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PHYSICAL-CHEMICAL ASPECTS OF VIRUS-CELL INTERACTIONS

by

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This Thesis for the Ph. D. degree, by

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Physical-Chemical Aspects of Virus-Cell Interactions

Thesis directed by Professor T. T. Puck

Experiments with E. Coli B. and its mutants B/1 and B/1,5 showed that the cells of each mutant strain will agglutinate and precipitate in certain definite ranges of concentration of H +, Na + and Ca $^{++}$. This precipitation is reversible, and is apparently due to the neutralization of surface charge by the cations in a manner comparable to the flocculation of colloidal particles.

The regions of cation concentration within which B/1 and B/1,5 precipitated were identical within experimental error; the concentration needed to precipitate these mutants is markedly less than that required to precipitate the wild-type B. This differentiation is the first one other than their difference in virus resistance to have been made.

The metallic cation molar concentration needed to pre-cipitate B and B/1,5 is given by the following tabulation:

	Na $^+$ conc.	Ca $^{++}$ conc.
B	0.6	0.06
B/1,5	0.25	0.01

The ratio of $\underline{/Na^+/}$ to $\underline{/Ca^{++}/}$ required to precipitate B is thus seen to be 1:0.1, while for B/1,5 this ratio is 1:0.04. Although the generally lower level of cation concentration

needed for B/1,5 precipitation may indicate that B/1,5 has a lower surface charge than B, the difference in their Na^+ : Ca^{++} ratios may also indicate a difference in the ion-binding properties of the surfaces of these mutant forms. The specific nature of this difference has not been identified.

In equilibrating T1 with B/1, to which it attaches in a completely reversible manner, it was found that the equilibrium constant was a function of cell concentration, which could be empirically approximated as $K = 1.9 \times 10^{-4} \times (B/1)^{-\frac{1}{2}}$. This same dependence of K on cell concentration was found to hold between T1 and wild-type cells in which the second, irreversible, step had been inhibited by exposure to ultra-violet radiation.

In an effort to determine the cause of this variation of K with cell concentration, the following factors were investigated, and all found to be incapable of providing a satisfactory explanation:

(1) The effect of exudation of cell metabolic products into the medium.

(2) Distortion of experimental data as a result of a shifting of the equilibrium between phage and cells as the cell concentration is reduced in the process of centrifuging.

(3) The effect of salt concentration. A reduction of
the concentration of $MgCl_2$ in the adsorption medium
from 10^{-3} to 3.3×10^{-4} molar caused an increase in K
at all cell concentrations, presumably by increasing
the rate of attachment. The value of K, however,
remained proportional to $(B/1)^{-\frac{1}{2}}$.

(4) The masking of accessible cell surface by the
clumping of the bacteria cells at high concentrations.
Although it is possible that this consideration may
have some slight contributing effect, it may be
ruled out as a major factor.

(5) The mechanical effect of cell-cell collisions in
enhancing the rate of elution of phage from the cell
surface.

(6) Electrostatic interactions between cells.

(7) Phage-phage interactions.

A pattern of heterogeneity of the phage population
was postulated, and a distribution function proposed, de-
scribing a phage population composed of elements having
different equilibrium constants. This was found to explain
satisfactorily the observed variation in the overall equili-
brium constant as a function of cell concentration.

To confirm the actuality of such a heterogeneity, T1

phage was equilibrated with B/1, centrifuged, the supernatant

discarded, and the cells with their reversibly attached phage

allowed to equilibrate again in a resuspension in fresh medium.

This cycle was repeated several times, the measured equilibrium

constant increasing in each cycle, due to the preferential ad-

sorption of phage of high K.

The nature of the heterogeneity was not determined.

This abstract of about 570   words is approved as to form and
content. I recommend its publication.

Theodore T. Puck

Instructor in charge of dissertation

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PART I

INTRODUCTION

Work done in studying the mechanism of virus multiplication in host cells has been focused for the most part on the series of bacteriophages parasitic on Escherechia Coli B. The ability to make accurate titers of these phages makes this a particularly favorable system for detailed study.

The parent, wild-type cell of E. Coli B is host to seven distinct strains of bacteriophage, which have been identified by the designations T1, T2, . . .T7. The seven phage strains exhibit wide variations in size, morphology, and rapidity of reproduction, despite their possession of a common bacterial host.

The host bacterium undergoes infrequent spontaneous mutations to forms resistant to the reproduction of one or more of these virus parasites. These mutated strains are customarily designated as B/1, B/2, B/1,3,4,5,7, etc., the numerals serving to identify the phages to whose reproduction they are resistant. These bacterial strains reproduce their mutant characteristics through many generations, so that stocks which represent a wide range of stable and highly specific host behaviors can be easily and dependably maintained.

The use of certain of these mutants has been a most useful tool in investigating the pre-reproductive interactions between phage and bacteria. It has been demonstrated by Puck, Garen, and Cline (1, 2) that the initiation of a fertile invasion of a bacterium by a phage includes two distinct steps: an initial reversible attachment of the phage to its host, which appears to depend on the formation of ionic bonds between complementary patterns of electrostatic charges on the virus and on the bacterial surfaces; and a subsequent irreversible attachment apparently of an enzymic nature, which leads to actual penetration of the bacterium and, under proper conditions, reproduction of the bacteriophage.

The two-step nature of this preliminary interaction may be clearly demonstrated by the behavior of T1 phage toward three different forms of E. Coli B. (2) T1 attaches rapidly to the wild-type bacterium, then proceeds to invade and reproduce. With the mutant B/1, the initial attachment proceeds at the same rate as to the wild form. This attachment remains a completely reversible one, however, no phage going on to the next, irreversible, step; practically all of the attached phage can be eluted apparently unaltered from the bacterial surface by a simple dilution. With the mutant B/1,5, however, T1 does not form even the first reversible attachment to any measurable degree.

Thus, two specific functional abilities of the bacterial

surface are demonstrated, which exhibit, in the various mutant strains of E. Coli B, radically different behaviors in their reaction to the invasion of various members of the T-series of bacteriophage. Bacterial strains differing in these surface properties may still exhibit identical growth, nutritional requirements, staining, and morphological characteristics. It accordingly appears that these mutations bear no obvious relation to commonly studied metabolic functions.

The proposal has been offered that such mutations to virus resistance involve changes in electrostatic configurations on the surface of the bacterial cell (3,4,5). The bond which must form between the phage and its host as the first step in the complicated series of reactions which leads ultimately to the synthesis of more virus material, has been shown to involve the interaction of strong electrostatic forces like those of ionic bonds (1,6). It has been shown further (7) that in the case of the bacterial virus T2 and its host, the bond forms only when ionized carboxyl groups on the surface of the bacterial cell are intact, and the pH dependence of the attachment reaction of both T1 and T2 phages to their host suggests that ionized carboxyl groups react with amino groups when union takes place.

Since cell mutants resistant to specific viruses may still bind others at the same rate as the wild type (2), the

surface changes arising from mutation must presumably be at specific sites. The new configuration, which is not complementary to that of the virus which can no longer attach, retains enough of its original pattern so that other viruses can still form bonds with the cell. Because these surface changes are electrostatic in nature, other reactions which depend on changed surface groups might be affected. In agreement with this prediction, it has been possible to demonstrate differences in properties dependent on the surface changes of bacterial mutants which exhibit various specificities to the T-viruses.

Most bacteria are strongly negatively charged at pH 7 (8) and hence do not spontaneously agglutinate, because the repulsive forces arising from the similarity of their surface charges prevent a close enough approach for short-range binding forces to become effective. As the electrolytic strength of the medium is increased, this excess charge is progressively neutralized by cation binding and/or shielding by double-layer formation until the forces keeping the cells apart are diminished to the point at which the cells can approach sufficiently close to establish binding forces between their surfaces. Hence the effect of inorganic cations and pH on the cell agglutination of various virus-resistant B mutants was studied in the hope of finding differences which might reflect the surface differences responsible for virus specificity. This study is described in

Part II.

Intimately associated with the surface characteristics of both bacteria and phage are the interactions which take place between them prior to the penetration of the phage to that point where deeper structures of the host cell are involved in an irreversible manner. This presumably surface-controlled interaction includes the initial, reversible, step in phage attachment, and has been the subject of extensive inquiry in this laboratory. Study of this primary step in the attachment of the bacteriophage to its host may be facilitated by employment of the mutant B/1, to which the phage attaches reversibly at the same rate at which it does to the wild-type B, but with which no irreversible step can follow (2). Thus the isolated first step can be separately studied without the more complex subsequent interactions which take place with the wild-type bacteria. One major investigative method lies in measurement of rates of interaction as a function of different aspects of environmental conditions.

With the T1-B/1 system a very simple kinetic scheme of interaction might be anticipated:

$$V + B \; \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \; VB \qquad \qquad \boxed{1}$$
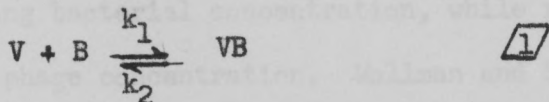
where the virus and bacterium are represented by V and B respectively, and VB stands for the reversibly bound virus-bacterium complex. From this relationship one could predict the

existence of a true equilibrium constant, K, such that

$$K = \frac{k_1}{k_2} = \frac{(VB)}{(V)(B)} = \frac{(V_o)-(V)}{(V)(B)} \text{ , where } [2]$$

(V)= concentration of free, unbound virus
(B)= concentration of bacteria
(VB)= concentration of reversibly bound virus
(V_o)= concentration of total virus

K, in this kinetic scheme, would be expected to be unchanged in value throughout a wide range of concentrations of both phage and bacteria.

This simple thermodynamic equilibrium has been assumed in the computation of standard free energy, heat of reaction, and entropy involved in this phage-bacterium interaction (6). In this analysis it was demonstrated that the steady state attained in the mixture of T1 and B/1 was a true equilibrium in which the same final value was attained, regardless of from which direction equilibrium was approached. In this work, however, it was noted that the equilibrium constant, K, depended to some extent on the bacterial concentration; it has also been qualitatively noted in this laboratory (1), that the measured value of k_1 decreases with increasing bacterial concentration, while remaining independent of the phage concentration. Wollman and Stent (9,10) also have recorded that the attachment rate of a certain T4 strain to E. Coli B, while varying linearly with bacterial concentration at low concentrations, becomes apparently independent of it at higher concentrations.

These variations of K and k_1 in response to variations in
(B) serve to make questionable the validity of the simple scheme
of kinetic interaction diagrammed above, which assumes k_1 to be a
true constant, and hence implies the independence of K and (B).
Apparently some modification of this model is required, and without
a more trustworthy picture of the mechanism involved, any inter-
pretation of data obtained from the kinetics of phage-bacteria
interactions must be regarded as dubious.

It was thus felt that a study of K as a function of (B)
in the T1-B/1 system should be rewarding in shedding further
light on the elements involved in this particular reaction between
virus and bacterial surfaces. This study is described in Part III.

this range the density of the cell suspension had no measurable
effect on its agglutination by either H⁺ or metallic cations.
A single experiment utilized a combination of 40 individual
tubes in order to cover adequately the effect of cation concen-
tration at various pH's. It was found that in certain ranges of
cation concentration we may obtain reproducible...

PART II

ELECTROSTATIC ASPECTS OF CELL SURFACES OF E. COLI B MUTANTS

A. Experimental Procedures

All bacteria used in this work were harvested in their
exponential growth phase. Overnight (approximately 16-hour)
cultures in 0.8% Difco Nutrient Broth + $\frac{1}{2}$% NaCl were grown at
37° with aeration, inoculations being made from laboratory stock
slants. Inoculations of $\frac{1}{2}$% by volume of this culture were trans-
ferred to culture flasks containing 0.8% Difco Nutrient Broth +
$\frac{1}{2}$% NaCl, and the cells grown for about $2\frac{1}{2}$ hours at 37° with
aeration. These cultures were then centrifuged, washed as
described in detailed experimental procedures, and resuspended
in the appropriate medium in sufficient quantity to give the
desired final titer.

Such cultures of various B mutants were washed in dis-
tilled water and resuspended at a concentration of approximately
10^9/cc in sets of culture tubes containing a range of concen-
trations of calcium chloride or of sodium chloride, buffered at
different pH values. Cell density in these settling tubes, since
it is not critical, was based on an estimate of the bacterial
titer at the time of centrifugation of the culture. Checks made
at various concentrations from 10^8 to 10^{10}/cc indicated that in
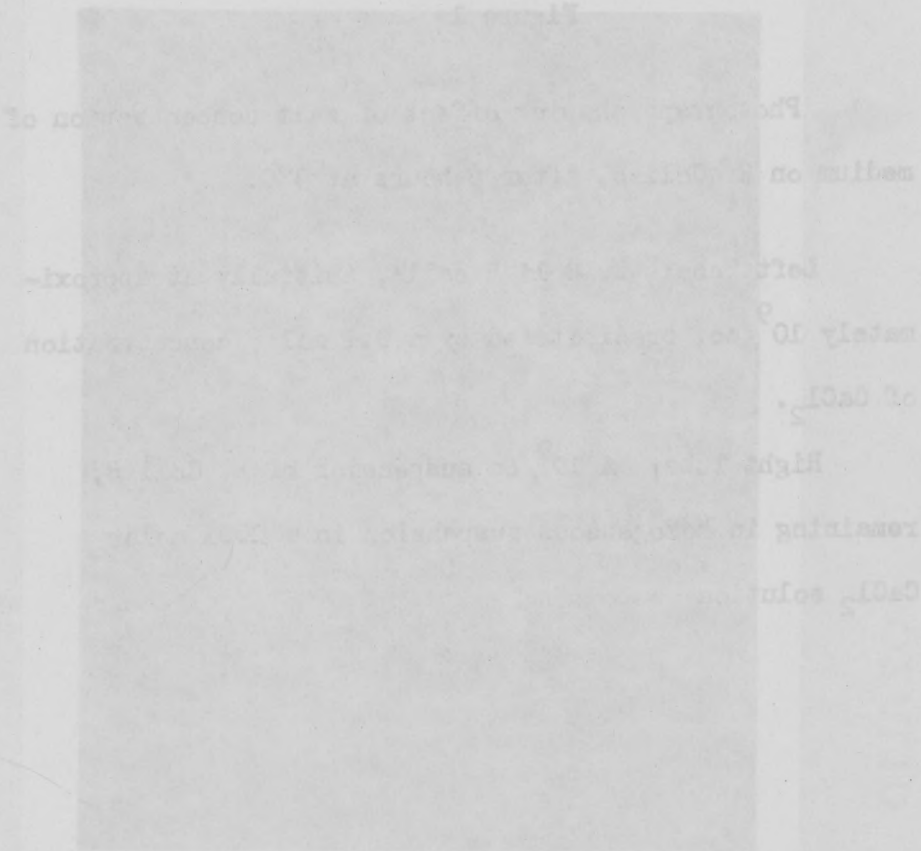
LEFT: Culture tube containing E. Coli B cells
   precipitated by CaCl2.

RIGHT: Tube containing a suspension of
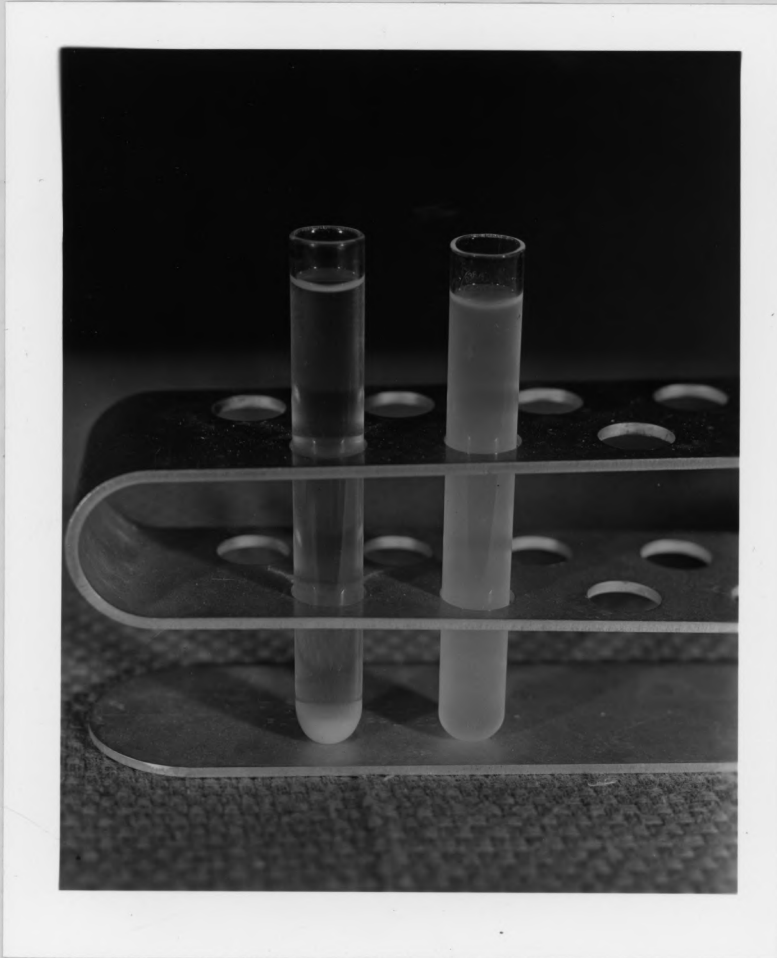   E. Coli B cells in a ... concentration of CaCl2

Figure 1

Photograph showing effect of salt concentration of medium on E. Coli B, after 6 hours at $3^{\circ}C$.

Left Tube: E. Coli B cells, initially at approximately 10^9/cc, precipitated by a 0.1 molar concentration of $CaCl_2$.

Right Tube: A 10^9/cc suspension of E. Coli B, remaining in homogeneous suspension in a 0.01 molar $CaCl_2$ solution.

Figure 1.

LEFT: Culture tube containing E. Coli B cells
 precipitated by CaCl₂.

RIGHT: Tube containing a suspension of
 E. Coli B cells in a sub-agglutinating
 concentration of CaCl₂.

suspension.

Figure 2 shows the network of points examined with E. Coli B in media containing various concentrations of Ca^{++} and H^+, with a contour line drawn to separate the regions of stability and agglutination. This represents conditions at the end of 15 hours' settling. The contour line was arbitrarily drawn at an optical density equal to 50% of that of a group of unagglutinated control tubes containing an equal cell density, with no Ca^{++}, 0.01M Na^+ (in the buffer), and at pH's in the neutral range from pH6 to pH8.

Microscopic examinations were made of cells from tubes on each side of this sedimentation contour. In every case, cells from the stable suspension showed no evidence of clumping, while cells from agglutinated tubes without exception showed conspicuous aggregation into clumps, some of massive size. The clumps were roughly spherical, with no indication of preferential orientation of the bacterial cells along any particular axis. The fact of this three-dimensional polymerization of the bacteria into clumps suggests that structures which may enter into this cell-cell binding are distributed over a large part of the surface of the cell.

It can be seen from Figure 2 that precipitation of E. Coli B occurs whenever the pH falls below a value in the neighborhood of 5, regardless of the cation concentration. At higher pH's
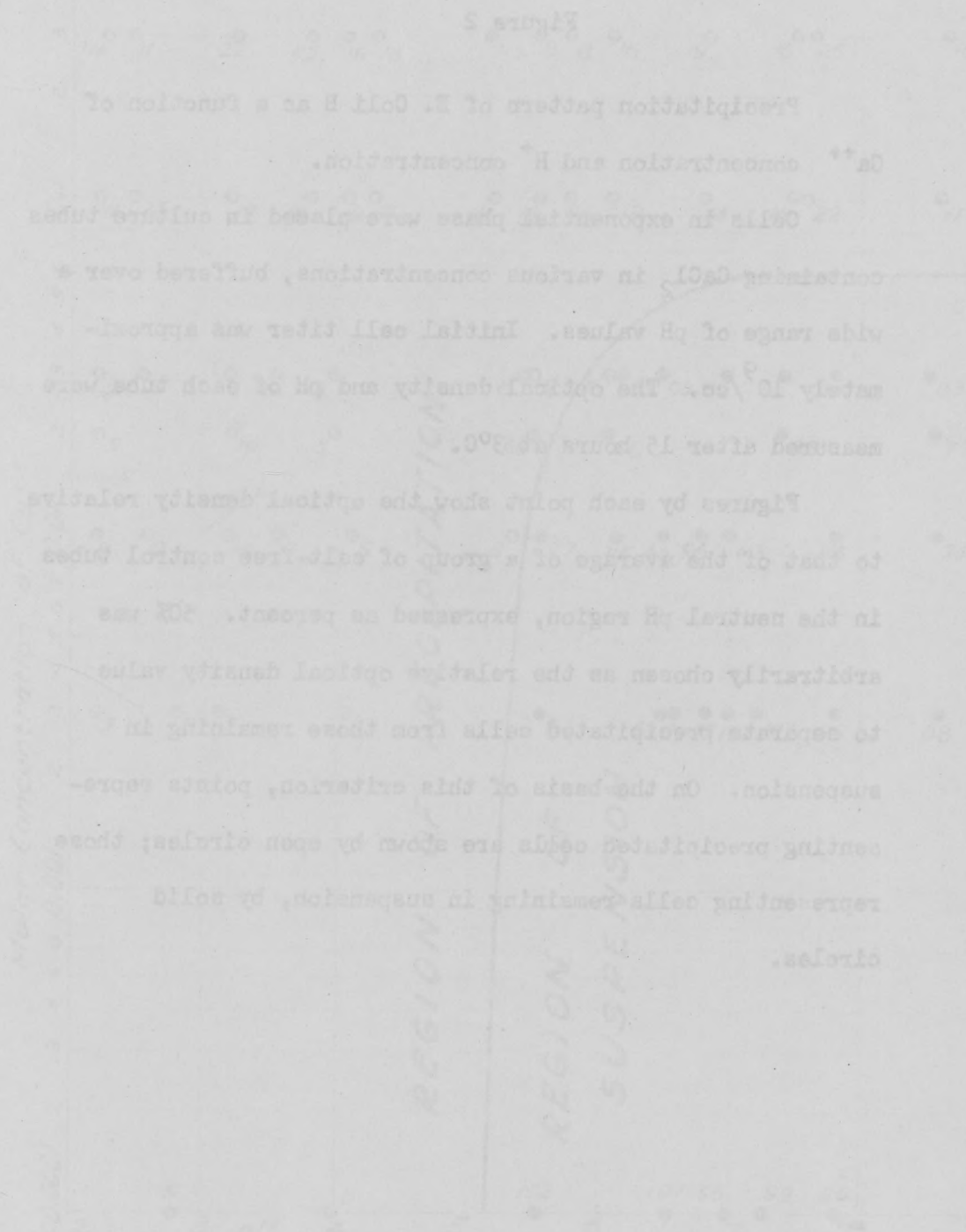
Figure 2

Precipitation pattern of E. Coli B as a function of
Ca^{++} concentration and H^{+} concentration.

Cells in exponential phase were placed in culture tubes
containing $CaCl_2$ in various concentrations, buffered over a
wide range of pH values. Initial cell titer was approxi-
mately 10^9/cc. The optical density and pH of each tube were
measured after 15 hours at $3^{o}C$.

Figures by each point show the optical density relative
to that of the average of a group of salt-free control tubes
in the neutral pH region, expressed as percent. 50% was
arbitrarily chosen as the relative optical density value
to separate precipitated cells from those remaining in
suspension. On the basis of this criterion, points repre-
senting precipitated cells are shown by open circles; those
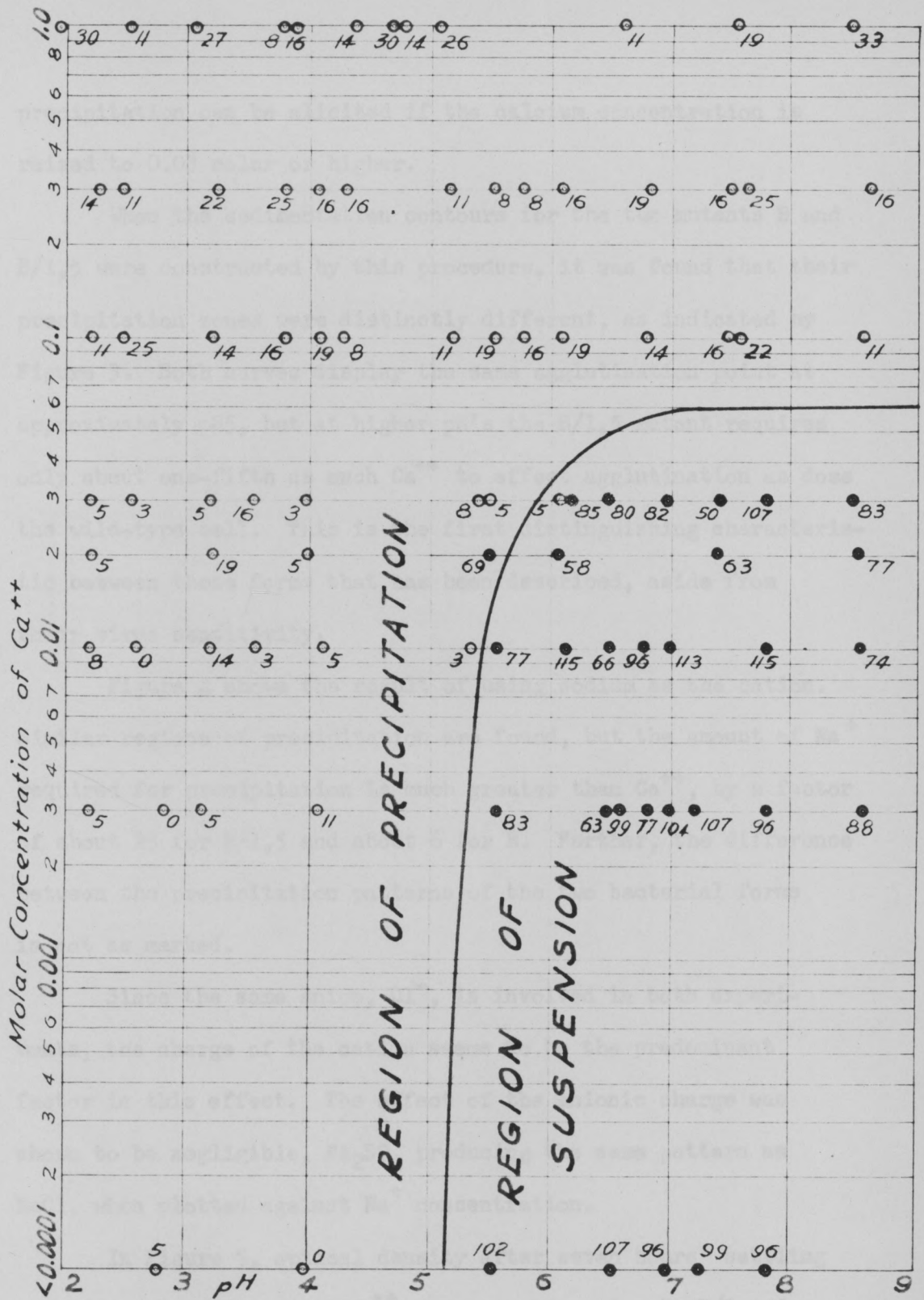representing cells remaining in suspension, by solid
circles.

FIGURE 2: Precipitation Pattern of E. Coli B in Media of Various Ca⁺⁺ concentrations and pH's.

precipitation can be elicited if the calcium concentration is raised to 0.03 molar or higher.

When the sedimentation contours for the two mutants B and B/1,5 were constructed by this procedure, it was found that their precipitation zones were distinctly different, as indicated by Figure 3. Both curves display the same agglutination point at approximately pH5, but at higher pH's the B/1,5 mutant requires only about one-fifth as much Ca^{++} to effect agglutination as does the wild-type cell. This is the first distinguishing characteristic between these forms that has been described, aside from their virus sensitivity.

Figure 4 shows the result of using sodium as the cation. Similar regions of precipitation are found, but the amount of Na^{+} required for precipitation is much greater than Ca^{++}, by a factor of about 25 for B/1,5 and about 6 for B. Further, the difference between the precipitation patterns of the two bacterial forms is not as marked.

Since the same anion, Cl^{-}, is involved in both experiments, the charge of the cation seems to be the predominant factor in this effect. The effect of the anionic charge was shown to be negligible, NA_2SO_4 producing the same pattern as NaCl, when plotted against Na^{+} concentration.

In Figure 5, optical density after seven hours' settling at pH8 is plotted against Ca^{++} concentration, for B, B/1 and

REGION OF PRECIPITATION

REGION OF
SUSPENSION

Figure 3

Contours dividing regions of precipitation of cells from regions of stable cell suspensions, for B and B/1,5, shown as functions of Ca^{++} and of H^+ concentrations. Initial titer of suspensions was approximately $10^9/cc$; settling for 16 hours at $3°$. Procedure employed is the same as in Figure 2.
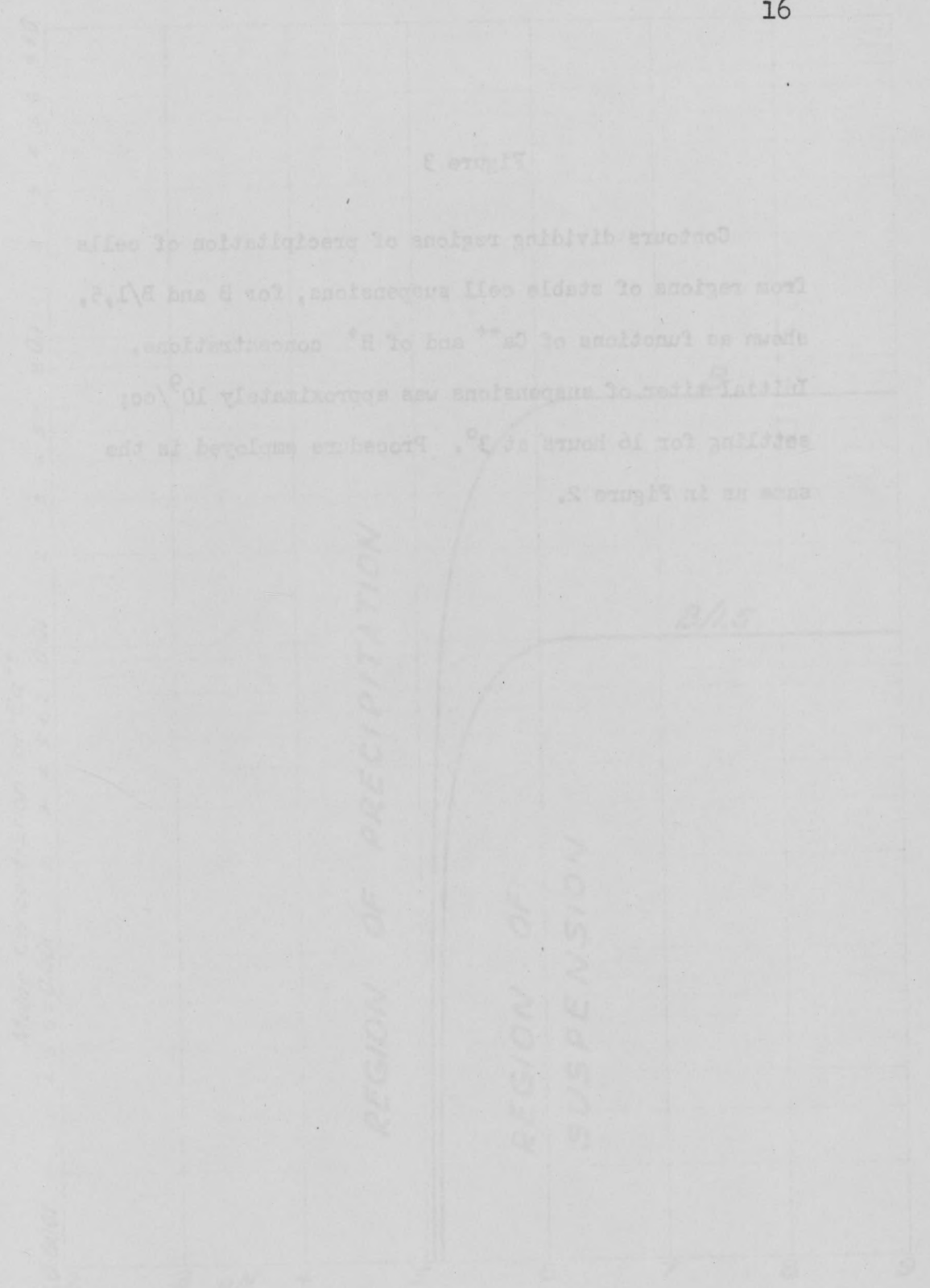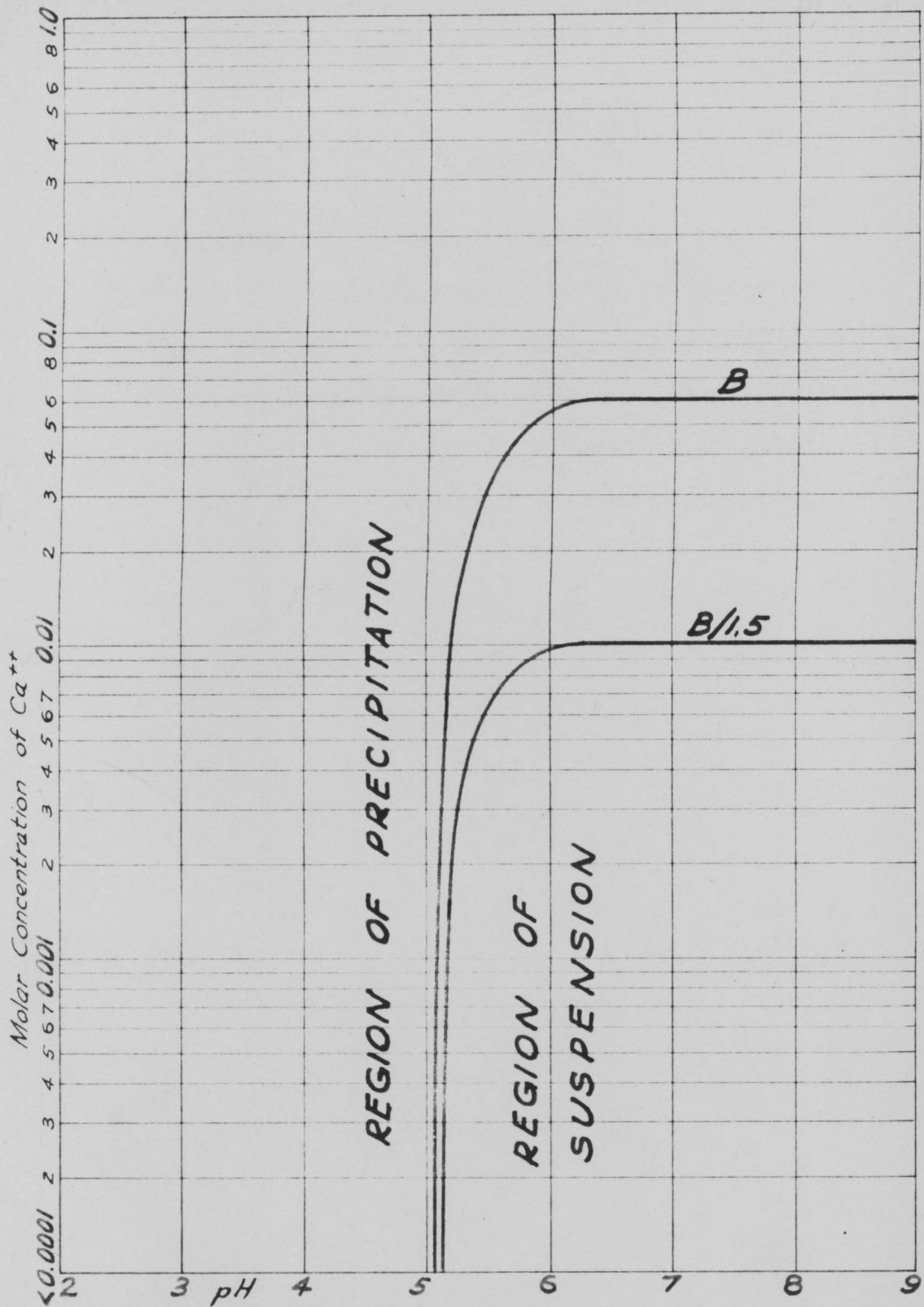
FIGURE 3: Precipitation Contours of E. Coli B and B/1.5 in media of various Ca⁺⁺ concentrations and pH's.
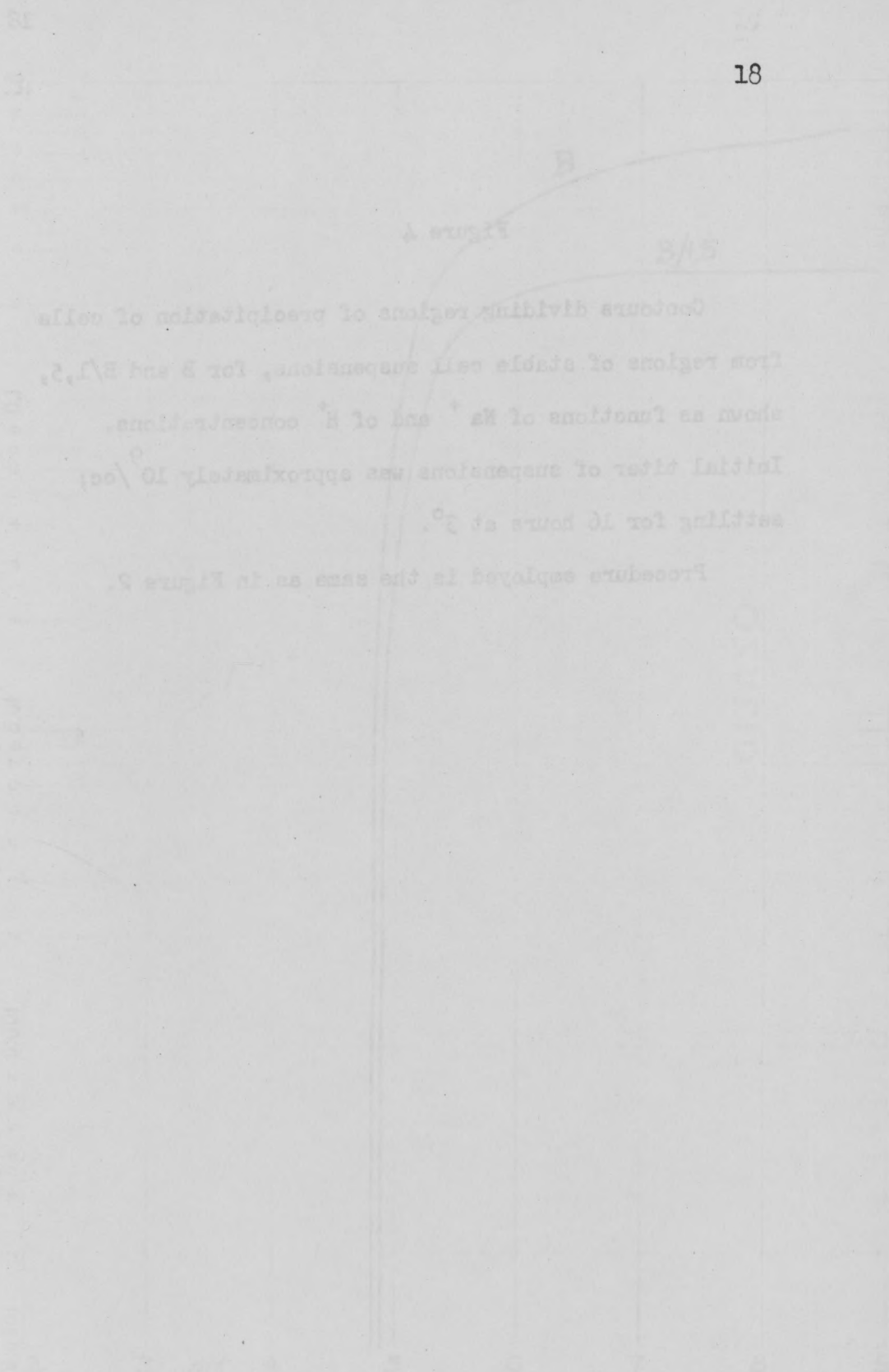
Figure 4

Contours dividing regions of precipitation of cells
from regions of stable cell suspensions, for B and B/1,5,
shown as functions of Na^+ and of H^+ concentrations.
Initial titer of suspensions was approximately 10^9/cc;
settling for 16 hours at 3°.

Procedure employed is the same as in Figure 2.
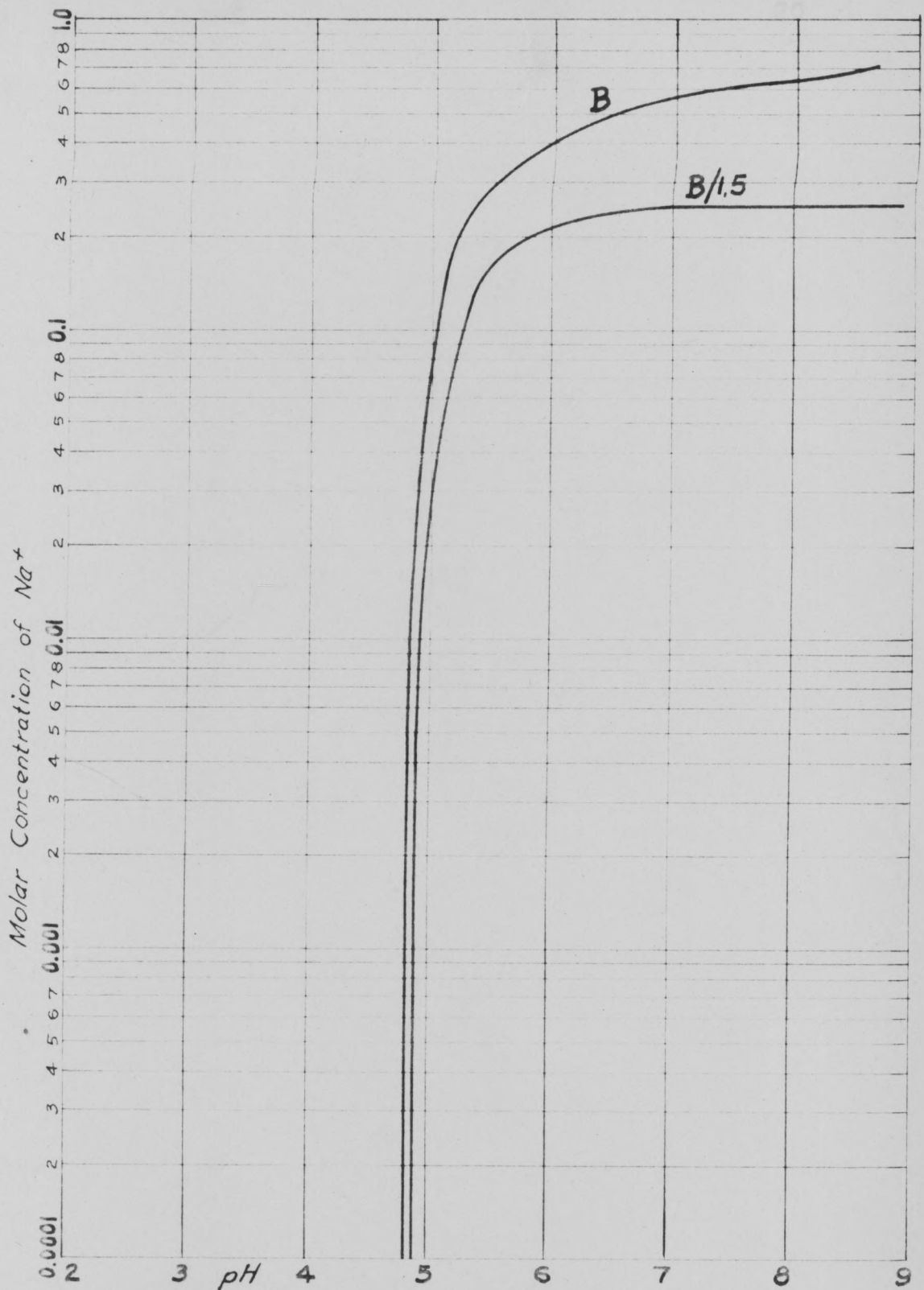
FIGURE 4 : Precipitation Contours of E. Coli B and B/1.5 in media of various Na+ concentrations and pH's.

Figure 5

Optical density of suspension of B, B/1 and B/1,5 as a function of Ca^{++} concentration, after 7 hours' settling at 3°, in media of pH 8.

Optical densities are expressed as the percent of initial optical density.

FIGURE 5
OPTICAL DENSITY vs. [Ca⁺⁺]
FOR B, B/1, B/1.5.
7 hours' settling; pH 8.
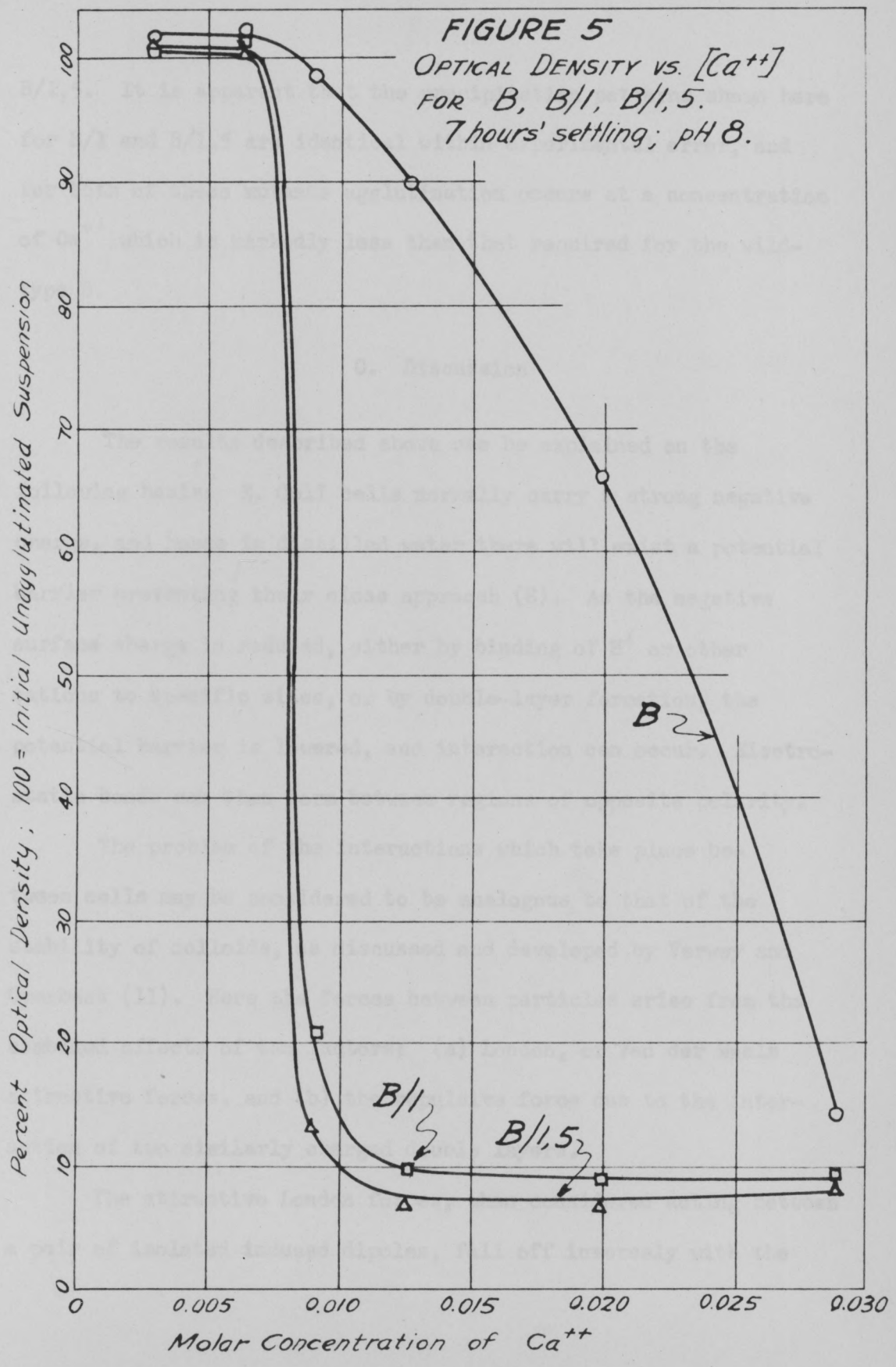
B/1,5. It is apparent that the precipitation patterns shown here for B/1 and B/1,5 are identical within experimental error, and for both of these mutants agglutination occurs at a concentration of Ca^{++} which is markedly less than that required for the wild-type B.

C. Discussion

The results described above can be explained on the following basis: E. Coli cells normally carry a strong negative charge, and hence in distilled water there will exist a potential barrier preventing their close approach (8). As the negative surface charge is reduced, either by binding of H^+ or other cations to specific sites, or by double-layer formation, the potential barrier is lowered, and interaction can occur. Electrostatic bonds can then form between regions of opposite polarity.

The problem of the interactions which take place between cells may be considered to be analogous to that of the stability of colloids, as discussed and developed by Verwey and Overbeek (11). Here the forces between particles arise from the combined effects of two factors: (a) London, or Van der Waals attractive forces, and (b) the repulsive force due to the interaction of two similarly charged double layers.

The attractive London forces, when considered acting between a pair of isolated induced dipoles, fall off inversely with the

seventh power of the distance. Since these universal forces act between each atom and every other atom in its neighborhood, essentially unaffected by intervening material, the net attraction between two particles may be computed by summing the inter-atomic forces between each atom in one particle and every atom in the other particle. When this is carried out for the case of large particles with parallel flat faces, the total force is found to change inversely as the square of the distance. In the case of spheres, the attractive force initially drops off inversely with the distance, then more quickly; but at a separation of nearly a half-radius the rate of decrease of the force is still less than inversely as the square of the distance.

The repulsive forces between particles, based on the interaction of their diffuse ionic double layers, drop off in an exponential manner with increasing distance.

Hence, the behavior of neighboring particles may be considered to be governed by a superposition of these two opposing effects. If this superposition results in a zone within which the repulsive forces are stronger, the particles will be unable to make a sufficiently close approach to enable the attractive London forces, which are strong at close range, to effect a binding.

Neglecting any energetic binding of specific ions to oppositely charged sites on the cell surface, the primary effect

of changes in the salt concentration of the medium may be considered
to be upon the thickness of the double layer. This thickness,
measured from the surface to the center of gravity of the dif-
fuse ion atmosphere, is given as $1/\kappa$, where

$$\kappa^2 = 8\pi ne^2z^2/DkT \qquad , \text{ and}$$

$n =$ ion concentration per cc
$e =$ electronic charge
$z =$ ion valence
$D =$ dielectric constant

This evaluation of κ is an approximate one, based on the assumption
made by Debye and Hückel (12) that the electric potential, ψ ,
is sufficiently small that $ze\psi/kT \ll 1$. It can be shown
that the ions whose charge is of the same sign as the surface
of the particle have a negligible effect upon the formation of
the double layer. Accordingly, z may be taken as the valence
of the ions bearing a charge opposite to that of the surface
without introducing any appreciable error in determining the
magnitude of κ . The double layer thickness is thus seen to
be inversely proportional to the valence, and inversely pro-
portional to the square root of the concentration. Reduction
of this thickness reduces the range of the repulsive forces,
and in excess of some critical concentration, attraction will be
predominant at all distances, thus leading to a binding contact
between particles.

The effect of ion valence on colloid coagulation is quali-
tatively expressed by the rule of Schultz and Hardy which states

that the flocculating concentration is determined primarily by the
valency of the ions bearing a charge opposite that of the sol
particles, their specific nature being of secondary importance.
Verwey and Overbeek have computed the effect of the valency of
oppositely charged ions in the case of flat surfaces of high po-
tential to which the approximating assumptions of Debye and Hückel
are inapplicable, and to which no ion binding takes place. They
find that the coagulating concentrations for univalent, bivalent,
and trivalent salts should be in the proportions

$$1: (\tfrac{1}{2})^6 : (1/3)^6$$

$$= 1: 0.016 : 0.0014.$$ This is found to be

substantially true for the flocculation of a number of inorganic
crystalline sols.

In the case of spheres, however, of small surface potential,
to which the approximate methods of Debye and Hückel are appli-
cable, the flocculating concentration ratios can be theoretically
expected to be

$$1: (\tfrac{1}{2})^2 : (1/3)^2$$

$$= 1: 0.25 : 0.11.$$

For approximately cylindrical bacterial cells it might be
anticipated that the corresponding ratios should fall somewhere
between these extreme values. Taking the agglutinating concen-
tration of Na^+ for E. Coli B to be 0.6 molar, and of Ca^{++} 0.06
molar; and for B/1,5 0.25 and 0.01, respectively, we get the ratios

for monovalent NA$^+$ to divalent Ca^{++},

for (B) 1: 0.1

for (B/1,5) 1: 0.04, both of which fall within the range bounded by the two cases considered by Verwey and Overbeek.

The fact that B/1,5 is agglutinated by lower concentrations of both Na$^+$ and Ca^{++} than are required by B might be qualitatively accounted for by postulating that the surface charge of B/1,5 is less, or that it contains a larger number of sites capable of forming an energetic bond with the metallic cations, or both.

These considerations alone, however, provide no accounting for the wide difference in the coagulating concentration ratios of Na$^+$ and Ca^{++} between B and B/1,5. Manifestly, the two cell surfaces exhibit different behaviors toward these two ions, which must reflect differences in chemical composition.

A number of naturally occurring long-chain polyelectrolytes have been found to have a marked ability to bind Na$^+$ and K$^+$ (13); *arabic acid, agar, and nucleic acids all display this* behavior. The presence of a larger number of such molecular configurations on the surface of B could explain the relatively smaller amount of Na$^+$ required to effect its coagulation. Sites on the surface of B/1,5 exhibiting a stronger binding of Ca^{++} could also produce this effect. Steric and structural differences, differences in the configurations of charged sites, and/or differences in the deformability of these surface

characteristics in the presence of an ionic atmosphere might also be contributing factors.

No quantitative analysis of the relative importance of double-layer formation and of ion-binding can be made on the basis of the data here presented. Supplementary micro-electrophoretic measurements which this laboratory expects to carry out, however, may lead to a quantitative comparison of the charges on the surfaces of various bacterial mutants previously known to differ only in their patterns of virus resistance.

The experimental data here presented have not closely defined the pH below which agglutination occurs in the presence of low concentrations of metallic cations. Its location in the neighborhood of 5, however, points to the conclusion that neutralization of ionized carboxyl groups by H^+ is the mechanism that is effective in the reduction of the negative charge on the surface of the cell.

Edsall (14) gives values of the pK for the aspartyl carboxyl from 3.0 to 4.7, and for the glutamyl carboxyl as about 4.4, as measured for these groups when present in peptides of known composition. Although these values are somewhat less than the pH at which neutralization of the cell surface appears to take place, a deviation in this direction is to be expected. Interaction with positive groups on the surface, which would be expected to raise the required concentration of H^+ above that

for relatively isolated groups, is probably an unimportant factor, as evidenced by work in this laboratory (15) demonstrating that there are relatively few positive groups on the surface of E. Coli B. Tolmach has shown (15) that between 10^7 and 10^8 positively charged dye molecules can be bound per E. Coli cell. Since this attachment presumably can take place only onto negative groups, an average spacing of about 5Å between negative groups follows from assuming the bacterial surface to be equivalent to that of a smooth cylinder 1 micron in diameter and 2 microns long. Interaction with these closely neighboring negative groups could be expected to diminish the concentration of H^+ needed for neutralization, and hence materially to raise the pH at which cell agglutination occurs.

Tolmach and Puck have shown (7) cellular surface carboxyl groups to be functional in the attachment of T2 to E. Coli B, and that this attachment is almost completely inhibited at pH's below 4.8. The approximate coincidence of this pH value with that below which the agglutination of cells occurs strengthens the assumption that ionization of carboxyl groups is a determining factor in the attachment of this phage, just as their neutralization is for cell precipitation.

Because B/1 and B/1,5 were found to precipitate at

lower Ca^{++} concentrations than wild-type B, the negative surface potential on these resistant mutants must be smaller than on the wild type. It can be concluded, however, from these data that charge alone does not determine virus sensitivity: B/1 and B/1,5 exhibit precipitation patterns that are identical within experimental error, and yet the former attaches T5 virus, while the latter does not. Also T1 reversibly attaches to B/1 at a rate identical with that of its attachment to the wild type, while no measureable attachment to B/1,5 is observable (2). It therefore appears inescapable that it is the specific pattern of charge distribution, rather than the gross charge, which is the factor that determines whether or not a given virus will be able to attach to a particular host cell. A similar conclusion was reached by Puck and Sagik (16) on the basis of the interaction of bacteriophage with anionic and cationic exchange resins.

It is possible to present a purely schematic picture of this conception of the differences in the surfaces of the resistant mutants on the basis of a theory proposed in this laboratory (4,5). Figure 6 shows how the wild-type cell surface might be visualized as bearing a repeating pattern of charged groups. Attachment of a particular virus, say T2, might involve specific charged groups in this pattern, for example, groups a, b, and c. Another virus, say T4, could make use of groups c, d, f, and h when it attaches. Mutation to B/2 could then come about by a

Figure 6

Hypothetical, schematic repeating pattern of electro-
static charges on a cell surface.

A phage strain requiring the pattern of groups a,
b, and c to effect its attachment would be unable to attach
to a bacterial mutant in which any of these groups were
eliminated or altered in charge or position. Simi-
larly, a mutation altering or eliminating any one of
the groups c, d, f, or h would bring about resistance
to a phage strain requiring these groups to effect
attachment.

Alteration of group c would thus cause a resistance
to both phage strains.

FIGURE 6
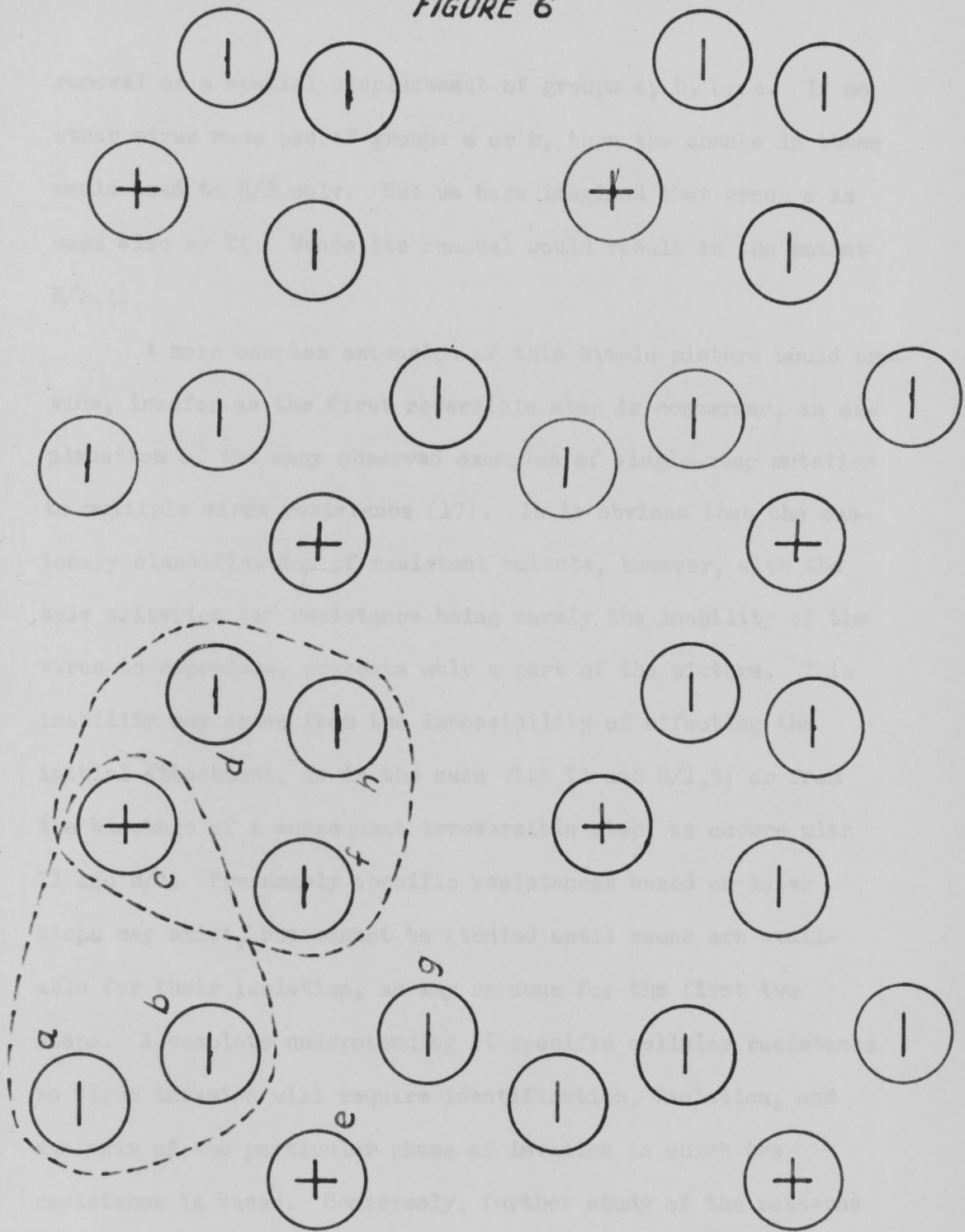
Hypothetical Schematic Pattern of Charges on Cell Surface

removal or a spatial displacement of groups a, b, or c. If no other virus made use of groups a or b, then the change in these would lead to B/2 only. But we have imagined that group c is used also by T4. Hence its removal would result in the mutant B/2,4.

A more complex extension of this simple picture would provide, insofar as the first reversible step is concerned, an explanation of the many observed examples of single-step mutation to multiple virus resistance (17). It is obvious that the customary classification of resistant mutants, however, with the sole criterion for resistance being merely the inability of the virus to reproduce, presents only a part of the picture. This inability may arise from the impossibility of effecting the initial attachment, as is the case with T1 and B/1,5; or from the blockage of a subsequent irreversible step, as occurs with T1 and B/1. Presumably specific resistances based on later steps may exist, but cannot be studied until means are available for their isolation, as may be done for the first two steps. A complete understanding of specific cellular resistance to virus invasion will require identification, isolation, and analysis of the particular phase of invasion in which the resistance is based. Conversely, further study of the patterns and nature of virus resistance may be expected to point the way to a fuller understanding of the entire process of virus invasion.

These experiments have emphasized the role of the ionic
groupings on the surface of the virus and of the host cell.
Multipolar and Van der Waals' forces probably are also involved,
but different experimental techniques will be required to es-
tablish their role.

Elaboration of this theory must await a more detailed molecu-
lar picture of the bacterial surface, as well as that of the
virus. This can be obtained by means of the direct titration
of the active surface groups. These studies are now under way
by others in this laboratory.

PART III

INVESTIGATION OF THE KINETICS OF THE REVERSIBLE ATTACHMENT OF T1 TO E. COLI B AND ITS MUTANTS

A. K as a Function of Cell Concentration

For the experimental investigation of the observed decrease
in the equilibrium "constant," K, with increasing cell titer, the
T1-B/1 system was employed as the primary tool. Since T1 engages
in only a reversible attachment to this bacterial mutant (2)
the complicating factor of the rate of formation of a subsequent
irreversible bond is not present in this system. Accordingly, it
is necessary to consider only the rate of attachment, k_1, and
the elution rate, k_2, as factors governing the value of K under
any given circumstances, as given by equation $\boxed{2}$ of Part I,
for the steady-state condition.

Bacteria used in this series of experiments were grown and
prepared as described in Section A of Part II.

The particular T1A8 strain used here was grown on E. Coli
B in nutrient broth + $\frac{1}{2}$% NaCl, then purified by slow centri-
fugation and filtration through a Mandler Candle filter. The
stock phage titer was 3.0×10^9/cc.

Cell titers were made by making a suitable dilution in
nutrient broth + $\frac{1}{2}$% NaCl, suspending aliquots of this in 4 cc.

of 0.6% agar ("soft" agar) at 45°, and immediately pouring onto
1.2% agar ("hard" agar) plates in Petri dishes. Incubation was
from 16 to 24 hours at 37°. Both soft and hard agars contained
0.8% nutrient broth + ½% NaCl.

Phage titers were made in the same manner, except that a
few minutes previous to pouring, the 45° soft agar tubes were
inoculated with approximately 10^8 cells of a 24-hour E. Coli B
culture. Such T1 plates were incubated about 16 hours at 19°.

In early experiments concerning T1-B/1 equilibrium, pro-
gressive inactivation of the phage in a number of synthetic
media was observed, which did not occur in broth. Examples of
this inactivation are shown in Figure 7. Inasmuch as the nature
of the inactivation was not known, it was felt that the possi-
bility existed that in addition to a portion of the phage being
inactivated, the survivors might suffer some alteration which
would make their behavior not representative of normal phage.
The inactivation is not reversible; no measureable reactivation
appears even after transfer of the inactivated phage to nutrient
broth + ½% NaCl for 24 hours at room temperature.

It was found that addition to the adsorption medium of 1
part in 800 of standard 0.8% Difco Nutrient Broth (10 γ/cc of
the dehydrated broth) sufficed to prevent inactivation, and yet
provided insufficient nutrient to permit bacterial multiplication
in appreciable amount during the course of any experiments
undertaken. The factor or factors in the broth serving to offer

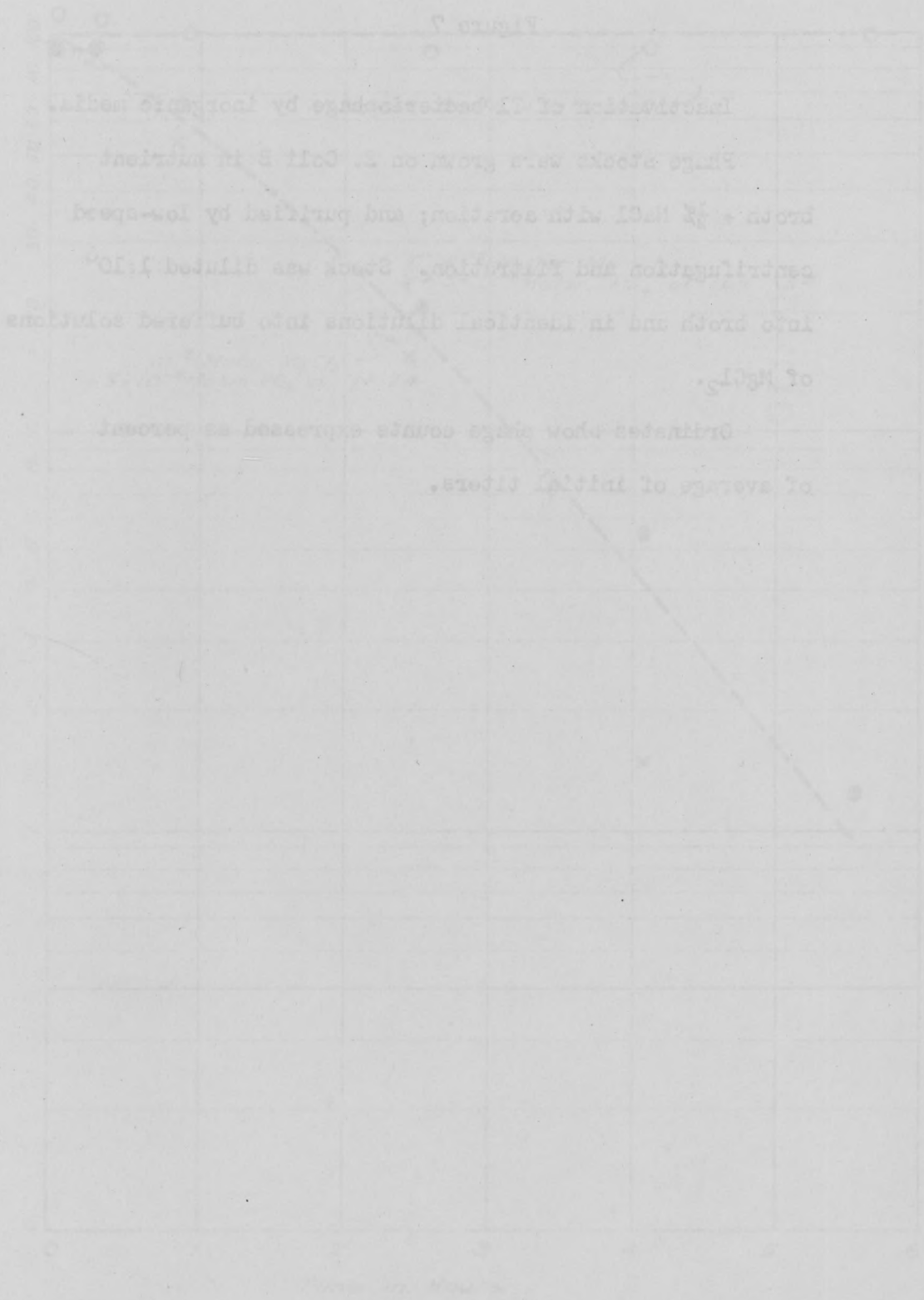Figure 7

Inactivation of T1 bacteriophage by inorganic media.

Phage stocks were grown on E. Coli B in nutrient broth + $\frac{1}{2}$% NaCl with aeration; and purified by low-speed centrifugation and filtration. Stock was diluted $1:10^6$ into broth and in identical dilutions into buffered solutions of $MgCl_2$.

Ordinates show phage counts expressed as percent of average of initial titers.

FIGURE 7: Inactivation of T1 Bacteriophage by Inorganic Media.
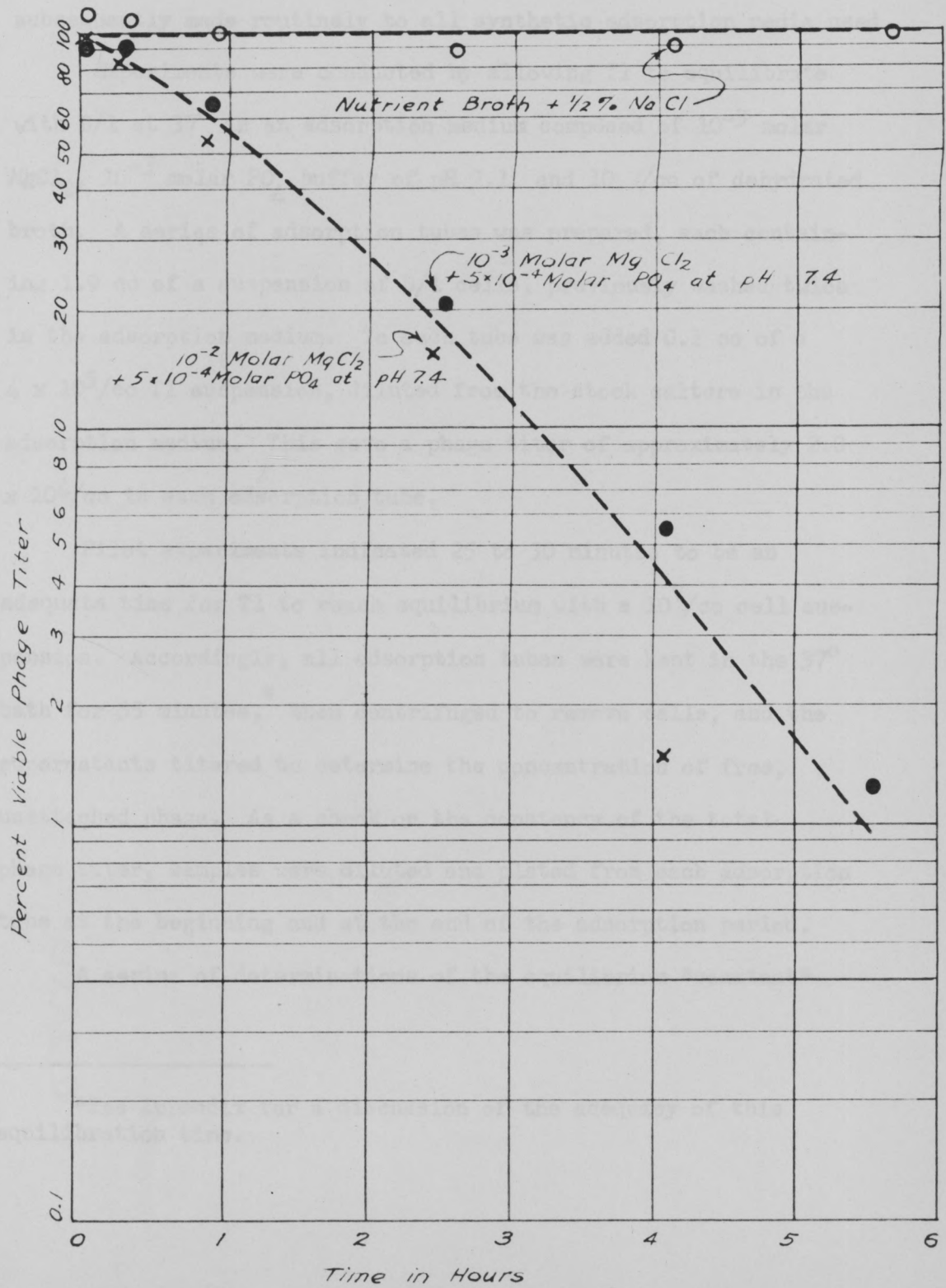
this protection were not identified, but this broth addition was subsequently made routinely to all synthetic adsorption media used.

Experiments were conducted by allowing T1 to equilibrate with B/1 at 37° in an adsorption medium composed of 10^{-3} molar $MgCl_2$, 10^{-3} molar PO_4 buffer of pH 7.1, and 10 γ/cc of dehydrated broth. A series of adsorption tubes was prepared, each containing 1.9 cc of a suspension of B/1 cells, previously washed twice in the adsorption medium. To each tube was added 0.1 cc of a 4×10^5/cc T1 suspension, diluted from the stock culture in the adsorption medium. This gave a phage titer of approximately 2.0 $\times 10^4$/cc in each adsorption tube.

Pilot experiments indicated 25 to 30 minutes to be an adequate time for T1 to reach equilibrium with a 10^7/cc cell suspension. Accordingly, all adsorption tubes were kept in the 37° bath for 35 minutes,[*] then centrifuged to remove cells, and the supernatants titered to determine the concentration of free, unattached phage. As a check on the constancy of the total phage titer, samples were diluted and plated from each adsorption tube at the beginning and at the end of the adsorption period.

A series of determinations of the equilibrium "constant"

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*See Appendix for a discussion of the adequacy of this equilibration time.

K by the experimental procedure outlined above shows it to have
an apparent relationship to bacterial concentration as indicated
in Figure 8. K here has been computed in accordance with equation
$\underline{/2/}$ of Part I. The functional relationship between K and (B/1)
can be approximated by the empirical relationship

$$K = 1.90 \times 10^{-4} \, (B/1)^{-\frac{1}{2}}, \qquad \underline{/3/}$$

which represents the data with an uncertainty probably no greater
than that of the experimental measurements.

No theoretical justification for equation $\underline{/3/}$ has been
discovered, and arbitrarily curved lines could be drawn which would
fit the plotted points equally well or better. It may be assumed
that this simple linear relationship between log K and log (B/1)
is a fortuitous one. It is nevertheless a useful, and hitherto
unavailable, tool for the phage experimenter, to serve as a guide
in the design of experiments involving T1 adsorption.

It has been shown that arrest of the second, irreversible,
step of virus invasion can also be accomplished by exposure of
wild-type cells to ultra-violet radiation (2). It was therefore
important to determine whether these cells would also exhibit the
characteristic depression of K with increase of cell concentration.
If this were the case, the conclusion could be drawn that the
factors responsible for this action lie entirely within the
parameters controlling the first step of the invasion cycle.

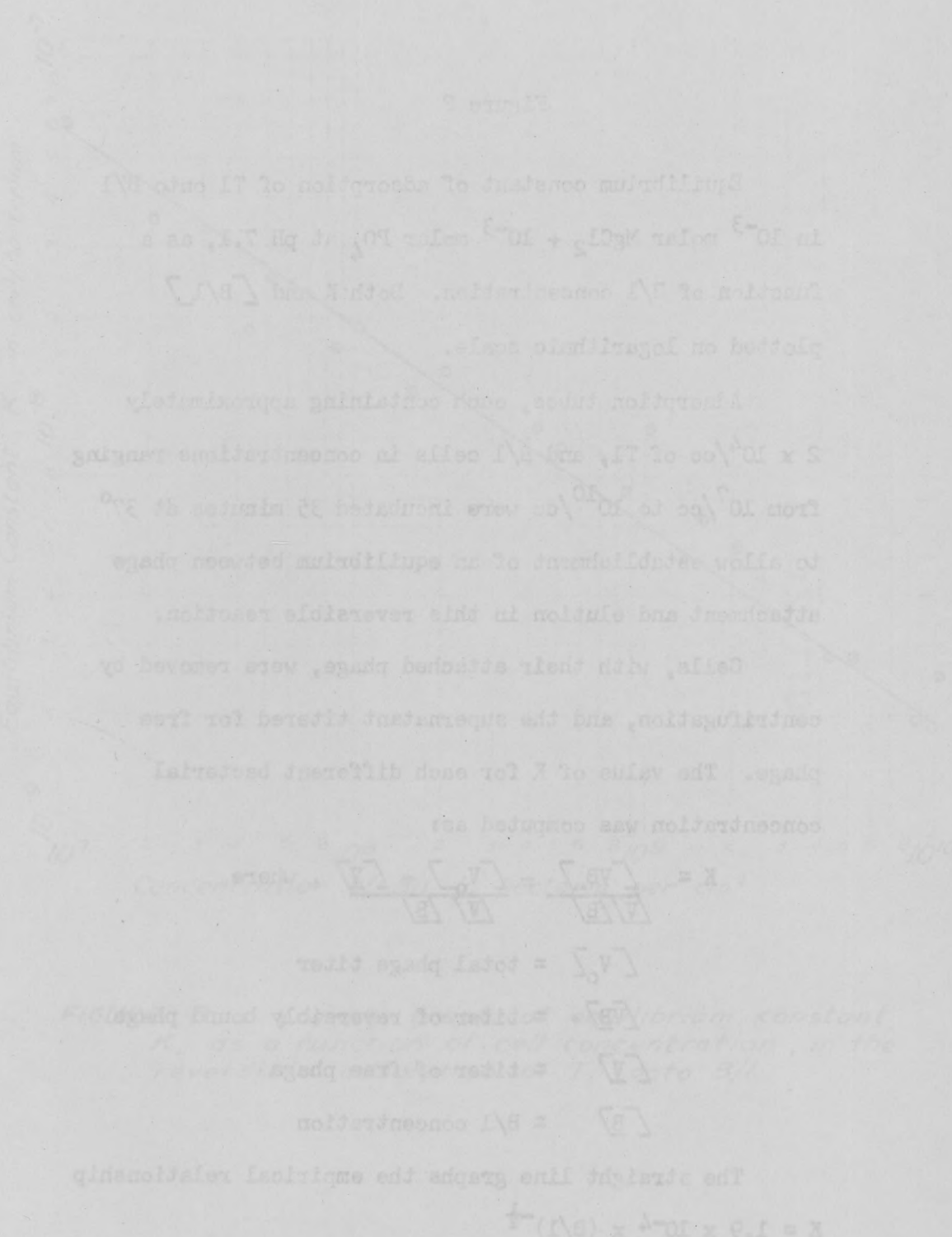Cells at a concentration of about 10^{10}/cc were exposed to

Figure 8

Equilibrium constant of adsorption of Tl onto B/1 in 10^{-3} molar $MgCl_2$ + 10^{-3} molar PO_4 at pH 7.1, as a function of B/1 concentration. Both K and $[B/1]$ plotted on logarithmic scale.

Adsorption tubes, each containing approximately 2×10^4/cc of Tl, and B/1 cells in concentrations ranging from 10^7/cc to 10^{10}/cc were incubated 35 minutes at 37° to allow establishment of an equilibrium between phage attachment and elution in this reversible reaction.

Cells, with their attached phage, were removed by centrifugation, and the supernatant titered for free phage. The value of K for each different bacterial concentration was computed as:

$$K = \frac{[VB]}{[V][B]} = \frac{[V_o] - [V]}{[V][B]} \text{, where}$$
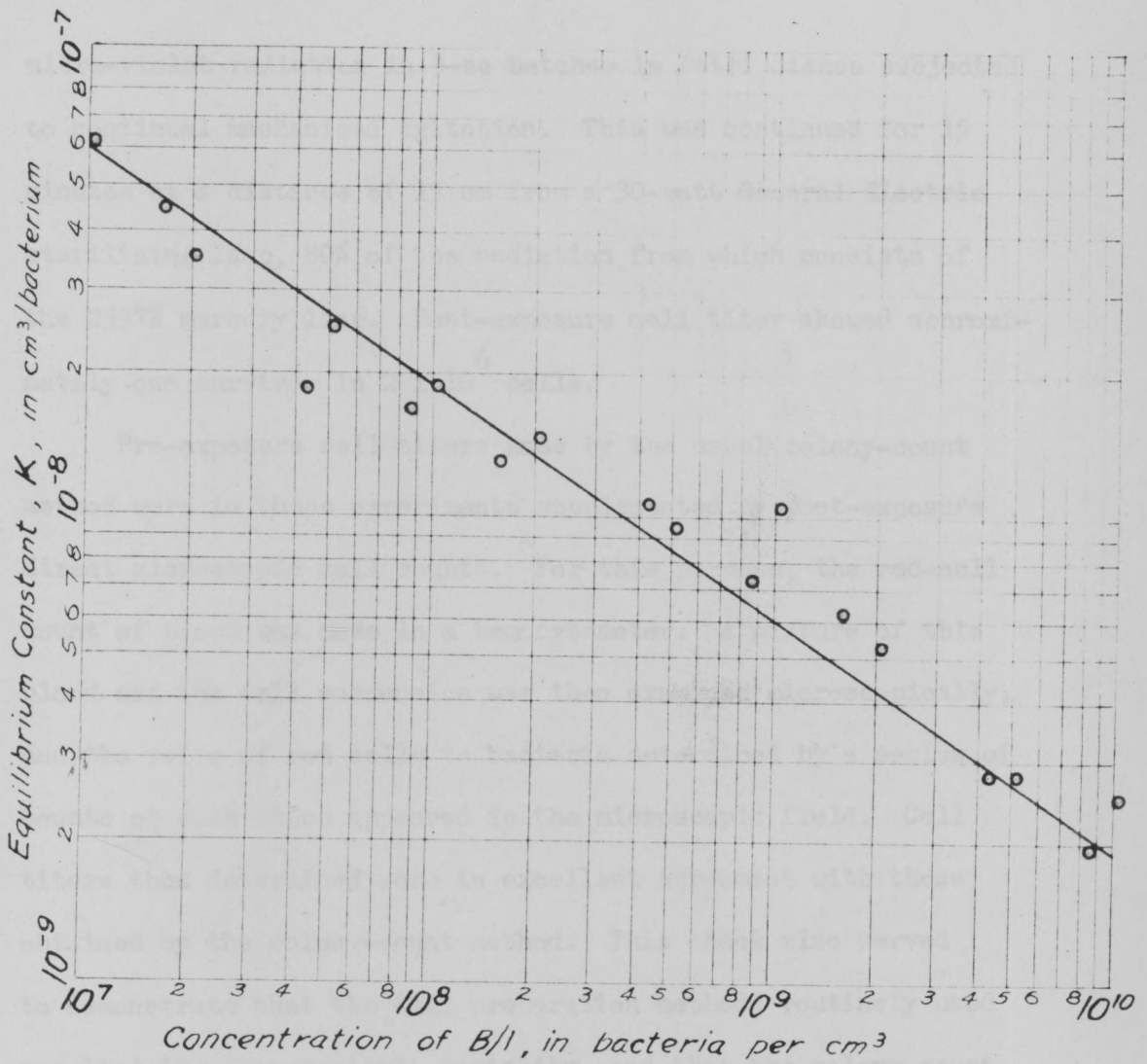
$[V_o]$ = total phage titer

$[VB]$ = titer of reversibly bound phage

$[V]$ = titer of free phage

$[B]$ = B/1 concentration

The straight line graphs the empirical relationship

$K = 1.9 \times 10^{-4} \times (B/1)^{-\frac{1}{2}}$

FIGURE 8 : Log-log graph of equilibrium constant,
K, as a function of cell concentration, in the
reversible adsorption of Tl onto B/1.

ultra-violet radiation in 3-cc batches in Petri dishes subjected to continual mechanical agitation. This was continued for 15 minutes at a distance of 15 cm from a 30-watt General Electric sterilizing lamp, 80% of the radiation from which consists of the 2537Å mercury line. Post-exposure cell titer showed approximately one survivor in 2×10^6 cells.

Pre-exposure cell titers made by the usual colony-count method were in these experiments supplemented by post-exposure direct microscopic cell counts. For this purpose, the red-cell count of blood was made in a hemocytometer. A mixture of this blood and the cell suspension was then examined microscopically, and the ratio of red cells to bacteria determined by a series of counts of each which appeared in the microscopic field. Cell titers thus determined were in excellent agreement with those obtained by the colony-count method. This check also served to demonstrate that the cell preparation methods routinely used resulted in no appreciable mortality, and that the colony-count, which provides viable cell titers, is, within the limits of experimental error, an accurate representation of the total cell concentration.

Determination of K at various cell concentrations was carried out according to the identical procedure followed for previous similar experiments with B/1. Results obtained are, within experimental error, the same as those obtained with B/1, as is

indicated in Figure 9.

B. Investigation of the Possible Influence of Various
Factors on the Dependence of K on Cell Concentration

(1) Effect of Metabolic Products in the Medium

An experiment was designed to determine whether the observed

relationship between K and (B) is dependent upon the cell concen-

tration itself, or results from effects of cell metabolism on the

medium. Bacterial metabolic products capable of influencing the

attachment characteristics of the phage might be present in

larger quantities in media in which higher bacterial concentrations

had been present. Therefore the effect of such products on the

attachment was tested by the following experiment:

a) A B/1 culture was washed twice in the standard

adsorption medium, and resuspended in this same medium

at a titer of approximately 5×10^9/cc. This was

designated as "H" (for "high") titer. A 1:100 dilution

of this suspension in adsorption medium was prepared and

designated as "L" (for "low" titer).

b) Equal portions of H and L were centrifuged; the

H cells were resuspended in the supernatant from L and

designated as "HT." Similarly, a resuspension of the L

cells in the supernatant from H was designated as "LT."

c) T1 was added to each tube to give a phage concen-

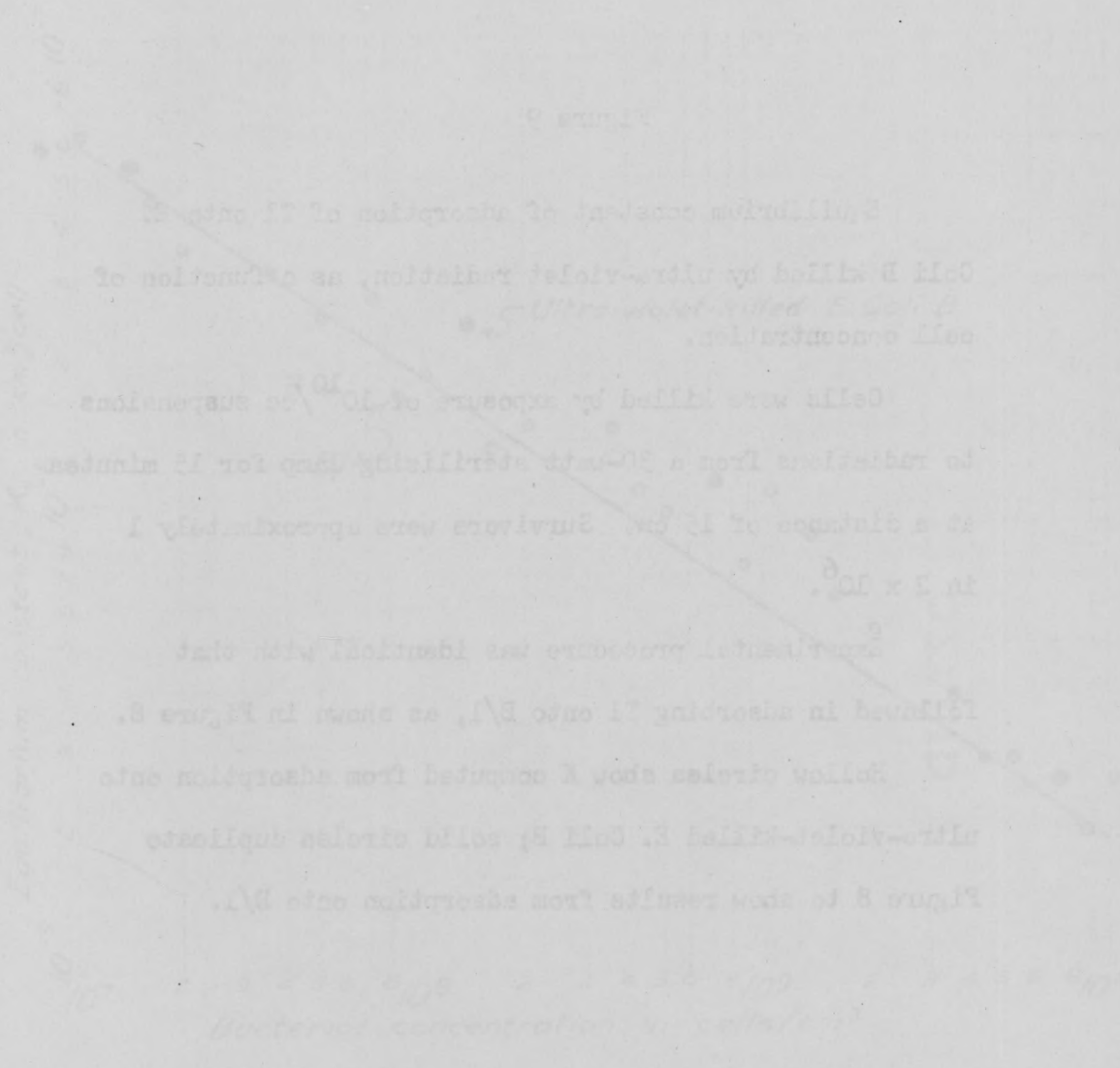tration of approximately $2 \times 5 \times 10^4$/cc in each; each tube

Figure 9

Equilibrium constant of adsorption of T1 onto E. Coli B killed by ultra-violet radiation, as a function of cell concentration.

Cells were killed by exposure of 10^{10}/cc suspensions to radiations from a 30-watt sterilizing lamp for 15 minutes at a distance of 15 cm. Survivors were approximately 1 in 2×10^6.

Experimental procedure was identical with that followed in adsorbing T1 onto B/1, as shown in Figure 8.

Hollow circles show K computed from adsorption onto ultra-violet-killed E. Coli B; solid circles duplicate Figure 8 to show results from adsorption onto B/1.
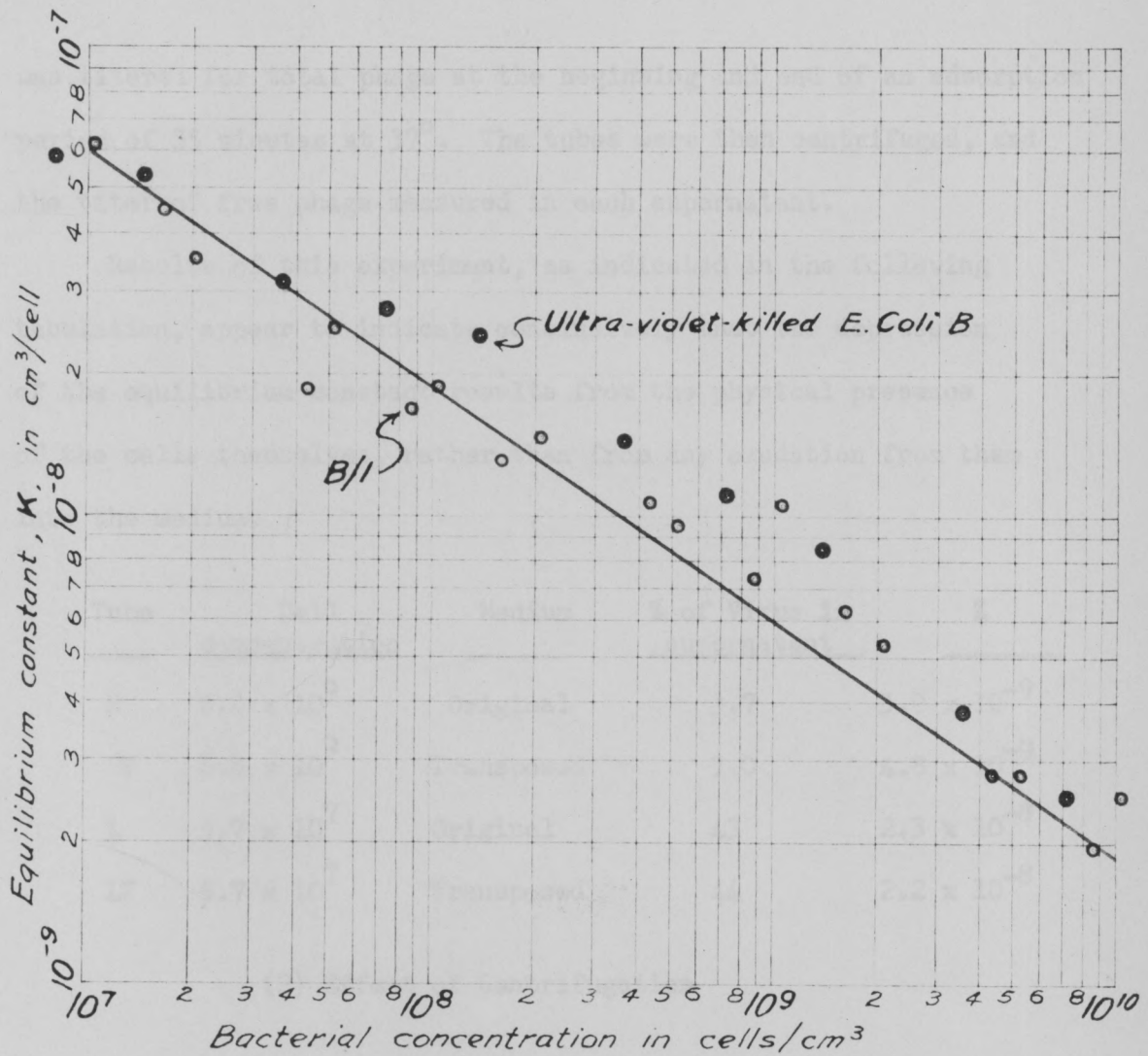
FIGURE 9 : Log-log graph of equilibrium constant, K, as a function of cell concentration, in the reversible adsorption of TI onto E. Coli B killed by exposure to ultra-violet radiation. Similar points for adsorption onto B/1 are included for comparison.

was titered for total phage at the beginning and end of an adsorption period of 35 minutes at 37°. The tubes were then centrifuged, and the titer of free phage measured in each supernatant.

Results of this experiment, as indicated in the following tabulation, appear to indicate conclusively that the depression of the equilibrium constant results from the physical presence of the cells themselves, rather than from any exudation from them into the medium.

Tube	Cell concentration	Medium	% of Virus in supernatant	K
H	6.8×10^9	Original	3.7	3.9×10^{-9}
HT	6.8×10^9	Transposed	3.0	4.8×10^{-9}
L	5.7×10^7	Original	43	2.3×10^{-8}
LT	5.7×10^7	Transposed	44	2.2×10^{-8}

(2) Effect of Centrifugation

In the unrealized circumstance of the centrifugation of an ideally homogeneous cell suspension, the phage would undergo an abrupt transition from an environment of constant cell titer with which it was in equilibrium, to a completely cell-free environment as the boundary descended past it. This would give an accurate and undistorted titer of free phage from the supernatant, no matter at what speed the centrifugation were carried out. In the real situation, however, with the cell population exhibiting wide

variations in size and shape, the phage finds itself in an environ-
ment of gradually decreasing cell titer, as can be attested by
periodic inspection of a centrifuging suspension. In the event
that this decrease were sufficiently slow for the phage population
to maintain a changing equilibrium with it, the end result would
be an increasing proportion of free phage during the course of
the centrifugation. Qualitatively, then, the titer from the
final supernatant should be expected to contain more free phage
than the initial suspension, and this discrepancy should be more
pronounced at higher cell concentrations, in which the phage-
bacterial equilibrium is more rapidly established. The direction
of this effect is, then, in the proper direction to cause a de-
pression of K at high (B), as is observed.

This possibility was experimentally checked by centri-
fugation at two speeds: (a) for 4 minutes in 1-cc tubes in a
high-speed Micro centrifuge at approximately 5000g, which can
clear a dense bacterial suspension in less than 30 seconds; and
(b) in the manner employed routinely in the determinations of K:
for 20 minutes in a Sorvall angle-head centrifuge at approxi-
mately 600g. This procedure was carried out at four bacterial
concentrations: approximately 10^7, 10^8, 10^9, and 10^{10}/cc.
Discrepancies between K's determined at the two centrifugation
speeds were within the range of experimental error.

Garen (6) has determined the value of the dissociation rate,
k_2, to be approximately 0.1 min^{-1} for the T1-B/1 system in 10^{-3}

molar MgCl$_2$, as was used in this experiment. It is apparent that the dissociation reaction with a rate constant of this magnitude cannot deviate appreciably from its initial state of equilibrium during the fraction of a minute required to remove the cells from suspension in the high-speed Micro centrigue. Since K values determined in this centrifugation deviate by no more than normal experimental error from those obtained by centrifuging in the manner used throughout this work, we may be confident that any error introduced hereby cannot be appreciable.

(3) Effect of Salt Concentration

It has been shown (2) that the ionic strength of the adsorption medium has a profound effect on the rate of T1 attachment to wild-type E. Coli B, this rate decreasing markedly with a variation of salt concentration in each direction from a well-defined optimum attained at approximately 5 x 10^{-4} molar Ca^{++} or Mg^{++}. Experiments by Puck and Sagik (16) have indicated that at least the initial increase of the equilibrium constant for attachment, as the salt concentration is raised from zero to its optimum value, is due to electrostatic interaction of the inorganic ions with the bacteriophage surface. Hence it was important to determine whether the change in K with bacterial concentration is also influenced by the ionic constitution of the medium. If such an effect were found, it would raise the

possibility that the lowering of K produced by a high bacterial concentration is an effect which operates through the same molecular groups that display the strong salt-sensitivity of virus attachment.

Accordingly, in order to investigate the effect of salt concentration on the variation of K with cell concentration, equilibration experiments were performed, identical with those previously described, but with a concentration of Mg^{++} equal to 3.3×10^{-4} molar instead of 10^{-3} molar. This, as may be seen from Figure 10, resulted in an approximate doubling of the equilibrium "constant," an effect consistent with the changes observed by Garen (6) at a constant bacterial concentration. However, the curve obtained possesses substantially the same form and slope, and hence the equilibrium constants are exhibiting the same variation with bacterial concentration as in the 10^{-3} molar medium. Therefore the effect of the increased bacterial concentration does not seem to involve the same mechanism responsible for the variation of attachment rate with the ionic constituents of the medium.

(4) Effect of Bacterial Clumping

Another factor whose possible role in reducing K with increasing cell concentration was investigated, was that of actual reduction of effective cell surface as a result of clumping.
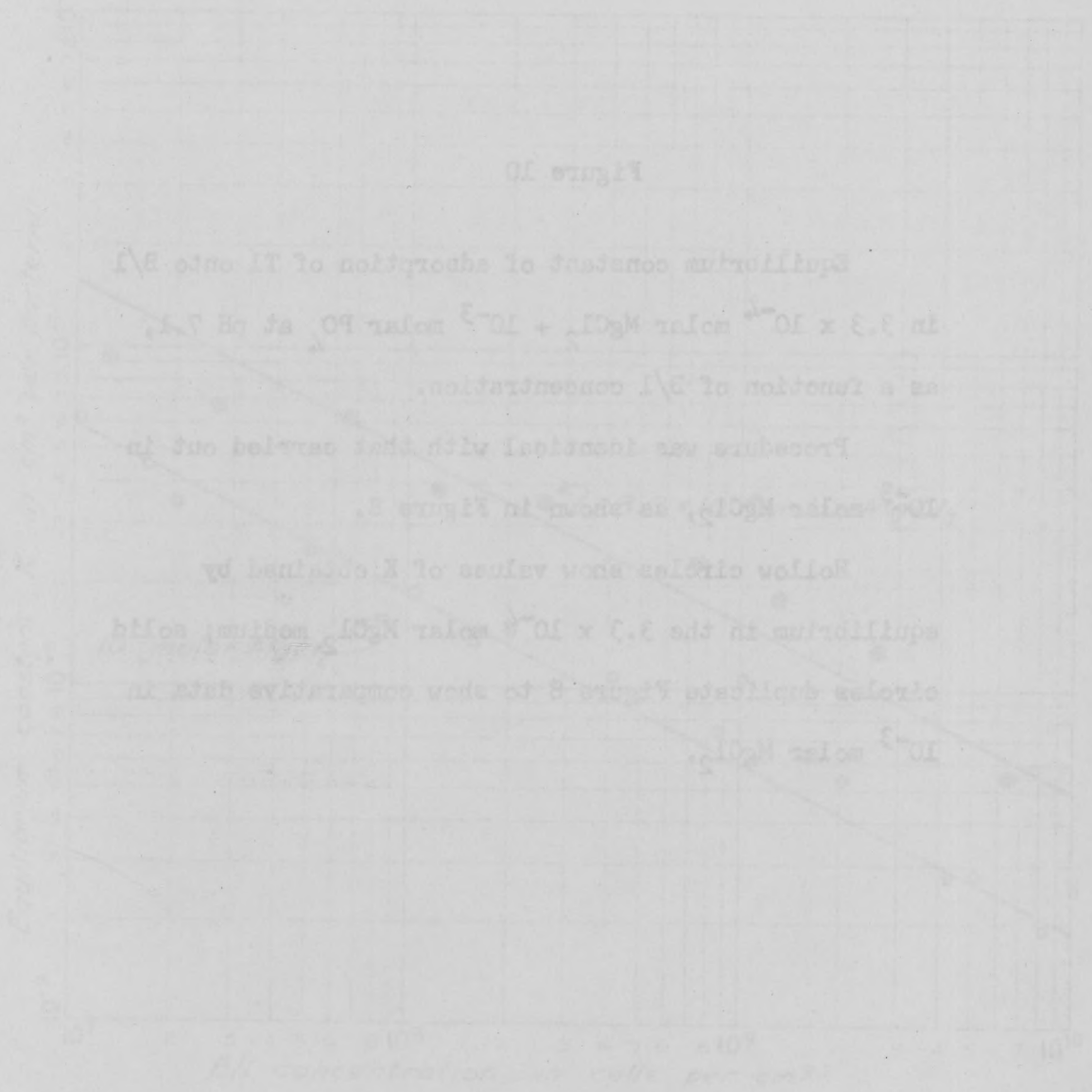
Figure 10

Equilibrium constant of adsorption of Tl onto B/l in 3.3 x 10^{-4} molar $MgCl_2$ + 10^{-3} molar PO_4 at pH 7.1, as a function of B/l concentration.

Procedure was identical with that carried out in 10^{-3} molar $MgCl_2$, as shown in Figure 8.

Hollow circles show values of K obtained by equilibrium in the 3.3 x 10^{-4} molar $MgCl_2$ medium; solid circles duplicate Figure 8 to show comparative data in 10^{-3} molar $MgCl_2$.
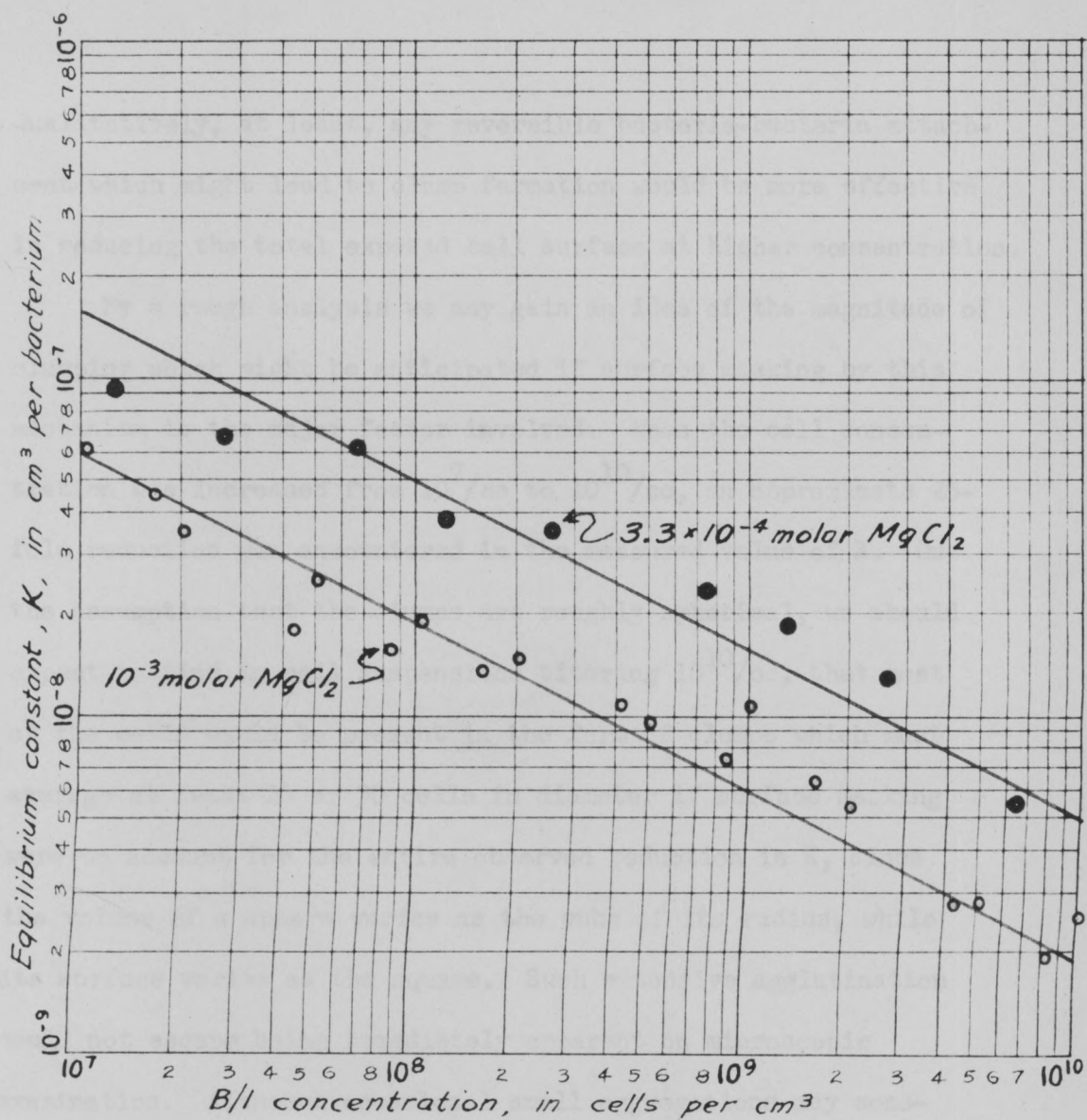
FIGURE 10 : Log-log graph of equilibrium constant, K, as a function of cell concentration, in the adsorption of TI onto B/I in a medium containing 3.3×10^{-4} molar $MgCl_2$. Similar points for a medium containing 10^{-3} molar $MgCl_2$ are included for comparison.

Qualitatively, at least, any reversible bacteria-bacteria attachment which might lead to clump formation would be more effective in reducing the total exposed cell surface at higher concentration.

By a rough analysis we may gain an idea of the magnitude of clumping which might be anticipated if surface masking by this mechanism is the major factor involved. When the cell concentration was increased from 10^7/cc to 10^{10}/cc, an approximate 25-fold reduction was encountered in the measured value of K. On the assumption that the clumps are roughly spherical, we should expect to find in cell suspensions titering 10^{10}/cc, that most of the cells would be present in the form of clumps which must average at least 20 or 30 cells in diameter if surface masking were to account for the entire observed reduction in K, since the volume of a sphere varies as the cube of its radius, while its surface varies as the square. Such extensive agglutination could not escape being immediately apparent on microscopic examination. Although occasional small aggregations may sometimes be observed in these suspensions, the amount is far too little to account for the observed decrease in the virus attachment constant. Therefore, although it may be to some extent contributory to the observed effect, cell aggregation cannot constitute the major factor involved.

(5) Mechanical Cell Interactions

Investigation was made of the possibility that the reduction of K with increasing cell concentration might be due to an increase in the phage-bacteria dissociation rate, k_2, through the purely mechanical action of the increasing frequency of inter-bacterial collisions.

It has been established (2) that T1 does not exhibit any measurable reaction of any sort with B/1,5. Accordingly, the only effect this bacterial mutant might be expected to exhibit would be such a purely mechanical one as was being investigated. The experimental procedure was as follows:

    a) B/1 and B/1,5 were centrifuged and washed twice in the standard adsorption medium, and then resuspended in the same medium.

    b) To each of a series of tubes at 37°, containing dilutions of B/1,5 ranging in titer from 7×10^9 to 7×10^7, was added an equal aliquot of B/1 and of T1.

    c) After standing 35 minutes, the tubes were centrifuged and phage titers made from each supernatant. Results are indicated in the following tabulation:

Tube Number	B/1 titer	B/1, 5 titer	Supernatant T1 titer
1	5.1×10^7	7.0×10^9	7950
2	5.1×10^7	3.5×10^9	7800
3	5.1×10^7	1.4×10^9 0	7300
4	5.1×10^7	7.0×10^8	8200
5	5.1×10^7	3.5×10^8	9100
6	5.1×10^7	1.4×10^8	8150
7	5.1×10^7	7.0×10^7	7500

Since the quantity of supernatant phage remained constant within the limits of experimental error over this wide range of added B/1,5, which may be presumed to have the same purely mechanical effect as an added equal amount of B/1, it appears inescapable that the mechanical effect of increased bacterial collisions cannot be a pertinent factor in the observed variations of K with (B).

(6) Electrostatic Cell Interactions

Since electrostatic forces play a major part in the primary, reversible, phage-bacterium attachment, the possibility of this attachment being seriously affected by the proximity of other, and in this medium, strongly charged, bacteria cannot be overlooked. Several pieces of experimental evidence, however, make this an untenable hypothesis.

In Part II it has been shown that, since B/1 and B/1,5 agglutinate at approximately the same concentration of Ca^{++} and of H^+, these two mutant forms have, at these points at least, the same surface charge. In the absence of any contradictory indications, the assumption may be made from this evidence that these two mutant bacterial forms also possess the same overall surface charge at the lesser cation concentrations used in the phage adsorption experiments. Yet the presence of B/1,5 has no observable effect on the attachment equilibrium of T1 with B1, which is so strongly dependent on the B/1 concentration. Hence, this fundamental difference in behavior cannot have been brought about through the action of quantitatively different surface charges.

It is true that B/1 and B/1,5, since they display different behaviors with respect to the attachment of different viruses, must have differences in the detailed configurations of their surface charges. Such configurational differences can be represented as alterations of magnitude, location, or orientation of electric multipoles on the cell surface. Forces existing as a result of electric fields arising from multipolar effects decrease rapidly with increasing distance, in accordance with the following tabulation (18, 19):

Nature of force	Rate of decrease with distance
ion – dipole	inversely as d^3
dipole–dipole	inversely as d^4
ion–quadrupole	inversely as d^4
ion–induced dipole	inversely as d^5
dipole–induced dipole	inversely as d^7
Van der Waals'	inversely as d^7

Experimental work presented here shows the increase in K with decreasing (B) to be effective at cell concentrations as low as 10^7/cc. Even at concentrations of 10^8/cc, inter-cellular distances are of the order of 20 microns, or 2 x 10^5 Å. These distances are of the order of 10^4 to 10^5 times as large as the separation of charges in an amino acid dipole, or the estimated 5 Å separation between negative sites on the E. Coli B surface. That any multipolar forces which decrease so rapidly with distance might have appreciable effect at such distances seems highly improbable.

In further support of this argument, it has been shown in Section A that the magnitude and variation of K is the same for B/1 and for wild-type B in which the irreversible second step of attachment has been inhibited by ultra-violet irradiation. In Part II, however, it was demonstrated that a considerable difference in overall electric charge must exist

between B/1 and the wild-type B. Forces arising from actual charge magnitude vary inversely as d^2; since these have no appreciable effect on K, it follows that the even more rapidly diminishing multipolar forces must likewise be ineffective. It thus appears impossible that the observed variation in K can be due to any form of electrostatic cell-cell interaction.

(7) Phage-phage Interaction

The frequency of phage-phage collisions, based on the assumption that no repulsive forces act between them, can be computed from the relation (20):

$G = 8\pi a D' n$, where G=collisions/sec/phage particle
a=radius of particle
$D' = 2D = 2$ x diffusion constant
n=number of particles/cc

Using the established value $D = kT/6\pi\eta a$, this gives us

$$G = \frac{8kTn}{3\eta}$$

Taking k=1,38 x 10^{-16} erg-deg^{-1}
T=298 deg
η=9 x 10^{-3} poise, this gives

G=1.2 x 10^{-11}n collisions per second per phage particle. This corresponds, for any single phage particle, to an interval of more than a month between collisions with another phage particle, at a concentration of 2 x 10^4/cc, as was used in these experiments. In 2 cc of such a suspension, a phage-phage collision occurs once in 10 seconds. It is obvious that any effects which might arise

from such infrequent encounters may be neglected.

(8) Phage Heterogeneity

Heterogeneity of phage populations, with respect to the rates of adsorption onto their hosts, has been previously noted by others. Garen (6), in experiments with T1, determined that at least several percent of the phage attached at a rate appreciably slower than that of the rest of the population. Sagik (2) found that freshly prepared T2 stocks contained as much as 80% of the phage particles which attached at a very low rate, apparently due to the association with the phage of inhibitory material which could be removed by proper treatment.

It may immediately be seen that the presence of a portion of the phage population which has a low K (whether due to a small k_1 or a large k_2, or to both) will result in a larger fraction of free phage at high cell concentration than would be the case if the population were a uniform one.

From the fundamental equilibrium conditions, we can define an equilibrium constant for each section of the population, taken sufficiently small as to be considered homogeneous:

$$K_i = \frac{(VB_i)}{(V_i)\,(B)} = \frac{(V_{oi}) - (V_i)}{(V_i)\,(B)}$$, from which the fraction

of this section of the phage population free at equilibrium is

$$\frac{(V_1)}{(V_{oi})} = \frac{1}{K_1(B) + 1} \qquad [5]$$

Computed on this basis, Figure 11 shows the fraction of free phage to be expected in equilibrium with B/1 concentrations ranging from 10^7/cc to 10^{10}/cc, for homogeneous phage populations having K's varying by decades from 10^{-7} to 10^{-11} cm^3/min, together with the fraction of free phage as has been experimentally measured.

The problem thus resolves itself into the determination of a distribution function describing a phage population divided into elements, each having a different K, in such a manner as to give the experimentally determined amount of free phage at all cell concentrations from 10^7/cc to 10^{10}/cc. For this purpose, the population was arbitrarily divided into groups a, b, c,..., with $K_a = 10^{-7}$, $K_b = 10^{-8}$, etc. The following tabulation shows the fraction of each group remaining unattached when in equilibrium with various concentrations of B/1:

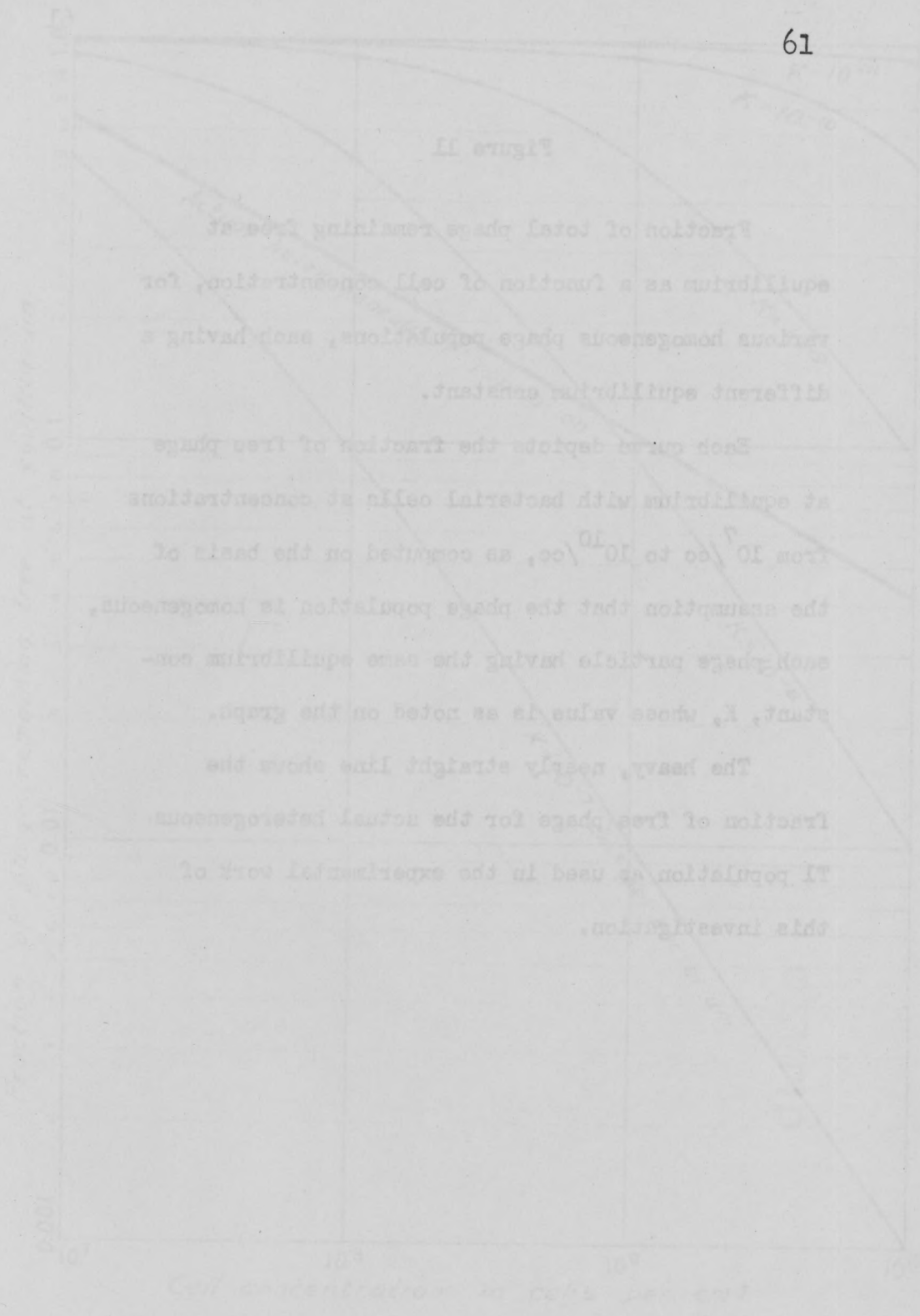| Group	K	(B) 10^7	10^8	10^9	10^{10}
a	10^{-7}	0.500	0.091	0.010	0.001
b	10^{-8}	0.909	0.500	0.091	0.010
c	10^{-9}	0.990	0.909	0.500	0.091
d	10^{-10}	0.999	0.990	0.909	0.500
e	10^{-11}	1.000	0.999	0.990	0.909
f	10^{-12}	1.000	1.000	0.999	0.990

Figure 11

Fraction of total phage remaining free at equilibrium as a function of cell concentration, for various homogeneous phage populations, each having a different equilibrium constant.

Each curve depicts the fraction of free phage at equilibrium with bacterial cells at concentrations from 10^7/cc to 10^{10}/cc, as computed on the basis of the assumption that the phage population is homogeneous, each phage particle having the same equilibrium constant, K, whose value is as noted on the graph.

The heavy, nearly straight line shows the fraction of free phage for the actual heterogeneous T1 population as used in the experimental work of this investigation.
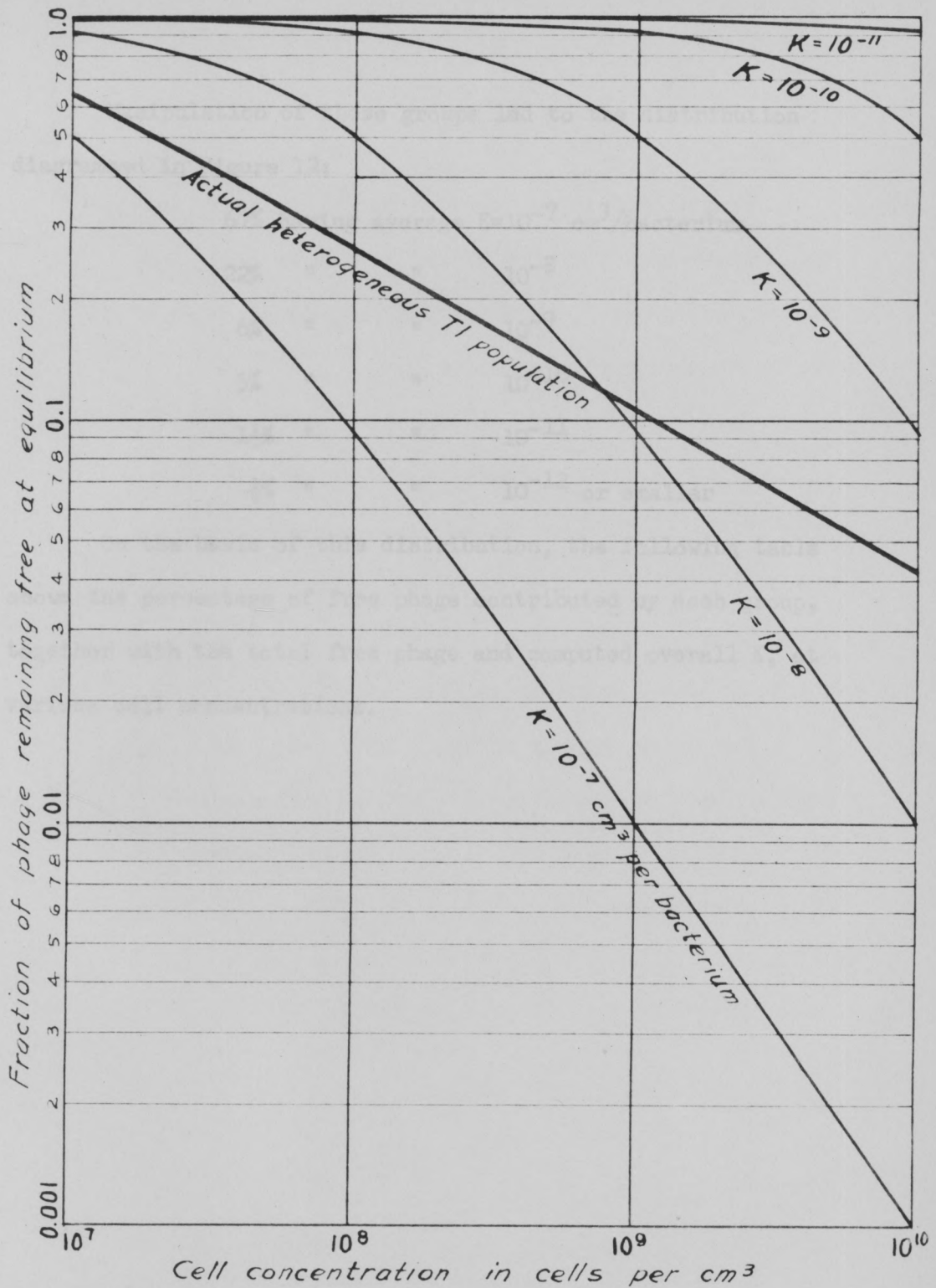
FIGURE 11 : Fraction of total phage remaining free at equilibrium as a function of cell concentration ; for various homogeneous phage populations, each character- ized by a different equilibrium constant, K. The actual heterogeneous T1 population is also included.

Manipulation of these groups led to the distribution diagrammed in Figure 12:

67% having average $K=10^{-7}$ cm^3/bacterium

22% " " 10^{-8}

6% " " 10^{-9}

3% " " 10^{-10}

$1\frac{1}{2}\%$ " " 10^{-11}

$\frac{1}{2}\%$ " " 10^{-12} or smaller

On the basis of this distribution, the following table shows the percentage of free phage contributed by each group, together with the total free phage and computed overall K, at various cell concentrations.

Group	Percentage free phage at $(B) = 10^7$	Percentage free phage at $(B) = 10^8$	Percentage free phage at $(B) = 10^9$	Percentage free phage at $(B) = 10^{10}$
a	$67 \times 0.50 = 33.5$	$67 \times 0.09 = 6.0$	$67 \times 0.01 = 0.7$	$67 \times 0.001 = 0.07$
b	$22 \times 0.91 = 20.0$	$22 \times 0.50 = 11.0$	$22 \times 0.09 = 2.0$	$22 \times 0.01 = 0.22$
c	$6 \times 0.99 = 6.0$	$6 \times 0.91 = 5.5$	$6 \times 0.50 = 3.0$	$6 \times 0.09 = 0.54$
d	$3 \times 1.00 = 3.0$	$3 \times 0.99 = 3.0$	$3 \times 0.91 = 2.7$	$3 \times 0.50 = 1.50$
e	$1.5 \times 1.00 = 1.5$	$1.5 \times 1.00 = 1.5$	$1.5 \times 0.99 = 1.5$	$1.5 \times 0.91 = 1.37$
f	$0.5 \times 1.00 = 0.5$	$0.50 \times 1.00 = 0.5$	$0.5 \times 1.00 = 0.5$	$0.5 \times 0.99 = 0.50$
Total free phage	64.5%	27.5%	10.4%	4.2%
K	5.5×10^{-8}	2.6×10^{-8}	8.6×10^{-9}	2.3×10^{-9}

The K's above, computed from the theoretical total free phage, are shown as solid circles on Figure 13.

Figure 12

Proposed distribution function of equilibrium constants in a heterogeneous phage population whose behavior will conform to the actual observed data.

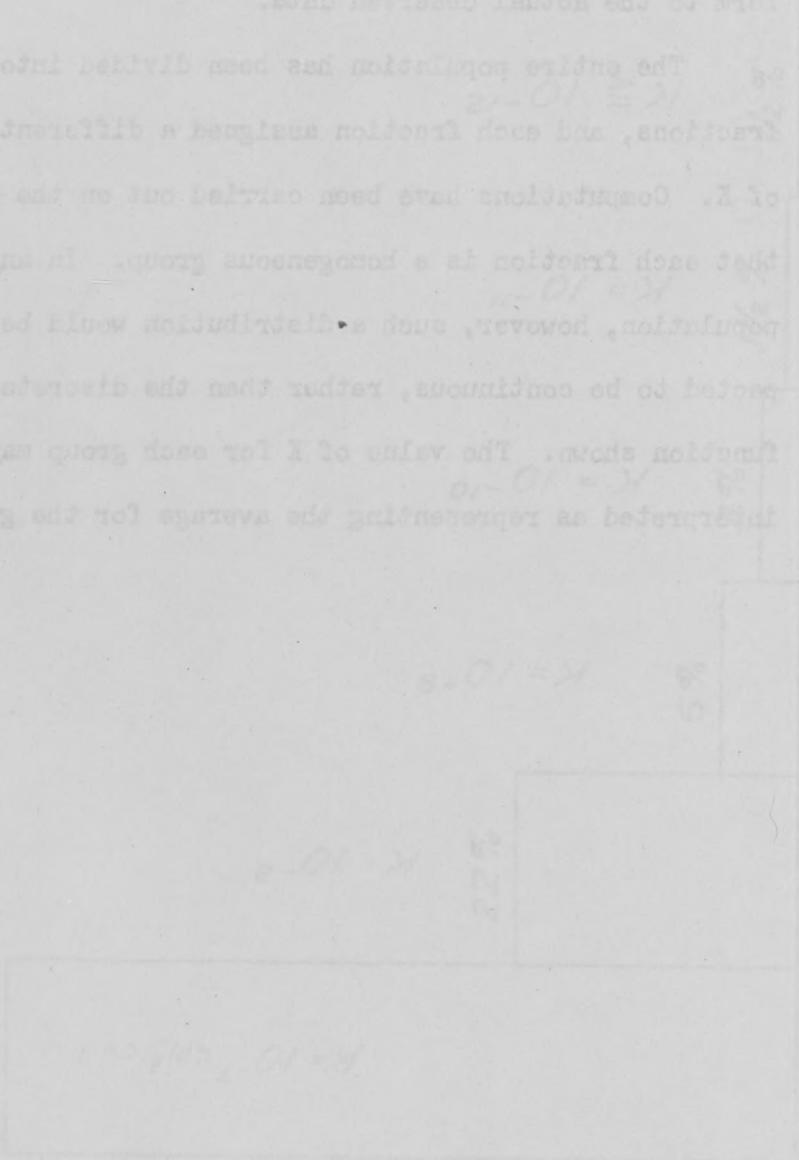The entire population has been divided into six fractions, and each fraction assigned a different value of K. Computations have been carried out on the assumption that each fraction is a homogeneous group. In any real population, however, such a distribution would be expected to be continuous, rather than the discrete step-function shown. The value of K for each group may be interpreted as representing the average for the group.

FIGURE 12

Distribution of equilibrium constants, K, in a heterogeneous phage population whose behavior is consistent with experimental data.
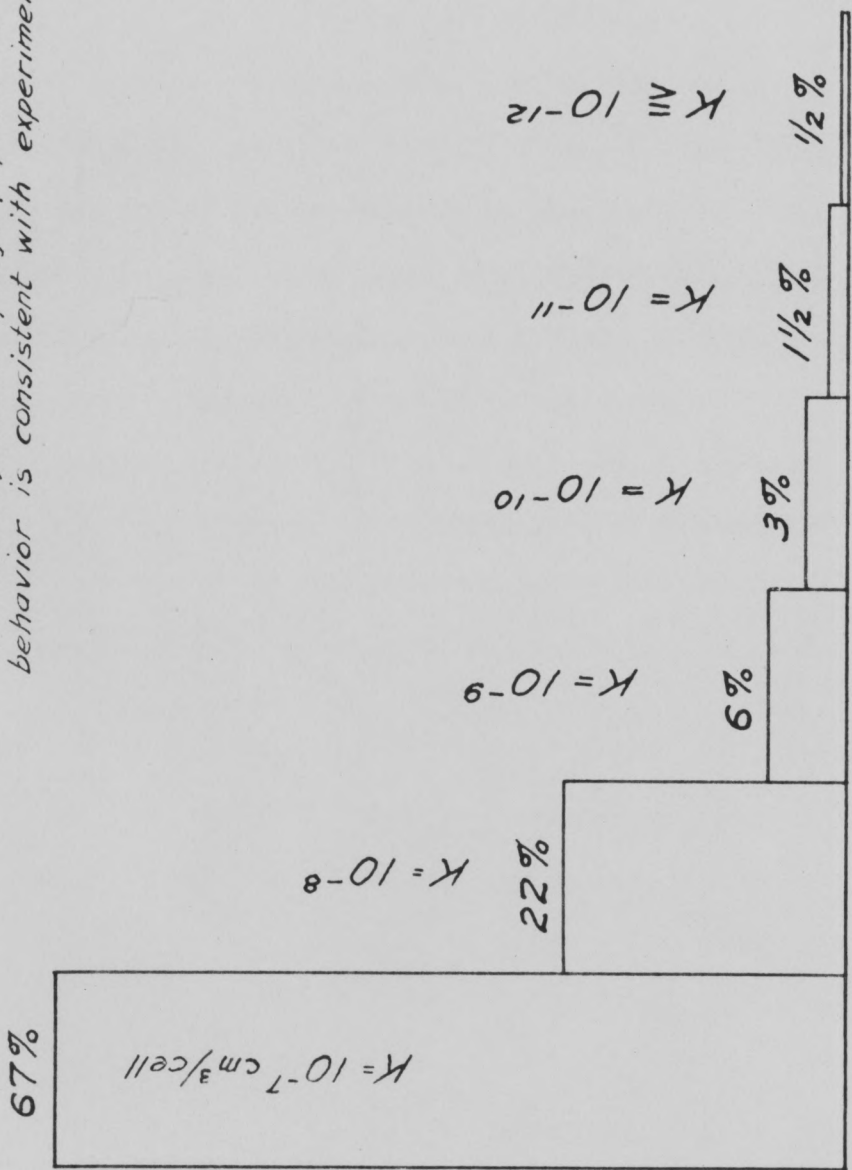
Figure 13

Equilibrium constant as a function of cell concentration
for the heterogeneous phage population described in Figure 12.

The fraction of free phage at equilibrium for each of the
six groups shown in Figure 12 was computed at cell concen-
trations of 10^7, 10^8, 10^9, and 10^{10}/cc. For each cell con-
centration, the free phage contribution of all the groups was
summed to give a total fraction of free phage, $[v]$. K at
each cell concentration was then computed as

$$K = \frac{1 - [v]}{[v][B]}$$

These points are shown as heavy filled circles. The hollow
circles represent the experimentally determined points ob-
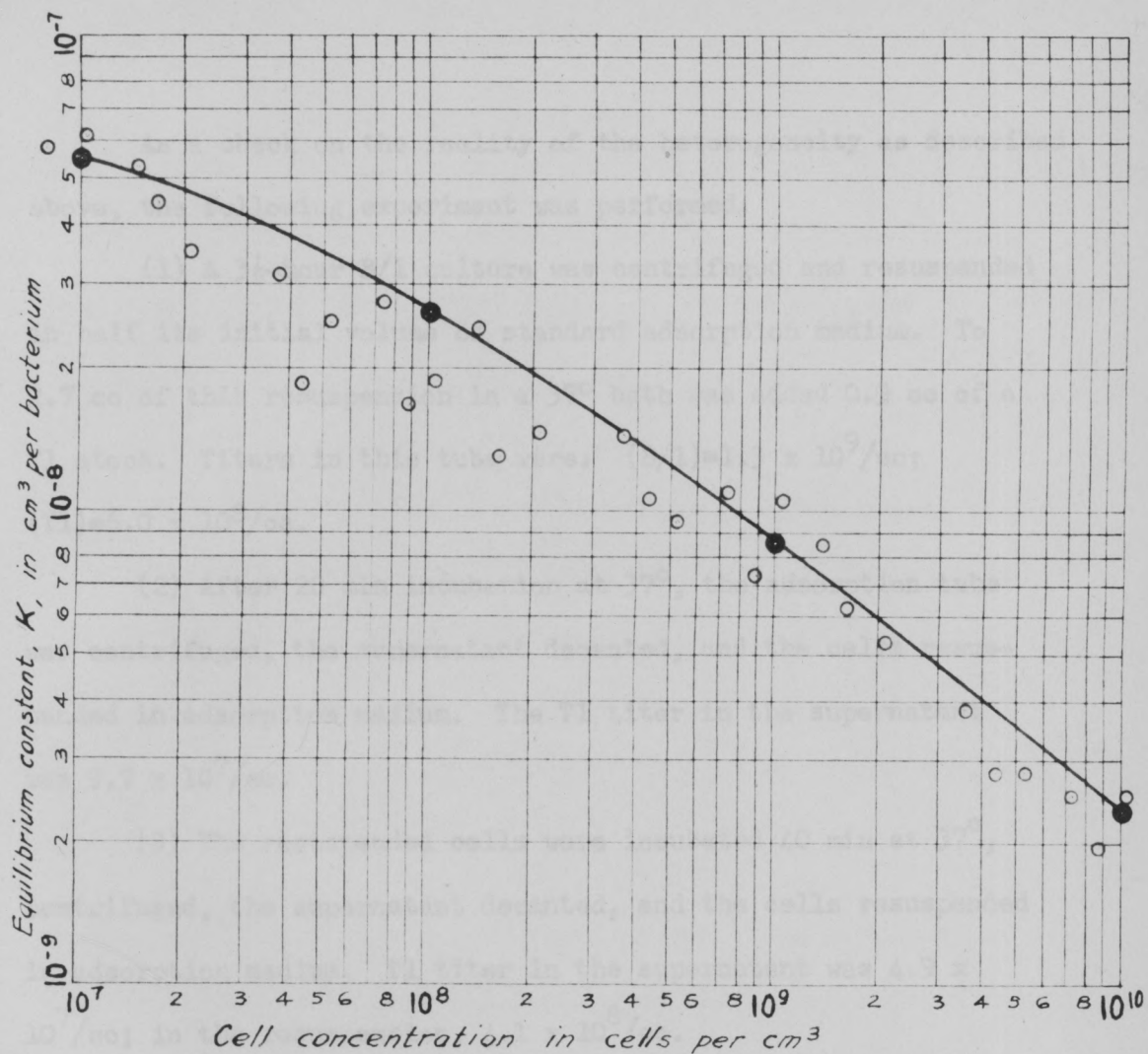tained with both B/1 and ultra-violet-killed E. Coli B,
as shown in Figure 9.

FIGURE 13: *Log-log graph of equilibrium constant, K, as a function of cell concentration, computed for the heterogeneous phage population described in Figure 12. (Solid circles)*
Experimentally determined points for T1 equilibrium with B/1 and ultra-violet-killed B are included for comparison.

As a check on the reality of the heterogeneity as described above, the following experiment was performed:

(1) A $3\frac{1}{2}$-hour B/1 culture was centrifuged and resuspended in half its initial volume of standard adsorption medium. To 2.7 cc of this resuspension in a 37° bath was added 0.3 cc of a T1 stock. Titers in this tube were: (B/1)=1.3 x 10^9/cc; (T1)=6.0 x 10^8/cc.

(2) After 20 min incubation at 37°, the adsorption tube was centrifuged, the supernatant decanted, and the cells resuspended in adsorption medium. The T1 titer in the supernatant was 9.7 x 10^7/cc.

(3) The resuspended cells were incubated 40 min at 37°, centrifuged, the supernatant decanted, and the cells resuspended in adsorption medium. T1 titer in the supernatant was 4.9 x 10^7/cc; in the resuspension, 4.1 x 10^8/cc.

(4) The resuspended cells were again incubated 40 min at 37°, centrifuged, and the supernatant decanted. T1 titer in the supernatant was 2.8 x 10^7/cc.

In the three adsorption cycles performed, it would be anticipated that those phage having the highest K would be selectively adsorbed, leaving those of lower K to be decanted in the supernatant. Thus, in each cycle, the fraction of free phage should decrease, and the computed K should rise. The results of this experiment are summarized in the following tabulation:

Cycle	Total phage	Supernatant phage	Fraction of free phage	Apparent K
1	6.0×10^8	9.7×10^7	0.161	4.0×10^{-9}
2	5.0×10^8	4.9×10^7	0.098	7.1×10^{-9}
3	4.1×10^8	2.8×10^7	0.068	1.0×10^{-8}

This trend of values for K constitutes definite evidence of the heterogeneity of the phage population, as postulated on the basis of the previous experiments.

C. Appendix

The 35-minute period chosen for the establishment of equilibrium conditions between T1 and its cellular environment was based on adsorption curves such as the one shown in Figure 14. It was later noted that on the basis of the heterogeneity postulated in Part III, a much longer time might be required for the more slowly adsorbing components of the phage to attain even a roughly approximate equilibrium state. An analysis was accordingly made to determine the extent of the error which might have been thus introduced.

The assumed model for interaction between virus and bacteria (equation $\boxed{1}$, page 5), gives us

$$\frac{d(V)}{dt} = -k_1(V) + k_2(VB) = k_2 - (k_1 + k_2)(V),$$ from which,

at the steady state in which $d(V)/dt = 0$, we have that $(V) = k_2/(k_1 + k_2)$.

In the attainment of equilibrium, (V) changes from a value of 1 to $k_2/(k_1 + k_2)$. Denoting by x the fraction of this total adsorption which will have taken place at time t, we then have at this time

$$(V_t) = 1 - \frac{xk_1}{k_1 + k_2} = \frac{k_2 + (1 - x)k_1}{k_1 + k_2} \ .$$

The differential equation describing (B) is given by

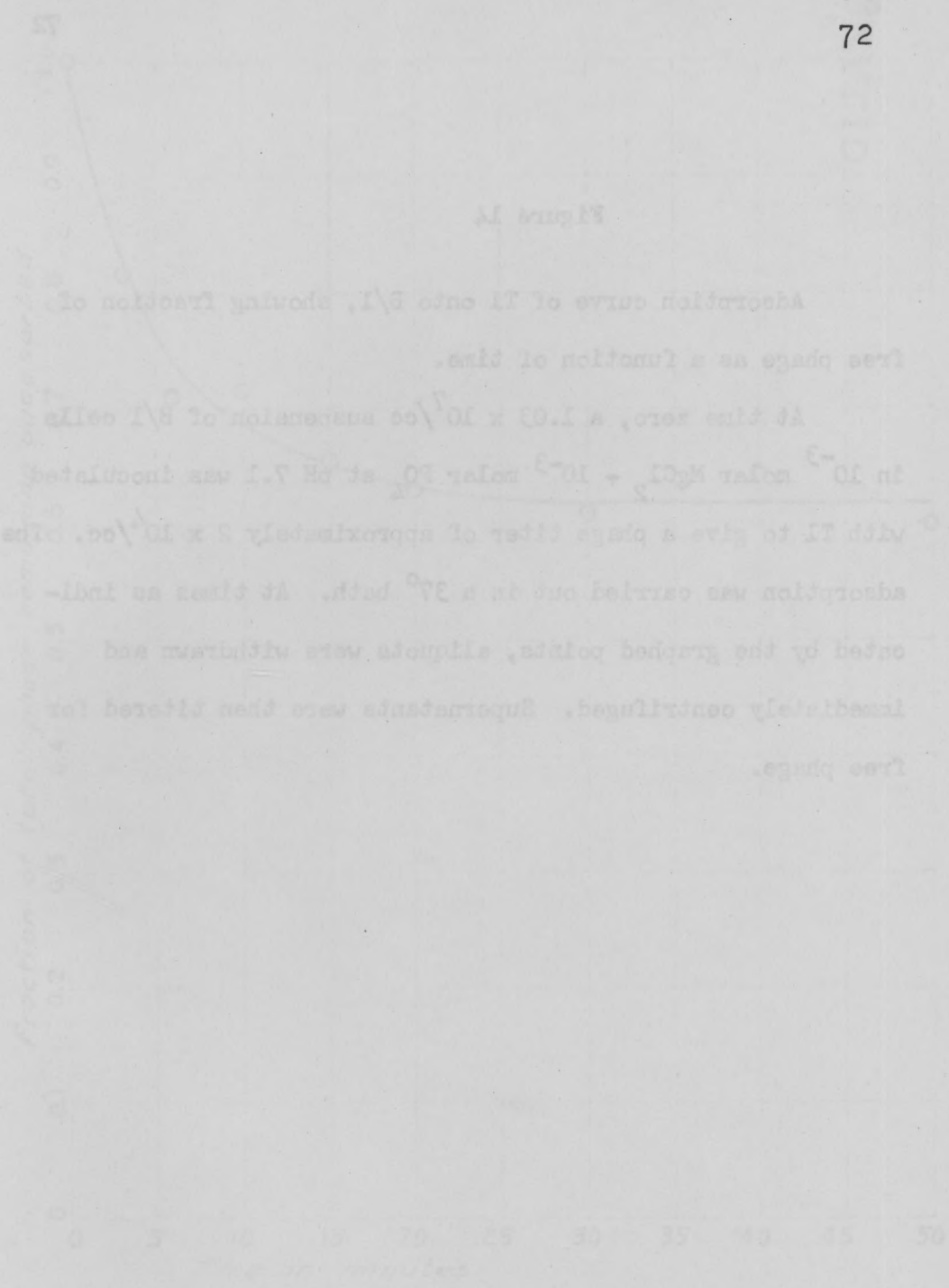$$\frac{d(V)}{k_2 - (k_1 + k_2)(V)} = dt,$$ which integrates to

Figure 14

Adsorption curve of T1 onto B/1, showing fraction of free phage as a function of time.

At time zero, a 1.03×10^7/cc suspension of B/1 cells in 10^{-3} molar $MgCl_2$ + 10^{-3} molar PO_4 at pH 7.1 was inoculated with T1 to give a phage titer of approximately 2×10^4/cc. The adsorption was carried out in a 37° bath. At times as indicated by the graphed points, aliquots were withdrawn and immediately centrifuged. Supernatants were then titered for free phage.
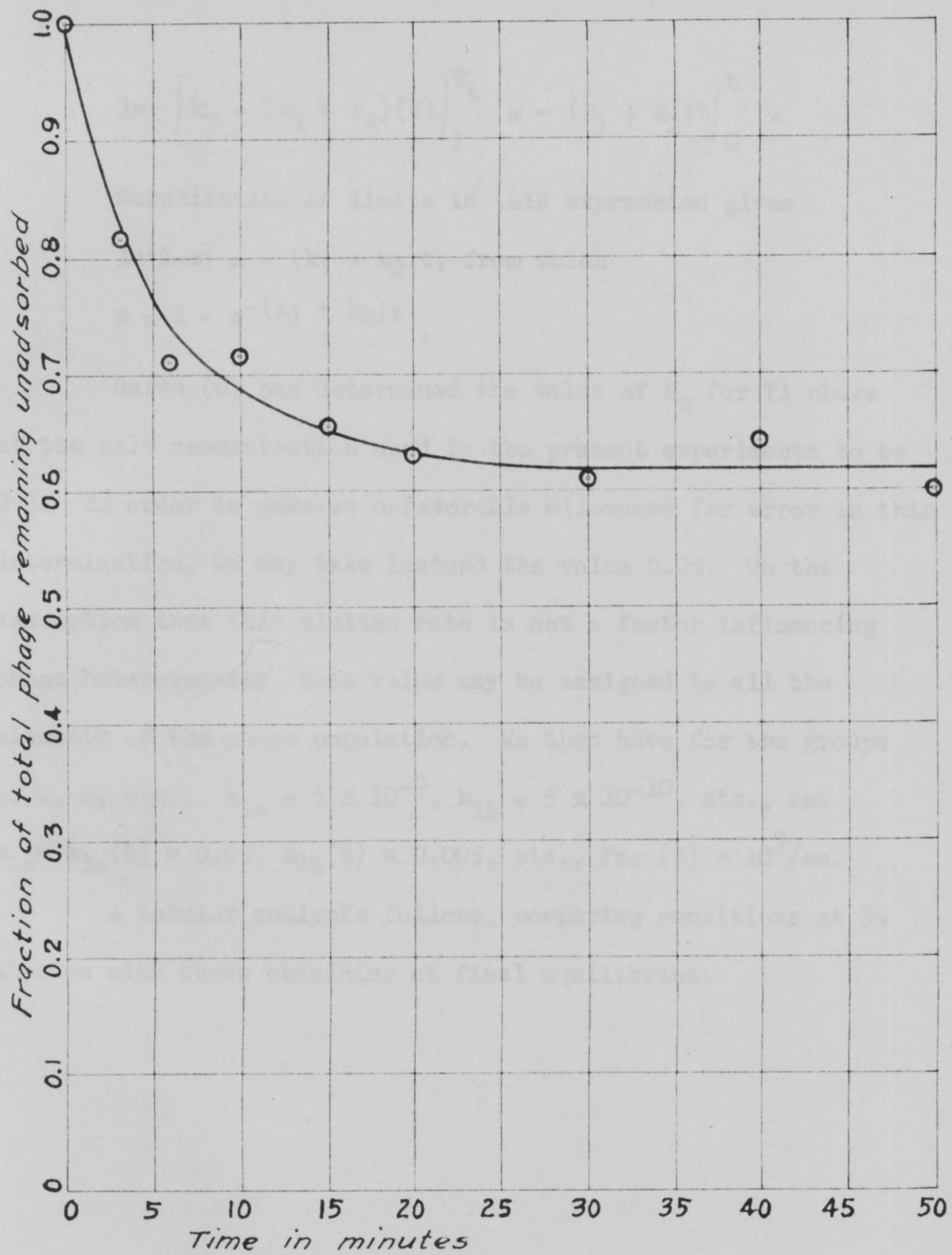
FIGURE 14 : Rate of adsorption of T1 onto B/1.
B/1 concentration = 1.03 × 10⁷ per cm³.

$$\ln \left[k_2 - (k_1 + k_2)(V) \right]_1^{V_t} = - \left(k_1 + k_2 \right) t \Big]_0^t .$$

Substitution of limits in this expression gives

$\ln(1-x) = - (k_1 + k_2)t$, from which

$x = 1 - e^{-(k_1 + k_2)t}$.

Garen (6) has determined the value of k_2 for T1 phage at the salt concentration used in the present experiments to be 0.1. In order to make an unfavorable allowance for error in this determination, we may take instead the value 0.05. On the assumption that this elution rate is not a factor influencing phage heterogeneity, this value may be assigned to all the elements of the phage population. We then have for the groups a, b, c, etc., $k_{1a} = 5 \times 10^{-9}$, $k_{1b} = 5 \times 10^{-10}$, etc., and $k_{1a} = k_{1a}(B) = 0.05$, $k_{1b}(B) = 0.005$, etc., for $(B) = 10^7/cc$.

A tabular analysis follows, comparing conditions at 35 minutes with those obtaining at final equilibrium:

Group	k_1	$k_1 + k_2$	$(k_1 + k_2) \times 35$	x, at $t = 35$ min	Fraction of phage in each class adsorbed at $t = \infty$	Fraction of phage in each class adsorbed at $t = 35$ min	Fraction of phage in each class free at $t = \infty$	Fraction of phage in each class free at $t = 35$ min	Fraction of total phage free at $t = \infty$	Fraction of total phage free at $t = 35$ min
a	0.05	0.10	3.50	0.97	0.500	0.485	0.500	0.515	0.335	0.345
b	0.005	0.055	1.93	0.86	0.091	0.077	0.909	0.923	0.200	0.203
c	0.0005	0.0505	1.77	0.83	0.009	0.008	0.991	0.992	0.059	0.059
d	0	0.05	1.75	0.83	0	0	1.00	1.00	0.030	0.030
e	0	0.05	1.75	0.83	0	0	1.00	1.00	0.015	0.015
f	0	0.05	1.75	0.83	0	0	1.00	1.00	0.005	0.005
							Total fraction free phage		0.644	0.657

The difference between the computed free phage titer at 35 minutes and that to be expected at equilibrium is thus seen to be about 2% of the measured value, an amount well within the attainable limits of experimental accuracy.

SUMMARY

Experiments with B, B/1 and B/1,5 showed that these bacterial cells will agglutinate and precipitate in certain ranges of concentration of H^+, Na^+, and Ca^{++}. This precipitation is reversible, and is apparently due to the neutralization of the surface charge by the cations, in a manner comparable to the flocculation of colloidal particles. The data available did not permit distinction to be made between the effects of ion binding and of double layer formation.

The regions of cation concentration within which B/1 and B/1,5 precipitated were identical within experimental error; the concentration needed to precipitate these mutants is markedly less than that required to precipitate the wild-type B. It can hence be concluded that the total surface charge of B/1 and B/1,5 is less than that of B, or that their ion-binding properties are markedly different. This differentiation is the first one other than their difference in virus resistance to have been made.

In equilibrating T1 with B/1, it was found that the equilibrium constant was a function of cell concentration, which could be empirically approximated as $K=1.9 \times 10^{-4} \times (B/1)^{-\frac{1}{2}}$, a hitherto undescribed relationship which should be of use to the phage experimenter. This same dependence of K on cell concentration

was found to hold between T1 and wild-type cells in which the second, irreversible, step had been inhibited by exposure to ultra-violet radiation.

In an effort to determine the cause of this variation of K with cell concentration, the following factors were investigated, and all found to be incapable of providing a satisfactory explanation:

1) the effect of exudation of cell metabolic products into the medium.

2) distortion of experimental data as a result of the shifting of the equilibrium between phage and cells during the process of centrifuging.

3) the effect of salt concentration.

4) the masking of accessible cell surface by the clumping of the bacterial cells at high concentrations.

5) the mechanical effect of cell collisions in enhancing the rate of elution of phage from the cell surfaces.

6) electrostatic interactions between cells.

7) phage-phage interactions.

A pattern of heterogeneity of the phage population was postulated, and a distribution function proposed, describing a phage population composed of elements having different K's, which was found to explain satisfactorily the observed variation in the measured overall equilibrium constant. Experimental evidence was presented to show that such a heterogeneity does exist.

REFERENCES

(1)   Puck, T. T., Garen, A. and Cline, J., Journal of Experimental Medicine 93 (1951), 65.

(2)   Garen, A. and Puck, T. T., Journal of Experimental Medicine 94 (1951), 177.

(3)   Puck, T. T., Science 114 (1951), 3.

(4)   Cleveland, J. M., Puck, T. T. and Tolmach, L. J. (to be Published)

(5)   Puck, T. T., Cold Spring Harbor Symposium on Quantitative Biology (1953), in press.

(6)   Garen, A., Ph. D. Thesis, University of Colorado, 1953.

(7)   Tolmach, L. J. and Puck, T. T., American Chemical Society Journal 74 (1952), 5551.

(8)   Dubos, R. J., The Bacterial Cell (Cambridge, Mass., Harvard University Press, 1946), 57.

(9)   Wollman, E. L. and Stent, G. S., Biochimica et Biophysica Acta 6 (1950), 292.

(10)   Wollman, E. L. and Stent, G. S., Biochimica et Biophysica Acta 8 (1952), 260.

(11)   Verwey, E. J. W. and Overbeek, J. Th. J., Theory of the Stability of Lyophobic Colloids (New York, Elesevier Publishing Co.).

(12)   Debye, P. and Hückel, E., Physik. Z. 24 (1923), 185.

(13)   Klots, I. M., in Trends in Physiology and Biochemistry, ed. by Barron, E. S. G. (New York, Academic Press, 1952), 427.

(14)   Edsall, J. T., in Cohn, E. J. and Edsall, J. T., Proteins, Amino Acids and Peptides (New York, Reinhold Publishing Company, 1943), 445.

(15)   Tolmach, L. J., to be published.

(16)   Puck, T. T., and Sagik, B., Journal of Experimental
       Medicine $\underline{97}$ (1953), 807.

(17)   Luria, S. E., Cold Spring Harbor Symposium on Quantitative
       Biology XI (1946), 130.

(18)   Kennard, E. H., Kinetic Theory of Gases (New York, McGraw-
       Hill Book Company, 1938), 228.

(19)   Margenau, H. and Murphy, G. M., The Mathematics of Physics
       and Chemistry (New York, D. Van Nostrand Co., 1943), 223.

(20)   Verwey and Overbeek, op. cit. (Ref. 11), 166.

(21)   Sagik, B., Ph. D. Thesis, University of Illinois (1952).