

INDOLE INHIBITION OF COFACTOR ACTIVATED BACTERIOPHAGE

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ABSTRACT

Certain bacteriophage strains must be activated by a cofactor before attachment to their host cells is possible. In some of these strains indole competitively inhibits the activation. This inhibition by indole is the subject of the present study.

The following sets of measurements were made:

- 1) Kinetics of the approach to equilibrium from both sides.
- 2) Deactivation in the presence of indole.
- 3) Activation in cofactor after pre-equilibration with indole.
- 4) Measurements of the equilibrium activity in the presence of various cofactor-indole mixtures.

The following principal conclusions may be drawn:

- 1) A true thermodynamic equilibrium exists between indole-deactivated virus and active virus.
- 2) The presence of indole increases the rate of deactivation of the virus population in the absence of external cofactor, over that found when only cofactor is removed.
- 3) Indole combines only with active phage particles.
- 4) At equilibrium in the presence of high concentrations of cofactor and in the range of strong inhibition, the activity is inversely proportional to the concentration of indole.

These findings make it necessary to modify the theory of activation previously developed by Stent and Wollman. It is assumed that the surface of the phage particle, during the process of activation, undergoes a structural change perhaps similar to the reversible denaturation of proteins.

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I INTRODUCTION

The bacterial virus used in this work is a submicroscopic, tadpole-shaped particle with a nearly spherical head piece and a club-shaped tail. The head piece consists of a protein coat and a desoxyribosenucleic acid core. The virus reproduces only by introduction of the core into a specific bacterial cell, to which it attaches by means of the tail (1). After replication of the virus within the host, the cell lyses liberating many progeny particles similar to the infecting particle.

Certain of these bacterial viruses are unable to attach to their host unless brought into an active state by first reacting reversibly with an agent called a cofactor. This phenomenon, called cofactor activation, was discovered by T. F. Anderson in 1944 (2). The amino acid l-tryptophane was found the most active of a series of compounds with cofactor activity. The d enantiomorph was found to be completely inactive and not inhibitory (3). In 1948 M. Delbrück discovered that indole, a homologue of tryptophane, inhibited the ability of l-tryptophane to activate some of these viruses (4).

This series of discoveries set the stage for more thorough work on this problem. During the years 1948 through 1950 G. S. Stent and E. L. Wollman carried on a

detailed analysis of the kinetics of cofactor activation (5,6,7). Anderson had previously described the threshold nature of the action of tryptophane. He had found that the fraction of particles able to form a union with cells in the presence of cofactor was proportional to the fifth or sixth power of cofactor concentration. Anderson concluded that activation required the cooperation of at least five or six l-tryptophane molecules (2). It was then surprising that Stent and Wollman found that activation proceeded at a rate which was constant with time after mixing cofactor and inactive bacteriophage. For the rate would be expected to increase as the number of bacteriophage particles with one less than the required number of adsorbed molecules increased. Stent and Wollman postulated that a further, rate limiting, reaction occurs among five molecules adsorbed to a local area (site) on the surface. This further reaction locks the cofactor molecules in a fixed pattern and then produces an area complementary (active site) to the bacterial cell membrane, thereby promoting attachment of virus to cell.

In this work Stent and Wollman had taken advantage of an unexpected property of these cofactor requiring phage particles. It had been known that an infected cell could in some manner form a viral colony in a confluent bacterial growth on solid medium without the presence of cofactor. On the other hand a free phage particle under similar condi-

tions will not form such a colony. This allowed a means of measuring the number of virus-infected cells in the presence of free virus, therefore the rate at which phage particles attach to cells. In a subsequent study of this property Stent and Wollman have shown that phage particles are liberated from bacteria in an active state (8). They have called this property nascent activity. A nascent phage population loses activity slowly to become a quiescent phage suspension. It was shown that each nascent phage particle is in a state intermediate between full activity and complete inactivity. In the case of quiescent phage particles methodological reasons prevent at present a similar determination of the meaning of activity with respect to individual particles.

M. Delbrück and A. D. Hershey had found that small concentrations of residual tryptophane, though unable to impart significant activity to inactive phage particles, could nevertheless retard the initial rate at which a fully active suspension loses activity. The actual retardation of the rate was found by Stent and Wollman to be approximately proportional to the first power of the residual tryptophane concentration. This effect allowed a decision in favor of cooperation of molecules on the surface of phage particles rather than formation of micelles of five tryptophane molecules in solution (6). Stent and Wollman presented a model of cofactor activation (6) permitting a unified interpreta-

tion of their experimental results. The work described here was undertaken in order to test the ability of this model to account also for the kinetics of indole inhibition.

At the beginning it was realized that a convenient, rapid method of determining the rate at which phage becomes active would be desirable. T. T. Puck had found that l-tryptophane could activate phage particles to attach to fritted glass filters (9). J. J. Weigle noticed that active phage particles could be observed attaching to a glass cover slip under the dark-field microscope (10). By combining these findings it would be possible in principle to develop a technique for studying quantitatively the activation process. A chamber is filled with a suspension of inactive phage particles and viewed with the dark-field microscope while cofactor is added. The number of particles attaching to a well cleaned glass cover slip after a given time would be a measure of the rate of activation if diffusion to the glass surface were not limiting. In practice this technique did not provide the necessary time resolution due to the long exposure times required to obtain permanent photographic records. It was therefore abandoned and the more usual phage techniques applied to the problem.

Preliminary results by M. Delbrück (11) had demonstrated that the equilibrium fraction of active particles in the presence of tryptophane and various quantities of indole depended on the inverse first power of indole con-

centration. He had also shown that this active fraction remained approximately constant in the presence of a constant ratio of tryptophane to indole concentration when the tryptophane concentration alone would impart full activity. In addition to this he had demonstrated that this equilibrium could be approached from both directions.

After confirming these findings our attention turned to their meaning in relation to the Stent-Wollman model. Very early we felt that the dependence of the equilibrium active fraction on indole concentration provided data which would not fit into the concepts which they proposed. An analysis of their model in terms of chemical probabilities which included the competitive effect of indole on the phage-tryptophane reaction did, indeed, predict an entirely different dependence of this active fraction on indole concentration. Other experiments demonstrated that indole had ability to increase the deactivation rate over that found when only tryptophane was removed.

It was at this point that an entirely new approach to cofactor activation was made. Previous approaches to this problem have been made from the assumption of an essentially rigid structure on the viral surface to which cofactor adsorbed. M. Delbrück suggested the existence of an equilibrium between various alternative structural configurations of the tail protein, analogous to what is known to exist in the case of reversible denaturation of various proteins.

We assumed that some of these configurations might be complementary, some not complementary to the bacterial cell membrane and that the complementary structure is stabilized by the adsorption of tryptophane. Shortly after this proposal was made Sato (12) found that a 14% urea solution at 0°C, a powerful denaturing agent, is able to bring cofactor-requiring phage particles into an active state. This discovery may therefore be considered as proof that the proposed structural changes leading to activation actually occur. Other findings also indicate that structural changes may well be associated with the activation process. Mandell (13) has shown that the rate of inactivation of phage by specific antibody at 37°C is approximately 2.5 times greater for phage particles equilibrated with l-tryptophane than for inactive phage. Mandell has demonstrated that indole antagonizes this effect of tryptophane. The measurements to be presented also suggest that the ideas of Stent and Wollman are not adequate to interpret the effects of indole.

Aside from its importance in the field of virology this phenomenon may provide a new approach to problems of general biological interest. A critical problem in biology centers on the ability of small molecules to regulate reactions within the cell. These molecules may be provided to the cell in the form of drugs or hormones, but they may also be synthesized within the cell at some phase of the division cycle and utilized for the performance of some

specific function. We recognize in this system a model of great utility, partly because of the lack of any known metabolic action of the virus on the cofactor, partly because of the lack of uncertainties introduced by cell membranes but mostly because of the relative ease with which quantitative results may be obtained.

II MATERIALS AND METHODS

1. Medium

A synthetic salt-lactate medium designated as F medium was used in all experiments where a cofactor-free medium was required. F medium has the following composition: Na₂HPO 3.5 g/l; KH₂PO₄ 1.5 g/l; NH₄Cl 1 g/l; NaC₃H₅O₃ 15 ml of a 60% solution/l; CaCl₂ 0.08 ml of a 10% solution/l; MgSO₄ 0.5 ml of a 1 M solution sterilized separately/l. F medium was made up in 18 liter lots to provide constant conditions from day to day. A uniform source of glass-distilled water is necessary.

N medium containing 10 g/l Difco bacto tryptone and 5 g/l NaCl was used when a medium requiring excess cofactor was required.

2. Bacteriophage stocks

Bacterial virus T4.38r⁺ (5) was used throughout this work. A stock originally made by Stent and Wollman was used until depleted. This stock, stored in N medium, had a very stable titer of 2×10^{10} particles/ml. A new stock was obtained from this by mass lysis of a bacterial culture grown in Fraser's medium.* This crude lysate was centri-

*Fraser's medium has the following composition: KH₂PO₄ 4.5 g/l; Na₂HPO₄ 10.5 g/l; NH₄Cl 3 g/l; Glycerine 30 g/l; Casamino acids 15 g/l; Gelatin 3 ml of a 1% solution; CaCl₂ 3.0 ml of a 0.1 M solution sterilized separately; MgSO₄ 3.0 ml of a 1 M solution sterilized separately.

fuged to remove bacterial debris and dialyzed against F medium in which it was stored at 10°C. This stock had a stable titer of 2×10^{11} particles/ml of which 2×10^6 particles/ml were tryptophane independent mutants.

3. Bacteria

Escherichia coli strain B was employed in the experiments described here. Cells were grown in F medium with moderate aeration to 5×10^9 cells/ml, requiring about 12 hours. It was found necessary to use such a culture, which is nearly saturated, as soon as it attained this concentration. A standard bacterial suspension is obtained by washing (centrifuging twice) and resuspending these cells in a volume of F medium twenty times the original volume. Data obtained using cells from the same culture were reproducible to about $\pm 25\%$, from different cultures to about $\pm 50\%$.

4. Cofactor and Inhibitor Stocks

Commercially available crystalline l-tryptophane and indole were dissolved with moderate heating in sterile distilled water to provide solutions of cofactor and inhibitor. Solutions were stored at 10°C in glass stoppered bottles. A fresh solution of indole was made monthly, of l-tryptophane once every two months.

5. Assays

A bacterial virus suspension may be assayed by first

placing a small volume of it on the surface of solid medium in a Petri dish. This is followed by overlaying with approximately 2×10^7 bacterial cells in melted agar medium.* After incubation at 37°C for 10 hours small clear areas, known as plaques, are found in an otherwise confluent bacterial growth. Each plaque corresponds to either an infected cell or to a free virus particle originally present in the volume placed on the solid medium. Such an assay may be performed under essentially cofactor-free conditions (F assay), or under conditions in which cofactor is in excess (N assay).

Unattached virus particles do not form plaques on cofactor-free medium, but infected cells do form plaques. The F assay therefore determines the titer of attached virus in a mixture of free and attached virus. Both attached and free virus particles form plaques on N medium. If the fraction of multiple infected bacteria is negligible, the ratio of F assay to N assay is a measure of the fraction of virus which have attached to cells. This ratio of assays is called the F/N ratio.

* We have departed from the usual technique of phage assay wherein phage and bacteria are mixed in 45°C top agar before being poured over the solid medium in a Petri dish. This departure has been helpful in preventing premature bursts of virus infected cells caused by sudden changes in temperature. We have also used less plating bacteria to minimize the appearance of small, turbid plaques when the activity is depressed to values in the range 10^{-3} . All other techniques have been according to standard practice (14).

6. Measurements of the Degree of Activity

The degree of activity, D , is a variable defining the state of a virus population relative to the ability of its members to attach to bacteria. It ranges in value from zero to one. It is operationally defined in an experiment of the following kind: A small volume of virus suspension at activity D is rapidly diluted with a large volume of the standard bacterial suspension. Cofactor and inhibitor are reduced to low residual concentrations, c_r and i_r , respectively. Values of c_r below 0.2 mg/l and i_r below 0.005 mg/l have no effect on the subsequent events. Immediately upon dilution a competition sets in between loss of activity and attachment of the virus. The fraction of the total phage population which forms an essentially irreversible union with cells is determined by an F/N assay after sufficient time has been allowed for the mixture to come to equilibrium, normally about 10 minutes. Under certain conditions this fraction is proportional to the degree of activity of the virus population at the instant of the dilution. The requirements for this proportionality are (a) that the virus population lose activity at a first order rate, (b) that the fraction attaching be small compared with unity, and (c) that this rate be independent of the previous history (see Appendix I). Conditions (a) and (b) are met in practice. Measurements of the rate of inactivation of an indole inhibited phage suspension when both cofactor and

indole are diluted indicate that condition (c) is also approximately met (Fig. 1).

The accuracy of measurements of activity below 5×10^{-3} is limited by the occurrence of small, turbid plaques on plates yielding data in this range. Some of these plaques appear mottled with r clones, but single cell burst experiments failed to show any r particles in the immediate progeny. The presence of these plaques has caused measurements of D below 10^{-3} to be approximately 10% too high; the error slowly increases with smaller values of activity.

In each experiment a fully active suspension of virus (equilibrated with 20 mg/l l-tryptophane) is diluted with a standard bacterial suspension under the same conditions as those in which D is to be determined. The fraction of virus ultimately adsorbed in this case (ca. 0.05) is divided into each of the other fractions to determine their respective values of D at the time of the dilution.

The method described above is called the dump technique (2). Its chief utility in this work stems from the possibility which it affords in carrying out instantaneous measurements of the degree of activity. Stent and Wollman determined the degree of activity by measurements of attachment rates and showed these measurements to be in agreement with those found in the dump experiment (5). We have used the dump technique exclusively in this work because of the surprisingly high rate at which tryptophane is degraded to

indole by bacteria. This effect may be demonstrated to play an extreme role in the life cycle of these tryptophane-requiring phage particles. After exposing 20 mg/l l-tryptophane to the standard bacterial suspension at 15°C and 37°C the cells are removed by filtration. The filtrate is used to activate a suspension of phage particles at 15°C. The final fraction attaching in a dump experiment employing these phage particles is much less than a suspension previously activated with fresh cofactor. We have the following results:

Table I

Bacterial concentration	Time tryptophane incubated (min)	Temperature of incubation	Fraction attaching	D
2.5×10^8	10	15°C	0.0084	0.11
2.5×10^8	25	15°C	0.0020	0.026
2.5×10^8	8	37°C	0.0018	0.023
2.5×10^8	20	37°C	0.0018	0.023
--	(fresh cofactor)	--	0.077	1

In 10 minutes enough inhibitor has been formed by bacteria at 15°C to depress the activity to 0.11. The dump technique is of great value in overcoming this difficulty for it offers instantaneous measurements of the attachment rate under conditions in which the conversion of cofactor to indole is negligible.

7. Measurements of Rates of Activation and Deactivation

Loss or gain of activity has been followed with the dump technique. Some deactivation and activation experiments require dilution of cofactor or indole. In these experiments phage suspensions are blown with a temperature equilibrated pipette into a reaction tube containing a suitable concentration of indole or cofactor. In experiments where time allows small aliquots of this reaction mixture are distributed to a series of empty tubes. The standard bacterial suspension is poured over the aliquot to perform the dump. Rapid reactions do not permit this distribution; in such cases the aliquot is blown with a pipette, previously cooled to the temperature of the aliquot, into the standard bacterial suspension at the time of the dump.

In other experiments only indole is added while the cofactor concentration is kept constant. These experiments were performed by placing a drop of an active phage suspension in the bottom of a flat bottom beaker. The deactivation is started by allowing another drop with indole at twice the desired final concentration to mix with the first drop keeping the cofactor concentration constant. Separate mixtures are allowed to deactivate for various times before being diluted with a suitable volume of the standard bacterial suspension to perform the dump. All reactions and dump experiments have been carried out at the standard temperature of 15°C.

III EXPERIMENTAL RESULTS

1. The Approach to Equilibrium Activity

We shall first consider the kinetics of the approach to a constant value of activity. Either cofactor or indole was added to a suspension of either inactive or active phage particles. The resulting changes in activity were followed with the dump technique. Measurements of this approach were made using a value of cofactor concentration (20 mg/l) which would elicit maximal activity in the absence of indole.

A suspension of active phage particles in the presence of 20 mg/l 1-tryptophane was diluted into a mixture of cofactor and indole keeping the cofactor concentration constant. When the indole concentration added is 0.05 mg/l the activity is depressed until it reaches a constant value of 0.34 in approximately 2 minutes (fig. 2, upper curve). The same value of activity is approached from below when phage particles previously equilibrated with 0.05 mg/l indole are exposed to 20 mg/l 1-tryptophane (fig. 2, lower curve) keeping the indole concentration constant. A true thermodynamic equilibrium exists between the active and inactive phage particles.

Larger values of indole concentration have been employed to depress the activity in the presence of 20 mg/l

l-tryptophane (fig. 3). The initial rate of deactivation is increased by only a relatively small amount over that found when 0.05 mg/l indole is added. An eighty-fold increase in indole concentration results in an increase of the initial rate by a factor of four. The equilibrium value of activity, however, is depressed severely. In the case of an eighty-fold increase in indole concentration the activity is depressed by a factor forty.

The results of the inverse experiment in which 20 mg/l l-tryptophane is added to an indole-equilibrated bacteriophage suspension are shown in figure 4. In these experiments the indole concentration remains constant. By comparison with figure 3 we note that the equilibrium value of activity of 0.007 is approached from both directions irrespective of the order in which cofactor at 20 mg/l and indole at 4 mg/l are added. If the indole concentration is 0.5 mg/l the activity rises from 0 to 0.1 when cofactor is added last, and falls from 1.0 to a value 0.07 when indole is added last. The difference in these values is within experimental error. Thus a true equilibrium exists also at higher values of indole concentration.

2. The Effect of Indole on the Deactivation Rate when Cofactor is Removed

During experiments designed to determine a safe upper limit of residual indole concentration in the dump mixture

it was noticed that small amounts of indole added directly to the dump bacteria were able to significantly lower the fraction of phage ultimately attaching in a dump experiment. If larger and larger amounts of indole are added to the dump bacteria the ratio of the fraction attaching without indole to that attaching with indole increases rapidly to the value 42 at 20 mg/l indole (fig. 5). At this point its value remains essentially constant even though higher values of indole concentration are added to the dump bacteria. The ability of indole to increase the rate of deactivation over that found when only cofactor is diluted was believed to be responsible for this reduction of the fraction attaching with indole mixed with dump bacteria. A direct test of this was undertaken by measurements of deactivation rates when cofactor is removed and indole is added. The results did, indeed, show an increase in this rate.

When l-tryptophane is removed by dilution from a suspension of cofactor-requiring bacteriophage, activity is lost at a first order rate with a half-life of 34 seconds (5). It is possible to follow the deactivation of a fully active phage suspension when simultaneously cofactor is removed and indole is added. Virus is first equilibrated with 20 mg/l l-tryptophane to provide unit activity. This mixture is diluted into an indole solution of suitable concentration reducing the cofactor concentration below 0.2 mg/l. The activity is estimated at various times by use

of the dump technique. As predicted, deactivation proceeds at a greater rate than that found when only cofactor is removed (fig. 6). Indole in a concentration of only 0.01 mg/l causes a doubling of the initial deactivation rate.

When larger values of indole concentration are used the rate decreases during the deactivation process causing a bending of the curves. A definite break in the curves first occurs for values of indole concentration near 0.1 mg/l. After the break in the curves the deactivation proceeds in typical first order manner (straight line on a log activity vs. time plot) with an approximately constant deactivation rate in the range 0.1 to 4 mg/l indole. The ultimate deactivation rate constant has a value 6/min at an indole concentration of 4 mg/l.

The decrease in the ability of indole to affect the rate of deactivation for larger and larger values of indole accounts for the fact noted above that larger values of indole in the dump bacteria do not continue indefinitely to reduce the fraction attaching.

The behavior of a phage suspension when simultaneously cofactor is removed and indole is added suggest two processes by which a phage particle may become inactive. A particle may become inactive spontaneously or it may be induced to become inactive by indole. In order to more clearly show this division we may plot the fraction of particles which have become inactive by the induced route

after any given time. This is accomplished by calculating the fraction: activity with indole/activity without indole (fig. 7) from the curves of figure 6. After the initial break these curves are straight lines indicating that the indole-induced deactivation process is first order.

3. Activation after Pre-equilibration with Indole

The results of experiments in which cofactor is added to an indole-equilibrated phage suspension (fig. 4) show an initially rapid rise of activity for all but the highest indole concentration of 4 mg/l. We suspected that the presence of indole had only a small effect on the rate of this initial rise. An experiment designed to show more clearly this feature of the kinetics was conducted. A phage suspension was pre-equilibrated with 0.5 mg/l indole. At the start of the experiment indole was diluted to a very small residual concentration and 5 mg/l l-tryptophane was added. The rate of activation was approximately the same as that of a control experiment in which the phage suspension was not pre-equilibrated with indole (fig. 8) and the same amount of tryptophane was added. In the control a small amount of indole was added equal to the residual indole concentration of the experiment. The results are very suggestive that indole is not adsorbed by the inactive phage particles.

4. The Value of the Equilibrium Activity in Various Mixtures of Cofactor and Indole

After equilibrating virus with l-tryptophane and indole, dump experiments are performed to determine the value of the equilibrium activity. The results of these measurements are shown in figure 9 where indole is varied for constant values of cofactor, producing a family of curves. For values of indole in the range 1 to 100 mg/l at 20 mg/l l-tryptophane, the decrease in activity produces a straight-line dependence with a slope -0.92 on a log activity vs. log indole concentration plot. The difference between -0.92 and -1.00 is within experimental error. Experimental values of activity in the range of 5×10^{-3} are in error because of the occurrence of the small, turbid plaques mentioned earlier (Section II, 6). These extraneous plaques have caused values of measured activity to be greater than the true activity. Since the error increases with decreasing activity the experimental curves are not as steep as the true values of activity would indicate. This correction would tend to change the value of the slope toward -1.0. In the straight line region the other curves have values of slope equal to 0.71, 0.72, 0.25 for values of l-tryptophane concentration 2, 1, and 0.5 mg/l, respectively.

IV DISCUSSION

1. The Attachment Process.

The process by which active bacteriophage particles attach to bacteria displays properties of a typical reaction involving ionized groups. The initial attachment rate is exceedingly rapid, being under some conditions diffusion limited (15). The rate is dependent on the ionic constitution of the medium (9). The properties of this attachment reaction are similar to attachment of phage particles to cationic exchange resins (16) and to glass (9).

After the initial binding a further step in the life cycle of the virus may be detected. During the time between initial attachment and this second step the phage particle may be readily removed from the bacterial membrane (17). There is, in fact, evidence that this second step may become rate limiting at high bacterial concentrations (18). Puck (17) has shown that cofactor is required for the primary attachment. It is not known whether it is also required for the second step. Working with phage T2 (closely related to T4) Puck found suggestive evidence that carboxyl and amino groups play a role in this primary electrostatic reaction (19). Using arguments relating principally to experiments on the physico-chemical properties

of the bacterial membrane and on host-virus specificity Puck (16) and Weidel (20) have concluded that these groups lie imbedded in a regular, repeated pattern throughout the exterior of the cell. When a virus having a complementary pattern on its surface is brought by collision into proximity with this structure binding occurs. These findings strongly suggest that the process of activation is essentially a reaction by which the virus gains this complementarity for primary attachment. This complementarity was believed to be brought about by the presence of cofactor molecules on a static surface which were themselves a necessary part of the pattern.

We shall first discuss how our results led us to believe that the Stent-Wollman concepts would have to be modified. Then we shall discuss qualitatively the more recent proposals about the processes by which complementarity is gained.

2. The Stent-Wollman model

Stent and Wollman (6) interpreted their results in terms of a formal model. In this model cofactor molecules rapidly adsorb to and desorb from the surface of the virus. If five cofactor molecules happen to lie in close proximity on this surface they interact and lock into some kind of special configuration which is bound to the surface more firmly than the separate cofactor molecules. Such rings of

cofactor molecules are active sites. Sites become inactive by loss of a single cofactor molecule from the ring. The sites with four bound tryptophane molecules are called "sub-active" sites. "Sub-active" sites display no physiological activity but may be activated by adding a single cofactor molecule to complete the ring. The "sub-active" sites were introduced to account for the departure from first order kinetics found when l-tryptophane was reduced from high values to values in the range 0.5 to 1.5 mg/l.

It was also found that the initial rate of deactivation was dependent on the residual cofactor concentration in the range 0.5 to 1.5 mg/l. To account for this a further kind of site was proposed: a "protected active site." This is an active site (having five bound cofactor molecules) to which an additional cofactor molecule has adsorbed. An equilibrium between this site and the active site is assumed to exist at all times because of the rapidity with which the single molecule adsorbs and desorbs. Thus when tryptophane is diluted to values in the range 0.5 to 1.5 mg/l only a fraction of the total active sites will be unprotected and lose activity. For even at these low values of cofactor concentration there will be an appreciable fraction of initially active sites with one additional adsorbed cofactor molecule and therefore, in the protected state.

3. Analytical Formulation of the Stent-Wollman Model:

Introduction of Indole

In their analysis of these ideas Stent and Wollman assumed that cofactor molecules were distributed at random among the sites in the pre-equilibrium before "ring" formation. They assumed that saturation of sites with cofactor molecules did not occur. They applied a Poisson distribution to determine the fraction of inactive sites with five adsorbed l-tryptophane molecules. We will now show the competitive effect of indole on this system and will take into account saturation.

We wish to determine the number of sites which can become active, i.e. which have adsorbed five l-tryptophane molecules and no indole molecules. Assume that all molecules adsorb and desorb rapidly so that the processes are in equilibrium at all times. In this pre-equilibrium let Π_i be the fraction of loci occupied by species i, let n_i be the concentration of species i. We assume that tryptophane and indole in the medium are not significantly depleted by adsorption. We then have

$$\Pi_i = \frac{b_i n_i}{1 + b_1 n_1 + b_2 n_2} \quad \cdot \quad i = 1,2 \quad (1)$$

We will call b_i the intrinsic equilibrium constant.

Expressions of this functional form have been derived for one adsorbent using the law of mass action (21,22). It is much simpler to approach the problem as we have done here with an analysis similar to the derivation of Langmuir's adsorption isotherm (23).

Inactive sites, having adsorbed five tryptophane molecules, activate at the specific rate k_a . We will omit from this consideration both the "sub-active" sites and the "protected" active sites of Stent and Wollman. The probability that five cofactor molecules will adsorb at the same site will be \prod_1^5 . The rate of activation will then be proportional to $k_a \prod_1^5$. We will assume that indole simply competes for points of adsorption within the site and therefore does not affect the deactivation rate*. Under these conditions the equilibrium degree of activity will also be proportional to $k_a \prod_1^5$. At large indole concentration this becomes $k_a (b_1 n_1 / b_2 n_2)^5$. This analysis, in which indole competes with l-tryptophane at each of its points of adsorption in the "rings" is not in agreement with experiment. At high indole concentration in the presence of 20 mg/l l-tryptophane the equilibrium activity is found experimentally to be proportional to $1/n_2$. As the indole concentration is increased

*The possibility that indole may adsorb at the super-numerary locus to inactivate the site will be considered later.

under the assumptions we have made the equilibrium activity will depend on the inverse fifth power of indole concentration contrary to the experimental finding. We conclude that the effect of indole cannot be understood on the basis of purely competitive inhibition in the Stent-Wollman model.

4. A Review of the Basic Assumptions of the Stent-Wollman Model

The model developed by Stent and Wollman has accounted for the large variety of experimental results found by these workers. The basic assumption of a surface of constant structure to which cofactor adsorbed and then reacted must now, in the light of the previous section, be re-examined. Once this assumption is made their model in all its detail appears obligatory. They have accounted satisfactorily for the extreme dependence of the fraction of active phage particles on tryptophane concentration, for the first order rate by which activity is gained, for the effect of residual tryptophane on the initial deactivation rate, and for the effect of temperature on the activation rate. These results appear not to be directly related and a model which simultaneously accounts for all of these findings must be considered satisfactory.

We are thus reluctant to abandon this description even though in some of its details it seems forced. At first we attempted to show how this model might be extended to the

indole findings. We considered the possibility that a single indole molecule having adsorbed to the supernumerary locus caused the adjacent site either to be immediately inactive, or to rapidly become deactivated. To avoid an inverse fifth power dependence on indole in such a model we assumed that indole did not compete with cofactor in the pre-equilibrium before "ring" formation. This approach would qualitatively account for the known data if we assumed that the indole-inactivated phage particles returned very rapidly to the pool of virgin phage particles. A series of preliminary experiments were undertaken involving a second activation after deactivation by indole. Unfortunately, this point could not be resolved due to the difficulty in obtaining consistent data and will have to be the subject of later work. In any case, we feel that the assumption regarding the inability of indole to compete with cofactor in the pre-equilibrium is extremely forced. Because of structural similarities indole and the indole moiety of tryptophane must be considered to adsorb to the same locus on the protein surface. A paradox thus arose in which, to satisfy the requirement of competition of indole with cofactor at the same locus and to avoid an inverse fifth power dependence of activity on indole concentration, a completely new approach to the cofactor problem became mandatory. It was at this point that the suggestion was made that the basic assumption of a static surface may be in error. We

would now like to discuss, after a brief description of current concepts of protein structure and of reversible denaturation, how the data available at present could be interpreted when this assumption of constant structure is dropped.

5. Current Concepts of Protein Structure and of Denaturation of Proteins

Both fibrous and globular proteins are composed of chains of peptide bonds linking together a large number of the various amino acids. These chains of peptide linkages are coiled into a helix in which a maximum amount of hydrogen bonding occurs along the direction of the axis of the helix (24). These helices are believed to be folded back and forth to form a single protein molecule. The means by which these helices are bound together is still not definitely known. It is likely that they are held in a stable side-by-side array by a variety of bonds. These may be peptide bonds between amino acids having two carboxyl and amino groups each, they may be salt bridges, hydrogen bonds, or bonds of the van der Waals type. Although a complete understanding of reversible denaturation does not exist, we may conclude that rupture of these interhelix bonds would lead only to a relatively minor disorganization of the pattern and would likely be reversible (25). More extensive disorganization of the pattern, involving the

rupture of intrahelix bonds, (such as the rupture of hydrogen bonds by urea), are not likely to be reversed when the denaturing agent is removed. The effect of this disorganization has been shown to alter a great variety of the physico-chemical properties of these molecules (25). Up to now no one has proved that structural changes of the reversible type produced by specific cofactors play a physiological role, but it seems probable that this could occur and that nature may have made use of this possibility. For these reasons we have the possibility of a new approach to one of the most important problems in modern biology--the regulation of the functional characteristics of proteins.

6. Application of These Concepts to the Present Data

The protein in question is considered to exist in two physiological states, one active (A) and one inactive (I). These states may both be represented by a large number of configurations. We should envision that cofactor combines more readily with the active state and for this reason shifts the equilibrium toward state A. In the absence of cofactor we must assume that there is practically no protein in state A. As the structure opens up we would expect a great many more cofactor molecules to adsorb to hydrophobic groups which have become more readily accessible. The adsorption and desorption of cofactor is considered to be rapid, the relatively slow rate of 1.2/min found during the deactiva-

tion process when cofactor is diluted is then ascribed to the rate at which the protein returns to the inactive state.

At the present time this picture does not seem susceptible to a quantitative analysis. We would have to introduce many rate constants and affinity constants each of which would undoubtedly depend upon the structure of the protein at any given moment. Nevertheless, it is possible to qualitatively examine the implications of this approach. Certain features of the data are surprisingly well explained within the framework of the assumptions we have made.

The fifth power dependence of the rate of activation on the cofactor concentration means that after the configuration is sufficiently open for five tryptophane molecules to adsorb (and these are actually adsorbed), it is likely to go rapidly and completely to the active state. The activation rate is expected to be first order in time in this picture only if the first cofactor molecules do not impart significant activity. This leads us to believe that the simultaneous action of four to six cofactor molecules is necessary to wedge open the structure.

Since the transition from I to A should involve a large number of bonds, we may expect the transition to be analogous to a change in physical state of a solid or a liquid, and therefore to be abrupt when external parameters such as the temperature are varied. This is very much the case in cofactor activation. The activation rate in the presence of

limiting cofactor concentration was found by Stent and Wollman to increase by a factor 10^5 over the temperature range 0°C to 37°C (7). They found that this rate was less dependent on temperature at high cofactor concentrations and interpreted this difference as due to a variation with temperature of the affinity of cofactor with sites. In our picture we must attribute the great temperature sensitivity to the actual structural change. The variation of this sensitivity with the cofactor concentration is also amenable to interpretation. At low cofactor concentration it will be necessary to rupture thermally a larger number of bonds than at high cofactor concentration to impart activity. We have consequently accounted for the greater dependence of the activation rate on temperature when cofactor is limiting.

An effect which is also adequately explained in our interpretation is the property displayed by residual tryptophane of slowing down the initial rate of deactivation. As the protein is wedged open and other tryptophane molecules adsorb, the ability of smaller numbers of these cofactor molecules to retain the unfolded configuration appears likely to be great. Stent and Wollman found this retardation in the rate to be approximately proportional to the first power of the residual tryptophane concentration (5). We could interpret this as a manifestation of the ability of a single tryptophane molecule to prevent refolding when adsorbed at a point where refolding would

otherwise begin.

Some of the processes involved in deactivation and inhibition do not occur at a first order rate. Thus when a fully active suspension of phage particles is deactivated either by adding a relatively large amount of indole or by lowering the tryptophane concentration to 0.5-1.5 mg/l, a slowing down of the rate occurs. Departure from first order kinetics may be caused by inhomogeneities in the phage population or by a succession of reactions wherein the rate limiting step shifts from one reaction to another. In our interpretation it would appear probable that the processes which lead to activation would occur in a number of different ways. This would give rise to an inhomogeneous population. At the same time, since many transitions are involved, it would seem that during the over all reaction from A to I some steps would limit during certain times, while other steps would limit at other times. Thus both of the causes of departure from first order kinetics are, in our interpretation, distinct possibilities.

We have yet to interpret the effect of indole within the framework of a structural change of the bacteriophage protein. We must explain the following principal facts:

- 1) The presence of indole shifts the equilibrium in the direction of physiological inactivity.

- 2) Indole increases the rate of deactivation in the absence of external cofactor over that found when only cofactor is removed.
- 3) Indole does not appear to combine with inactive phage particles.
- 4) Indole appears not to have a pronounced effect on the initial activation rate when cofactor is added to an equilibrated mixture of indole and phage particles. The action of indole on the equilibrium activity appears to reside to a great extent in the effect of indole on the deactivation rate. This action is proportional to the first power of the indole concentration, and the proportionality constant is such that indole concentrations about 100 times lower than the relevant cofactor concentrations produce a pronounced effect.

Indole apparently acts only on the active form of the protein. When a single indole molecule is adsorbed near an area previously unfolded by the action of cofactor, this area either is ipso facto inactive or becomes inactive at a high rate. If, in the first case, the indole molecule were to desorb the population would pass transiently through the physiologically active state and decay with the usual half-life (at 15°C) of 34 seconds. This has not been observed and we may conclude that the second alternative is the correct explanation. The ability of indole to increase the rate of deactivation over that found when cofactor is

removed is reasonable on the basis of either description. A separate reaction step either to state I or to another physiologically inactive state A is involved. The deactivation rate of this step is proportional to the first power of indole.

In figure 9 we notice that in the straight-line region of the curves for a given value of indole concentration the activity varies directly with the tryptophane concentration. We may explain this as being due to the competition of indole and tryptophane for loci on the physiological state A. We cannot decide whether these loci are identical with loci to which tryptophane originally adsorbs to cause unfolding. This deactivation rate then is proportional to the quantity n_2/n_1 . This is consistent with the data of figure 6 since the initial rate of deactivation may well depend on the first power of indole concentration. It is difficult to conclude positively that this is the case for the initial rates are very large.

Up to this point we have left open whether cofactor must necessarily be adsorbed to impart physiological activity to state A. We could assume that the polar groups of tryptophane are actually necessary to impart complementarity of the phage surface to the bacterial surface. Anderson has shown that alterations of these polar groups destroy cofactor activity of l-tryptophane (3). On the other hand an

assumption of this form would make it difficult to understand how denaturing agents such as urea (12) promote activity. In our picture we would then ascribe the function of promoting the proper kind of structural change to the polar groups.

A striking feature of our picture is the prediction that the surface should have different affinities for either cofactor of indole depending on its state. In fact, we have proved that indole has little or no affinity for the inactive structure. This must be considered as strong evidence that our interpretation is the correct one.

7. Interpretation of the Nascent State

Phage particles newly liberated from bacterial cells are called nascent phage particles. They possess an activity which is lost only very slowly compared to tryptophane-induced activity of the parental types.

Stent and Wollman were able to interpret their results concerning this phenomenon by proposing a change in the surface of nascent phage particles as they decayed to a quiescent phage population. This process was considered either a modification of the properties of the key sites under the influence of the nascent surface or due to a high affinity of cofactor for the nascent surface (8). In our interpretation this phenomenon appears as a more natural consequence of the basic assumptions. We should envision

that during synthesis of the bacteriophage protein some of the interhelix bonds are not formed, although probably not those which are ruptured by cofactor during normal activation. The nascent activity is lost as these incompleated structures form the various bonds typical of the quiescent phage protein. It is significant that the later data of Stent and Wollman pointed toward our interpretation.

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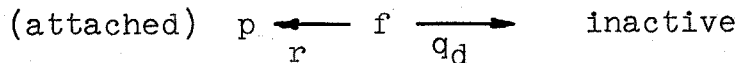
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APPENDIX I

Analysis of the Dump Experiment

In the all-or-none description the virus population is envisioned as being a mixture of completely active and completely inactive particles. The degree of activity is then the fraction of particles in the active state. In the intermediate state description the virus population is viewed as being homogeneous, each particle having a probability of attachment per time unit depending on the state of its activity. The degree of activity is then the ratio of this probability to the probability of attachment per time unit of maximally active particles.

The all-or-none description: In this description we consider the fraction f , of free, active particles. These particles attach at a specific rate r to bacteria.* During the dump experiment in which $c_p < 0.2$ mg/l the fraction f decreases due to attachment and due to loss of activity.



Thus if B is the concentration of bacteria, the fraction of free, active particles decreases at the rate

* Active bacteriophage particles attach to cells at a rate proportional to the bacterial concentration (26) if the latter is below 10^9 cells/ml (18).

$$\frac{df}{dt} = -rBf - q_d f . \quad (1)$$

Simultaneously the rate at which virus attaches to cells is given by:

$$\frac{dp}{dt} = rBf \quad (2)$$

A solution of (1) having the desired initial conditions, $f = f_0$ at $t = 0$, is $f = f_0 e^{-(rB + q_d)t}$. Substituting this solution into (2) and integrating from $t = 0$ (the time of the dump) to $t = \infty$ (the time of the F/N assay) we have

$$F/N = \int_0^{\infty} dp = \frac{rBD_0}{rB + q_d} , \quad (3)$$

where we have identified f_0 with the initial degree of activity D_0 . For large B the fraction $F/N \rightarrow D_0$. Furthermore, if $q_d \gg rB$ then we have $F/N = \frac{rBD_0}{q_d}$.

The intermediate state description: Virus particles have equal probability of attachment per time unit. Let this probability be g . Now during the dump experiment this probability will decrease at the rate

$$\frac{dg}{dt} = -q_d g . \quad (4)$$

The rate at which virus attaches to cells is

$$\frac{dp}{dt} = \frac{g}{g_{\max}} rB(1-p) . \quad (5)$$

A solution of (4) with the initial conditions $g = g_0$ at $t = 0$ is $g = g_0 e^{-q_d t}$. Substitution in (5) and integration

gives

$$\int_0^{F/N} \frac{dp}{1-p} = \int_0^{\infty} \frac{g_0 r B e^{-q_d t}}{g_{\max}} dt,$$

and

$$\ln(1-F/N) = \frac{-g_0 r B}{g_{\max} q_d} = \frac{-r B D_0}{q_d}. \quad (6)$$

Where we have identified D_0 with g_0/g_{\max} . If $F/N \ll 1$, we have

$$F/N = \frac{r B D_0}{q_d}.$$

From equation (6), for large B , $F/N \rightarrow 1$.

We note that conditions (a), (b), and (c) of section II, 6 are sufficient to insure that F/N is proportional to D_0 in the intermediate state description. Conditions (a) and (c) are sufficient for this proportionality in the all-or-none description.

It is not possible to determine which description obtains in the case of quiescent phage. In this case we have $q_d \geq 1.2$; rB is limited to below 0.4, set by the failure of equations (2) and (5) at higher bacterial concentrations (18). The difference between $\frac{0.4}{1.2 + 0.4} = 0.25$ and $1 - e^{-0.4/1.2} = 0.28$ (for $D_0 = 1$) is within experimental error. Nascent bacteriophage particles are best described by the intermediate state formulation (8).

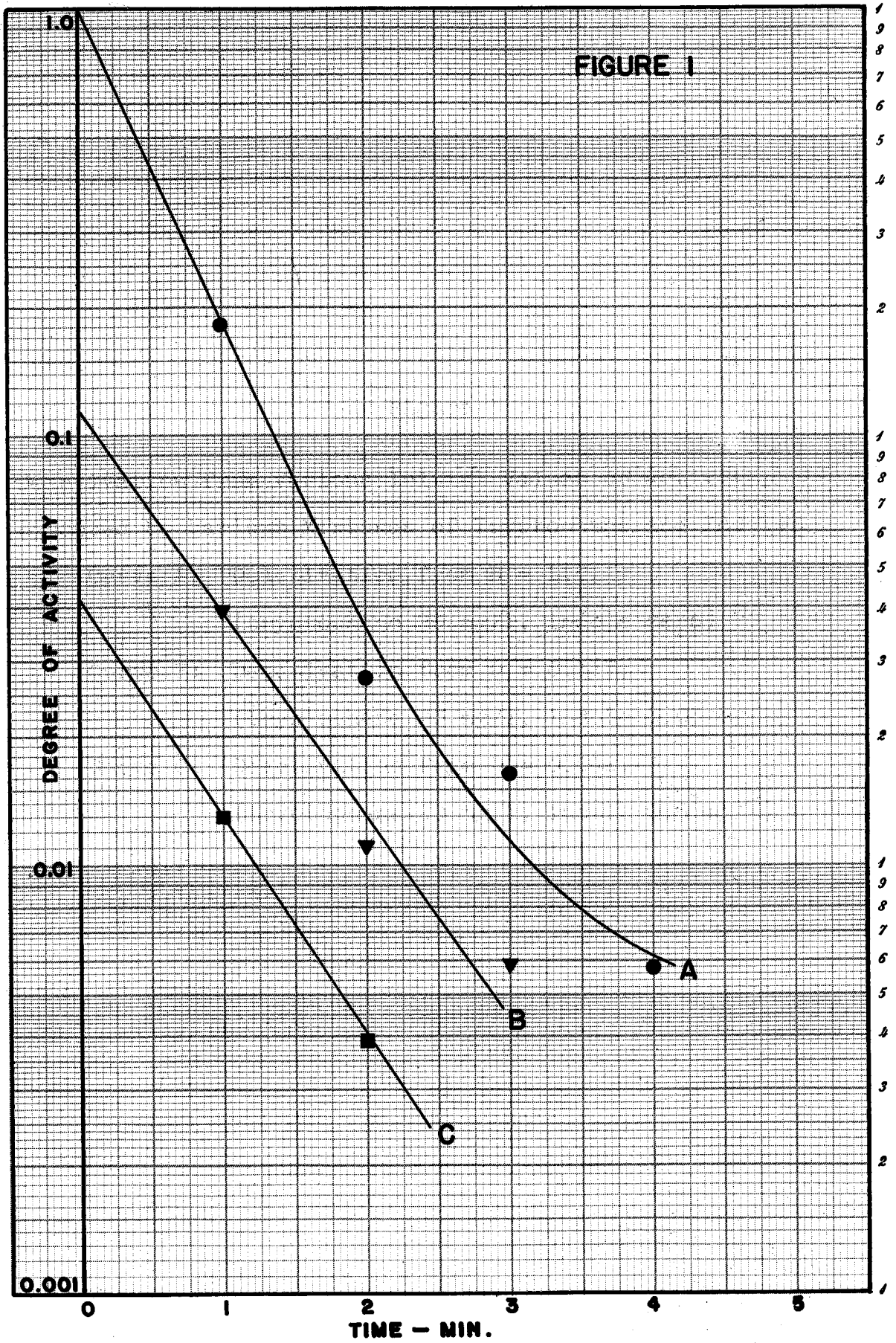
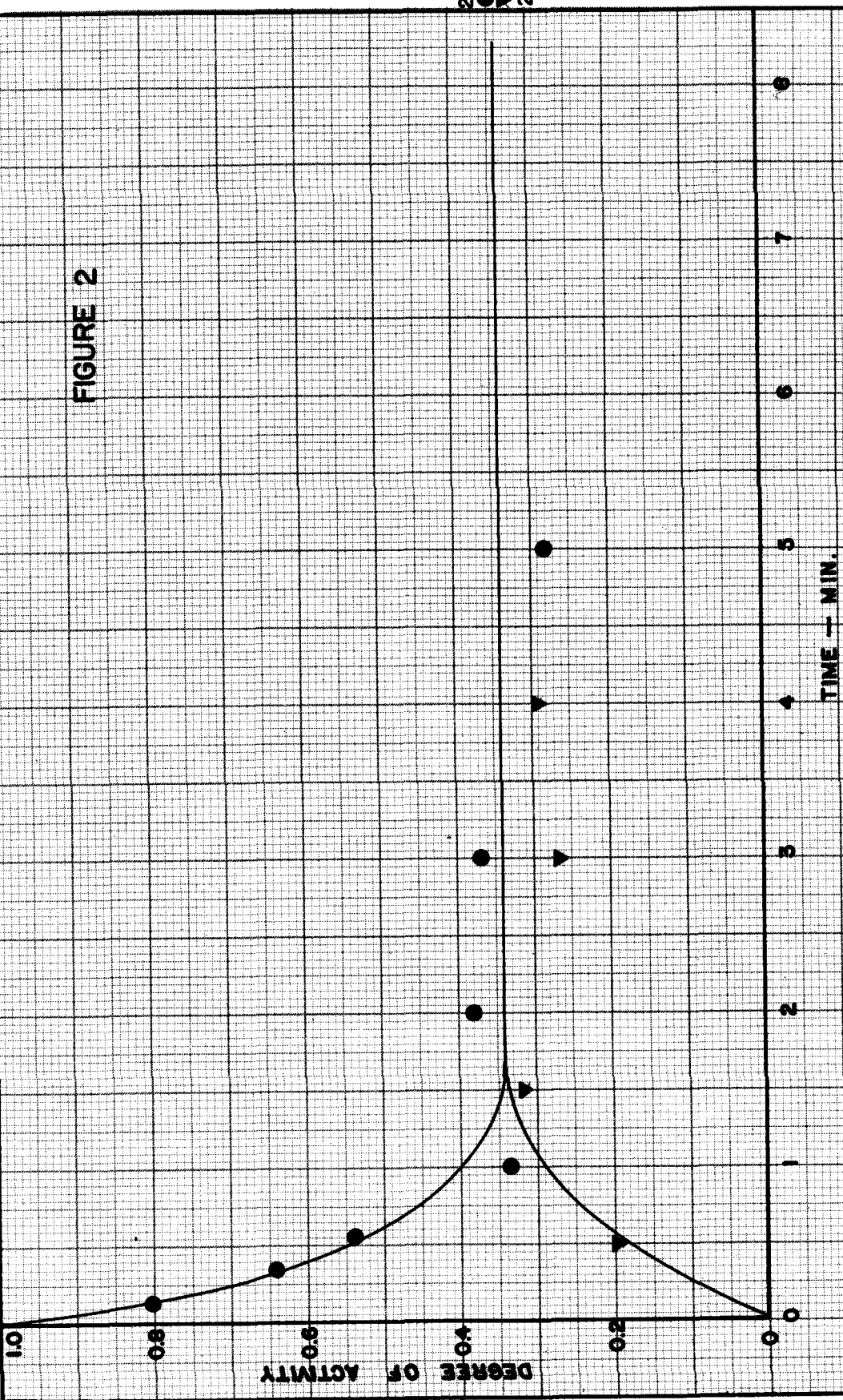


Figure 1

Deactivation from Various Initial Values of Activity,
Produced by Various Mixtures of Tryptophane and Indole.

- Curve A: Deactivation from $D = 1.0$,
(tryptophane 20 mg/l, indole zero)
- Curve B: Deactivation from $D = 0.117$,
(tryptophane 20 mg/l, indole 0.05 mg/l)
- Curve C: Deactivation from $D = 0.042$,
(tryptophane 20 mg/l, indole 0.5 mg/l)

FIGURE 2



20 ● 20 ▲

Figure 2

Approach to Equilibrium in a Mixture of 20 mg/l Tryptophane and 0.05 mg/l Indole.

Lower Curve: Tryptophane added at $t = 0$, after pre-equilibration in the presence of 0.05 mg/l indole.

Upper Curve: Indole added at $t = 0$, after pre-equilibration in the presence of 20 mg/l tryptophane.

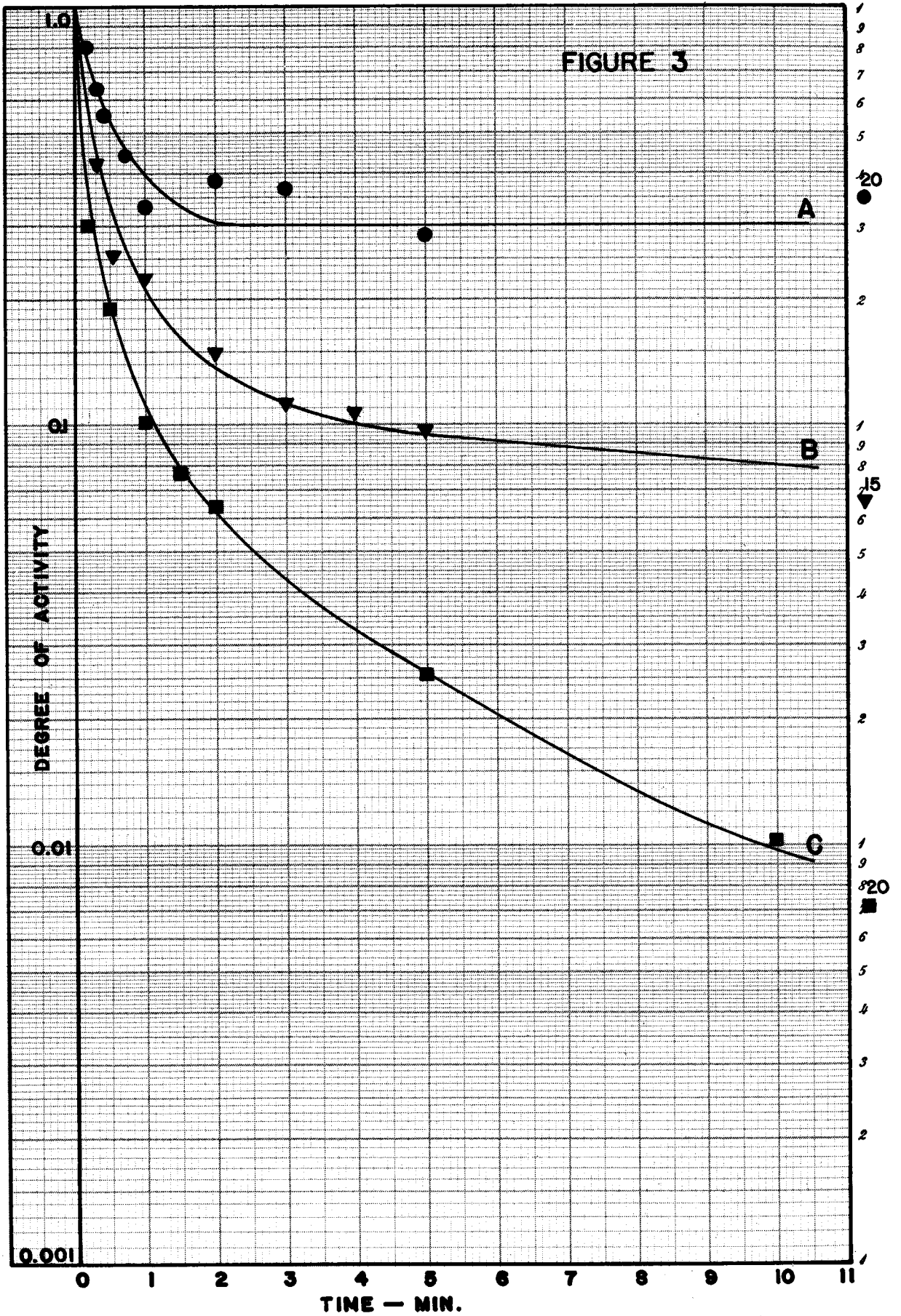


Figure 3

Approach to Equilibrium in a Mixture of 20 mg/l Tryptophane and Varying Amounts of Indole. Suspensions Pre-equilibrated with Tryptophane before adding Indole.

Curve A: 0.05 mg/l indole.

Curve B: 0.5 mg/l indole.

Curve C: 4.0 mg/l indole.

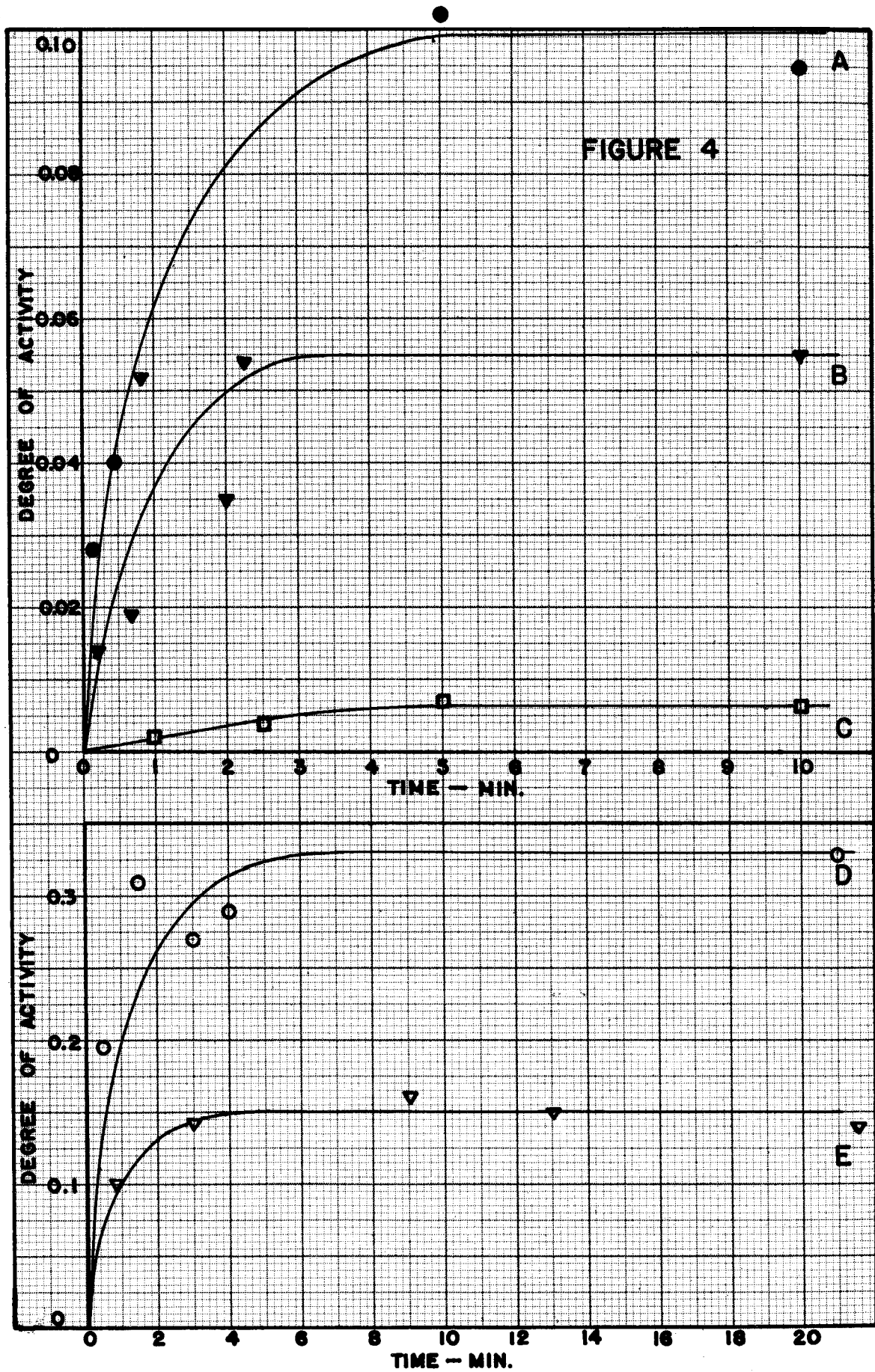


Figure 4

Approach to Equilibrium in a Mixture of 20 mg/l Tryptophane and Varying Amounts of Indole. Suspensions Pre-equilibrated with Indole.

Curve A: 0.5 mg/l indole.

Curve B: 1.0 mg/l indole.

Curve C: 4.0 mg/l indole.

Curve D: 0.05 mg/l indole.

Curve E: 0.1 mg/l indole.

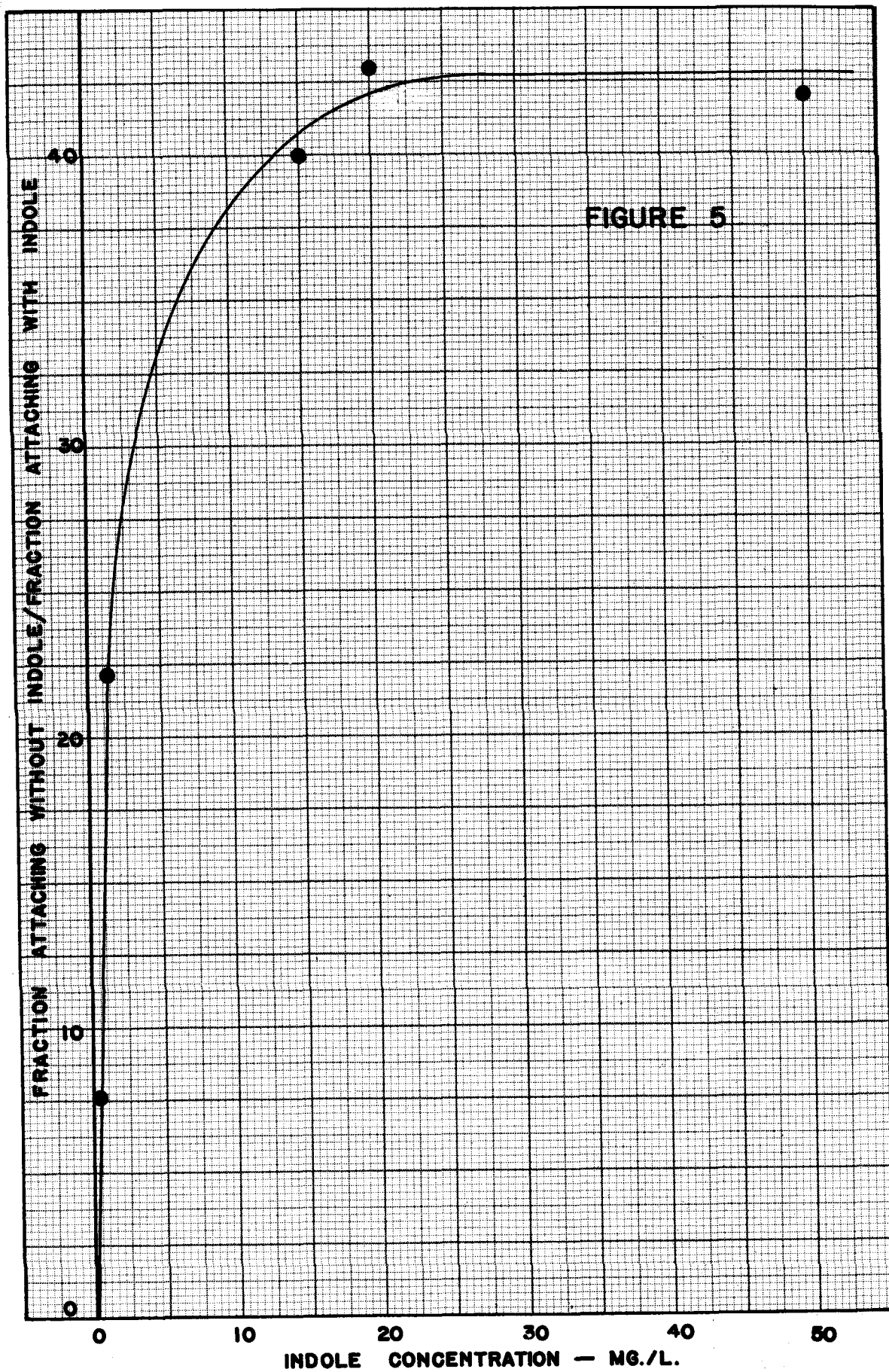


Figure 5

The ratio of the fraction attaching without indole in the dump tube to the fraction attaching with indole in the dump tube, vs. concentration of indole.

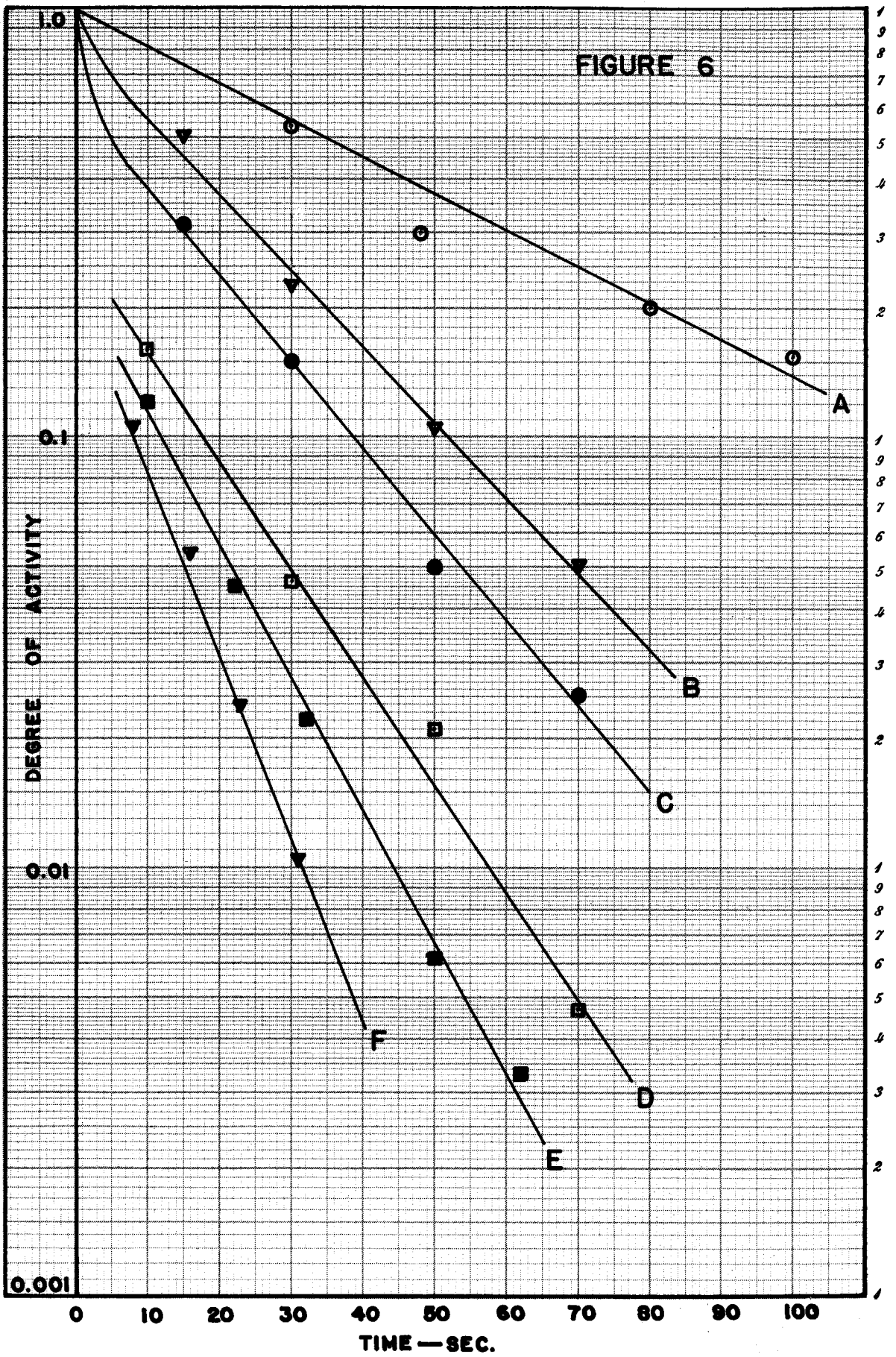


Figure 6

Deactivation in the Absence of Tryptophane and Presence of Various Concentrations of Indole.

Curve A: No indole.

Curve B: 0.01 mg/1 indole.

Curve C: 0.05 mg/1 indole.

Curve D: 0.1 mg/1 indole.

Curve E: 1.0 mg/1 indole.

Curve F: 4.0 mg/1 indole.

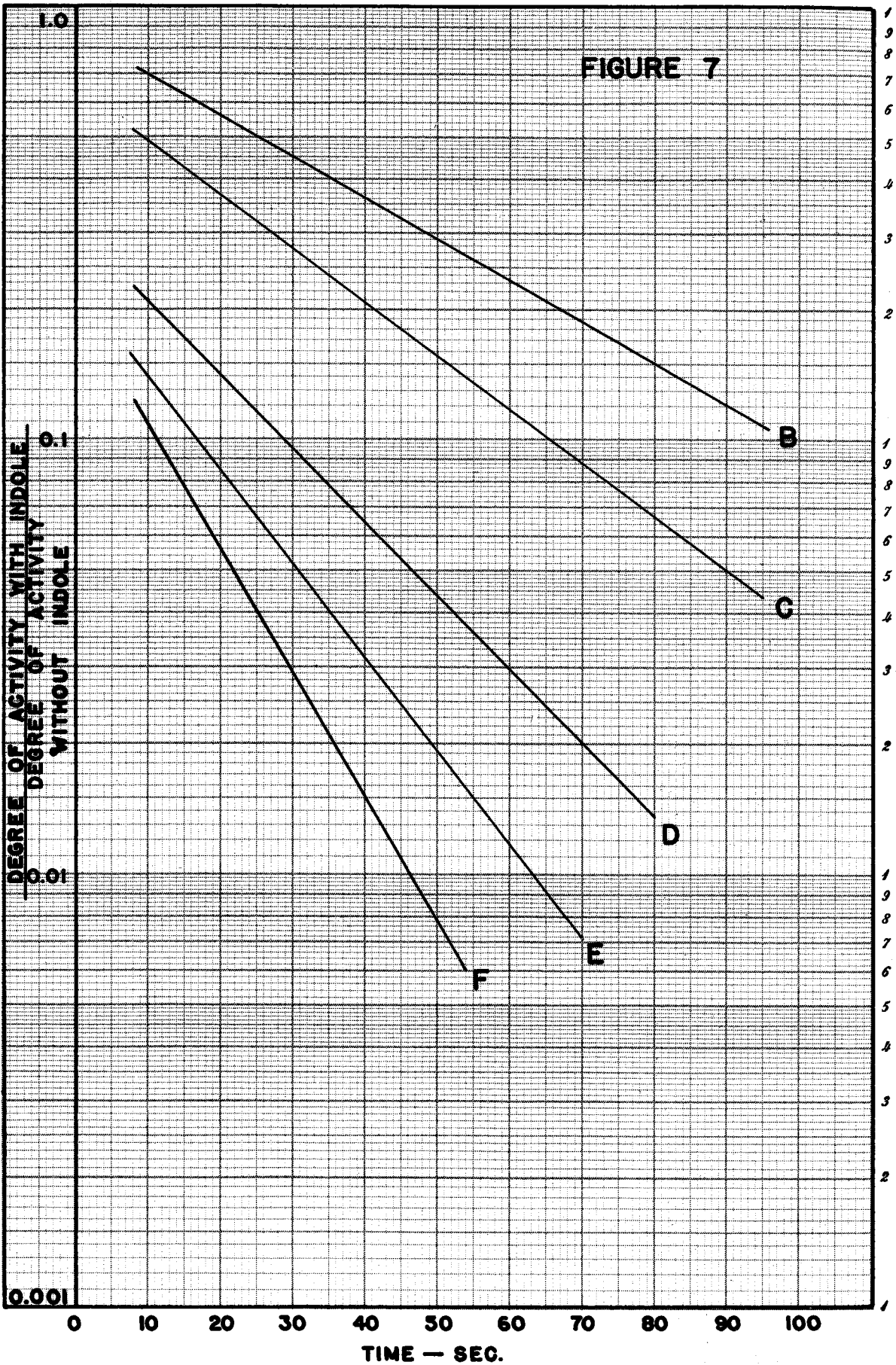


Figure 7

The Deactivation Due to Indole.

The ratio of the activity with indole to the activity without indole vs. time. Ordinates calculated from the curves of figure 6.

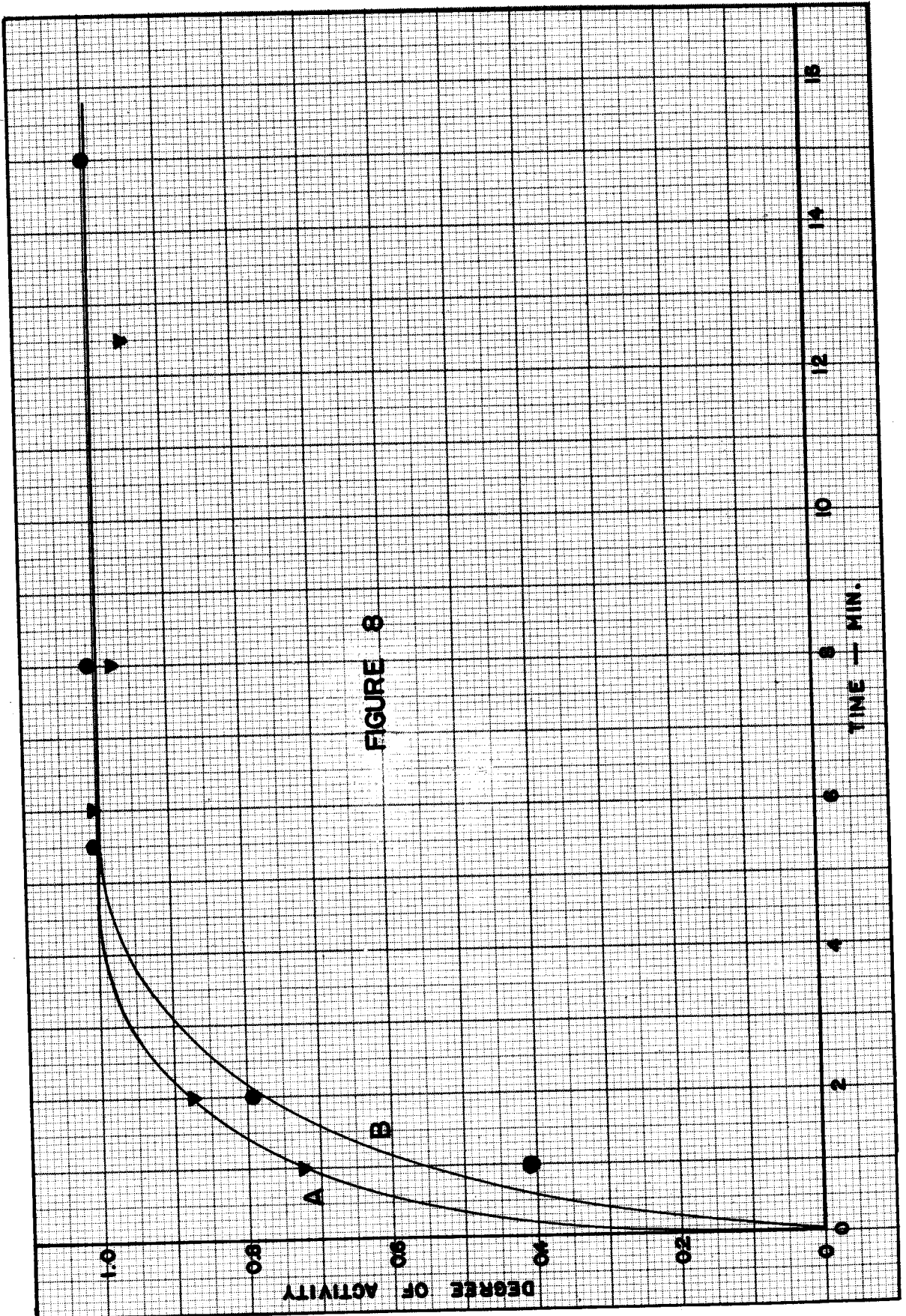


Figure 8

Activation with Tryptophane after Pre-equilibration with Indole.

Curve A: 5 mg/l tryptophane added at the moment of diluting out 0.5 mg/l indole. Final indole concentration: 0.0025 mg/l.

Curve B: Control activation with 5 mg/l tryptophane and 0.0025 mg/l indole.

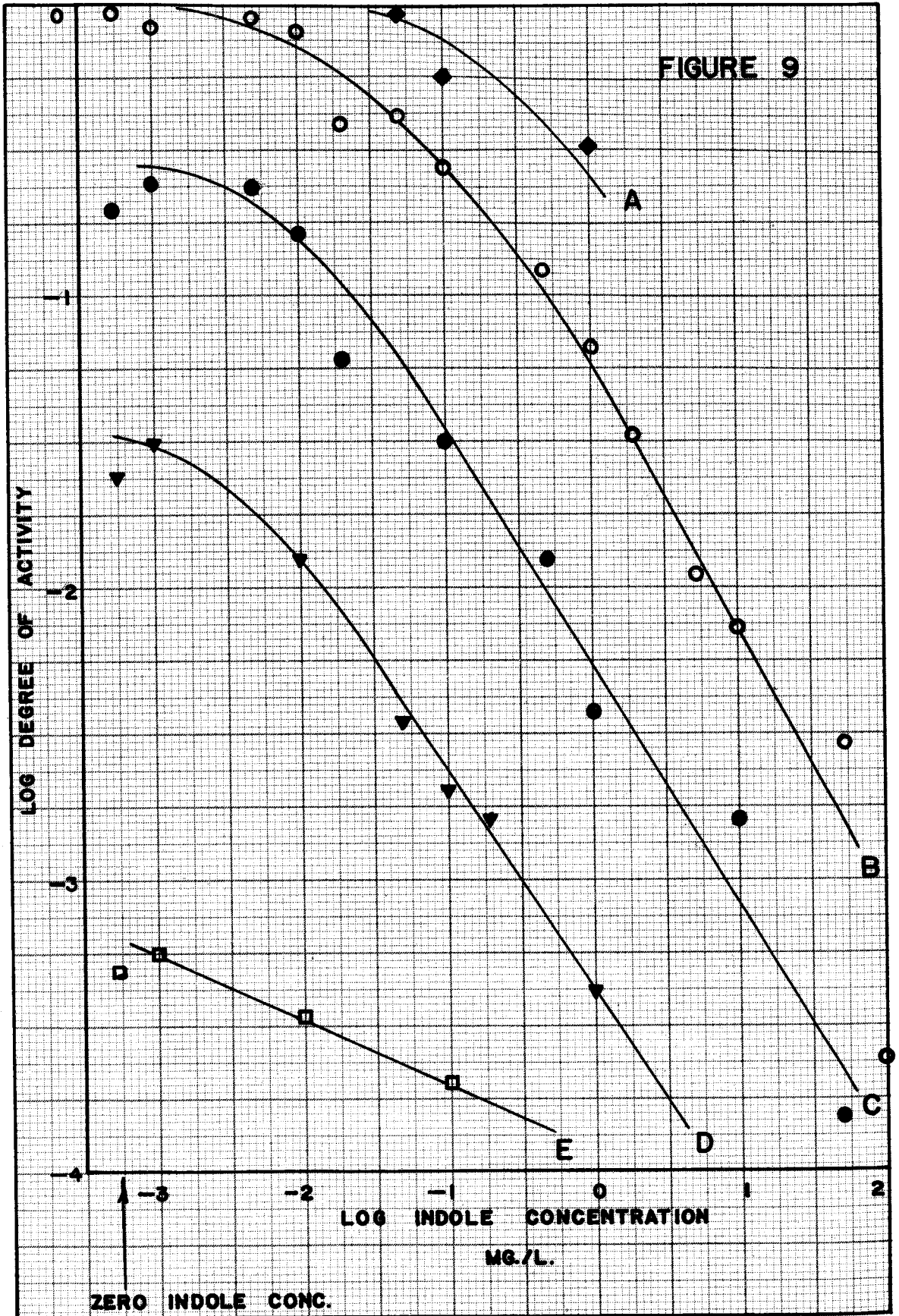


Figure 9

Equilibrium Activity vs. Tryptophane and Indole Concentrations

Curve A: 100 mg/1 tryptophane.

Curve B: 20 mg/1 tryptophane.

Curve C: 2 mg/1 tryptophane. .

Curve D: 1.0 mg/1 tryptophane.

Curve E: 0.5 mg/1 tryptophane.