1-phosphate;   G-6-P: glucose-6-phosphate; M_1:   maltodextrin; M_2 or M-M: maltose (maltodextrin dimers);   Mn, Mn-1 or M_1-M_2-...-M_n: maltodextrin oligomers;   P: maltodextrin phosphorylase; P-circled: phosphate ions;   PEP: phosphoenolpyruvate;   Q: amylomaltase;   R: λ-receptor;   T: *malTp* regulatory protein.   Other symbols are explained in the text.

mal B REGION   47 min

mal A REGION   90 min

T–M₂

malG?   F?   K?

◄malG malF malE   i_{B1:B2}   malK   i_{B3}   lamB ►   ◄malT   i_A   malP malQ ►

⊕   ⊕

E

R

E

?   ⊕

RPase + cAMP-CRP   T   Q

CPS Synthesis

crp

M_n → M_{n-1} → M_2 → M_1 P   P   G-1-P

CRP   +   +   +
G (n-2)G (n-1)G   G-6-P

R

R   cAMP ⇌ AMP   cya   ATP   PEP   Pyruvate   ADP   Glycolysis

CYA   PTS.I   (Acid Production)

CYA·CRR ⇌   pts I

?   CRR

crr ⊖

P
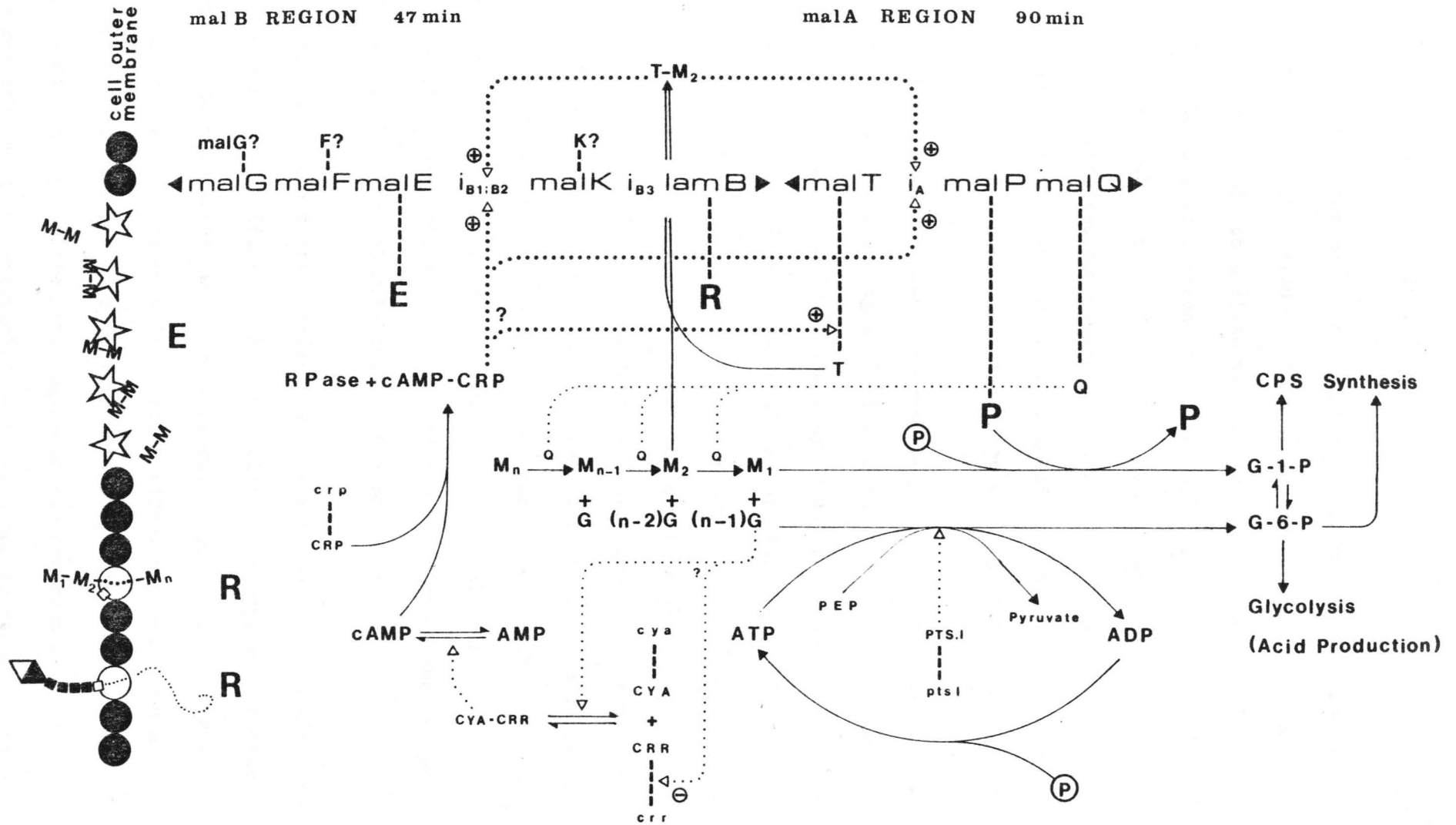
Beckwith, 1970; Schwartz, 1976).

The great majority of the mutations conferring resistance to phage λ
in *E. coli* map on either region of the maltose regulon. About 80% of
these mutants are unable to utilize maltose (mal⁻ phenotype) while
the remaining 20% have an apparently normal (mal⁺) maltose metabolism
(Lederberg, 1955; Schwartz, 1967). The synthesis of the
λ-receptor can be affected by mutations occurring in the gene *lamB*
itself, by mutations in *malT*, or by polar mutations in *malK* (Hofnung
et al, 1971; 1976; Raibaud *et al*, 1979; Thirion and Hofnung, 1972).

The *lamB* nonsense mutants produce no λ-receptors; they are, therefore,
resistant to all host-range phages (including the classical *h*-mutant
isolated by Appleyard *et al*, 1956) and they do not grow on maltodextrins
of more than three units (Szmelcman *et al*, 1976; Wandersman *et al*,
1979). In contrast, the missense mutants produce altered *lamB*-proteins
which are recognised by at least one host-range mutant phage each. This
was confirmed by Roa (1979) who demonstrated that from a wide range of
λ-resistant *E. coli* mutants only *lamB* missense mutants were susceptible
to host-range mutants of either λ or the coliphage K10, which also uses
the *lamB* product as receptor (Hancock and Reeves, 1976). Roa also
demonstrated that both, the specificity and the activity of the receptor
in vitro vary with different mutations of the *lamB* gene.

Several mutations which confer resistance to λ map, however, outside the
maltose regulon. The effect of these mutations is always pleiotropic,
implying that they interfere with the λ-receptor synthesis, or function,
in some indirect way. For example, Curtiss (1965) has described a
λ-resistant mutant which is also resistant to phages of the T-series
and whose mutation maps in the *proA* region of the chromosome. Such
multiple resistance may be attributed to alterations in other membrane

components which interact with phage-receptors (Bassford *et al*, 1977).

An important aspect in this discussion of λ-resistance is its regulation by the *catabolite repression system* which also controls a great number of operons and inducible genes (its functions, the genes involved in and controlled by, it and the effects of mutations are reviewed by Pastan and Adhya, 1976). This regulation is mediated by the CRP, product of the gene *crp* (also known as *cap*) which, when activated by cAMP, reacts directly with special DNA sites at the promotors of the operons to facilitate the binding of the RNA-polymerase (RPase) and the initiation of transcription. The intracellular concentration of cAMP is inversely correlated with that of glucose. Thus, when the glucose level is low the catabolite repression system stimulates the cell's mechanisms of searching and consuming alternative resources, whereas when glucose concentrates, the cell economizes in energy by turning off the synthesis of enzymes which are not to be used. It is not yet known whether the CRP-cAMP complex exerts its control directly on each maltose operon or regulates the whole maltose regulon indirectly, by controlling the transcription of the *malT* gene (Raibaud and Schwartz, 1980), but it is known that both, the catabolite repression system and the control mediated by the maltose-*malT*p complex need to be in the "on" position for full function of the regulon (Schwartz, 1976).

The mechanism by which the concentrations of glucose influences the concentration of cAMP in the cell is not yet conclusively established. There is evidence that the catabolite repression is linked with another multi-functional central regulatory system, the *phosphoenolpyruvate : glucose phosphotransferase system* (PTS) which is involved in the phosphorylation of the so called PTS-sugars and their transport through the cell's membrane, as well as in other, less well defined, physiological,

regulatory processes (Saier and Roseman, 1976a). From studies in *Salmonella typhimurium* it has been suggested that this link is the product of the gene *crr* (carbohydrate repression resistance), protein which allosterically regulates the activity of the adenylate cyclase (product of the gene *cya*) which, in turn, catalises the synthesis of cAMP from its non-cyclic form, AMP (Saier and Feucht, 1975). Mutants of the PTS have been reported which influence the inducibility of the maltose regulon and of other non-PTS-sugar operons. For example, mutants of the *ptsI* gene (coding for Enzyme I of the PTS system) appear to be much more sensitive to catabolite repression than the wild-type parents while this sensitivity is overcome by mutation in the *crr* gene (Saier and Roseman, 1976b; Saier *et al*, 1976).

In any case, the maltose regulon seems to be part of a complex network of functions involved in the utilisation of carbohydrates. Consequently, the synthesis of λ-receptor is likely to be affected by alterations in genes and proteins which are even less closely related to it than are the *crp* and *cya* genes and their products. Several λ-resistant *crp* or *cya* mutants have already been isolated. (de Crombrugghe *et al*, 1971; Pearson, 1972; Yokota and Kasuga, 1972).

2.1.4 λ-*Resistant Populations*

Evidence has been presented that adsorption occurs in at least two steps: (i) a reversible reaction of the phage's adsorption site (P) with the bacterial receptor (R) is followed by (ii) an irreversible, specific reaction of the two bodies leading to the formation of a stable complex PR (Puck *et al*, 1951; Stent and Wollman, 1952; Tolmach, 1957; Schwartz, 1975; 1976). Roa and Scandella (1976) showed that, at least *in vitro*, irreversible adsorption and DNA injection are two distinct steps of the infection process and suggested that the irreversible adsorption reaction

itself is also a multi-step process; but for the sake of simplicity let us consider only the first two-step reaction which may be represented by the equation

$$2.1: \qquad P + R \mathrel{\mathop{\rightleftharpoons}^{k_1}_{k_2}} (PR) \xrightarrow{\quad k_3 \quad} PR$$
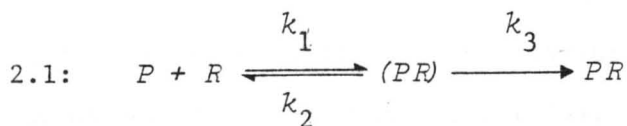
where k_1 is the phage-receptor collision constant and k_2 and k_3 are the dissociation and association constants respectively. k_1 depends mainly on the densities of P and R in the mixture while k_2 and k_3 depend on the phage-receptor affinity and on the factors which can influence it (eg the ionic composition of the medium, any adsorption co-factors and so on).

Population resistance may, therefore, be understood within the framework of two basic sets of terms. The first may be called *ecological resistance* and is equivalent to Chao's *et al* (1977) concept of a numerical refuge. The population becomes essentially resistant by reducing its density and, consequently, k_1. In this sense resistance is inversely dependent on density. Instead, *evolutionary resistance* is the result of mutations which reduce k_3, or increase k_2, by altering the molecular structure of the receptor or by interfering with its biosynthesis. Such resistance is density independent; a *lamB* nonsense-mutant population, for example, is equally resistant at low and high densities.

These two views, taken together, give rise to a third and, perhaps, more realistic interpretation of population resistance. This is a density-dependent resistance of an evolutionary or physiological nature. k_1 may be reduced not only by reducing the density of the population but also by reducing the *mean number of receptors per cell*. This

number is determined by the level of expression of *lamB* allowed by each regulatory mechanism (maltose-*malT*p and cAMP-CRP mediated) and, consequently, depends on the nutrient environment and the physiological state of the cells. In a wild-type population, it can reportedly vary from fewer than 30 to 6000, or possibly more, receptors per cell (Ryter *et al*, 1975; Schwartz, 1976). Thus, even a wild-type population may, under certain physiological conditions, contain cells with no receptors at all (physiological resistance).

That the phenotypic effects of mutations on a particular gene may differ quantitatively needs no further support. "Leaky" mutations, for example, may greatly increase the frequency of cells without receptors, by drastically reducing the rate of the receptor synthesis, but do allow (by definition) for some mutant cells to produce few receptors. A population may, thus, be phenotypically heterogeneous while being genotypically homogeneous. The resistance of this hypothetical population is density-dependent. The population may become totally resistant by reducing its density so that sensitive cells appear at a frequency of less than one per generation.

The beauty of the genetic and physiological control of λ-resistance is that it allows for all k_1, k_2 and k_3 values varying continuously from zero to a maximum. Thus, for a bacterial population which grows in the presence of a phage there exists not only few, but an infinite number of possible strategies. In other words, the population may accurately adjust its resistance, qualitatively and quantitatively, according to the type and density of the coexisting phage. Any such intermediate resistance may be called *semiresistance*.

Some cases of semiresistance at the level of infection are documented by the empirical analysis of the selected populations that follows and several other examples are discussed. The broader meaning of semiresistance and its dialectic relation with λ-virulence (whose quantitative control is studied in the subsequent chapter) are considered in chapter 5.

PAGE NUMBERING AS

ORIGINAL

Colonies which are unable to utilize the available sugar are colourless. The sugars were autoclaved separately and added to the basal medium after cooling at 60°C. The pH of these media was 7.6 when K_2HPO_4 was used and 6.8 when KH_2PO_4 was used. In some cases the pH was adjusted to 8, 7 or 6 with KOH or HCl before autoclaving. EMB will be referred to as EMB (sugar) I when the pH was 7.6 or 8 and as EMB (sugar) II when the pH was 7 or lower. Filter-sterilized, sodium salt of adenosine 3':5'-cyclic monophosphoric acid (cAMP; *Sigma Chemical Co*) was added on plate, just before pouring the medium, at a final concentration of 5mM. At this concentration, cAMP greatly induces the synthesis of λ-receptor in *E. coli* even in the absence of maltose (Schwartz, 1976).

TA2 medium (described in section 1.2.3) was adjusted to pH 6, 7 or 8 and supplemented with maltose, KH_2PO_4 and cAMP exactly as the EMB basal medium was.

The minimal salt medium M63malI was based on the composition of M63 (Pardee *et al*, 1959) but $FeSO_4$ was omitted. It contained final concentrations of potassium *di*-hydrogen-orthophosphate 13.6g, ammonium sulphate ($[NH_4]_2SO_4$) 2.0g, magensium sulphate ($MgSO_4.7H_2O$) 0.2g, maltose (*Fisons*) 1mg, leucine 10mg, threonine 10mg and thiamine 1mg per 1000ml of distilled water. The pH was adjusted to 7.5 with KOH. This medium was used in a chemostat, identical to those described in chapter 1, for selecting λ-sensitive, S bacteria.

Nutrient Broth No 2 (NB; OXOID CM67), Tryptone Water (TW; OXOID CM87) and *Tryptone,* 1 per cent solution (TS), were also used for preparation of lysates.

2.2.3 *Induction of λ-Receptor Synthesis*

For induction of phage receptor synthesis, overnight cultures were diluted 5-fold into maltose (0.5%) and KH_2PO_4 (0.2%) containing TB1 (pH adjusted to 7.5 with KOH) and incubated further at $37^{\circ}C$ for 4-5 hours.   At the end of the adsorption experiments the induced bacterial populations were screened on EMB media to confirm that the addition of the inducers did not change their genotypic composition, by favouring growth of rare mutants, but simply altered the phenotype of the dominant genotype.

The other strains, media and techniques used were as described in section 1.2.

2.3 *ISOLATION AND CHARACTERISATION OF SELECTED BACTERIAL SUBPOPULATIONS*

2.3.1 mal-*Polymorphorism*

The ability of a particular bacterial type to utilize maltose is usually designated as *mal*$^+$ (for the wild type), *mal*$^-$ (negative) or *mal*$^{\pm}$ (doubtful). Since, however, most of the selected types were neither typically positive nor typically negative but differed from each other in some respect, this designation will not be used. Instead, the selected phenotypes will be described in detail and will be designated conveniently as information about them is being presented.

On EMBmalI plates the selected bacterial population appeared to consist of three colony-phenotypes. Type n (for negative) were small colourless colonies of regular shape which, however, appeared mucoid when only few were on the plate. The development of mucoidity was presumably dependent upon the availability of nutrients. On TA2 medium the n-type colonies were larger than the wild type (C600) but not mucoid. Type p (for positive) formed small black colonies with metallic green surface; p types were indistinguishable from control colonies on either EMBmal or on TA2 medium. Similar colonies which lacked green colouration on their surface were also isolated but these were considered as p because (i) they also appeared in control populations, (ii) they were always unstable, giving rise to p populations and (iii) they were found identical to genuine p in all subsequent tests. The third type, m (for mucoid) were very mucoid, colourless colonies which retained their mucoid appearance even when plated in large numbers and overgrew on the lawns of the other selected types.

The great majority in the stock selected mixture were n-type bacteria while types p and m were at similarly low frequencies of about 10^{-4} to 10^{-3}. The number of mucoid colonies in the mixture increased considerably after prolonged incubation at room temperature. Routine identification tests confirmed that all the above isolates belonged to the original strain, *E. coli* C600.

Reportedly (Szmelcman and Hofnung, 1975) *lamB* mutants have a profound competitive disadvantage when cultured with their isogenic, parental strain in chemostats where maltose is provided as the sole carbon source and at very low concentrations (3.5 μM); in particular, the wild-type population quickly eliminates the mutant even when the latter has a frequency-advantage in the inoculum. With this observation the above authors established the importance of the λ-receptor in maltose utilisation, which is not obvious on EMB mal or any other medium with maltose provided in routinely used concentrations.

The above technique was employed here to select for sensitive ($lamB^+$) bacteria from the selected mixture. A M63 mal I chemostat was inoculated with 10ml of the selected mixture and run for approximately 15 generations. Surprisingly, the harvested population consisted of the same phenotypes, n, p and m on EMB mal I, though at similar frequencies. This indicated that either all these types were equally, or similarly, fit in this medium, or that one of them, the dominant, gave rise to the others at high frequencies or that the n-p-m polymorphism was simply an artifact of EMB mal I. Isolates from this chemostat will be referred to as M63 mal I chemostat isolates and those from the original selected mixture as direct isolates.

Ten colonies of each type were diluted in TB1 and aliquots were immediately plated on EMB mal I agar to test their purity. All the

M63 mal I chemostat isolates and also all the *n* and *p* direct isolates were pure and were subcultured for further tests.   The populations resulting from *m* direct isolates, however, were heterogeneous consisting of the expected *m* and another type of colony at similar frequencies.   This new type, designated as *Sm* (for small) were small identical, in size and shape, to the type *p* but colourless on EMB mal I and differed from the *n* type being small even when they were few on plates.   The second round of purification from single colony subcultures resulted in, apparently, pure *m* and *Sm* populations in TB1. Colonies of the *Sm* type were not found in the cultures of *m* colonies isolated from the M63 mal I chemostat mixture.

It was found that all the *m* types and also the *Sm* types were able to utilize maltose though to a lesser extent than the control and *p* bacteria.   This was shown on EMB mal II agar where *m* colonies turned black, and developed green colouration on their surface, after two days of incubation at 37°C while after the same period of incubation *Sm* colonies appeared only with a dark centre.   On this medium *m* types showed spreading growth;   both growth and the development of the mucoid character stopped, however, as soon as the colonies developed the characteristic black-green colouration.   Their final size was, nevertheless, distinctly larger than that of control and *p* types on the same medium.   This appearance will be referred to as *pL* (for positive-large).   The *Sm* colonies continued growing after the appearance of dark colour in their centre and occasionally they turned mucoid after prolonged storage at room temperature.   Most of them, however, remained as small as control and *p* colonies incubated at the same temperature for the same time.

Two explanations could be given for the effect of the pH on mucoidity: maltose is used for capsular polysaccharide synthesis (see below) at high pH and for acid production (glycolysis) at low pH or, simply, growth is soon impaired at low pH due to the extreme acidity at the neighbourhood of the colony.

2.3.2 *Polymorphism of λ-Resistance*

The sensitivity of these types to phage under standard growth conditions was tested on TA2 plates by spot tests and in TB2 cultures. All n and p type isolates were completely resistant to both λv and λs which could neither form plaques on lawns nor increase their titre in liquid cultures of these bacteria. The isolates of the *Sm* type showed some kind of semiresistance; although none of the phages could form visible plaques on *Sm* lawns, in liquid cultures of these bacteria both phages grew to densities of 10^6 to 10^7 pfu/ml (20-fold increase) overnight.

In spite of their identical colony morphology m isolates had different sensitivities depending on their origin. All the direct m isolates were sensitive to both phages. On plates they supported clear-plaque growth of λv as well as of λs and in TB2 both phages formed high titre lysates after overnight incubation. On lawns of M63 mal I chemosat m isolates the selected phage could form turbid, but easily visible, plaques while when undiluted λv lysate was spotted on these lawns only small, countable numbers of distinct turbid plaques appeared. In liquid cultures of these bacteria λs could multiply well, achieving lysates of 10^3 pfu/ml, while growth of λv was evident only when the inoculum exceeded the 10^6 pfu. This suggests that only a small proportion of λv population, having common properties with λs, was able to grow on

these bacteria.   On the basis of the above tests the selected

bacterial population was divided into five distinct subpopulations

(S1-S5) the properties of which are summarised in *table 2.1*.

TABLE 2.1:   Properties of the First Selected Bacterial Isolates[a]

Phenotype	Colony Morphology[b] on		Phage Sensitivity[c] to	
	EMB mal I	EMB mal II	λv	λs
S1	*nL* or *S*[e]	*nL* or *S*[e]	*rr*	*rr*
S2	*pS*	*pS*	*rr*	*rr*
S3	*m*	*pL*	*rr*	*ss*
S4	*m*	*pL*	*ss*	*ss*
S5	*nS* or *m*	*dS* or *m*	*sr*	*sr*

[a]   Precise description is given in the text.

[b]   *n* = negative (colourless); *p* = positive (greenish black), *S* = small, regular growth; *L* = large, spreading growth; *m* = mucoid; *d* = dark centre.

[c]   The first letter refers to sensitivity in liquid culture, the second to sensitivity on plate; *s* = sensitive, *r* = resistant.

[e]   Depending on the number of colonies on plate.

2.3.3   *Host Range and Medium Preference of the Selected Phage*

The sensitivity of the S3 and S5 bacteria to phage was further investi-

gated.   The relative efficiency of plating (r.e.p.; the ratio of the

titre of the particular phage lysate measured on a particular host

lawn over the titre of the same lysate measured on control bacterial

lawns) of the selected phage on S3 lawns was 0.5 while that of the

control phage was only 2×10^{-5}. λv plaques isolated from S3 lawns

grew well on either control or S3 cultures in TB1 (or TB2) but lysates

obtained with control bacteria were significantly denser ($\pm 5 \times 10^{9}$ pfu/ml)

than those obtained from S3 cultures ($\pm 10^8$ pfu/ml). The plating
efficiency of these lysates, from either bacterial culture, on S3
lawns was largely, but not fully, restored to about 0.35, which
however was still significantly lower than that of λs, suggesting
that such λv plaques were host-range mutants able to grow on S3
bacteria but not identical to λs. These plaques were also turbid
on S3 but their size was much larger than that of λs-plaques. The
virulent character of these mutants was confirmed on K12(λ) lawns on
which they had full plating efficiency. The lysate of the highest
density, prepared with control bacteria in TB1, was stored for further
analysis and will be referred to as λvh.

Except for its ability to grow on S3 bacteria, λvh showed the same
growth ability as λv on the rest of the selected bacterial types and
formed identical to λv plaques on control lawns. The r.e.p. of all
phages on S1, S2 and S5 was zero implying that none of the lysates
contain host-range mutants able to form plaques on these bacterial lawns
under standard growth conditions.

Another interesting observation was made in a series of trials to find
the optimum conditions of growth for each phage. It appeared that λs
grew to its highest titres in S3 and S4 bacteria ($\pm 5 \times 10^9$ pfu/ml) but
only in TB1 or TB2 media. When NB, TW or TS was used λs lysates never
exceeded 5×10^7 pfu/ml and were sometimes as weak as 5×10^5 pfu/ml.
Similarly, when the control host was used, the denser lysates were
obtained in TB media but were still of lower titre ($\pm 5 \times 10^8$ pfu/ml) while
growth was, again, very poor in NB, TS and TW. The growth requirements
of λv and λvh were different; both these phages gave their highest
lysates with control bacteria (5×10^9 pfu/ml) but TB had only a slight
effect on their titre (5×10^8 to 10^9 pfu/ml). Consequently, not only

the host but also the medium had different effect on the growth of λs compared with that on the growth of λv and λvh. The effect of the medium was focussed on the concentration of Mg^{++} ions which is the only qualitative difference between TB and TW or TS media (according to *OXOID* manual Mg^{++} is only found in trace quantities in *Tryptone*).

2.3.4 *The Semiresistance of Selected Type 5 (S5)*

The above analysis gave direct evidence of partial resistance and host-range mutation as well as of heterogeneity of the selected bacteria in respect of their sensitivity to phage. The problem of the "semiresistance" of the selected bacteria was reduced to the problem of the "semiresistance" of the S5 phenotype. However, there was still a need to solve the problem of whether this was due to some undetected genetic heterogeneity or to some genotype which confers phenotypic heterogeneity to the population, as explained in section 2.1.3.

The evidence so far cannot exclude either possibility. S4 and S5 could, for instance, give rise to one another at a high frequency so that either population is in fact a mixture of the two at proportions depending on the type of the inoculum colony. This was supported by the facts that (i) both types were simultaneously isolated from single colonies, (ii) S5 occasionally develops mucoidity, (iii) S5 is sensitive to λv and (iv) both S4 and S5 were seemingly selected against in the M63 mal I chemostat. These were actually the reasons why the S4 and not the S3 types were thought to be the "partners" of S5 if such genetic dimorphism really existed. The following experiments were designed in order to obtain evidence for, or against, each of these hypotheses.

Ten typical S5 colonies with dark centre and five old, mucoid colonies,
all from EMB mal II plates, were diluted in TB1 and their contents
screened on EMB mal II. Only one of the ten typical S5 but,
surprisingly, none of the mucoid colonies contained some mucoid
daughter colonies which differed, however, from the S4-type being
colourless; this will be referred to as S51. Except the great
majority of small S5-like colonies, which presumably were the main
ingredient of all S5 populations, two other types were common, though
at lower frequencies (approximately 1 in 100 typical S5): S52, dark
colonies, which, however, did not exhibit greenish colouration, were
present in all isolates. S53, orange-coloured colonies, appeared in
most but not all plates. S52 and S53 had similar size and shape but
were distinctly different from the majority of the daughter colonies, .
and also from the S4 type showing no sign of mucoidity. The
frequency of S53 increased considerably in EMB mal II when this was
supplemented with $MgCl_2$ on which medium even some typically S5 colonies
from a highly diluted liquid culture developed orange colouration.
The difference between S52 and S53 colonies diminished after prolonged
incubation suggesting changes in their physiological state.

One colony of each type from each plate was chosen at random (but only
well separated ones were considered) and used for further characterization.
This was performed in the same manner as for the original S5 colonies;
that is, by screening on EMB mal media the contents of each colony, and
the contents of the populations resulting from these colonies after
overnight incubation in TB1. It appeared that only the S51 mucoid
colonies were homogeneous and stable on subculture. The other three
types gave rise to one another and to typical S5 populations. Moreover,
all S52, S53 and even S51 populations supported growth of λv and λs in

TB1 but their lawns (in TA2) did not allow either phage to form plaques. The evidence was, therefore, in favour of phenotypic rather than genotypic heterogeneity of the S5 populations in respect to their sensitivity to phage since the only stable type, S51 exhibited the typical S5-semiresistance.

That the semiresistance of S5 populations was at least partially phenotypic was shown when the inducing effect of maltose was studied. Both λv and λs formed turbid but easily visible plaques on S5 lawns when maltose and phosphate were added to TA2 while the effect of either maltose or phosphate added alone was very little if any at all.

Another type of colony (designated as S521) appeared on lawns of S52 when these lawns developed on maltose-containing TB2 media. These colonies appeared at a frequency of 10^{-3} and overgrew their lawns, presumably consuming maltose at higher rates. They were absent, however, in the areas covered by the drops of either phage even when no clearance of the lawn was apparent. S521 were, therefore, sensitive to both phages at least in the presence of maltose. Such colonies did not appear in ordinary, S5, S51 or S53 lawns.

By diluting the lawns to obtain well separated S521 colonies these could be isolated. 5 isolates were subcultured in TB1 and tested for their sensitivity to phage. They all supported growth of both λv and λs in TB1 but none permitted plaque formation on standard, TA2. Maltose and phosphate increased their sensitivity to both phages considerably when added separately but even more when added together. On EMB mal media they form colonies with typical S5 morphology. This behaviour clearly differentiated them from the S4 types.

Similar tests applied to S4 populations showed that small, non-mucoid colony formers were to be found after prolonged storage; their frequency was very similar to that of their parents. These small S4 types (designated as S41) were stable on subculture but so were their mucoid S4 parents. On TA2, S41 lawns were fully sensitive to both λv and λs. Maltose increased the turbidity of the plaques of both phages on both S4 and S41 lawns. Phosphate had no effect when added alone but cancelled the effect of maltose when these were added together. The only difference, apart from their colony morphology, between S4 and S41 types concerned the effect of maltose on the size of λs plaques. Maltose with phosphate increased the size of the plaques formed by both λv and λs-lysate spots on S41 but decreased the size of λs plaques and increased the size of λv plaques on S4 lawns. These tests were performed on 5 isolates of each type.

No polymorphism was traced in S1, S2 or S3 bacterial populations using the same methods. Maltose increased the turbidity of both λvh and λs plaques on S3 lawns when added alone and phosphate seemed to cancel the effect of maltose in both cases. Diluted λv lysate which contained no λvh mutants failed to form plaques under any conditions. Also, neither maltose nor phosphate could restore sensitivity of S1 and S2 lawns.

2.3.5 *Survival of Selected Sensitive Bacteria to Phage*

When added alone, maltose increased the turbidity of the plaques of all phages not only on selected (sensitive type) but also on control bacteria. This phenomenon is easily understood as an effect of intracellular accumulation of glucose, an intermediate metabolite of maltose catabolism (*figure 2.1*) which cannot be phosphorylated and used

further unless phosphate is available.   In the absence of phosphate

the intracellular glucose accumulates and activates the catabolite

repression system which in turn represses the maltose regulon and,

consequently, the production of λ-receptors;   thus, the bacterial lawns

develop physiological (phenotypic) resistance, and the plaques are

turbid.   In the presence of phosphate the intracellular glucose

remains at its normal steady-state concentration and physiological

λ-resistance does not develop.

Under no circumstances, however, were the plaques of any phage on

selected bacteria as clear as they were on control lawns.   This

indicated a difference between selected and control hosts concerning

their survival in dense phage populations.   To test this the

frequencies of survivors to each phage in each sensitive bacterial

stock were compared on TA2 plates containing 10^9 pfu/ml.   As expected,

significant mortality was found only in bacteria on which the particular

phage could form visible plaques.   Thus, although S5 bacteria supported

growth of all phages in liquid cultures, and presumably on plates,

they did so in the expense of an invisible (on plates), non-measurable

(by this technique) fraction of their populations;   but even the lowest

value of survival measured in selected bacteria (S4) was far greater

than that of the control bacteria.   The results of these measurements

are shown in *table 2.2*.

A more detailed experiment was performed with S5 bacteria in an attempt

to isolate genetically sensitive individuals of this type.   100 young

colonies from a phage-free plate were transferred to plates containing

λs at 10^9 pfu/ml.   This method proved to be inadequate, however,

since not only all the S5 but also 10 S4 and 10 control colonies, from

which most, if not all, were statistically expected to have had

TABLE 2.2 : Survival of Sensitive Selected Bacteria on Phage Containing Plates[a]

Phage	Bacteria Control[b]	S3	S4[b]	S5[c]
λv[d]	1.4×10^{-4}	1.00	0.14	1.00
λvh[d]	2.0×10^{-4}	0.74[e]	0.10	1.00
λs[d]	1.6×10^{-4}	0.62[e]	0.13	1.00

[a] The frequency of survivors shown is the ratio of the bacterial titre measured on plates containing approximately 10^3 pfu/ml of the corresponding phage over the titre of the same bacterial culture measured on phage-free plates; each titre was the mean of 5 replicate counts.

[b] Differences are not significant.

[c] Mortality was not traced but does occur (see text).

[d] $P < 0.001$

[e] $P < 0.05$

originated from sensitive cells, survived on such λs lawns. This was presumably because even colonies of control bacteria may well contain resistant mutants by the time they become visible.

2.3.6   *Resistant Mutants of Sensitive Selected Parents*

To investigate the nature of the survivors 10 colonies of each selected type (S3, S4 and S5) and 10 control colonies from λs-containing plates were screened on EMB mal plates in the usual manner. All colonies appeared homogeneous in terms of the size and shape characterising their type. However, changes in their colour and, therefore, in their ability to utilise maltose were observed. The S5 isolates were not polymorphic and had lost their dark centre character on EMB mal II.

All control surviving colonies contained only small colourless members, on EMB mal I, but appeared to be mixtures of small black (*pS*-type) colonies (R1) with also small but colourless types (R2; at frequencies of the order of 10^{-2}) on EMB mal II. This was in good agreement with the finding that most (80%) of the λ-resistant *E. coli* have a mal⁻ phenotype on the commonly used EMB media, as EMB mal I (Thirion and Hofnung, 1972); the more sensitive test on EMB mal II media demonstrated, however, that these mal⁻ mutations reduce, rather than eliminate, maltose utilisation and the sensitivity to λ and that these properties may be reduced further by secondary mutations.

Such was also the situation in all tested S3 and S4 surviving colonies which contained S1-looking daughter colonies (S311, S421) and darker, but not typical *pL* types, (S312, S422) at similar frequencies when screened on EMB mal II; though they appeared homogeneous, containing only large colourless colonies, when screened on EMB mal I.

From 6 S311, 7 S312, 11 S421, 6 S422 and 4 S54 isolates tested for their sensitivity to λv, λvh and λs spots, only one, designated S313 showed some sensitivity. It allowed turbid plaques of only λvh and λs and only in presence of maltose and phosphate. It was one S312-type colony. Although their sensitivity was not tested in liquid cultures their resistance on plate suggests that these populations consisted of, or were at least enriched in, resistant mutants of non-S5 type. The secondary isolates and their phenotypes are shown in *table 2.3*. *Table 2.4* summarises the effect of maltose on the sensitivity of all isolates on plate.

TABLE 2.3 : Properties of Secondary Isolates[a]

Designation	Parent Population	Colony Morphology on		Sensitivity to		
		EMB mal I	EMB mal II	λv	λvh	λs
S311	S3	nL	nL	r	r	r
S312	S3	nL	$(p)L$	r	r	r
S313	S3	nL	$(p)L$	r	r	r
S41	S4	nS	pS	s	s	s
S421	S4	nL	nL	r	r	r
S422	S4	nL	$(p)L$	r	r	r
S51	S5	m	m	rs	rs	rs
S52	S5	nS	dS	rs	rs	rs
S521	S52	nS	dS	rs	rs	rs
S53	S5	nS	dS	rs	rs	rs
S54	S5	ND	ND	r	r	r

[a] Precise description is given in the text; n = negative;
p = positive; (p) reduced colouration; d = dark centre,
colonies heterogeneous; r = resistant on plate; S = small,
regular shape; L = Large, spreading growth; m = mucoid;
rs= resistant on plate but supports phage growth in liquid cultures;
ND = not done.

TABLE 2.4 : The Effect of Maltose on the Sensitivity to Phage of

Selected Strains.[a]

Bacterial Type	Sensitivity to λv with		Sensitivity to λs and λvh with	
	Maltose	Maltose+PO_4	Maltose	Maltose+PO_4
S1	O	O	O	O
S2	O	O	O	O
S3	O	O	−	O
S311(2)	O	O	O	O
S313	O	O	O	+
S4	−	+	−	−
S41	−	+	−	+
S421	O	O	O	O
S422	O	O	O	O
S5	O	+	O	+
S51	O	+	O	+
S52(3)	O	+	O	+
S521	+	+	+	+
S54	O	O	O	O

[a] Maltose and KH_2PO_4 were added to TA2 just before plating. One drop each of stock λs-lysate, stock λv-lysate and diluted (to avoid the presence of λh) λv-lysate was placed on each lawn and the clearness and size of the phage plaques (when developed) were compared with those on control plates, in which both maltose and phosphate were omitted, after overnight incubation; O : plaques as on the control plates; + : increase in clearness or size; − : decrease in clearness or size.

2 .3.7   *Growth on Sugars and the Effects of Temperature, pH and cAMP*

In an attempt to characterize further the five main selected mutants, their colony phenotype was tested on maltose-galactose-arabinose- and glucose- containing EMB and their sensitivity to high spots of λv, λvh and λs was tested on TA2 plates.   Both series of tests were performed at three pHs, 8, 7 and 6, at two temperatures, 37 and $42^{o}C$ and in the presence and absence of cAMP.

The purpose of this series of tests was to see whether any of these mutants was defective in some central mechanism affecting the utilization of sugars other than maltose (catabolite repression system or PTS) and whether these defects were temperature- or pH-dependent.   For example, on binding with cAMP, CRP undergoes conformational changes (Krakow and Pastan, 1973;   Wu and Wu, 1974; Wu *et al*, 1974) which allow binding with DNA and stimulation of trans-cription.   Krakow and Pastan (1973) found that the reaction between the DNA-binding site of this protein and the DNA *in vitro* depends on cAMP at pH 8 but not so at pH 6.   Some of the isolates which were negative on EMB mal I but positive on EMB mal II might have, therefore, been *crp* mutants with defective cAMP-binding site of the CRP.   The pH values, temperatures and concentrations were chosen as those which have been reported to have some effects on the expression of such mutations.

All strains except S5 formed wild-type (small or large) black colonies on all, EMBgal, EMBara and EMBglu media at both temperatures and no change in their colony-forming ability was observed.   Neither temperature nor cAMP changed the effect of the pH, as already described on the colony morphology of any bacteria on EMB media.   The S5 bacteria formed colourless small colonies on EMBara ar pH 8 and wild-type colonies on all other media.   Again neither the temperature nor the

cAMP had any effect on their phenotype nor was any loss in their colony-forming ability observed. Mucoidity did not develop in any of the S5 plates during incubation but it did so, exactly as on EMB mal plates, on EMB ara I plates when these were stored at room temperature. Whether the development of mucoiduty was a temperature-dependent phenomenon or simply occurred late in time was impossible to know, since if incubation continued at elevated temperatures the plates were drying before development of mucoidity was due. Plates incubated at 42°C also dried quickly on storage and no mucoidity could be seen.

2.3.8 *Effects of Temperature pH and cAMP on Phage Growth*

All bacteria had the same pattern of sensitivity to phage shown in *table 2.3* at both incubation temperatures and the acidity and cAMP did not seem to affect the turbidity of the plaques or change the effect of maltose and phosphate. Moreover, growth of all phages on all sensitive bacteria (including the control) was largely accelerated at 42°C, as their plaques appeared much earlier than at 37°C, and none of the r.e.p. of the phages altered at this temperature. Thus, there was no evidence of thermosensitivity in phage growth.

2.3.9 *Phage Adsorption on Sensitive Selected Strains*

The 15 min adsorption rates of phages λv, λvh and λs on the main selected bacterial populations are shown in *table 2.5*. The number of total and free plaque-forming units was measured in each adsorption mixture using the streptomysin-protocol as described in section 1.2.7. Significant drop in total titre was evident only in mixtures with S3 bacteria. In the case of λv the total titre was not different from

TABLE 2.5 : Phage Adsorption on Selected Bacteria [a]

Bacteria	Phage λv	λvh	λs
Control	76	45	94
S1	0	0	0
S2	0	0	0
S3	75[b]	77[c]	85[d]
S4	64	73	80
S5	0	0	0
S1 Induced[e]	0	0	0
S2 Induced[e]	0	0	0
S5 Induced[e]	92	92	96

[a] Values are proportions of the input phage titre found as infected cells at 15 min from mixing, and are the means of at least two replicate measurements; 0 indicates non-measurable adsorption.

[b] Adsorption is totally non-productive.

[c] Only 71% of the total adsorption is productive.

[d] Only 75% of the total adsorption is productive.

[e] Induction was by maltose and phosphate (see section 2.2.3).

the free phage titre and, therefore, productive adsorption was not measurable. The similarity of its rate of inactivation with the rate of total adsorption of λvh on this bacteria, however, indicates that λv did adsorb irreversibly on S3 bacteria but that its infective process was blocked at a later stage. λvh and λs carry mutations which overcome this block but only partially, since these too are inactivated by S3 bacteria at a low but significant rate. The

partially productive adsorption of these phages on S3 bacteria is in agreement with the partial restoration of their relative efficiency of plating on lawns of this host. The consistently higher rate of total adsorption of λs against those of λv and λvh on these and all the other sensitive bacteria, on the other hand, supports the hypothesis that mutations in λs have altered its adsorbing machinery so as to make the formation of phage-receptor complex easier and more frequent.

Whereas S5 bacteria grown under standard conditions did not allow measurable adsorption of any phage, all phages could very efficiently adsorb in populations grown with maltose and phosphate demonstrating that λ-receptor synthesis was greatly induced under these conditions. In contrast, such induction did not occur in S1 and S2 bacteria.

2.4   *O N   T H E   G E N O T Y P E S   O F   T H E   S E L E C T E D   I S O L A T E S*

2.4.1   *Phenotypic and Genotypic Polymorphism in the* mal *Regions*

On the basis of their sensitivity to phage the selected bacterial
isolates were found to comprise four major groups: (i) *Totally
resistant* (to both phages) which show no ability to produce functional
receptors; (ii) *partially resistant* which are susceptible to the
coexisting but not to the original phage; (iii) *semiresistant* in
which the receptor synthesis is greatly but not fully inhibited and
are, therefore, slightly susceptible to both the original and the
coselected phage; (iv) *sensitive* to both phages with normal production
of "wild-type" receptors.   This analysis is of course insufficient
to elucidate the precise nature of each isolate but it does fulfill
the purpose of demonstrating the dual, genotypic and phenotypic,
nature of the heterogenity of the selected bacterial population.

The mutations carried by the first four selected types (S1, S2, S3
and S4) concerning their ability to utilise maltose seem to lie within
the maltose regulon rather than any central regulatory system as
there was no evidence of defects in the utilisation of other sugars
(page 67).   The phenotype of S1 is reportedly produced by mutations
on the *malT* or strong polar mutations on the *malK* gene while that of
S2 is typical of mutations in the gene *lamB* itself.   The failure to
isolate phage mutants which could infect S2 bacteria indicates that in
this type too λ-receptors are absent from the outer membrane or they
have completely lost their λ-binding ability.   Resistance of this
type, however, has always been associated with the absence of

receptors from the cell surface and is usually of the nonsense type

of mutants (Hofnung *et al*, 1976) or mutants, such as those described

by Emr *et al* (1978) and Emr and Silhavy (1980), which synthesize a

lamB-product which remains in the cytoplasm.

2.4.2   *Partial Resistance and Host Range*

The only case of partial resistance found was that of the S3-type.

The fact that λv is able to carry out irreversible adsorption on S3

host just as well as the λvh mutant indicates that the S3 mutation

blocks some step of infection subsequent to the irreversible adsorption

process.   The plating efficiencies of λv and the host-range mutant,

λvh, on S3 bacteria are remarkably similar to those of the "wild-type"

λ and of *H* and *V* λ-mutants infecting *pel⁻* bacteria (recently renamed

ptsM by Elliott and Arber, 1978, and Bachmann and Low, 1980) which

inhibits λ-DNA injection (Scandella and Arber, 1976).   Mackay and

Bode (1976a) presented evidence that there were at least three distinct

steps occurring *in vivo* after irreversible adsorption and before the

phage DNA finds itself in the cytoplasm ready to replicate.   Whether

any bacterial or phage proteins other than the mannosephosphotransferase

enzyme II (*ptsM*-product) and the phage *H* and *V* proteins are involved

in any of these post-irreversible adsorption events is not yet known but

it seems that either this is the case or the *lamB* product itself

undertakes additional functions after its irreversible binding with the

tip of the λ-tail (Roa and Scandella, 1976).   Thus, although the S3

defect would seem to concern some step of the phage-host interaction

subsequent to DNA injection, the possibility that it concerns the phage-

receptor reaction cannot be ruled out.   On the other hand, the

mutation(s) which enable λvh and λs to grow on S3 is (are) clearly not

concerned with the phage-receptor irreversible reaction since λv too can adsorb irreversibly on these bacteria.

The inactivation of λs and λv observed upon mixing with selected bacteria may be partially attributed to the existence in the bacterial mixture of S3 types at relatively high density. There is, however, an important inconsistency; in mixture with S3 bacteria alone productive adsorption of λs occurred at a relatively high rate whereas when added into the mixed, selected bacterial population both phages were inactivated at almost identical rates (*figure 1.11*) and productive adsorption of λs was much rarer. To explain this one would assume that the selected bacterial mixture was enriched in S3-like mutants which, although they permitted irreversible adsorption, inhibited propagation of both phages completely. The difference in rates of productive adsorption between λs and λv, found with the streptomycin-chloramphenicol protocol, may well have been due to the presence of S3 bacteria on which λs does adsorb productively and λv is inactivated. This also implies that the frequency of S3 bacteria in the selected population was low, which is probably the reason why these types were not among the first isolates from EMB mal plates.

2.4.3 *Causes and Effects of Evolution in the Phage Gene* J

Buchwald and Siminovitch (1969) found that *J* was the only known λ-gene involved in the production of serum-blocking material. Their findings remain unchallenged. If this is so, the change in the antigenic properties of the phage during selection could only be explained as a pleiotropic effect of the host-range mutation(s) which allow growth on S3 if the alterations in the S3-λs relationship concerned the reaction between the *J* and *lamB* proteins; but such alterations which

do not affect the irreversible step of adsorption have not been reported. The following proposition is to explain the changes in the antigenic properties of λs, as well as a number of other observations, without making forced assumptions of this kind.

Schwartz (1976) found that high concentration of Magnesium ions (Mg^{++}), as in the commonly used for λ-growth TB media, has an inhibitory effect on the adsorption rate which was identified as an increase of the dissociation constant (k_2) of the *equation 2.1*. This effect was not seen however when the cells were induced to have large numbers of receptors on their surface. The explanation given was that when the phage adsorbs on induced host the high rate of dissociation due to the effect of Mg^{++} is compensated by the high probability that the particles will re-adsorb to another receptor before leaving the cell surface; in other words, with induced cells the reversible reaction moves to the right not because k_2 decreases but because k_1 increases.

In the chemostat, the presence of phage is a pressure selecting for hosts with reduced phage receptors on their surface. With such hosts the high concentration of Mg^{++} selects for phage *J*-mutants whose affinity for the receptor is enhanced or, to a greater extent, for mutants whose complex with the receptor is stabilised (as Puck *et al*, 1951, have shown in other cases) rather than destabilized by Mg^{++}.

This view is consistent (i) with the observation that λs forms high-titre lysates only in presence of Mg^{++} (though these ions may be involved in some other phage-host interactions) in contrast with λv which forms its densest lysates in Mg^{++}-free media; (ii) with the difference in rates of adsorption between λs, on the one hand, and λv

and λvh, on the other, onto all sensitive bacteria (including the
control; *figure 1.10*); (iii) with the fact that this difference (if
at all) is far less in the case of induced S5 bacteria, in which the
adsorption rates are predominantly determined by K_1 *(equation 2.1)*.

If gene *J* is, as it seems to be, the only phage-gene responsible for
irreversible adsorption (Roa and Scandella, 1976; Szybalski and
Szybalski, 1979) then changes in the phage-receptor affinity are
expected to arise from mutations·on this gene.   It has not been
reported whether host-range mutants show increased affinity for wild-
type receptors, but mutants showing increased affinity are not
necessarily host-range mutants.   Because adaptation of the organisms
to their chemical environment is possible anyway, a special warning
should be made;   that, when studying the properties and interactions
of selected organisms, the difference between bench and chemostat media
must either be eliminated or taken into account.

There is another aspect in which λs mimics the behaviour of a typical
host-range mutant.   The increased sensitivity of host-range λ to
chloroform has been repeatedly observed but is not well understood
(Randall-Hazelbouer and Schwartz, 1973;   Hofnung *et al*, 1976;   see also
section 1.4.2).   Authors tend to interpret this phenomenon as a direct
"destabilizing" effect of chloroform on the "unstable" host-range
mutants (see for example Wandersman and Schwartz, 1978).   It is more
reasonable to suppose, however, that the effect is indirect.

It is known that chloroform (or ethanol) catalyses in some way the
irreversible adsorption of wild type λ with purified receptor *in vitro*
(Randall-Hazelbauer and Schwartz, 1973;   Mackay and Bode, 1976b).
Host-range λ can bind to purified receptors in the absence of chloroform
but the rate of irreversible adsorption increases further when

chloroform is added. The paradox of inactivation by chloroform of host-range lysates to which purified receptor has not been added can be simply solved if one accepts that lysates contain, by nature, large amounts of receptors, free or bound to membrane debris, as a result of bacterial lysis. It would be interesting to see whether extensively purified lysates still show sensitivity to chloroform.

Further, Mackay and Bode (1976b) suggested that chloroform may favour a conformational state of the tail fibre and/or the receptor protein essential for their irreversible interaction and that a membrane component might perform the same function. If this is the case, large membrane fragments which would contain all the necessary components for adsorption may inactivate even wild-type phage. Analogous observations were made by Hantke (1978) who found that protein Ia, which serves as a receptor for T_2 and several other phages, inactivated the wild-type phage only after the addition of lipopolysaccharide while a host-range mutant (T_2h) was equally well inactivated with pure protein Ia. Thus, the well-known, yet little understood, phenomenon that there is a substantial drop in phage titre after its exponential growth (Delbruck, 1940; Cohen, 1949) can be explained.

In this case the above hypothesis can explain the difference in post-peak inactivation of λv and λs in lysates formed on control bacteria (see *figure 1.9*) and the difference in their sensitivity to chloroform (*table 1.2*). It should be re-stated that an increased phage-receptor affinity but not necessarily a typical host-range mutation would be sufficient for such behaviour. It should also be pointed out for further discussion that binding to free receptors can, under some circumstances, be a serious cause of "death" for the phage, as is irreversible adsorption to bacteria which do not permit intracellular growth.

2.4.4 *Genotypic and Phenotypic Polymorphism in Selected Semiresistant Populations and the Nature of S5 Genotypes*

The evidence obtained for the nature of the S5 types and their semi resistance is far too little to allow specific propositions to be made.   Some explanations are preferred, however, while others can be ruled out.   An important characteristic of S5 populations is their phenotypic heterogeneity, some of which may be physiological but some is certainly genetic.   The evidence of genetic heterogeneity was provided not only by the stable S51 and S54 types, which are most probably heterogeneous themselves, but also by S52, which were the only ones to give S521 types.   Given such plastisity, it would not seem improbable that the semiresistance of S5 populations is partially due to the presence of stable or unstable mutants, originating from a dominant S5 genotype.   For example, although S4 bacteria were not found in any of S5 populations, it remains possible that the mucoid λ-sensitive colonies observed on plates with the original selected mixture arose from S5 bacteria, which might well have been plentiful, through one or more of the genotypes with intermediate phenotype (S51, S521 or S41).

There are, however, several other ways that semiresistance may be understood.   One is the semiresistance of the S3 type and, generally, of bacteria which are susceptible to host-range phage.   In population terms, the probability that a collision of a partially resistant cell with a host-range phage mutant will result in cell lysis depends on how efficient the host-range mutation is.   As for S3 bacteria, host-range mutations are not always 100 per cent efficient.   Two other cases of semiresistance of homogeneously resistant to λ genotypes have been suggested.   (i) A weak promoter site, located between *malK* and *lamB*

genes may be responsible for a residual expression of the *lamB* gene
(2 to 6×10^{-2} λ-receptor molecules/cell) in *malT* or *malK*-polar mutants
(Braun–Breton and Hofnung, 1978). (ii) The presence of a secondary
receptor structure of low reactivity towards the phage (Braun–Breton
and Hognung, 1978; Wandersman and Schwartz, 1978). The authors of
the later paper also considered the possibility that, as a result of
their evolution, many phages are able to use more than one unrelated
outer-membrane component as their receptors.

None of these explanations seems necessary for the semiresistance of S5
types although all may be considered as potential solutions under other
circumstances. After induction with maltose and phosphate, S5 bacteria
become sensitive to both the original and the selected phage. This
indicates that neither the structure of the *lamB* product has altered
drastically nor is its synthesis eliminated. Also, the *malT* gene is
transcribed and its product protein is functional, at least as far as
its sites for maltose binding and activation of transcription (DNA-
binding) are concerned. The initiator site (i_{B2}) of the *malK-lamB*
operon which the *malT*p recognizes can be considered intact too.

Mutants of the structural genes of each operon have been found to
affect the expression of the other operons of the maltose regulon.
For example, some *malK* mutations result in a constitutive expression of
all three *mal*-Operons (Hofnung *et al*, 1974) while some *lamB* mutations
and some mutations of the *malEFG* operon genes (then known as *malJ* gene)
may result in a silent *malPQ* operon (Thirion and Hofnung, 1972;
Hofnung, 1974). Yet, sensitivity to maltose, caused by mutations in
malQ is compensated by mutations in the *malB* region or the *malT* gene
(Hofnung *et al*, 1971; Raibaud *et al*, 1979). Despite these observations
the gene-gene interactions within the maltose regulon remain obscure;

also obscure are the functions of three *malB* products, namely the proteins coded by *malK, malF* and *malG* (Silhavy *et al*, 1979). It is because of the lack of this information and because of the peculiarities of the S5 phenotypes that speculation on their genotypes can be very wide and at the same time very difficult.

The peculiarities of the S5 phenotype except its polymorphism are: (i) that λ-receptor synthesis, but not acid production, is fully induced by maltose; (ii) that arabinose, but not galactose (or glucose), utilisation is also affected; (iii) that maltose utilisation is affected more than arabinose utilisation. To explain this behaviour one confronts the dilema of whether hypothesise a single mutational event and a very complex functional network which extends outside the maltose regulon into the arabinose operon, or assume multiple mutational events within the already known functional inter-relationships. The complexity of both, the central regulatory network of the carbohydrate utilisation system and the maltose regulon (no other transport system in *E. coli* is known to require five gene products; Silhavy *et al*, 1979), is great enough to allow for speculation on additional functions of their genes and products. Conversely, and for the very same reason, it seems very likely that the S5 phenotype is the result not of one but of multiple mutational events; that is, the result of extensive evolution.

Based on the existing information, the only central factor of the carbohydrate utilisation network involved in the regulation of both the maltose regulon and arabinose operon is the CRP-cAMP complex. The tests performed failed to show any dependence of maltose or arabinose utilisation by S5 on the concentration of cAMP (page 67) but a number of other possibilities remain. For example, CRP may fail to bind cAMP

either because of structural faults of its cAMP binding site or because
it cannot be properly activated.   The same can also be said for its
DNA-binding site;   failure of the CRP to undergo any of the necessary
successive conformational changes will affect its overall efficiency
as an inducer of transcription.   Different operons may then have
different sensitivities to such mutational changes, determined by the
particular base-sequence of their CRP-binding DNA sites.   The same
arguments could in fact be used for supporting the idea that the
differences in the expression of arabinose and maltose operons reflect
different sensitivities to changes in the RNA-polymerase subunits (see
Doi, 1977) or, within the maltose regulon, to changes in the *malT*
regulatory protein.   If one more *mal*-gene product played some regulatory
role the attribution of the S5 phenotype to a single mutation might
have been easier.

2.4.5   *Mucoidity: A Phage-Independent Response*

While all efforts to explain the phenotypic characters of the selected
organisms have been on the conservative side, that is always assuming
the smallest number of mutational/evolutionary events, the evidence of
genetic heterogeneity should by no means be neglected.   The mucoidity
of S3, S4 and S51 is a stable character of these bacteria and it is
most probably caused by an independent mutation.   Mucoidity is
reportedly caused by mutations in the gene *lon*, which controls the *gal*-
operon, the capsular polysaccharide synthesis and the process of cell-
division (for references see Bachmann *et al*, 1976, and Bachmann and
Low, 1980), or by mutations in *capS*, also involved in the regulation of
the *gal*-operon (Hua and Markovitz, 1972).   Although such mutations
have a wide range of pleiotropic effects (particularly those on *lon*;   see,
for example, Gayda and Markovitz, 1978), none is known to interfere with

the expression of the maltose regulon and with the function of the λ-receptor
Moreover, none of the reported mutations in the maltose regulon knowingly
result in mucoidity. In support of the above statements the conversion
of S4 to S41 and of S5 to S51 was not accompanied by any obvious
changes in the maltose phenotype of these bacteria. It seems, therefore,
that at least in the case studied here, mucoidity developed in response
to some selective pressure other than the presence of phage in the
culture.

The S3 and S4 types appeared to have a reduced rate of acid production
from maltose. The normal production of λ-receptors, at least in S4, on
the other hand, would suggest that the maltose regulon functions at a
normal, or approximately normal, rate. Unless the production of
λ-receptor has been restored by a second mutation at a misfunctioning
maltose regulon, or, if the mutation affects more maltose utilisation
than the transcription and/or the translation of *lamB*, the peculiarity
of their maltose phenotype can be explained by assuming that maltose is
partially, or at pH 8 totally, used for capsular polysaccharide (CPS)
synthesis to which these bacteria presumably owe their mucoidity.

However, both these types formed large black colonies on galactose at
pH 8 and smaller black colonies on arabinose and glucose at the same pH.
It is unlikely that maltose is preferably used for CPS synthesis rather
than galactose, which can provide two of the building-blocks of the
CPS (Hua and Marcovitz, 1972) or glucose, which would need fewer
enzymes to be converted into any of the CPS's building-blocks. Against
the hypothesis of an altered biochemistry of maltose utilisation
towards CPS synthesis is also the isolation of S41 types which are
similar to S4 but not mucoid. It is, therefore, most probable that
the S3 and S4 colony phenotype is due to independent mutations within

and outside (in *lon* gene?) the maltose regulon.

2.4.6   *High Modulated Mutability*

Probably one of the most important observations made here is the
unusual rates of survival to high titre phage surrounding of all
sensitive selected bacteria and particularly of the most sensitive
S4 type.   A cell, plated on a medium which contains 10^9 pfu/ml of
phage, will escape lysis either because it is genetically resistant
(is defective in phage-receptor synthesis or function) or because it
happened to have few or no receptors available to phage on its surface.
Part of the possibility of escape can also be attributed to its low
mobility (diffusion) in solid media; but this possibility becomes
insignificant when the produced colony reaches a certain size and the
first cell is infected.   None of the selected bacteria are mucoid on
TA media and therefore a possibility of phenotypic resistance due to
over production of polysaccharide is also limited.   The following
arguments are to show that the difference between S4 and the original
strain is more complex than a single mutational event resulting in
mucoidity.

Schwartz (1976) found thata200-fold change in the average number of
receptors per cell corresponds to approximately 10 per cent change in
adsorption rate.   She also reported that the maximum capacity of the
cell surface under full induction is about 2000 phage particles.
Under conditions in which λv adsorbs at a rate of around 70 per cent at
15 min (in TB1) one should expect that both control and S4 bacteria
have similar, very low numbers of receptors per cell on their surface.
Having low numbers of receptors, single cells may temporarily survive
phage attack on plate, for the reasons given in the previous
paragraph, and give rise to a small colony.   The survivors are, thus,

either genetically resistant cells or cells which succeed in giving resistant progeny before the colony was attacked by phage.

Whatever the case is the S4 populations seem to contain resistant forms at unusually high proportions. Assuming that resistant forms appear at equal rates from S4 and original bacteria, the S4-resistant ones must have a selective advantage to achieve these unusual numbers in S4 cultures which advantage the resistant forms arising in control cultures do not have. This would be in contrast with the general belief that resistant bacteria are less fit than their sensitive parents in the absence of phage, but it would also indicate that the λ-resistant S4, and their isogenic, parental genotypes, differ from the corresponding original genotypes in genes outside the *mal*-regulon. Alternatively, mutations to resistance occur in S4 types at unusually high frequency.

This latter proposition is, in fact, strongly supported by the fact that the surviving colonies contained at least two types of resistant bacteria. The same was observed in S3 surviving colonies. Interestingly enough, surviving colonies of the S5 strain were enriched in forms whose sensitivity was not inducible by maltose, although additional mutations would not seem necessary for survival in S5 types.

Instability of mutants is a widely observed phenomenon. It is usually attributed to reverse mutations occurring at high frequencies. If this is the case of the sensitive selected isolates of this study then these had arisen from some resistant forms which may, or may not, be some of the resistant isolates. Although there is no serious objection to that sensitive mutants arising from resistant parents may be unstable (i.e. may revert at high frequency), the isolation of more than one type of "revertants" from S3 and S4 accounts strongly against the view that the sensitive selected bacteria were unstable mutants of the coexisting types.

Alternative explanations for this "instability" are (i) that the maltose regulon has suffered such rearrangements that any single further mutational event is critical, or (ii) that such frequent changes in the *mal*-phenotype and λ-resistance are part of a generalized high-rate mutability caused by a "mutator" gene (for literature on mutators in *E.coli* see Backmann and Low, 1980) or analogous phenomenon. This last hypothesis is not in contrast with, but rather contains, the other two and predicts that high rates of changes are to be found in other regions of the chromosome of the selected bacteria given appropriate selective techniques; it is supported by a number of other observations presented in the following chapters and it is given a fuller account in the last chapter.

It can be concluded from the above that (i) polymorphism existed in almost every examined aspect of the biology of the selected bacteria; (ii) in many instances the phenotypic differences among selected and control bacteria are better explained as effects of multiple mutational effects, implying the occurrence (in culture 1) of multiple evolutionary steps; and (iii) that at least some of the observed variation in bacteria is apparently subject to gene-specific or general modulation. Concerning the phage, there was direct evidence of host-range evolution and indirect evidence of evolution of the phage's adsorption site. The two sets of evolutionary events are however suggested to be quite independent.

EVOLUTION OF VIRULENCE

3.1 G E N E T I C   C O N T R O L   O F   λ-D E V E L O P M E N T

3.1.1   Development and Virulence

The mode of intracellular multiplication is one of the most important
aspects of traditional ECE systematics.   Thus, lytic and temperate
bacteriophages and bacterial plasmids are still, for most biologists,
well defined taxonomic concepts.   All phages whose DNA *has been found*
to be able to integrate into the chromosome of a host are classified
as temperate, irrespectively of the frequency of lysogenisation,
relative to that of lysis, of the host.   Mutations may increase or
decrease the ability of a temperate phage to lysogenise its host(s).
Unlike ordinary clear-plaque mutants, however, virulent mutants are
never seen to undertake the lysogenic developmental pathway and can
multiply in lysogens carrying the parent prophage.   Phages which
have not been found to be able to integrate their genome into the
bacterial chromosome are classified as lytic unless they are known to
be descended from temperate ones, in which case they are designated as
virulent mutants.

The above taxonomy is on a functional (ecological) rather than an
evolutionary basis; it has not yet established satisfactory phylogenetic
links among various ECEs.   The existence of ECEs, like λ, which may
follow any of the three developmental pathways (depending, to a considerable
extent, on the host genotype) suggests that the mode of intracellular
development *per se* is of relatively minor taxonomic importance.

Virulence and temperance are regarded here as the extremes of a
continuous range of developmental strategies, designed by collaboration

of the phage and host genomes, rather than as fixed, "special",
qualitative characters of phages.   In this sense, virulence is a
quantitative parameter related to the intensity of the lytic
developmental pathway, (i.e. the proportion of propagules which opt,
or are allowed, to multiply lytically). Lytic or temperate phages
and plasmids may evolve from each other through successive inter-
mediate steps of (co)evolution of the ECE and the host genes which
control and channel the ECE's development.   Thus, changes in
developmental parameters are not merely changes in ecological fitness
but also manifestations of   micro-evolution.

The above concepts were implicit in Horne's (1970a; 1971) suggestion
that lysogeny may be the ultimate outcome of the evolutionary decrease
of the phages' reproductive potential (efficiency of lytic development).
The same ideas were behind the interpretation of the unusually high
burst-size of the selected phage on selected bacteria as a sign of
evolution of virulence.   The isolation of sensitive selected host
strains (presented in the preceeding chapter) resolved the technical
difficulties encountered in the preliminary experiments (see page 38)
and allowed a more detailed study to be made of the phenotypic changes,
in phage and bacteria, which are associated with evolution of post-
infectional phage-host interactions.   This study is presented in
section 3.2.   The subsequent section (3.3) deals in more detail with
the quantitative concept of λ-virulence, its control and its
evolution.   The following overview of the genetics of λ-development is
to assist this discussion.

3.1.2 *The Genes of* λ

The genetic map of λ, as recently reviewed by Szybalski and Szybalski
(1979), is divided into 7 regions.   The first two regions from the
left end of the linear map of the mature λ-DNA (*figure 3.1*) code for
proteins of the head and tail morphogenesis;   their products are
either structural proteins of the virion or enzymes of the virion-
morphogenesis pathways.   The *b*-region contains genes non-essential
for lytic growth most of which are as yet unidentified;   it ends at
the site *att*, which is essential for the site-specific recombination
between phage and bacterial DNA that is required for integration and
excision of the λ-prophage.   The recombination region codes for site-
specific (*int, xis*) and general (*exo, bet*) recombination proteins, for
a protein which inhibits the host *recBC*-coded nuclease (*gam*) and at
least two other proteins of unknown function (Ea8.5, Ea22).   The
regulation region, from gene *cIII* through *cII*, contains sites and
genes which regulate the intracellular development of the phage;   this
is reviewed in more detail below.   The genes *O* and *P* of the replication
region code for proteins which promote initiation of DNA replication
from the site *ori* which lies in the middle of the gene *O*.   *Q* is a
regulatory gene whose protein promotes late transcription (see below).
The last, lysis region contains at least three genes, *S, R* and *Rz*
coding for proteins which cause lysis of the infected cell.

3.1.3 λ-*Development*

This is extensively reviewed by Lewin (1977) and very recently by
Herskowitz and Hagen (1980).   Summaries of the literature and complete
lists of references on the developmental functions of the genes can be
found in Szybalski and Szybalski (1979).

FIGURE 3.1:   *Genetic Map of the Mature λ-Chromosome*

This is a simplified version of the map by Szybalski and Szybalski
(1979).   The heavy lines symbolise the two strands of the λ-DNA.
The scale, in map units, is given on the left strand (L) whereas
the major functional regions are indicated on the right strand (R).
Non-coding sequences (promoters/operators (△), terminators (○) and
other (□) are indicated with small lettering in the area inside
the strands;   the mutations of λvir are shown also with small
lettering in the area outside the strands;   genes essential for
lytic growth are symbolised with capital letters and non-essential
genes with small letters.   The genes *exo* and *bet* are collectively
referred to as *red*;   the function *kil* seems to be coded for
partially by the gene *gam* and partially by *cIII*.

Dotted lines symbolise scriptons and the thickness of the dots
indicates various levels of transcription.   *L1:* immediate early
transcription;   *L1E:* scripton for the establishment of lysogeny;
L1M: scripton for the maintenance of lysogeny;   *INT:* integration
transcript;   *L2:* major leftwards delayed early transcription which
terminates at several sites along the left strand;   *R1:* immediate
and delayed early transcription the bulk of which terminates at
several terminator sites in the t_{R_2} region;   *R2:* late transcription
dependent on the Q-protein and led by a small transcript from p_Q to
t_Q.

0

Nu1

A
W
B
C
Nu3
D
E
F II
F I
Z
U
V
G
T
H
M
L
K
I

J

10

20

30

40 Iom

bEa47

50 bEa31

bEa59
bEa24

att

int
xis
Ea8.5
Ea22
Ea9
exo
bet
gam
cIII
Ea10
ral
N
rex

cI
cro
cII

O
P

Q
S
R
Rz

60 P_I

t_{L3}

t_{L2}

t_{L1}

V_2 $P_L \cdot O_L$
t_i

P_M
P_E

80 ori

90

100

5'm
cosL
3'

HEAD

TAIL

b-REGION

att

red
kil

RECOMBINATION

REGULATION

$P_R \cdot O_R$ $V_1 \cdot V_3$
t_{R1}

ori REPLICATION

t_{R2}
Region REGULATION

P_Q
t_Q

LYSIS

cosR
m' 5'

3'

R1 R2

INT

L2 L1

L1M
L1E

R1

R2
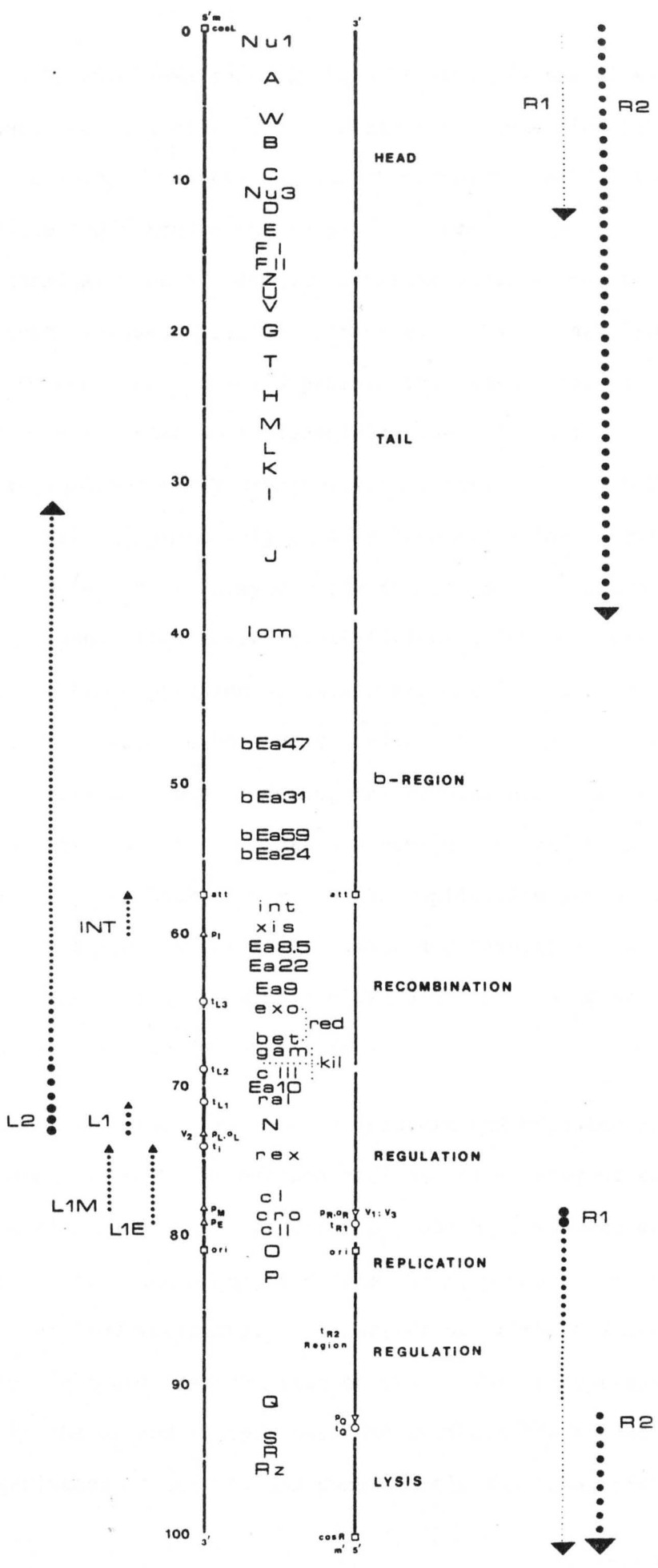
Upon penetration of the λ-DNA through the host membrane the linear molecule is converted to a closed, covalently continuous circular one. Cohesive termini (*cos*) are necessary and sufficient for this event. *Immediate early* transcription proceeds from p_L and p_R promoters and terminates at t_{L1} and t_{R1} termination sites due to the action of the host, RPase-associated, ρ factor. Thus, N and CRO proteins are produced first. The N protein acts immediately as an antiterminator of transcription (antagonizing the ρ factor) and allows post-t_{L_1} and -t_{R_1}, delayed early transcription to proceed. CRO seems to act at a later stage, presumably when its concentration is sufficiently increased. The N-dependent delayed early transcription produces messages from all genes (low scale transcription) which, however, is not sufficient for the expression of late functions (genes *S* through *J*). Transcription at the neighbourhood of the site *ori* and the amounts of *O*- and *P*- coded proteins produced during the delayed early stage of development are sufficient to initiate DNA replication which is thereafter undertaken by host enzymes. DNA replication proceeds bidirectionally in a semi-conservative manner and results in two daughter circular molecules which may initiate another round of replication in the same way (θ-*replication*).

As delayed early development proceeds *cro* protein and circular copies of the genome accumulate. The *cII* and *cIII* proteins interact and stimulate transcription from the promoter p_E, which terminates at the terminator site t_i (*L1E* scripton), and from the p_I promoter to just after the gene *int* (*INT* scripton). Transcription of the *L1E* scripton antagonizes the rightward transcription of *cro*. The *cI*-repressor protein binds to the o_L and o_R operators and inhibits the transcription of the major scriptons *L2* and *R1*, and consequently the transcription of

cro and *N*, and the initiation of DNA replication. Inhibition of the
leftward transcription prevents killing of the host (inhibition of
the process of cell-division) by the products of genes *kil*, *Ea10* and *N*.
The *int*-protein integrates one of the circular copies of the genome
into the bacterial chromosome and creates a prophage. When total
inhibition of late functions is achieved by *cI*-repressor the prophage
may divide synchronously with the host chromosome. Maintenance
of the prophage requires continuous production of the *cI*-repressor to
keep the excision and developmental functions repressed. In lysogens,
low-scale transcription of the prophage *cI* gene starts at the
promoter p_M and terminates at the terminator t_i (*L1M* scripton); this
transcription is stimulated by the *cI*-protein itself (autoregulation).
These events underlie the lysogenic response.

The *cro* protein recognizes different sequences in o_L and o_R but its
binding antagonizes the binding of the *cI*-repressor to these operators.
Moreover, the binding of CRO onto o_L and o_R prevents the transcription
of the *cII* and *cIII* genes and, consequently, the transcription of *cI*.
However, CRO does not inhibit the early transcription as efficiently
as the *cI*-repressor does and allows some 10 per cent residual
transcription of the rightward operon. It has been postulated
(Lewin, 1977) that this residual transcription leads to sufficient
synthesis of *Q*-protein which stimulates transcription of the late
genes *S* through *J* and that this is probably why CRO always channels
to lytic development. The transition from early transcription to
late transcription and from early θ-replication, to late rolling-circle
replication of the phage DNA, which both occur after *cro*-repression
but not after *cI*-repression, remains the least understood aspect of
λ-development. When this transition occurs development enters

irreversibly into the lytic pathway.

The rolling-circle replicating genomes produce linear poly-genomes, the *concatemers*, which are essential precursors of the mature DNA molecules found inside virions. The Q protein stimulates transcription of the late genes from the promoter p_Q (full scale *late* transcription) and particle morphogenesis proceeds. The A protein cleaves the concatemers into single units, creates cohesive termini (DNA *maturation*) and packages the mature, infective genomes into phage heads. Particle morphogenesis is completed with the specific joining of tails onto heads containing infective DNA. The lysis-proteins interact specifically with components of the cell membrane and cause its disruption. Lysis is timed by, at least, the gene *rex* whose product has been found to regulate the activity of the S lysis protein (Rolfe and Campbell, 1977).

3.1.4 *Mutants of* λ

Most of the known λ-mutations belong to one of the following categories: (i) lethal, that can be maintained only in the state of prophage (*defective lysogeny*); (ii) mutations, usually deletions, which interfere with both the lytic and the lysogenic pathway but not with plasmid perpetuation, designated as λ*dv*; (iii) conditionally lethal, allowing plaque formation only on permissive host and/or under permissive conditions; (iv) deleterious, reducing the plaque size, the plating efficiency or the reproductive potential of the phage; (v) affecting the establishment, maintainance (clear-plaque and virulent mutations) or excision of prophage; (vi) mutations which offer new properties to the phage and are detectable on mutant host while not affecting the functions of the phage or its interaction with standard

hosts in an obvious manner (like, for example, the host-range
mutations). The only class of mutations reported to increase the
phage yield are those on the lysis gene S; these mutants also show
a prolonged latent period (Reader and Siminovitch, 1971a; b). Of
course the above classification is arbitrary; a mutation which is
lethal in one genetic background may be innocuous or even beneficial
in another.

The phage used here, λvir, carries three point mutations: v_1 and v_3
on the o_R operator and v_2 on the o_L operator (Maniatis et al, 1975).
These mutations lie on the cI-repressor's recognition sites but do not
affect the recognition sites of CRO; thus λvir always follows the
lytic pathway and forms clear plaques. Unlike clear-plaque mutants
which produce no functional cI-repressor (eg cI, cII or $cIII$ mutants)
the virulent mutants develop lytically and form plaques even on lawns
of lysogenic bacteria where repressor is constitutively produced by
the prophage. Virulent mutants fail, however, to grow lytically on
plasmid-carrying bacteria due to early inhibition by CRO (synthesized
by the plasmid) of essential lytic functions. Mutations of the CRO-
recognition sites of o_L and o_R enable the phage to grow on plasmid-
carriers; such mutants are called $supervirulent$.

3.2   E V I D E N C E   F O R   E V O L U T I O N ·O F   P O S T -

I N F E C T I O N A L   P H A G E - H O S T   I N T E R A C T I O N S

3.2.1   *The High-Burst-Size Phenotype*

The pilot one-step growth experiments presented in section 1.3.3 indicated differences in the average burst sizes, and in the latent period of the control and selected phage-host systems.   The same experiments suggested that there might have been some genuine heterogeneity in either the selected host or the selected phage population concerning these growth parameters (section 1.4.3). Due to technical complications, however, those results were not conclusive.

The one-step growth experiments presented here were performed separately on each isolated selected host-type using the same, straightforward protocol (without chloramphenicol) as with control bacteria.   The phage (λv, λh or λs) was added into the host culture which was produced from a single colony after overnight incubation in TB1.   The multiplicity of addition was about 0.01.   After 15 min the adsorption mixture was diluted appropriately and the titres of residual free phage and of infected bacteria were estimated from plaque counts on plates with, and without, streptomycin.   From then on phage growth was followed at regular time-intervals.   The burst size and the minimum and mean latent periods were estimated as described in section 1.2.8.   For the experiments with S5 bacteria, receptor synthesis was induced by maltose and phosphate which were added a few hours before the experiment and were removed by centrifugation immediately before the addition of phage.   Each

experiment was replicated using three colony-isolates of the same type.

The purpose of this design was to increase the sensitivity of the method and discriminate the variation between phages and between hosts from that between experiments. Thus, it became possible not only to confirm the difference in burst size between the selected and control phage-host systems but also to investigate which organism's evolution was responsible for this difference, and whether there was any correlation between the burst size, on one hand, and the latent period, the h mutation or the small-plaque character of the selected phage, on the other.

The obtained growth curves are shown in *figures* 3.2 to 3.12 while the burst size, minimum and mean latent period estimates are compared in *tables* 3.1 to 3.3. From *table* 3.1 it is evident that the burst-sizes of all three selected systems (λs with S3, S4 and S5 bacteria) were consistently higher than those of all other phage-host combinations. Most interestingly, neither λs nor any of the selected bacterial genotypes was alone sufficient to produce a high burst-size phenotype. On control bacteria the yield of λs was essentially the same as that of λv whereas neither of the two selected bacterial genotypes which supported growth of λv (S4 and S5) had any significant effect on its burst size. Comparison of the busrt sizes of λh and λs shows, on the other hand, that the high-burst-size phenotype of λs is not a pleiotropic effect of its h mutation(s), conferring ability to grow on S3 bacteria, but rather the effect of an independent (set of) mutation(s). This (set of) mutation(s) of λs is necessary but not sufficient for the phage's high-burst-size phenotype; it is expressed only on the genetic background of the selected hosts. The

latter statement implies that the tested selected types also carry a (set of) mutation(s), other than those which differentiate them from each other (described in chapter 2), which is necessary for the expression of the high-burst-size mutation(s) of λs but not sufficient to increase the progeny of the control phages (λv and λh).

TABLE 3.1:   Comparison of Burst Sizes of Selected and Control Phage-Host Systems

Phage	Host Bacteria			
	Control	S3	S4	S5
λv Rep 1	185		180	169
Rep 2	153		161	123
Rep 3	135		163	128
Mean	158		168	140
λh Rep 1	102	95	92	148
Rep 2	98	119	87	122
Rep 3	124	82	100	108
Mean	108	99	93	126
λs Rep 1	164	487	519	414
Rep 2	117	407	493	320
Rep 3	145	389	453	333
Mean	142	428	488	356

[a] Values obtained from the one-step growth experiments shown in *figures 3.2 to 3.12*; Rep = replicate.

The factors, whether bacterial or viral, which control the latent period do not seem to have evolved. At least, this is how one can interpret the wide overlap of the time-intervals within which the minimum latent periods were estimated from these experiments to lie (*table 3.2*).

TABLE 3.2: Comparison of Minimum Latent Periods of Selected and Control Phage-Host Systems[a]

| Phage | Host Bacteria | | | |
	Control	S3	S4	S5
λv Rep 1	58-64		54-60	66-72
Rep 2	60-66		56-62	62-68
Rep 3	62-68		58-64	64-70
Combined[b]	58-68		54-64	62-72
λh Rep 1	60-65	60-66	54-60	54-60
Rep 2	62-67	62-68	62-68	62-68
Rep 3	62-68	64-70	64-70	64-70
Combined[b]	60-68	60-70	54-70	54-70
λs Rep 1	60-66	54-60	60-66	54-60
Rep 2	68-74	62-68	68-74	62-68
Rep 3	58-64	64-70	70-76	64-70
Combined[b]	58-74	54-70	60-76	54-70

[a] The values are minutes from infection between which the first burst must have occurred (according to data shown in *figs 3.2* to *3.12*);

[b] The lowest minimum and the highest maximum replicate estimate of the minimum latent period of the particular phage-host system are used to define a wider, more reliable, time-interval within which the first burst is expected to occur.

More representative of the genetic composition of a phage population, and amenable to statistical analysis, is the mean-latent-period parameter, i.e. the time from infection required for 50% of the progeny phage to be released. This parameter was estimated from the one-step growth data by transforming the rise period into a probit regression line (see appendix). The mean latent period of each phage-host system was calculated independently from each replicate experiment and also after averaging the replicate data (every three consequent measurements) so as to obtain an "average" rise-curve.

The obtained values with 5 per cent confidence limits are shown in *table 3.3*; the probit-transformed, combined rise periods are shown with 5 per cent confidence zones in the b-parts of *figures 3.2* to *3.12*. Although some significant differences were found when the various phage-host systems are compared in pairs, two-factor analysis of variance revealed no significant overall variation between phages (rows) or hosts (columns). The goodness of fit of probit regression lines to combined data suggested that all the phage and host populations were homogeneous concerning the factors determining the latent period, although again, some individual replicate experiments suggested hetero-geneity.

When the preliminary estimates of the burst sizes (181 and 154), the minimum latent periods (61-64 and 66-72 min) and the mean latent periods ($83.9^{+}_{-}2.5$ and $91.6^{+}_{-}1.2$ min) of λv-C and λs-C systems are incorporated into the *tables 3.1* to *3.3* as additional replicate measurements their differences seem insignificant .

The results of the one-step growth experiments were analysed in a different way to see whether there was any correlation between the

TABLE 3.3: Comparison of Mean Latent Periods of Selected and Control Phage-Host Systems[a]

Phage		Host Bacteria Control	S3	S4	S5
λv	Rep 1	$90.5^{+}_{-}1.1$		$88.2^{+}_{-}6.3$	$93.7^{+}_{-}5.0$
	Rep 2	$90.4^{+}_{-}4.4$		$85.1^{+}_{-}4.4$	$89.4^{+}_{-}5.6$
	Rep 3	$90.7^{+}_{-}6.1$		$90.2^{+}_{-}4.0$	$91.0^{+}_{-}5.2$
	Combined[b]	$90.3^{+}_{-}1.1$		$87.2^{+}_{-}1.0$	$91.1^{+}_{-}4.3$
λh	Rep 1	$90.4^{+}_{-}1.5$	$84.1^{+}_{-}1.5$	$82.8^{+}_{-}5.2$	$90.2^{+}_{-}5.8$
	Rep 2	$91.8^{+}_{-}9.8$	$94.0^{+}_{-}7.4$	$87.1^{+}_{-}8.3$	$82.6^{+}_{-}6.9$
	Rep 3	$95.4^{+}_{-}1.5$	$92.5^{+}_{-}2.6$	$88.4^{+}_{-}1.4$	$85.6^{+}_{-}5.6$
	Combined[b]	$89.6^{+}_{-}4.1$	$87.8^{+}_{-}1.2$	$85.1^{+}_{-}1.3$	$85.5^{+}_{-}1.2$
λs	Rep 1	$84.2^{+}_{-}0.8$	$93.3^{+}_{-}2.3$	$88.7^{+}_{-}2.9$	$79.7^{+}_{-}3.7$
	Rep 2	$83.7^{+}_{-}10.1$	$94.5^{+}_{-}2.8$	$91.0^{+}_{-}3.8$	$81.9^{+}_{-}4.0$
	Rep 3	$82.9^{+}_{-}1.0$	$92.5^{+}_{-}2.4$	$91.5^{+}_{-}3.5$	$88.1^{+}_{-}3.7$
	Combined[b]	$83.5^{+}_{-}4.1$	$93.2^{+}_{-}2.3$	$90.1^{+}_{-}2.2$	$82.9^{+}_{-}2.3$

[a] The estimates (min) were made by probit analysis of the rise periods of the one-step growth experiments shown in *figures 3.2 to 3.12*; confidence limits are at P = 0.05.

[b] These values were obtained from the analysis of averaged rise-period data.

observed burst sizes and the corresponding latent periods. For this purpose the burst sizes were plotted against the corresponding mean latent periods (*figure 3.13*). The correlation coefficient (r) value of 0.121 indicates no significant linear regression (P>0.1) and the overall pattern suggests no other function which could possibly relate the two parameters. Therefore, the differences in burst size cannot be attributed to differences in latent period (as if they were due for example to lysis inhibition), nor *vice versa*.

3.2.2   *The Small-Plaque Phenotype*

That λs formed smaller plaques than λv and λhv even on its coselected hosts was one of the first observations following the isolation of these bacteria. This was true for all S3, S4 and S5 types but the S4 type was chosen as a representative of the selected hosts on which to compare the plaque forming abilities of λs, λv and λvh. This choice was for two reasons: (i) only S4 bacteria supported growth of all three phages under standard growth conditions; (ii) these bacteria offered the highest reproductive potential and adsorption capacity to λs.

The time-period of incubation required for 50 per cent of the plaques (on TA1) to become visible was estimated from the time-distribution of plaque appearance. The experiment and the probit-transformation of the data (shown in *figures 3.14* to *3.17*) were performed as described in section 1.3.3 and the appendix. The 50 per cent plaque appearance times on control and S4 bacterial lawns are compared in *table 3.4*.

It became apparent that one cannot directly associate the differences in the parameters of population growth, (i.e. the adsorption rate),

TABLE 3.4   Half Plaque Growth Times on Control and Selected Hosts[a]

	Phage		
Host Bacteria	λv	λvh	λs
Original	18[b]	20	50[b]
Selected Type S4	18	19	50

[a] Values, in hr, express the length of incubation (at $37^{\circ}C$) required for 50 per cent of the plaques to become visible when growing on TA1 medium.   The 5 per cent confidence limits of all estimates were $\pm$1 hr.

[b] Brought forward from section 1.3.3 for comparison.

the minimum or mean latent period and the average burst size, with the differences in size, or rate of growth, of plaques.   The high burst size and adsorption rate of λs on S4 bacteria do not seem to compensate at all for its delay in forming plaques.   Moreover, the small-plaque phenotype cannot be attributed to the h mutation alone, since λvh forms plaques of normal size, which grow at the same rate as the plaques of its isogenic parent, λv, on both hosts.   It is evident, therefore, that plate-growth *is* different from "one-step" growth.

3.2.3   *Lysogenisation of Selected Bacteria*

All, main and secondary, selected isolates were submitted to the tests of prophage induction by mitomycin C described in section 1.2.6 to confirm that none of them was a typical λ-lysogen.   It is very difficult, however, to prove that no phage DNA has become stably associated with a host chromosome or replicates as a plasmid.   Instead, a set of simple experiments were performed to investigate whether any

of the selected bacteria which allowed phage adsorption contained cI- or cro-repressors.

The efficiency of plating of "wild-type" λ (produced by strain K12(λ) induced by mitomycin C) on S3 bacteria was as low as that of λv (approximately 10^{-6}). λ Plaques isolated from S3 lawns gave dense lysates when grown on non-lysogenic original C600 and S3 bacteria but fail to grow on the lysogenic K12(λ) and C600(λ). They, thus, differed from the virulent λvh, which formed plaques and lysates on both lysogenic hosts, and will be referred to as λh. The temperate nature of λh was confirmed by its plaques on control C600 strain which had the characteristic turbid centre. Bacteria, isolated from the centre of a λh plaque were found to be lysogenic (C600(λh)); (i) they showed spontaneous prophage induction; (ii) they were largely induced by mitomycin C (*table 3.5*) to produce phage which retained the h-phenotype, and (iii) they supported growth of all virulent phages while they were immune to superinfecting λ or λh. These results demonstrate that λh was homoimmune to λ and indicate that the mutation(s) h did not interfere with the processes of integration, maintenance, and excision of the prophage.

The plaques formed by individual λ and λh particles on S4 lawns were, interestingly, as clear as the plaques of the virulent phages. This indicated a further difference between S4 and original bacteria concerning their ability to be successfully lysogenised. S4 lysogens did develop, however, in the centre of the large plaque created by spots of undiluted, λ and λh, lysates. Spots of λ and λh on S5 lawns, with maltose, and of λh on S3 lawns produced uniformly turbid plaques, identical to those of virulent phages.

Because of the large proportions of resistant cells in all selected host populations (see section 2.3.5) the abilities of these bacteria to be lysogenised could not be quantitatively compared. Lysogenic S3(λh), S4(λ) and S4(λh) bacteria were, however, easily isolated by subculturing bacteria from the centre of turbid λ and λh plaques and screening the contents of the resulting populations. Most of the isolates from such populations did not support growth of any phage and were, therefore, resistant. The lysogenic isolates supported clear-(S4) or turbid-(S3) plaque growth of λv, λvh or λs but no growth of λ or λh.

The cultures of the lysogenized selected bacteria contained a small number of the corresponding phage, which could not be eliminated by subculture and was presumably produced by spontaneous induction of the carried prophage. In contrast with the control strains (K12(λ), C600(λ), C600(λh) the selected lysogens did not increase their phage content after 2 hours of incubation with mitomycin C (*table 3.5*). This implied a defect in either the excision of the prophage or in the lytic development in presence of the drug.

Information in favour of the second hypothesis, i.e. that lytic development of the phage was impaired in selected bacteria in presence of mitomycin C, was obtained from a set of experiments with mixed plating of lysogenic and non-lysogenic sensitive bacteria (*table 3.6*). When a diluted lysogenic culture is plated onto a sensitive indicator lawn most of the lysogenic colonies are surrounded by a plaque which is produced by spontaneously released phage growing on the sensitive lawn. In some cases (e.g. K12 on C600 lawns, selected strains on control lawns) the colonies from the diluted culture overgrew on the lawn and were easily distinguishable

TABLE 3.5:   Prophage Induction by Mitomycin C in Control and

Selected Lysogens.

| Lysogen | Phage Content (pfu/ml) | | P |
	Before Treatment	After Treatment	
K12(λ)	3.2×10^4	2.5×10^8	< 0.001
C600(λ)	1.0×10^4	2.0×10^9	< 0.001
C600(λh)	5.3×10^4	2.7×10^9	< 0.001
S3(λh)	4.1×10^4	3.9×10^4	> 0.05
S4(λ)	2.5×10^4	2.9×10^4	> 0.05
S4(λh)	8.9×10^4	1.0×10^5	> 0.05

[a]   Values are the means of three replicate experiments (the protocol
is described in section 1.2.6).

even when they were not surrounded by plaque.

On C600 lawns containing mitomycin C the K12(λ), C600(λ) and C600(λh)
colonies were replaced with clear plaques of normal size; prophage
induction occurred and the released phage followed exclusively lytic
development on the sensitive lawn.   When these control lysogenic
strains were plated onto S3 or S4 indicator bacteria with mitomycin C
no colonies overgrew, presumably because the lysogens lysed, but
neither did any plaques appear indicating that the released phage
could not efficiently reproduce on these lawns.

Similar platings of S3(λh), S4(λ) and S4(λh) selected lysogens on
C600 control lawns focussed the defect on the release of infective
phage particles from the lysogenic selected strains; although C600
lawns could support plaque growth of the prophage carried by control
lysogens the selected lysogens carrying the same prophage did not

form plaques.

3.2.4 *Inhibition of Lytic Growth by Mitomycin C.*

That the selected strains (S3 and S4) were somehow defective in
allowing lytic phage development was also confirmed by the
behaviour of the virulent phages λv, λvh and λs on lawns containing
mitomycin C.   When control indicator lawns were used the drug did
not affect the size and clearness of any of these plaques but reduced
the sharpness of their periphery.   On S3 and S4 lawns, however,
mitomycin C reduced the size of all virulent phage plaques so severely
that the latter could be seen only with very careful examination.
These tiny plaques appeared after overnight incubation (TA2 was used)
and did not grow further, indicating that the effect of the drug was
more severe on the older lawns.

TABLE 3.6: The Effect of Mitomycin C on the Ability of Bacteria to Support Phage Growth on Plates.[a]

Plaque Forming Particles	Indicator Lawn	Plaque Growth Without Mitomycin	With Mitomycin
K12(λ), C600(λ) or free λ	C600	+	+
	S3	$-$[b]	$-$
	S4	+	$-$
C600(λh) or free λh	C600	+	+
	S3	+	$-$
	S4	+	$-$
S3(λh) or S4(λh)	C600	+	$-$
	S3	+	$-$
	S4	+	$-$
S4(λ)	C600	+	$-$
	S3	$-$[b]	$-$
	S4	+	$-$
λv, λvh or λs	C600	+	+
	S3	+[c]	$\pm$[c]
	S4	+	$\pm$

[a] Mitomycin C was added at a final concentration of 1µg/ml. +, plaques of normal size; $\pm$, tiny plaques; -, no plaques.

[b] Only h-mutants produce plaques.

[c] These indicate growth of λvh and λs; λv does not form plaques.

FIGURES 3.2 to 3.12 (a):

One-Step Growth of λv, λh and λs on Control and Selected Bacterial Isolates.

Counts of infected bacteria were taken throughout the latent period, using the standard (streptomycin) protocol, and their average (N_o) was used for the estimation of the burst size. N_t is the number of total pfu/ml in the growth tube at time t. Large dots: replicate 1; medium size dots: replicate 2; small dots: replicate 3.

FIGURES 3.2 to 3.12 (b):

Distriubtion of Latent Periods of λv, λh- and λs-Infected Control and Selected Bacterial Isolates.

The latent period is from infection and N.E.D.s are the Normal Equivalent Deviates of the percentage of progeny phage particles liberated by the time of sampling. Each percentage value is the average of three replicate measurements taken at subsequent intervals of 2 min; eg the N.E.D. value at 80 min corresponds to the average of the percentages of the final yield released by 78 (replicate 1), 80 (replicate 2) and 82 min (replicate 3). The upwards arrow in figure 5.3(b) indicates a positive infinite N.E.D. value which corresponds to 100% response (ie that count did not differ from the final yield count). The confidence limits of the slopes β, given in the legends, and the confidence zones of the distribution are at P = 0.05.

FIGURE 3.2: λv *with Control Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	5
χ^2	5.256
P	0.385
α	-7.939791984
β	0.0879494606 $\pm$0.0092436332
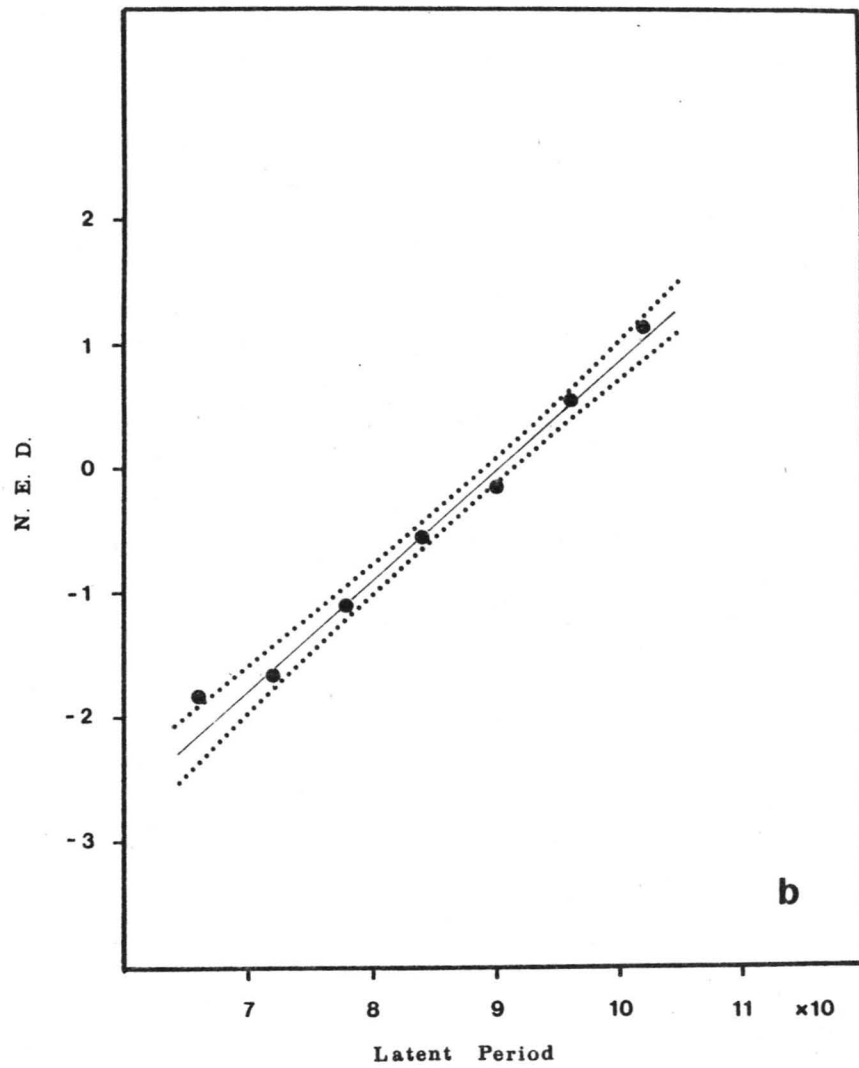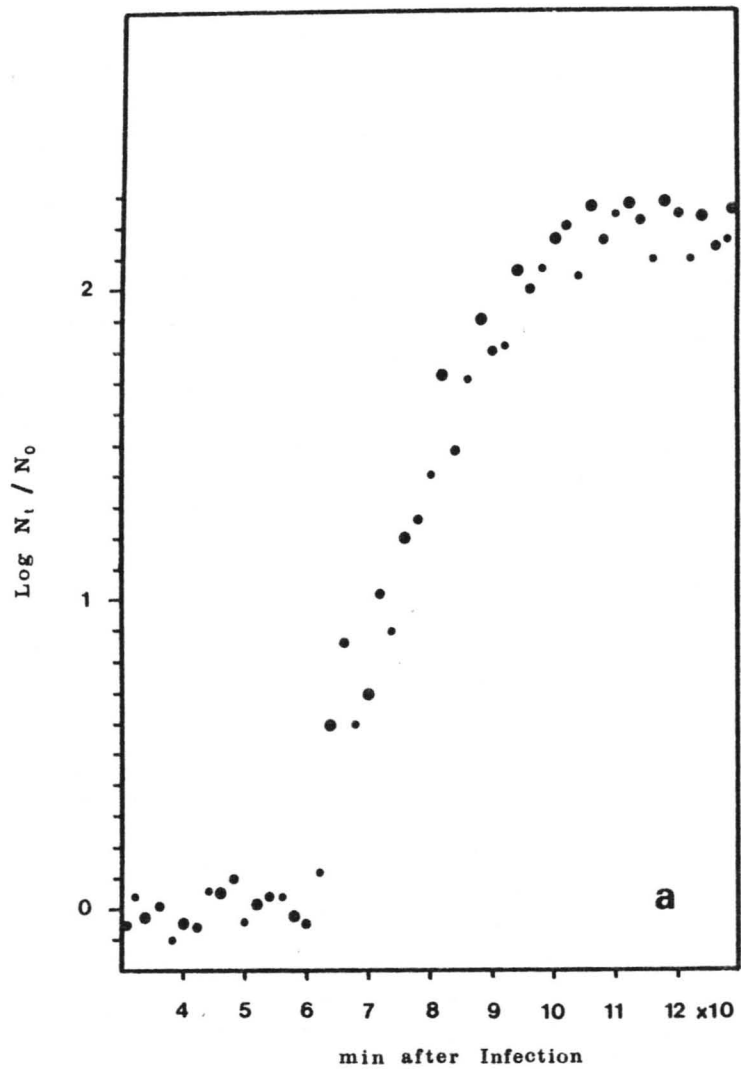
FIGURE 3.3:   λh *with Control Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	6
χ^2	5.021
P	0.541
α	-7.289626379
β	0.0813221705 ± 0.0286838734
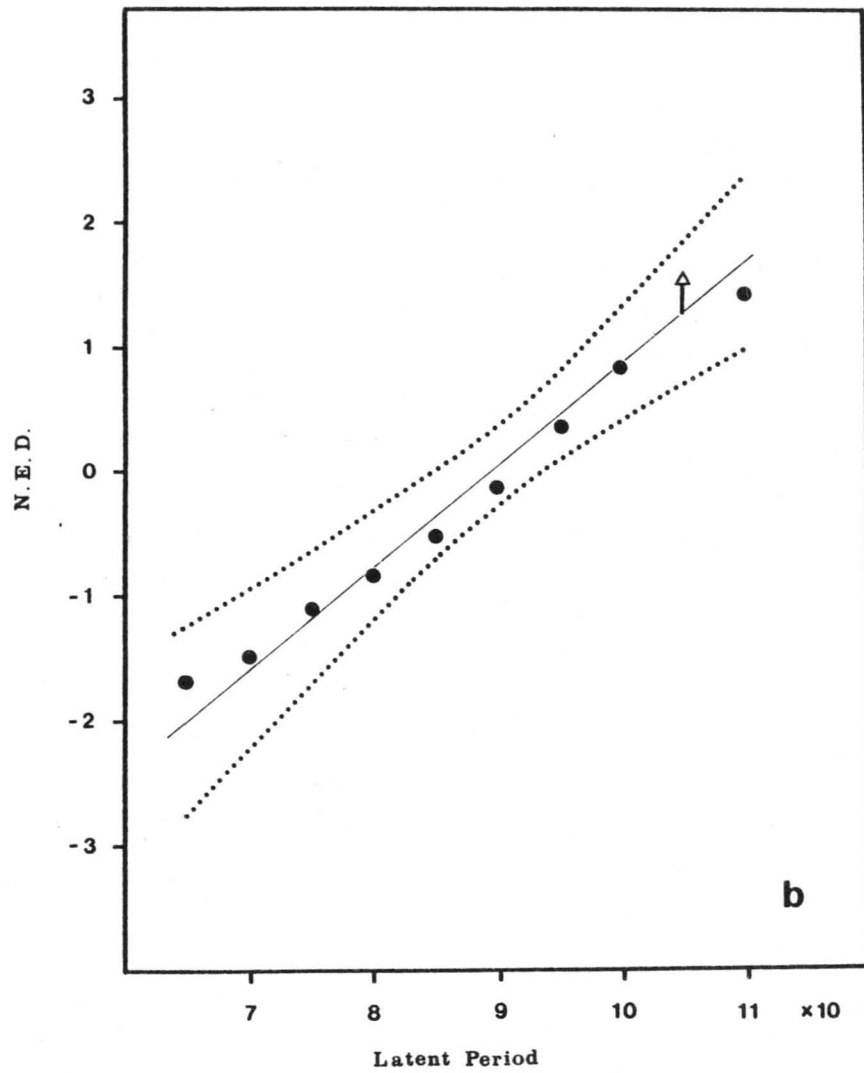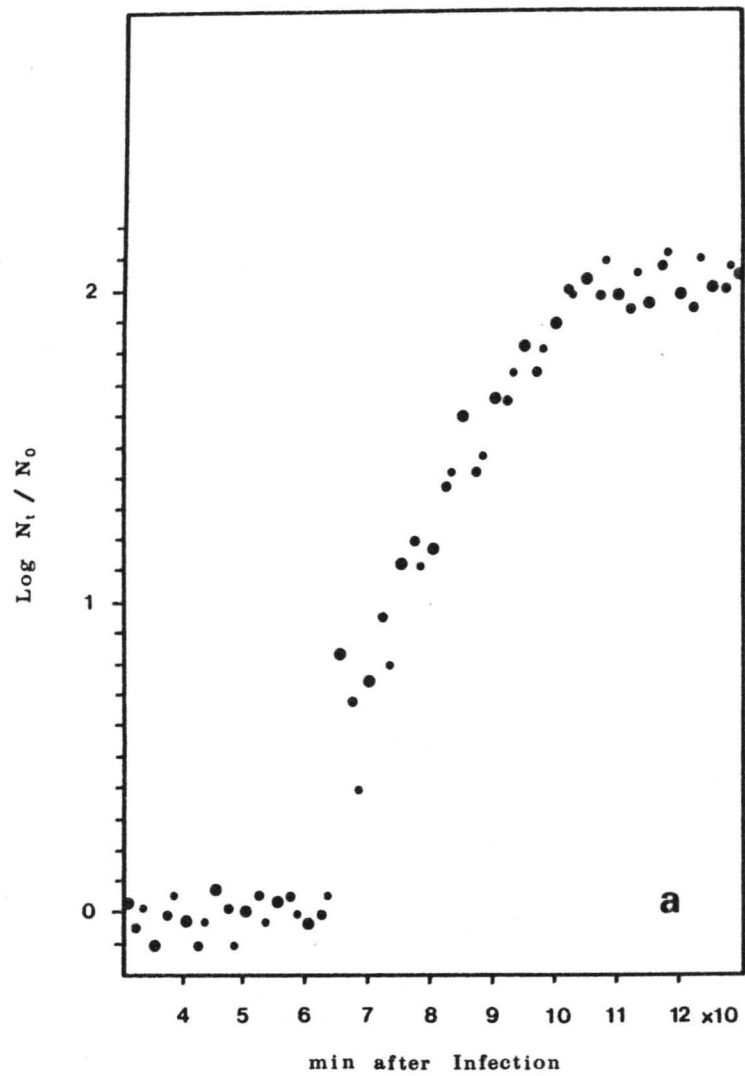
FIGURE 3.4:   λs *with Control Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	3
χ^2	1.243
P	0.743
α	-8.884281146
β	0.1063528505 $\pm$0.0440979351

a

b
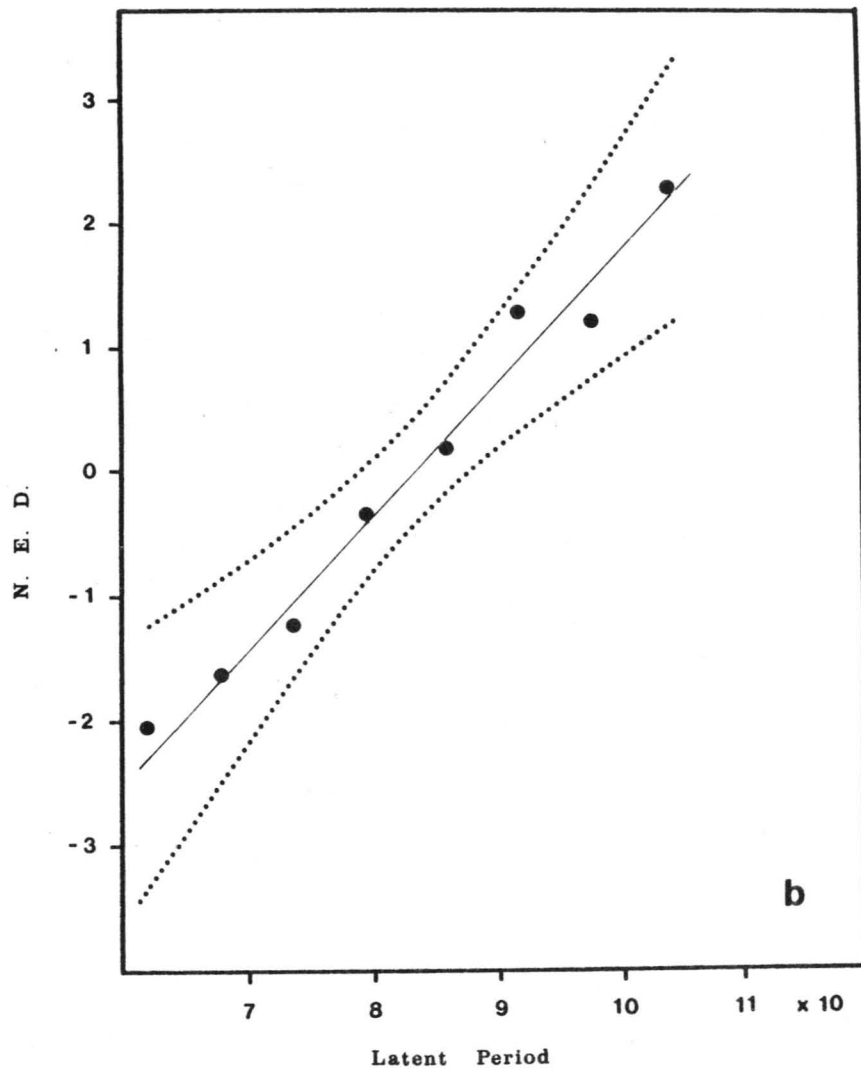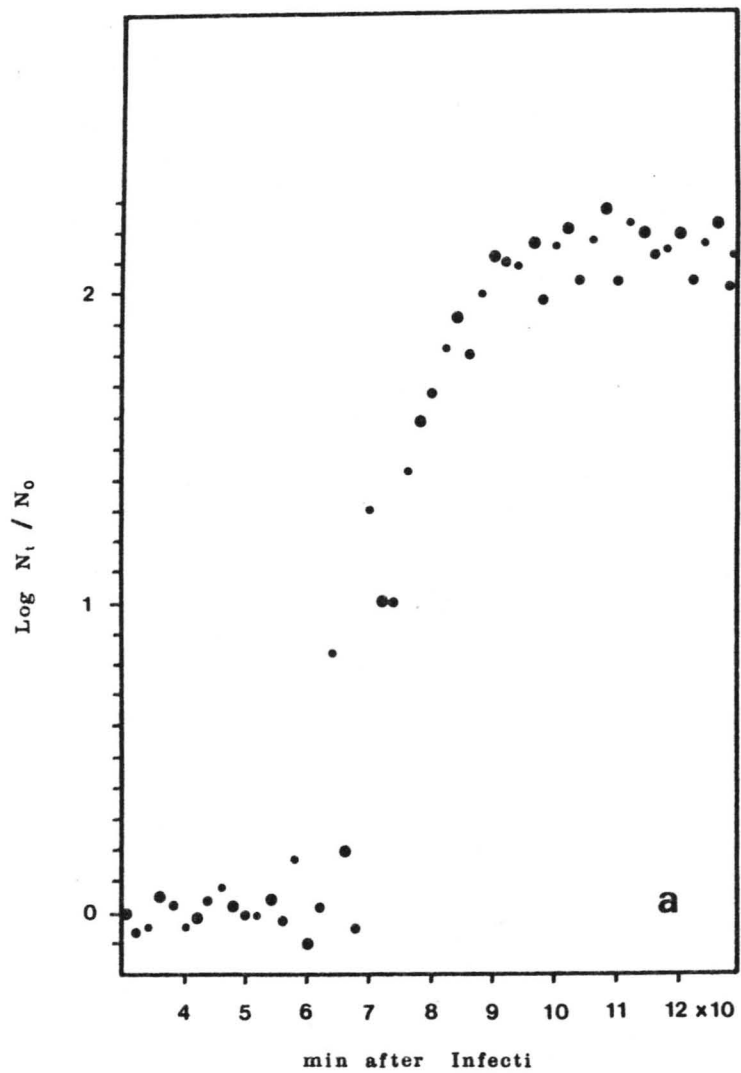
FIGURE 3.5: λh with S3 Bacteria

a.   One-Step Growth

b.   Distribution of Latent Periods

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	5
χ^2	10.993
P	0.052
α	-8.673439077
β	0.0987719813 $\pm$0.0119607429

Log N_t/N_0

min after Infection

N.E.D.
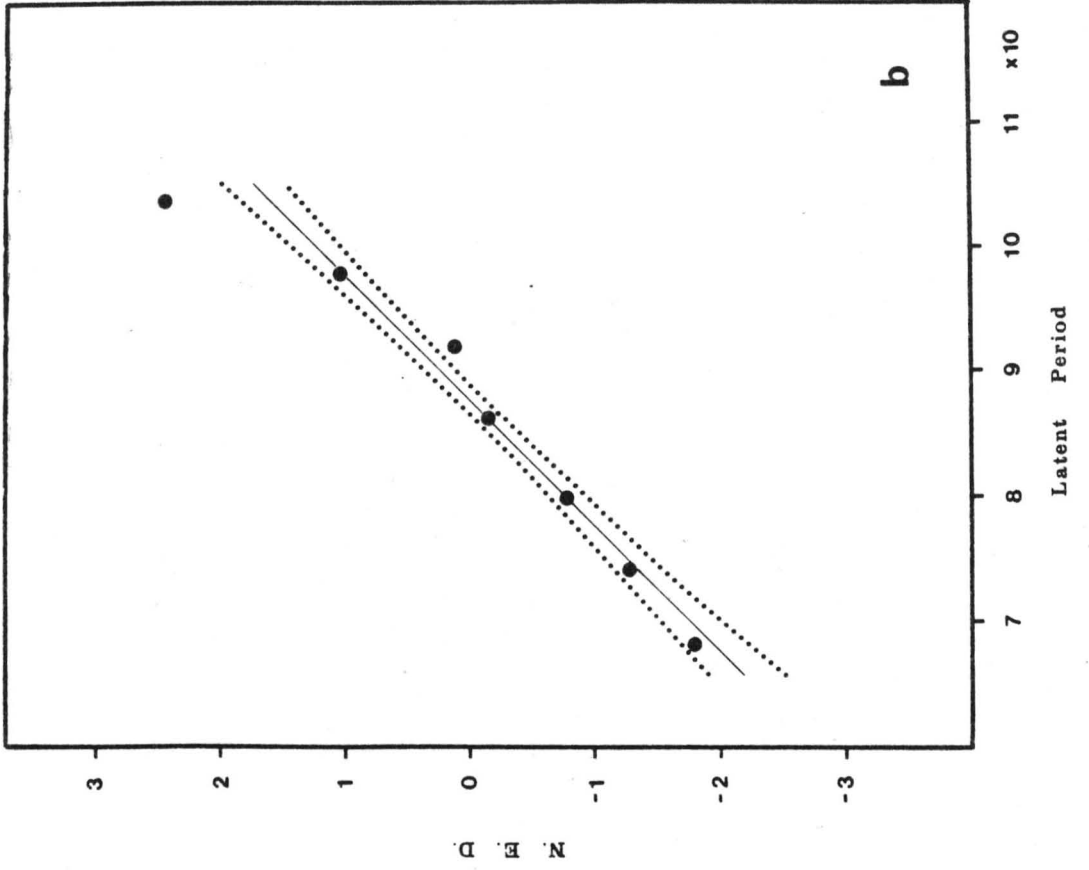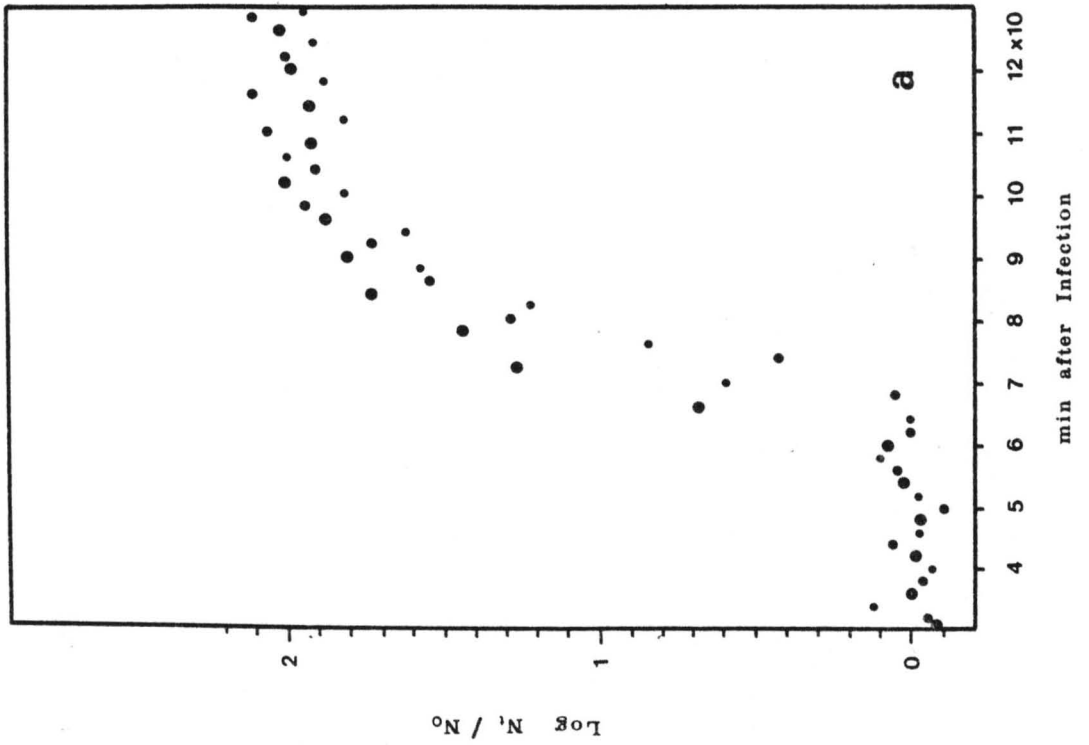
Latent Period

FIGURE 3.6:   λs *with S3 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	3
Degrees of Freedom	4
χ^2	5.620
P	0.229
α	-8.5487959700
β	$+0.0917082673$ -0.0194289327

Figure a: plot of Log N_t / N_o versus min after Infection. Figure b: plot of N. E. D. versus Latent Period.
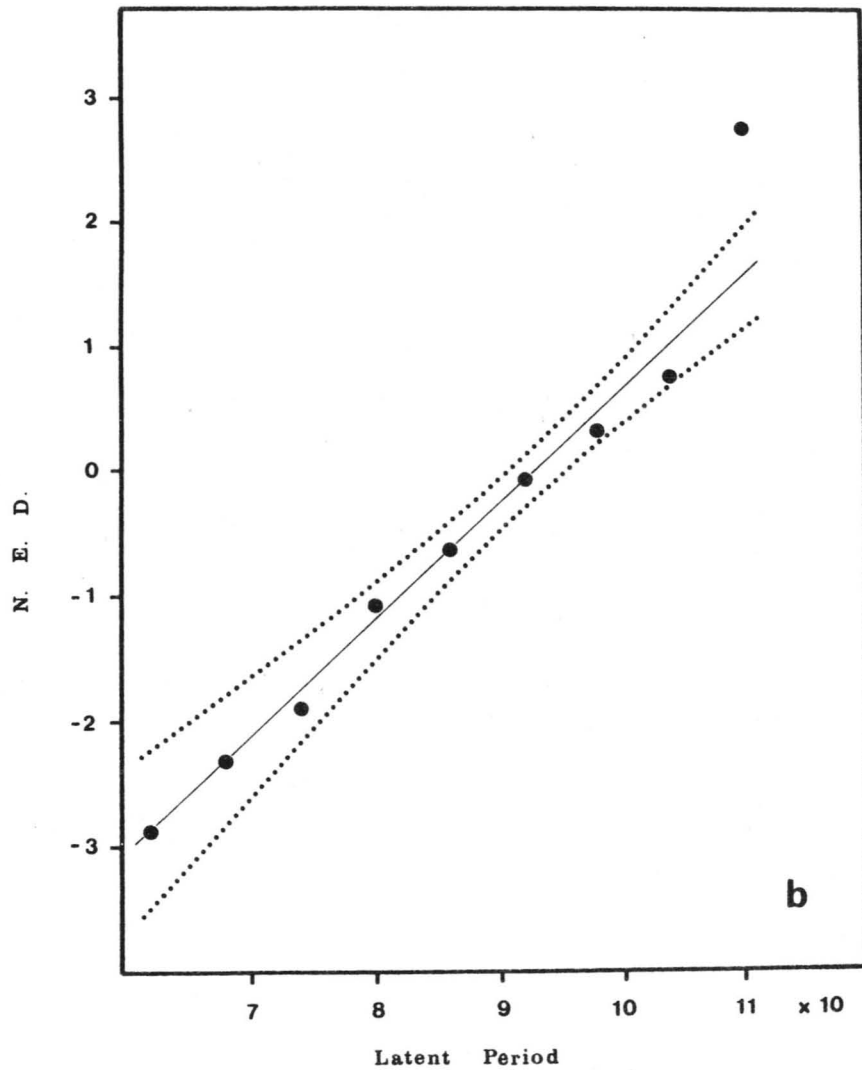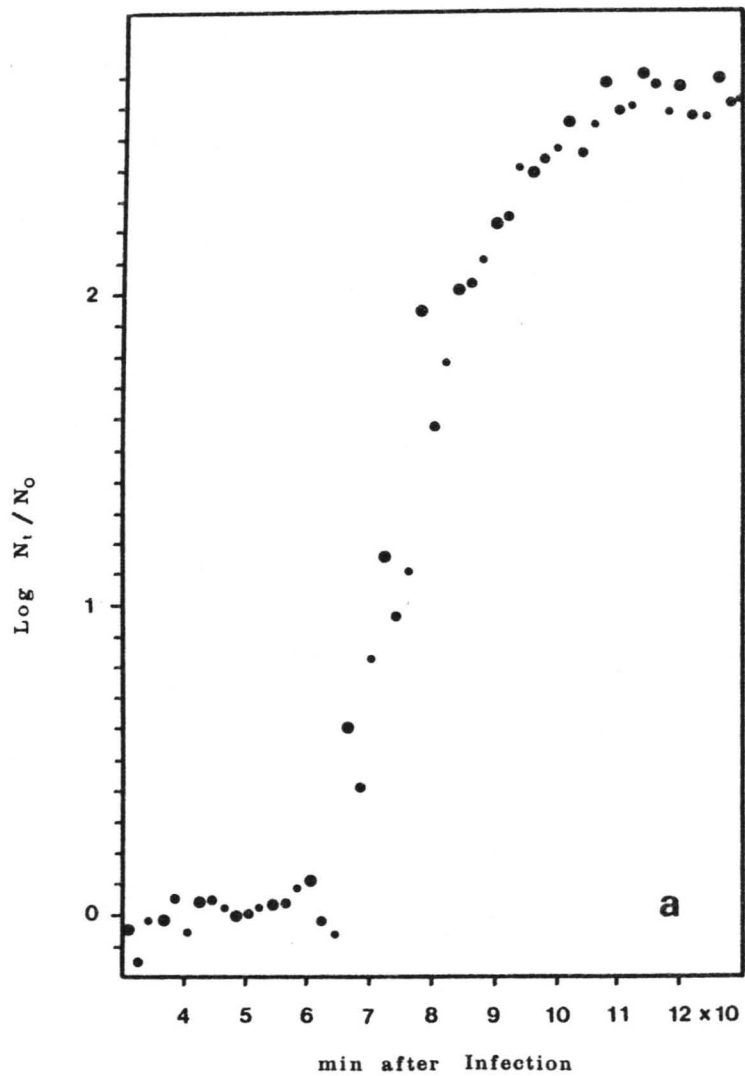
FIGURE 3.7:   λv *with S4 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	6
χ^2	6.132
P	0.409
α	-7.059709335
β	0.0809722709 $\pm$ 0.0073293365
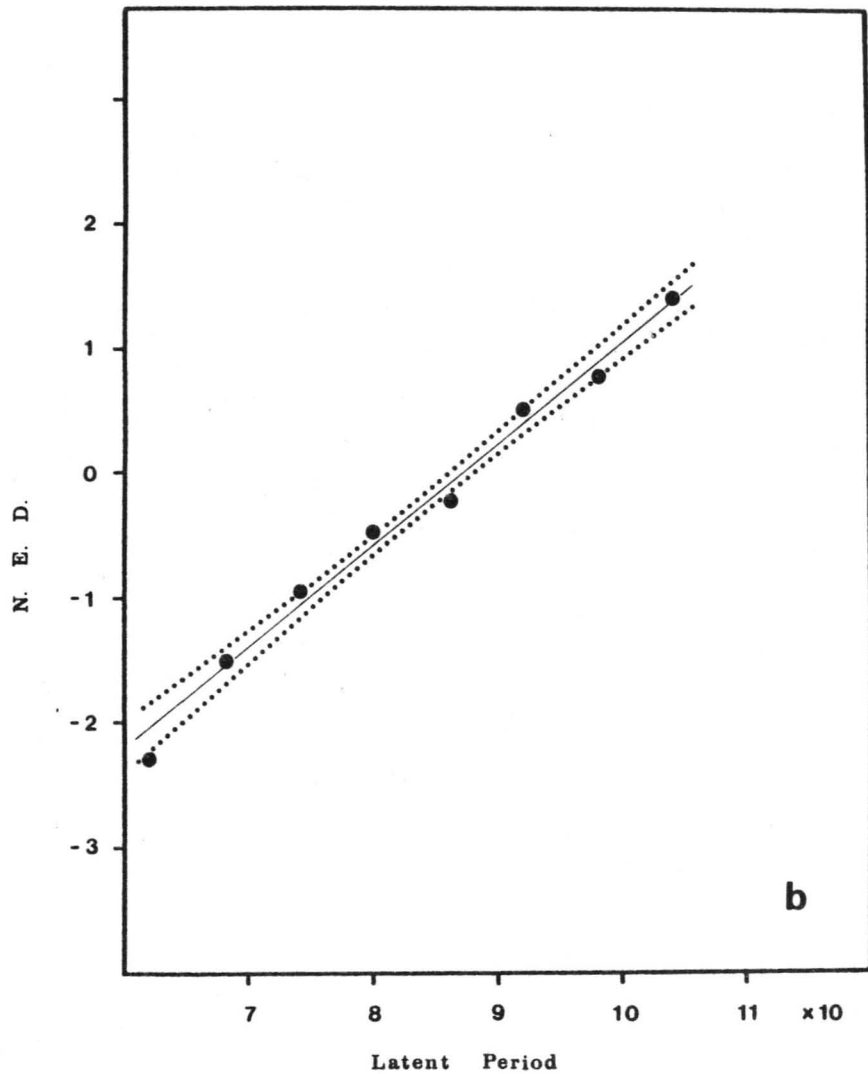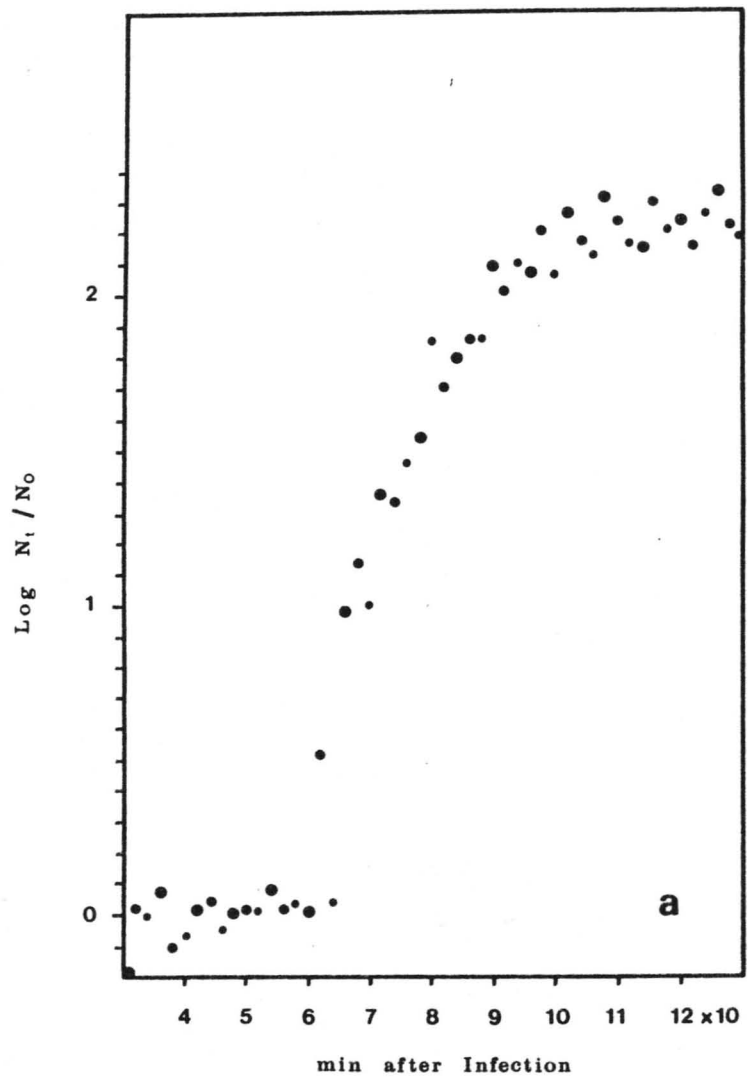
FIGURE 3.8:   λh with S4 Bacteria

a.   One-Step Growth

b.   Distribution of Latent Periods

Probit Analysis

M.L.-Rounds	1
Degrees of Freedom	7
χ^2	5.418
P	0.609
α	-7.494398806
β	$\pm^{0.0880206949}_{0.0098171477}$
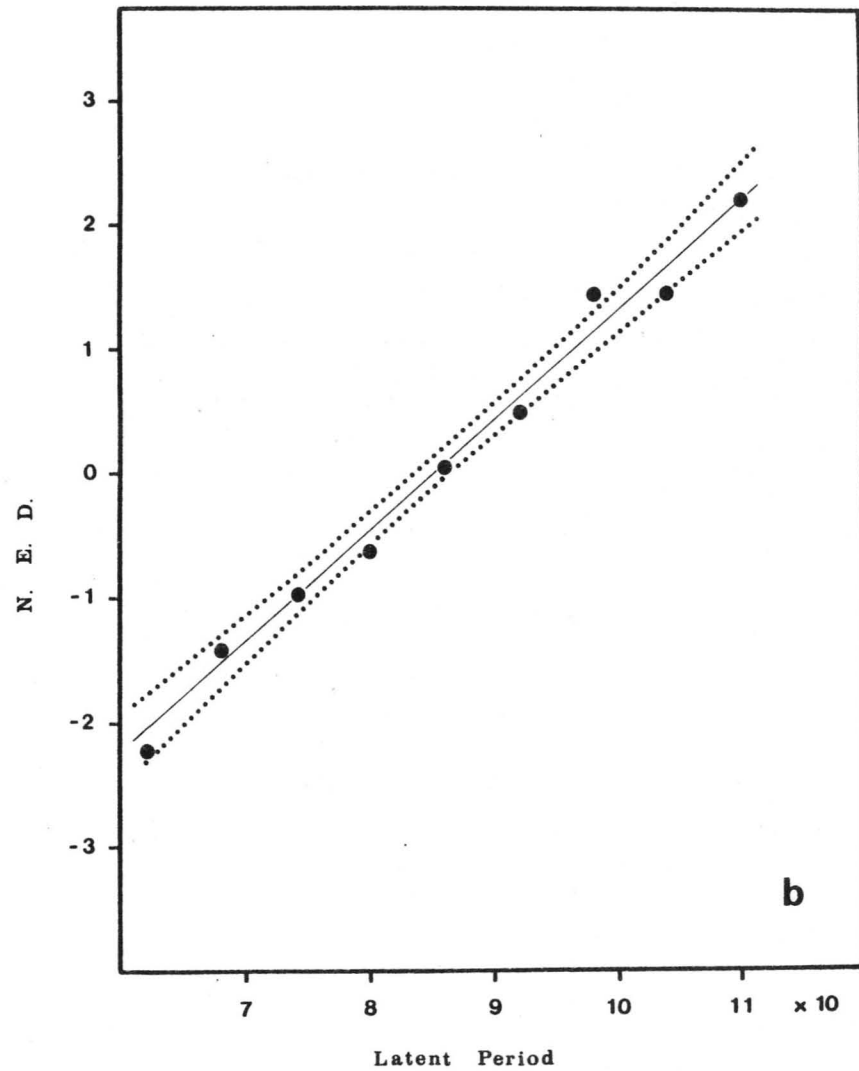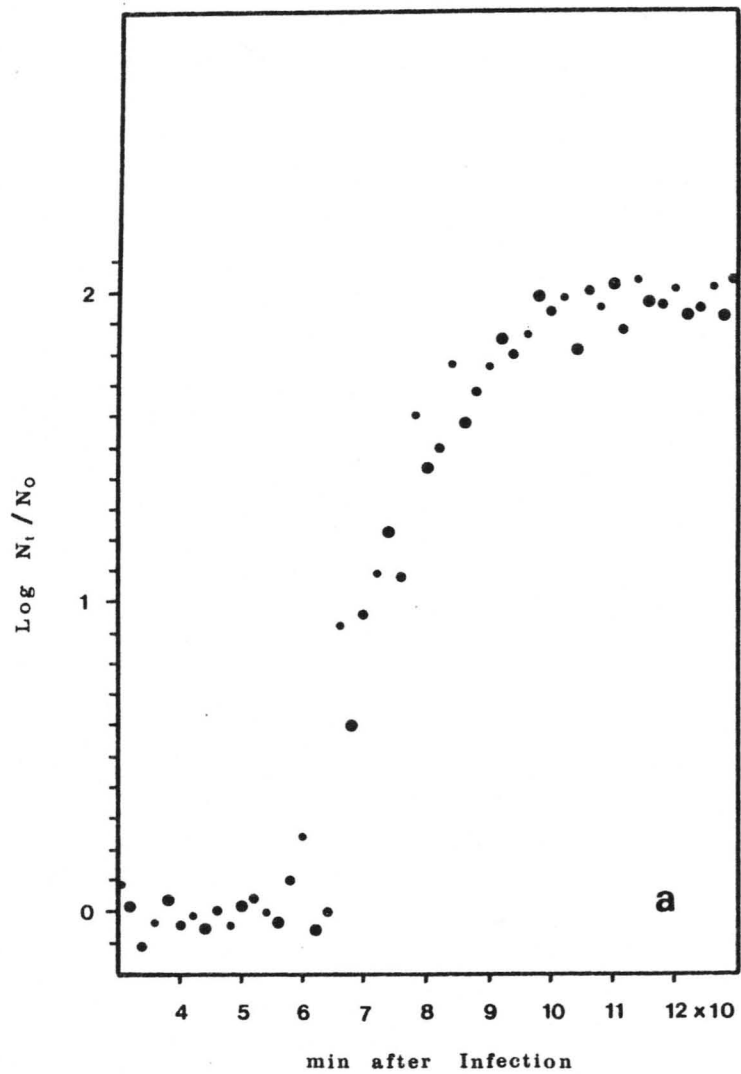
FIGURE 3.9:   λs *with S4 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.−Rounds	2
Degrees of Freedom	4
χ^2	4.623
P	0.328
α	−8.042769763
β	0.0892565666 $\pm$ 0.0174203241

Figure a: Plot of Log N_t/N_0 against min after Infection.

Figure b: Plot of N. E. D. against Latent Period.
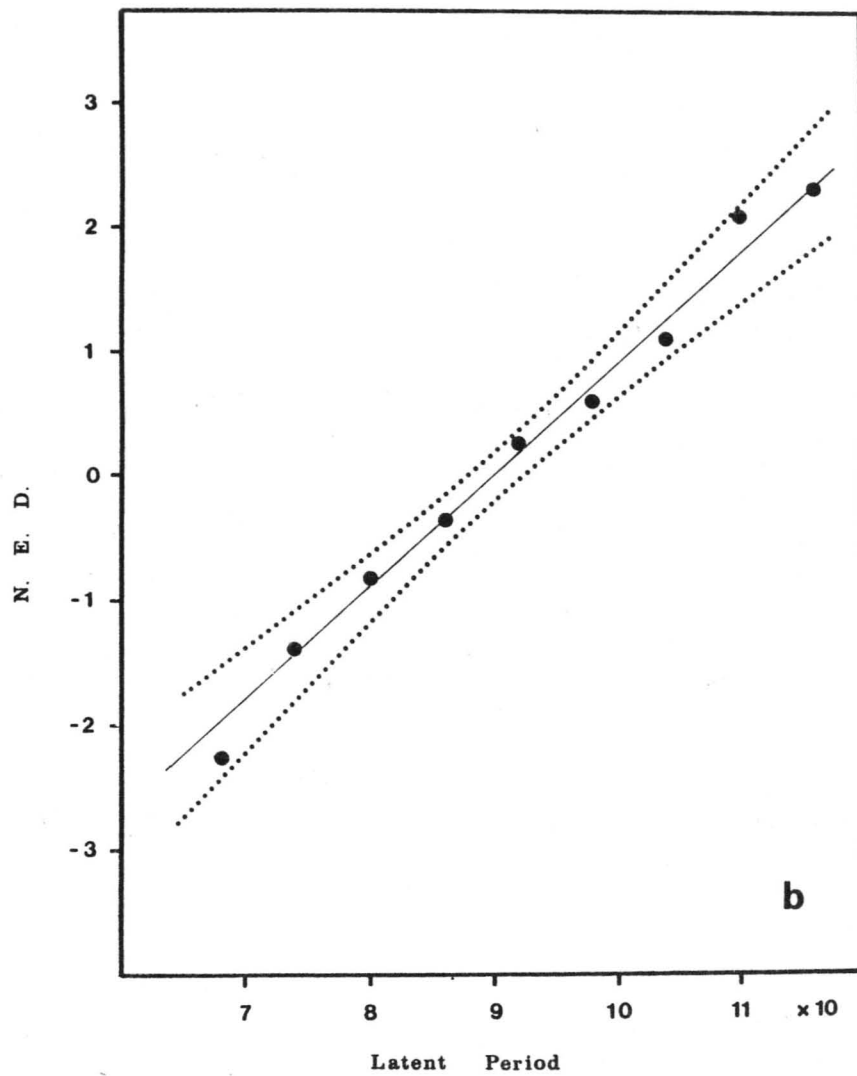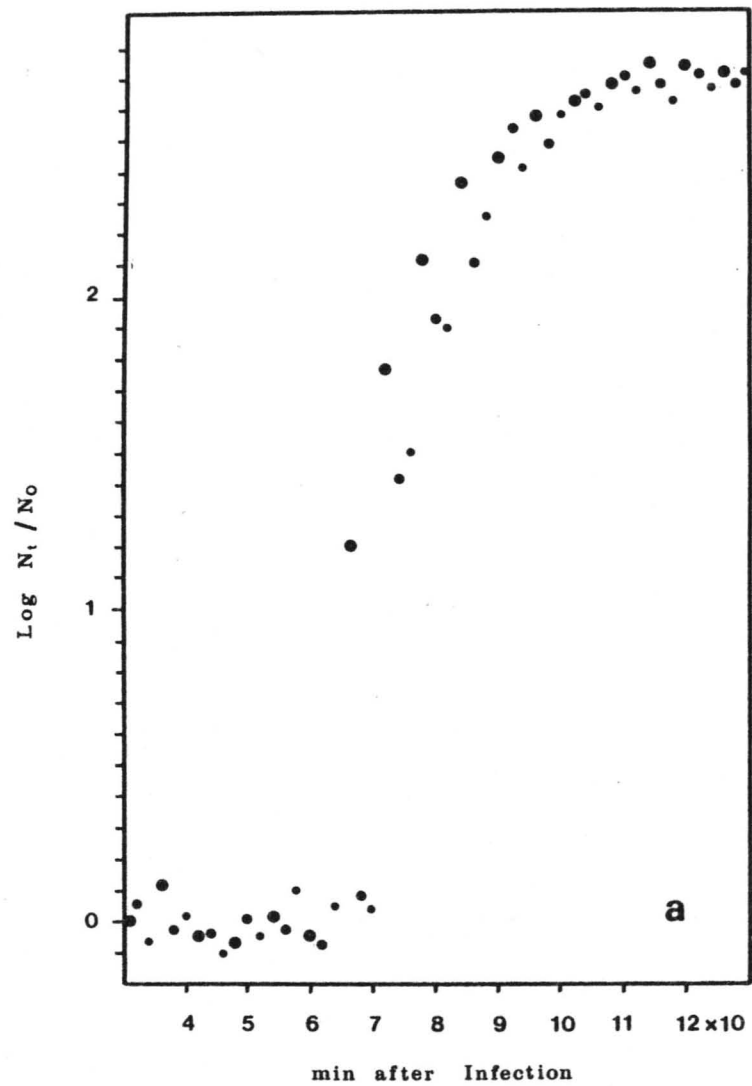
FIGURE 3.10:   λv *with S5 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	4
χ^2	6.257
P	0.181
α	-7.828384142
β	0.0859211370 $\pm$0.0310072365

Log N_t / N_0

min after Infection

N. E. D.

Latent Period

a

b
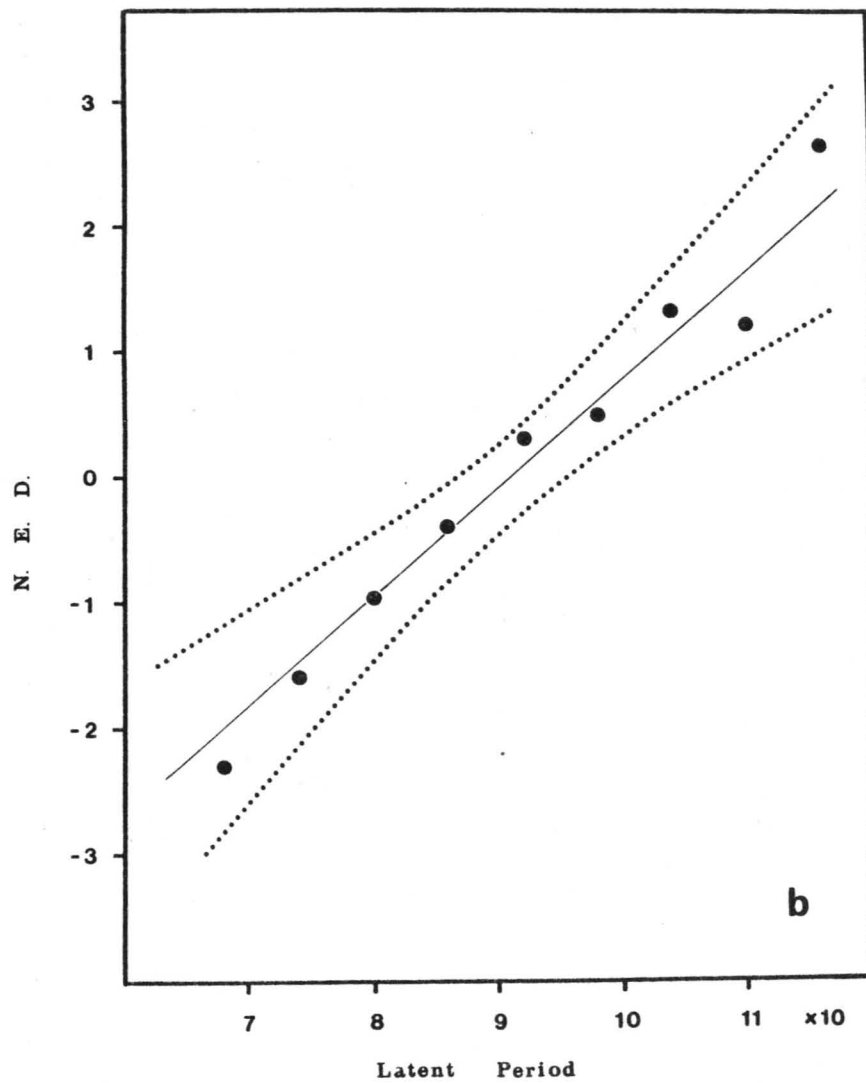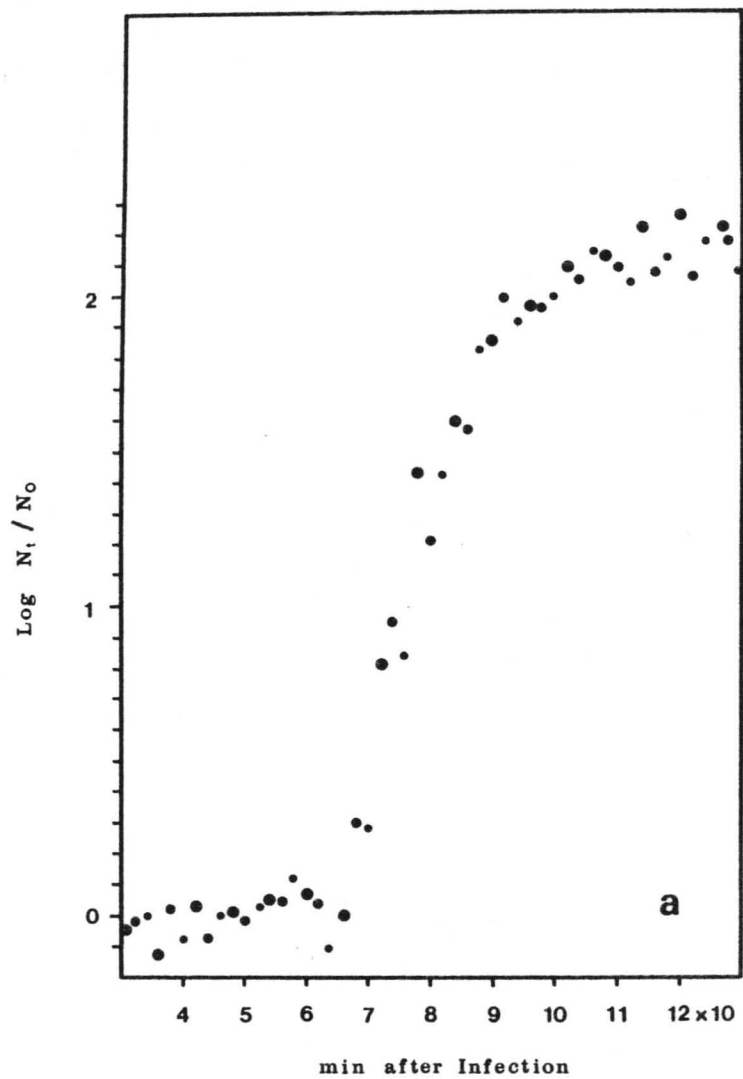
FIGURE 3.11:   λ*h with S5 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	6
χ^2	8.056
P	0.234
α	-7.645159838
$\tilde{\beta}$	0.0894745895 $\pm$0.0090425597
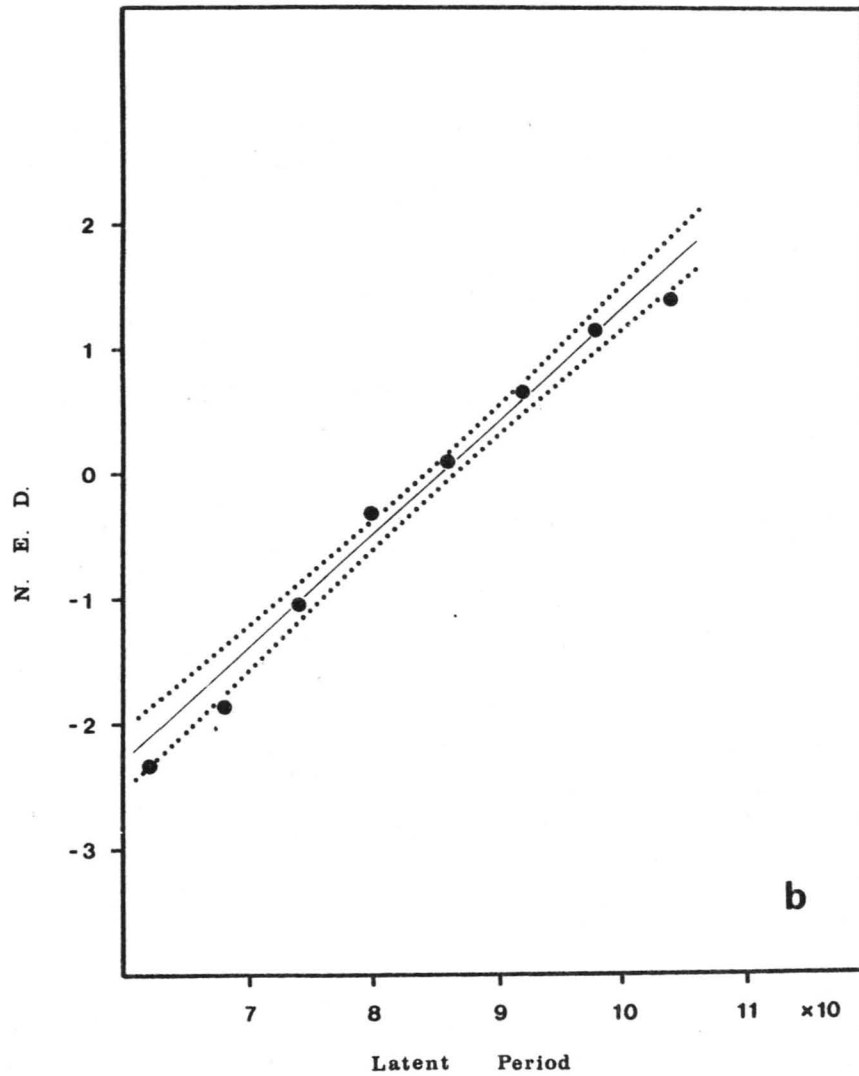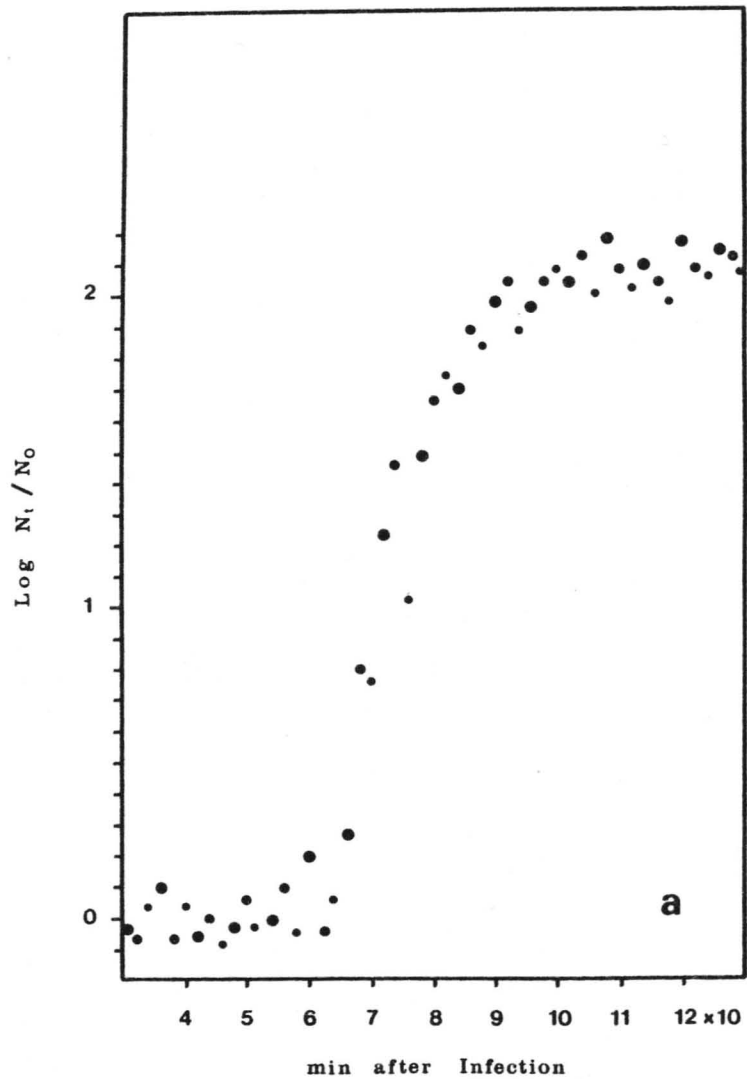
FIGURE 3.12:   λs *with S5 Bacteria*

a.   *One-Step Growth*

b.   *Distribution of Latent Periods*

Probit Analysis

M.L.-Rounds	2
Degrees of Freedom	3
χ^2	5.562
P	0.135
α	-8.610289378
β	0.1039235359 $\pm$0.0257083312

a

b
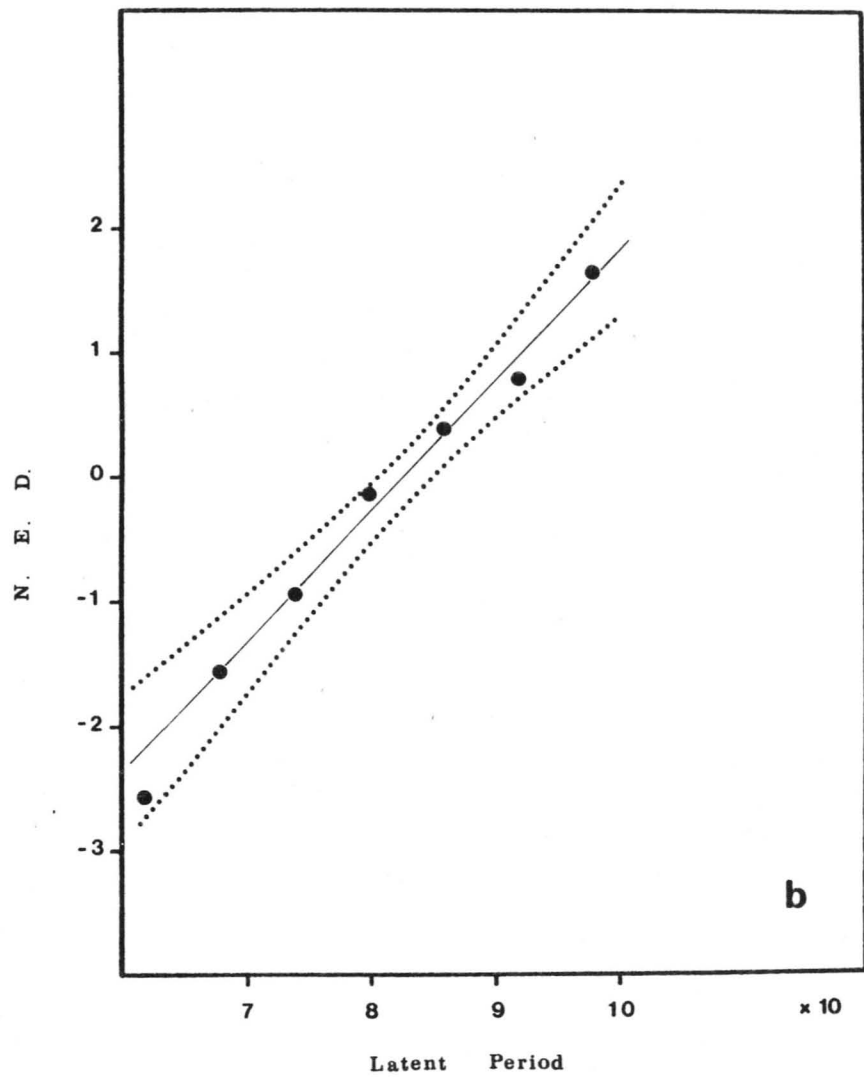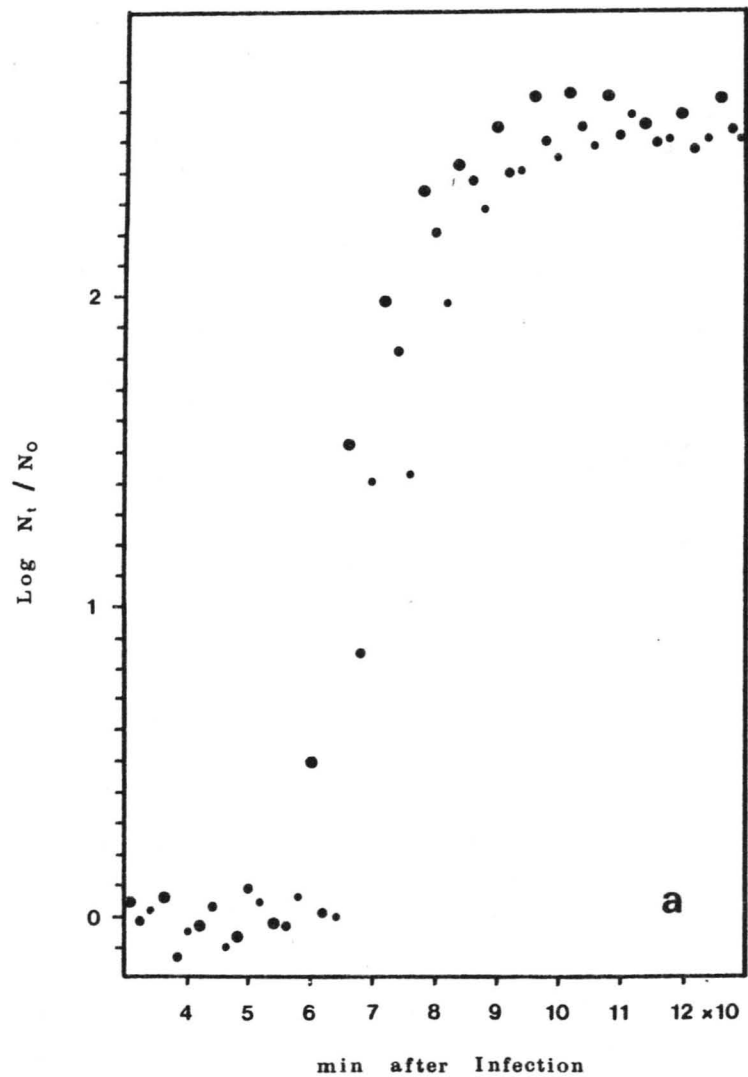
FIGURE 3.13:   *Relation Between Burst Size and Latent Period*

The coordinates of each point are the mean latent period and the average burst size estimates from each replicate experiment shown in figures 3.2 to 3.12(a);   the mean latent period of each replicate was estimated as usual by the probit transofrmation method but analytical results are not shown.
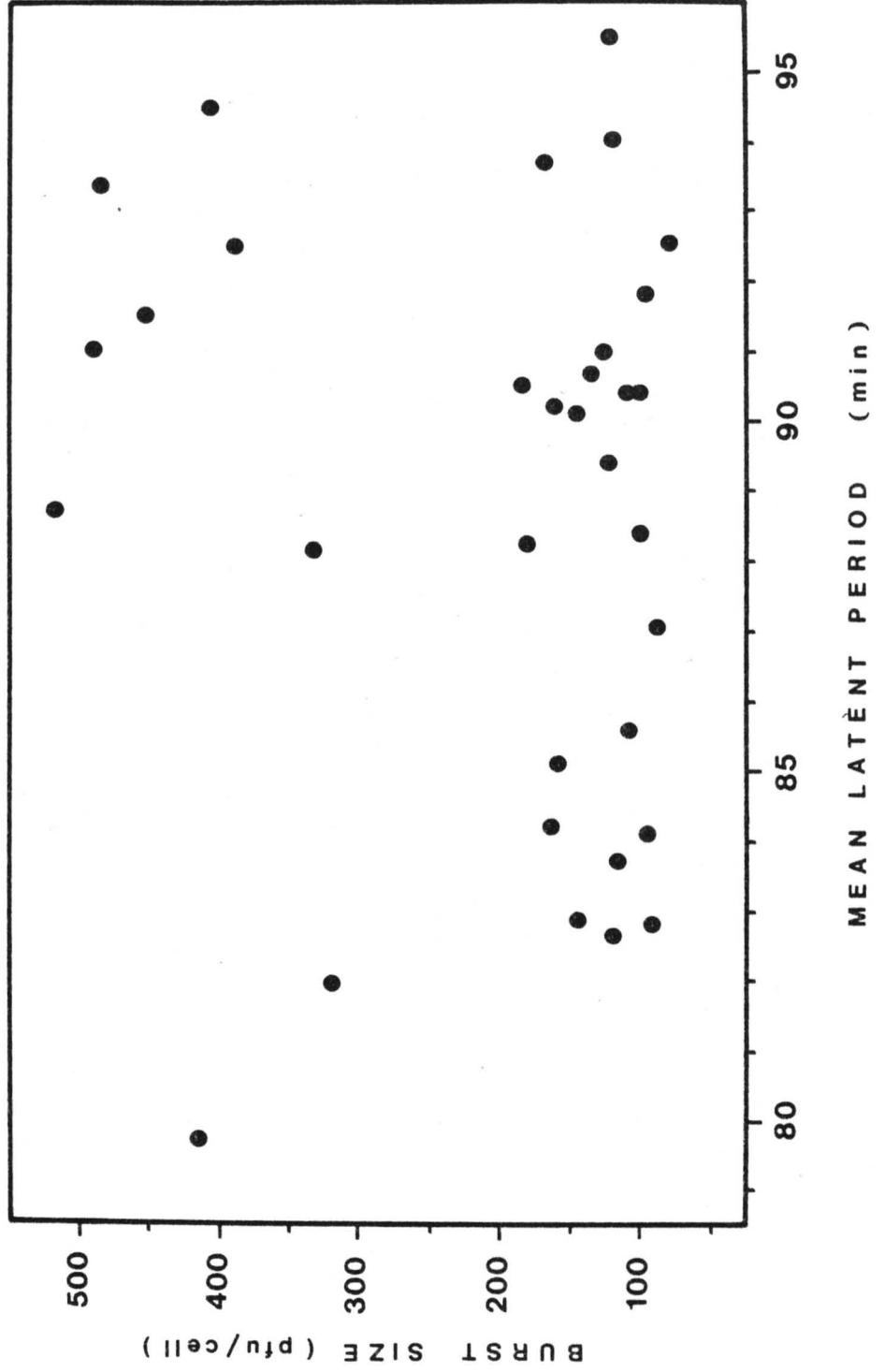
Distributions of Plaque Formation Times of λh on Control Bacteria and λv, λh and λs on S4 Bacteria

N.E.D.: Normal Equivalent Deviate of the percentage of plaques, on TA1, which become visible by the end of a particular period of incubation.   Confidence zones and confidence limits of the slopes β are at P=0.05.

FIGURE 3.14: *λh on Control Bacteria*   FIGURE 3.15: *λh on S4 Bacteria*

Probit Analysis

M.L.-Rounds	2	2
Degrees of Freedom	7	6
χ^2	2.075	2.414
P	0.956	0.878
α	-4.186934631	-3.935682987
β	3.235900047 $\pm$0.3055519073	3.101003203 $\pm$0.3148992413

FIGURE 3.16: *λv on S4 Bacteria*   FIGURE 3.17: *λs on S4 Bacteria*

Probit Analysis

M.L.-Rounds	2	2
Degrees of Freedom	6	11
χ^2	4.712	4.931
P	0.581	0.934
α	-4.032665724	-8.426560504
β	3.179928754 $\pm$0.2732147102	4.958146520 $\pm$0.3240365639
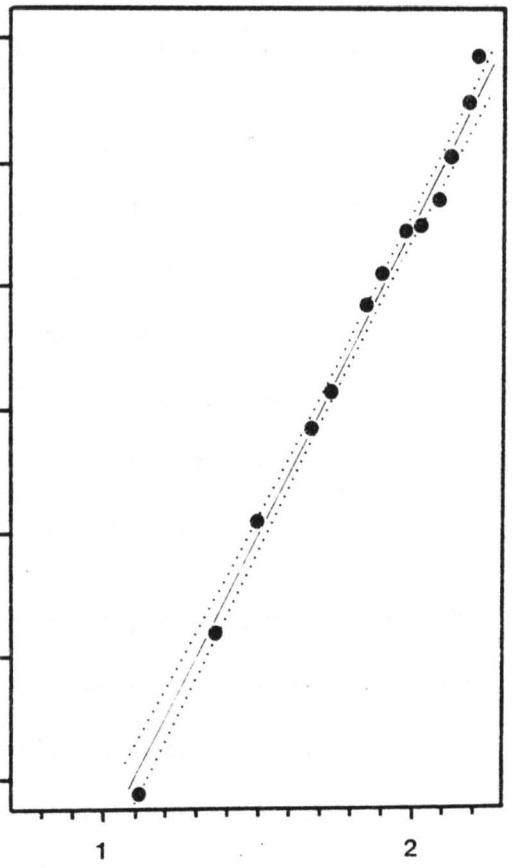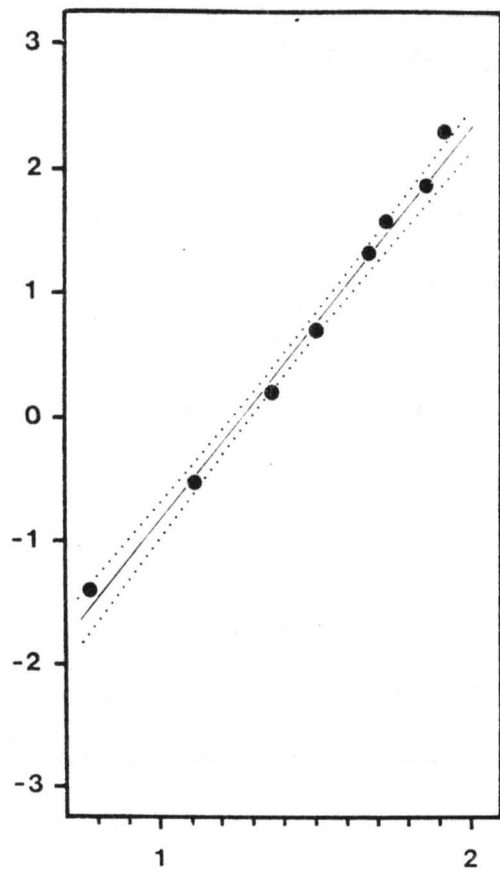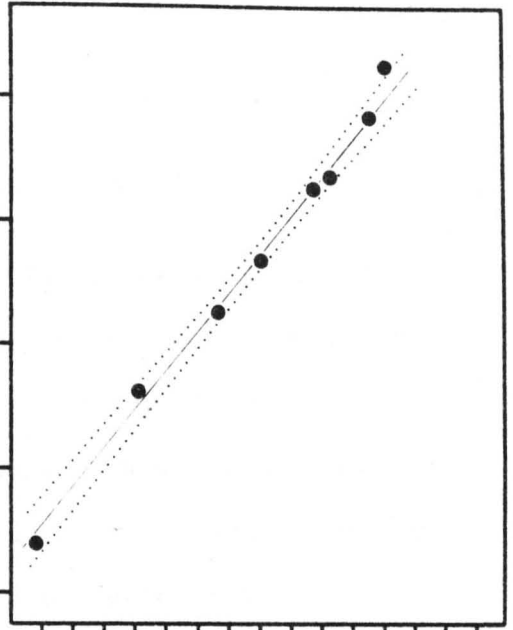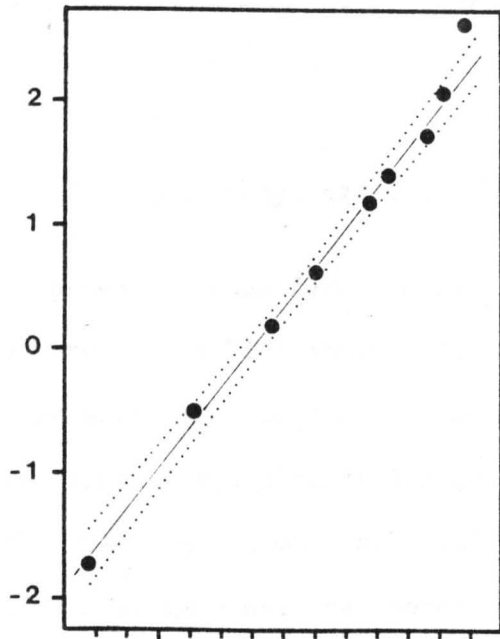
Log hr OF INCUBATION

3.3 *THE DEVELOPMENTAL STRATEGIES OF*
λ AND THEIR MICROEVOLUTION

3.3.1 *The "Selected" Phage has Indeed been Selected, But....*

The results presented thus far, and particularly the profound difference between the "intrinsic rates of increase" (i.e. rates of intracellular multiplication) of λs and λv infecting S bacteria, justify the originally arbitrary designation of chemostat phage as "selected". Although λs may carry selectively neutral mutations or be neutrally heterogeneous, one cannot help but attribute its evolution to natural selection for those mutations which differentiate it from its ancestor (λv) on the basis of infectivity, reproduction and survival.

Is, however, λs a new "wild-type", fit to the conditions of culture 1, or is it an "opportunist" whose advantages would diminish in the future? To answer this we need to know which selective forces created the λs-response and how would the λs-response affect (if at all) the occurrence and intensity of these selective forces. But first of all we need to know what the λs-response is.

Concepts like "intrinsic rate of increase" or "reproductive potential" are of overwhelming ecological importance but mean too little to a geneticist who is interested in their genetic variation. They are always assumed to be genetically determined but they can hardly ever be realised in nature, measured and mapped in the organism's genome. For this they have always been criticised by anti-selectionists as tautologies and untestable pseudo-explanations of natural selection.

The accumulated knowledge on λ genetics and development allows,
however, specific and testable propositions to be made about the
genetic/evolutionary basis of the phenotypic differences between
λs and λv, presented in the preceeding section (1.2), and further,
about the selective forces which have promoted such evolution.

3.3.2   *Is the Burst Size Determined Genetically?*

Phage workers are accustomed to the view that the reproductive
potential of a phage is determined by its genome, its host's genome
and physiological condition, and by the nutrient and physical
environment (Adams, 1959).   This is because different phages have
different reproductive potentials on the same host   (Delbruck, 1946),
while the same phage may have different reproductive potentials on
different host strains (Barry and Goebel, 1951) or, even, on the
same strain growing under different environmental conditions or being
in different physiological state (Delbruck, 1940; Heden, 1951).
All these old observations are now well established and there is
experimental evidence that lysis of the infected cell is genetically
timed.   In the case of λ,lysis is timed by, at least, the gene *rex*
as mentioned in section 3.1.3.

Premature lysis or lysis inhibition as well as defects in any of the
essential genes may considerably alter the burst size; also reduction
or increase of the genome size, by deletion or insertion of non-
essential sequences, repetitions and so on, may have some effect on
the rate of intracellular growth, and when such effects are accumulated
they may produce obvious alterations in the burst size.   It is even
easier to imagine why the burst size of a particular phage varies with
the availability of nutrients and the physiological condition of the host.

In this sense, the above view is justified and this is probably the reason why it is rarely questioned.

While the latent period is a remarkably constant parameter, the burst size of a particular phage varies widely between laboratories, between experiments and, most interestingly, between infected bacteria of the same culture. In single-burst experiments Delbruck (1945) recorded burst sizes of individual bacteria of the same culture, infected with phage T1, which ranged from less than 20 to more than 1000. Because this variation was far greater than the variation in the lysis times or the sizes of the infected cells, he concluded that the phage yield cannot be associated with either of these two factors. A similar conclusion was reached in this study though with different experimentation (section 3.2.1). Delbruck's experiment, together with the fact that no gene has yet been reported in λ, or probably in any other phage, which specifically determines the burst size, raises the questions of how this parameter is determined, or whether it is genetically determined at all. It is important to provide a solution to this question before claiming that the observed differences in burst size between the selected and control phage-host systems was the outcome of selection acting on the components of a real, genetic, burst size-determining mechanism.

3.3.3   *Dependence of the Burst Size on the Number of Rolling Circles*

While a deficiency in any of the genes of the phage is sufficient to limit the number of progeny particles, to explain an increase of the latter number one must assume that the production of all the phage components has been amplified proportionally. The easiest way that this can be achieved is by an increase in the number of copies of the phage genome (Thomas, 1970). The purpose of the following model is to

explain the variation in burst size of individual cells infected with λ without assuming genotypic variation in either the phage or the host.

During the early, θ-mode of replication the number of copies of the genome increases exponentially. At the end of this period the number of circular DNA molecules ready to undertake rolling-circle replication will be 2^{n_1}, where n_1 is the number of rounds of θ-replication. Then replication switches to the late, rolling-circle mode during which each circle produces linear copies of the genome, the number of genome copies increases linearly, or approximately linearly, with the time. At the end of the latent period, therefore, there will be 2^{n_1} x n_2 genomes where n_2 is the number of rounds of rolling-circle replication.

Assuming, for simplicity, (i) that the switch to the rolling-circle replication is synchronous and fully efficient; (ii) that all the linear copies of the genome are matured and packaged into infective virions; (iii) that replication forks move at the same speed, no matter what the mode of replication is, i.e. one round of θ-replication needs the same time (t) to be completed as one round of rolling-circle replication does, and (iv) that there are no lag periods at the beginning of each process, i.e. the latent period (L) equals $t(n_1+n_2)$; then the burst size (B) is:

3.1: $B = 2^{n_1}$ x $(\frac{L}{t} - n_1)$

If the switch from the θ-mode to the rolling-circle mode of replication delayed t minutes the burst size would virtually double, whereas if the same delay was in time to lysis the burst size would only increase by 2^{n_1}. A numerical example of how the burst size varies with the rounds of θ- replication achieved before the switch to the late mode (n_1) and

with the rate of DNA replication (t), as determined by the genome size, the available nutrients efficiency of the enzymes involved, is given in *table 3.7*.

TABLE 3.7: Variation in Burst Size with Varying Numbers of Theta-Replication Rounds (n_1) and Rates of DNA Replication (t).[a]

n_1 \ t(min)	1	2	3	4	5	8	10
1	158	78	51	38	30	18	14
2	312	152	99	72	56	32	24
3	616	296	189	136	104	56	40
4	1216	576	363	256	194	96	64
5	2400	1120	693	480	352	160	96
6	4736	2176	1323	896	640	256	128

[a] Values are the expected burst size (from *equation 3.1*) when the latent period (L) is 80 min.

It has been reported that about 20 circular DNA molecules are accumulated by 15 min from infection (Young and Sinsheimer, 1968; Carter and Smith, 1970).   These correspond to 4.32 rounds of θ-replication suggesting that each round is completed in about 3.5 min.   These values are within the range of n_1 and t shown in the *table 3.7* and therefore the example seems quite realistic to work with.

The available information on the exact time when the transition from one mode to the other occurs, on how many circular genomes are accumulated and how many of them are actually converted into rolling-circles, and on the exact rate of each mode of replication, is far from

conclusive. The same authors (Carter and Smith, 1970) observed about 60 molecules per cell of linear DNA accumulated by 20 min, i.e. about 5 min after the transition to the late mode. If all the accumulated circles were converted into rolling circles these data would imply a minimum rate of rolling-circle replication of 1.7 min per round.

Takahashi (1974) presented very different data; at 15 min 85% of the replicating molecules were θ-structures and at 30 min not all the molecules were rolling circles which means that not only the rate of θ-replication was much slower, or the number of rolling circles during the late period was much higher, but also that the transition was not synchronous. Bastia *et al* (1975) observed rolling circles with "tails" from less than 1 to about 5 genomes long only after 20 min from infection, before which time the predominant replicating forms were θ. Apart from the error and the artifacts involved in these delicate experiments, most of the variation in the reported DNA replication rates is probably significant; it is, like the variation in reported burst sizes, due to differences in the circumstances of experimentation, i.e. in the phage and host strains or stocks used and in the physical conditions of measurement.

3.3.4 *Control and Microevolution of λ Development: A Model of "Indecision"*

Equation 3.1 shows the importance of the number of rolling circles (2^{n_1}) in determining the burst size but it generally predicts higher yields than those usually observed. In fact, the assumptions on which this equation is based are quite unrealistic. The model of intracellular development in *figure 3.18* describes events of which the frequency may determine the burst size as well as the outcome of infection. These events are as follows:

The diagram represents a hypothetical wild-type infection which proceeds indecisively towards all developmental routes and results in lysis and liberation of mature phage particules, prophage and plasmids. The latent period is divided into time units which represent the time needed for one replication round to be completed; replication is assumed synchronous or delayed in a quantal manner.

a.   θ-replication (plasmid pathway).

b.   Circular genomes are arrested by λ-repressor and do not replicate further (lysogenic pathway).

c.   Circular genomes are arrested by CRO.

d.   CRO bound genomes may initiate rolling-circle replication.

e.   In the absence of IRC molecules, CRO delays replication.

f.   The tails of rolling circles are cleaved by A and packaged into mature virions (solid rombi); up to 100% of the genome units in a concatemer may be maturated in this way.

g.   Some genomes, however, are not properly processed or are defective (blank rombi).

h.   Linear concatemers are attacked and digested by the host RecBC exonuclease.

i.   The RecBC molecule may be inactivated by either the host RecA or the phage Gam protein, in which case the concatemer is allowed to grow again.

j.   Circular genomes are joint together by the action of phage and host recombination enzymes (*red* and *rec* products) and form circular concatemers of two, or more, units (secondary lytic pathway).

k.   Circular concatemers are processed by A just as the linear ones.

m.   λ-repressor bound circular molecules recombine with the *gal-bio* region of the host chromosome with the action of Int and Xis proteins; other, "illegitimate" recombinations with the bacterial chromosome also occur (transductions) but lysogens and transducents survive only when the lytic pathway is completely inhibited. The frequency of lysogenisation and the average infective progeny yield reflect the frequencies of the above events.
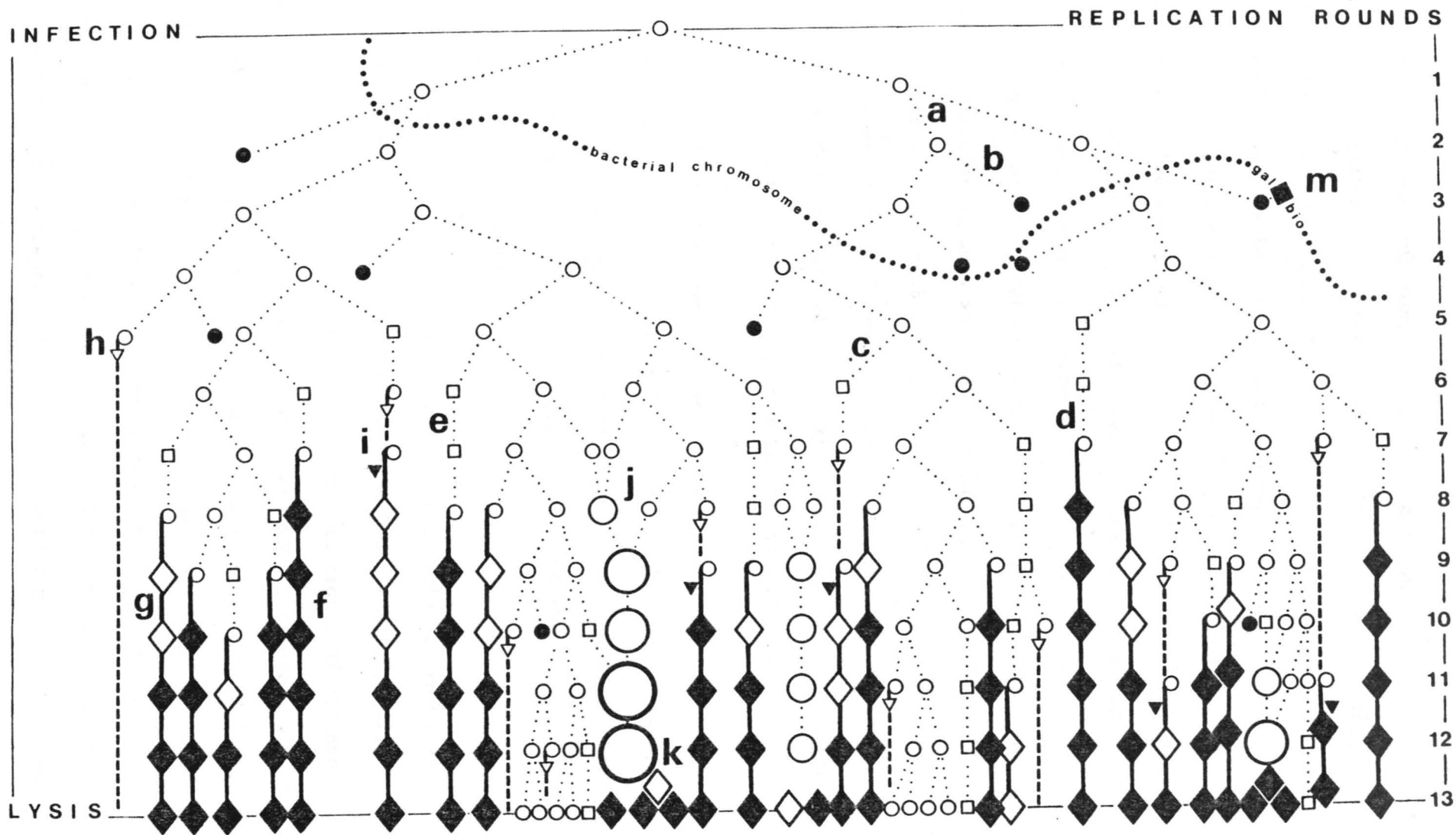
Inhibition of early transcription. Because initiation of θ-replication necessitates transcription of the site *ori* (Dove *et al*, 1971), the rate of transcription of the *R1* scripton determines the length of the lag periods between rounds of θ-replication. Genomes arrested by *cI*-repressor can no longer initiate θ-replication, whereas those arrested by CRO may continue to replicate in the θ-mode, though at a much slower rate, if rolling-circle initiation failed (plasmid replication). Thus, the rate of early transcription and its controllers, cI and CRO, determine the burst size by determining the number of circular genomes available for initiation of rolling-circle replication. This number and, therefore, the burst size, is expected to be greater when *cI*- and/or *cro*-products are less active (e.g. in clear-plaque and virulent mutants) or when the transcriptional activity of the host is enhanced (under unlimited growth conditions).

Initiation of rolling-circle replication. The molecular nature of this event and the factors evolved in it are still unknown. However, because this event is the first step of the lytic pathway and, as such, it plays a primary role in determining the outcome and the yield of infection, it deserves examination.

Although not proved, it seems very likely that initiation of rolling-circle replication takes place at the *ori*-locus (Bastia *et al*, 1975; Takahashi, 1976). Furthermore, the only known functional difference between the *cI*- and *cro*-repressors, to which one may attribute their effects on development, is that CRO allows for a residual transcription of the *R1* scripton (Lewin, 1977). It is reasonable, therefore, to assume that this residual transcription of the *ori*-site is essential for initiation of rolling-circle replication and that circular genomes which are arrested by *cI*-repressor cannot replicate further, in either

mode. Because, however, rolling circles can be observed only at times later than 10 min from infection, that is after CRO has reached its maximum activity (Champoux, 1970; Szybalski *et al*, 1970), transcription of the *ori* alone is not sufficient for initiation of rolling-circle replication. Yet, this event is not a late, *Q*-dependent one (Skalka, 1971).

It is possible that a rolling-circle initiation machinery (consisting perhaps of nucleases of the "control" and/or "*b*" region) which will be referred to as IRC is synthesised during early transcription but remains inactivated by some other early protein(s) which decay(s) faster than the components of the IRC after the turn-off of the early transcription. Possible inhibitors of the IRC are the products of the genes *cII* and *cIII* which are both known to decay rapidly after CRO-inhibition (Reichardt, 1975).

If such a mechanism does exist, its efficiency would determine the proportion of the repressor-free genomes entering the lytic pathway and, therefore, the outcome and yield of infection. Note that, if all the components of the IRC acted in *trans*, initiation of rolling-circle could also occur in CRO-free genomes.

Destruction of linear concatemers. The tails of the rolling circles are sensitive to the degradative action of the host, RecBC nuclease (exonuclease V). This degradation is inhibited by the phage, *gam* protein (Greenstein and Skalka, 1975). Also, the action of the RecBC nuclease is limited in hosts with a wild type *recA* gene (Barbour and Clark, 1970). Thus the number of "successful" rolling circles and, perhaps, the length of the linear concatemers depend on the relative activities of the genes *recA*, *recB*, *recC* and *gam* and of their products.

Formation of circular concatemers. The phage and host recombination systems (*red* and *rec*) provide an alternative mode of concatemer formation by joining together circular genomes (Weissbach *et al*, 1968; Skalka, 1971; Skalka *et al*, 1972, McClure *et al*, 1973; Takahashi, 1975). This pathway is of vital importance under conditions which do not permit rolling-circle replication but, otherwise, it reduces the number of rolling circles and may influence the burst size.

What this model suggests, in summary, is that all three developmental pathways proceed simultaneously upon infection and counteract until the final victory of one. The average burst size and the frequency of lysogenization depend on the average frequencies of the above events in the infected population. In the hypothetical cell, shown in *figure 3.18,* no decision is taken on which pathway to be followed and the cell lyses liberating prophage and plasmids together with mature phage particles. Selection alters the burst size and, as it will be argued, the frequency of lysogenization by altering the phage genotype so as to increase the frequency of events which favour the lytic pathway, or to decrease the frequency of those which inhibit it, in the particular genetic background of the coexisting hosts.

The model in its general form shown in *figure 3.19* describes the effect of mutations in those "key genes" on the final outcome of infection. Furthermore, it will be argued in the last chapter that this same model describes a steady-state coexistence of prophage, plasmids and lytic phage the proportions of which are shifted appropriately by the environment.

3.3.5 *Phage Adaptation to Bacterial Physiology*

Not much speculation can be made on the evidence here about the genetic

FIGURE 3.19:   *Genetic Control of λ-Development*

The diagram shows the channelling of replication of individual
λ-DNA molecules by the key-gene products as well as the
overall effect of mutations of these genes on the intensity
of each developmental pathway.

Circles represent the main and the secondary lytic pathway;
triangles represent the plasmid pathway and squares the
lysogenic pathway.

Prophage
Released
△
□
Lysis
□

No Growth | PROPHAGE □ □ □ Division □ □ ▷ | LYSOGEN
□ | □ | □ □
int⁻ | int⁺ | cI⁻(₌,recA⁺)
| □ | □ □
| □ | No Excision ◁ □ xis⁻ □ ◇ □
| □ | □
| □ □ | xis⁺
| cI⁺ | □
| □ □ |

INFECTION • • • • • • • • • PLASMID ◁ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ □ ◇
| ▽ • • ▽ | ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ◁ ▽
| cI⁻ | △
| ▷ • • ▽ | △
△ ◁ ◁ ◁ ◁ ◁ | ▽ | △
▽ | ▽ | recA⁻
cro⁻ | | red⁻
▽ | cro⁺ | △
▽ | | ◁ ▽
Uncontrolled | ▷ ▷ ▷ ▷ ▷ ▷ IRC⁻ ▷ ▷ ▷ ▷ ▷ ▷ ▽
Plasmid | •
Replication |
Cell Death ? | • | red⁺
• | IRC⁺ | recA⁺
• | • |

INFECTIVE | ROLLING | CIRCULAR
VIRIONS | CIRCLES | CONCATEMERS
• | • • • • |
• | recBC⁺ |
• | recA⁻ | recA⁺
• | gam⁻ | or
• | • | gam⁺
• | No Growth |
• | |
• | LINEAR |
• | CONCATEMERS |
• | • |
• • • (S to J)⁺ • • • Q⁺ •
• | • |
(S to J)⁻ | Q⁻
• | •
• | •
▼ | ▼
Lysis Inhibition | No Growth
or
Defective Virions

basis of the small-plaque phenotype developing in chemostat phage populations. However, there are a few important points which these results suggest and which should be emphasised. The selected phage grows relatively poorly on plates but normally, or even better than the original (depending on the host), under the conditions of a one-step growth experiment. This suggests that growth on plates is different from one-step growth and, possibly, from growth in chemostats.

Assuming that the growth parameters on plates are as measured by the one-step growth experiments, the only factor to which one may attribute differences in plaque-growth rates is the presence of agar; the solid media may amplify some diffusion deficiency of the phage. It is difficult to see, however, what the selective advantages of lower diffusion rates, or larger particle sizes, could be in a chemostat; thus this possibility seems remote.

Alternatively, the parameters of growth on a plate are not as measured by the one-step growth experiments. This seems quite sensible if one considers the extensive differences in gene expression and cell biochemistry between hosts of different physiological states (Nierlich, 1978).[1] Usually stock phages multiply better on growing bacteria than on bacteria in stationary phase (Delbruck, 1940). This is to be expected since the preparation of lysates is, itself, a selective technique for genotypes which multiply better on growing bacteria.

The one-step growth protocol measures the growth of phage on a synchronously dividing host population and, particularly, during the transition of the host population from the late logarithmic or stationary phase to the early logarithmic phase of growth. In contrast, plaque growth has two phases: at first, the phage grows on

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[1] see also Milne *et al* (1975)

exponantially growing bacteria and, later, on bacteria with a
stationary phase physiology.   Neither of these stages resembles
the "one-step" growth.   Growth in the chemostat resembles the late
stage of plaque-growth in that phage infects and multiplies in hosts
which are very near the stationary phase.

Then, the inefficiency of the selected phage in forming plaques on
young lawns may be interpreted as a result of its "specialisation"
on the particular physiological state of its coexisting hosts.   This
implies that a phage component (or set of components; proteins or DNA
sequences) has evolved so as to interact efficiently with bacterial
factors which are abundant at the stationary phase but absent during
the exponantial phase of bacterial growth.   Thus, *it is not that
deleterious mutations were selected in the chemostat, but that the
plate conditions were deleterious for the selected phage.*

Based on the assumptions (i) that gene expression and cell physiology
are very different in unlimited and limited growth conditions,
(ii) that all phages use host machineries for their reproduction and
(iii) that the common selective force in all chemostats, independently
of their design, dilution rate and limiting substrate, is the limitation
of the host's growth, the above explanation predicts that any phage
adapted to a host which grows under limited conditions in the manner
explained above, would develop a small-plaque phenotype after
sufficiently long periods of coexistence with its host in any
chemostat.   This is, thus far, justified (see section 1.1).   It can
also be predicted that, in contrast with the suggestions of Paynter
and Bungay, and Horne (Paynter and Bungay, 1971) of gradual accumulation
of distinct deleterious mutations, the development of the small-
plaque forming population would be instant, and also that the reduction

in plaque size would not continue further.

If things are so, the development and persistance of the small-plaque phenotype could be taken as a good example of directional selection followed by stabilizing selection. The distinctness of this phenotype offers possibilities of mapping its causal mutation and investigating further the nature, the causes and the effects of this evolutionary response.

3.3.6 *Are there Lysogens and Plasmid Carriers among the Selected Bacteria?*

Reference is made again to the model in *figure 3.19* to see how lysogenic, or plasmid-carrying clones, can arise; then the discussion can proceed on whether the selected bacterial isolates were such clones and, generally, whether and how such clones can be fixed in a chemostat, or natural population. The model suggests that in a wild-type situation prophages and plasmids are created at frequencies considerably higher than those at which they are observed. The reason is that, for the prophages to be cloned, the lytic pathway must be completely inhibited or, for the plasmids to be cloned, both the lytic and the lysogenic pathway must be inhibited (the repressor produced by a prophage inhibits replication of plasmids).

In the virulent mutant the lysogenic pathway is practically eliminated and, therefore, any phage or host mutation which interferes with the lytic pathway without affecting the action of *cro* and the θ-mode of replication may give rise to a plasmid-carrying clone. Stable plasmids may be cloned, for example, simply by inactivation of the rolling-circle initiation mechanism following an inactivation of the general-recombination mediated, lytic pathway. On the other hand, a

back mutation in the o_L or o_R operators (v_1, v_2 or v_3 to v^+) or a mutation in *cI* gene which would restore the ability of the repressor to recognize the operators would result in the formation of lysogenic clones. If such mutations are possible, the model predicts that lysogenic and plasmid-carrying clones would eventually arise, particularly if hosts with increased mutability had been selected, in the chemostat (see section 2.4.6).

There was, however, no evidence that the selected isolates were lysogens or plasmid carriers (section 3.2.3). The sensitive strains S3, S4 and S5 supported growth of all the original and selected phages. If any of these bacteria carried a prophage or plasmid the latter should have been heteroimmune to all the superinfecting phages, i.e. the o_R, o_L and *cI* or *cro* sequences of the hypothetical carried phage should have been different from those of the superinfecting phages. In addition, although mitomycin C induction proved inadequate test of the inducibility of hypothetical selected prophages (as it prevents phage growth in selected hosts), the latter should have had deficiency in excitation or intracellular multiplication.

Cryptic phage genomes may, nevertheless, coexist not only with their host bacteria but also with their virulent competitors, simply when their host is resistant to the virulent phage. A detailed genetic analysis of the selected organisms may provide information on whether or not evolution has occurred (or occurs) towards stable phage-host associations but the problem would remain: is any of the modes of λ-development "superior" to be expected to be the ultimate outcome of coevolution in chemostats? This question is dealt with in the last chapter.

3.3.7   *Evolution of DNA Metabolism and Cell Division Genes*

Finally, some attention should be paid to the observed effects of mitomycin C on the ability of the selected bacteria to support phage growth.   This drug is known to damage DNA molecules causing formation of pyrimidine dimers (like ultraviolet light) and cross-linkage between their complementary strands (Otsuji and Murayama, 1972).   Such damages induce the so called "S.O.S. repair" enzymatic mechanism.   The genes involved the S.O.S. repair are genes of DNA metabolism, general recombination and cell division.   The exceptionally wide range of phenomena in which the S.O.S. repair genes are involved was reviewed by Swenson (1976) and by Witkin (1976); Goudas and Pardee (1975) proposed a model for the genetic control of these functions.

When λ-lysogens are treated with a sublethal (in terms of DNA damage) dose of mitomycin C the S.O.S. system repairs the chromosome and enables its replication but the prophage is induced to grow lytically due to the proteolytic cleavage of its repressor (possibly other operon repressors are also inactivated) by a component of the S.O.S. repair system, the *recA*-nuclease/protease (Roberts *et al*, 1978; Mount, 1980)

It is, thus, reasonable to suppose that the inhibition of phage growth in selected bacteria caused by mitomycin C is related to some deficiency of the process of synthesis of infective phage DNA.   This may be understood in two ways: firstly, assuming that the selected S.O.S.-repair system is defective in repairing the damages caused by the drug on the phage DNA increasing thus the frequency of defective propagules and phage particles;   secondly, assuming that the selected S.O.S. repair system antagonises lytic replication (by interfering, for example with the process of initiation of rolling circles).

Despite the apparently normal survival of the selected bacteria themselves to mitomycin treatment there seems to be some truth in the first explanation; the selected S.O.S. repair system, and in particular the *recA*-product, seems to allow some residual activity of the *cI*-repressor which is presumably responsible for the stronger inhibition of lytic (plaque-) growth of λ as compared with that of λv (*table 3.6*; see also sections 3.2.3 and 3.2.4).

Nevertheless, considering the ecological importance of DNA replication and cell division, one may expect that the S.O.S. repair genes would be one of the first targets of selection in the chemostat (and in any environment) and would have suffered major rearrangements with pleiotropic effects on all their functions. These are, perhaps, the host rearrangements to which the phage has adapted its genes to increase its lytic reproductive rate and to decrease (as a side effect) its plaque-forming efficiency on rapidly growing bacterial lawns. Three S.O.S. repair genes (*recA, recB* and *recC*) are already known to interact with each other and with phage genes (*gam, cI*) to determine the burst size (section 3.3.4); yet all these genes are inducible, and their function may well depend on the physiological state of the host.

If the S.O.S. repair genes had indeed evolved one might also expect that the mutability of the selected bacteria (sections 2.3.5 and 2.4.6) is not simply modulated by a "mutator" but reprogrammed by natural selection on the recombination genes; if the new "program" is for higher mutability, as the results in section 2.3.5 suggest, frequent recombinational rearrangements are to be expected in chemostat phages as well as in their coexisting hosts.

FITNESS

4.1   *F I T N E S S   A N D   R E P R O D U C T I V E   R A T E S*

The general conclusion one can draw from the results presented thus far, is that both species evolved during the course of their coexistence in culture 1.   Evolution occurred beyond, or perhaps far beyond, those genes which determine resistance and host range, as these terms are usually conceived.   Some evolutionary events might have been simple adaptations to the abiotic environment of the chemostat but most seem to be interrelated and are therefore better explained as coevolutionary responses.   Although several interesting questions arose concerning the precise nature and extent of this evolution, further investigation on these lines would take this thesis away from its central subject.   The original question, how *E. coli* and λvir coexist in continuous culture, is an ecological one;   it is a question of maintenance of allelic polymorphisms and species diversity as well as a question of what determines the population density and how densities are stabilized or change in time.   Fitness is the central concept and concern of the relevant theories.

In classical Darwinism "fitness" was synonymous with "existence"; only fit species existed whereas less fit ones were eliminated by natural selection.   This conception has now been abandoned by most ecologists who, instead, define the fitness parameter as a function of the rates of reproduction and survival.   However, the rates of reproduction and survival may be conceived, and have been used by ecologists, in several different ways (Murray, 1979).   There is, for example, a parameter which may be called the *birth rate*, in a particular set of environmental conditions, and which expresses the number of

viable progeny per generation, or the appropriate time unit (like,
for example, the clutch size, litter size or in this case the burst
size of a particular phage genotype;

similarly the *death rate* (or *mortality*) expresses the number, or
proportion, of the viable individuals which die, per generation or
time-unit; the death rate subtracted from the birth rate gives the
rate of *survival*. The *natural* growth rate of a clone is the
difference between birth and death rates per individual of the clone
in a particular environment; the product of the natural growth rate
and the density of the clone will be referred to below simply as the
growth rate of the clone. It should be pointed out that the growth
rate and natural growth rate are the only reproductive parameters
that can take negative (the density decreases) or zero (numerical
stability) values. The *intrinsic* (or *inherent*) rate of increase
refers to growth in an uncrowded habitat (where competition does not
occur) and under a particular set of environmental conditions; the
term *reproductive potential*, used here for phage, is equivalent but
not synonymous with the intrinsic rate of increase since the latter
depends, in the case of phage, on the rate of adsorption on, and
infection of, the hosts (intrinsic rates of phage growth are shown,
for example, in *figure 1.9*). Finally, the *maximum* growth rate is the
intrinsic rate of increase in *optimum* environmental conditions.

Which reproductive rate is measured and related to fitness and natural
selection has too often been a matter of convenience rather than one
of principle. In general, experimentalists find it easy to measure
intrinsic, or, even, maximum, growth rates whereas for field ecologists
such values are neither measurable nor testable in the wild and of
little utility. It has been a standard practice among microbiologists
who work with chemostats to compare the fitness of chemostat and control

organisms by comparing their maximum, or intrinsic (in the physical and nutrient conditions of the chemostat) growth rates, or other intrinsic attributes (see, for example, Nestmann and Hill, 1973; Cox and Gibson, 1974).

The above practice is totally inadequate for estimating and comparing fitness. Take, for example, the control and selected phages of this study and assume that they both coexist with control and S3 bacteria. At a particular ecological moment, the control bacterial population might be very large whereas the S3 might only be a tiny fraction of the total host population. In such a case the superior intrinsic properties of λs would hardly matter for its survival as a competitor; what would matter would be the frequencies of the two phages and the two hosts as well as the density of each phage and host population (density- and frequency-dependence of fitness). This is to say that, although the reproduction and the survival (neo-Darwinian fitness) of each phage in a particular environment are determined entirely on its genotype, which genotype will perpetuate itself and which will become extinct (classical Darwinian fitness) entirely depends upon the environmental conditions.

The classical view suggests that fitness cannot be compared unless measured *in situ*, i.e. at a particular ecological moment under particular environmental circumstances; the only concept of reproductive rate related to fitness is the clone's growth rate or natural growth rate (the fitter clone increases, the less fit decreases; differences in fitness are correlated with differences in rates of changes in density); growth rates measured in the laboratory do not correspond to those in the wild, and fitness in the wild may change from one ecological moment to another. It is mainly for its latter principle that Darwinism has

been called a "metaphysical research programme" (Popper, 1934; 1974 quoted by Wassermann, 1978).

In essence the classical and the neo-Darwinian views of fitness are identical. Both "fitnesses" are functions of reproductive rates. It is only the emphasis on what determines the fitness that has changed place, from the environment and its aim to increase mortality to the genotype and its aim to increase reproduction. As a result the neo-Darwinian methodology has, in practice, been diametrically opposite to the classical one. The promise of neo-Darwinism is that one can set an artificial environment, simulating a natural situation, in which to measure and compare the fitness of different genotypes; by altering the conditions of the experiment one can investigate on what fitness depends.

This technique has also been practiced by virtually all microbiologists who were interesting in relative fitness in competition in continuous cultures (typical of this kind are the competition experiments performed by Chao *et al*, 1977). To "prove" frequency-dependence of fitness, for example, several identical chemostats are set and are inoculated with inocula which contain the competitors at different frequencies. A "satisfactory" result is that different competitors win in different cultures. This may be a proof that fitness depends on frequency but not that frequency dependent selection is an important stabilising mechanism in nature, as the theoretical models suggest.

Extrapolation from such experiments to natural situations cannot, *in principle*, be verified. One natural condition which has not been simulated in the laboratory is sufficient to invalidate any comparison, of fitness, of this kind. The neo-Darwinian methodology has, therefore,

often been a "metaphysical programme of research".

The principles of the classical view, that fitness estimated *in situ* (from density changes) does not correspond to fitness measured in any other way, and that fitness in the wild may change from one ecological moment to the next, are also testable predictions of the classical theory. The experiments presented in the following section were designed (i) to compare the fitness in competition of the selected and control bacteria as well as those of λvir-resistant and λvir sensitive bacteria, by observing density changes in chemostats; (ii) to compare the intrinsic rate of increase of the control bacteria with that of each one of the main selected strains growing under the chemostat's physical and nutrient conditions; (iii) to replicate culture 1 and study in more detail the bacterial polymorphism and the fate of identifiable traits. The first two sets of experiments were principally to test the fundamental assumptions of the model by Chao *et al* (1977; presented here in section 3.1.2) but the results, together with the results of the replication of culture 1, support the classical view of natural selection.

4.2 *C O M P A R I S O N   O F   T H E   F I T N E S S   O F   S E L E C T E D   A N D   C O N T R O L   T R A I T S*

4.2.1. *Competition of Control and Selected Bacteria in Phage-Free Chemostats*

Three replicate TB1-chemostats running at 37°C and at a dilution rate of 0.04 hr $^{-1}$ were inoculated with equal numbers of control, S1, S2, S3, S4 and S5 bacteria.   These populations were then allowed to compete for 15 days (approximately 21 generations).   Samples of the mixed population were seeded on TR2, with and without maltose and phosphate, and spotted with λv, λvh and λs to test the sensitivity pattern of the predominant type;   they were also titred for λv-resistant cells, on plates containing approximately 10^{9} pfu, and for total viable cells on phage-free plates.

The outcome of competition between these bacteria was identical in all three replicate experiments.   The turbidity of the plaques formed by the phages increased during the first 3 days and by day 4 the mixed populations appeared to be resistant to all three phages.   Also, except in samples taken during the first two days, the titre of λv-resistant types remained equal to the total bacterial titre.   This resistance was of the S5 type (see section 2.3.4) since all three populations retained their sensitivity to all phages on maltose-phosphate plates throughout the experiment.

Despite the clear phenotypic differences of the competitors on EMB media it proved impossible to follow the changes in their population density precisely using this criterion.   On EMB mal I all samples seemed to consist of mal^{-} types (other than control or S2 types)

whereas on EMB mal II they appeared as mixtures of several
unclassifiable intermediate types. This was not surprising since
the selected isolates themselves often gave rise to polymorphic
populations (section 2.3). It was quite clear, however, that
selected, semiresistant (S5) and possibly totally resistant, types
dominated all three cultures while the sensitive types (control, S3
and S4) were rapidly reduced to lower frequencies or perhaps eliminated.

4.2.2   *Competition of Control* λv*-Sensitive and* λv*-Resistant Bacteria*
         in Phage-Free Chemostats.

The above results suggested that the selected bacteria, or at least
some of them, are fitter in competition with the sensitive, original
ones even in the absence of phage. It is more sensible to suppose
that this higher fitness of the selected bacteria is the result of their
multi-directional adaptation to chemostat conditions rather than a
direct effect of their specific mutation(s) to phage-resistance on
their reproductive rate. To rule out the second possibility, two
control chemostat-cultures were set with the control bacteria
(original C600) alone; technically, these cultures were identical to
all the other. The inocula, of about 5×10^8 cells, contained λv-
resistant mutants at frequencies of 1.6×10^{-4} (culture A) and 1.2×10^{-4}
(culture B). Counts of resistant cells were taken, as previously,
on plates containing about 10^9 pfu of λv, and total counts on phage-
free plates.

Unexpectedly, the numbers of resistant bacteria rose, rapidly at the
beginning and slower later, to levels very near to those of their
sensitive competitors by day 10 (*figure 4.1*). From then onwards the
two populations seemed able to coexist indefinitely. Although the
changes in frequency of the resistant population were only recorded

FIGURE 4.1:   *Competition of Control, λv-Sensitive and λv-Resistant Bacteria in Phage-Free Chemostats*

The values are frequencies of the λv-resistant subpopulation.   .N_R: viable, λv-resistant cells/ml; N_T: total viable cells/ml.   Solid line: culture A; Dotted line: culture B.

DAYS AFTER INOCULATION

Log N_R/N_t
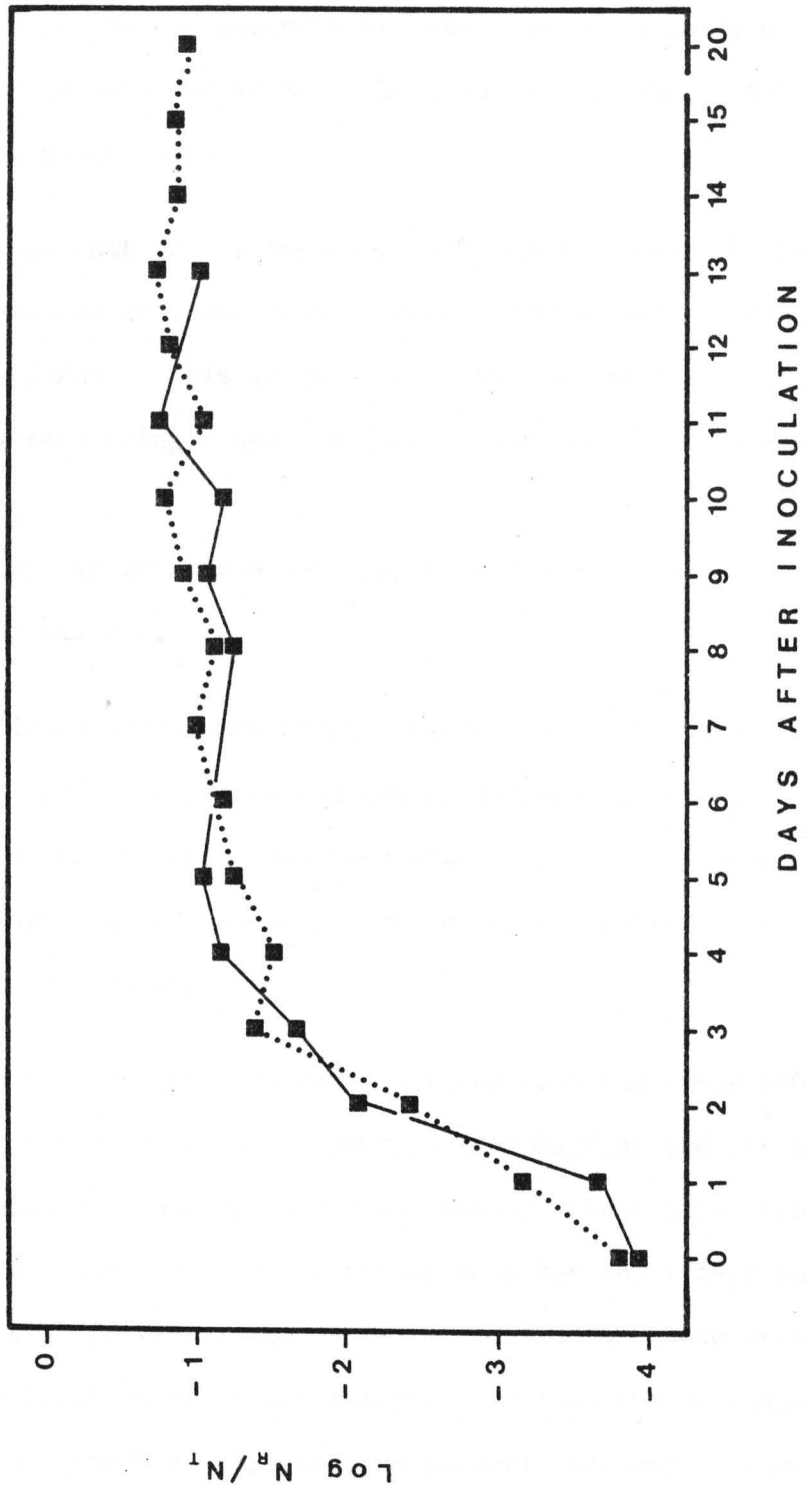
for a relatively short period of time, their pattern may be interpreted as that the growth rate and the fitness of the resistant bacteria did not depend on their frequency in the inoculum, though it may depend on, or be correlated with, their actual density or frequency in the culture and it may, thus, change in time. Other explanations are given below.

It is worth noting that the frequency of mal^- types (tested on EMB mal I media) remained at undetectable levels, far below the frequency of λv-resistant forms. This suggests that the observed λv-resistance was due to mutations lying either on $lamB$ or outside the mal-regulon.

4.2.3 *Comparison of Intrinsic Rates of Growth of Control and Selected Bacteria*

No significant differences were found between any of the main selected strains and the control bacteria concerning maximum, stationary phase density in TA1 medium. The intrinsic (exponential) rate of growth of the selected strains was, however, consistently lower than that of the control bacteria.

Two control λs-resistant strains were isolated from the stock C600 culture, of which one had a mal^- phenotype (C600mal/λs) and the other had a mal^+ phenotype (C600/λs) on EMB mal media; these were also to see whether or not mutations in the mal-operons had any effect on the rate of exponential growth. The values estimated for these strains were almost identical to the control indicating that the decreased intrinsic rate of growth of the selected bacteria was not a result of their mal-mutations, or their mutations to λ-resistance, *per se*. The same is suggested by the fact that, despite their clear phenotypic differences, the selected strains grew at similar rates.

The results discussed above are shown in *table 4.1* where the intrinsic rates of growth are expressed as intrinsic generation times. The later parameter was estimated as the reciprocal of the slope (k) of the logarithmic growth curve:

4.1 $\qquad \log_2 \dfrac{N}{N_o} = k\ t$

where N_o is the initial number of viable cells and N is the number of viable cells at time t.

TABLE 4.1: Intrinsic Generation Times of Control and Selected Bacteria

Bacterial Strain	Generation Time (min) [a]
C600 (Control)	28.3 ± 1.1
C600/λv[b]	27.5 ± 1.0
C600 *mal*/λv[b]	27.6 ± 1.1
S1	33.7 ± 1.4
S2	34.9 ± 1.8
S3	32.9 ± 1.6
S4	35.2 ± 1.8
S5	33.3 ± 1.5

[a] Values are estimated as k^{-1} from *equation 4.1*. The stocks were diluted in pre-warmed (37°C) TB1 to a density of approximately 10^4 cell/ml and growth was followed for 5 hr by viable-cell counts taken every 30 min. All strains showed an initial lag of approximately 1 hr. Confidence limits are at P = 0.05 and with 6 degrees of freedom.

[b] For description see text.

The colony morphology (mucoidity or spreading growth) of some selected strains and other results suggested that these bacteria carry mutations in genes which control cell division (see sections 2.4.5 and 3.3.7). It could well be, therefore, that the prolongation of the intrinsic generation time of at least some of the selected strains was not due to some deficiency in consuming the available nutrient but, simply, to a delay of the cell division process which would result in an increase in the cell's size. Indeed, although no precise measurements were made electromicrographs revealed that all main selected strains (including non-mucoid ones) grown in TB1 contained a small, but easily detectable proportion of long, sometimes filamentous, cells (*figure 4.2*). In contrast, long cells (of more than twice the average size) were not found in preparations of control bacteria.

4.2.4 *Culture 3: The Fitness of Control and Selected Traits* In situ

Culture 3 was a replicate of culture 1 (section 1.3.1) and was used to follow the population dynamics of all identifiable subpopulations. The phage (λv) was added five days after the inoculation of the bacteria (*E. coli* C600). Time was measured from phage inoculation.

The population dynamics of this culture are shown in *figure 4.3*. The phage population grew rapidly to reach a titre of 1.4×10^{10} pfu/ml by day 2. This first growth was considerably faster and the yield higher than in culture 1 (*figure 1.2*). During this first peak-growth of the phage the original bacterial population (λv-sensitive, mal^+) was replaced by a phage-resistant population which appeared homogeneously mal^- on EMB mal I medium but heterogeneous, consisting of several intermediate mal-phenotypes, on EMB mal II. A functional deficiency of the maltose regulon and, presumably, a reduction in the rate of
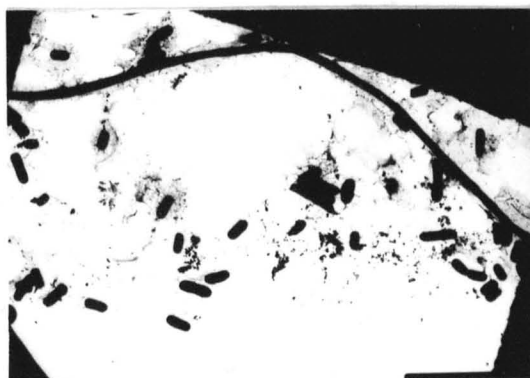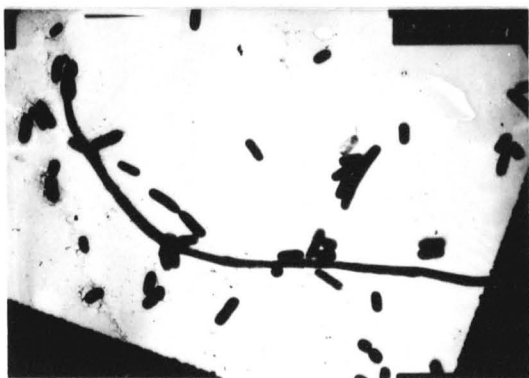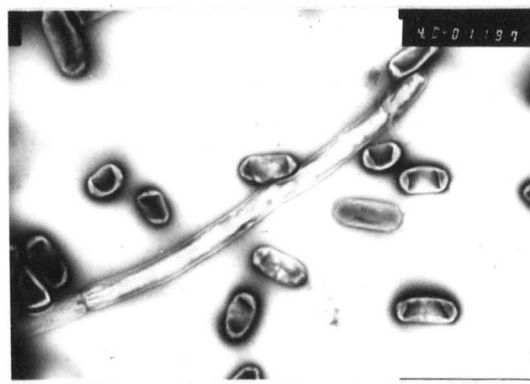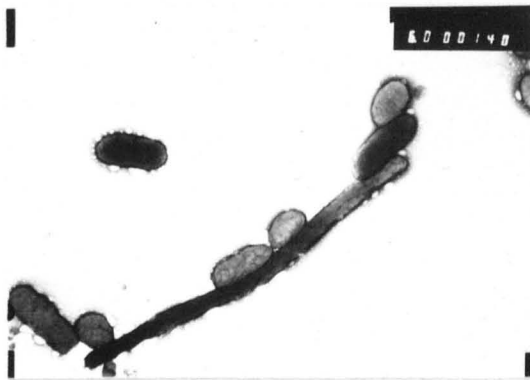
FIGURE 4.3:   *Population Dynamics in Culture 3*

o λv-sensitive, mal^+ bacteria

● Mixture of λv-resistant and -semiresistant mal$^-$ and mal$^+$ populations.

□ Large plaque forming phage (λv)

■ λh

△ Small plaque forming phage (λs3)

Dotted and dashed extensions represent hypothetical density changes'.

D = Dilution rate.

D = 0.04 hr⁻¹

DAYS AFTER PHAGE INOCULATION

Log PFU AND VIABLE CELLS PER ml

synthesis of λ-receptor was, therefore, the immediate response of the bacteria to the increase of the phage's density.

Unlike culture 1, culture 3 showed no obvious drop in density of the total bacterial population during the first peak of the phage population. The frequencies of mal^- types and of types which did develop some colouration on EMB mal II remained very similar throughout the experiment so that no definite statement can be made as to which mal-type was dominant in the culture. However, counts of resistant bacteria, taken on λv-containing plates at day 2 and afterwards were equal to those of total viable cells, taken on phage-free plates; this clearly showed that the great majority of the bacteria, irrespective of their mal-phenotype, were to some degree resistant to phage and that phage growth was supported by small subpopulations.

To test the overall pattern of resistance the mixed bacterial population of each sample was seeded on plates with, and without, maltose and phosphate and was spotted with dense lysates of λv, λvh and λs. All three phages formed visible plaques on all the induced lawns, and only on those, suggesting that semisensitive (S5?) types persisted at sufficiently high frequencies to allow plaque formation; the plaques, however, were much more turbid than when developed on pure S5 lawns indicating that non-inducible, totally resistant types were also very frequent or, perhaps, dominant in the culture. The persistant heterogeneity of the population concerning the mal-phenotype contradicts the alternative hypothesis, that there was only one semiresistant population and this this was more resistant than S5.

Immediately after its first peak the phage population declined at a rate equal to the dilution rate (D) but this decline stopped around day 10 and a second peak was observed around day 21. During the same

period, the density of the λvh population, measured on S3 bacterial

lawns, appeared to change in parallel with the total phage numbers.

Interestingly, however, its relative frequency in the total phage

population did not remain constant, as it would be expected if it was

only determined by the frequency of the h mutation; it dropped

significantly from 1.2×10^{-5}, at day 2, to 6.3×10^{-7} of the total

population, at day 21, and continued to decrease from then onwards.

In remarkable agreement with the results from culture 1, small-

plaque forming phage dominated the culture by day 38 and apparently

eliminated the original, large-plaque forming population by day 45.

This evolution was seen as a sudden increase in the length of time of

incubation needed for plaques to appear. Most of the plaques of the

original phage appeared before any plaques of the new type did so.

This allowed titration of the original phage and visualisation of

its decrease to extinction. At day 38 it comprised a 10^{-4} fraction

of the total population and it was undetectable (at densities below

10^2 pfu/ml) in all subsequent samples. Its h-mutant, λvh, was last

seen at day 26, at a density of 2×10^1 pfu/ml, and occurred at

densities below 1 pfu/ml, if at all, in subsequent samples.

The evolution of the small-plaque forming population, at day 38, was

associated with a considerable fall of the total bacterial titre, but

had no apparent effect on the composition of the bacterial population

in respect to its *mal*-phenotype. It is possible that this new phage

could multiply on, and lyse, some dominant (until day 38) bacterial type

which was resistant to all indicator phages, λv, λvh and λs. The

bacteria recovered, however, and the new phage grew to reach a density

of 6.3×10^8 pfu/ml by day 44. Subsequently the phage persisted at

high titre and fluctuated in parallel with the total bacterial

population.

4.3 *DEPENDENCE AND CHANGE OF FITNESS*

4.3.1 *Is there a Wild-Type?*

The presence of phage in the environment of bacteria differentiates
the survival (selective) values of phage-sensitivity and phage-
resistance.   Any population that persists in a chemostat or natural
environment must somehow balance its mortality and reproduction.
The mortality of a phage-sensitive bacterial population depends,
directly, on the density of the coexisting phage population and,
inversely, on the density of the bacterial population itself; it may,
thus, be equalised with the dilution rate of the chemostat (D) and,
consequently, with the mortality of any coexisting, phage-resistant,
competing population (*numerical refuge*; Chao *et al*, 1977).

This intraspecific and interspecific dependence of bacterial
mortality values on density does not explain the persistence of phage.
When bacterial mortality from phage predation is zero phage reproduction
is necessarily, also zero; dilution would eventually remove all the
phage from the culture.   Whenever the mortality values in sensitive
and resistant competitors are equal the fitness of each population
depends entirely on its reproduction.   The persistance of phage
suggests that sensitive bacteria do not remain in their numerical refuge
but tend to increase.   *The problem is, therefore, not how do sensitive
bacteria persist in the presence of phage but why their rate of increase
is higher than that of their resistant competitors.*

To explain this Chao *et al* assumed (as most neo-Darwinian selectionists
do) that the bacterial molecular components which are responsible for
sensitivity to phage offer high reproductive rates (i.e. have been

selected for), and that any mutational distortion of these components would have negative effects on the reproductive efficiency of the cell. For the reasons explained in section 4.1, the competitive superiority of the original strains in phage-free chemostats, reported by these authors, does not really verify their model of coexistence in presence of phage. In contrast, the results presented in section 4.2 suggest that wild-type genes responsible for sensitivity to phage *are selectively dispensable*.

If bacterial mutants with lower mortality may also have higher reproductive rates when competing with their parents, then evolution of competitive resistant bacteria and extinction of their sensitive ancestors should be expected in chemostats with phage. The techniques used here were not sensitive enough to demonstrate the extinction of control bacteria but they did demonstrate the extinction of the original phage, λv, from culture 3.

The results of the competition experiments in section 4.2.1, the persistance of the S5-type semiresistance of the bacteria in culture 3 as well as the fact that small-plaque forming phages evolved in both cultures (1 and 3) might be interpreted as meaning that new "wild types of bacteria and phage evolve, by coadaptation, to form stable chemostat communities. The *mal*-polymorphism, and the absence of the h character from the phage, in culture 3, contradict, however, the above interpretation and raise two important questions: (i) how much of the polymorphism is maintained by selection and how much is selectively neutral ; (ii) in how many alternative designs of maintainable communities may coevolution result.

In the next chapter the concept of the wild-type, with constantly higher reproductive ability, is disposed of and the emergence of

sensitive bacteria from their numerical refuge is explained as an effect of the evolution of host-range in phage; the polymorphism is accounted for and it is suggested that the communities studied here *are not maintainable* but *are continuously evolving*.

4.3.2 *Fitness and Mutability*

It was postulated in section 1.3.1 that the unusually high frequency of survival of the bacteria during the phage epidemic in culture 1 might have had some physiological, rather than mutational (genetic) basis. The observation made in cultures A and B (*figure 4.1*) suggest, however, quite the opposite. The frequency of λ-resistant mutants may be very high even in the absence of phage. If phage was added to culture A or B, 5 days or so after the inoculation of bacteria the density of the latter would only fall one log-unit; this is what was observed in cultute 1 (*figure 1.2*). In culture 3 (*figure 4.3*) the resistant mutants seem to have increased to even higher levels. Detailed characterization of these mutants is needed to discriminate among the following explanations of their increase in such chemostats.

Similar phenomena have already been extensively studied and are traditionally described as accumulation of mutants that have no apparent selective advantage. Novick and Szilard (1950a), Atwood *et al* (1951) and Kubitschek (1970), among many others, have interpreted such phenomena as genetic drift. This line of thought is, however, difficult to follow. Mutants are not "accumulated" in a chemostat unless their reproductive rate is higher than the dilution rate (plus any other cause of mortality) and, consequently, higher than the reproductive rate of their parents (natural selection). Mutations to resistance may accumulate on a genome, i.e. resistant bacteria may have suffered

multiple mutations on genes responsible for sensitivity. Still, however, multiple mutants would remain at low frequencies unless they were selected for. Confusion has, apparently, been due to the usual, narrow view of natural selection as differential mortality.

Do, however, mutations to resistance affect reproductive rates? In some cases they decrease reproductive rates and they do so for clear reasons (Szmelcan and Hoffnung, 1975; see page 43). Results here (in the same page, 43, and also in *table 4.1*) suggest, however, that it is not always so; different resistant mutants may take identical reproductive/selective values. There is no concrete theoretical basis for rejecting that some mutations to phage resistance may increase the reproductive rate under certain environmental circumstances, or that their effect may be density;, or frequency-dependent. Are, however, the particular λ-resistant mutants fitter (at particular densities or frequencies), or is it that the fitter mutants happened to be pleiotropically resistant to λ?

Gibson *et al* (1970), Nestmann and Hill (1973) and Cox and Gibson (1974) regarded the accumulation of mutants of no obvious selective advantage in the second of the above ways; their interpretation is adopted here because it is consistent with a number of other observations. These authors attributed the phenomenon to modulation of general-mutation frequency by mutator genes whose selective advantage is the adaptive flexibility they confer to their carriers. Their prediction, that the frequency of any kind of mutants, which the investigator happens to observe, will increase in chemostats, seems to be confirmed. There is, however, some inconsistency; mal^- forms did not increase proportionally (if at all) with λ-resistant forms in cultures A and B. Although the technique of measuring mal^- minorities in a mal^+

population is not reliable enough to account strongly against the general-mutation modulation hypothesis, a case of a site-specific modulation may also be considered as an alternative explanation. The modulated site, in this particular case of mutants, needs not be in the $lamB$ gene (to account for the mal^+-phenotype) since mutations which block steps subsequent to adsorption also confer λ-resistance without affecting maltose metabolism.

A last, but not least, possibility is that of a mutation which does not affect mutability but does increase competitiveness in TB1 chemostats and, by coincidence, blocks λ propagation at some stage subsequent to adsorption.   Such mutants are potentially sensitive to host-range phage and their fitness may take negative values when their specific predator-phages occur.

4.3.3   *Bacterial Fitness and Generation Time*

As argued in section 4.1 intrinsic generation times, or values of any other intrinsic attributes, *are not* measures of fitness.   However, comparison of intrinsic attributes of variants is an indispensable technique for tracing evolution.   The selective importance of the prolongation of the generation time of the bacteria in culture 1 can only be assessed in the light of the mutation(s) that caused it and of the other pleiotropic effects such mutation(s) may have on the general phenotype.

There is little doubt that the relatively long generation time of the selected bacteria is associated with their filamentation and mucoidity and, ultimately, with mutations in genes controlling cell division (see section 2.3.7).   In phage-containing chemostats mutants of cell division may be selected for, either because they reproduce

faster or because they suffer lower mortality than their parents. As explained earlier (section 3.3.7) cell-division genes are also involved in the control of phage-DNA replication and their mutants may increase survival to phage infection. Thus, the prolongation of generation time may well be a side effect of selection for resistance to phage (the latter term used with its broader meaning; see section 2.1.1).

Alternatively evolution of cell-division genes may be an adaptation to chemostat conditions other than the presence of phage. In chemostats, the size and the macromolecular composition of bacterial cells depend upon the dilution rate (that is, upon the generation time; Stanier *et al* 1977). Cell-division, and other central regulatory genes may, therefore, be expected to evolve in order to allocate more efficiently the limited chemostat resources. Whether the prolongation of the generation time, or, generally, any change in intrinsic attributes of the bacteria, is a coevolutionary (due to the presence of phage) or an adaptive response should not be difficult to test using control cultures without phage.

The above propositions do not exclude each other and should rather be incorporated into the following, repeatedly stated, but little exploited, generalisation of the theory of natural selection. The relative fitness of an allele depends on the allele's effect on *both* the reproduction and the survival of its carrier. In terms of this study, the bacteria evolve simultaneously towards maximum efficiency of utilisation of all the available resources and towards maximum resistance to phage.

4.3.4 *Fitness in Time*

Irregularity of Population Oscillations. The modern ecological
theory recognises that fitness is not a constant but a variable.
Evolutionary ecological models are to reconcile the observed
ecological stability with the concept of a changing fitness. The
major concern of these models are the factors which determine
fitness and the way their occurrence and intensity change in time.
Density and frequency have become of overwhelming importance.
Perhaps without exception, the models of density- and frequency-
dependence suggest that fitness oscillates in time and does so in a
regular manner. The results obtained here suggest otherwise.

Culture 3 confirmed many of the basic aspects of the analysis of
culture 1, like for example, the *mal*-polymorphism and the evolution
of semiresistance in bacteria as well as the evolution of small-plaque
forming phage. According to Wassermann (1978) these "... significant
correlations between particular character states associated with
specific loci and particular environements" would be methodologically
sufficient to demonstrate the occurrence of natural selection and its
importance in shaping the population dynamics of the chemostat
E. coli-λvir community. To what extent the above phenotypic
characters are associated with the same loci in the two cultures
remains, of course, to be seen by genetic analysis. More interesting,
at this point, are the differences between the two cultures.

The first striking difference is in the population dynamics. Much
stronger phage-host population interactions are manifested in culture 3
(*figure 4.3*) by (i) the wider fluctuations in total bacterial numbers,
particularly after day 30, and by (ii) the persistance of phage at
very high densities throughout this replicate experiment. Analogous,

very significant differences are to be found when comparing these
cultures (1 and 3) and cultures with other lytic phages (reviewed
in section 1.1) or when comparing the population dynamics in previously
reported replicate cultures with the same phage (see, for example,
the dynamics of "A" and "B" type reported by Chao *et al*, 1977). In
none of the examined phage-host cultures is there any obvious
regularity whatsoever. This is in contrast with the expectations of
population regulation models.

Dependence on the Present. The second very important difference
between the cultures 1 and 3 concerns the behaviour of the h phage
character and allows some extrapolations to be made on what determines
fitness. In culture 1 the h-allele is fixed in the phage genotype of
day 135; in culture 3 the same allele is practically eliminated by
day 38. Since S3 bacteria *were* isolated from culture 1, these results
are best explained by causally associating the fixation of the
h-allele with the coexistence of these bacteria, in culture 1, and its
failure to be fixed to the absence of the very S3 genotype, from
culture 3. The fitness of an allele (like h) depends, therefore, on
the environmental circumstances at the moment of its occurrence,
namely, on the co-occurrence of its specific host at sufficient densities.

Dependence on the Past. It is also very significant that, whereas in
λv populations viable   h-mutants occur at relatively high frequencies,
the phage selected in culture 3 (λs3) produces no viable h-mutants.
Almost certainly, λs3 carries another (set of) mutation(s) (s3) which
interacts with the h-mutation(s). This interaction may be understood
in three ways: (i) as that the s3 reduces the frequency of h (site-
specific modulation); (ii) as that the s3 does not allow the expression
of h (e.g. the s3 prevents transcription of the h-gene); (iii) as that
s3 renders h-particles non-viable.

The fitness of an allele (like h) depends, therefore, not only on the circumstances of the particular ecological moment of its occurrence but also on past ecological events recorded on its genetic background.

Unpredictability of the Future.   The s3 mutation(s) may, or may not, map on the same gene, and may, or may not, affect the same molecular phage-host interaction as the h-mutation(s); but it is very improbable that s3 would affect the population dynamics and the future evolution of the community in exactly the same way as h.   The density to which each mutant will grow depends on the abundance of its resources and its efficiency of utilisation of these resources.   Selectively different mutants reach, therefore, different densities and apply quantitatively and qualitatively different selective pressures on their biotic environment.   Each "present" evolutionary event in the phage, together with the evolutionary "past" of the bacteria, determines (i) the "present" evolutionary response of the bacteria and (ii) the range of "future" potential responses of the phage.   The "present" evolutionary response of the bacteria determines the fitness and the "future" density-distribution of the "presently" existing phage genotypes.   The range of "presently" available genotypes changes successively with each evolutionary event.

Prediction of future densities necessitates, therefore, a far larger amount of information than ecologists ever had; namely, it necessitates knowledge of the entire "present" biology of the community.   If the biological sciences could ever develop to such a degree that the study of the "present" could be completed before the "present" became "remote past" such predictions would be feasible.   Until then biologists may only provide untestable explanations of "past" population fluctuations,

or micro- and macro-evolutionary phenomena.   However, the above
coevolutionary view of population dynamics can be tested indirectly;
it is developed in detail through the general discussion of the phage-
host system's biology that follows.

COEVOLUTION OF

RESISTANCE AND VIRULENCE

5. *C O E V O L U T I O N   O F   R E S I S T A N C E   A N D   V I R U L E N C E*

The chemostat populations studied here confirm the predictions of the theoretical models of coevolution by Levin and Udovic (1977) and Wilcox and Walters MacCluer (1979; see page 3). The development of phage-resistance in bacteria and of the small-plaque character in phage are clear examples of evolution which causes changes in the population's densities. On the other hand, the clearest example of evolution having no effects on the total density of the population is the change in the frequency of phage-resistant subpopulations in phage-free chemostats (section 4.2.2). The results in chapters 2 and 3 showed that multiple evolutionary events have affected the survival and reproduction parameters of both, the bacterial and the viral populations. In view of these results, pure ecological modelling of the mechanism of phage-host coexistence becomes inadequate. The coevolutionary model that follows uses the quantitative concepts of λ-resistance and λ-virulence, as developed in chapters 2 and 3, to explain both the stability of the phage-host system and the randomness of the population dynamics.

The notion of semiresistance makes it unnecessary to assume that sensitive bacteria have a competitive advantage over resistant ones, in order to explain their persistence in the presence of phage. The constant production of sensitive cells insures the persistence of the phage whereas the low susceptibility of the host clone prevents its extinction at any phage density. To use Pimentel's (1968) expression, the phage multiplies at the expense of an "interest" and not by

consuming the "capital". The evolution of semiresistance is thus a sufficient condition for indefinite coexistence of bacteria with phage and it is expected to stabilise the densities of both populations; however, any significant deviation from such stability is proposed here to be an effect of coevolution of resistance and virulence.

For the purposes of modelling this coevolution *resistance* of a bacterial genotype A to a phage genotype a will be defined as the probability of an A-cell surviving a collison with an a-particle. The resistance of a semiresistant genotype is between zero and the unity. Similarly, virulence of a phage genotype a on a bacterial genotype A is the number of viable phage genomes produced per collision. Virulence is the product of two parameters: the *host range*, defined as the probability of an a-A collision resulting in at least one viable phage genome, and the actual productivity of a-A collisons, i.e. the number of viable phage genomes produced per productive collision.

If a highly virulent phage a is introduced into a culture of its host A then the ecological vacuum created by the lysis of A-cells is occupied by first order resistant and semi-resistant clones, B_i, deriving from A by mutation. Among these clones there may be: (i) clones to which a cannot adsorb (*surface resistance*); (ii) clones to which a adsorbs but fails to inject its chromosome through the cell membrane (*membrane resistance*); (iii) *a-immune* clones into which a injects its DNA but fails to kill the host cell (a-immune cells continue to divide after having been injected with a-DNA; *intracellular resistance*).

Collisions of a with a-immune cells may be, and collisions with B_i cells of the second type certainly are, lethal for the phage. This kind of active, differential removal of phage from the system is an important selective pressure, though it has been neglected in previous studies. The case of a-immune clones deserves more attention. There are four types of immunity, each with a different ecological significance. (i) The cell may digest the injected a-DNA and utilize its nucleotides for synthesising its own chromosome. The extent of the selective advantage of utilizing a-DNA depends, of course, on the density of a. It can be large when many a-A contacts occur per bacterial generation but diminishes as A grows and destroys a. (ii) The injected a-DNA may simply remain in a non-digestible, non-replicatable form and be diluted out by cell division. (iii) The a-DNA may perpetuate itself intracellularly in the form of a prophage or a plasmid, and perform some function of selective value for the host or (iv) just consume nutrients which could otherwise be used for cell synthesis. This latter type of immunity is rather unimportant since it is disadvantageous for both organisms under any circumstances.

Fundamental to the coevolutionary model that is being developed here is the rule that each one of the above a-resistant and a-hostile bacterial clones is susceptible, to one degree or another, to one or more potential derivatives of a (b_i) which are by definition more virulent than a on this particular B_i host. This rule finds a great deal of support in phage genetics (for examples see section 2.1.1). Exceptions to this rule, like for example the case of bacteria which do not produce appropriate receptors and are therefore susceptible to no a-mutant, should be seen as cases in which the required genetic rearrangements are *improbable within one generation* although not

impossible. The suggestion of Wandersman and Schwartz (1978) should be recalled for the above example, that phages may recognize secondary receptors and may specialize on them should their primary receptor become rare.

The fitness of each first order, a-resistant bacterial clone, B_i, depends primarily on its competitiveness and its resistance to a, which is the only abundant phage at the time when the B_i occur. These criteria will determine the frequency-distribution of the B_i clones which, in turn, will determine the fitness of the first order virulent a-mutants, b_i. As the density of fit b_i clones increases, the density of their hosts decreases. The ecological vacuum is occupied (i) by more resistant B_i clones (whose phages did not occur in the b_i generation, or were not fit) and (ii) by B_i-originated b_i-resistant clones (C_i).

The increase in density of a b_i clone corresponds, therefore, to an ecological, phenotypic decrease of its virulence (host range). Any b_i clones whose hosts do not emerge spontaneously from their numerical refuge decrease to extinction (section 4.3.1). The cause of the subsequent increase of sensitive bacteria, which will ensure persistence of the phage, is not ecological but evolutionary; it is the occurrence of second order virulent mutants, c_i, which arise from each dense b_i and which will redistribute the frequencies of B_i, C_i and D_i potential hosts and will so determine the fitness of their third order, d_i, mutant progeny.

Any increase in density of the phage selects for resistance in bacteria and the consequent increase in frequency of resistant bacteria selects for virulent mutants which undertake the next round. Thus, both virulence and resistance increase in a spiral of coevolutionary loops

without a measurable, predictable or describable end.   The interacting species remain semiresistant and moderately virulent (which is a sufficient condition for indefinite coexistence) though without ever stabilizing their genotypic distributions.

This idea is not entirely new.   Horne (1970a) discussed a similar model which he called successive cycles of bacterial resistance and phage host-range mutations.   Based, however, on the narrow concept of resistance, meaning simply surface resistance, and on the narrow concept of host-range mutation, meaning a mutation of the phage's adsorption site, he had difficulty in seeing how these cycles could occur indefinitely.   He also had the idea that the persistence of sensitive bacteria, required for the persistence of phage, was due to mutation rather than some arbitrary ecological advantage of sensitivity.   But again his problem was to match the successive evolution of resistance with "back mutation" to sensitivity which would seem too improbable.   Because of these theoretical gaps he was forced to reject the idea of continuous, indefinite coevolution in favour of an ultimate, stable solution of lysogeny. Today's knowledge of phage-host genetics, however, not only allows models of continuous coevolution to be suggested, but also raised the opposite problem as to whether ecological regulation is possible at all.

One of the implications of this coevolutionary model is that for no clone is the chemostat environment a stable one.   Although adaptive evolution may occur, this is not sufficient to stabilise the gene frequencies of the interacting populations.   The above model is consistant with Levin's and Udovic's (1977) prediction that coevolution may create the conditions for stable coexistence (as, for example, the evolution of semiresistance) but may also destabilise already existing equilibria.

According to this model, statistically significant population fluctuations are merely "explosion-crash" episodes produced by "opportunistic" mutants. Most of the time the mutation which caused a population peak becomes fixed and can be traced among the dominant genotypes of subsequent generations. However, other genes comprising its genetic background may continue to evolve. Sometimes, as in the case of the small-plaque forming phage, these mutations have an obvious pheotypic effect but most of the time they will go undetected. Obvious phenotypic effects may appear after several such mutations have been accumulated. The small-plaque phenotype, for example, may have been the result of the accumulation of the mutations which had caused previous density oscillations in the phage population.

In general, the amplitude and frequency of population fluctuations are genuinely random and unpredictable just as the process of coevolution. The reason that oscillations become less intensive with time is clear. The extraordinary stability of the bacteria is to be explained by the fact that resistant bacteria of whatever genotype are limited by the available abiotic resources; these, within the sensitivity of the methods used, remain constant. Slight increases in bacterial density, if not due to technical error, may be caused by the evolution of bacteria preying on phage, the evolution of bacteria with higher efficiency of resource utilization or with smaller cell size or, in general, to selective pressures which are not directly related to phage predation. Decreases in bacterial titre should principally be due to the evolution of specialist phages which prey upon previously resistant, and dominant, bacterial clones.

The phage density is determined primarily by the density of its hosts and secondarily by its productivity on them. The evolution of

virulence and semiresistance is the main cause of stability in phage numbers. A small enhancement in host-range may take generations before influencing the total density, if at all; meanwhile the parent phage may persist, stabilised by the semiresistance of its hosts, until competition between parent and mutant begins. Should our techniques become efficient enough to detect changes at low density levels (as, for example, in the case of λh and the original, large-plaque forming phage in culture 3) many more evolutionary rearrangements of the genotypic distributions of the populations would be detected.

A more feasible task is to test whether phages and hosts sampled during phage-populational peaks do differ, over periods of time in the same chemostat and at the same time in replicate cultures, or are identical, as the theories of ecological regulation would predict. It should be pointed out that the numerical stability observed in culture 1 is for some unknown reason exceptional, as also are its overall population dynamics. Normally phage fluctuations are quite clearly defined. A reference can be made for example to Horne's (1970a; b) cultures where the phages continued to fluctuate irregularly for more than two years.

In an environment where coevolution occurs in such a random manner survival depends on the adaptive flexibility of the populations. Mutations which increase mutability (mutators) should be preferred by selection. The selective advantage of mutators is not that they provide greater diversity *per se*. Mutators are fixed either because their effect on functions within their immediate influence is of selective value (as it seems to be here in the case of, possibly, the *recA*; see sections 3.3.7 and 4.33) or because they are carried over in

the genetic background of the mutations they produce (Cox and Gibson, 1974).

The above comment applies for the selective value of extrachromosomal genetic elements (ECE). There is a tendency to consider lysogeny as a fitter state than "virulence" (see for another example, Echols, 1972). This is in fact paradoxical. Since the discovery of lysogeny, the theory that phages originate from pieces of bacterial chromosome which evolved some independence has generally been accepted. If it is so, "virulence" is logically the most recent step of phage evolution and for this it must have, or have had, some selective advantage over lysogeny.

The selective value of each form in which a phage (or ECE) may exist is primarily determined by its effects on survival and reproduction of its host in particular environmental circumstances. None of the known λ-genes can be imagined at the moment to confer a growth advantage to lysogens (whether typical or defective) or to plasmid carriers. In fact, in the prophage state all genes, except *cI* and *rex*, are known to be totally repressed. This is also the case, during most of the cell-cycle, for plasmids which are demanding in energy and nutrients. Even if the intracellular phage conferred some growth advantage on its host, λ-lysogens and λ-plasmid carriers would be at risk of extinction. Since they are vulnerable to superinfecting "virulent" and/or "supervirulent" mutant phages (see section 3.1.4). To ensure survival, lysogens and plasmid carriers should mutate to forbid adsorption or penetration of cooccurring, extracellular phage.

It is suggested that all the developmental pathways shown in *figure 3.19* (and perhaps many more, yet unknown) are carried out in the chemostat by mutants of the dominant virulent phage. This means that the phage

tries out all its chances for survival, but, at least for as long as the cultures were run, the more virulent forms were always fitter. Successful transducents, defective lysogens and plasmid carriers may occasionally increase in density, cause evolution of the virulent form and the rest of the community and disappear. Neutral or detrimental prophages and plasmids are restricted to their mutation frequencies and await the abrupt evolutionary moment when the virulent forms may no longer be present and active extracellularly. Then some successful transduction may occur in circumstances where no alternative mutational solution is provided by the dense resistant bacterial clones.

This is perhaps the advantage of sensitivity that Campbell (1961) had in mind and not a higher growth rate of the transducent *per se*. In such cases the phage succeeds in obtaining the maximum possible perpetuation of its genes though, when the conditions allow, maximum perpetuation of phage genes is achieved by continuously increasing virulence. A consequence of this is that temperate phage carried by a lysogen should also increase its virulence in chemostats like those used here (though not necessarily becoming *λvir*).

How much of a generalisation can be made from the above discussion? Concerning the specific explanations given for *E. coli* and *λvir*, none. Each species has its unique solutions to problems of existence. The theory suggests that similarities will occur less frequently than differences, in both the population dynamics and the underlying evolutionary events, even when these are compared between identical replicate cultures, grown at the same time, from the same inoculum and with the same machinery. Thus, neither in space nor in time can one predict the genotype and its abundance. Is it, however, true that all, or most, abundance and distribution phenomena are merely

effects and causes of unseen, irreversible micro-coevolution?
This would be interesting and possible to investigate.   The chemostat
seems to be a very promising tool.

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APPENDIX

PROBIT ANALYSIS

Theory

Assuming that the burst-times (or the latent periods) of individual phage-infected bacteria are normally distributed around their mean and that the burst-size does not depend on the length of latent period, the sigmoid one-step growth curve represents the integral of a normal curve and as such it can be transformed into a straight line using the probit-transformation (Adams and Wassermann,1956). The numbers of phage particles released at each time-interval from infection are expressed as percentages of the final phage yield (average burst-size) which are then replaced by their corresponding normal equivalent deviates (NED) or probits.

The sigmoid curves obtained when the number of phage plaques formed on plates are plotted against the incubation time can be transformed into probit-lines in the same manner, assuming that the times at which individual plaques appear during incubation are normally distributed around their mean. In this case the number of plaques formed at each incubation-time interval are expressed as percentages of the total plaque forming units tested.

The NED of a probability value, P, is defined as the abscissa corresponding to probability P in a normal distribution with zero mean and standard deviation the unity. Probits are NEDs increased by 5 to avoid negative values. The probits can be found in Finney's (1971) table I.

The probits or NEDs (y) are related to the dose (x) or to the dose's logarithm (natural or 10-based) by means of the linear regression equation:

1 : $y = \alpha + \beta x$

The parameters α and β are related to the distribution's mean (μ) and
standard deviation (σ) by the equations:

2:    $\mu = -\alpha/\beta$

3:    $\sigma = 1/\beta$

The goodness of fit (χ^2) of a straight line to probit-transformed data
provides a test for the hypothesis of normality of the distributions
of latent periods, in a one-step growth experiment, and of plaque
formation times, on plates.

When no systematic departure from linearity (normality) is traced,
one can estimate the median effective dose (M), which here represents
the time needed for 50% of the total plaque forming units to form
plaques, or for 50% of the final phage yield to be released in a one-
step growth experiment (mean latent period), and its standard error.

The "Maximum Likelihood" (ML) Method

The method described by Finney (1971) was employed to fit NED-
regression lines with confidence zones to the one-step growth and
plaque-formation data, to test the homogeneity of the phage populations
in these respects and to estimate the mean latent periods and mean
plaque-formation times of the phages on their hosts. Provisional lines
were drawn by the method of the "least-squares" and ML-calculations were
continued until no calculated Y (see below) differed from its
corresponding expected one (from the provisional line) by more
than 0.01.   Between one and three cycles of ML-calculations
were needed for such agreement.   In the following n represents
the final counts of plaque-forming units, in plaque-formation

measurements, or the average burst-size, in one-step growth experiments.

A special program was devised for a *TI*-programable 59* desk calculator which carries out all ML calculations and takes all decisions from a minimum input of data.   The method used in the program is as follows:

A.   *Data transformation.*   For each entered x_i (dose), y_i (probit of the observed percentage of response) and n (total number of individuals tested) the following values are calculated:

4:    Y_i(NED) $= y_i$ (Probit) $- 5$

5:    $P_i = \dfrac{1}{\sqrt{2\pi}} \displaystyle\int_{\infty}^{-y_i} \exp\{-\tfrac{1}{2}u^2\}du$

P_i is the observed frequency of response (r_i/n)

B.   *The maximum-likelihood calculations.*   A provisional line is calculated with the "least-squares" method and the following are calculated for each, first order expected NED (Y_i):

    P_i (the expected frequency of response) from equation 5

6:    $Z_i = \dfrac{1}{\sqrt{2\pi}} \exp\{-\tfrac{1}{2}Y_i^2\}$

7:    $Q_i = 1 - P_i$

8:    $w_i = Z_i^2 / P_i Q_i$

w_i is the weighting coefficient for each expected Y_i

9:    $y_i = Y_i + \dfrac{P_i - P_i}{Z_i}$

    y_i is the working probit for each expected Y_i

Then the following sums are calculated:

10: $\quad \Sigma nw_i, \quad \Sigma nw_i y_i, \quad \Sigma nw_i y_i^2,$

$\quad \Sigma nw_i x_i, \quad \Sigma nw_i x_i^2, \quad \Sigma nw_i x_i y_i.$

and from these:

11: $\quad \bar{x} = \dfrac{\Sigma nw_i x_i}{\Sigma nw_i}$

12: $\quad \bar{y} = \dfrac{\Sigma nw_i y_i}{\Sigma nw_i}$

13: $\quad \Sigma xx = \Sigma nw_i x_i^2 - \dfrac{(\Sigma nw_i x_i)^2}{\Sigma nw_i}$

14: $\quad \Sigma yy = \Sigma nw_i y_i^2 - \dfrac{(\Sigma nw_i y_i)^2}{\Sigma nw_i}$

15: $\quad \Sigma xy = \Sigma nw_i x_i y_i - \dfrac{(\Sigma nw_i y_i)(\Sigma nw_i x_i)}{\Sigma nw_i}$

16: $\quad b = \dfrac{\Sigma xy}{\Sigma xx}$

17: $\quad a = \bar{y} - b\bar{x}$

A new first order NED, Y_i', is calculated for each x_i-dose from the slope (b) and intercept (a) of equations 16 and 17 as:

18: $\quad Y_i' = \bar{y} - b\bar{x} + bx_i$

Each Y_i' is compared with the first order, expected Y_i of the same corresponding x_i; when no difference equal or greated than 0.01 is found the calculations proceed to a χ^2-test of the discrepancy between the observed values of Y_i and the ones predicted by equation 18. However, when the difference between one, or more, pair(s) of $Y_i - Y_i'$ values is 0.01 or greater the calculations in part B are

repeated with Y_i' being used as a provisional second order Y_i.

C.   *The "goodness of fit" test.*   The value of χ^2 is calculated as

19:    $\chi^2 = \Sigma \dfrac{n(P_i - P_i)^2}{P_i(1-P_i)}$

and the degrees of freedom as $k-2$ where k is the number of doses. When the probability corresponding to the χ^2 value from equation 19 is lower than 0.05 a second value of χ^2 is obtained with all P_i lying in the intervals between 0 to 0.1 and 0.9 to 1.0 grouped:

20:    $\chi^2 = \Sigma \dfrac{n(P_i - P_i)^2}{P_i(1-P_i)} + \dfrac{n(\Sigma P_i - \Sigma P_i)^2}{\Sigma P_i(1-\Sigma P_i)} + \dfrac{n(\Sigma P_i - \Sigma P_i)^2}{\Sigma P_i(1-\Sigma P_i)}$

$0.1 < P_i < 0.9$ $P_i < 0.1$ $P_i > 0.9$

This is because the NED-dose relation itself departs from linearity at these extreme intervals and it may be the reason for the high value of χ^2 from equation 19.   The degrees of χ^2 from equation 20 are accordingly reduced.   If heterogeneity is found by equation 19 the heterogeneity factor:

21:    $h = \chi^2/(k-2)$

is calculated with the χ^2-value from equation 19 and the original degrees of freedom. h is used for estimating the variances below. When the value of χ^2 is still lower than 0.05 after grouping the P_is in equation 20, the data are considered genuinely heterogeneous.

D.   *The distribution's mean and cnfidence zone.*   The t-value taken from table VIII in Finney (1971) for the required probability-level and with infinite degrees of freedom, or with $k-2$ degrees of

freedom when heterogeneity is traced, is entered at this point to calculate the following.

The confidence limits (CL) of the slope (b) of the NED-regression line, corresponding to the distribution's standard deviation, as:

$$22: \quad \text{CL}(b) = \overset{+}{-} t \sqrt{h / [\Sigma n w_i x_i^2 - \frac{(\Sigma n w_i x_i)^2}{\Sigma n w_i}]}$$

The confidence limits of the expected Y_i for each dose x_i as:

$$23: \quad \text{CL}(Y_i) = \overset{+}{-} t \sqrt{h [\frac{1}{\Sigma n w_i} \quad \frac{(x_i - \bar{x})^2}{\Sigma xx}]}$$

The median effective dose M with its confidence limits as:

$$24: \quad M = m + \frac{g}{(1-g)} (m - \bar{x}) \overset{+}{-} \frac{t}{b(1-g)} \sqrt{h [\frac{(1-g)}{\Sigma n w_i} + \frac{(m-\bar{x})^2}{\Sigma xx}]}$$

where:

$$25: \quad m = \bar{x} - \bar{y}/b$$

$$26: \quad g = t^2 h / b^2 \Sigma xx$$

In equations 22-24 and 26 h takes the value 1 when no heterogeneity is found.

In the tables of the results of this analysis, given in the legends of the relevant figures, the h-values are given only when the heterogeneity was genuine; the "degrees of freedom" are $k-2$ where k is either the original number of doses or the reduced number of doses, when the χ^2 was calculated from equation 20.