THE EFFECT OF UREA ON COFACTOR REQUIRING BACTERIOPHAGE

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Gordon Hisashi Sato

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ABSTRACT

Some strains of bacteriophage must be activated by cofactor before they can adsorb to their bacterial hosts. Up to the present time, the only compounds shown to possess cofactor activity have been certain amino acids and amino acid analogues.

In the present study, it is shown that urea, a well known denaturing agent, is capable of activating cofactor requiring phage. By a process, statistically independent of the activation process, urea also kills the phage. Experiments are performed to characterize the properties of urea-activated phage, in regard to stability of urea induced adsorbability and in regard to adsorption rate. The kinetics of the urea activation process and of the urea killing process are studied in detail. Both processes are shown to depend on concentration of urea, temperature, and pH in a manner similar to denaturation of protein by urea.

It is concluded that urea effects the activation of phage by denaturing the protein of the phage adsorption organ. It is further postulated that the action of cofactors consists in a denaturation of phage protein. The biological implications of this hypothesis are discussed.

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INTRODUCTION

In 1945, it was discovered by T. F. Anderson that certain strains of bacteriophage, T4 and T6, give about a million times higher assays when plated on nutrient broth than when plated on synthetic medium agar (1). Anderson traced this phenomenon to the inability of phages to adsorb to bacteria in synthetic medium, and showed that these phages could adsorb to bacteria in synthetic medium in the presence of certain active compounds, which he designated cofactors. Among the compounds tested, L-tryptophane was found to be the most efficient cofactor, being demonstrably active at concentrations around 10^{-5} M. Phenylalanine and tyrosine were found to be much less active, while a large number of other amino acids, vitamins, purines and pyrimidines were found to be inactive. Investigations on the structural features of tryptophane necessary for activity showed: D-tryptophane is inactive both in promoting and inhibiting adsorption, any alteration of the alpha-amino group results in complete loss of activity, reduction or removal of the carboxylic group results in complete loss of activity, and substitution in the indole nucleus results in reduction of activity (2). Subsequent experiments showed that the action of the cofactor is on the virus and not on the bacteria, and that the cofactors activate the virus in a reversible man-

ner (2,3). The cofactor requirement is a stable, heritable property of the phage (4). Mutations occur which alter the cofactor requirement permanently, resulting in such changes as complete cofactor independence, dependence on cofactor only at certain temperatures, and requirement of both cofactor and calcium ions (4,5,6).

G. S. Stent and E. L. Wollman carried out a detailed analysis of the kinetics of the activation by L-tryptophane of phage T4 (7,8,9). They found that the adsorption rate of phage to bacteria in synthetic medium in the presence of low concentrations of tryptophane is proportional to the fifth power of the tryptophane concentration, and that it is independent of the tryptophane concentration at high concentrations. The phage were first equilibrated with the given concentration of tryptophane before addition of the bacteria. The adsorption rate constitutes a measure of the equilibrium degree of activity.

Stent and Wollman then showed that a technique devised by Anderson, and named by him the 'Dump Technique', is an adequate measure of the instantaneous adsorbability of a phage population in the process of gaining or losing tryptophane induced activity (7). The dump experiment is performed by quickly diluting a suspension of the phage to be tested with a large volume of a standard bacterial suspension. This dilution reduces the concentration of any cofactors present in the phage suspension to a level where they do not influence

subsequent events. At the instant of the dilution, two competing processes commence: active phage lose their activity, and active phage adsorb to bacteria. The phage-bacteria suspension is incubated for a time sufficiently long to permit maximum adsorption to occur, and the fraction of adsorbed phage is measured. This fraction will be referred to as the catch, C, of the dump experiment.

Stent and Wollman defined the degree of activity of a phage population as the ratio of the catch in a dump experiment involving the sample to be tested, to the catch involving maximally activated phage in a dump experiment. They showed that the degree of activity, so defined, is equal to the ratio of the instantaneous adsorption rate of that population, to the adsorption rate of a maximally activated phage population.

Using the dump technique, Stent and Wollman found that the rate of activation upon addition of tryptophane to an inactive phage population is proportional to the fifth power of the tryptophane concentration at limiting concentrations. They found that the degree of activity rises immediately, without lag, upon addition of tryptophane to phage. The difference between the final equilibrium degree of activity associated with the given concentration of tryptophane and the degree of activity at any time after addition of the tryptophane declines exponentially with this time at a rate proportional to the fifth power of the tryptophane concentra-

tion (8). Upon dilution with buffer of an equilibrated phage-tryptophane mixture, the degree of activity declines exponentially at a rate independent of the original degree of activity (7). These results were consistent with the observation that the equilibrium degree of activity is proportional to the fifth power of the tryptophane concentration, and prompted the notion that the equilibrium degree of activity is simply the result of two competing processes: an activation dependent on the fifth power of the tryptophane concentration, and a random deactivation of active phage. However, an unexpected complexity in the kinetics of deactivation made this simple interpretation impossible. Hershey and Delbrück discovered that small residual concentrations of tryptophane in the deactivation mixture slowed down deactivation to a much greater extent than could be explained by a competition between deactivation and activation by this residual concentration of tryptophane (10).

Despite these difficulties, Stent and Wollman devised a model of the cofactor activation process, which was consistent with all the results (8). According to this model, a phage possesses one or more sites which can exist in an active or inactive state. The degree of activity of a phage population is then the fraction of these sites which are active. Each site possessed a number of subsites. Upon addition of tryptophane to the phage, the tryptophane molecules adsorb and desorb rapidly from these subsites so that an equilibrium

is immediately established between the free tryptophane, unoccupied subsites and subsites occupied by tryptophane. A fraction of the sites will by fluctuation have five or more of their subsites occupied by tryptophane. These five occupied subsites undergo a slow, rate limiting step, resulting in the activity of the site. The active sites then deactivate spontaneously at the rate observed upon dilution of the tryptophane. To explain the protective effect of small residual tryptophane concentration in the deactivation mixture, Stent and Wollman assumed that an active site having a sixth subsite occupied by tryptophane does not deactivate.

Further studies by Stent and Wollman showed that at limiting concentrations of cofactor the degree of activity is proportional to the fifth power of the tryptophane concentration at all temperatures in the range tested (5° C - 45° C), and that the activation rate at limiting cofactor concentration rises by a factor of ten thousand when the temperature is raised from 5° C to 37° C (9). This enormous rise is accounted for in their theory very plausibly by a moderate temperature dependence of the equilibrium constant between free subsites, occupied subsites, and tryptophane. This equilibrium is supposed to shift toward dissociation upon lowering the temperature.

We intended originally to check the validity of the Stent and Wollman model by carrying out similar studies using phenylalanine instead of tryptophane as the cofactor. Phenlalanine was found to act as a cofactor, but only at concentrations

about a hundred times higher than in the case of tryptophane (1). It was thought that such a study might throw light on the real meaning of the "site," "subsite," "reaction between five subsites," etc. of the Stent and Wollman model. We found that T4.38, the strain of phage used by Stent and Wollman, attains the maximum adsorption rate in 15° C F medium when the concentration of L-phenylalanine is 1000 gamma/ml. Although a phage suspension thus activated adsorbs as fast as phage maximally activated by tryptophane, it gives catches in the dump experiment a hundred times smaller than those obtained with phage maximally activated with tryptophane. Phenylalanine-activated phage must then deactivate much faster than tryptophane-activated phage. Technical difficulties prevented us from measuring either the activation or the deactivation rate. At limiting phenylalanine concentrations, the adsorption rate is proportional to the fifth power of the phenylalanine concentration. If it is assumed that phenylalanine has a much lower affinity for subsites than tryptophane, and that sites having five or more of their subsites occupied by phenylalanine undergo the final step which results in activity at a much faster rate, both in the forward and reverse direction, than in the case of tryptophane, the results are consistent with the Stent and Wollman model.

Experiments by Puck et al. have defined more clearly the step in the life cycle of the phage where the cofactor

is required. They have shown that adsorption of phage to bacteria can be divided into at least two steps, the first of which is reversible (11,12). They conclude that the first step is an electrostatic binding between complementary patterns of ionized amino groups on the phage and carboxylic groups on the bacteria (13). Cofactor activation of phage is necessary before the first step can occur (12). In fact, cofactor requiring phage must react with cofactor in order to adsorb to glass filters (12).

Delbrück discovered another feature of the activation process which should be helpful in analyzing the mechanism (6). He found that in some strains of cofactor requiring T4, indole inhibits the activation by tryptophane. He showed that in indole-tryptophane mixtures, the fraction of active phage is proportional to the inverse first power of the indole concentration, and that this fraction remains constant when the ratio of indole to tryptophane is constant, providing the tryptophane concentration is sufficiently high to impart full activity to the phage in the absence of indole (14).

It was felt that the dependence of the degree of activity of phage in tryptophane-indole mixtures on the inverse first power of the indole concentration was not a foreseeable outcome of the Stent and Wollman model. In view of the structural similarity between indole and tryptophane, it seems reasonable to assume that the two substances compete for the

same subsites. If one calculates the degree of activity of phage in indole-tryptophane mixtures on this assumption, and on the assumption that a site having one or more of its subsites occupied by indole cannot become active, an entirely different relationship is derived.

At this point, the studies on the indole inhibition of tryptophane activation were extended by McKee (15). McKee was able to confirm and extend the original findings of Delbrück. McKee showed that the inverse first power relationship is due to the fact that indole reacts only with active phage, and that upon reacting with indole, active phage quickly deactivate. Only one molecule of indole is necessary to deactivate an active phage. Although the Stent and Wollman model could be modified by auxiliary assumptions to be consistent with both the indole and tryptophane findings, it was felt that the resulting model was extremely forced and artificial.

At this point, Delbrück suggested that the effect of cofactor on the phage might be of greater complexity than that envisaged by the Stent and Wollman model. The cofactor might affect the equilibrium between two structural configurations of the protein of the phage adsorbing site in a manner analogous to reversible denaturation of protein. This notion prompted us to test whether urea, a well known denaturing agent, is capable of activating cofactor requiring phage. We discovered that this is indeed the case, and the present

thesis is a report on a detailed study of activation by urea.

According to current theories of protein structure (16), a protein molecule is composed of amino acids joined by peptide linkages. The chain of atoms involved in the peptide linkages is coiled into a helix, which is stabilized by hydrogen bonding. These helices are folded into a configuration, which is stabilized by a variety of inter-helical bonds, whose nature is not known in detail. The inter-helical bonds are believed to be salt linkages, hydrogen bonds, disulfide bonds, and bonds of the van der Waals type. The consensus of opinion of workers in the field of protein denaturation is that denaturation is an intramolecular rearrangement caused by the breaking and reforming of the intrahelical and interhelical bonds. According to F. Putnam (17), "All the evidence indicates that denaturation is a physical or intramolecular rearrangement rather than a chemical alteration of native protein structure and it leads to a change in specific spatial configuration without hydrolysis of primary covalent bonds."

Any detectable change in the biological, chemical or physical properties of protein may serve as a criterion for denaturation. The most important changes are: 1) decrease in solubility, 2) loss of biological activity, 3) increased reactivity of constituent groups, and 4) changes in molecular shape or size (17). Due to limited methods of detection, denaturation was earlier defined as a change in the native

protein whereby it becomes insoluble in solvents in which it was previously soluble (18).

In some cases, upon cooling or removing the denaturing agent, the denatured protein regains the properties of the native protein (19,20,21). The criterion for reversibility is non-detection of any differences between native and renatured protein. In some cases, an equilibrium exists between native and denatured protein (22,23,24). The phenomenon of reversible denaturation has prompted many authors to propose that it might be a normal physiological process.

The denaturation of protein by urea is characterized by a peculiar temperature effect. The process is slowest at about 20° C, and increases in rate as the temperature is lowered or raised from this value (25,20,26). Hopkins (26) explained this temperature effect by postulating that the urea denaturation occurs in consecutive steps. The first step is the union of protein and urea to form a denaturable complex. The equilibrium concentration of this complex is increased by lowering the temperature. The second step is the denaturation of this complex, and has a positive temperature coefficient. Similar explanations have been offered by various authors (25,27). Urea denaturation is further characterized by a strong dependence on the urea concentration. In the case of denaturation of lactoglobulin, the rate is proportional to the eleventh power of the urea concentration (20).

Our studies aimed at characterizing the kinetics of the activation of phage T4 by urea, and in particular, at establishing whether there existed similarities to denaturation by urea with respect to concentration range, concentration dependence, and temperature dependence.

MATERIAL AND METHODS

1. Bacterium Escherichia coli, strains B and B/4, were used in all experiments.

2. Bacteriophage T4.38 (7) was used in these experiments. The stock was prepared by infecting a culture of E. coli, strain B, at a concentration of 2.0×10^8 bacteria/ml in M9 medium with a multiplicity of 1/100 of the stock of T4.38 originally used by Stent and Wollman. The lysate was purified and concentrated by two cycles of alternate low speed and high speed centrifugation, and stored in 0.1 M phosphate buffer. The stock gave a stable assay of 1.0×10^{12} phage/ml on nutrient broth agar, and an assay of 5.0×10^6 when plated on F' agar. The stock has the same properties with regard to cofactor requirement as the original stock used by Stent and Wollman.

3. The synthetic medium used is a modification of the F medium used by Stent and Wollman, and is designated F' medium. The modification, suggested to us by Dr. N. K. Jerne, consists in the addition of glucose to the F medium used by Stent and Wollman. The presence of glucose prevents the bacteria from producing indole (28). Exceedingly small traces of indole may diminish the degree of activity of phage populations (15). The use of glucose furnishes bacteria with more uniform properties with respect to adsorption

of phage (29). F' medium has the following composition: Na_2HPO_4, 3.5 grams; KH_2PO_4, 1.5 grams; NH_4Cl, 1.0 grams; $CaCl_2$, .008 grams; $MgSO_4 \cdot 7H_2O$, 0.1 grams; sodium lactate, 10 grams; glucose, 5.0 grams; and distilled water, 1000 grams. F' agar is prepared by adding 7.0 grams of agar to 1000 ml of F' medium. N agar is Difco nutrient broth agar.

4. The urea solutions were prepared by dissolving the desired amount of urea in phosphate buffer solutions, which were prepared as follows: 1.7 grams of K_2HPO_4 and 0.6 grams of KH_2PO_4 were dissolved in 1.0 liters of distilled water, and the pH adjusted with 1.0 M H_3PO_4 and 1.0 M KOH. The resulting pH of the urea solutions was the pH of the buffer used. The urea used was Mallinckrodt analytical reagent grade urea. In early experiments, the urea was recrystallized from water or ethyl alcohol, but it was found that recrystallization did not yield different results from those obtained when the stock urea was used. Solutions were used on the day of preparation.

5. All adsorptions were carried out at 15° C in a standard bacterial suspension. This was prepared from a saturated culture of E. coli, strain B, grown in F' medium with aeration at 37° C. This saturated culture was washed in fresh F' medium, and diluted twenty fold in fresh F' medium.

6. Standard methods of phage assay were used (30). To measure the activity of urea treated phage populations

we follow the procedure of Stent and Wollman, i.e., we use the technique of the dump experiment. The phage are diluted into the standard bacterial suspension, and after thirty minutes of incubation of this mixture, aliquots are plated on F' and N agar. The count on N agar measures the surviving phage, while the count on F' agar measures the fraction of the surviving phage which was adsorbed to bacteria during the thirty minute incubation period (1,7). The theories underlying the application of the dump technique for measuring the instantaneous state of urea treated phage are discussed in the next section.

7. The phage were stored at 4° C. Immediately before dilution into urea, the phage were chilled to 0° C, and maintained at this temperature. This procedure was used because of the discovery by T. F. Anderson that the sensitivity of phage T6 to osmotic shock is determined by the temperature at which they are incubated prior to the shocking (31). Anderson found that an equilibrium exists between two states of phage T6, one sensitive and the other resistant to osmotic shock. As the temperature is raised, the equilibrium shifts in favor of the shock resistant form. The equilibrium is established rapidly at high temperatures, and very slowly at 0° C, so that the ratio of resistant phage to sensitive phage in a population incubated at a high temperature is essentially maintained when the phage are rapidly chilled to 0° C. The equilibrium ratio of resistant to sensitive

phage increases a factor of a hundred when the temperature is raised from 36° C to 48° C. This temperature coefficient corresponds to an entropy increase of 250 calories/degree in the transition from sensitive to resistant phage. Entropy increases of this magnitude are associated with the transition from native to denatured protein.

Our phage exhibits similar properties. Phage equilibrated at 4° C and 37° C were treated with 2.5 M urea at 0° C and pH 6.5 for 35 minutes and then diluted into 15° C F' medium, and F' medium containing 2.0 moles/liter of glucose. Upon dilution into F' medium, .0005 of the 4° C phage survived, and .0012 of the 37° C phage survived. Upon dilution into glucose, both phage samples had a fraction of survivors of .0025. Our method of activating phage equilibrated at 4° C was chosen for standardization of procedure and not for obtaining maximum survival.

8. Horse anti-T4 serum was obtained from Dr. N. K. Jerne of the Danish State Serum Institute. This serum has an inactivation rate constant of 2800/min.

9. S35 labelled T4.38 was obtained by infecting a culture of E. coli, strain B, grown to a concentration of 10^8 cells/ml in M9 medium at 37° C with aeration, with a multiplicity of 1/10 of T4.38. The sulfur content of 100 ml of this medium was furnished by .001 grams of $MgSO_4 \cdot 7H_2O$ and 1.0 mc of S35 in the form of carrier free H_2SO_4. The phage was purified and concentrated by three cycles of

alternate low and high speed centrifugation, and stored in 0.1 M phosphate buffer. The final concentration of phage was 2.0×10^{11} particles/ml. Each phage particle gave 8.0×10^{-7} counts/min when assayed by drying the samples on discs of lense paper. This method gives a counting efficiency of about 5%. In the presence of 20 mg/liter of L-tryptophane, 76% of the radioactivity adsorbed to B, when more than 99% of the infective phage had adsorbed. Under the same conditions, no detectable adsorption to B/4 occurred.

THE INTERPRETATION OF THE EXPERIMENTAL DATA

Before proceeding with the presentation of our experimental data, we wish to discuss here certain theoretical aspects which one has to have clearly in mind in order to be able to give a meaningful interpretation to the experimental results. We recall that in the case of activation by tryptophane, the procedure for assessing the activating effect consisted in a dump experiment, and that the degree of activity was taken to be proportional to the catch, C, i.e., to the fraction of the phage population adsorbed to bacteria when a sample of the suspension is mixed with a cofactor-free suspension of bacteria and this mixture is allowed to stand for 30 minutes before plating. Stent and Wollman pointed out that the catch is a fair measure of the degree of activity at the time of the dump if, and only if, 1) tryptophane does not kill the phage, 2) the rate of deactivation is independent of the initial degree of activity, and 3) the rate of deactivation in the dump tube is fast compared to the rate of adsorption.

In the case of activation by urea we know in advance that at least one of these conditions is not satisfied, namely the first. We know that the urea treatment kills a progressively larger fraction of the population. It is clear, therefore, from the start that the plaque counts on F'

plates will be affected by this killing. We may conceive of the killing either as being a process which is statistically independent of the process of activation, perhaps not involving the same part of the phage, or as a process that is consecutive to activation, the urea first activating and then killing the phage. In either event, the killed phage may or may not be active, i.e., adsorbable. We will anticipate here the result of an experiment to be described later showing that a large proportion of the killed phage is adsorbable. In fact, the adsorbable fraction is as large for the total population as for the surviving one. This suggests that killing and activation are not consecutive processes, but largely statistically independent ones. If the phages were first activated and then killed without losing their activity, then the killed fraction should be 100% adsorbable. To account for the proportionality just mentioned, one would have to make quite artificial assumptions about loss of adsorbability when the killing has gone too far. We will disregard this possibility and will proceed on the assumption that killing affects the population homogeneously irrespective of the degree of activity any fraction of it may have attained. In other words we will refer all F' plaque counts to a plaque count on N agar taken from the same dump tube. It must be emphasized at this point that under the conditions of our experiments, an appreciable part of the killing is due to osmotic shock. The statistical

independence of killing and activation may not hold if the part of the killing due to osmotic shock were to be eliminated.

We now turn to the relation between the catch and the degree of activity, and present three interpretations of the catch in a dump experiment:

1) The all-or-none theory views a partially active population as an inhomogeneous population, consisting of two fractions, a completely active and a completely inactive fraction. Deactivation means, on this view, that the active fraction decreases, i.e., that active particles undergo a transition from the active to the inactive state, according to certain probability rules.

2) The intermediate state theory views a partially active population as a homogeneous population, each particle of the population having an intermediate degree of adsorbability, i.e., an intermediate probability of sticking to a bacterium when it collides with it. This probability is assumed to be the same for every particle of the population. Deactivation means a decrease in this probability.

3) The preactive state theory is a variation of the all-or-none theory. According to this view, treatment with urea divides the phage population into two fractions: a native fraction unaffected by the urea treatment and a preactive fraction possessing urea-induced preactivity. Preactive phage cannot adsorb to bacteria, but upon dilution

of the urea, the preactive phage undergo a transformation into active phage, and this may be the rate limiting step for the events in the dump tube. In cofactor free medium, active phage may adsorb to bacteria. They may change spontaneously into inactive phage which are cofactor requiring but retain their ability to form plaques on N agar. In setting up the theoretical treatment, no specific assumptions will be made at first concerning the rates of the adsorption process and of the deactivation process as compared to the rate of transformation from preactive to active phage. We will find, however, that the theory is compatible with the facts only if activation is assumed to be rate limiting.

The principal distinction between the all-or-none theories and the intermediate state theory lies in the predictions to which they lead when the adsorption rate is increased indefinitely, by increasing the density of the bacterial population in the dump tube. On the all-or-none theories one predicts a maximum catch equal to the fraction which is active or preactive at the time of the dump. On the intermediate state theory the maximum catch is equal to unity. In the case of activation by tryptophane it was not possible to decide between these two alternative views, because of limitations inherent in the technique. In our case we must consider all of these possibilities, hoping that the experimental results may enable us to decide between them.

In developing the implications of these views we will have to generalize slightly the treatment given by Stent and Wollman by not assuming that the rate of deactivation is fast compared to the rate of adsorption. Anticipating qualitatively a result to be presented later, we will state here that deactivation, although proceeding exponentially and at a rate which is independent of the degree of activity, is, in fact, not fast compared to adsorption.

Quantitative formulation of the all-or-none theory.

We assume that a phage population having received a certain urea treatment, at the time of being freed of urea (by dilution), contains a fraction, D_o, which adsorbs to bacteria at a standard rate, a. The active fraction, D, decreases from its initial value, D_o, with a rate constant, f. P_o is the number of surviving phage.

On these assumptions, the number of adsorbed phage, P_a, in the dump tube increases according to the differential equation

$$dP_a/dt = aP_oD_oe^{-(a+f)t}$$

Integration of this equation gives

$$P_a/P_o = aD_o/(a+f)\left[1-e^{-(a+f)t}\right] \qquad (1)$$

In our dump experiments the time, t, is thirty minutes, and it turns out that this time is sufficiently long to make the

exponential term negligible compared to unity. Therefore, on this theory the catch, C, is equal to the quantity

$$C = aD_o/(a+f) \tag{2}$$

We are also going to report adsorption rate measurements, i.e., values of the catch for short periods, T, of sojourn in the dump tube. These are conveniently referred to as C(T), while the catch after thirty minutes incubation of the dump tube is referred to as C(30). The present theory predicts that

$$\frac{C(30) - C(T)}{C(30)} = e^{-(a+f)t} \tag{3}$$

Finally, we want to consider the interpretation of experiments on the deactivation of a phage population first treated with urea and then permitted to deactivate for T minutes in F' medium before performing a titration by a standard dump experiment. In this situation the all-or-none theory predicts that the fraction of active phage, D, at the time of the dump, is $D_o e^{-fT}$. Substituting this value for D_o in equation (2) we obtain for the catch as a function of T the relation

$$C(T) = [aD_o/(a+f)]\ e^{-fT} \tag{4}$$

The all-or-none theory thus predicts a simple exponential decrease of the catch with the correct time constant, f.

Quantitative formulation of the intermediate state theory.

We assume that a phage population having received a certain urea treatment, at the time of being freed of urea (by dilution) is homogeneous with respect to its adsorbability to bacteria, adsorbing with a rate constant, a_o. Deactivation means that this rate constant decreases from its initial value with a rate constant, f.

On these assumptions the number of adsorbed phages in the dump tube increases according to the differential equation

$$dP_a/dt = (P_o - P_a)a_o e^{-ft}$$

Integration of this equation gives

$$P_a/P_o = 1-e^{-a_o A} \text{ , with } A = (1-e^{-ft})/f \qquad (1a)$$

In the dump experiment (t = 30 minutes), it turns out that e^{-ft} is negligibly small compared to unity so that one obtains for the value of the catch

$$C = 1-e^{-a_o/f} \qquad (2a)$$

Note that on this theory, in contrast to the all-or-none theory, the catch is, in general, not proportional to the degree of activity. Proportionality obtains only in the limiting case where a_o/f is small compared to unity. This is true under all practical circumstances in the case of activation by tryptophane, but, as will be seen, it is in general not true for our case.

For the adsorption rate measurements we combine equations (1a) and (2a) to obtain

$$\frac{C(30) - C(T)}{C(30)} = \frac{\left[-e^{-a_o/f} + e^{-(a_o/f)(1-e^{-fT})}\right]}{1-e^{-a_o/f}} \qquad (3a)$$

Finally, for the deactivation experiments, we obtain the prediction of the intermediate state theory by replacing a_o with $a_o e^{-fT}$ in equation (2a). Thus we obtain

$$C(T) = 1-e^{-(a_o/f)e^{-fT}} \qquad (4a)$$

Note that this relation does not represent a simple exponential decline. It does so only in the limiting case where a_o/f is small compared to unity.

Quantitative formulation of the preactive state theory.

We assume that a phage population having received a certain urea treatment, at the time of being freed of urea (by dilution), contains a fraction, D_o, of preactive phage. Upon dilution into F' medium, preactive phage are transformed into active phage at a rate, b. Active phage adsorb to bacteria at a rate, a, and are transformed into inactive phage at a rate, f. The rate constants a and f are large compared to b.

On these assumptions the number of adsorbed phages in the dump tube increases according to the differential equation

$dP_a/dt = aP_oA$, where A is the fraction of active phage.

In F' medium A satisfies the differential equation

$$dA/dt = bD-fA$$

and in the dump tube A satisfies the differential equation

$$dA/dt = bD-(a+f)A$$

Solving these equations, we obtain

$$P_a/P_o = \frac{abD_o}{a+f-b}\left[\frac{-e^{-bt}}{b} + \frac{e^{-(a+f)t}}{a+f} + \frac{1}{b} - \frac{1}{a+f}\right] \quad (1b)$$

The conditions of a standard dump experiment (t=30) make $bt \gg 1$ and $(a+f)t \gg 1$. Under these conditions the catch is simply

$$C = aD_o/(a+f) \qquad (2b)$$

Where adsorption is stopped after time, T

$$\frac{C(30) - C(T)}{C(30)} = \frac{b(a+f)}{a+f-b}\left[\frac{e^{-bT}}{b} + \frac{-e^{-(a+f)T}}{(a+f)}\right] \qquad (3b)$$

and if b is considered small compared to a and f, we obtain for $(a+f)T \gg 1$

$$\frac{C(30) - C(T)}{C(30)} = e^{-bT}$$

When the urea-treated phage are allowed to deactivate for T minutes in F' medium before performing the dump experiment

$$C(T) = [a/(a+f)][D(T) + A(T)]$$

$$C(T) = [a/(a+f)]\left[D_o e^{-bT} + \left(bD_o/(f-b)\right) e^{-bT} - e^{-fT}\right] \qquad (4b)$$

If the approximation is made that b is negligibly small compared to a and f, the following equation is obtained

$$C(T) = [a/(a+f)]D_o e^{-bT}$$

According to all three theories, the catch is a fair measure of the degree of activity of a urea-activated phage population. In the intermediate state theory, the catch is not a proportional measure of the degree of activity. This theory deviates from the others in that it does not predict a simple exponential decline of C in the deactivation experiments or of the quantity, C(30)-C(T), in the adsorption experiments.

The all-or-none theory is testable because it permits the measurement of three relations between the parameters a and f. According to this theory, the catch is a fraction, a/a+f, of the phage active at the time of the dump. In the deactivation experiments, C declines exponentially with the rate constant f. In the adsorption experiments, C(30)-C(T) declines exponentially with the rate constant, a+f. When these quantities are estimated from the data, they may or may not be found to be compatible with each other.

The preactivation theory predicts complex relations, which simplify when the assumption is made that activation is the rate controlling step. Specifically, this theory then predicts an exponential time dependence of the quantities C, and C(30)-C(T) in the deactivation and adsorption experiments, respectively. It also predicts that only the rate constant, b, is measured in both the deactivation and adsorption experiments. Since the adsorption rate of active phage is assumed to be large compared to the rate of trans-

formation of preactive phage to active phage, the adsorption rate of active phage could be as large as the adsorption rate of phage maximally activated by tryptophane. However, neither the deactivation nor the adsorption rate is directly measureable according to this view. The physical picture suggested by this theory is that urea must be desorbed from preactive phage or new secondary or tertiary bonds must be formed before preactive phage become adsorbable. Moreover, these steps must be rate limiting.

EXPERIMENTAL RESULTS

1. Activation of T4.38 by urea, at 0° C.

In this experiment, the rate at which T4.38 is activated by urea and the rate at which it is killed are studied as a function of urea concentration.

At t=0, 5.0 ml of urea solution at pH 6.5 and 0° C, is poured over 0.1 ml of a suspension of T4.38 in buffer. This reaction tube is maintained at 0° C. After t minutes, 0.1 ml of the reaction mixture is diluted into 10 ml of standard bacterial suspension. This adsorption tube is maintained at 15° C for thirty minutes, at which time maximal adsorption has occurred. Aliquots are then plated on F' and N agar.

The results are presented in figure 1a, in which F'/N is plotted against time of exposure to urea, and in figure 1b, in which the fraction of survivors as measured by the N assay is plotted against time.

It is seen that F'/N rises and then declines with time of exposure to urea. Initially, there is a linear rise, $F'/N = kt$. The rate constant, k, is strongly dependent on the concentration of urea. From figure 1c, in which log k is plotted against log urea concentration, it is seen that k is proportional to the fourteenth power of the urea concentration in the concentration range 2.0 M to 3.0 M.

Initially, the fraction of survivors satisfies the re-

lation, $\ln(N/N_o) = -ct$ (figure 1b). It is seen from figure 1d, in which log c is plotted against log urea concentration that c is proportional to the tenth power of the urea concentration.

2. Activation of S35 labelled T4.38

F'/N is the fraction of surviving phage which in our standard test adsorb to bacteria. The question arises whether the killed phage adsorbs to the same extent, i.e., whether killing and activation are statistically independent events. In order to determine whether F'/N is also the fraction of the total population which adsorbs to bacteria, an activation experiment was performed, using S35 labelled phage. For five different times of exposure to urea, the fraction of adsorbable radioactivity adsorbing to bacteria in F' medium was determined by spinning down the bacteria in the adsorption mixture and assaying both the bacterial pellet and the supernatant for radioactivity. From the same adsorption mixtures, aliquots were plated on F' and N agar. As a control, the experiment was repeated, using B/4 as the adsorbing bacteria. The activating solutions were 2.5 M urea solutions in distilled water at 0° C and unspecified pH.

The results are presented in figure 2a, in which the fraction of adsorbable radioactivity adsorbing to B and B/4, and F'/N is plotted against time. In figure 2b, the

fraction of survivors is plotted against time.

From figure 2a, it is seen that, at all five points, the fraction of the total population adsorbing to B is equal, within the limits of error, to the fraction of surviving phage adsorbing to B, indicating that killing and activation are statistically independent processes. Since the extent of the urea induced adsorbability to B/4 is very slight, it can be concluded that the adsorbability conferred upon the phage by urea is brought about by bringing the attachment organ into some configuration specific for allowing the phage to adsorb to B, rather than to B/4.

3. Deactivation of urea activated T4.38

F'/N is the fraction of surviving phage which adsorbs to bacteria in our standard test. This is not necessarily the fraction activated by the treatment, as explained in the preceding theoretical discussion. A larger fraction may have been made adsorbable by the urea treatment, but upon dilution into the bacterial suspension, some of the phage may lose this adsorbability before they manage to adsorb. In other words, our catch of activated phage may be below 100%, as it is in the case of activation by tryptophane. To get an estimate of this deactivation, the following experiment was performed.

The phage are activated for precise lengths of time in urea solutions at 0^{o} C and at pH 6.5. After t minutes of

activation, 0.1 ml of the urea-phage mixture is diluted into 10 ml of F' medium, maintained at 15° C. This is the deactivation tube. At T minutes after dilution into the deactivation tube, 0.1 ml from the deactivation tube is diluted into 10 ml of the standard bacterial suspension. Thirty minutes later, aliquots are plated on F' and N agar. The results are presented in figure 3 for four different activation programs. F'/N is plotted against deactivation time. The N assays remained constant in every case during the deactivation period, and so are not presented here. In each case, F'/N declines exponentially with the deactivation time with the same rate constant, f, of approximately 0.1 min. We conclude that upon dilution into F' medium, the phage population changes in the course of time with respect to its adsorbability in a subsequent dump experiment. Similar changes must take place in the dump tube also. Therefore, the dump experiment characterizes some kind of average of the properties of the phage population, averaged over the thirty minute period of sojourn in the dump tube, rather than its overt properties at the moment of the dump.

4. Adsorption rate of urea activated T4.38.

This experiment is designed to analyze the course of events in the dump tube, by plating samples from it at various periods.

The phage are activated in urea solutions at 0° C and pH 6.5. After t minutes of activation, 0.1 ml of the urea-phage mixture is diluted into 10 ml of the standard bacterial suspension. T minutes later, the phage-bacteria mixture is diluted into F' medium at 0° C to stop further adsorption. Aliquots from this final dilution are plated on F' and N agar.

The results are presented in figure 4a, where $\frac{C(30)-C(T)}{C(30)}$ is plotted against adsorption time. Initially this quantity declines exponentially with a rate of about .06/min.

5. Effect of temperature on the activation process.

A characteristic feature of denaturations of protein by urea is that the process is slowest at about 20° C, and increases in rate as the temperature is either raised or lowered from this value. This experiment was designed to test whether the activation and killing of phage by urea depend on the temperature in a similar manner.

Phage were activated in 2.5 M urea solutions at pH 6.5 and at temperatures ranging from 0° C to 47° C. The results are presented in figures 5a, 5b, and 5c.

It is to be noted that both the activation and killing rates decrease as the temperature is raised from 0° C to 15° C and increase as the temperature is raised from 37° C to 47° C, with a minimum occurring somewhere between 15° C and 37° C.

6. Effect of pH on the activation process.

In general, proteins are least susceptible to denaturation at their isoelectric point. Phage T2, which is closely related to T4, has an isoelectric point of about 4.2 (32). This experiment was designed to determine whether the urea activation process depends on the pH in a manner consistent with this general principle.

Phage were activated by 2.0 M urea solutions at 0° C and at pH's 6.0, 6.5, 7.0, and 8.0.

The results are presented in figures 6a and 6b. It is to be noted that both the activation and killing rates increase as the pH is increased from 6.0 to 8.0, consistent with the notion that proteins are most resistant to denaturation at their isoelectric point. It is assumed, of course, that the isoelectric point of the phage is identical with the isoelectric point of the phage attachment organ.

7. Effect of tryptophane on the urea activation process.

Since tryptophane and urea are both efficient in activating cofactor requiring phage, their interaction in an activating mixture would be of interest. To determine the nature of this interaction, the following experiment was designed.

Phage were activated in 2.5 M urea solutions at 0° C and pH 6.5 to which was added 20 gamma/ml of L-tryptophane. The experiment included controls measuring activation in the

presence of urea only, and in the presence of tryptophane only.

The results are presented in figure 7a in which the catch is plotted against activation time, and in figure 1b in which the fraction of survivors is plotted against activation time.

Much greater activation is achieved in the presence of urea alone than in the presence of tryptophane alone. An intermediate amount of activation occurs in the mixture of the urea and tryptophane. It is to be noted that tryptophane inhibits both the activation and killing of phage by urea. The simplest interpretation of this result is that tryptophane competes with the urea molecules for adsorbing sites on the phage. Tryptophane is remarkably efficient in this respect, in view of the great discrepancy in the concentrations of tryptophane and urea.

DISCUSSION

Our experiments clearly demonstrate that urea is capable of activating a cofactor requiring phage, imparting to it the ability to adsorb specifically to its normal host bacterium. That the effects we observe are not merely due to a urea-induced sensitivity to traces of tryptophane-like cofactor is shown by two facts: 1) the stock urea and freshly recrystallized urea give the same results, and 2) tryptophane inhibits both the activation and killing by urea.

The question arises whether urea produces this effect by a denaturing action. In comparing the kinetics of the urea activation process to the kinetics of denaturation by protein, we find an extensive similarity. The activation rate depends upon a high power of the urea concentration (circa 14th power), and increases when the reaction temperature is either lowered below 15° C or raised above 37° C. The rate also increases as the pH is raised from the isoelectric point of the phage. These properties are characteristic of denaturation of protein by urea (17). Therefore, it seems extremely probable that urea effects an activation of cofactor requiring phage by a denaturation of the phage attachment organ. The reversible nature of this denaturation process is to be inferred from the deacti-

vation experiments in which it is shown that urea-activated phage lose their urea induced ability to adsorb to bacteria in cofactor-free medium when the urea is removed by dilution, but still retain their ability to form plaques on N agar.

We may now compare the predictions of the three hypotheses, which were discussed in the theoretical section, with the experimental results. In the activation experiments, we find catches varying from unity to zero. In the deactivation experiments, we find that the catch declines exponentially with the deactivation time with a rate constant of approximately 0.1/min; while in the adsorption experiments, we find that the quantity, C(30)-C(T), declines exponentially with the adsorption time with a rate constant of approximately .06/min.

The intermediate state theory deviates from the experimental results in that it does not predict an exponential decline for the catch in the deactivation experiments. It does so only in the limiting case where a_o/f is small compared to unity. In the case where the catch does approach unity, a_o/f cannot be small compared to unity because this theory predicts a catch equal to $1-e^{-a_o/f}$. Thus, the intermediate state theory is unsatisfactory.

According to the all-or-none theory, the catch is a fraction, $a/(a+f)$, of the phage active at the time of the dump. For the deactivation experiments, this theory pre-

dicts that the catch should decline exponentially with the rate constant, f, and for the adsorption experiments, it predicts that the quantity, C(30)-C(T), should decline exponentially with the rate constant, a+f. When these rate constants are estimated from the data, it is found that a+f (.06/min) is actually smaller than f (0.1/min). If this discrepancy is attributed to experimental error, we would still have to infer that a is small compared to f. Since the maximum catch attainable according to this theory is a/(a+f), the catch should never attain values approaching unity as is found experimentally. We feel that the large errors in the determination of the catch, which must be assumed to make this theory compatible with the data, are unlikely.

The preactivation theory predicts an exponential decline of C and C(30)-C(T) in the deactivation and adsorption experiments, respectively, when the assumption is made that the transformation from the preactive to the active state is rate limiting. No difficulty is encountered in explaining catches which approach unity, as the catch depends on the relative magnitudes of the rate constants, a and f, and these constants are not directly measurable according to this view. In fact, this theory predicts that only the rate constant, b, is measured in both the deactivation and adsorption experiments, i.e., both C and C(30)-C(T) should decline exponentially with the same rate

constant, b, in the deactivation and adsorption experiments, respectively. Actually, the rate constant determined in the adsorption experiments is found to be slightly smaller than the rate constant determined in the deactivation experiments. This could be attributed either to experimental error or to the fact that some error was introduced in making the extreme assumption that the rate constant, b, is negligibly small compared to the constants, a and f.

Because the preactive state theory is best in agreement with the experimental results, its correctness will be assumed. However, it is only necessary to assume that the catch is a measure of the degree of activity to give a reasonable interpretation to the data. It must be borne in mind that a kinetic analysis can answer the question of correctness of a theory with certainty only in a negative manner, i.e., a kinetic analysis can only determine that a certain theory is wrong with certainty.

Certain experiments are suggested by the preactive state theory. For instance, the theory predicts that the rates observed in the deactivation and adsorption experiments should be coincident at all temperatures as long as changing the temperature does not alter the rate limiting character of the transformation from preactive to active phage. This must be left for future investigation.

The phenomenon of urea activation of cofactor requiring phage supports the hypothesis that the action of trypto-

phane on phage consists in controlling the equilibrium between two states of the protein of the phage attachment organ, analogous to native and reversibly denatured protein. Three questions are raised by this hypothesis: 1) how does one visualize an intramolecular rearrangement of protein conferring activity on cofactor requiring phage, 2) is the postulated denaturing activity of tryptophane consistent with the fact that tryptophane is not a known denaturing agent, and 3) how do we have to interpret the kinetic data of Stent and Wollman and of McKee in terms of this new notion?

A clue to the first question concerning the nature of the intramolecular rearrangement, is furnished by the work of Puck et al. (11,12,13). These workers have shown that the first step in the adsorption of phage to bacteria is an electrostatic binding between complementary charge patterns on the phage and bacterium. The charge pattern on the phage is composed of ionized surface amino groups, and in the case of T1 carboxylic groups are also involved (13). Cofactor requiring phage must first react with cofactor before this electrostatic binding to bacteria or even to glass filters can occur (12). The effect of urea and tryptophane can then be visualized as follows: these agents cause an intramolecular rearrangement of the protein of the phage attachment organ resulting in either a new spacing of the amino groups or an exposure of these amino

groups. Increased reactivity of constitutive groups, including amino groups, is a well known result of protein denaturation (17).

The second question concerning the postulated denaturing activity of tryptophane, can be answered by assuming that the action of tryptophane on protein is so mild and readily reversible that it is not detectable by the methods employed, or more likely, that tryptophane denatures only a few proteins like the protein of the attachment organ of T4.38, which possess some structural peculiarity which make them especially labile to denaturation by tryptophane. In this connection, we should recall the structural specificity required of tryptophane. D-tryptophane is inactive, alteration of the amino group or carboxylic group results in complete loss of activity and substitution in the indole nucleus results in reduced activity.

In the case of T4.38, the structural peculiarity is such that a specific group of inter- or intra-helical bonds are especially sensitive to tryptophane action, and that after these bonds are broken, the protein has the correct configuration for attachment to bacteria, and is resistant to further denaturation. That proteins are constructed in such a manner, that upon denaturation, certain specific, and relatively stable intermediates are formed, is shown by the work of Rothen and Landsteiner (33), and many others. Rothen and Landsteiner obtained rabbit antiserum against heat denatured hen ovalbumin. This antiserum specifically pre-

cipitated the denatured hen ovalbumin, and reacted only weakly with the native hen ovalbumin. Although this antiserum also precipitated denatured guinea hen ovalbumin, upon absorption with denatured guinea hen ovalbumin, it no longer precipitated denatured guinea hen ovalbumin, but retained its ability to precipitate denatured hen ovalbumin, thus showing that denaturation conferred new antigenic specificities upon the hen ovalbumin. In the case of urea activated T4.38, phage which are adsorbable to B and still possess plaque forming ability can be considered as intermediates in the denaturation process. Further mutilation by urea causes these phage to become adsorbable to B/4 or to lose their adsorbability to B.

Before proceeding with the interpretation of the kinetic data of Stent and Wollman and of McKee in the light of the denaturation notion, we would first like to recall some of their findings and review some of the implications of the Stent and Wollman model (7,8,9,15). Stent and Wollman found that the degree of activity of phage T4.38 equilibrated with tryptophane is proportional to the fifth power of the tryptophane concentration at low concentrations of tryptophane. Upon addition of tryptophane to inactive phage, they found that the degree of activity rises in a first order manner, at a rate proportional to the fifth power of the tryptophane concentration at low concentrations of tryptophane. Upon removal of the tryptophane by dilu-

tion, the degree of activity declines exponentially at a rate independent of the original degree of activity. Small residual concentrations of tryptophane can lower the rate of deactivation to a much greater extent than could be explained by a competition between deactivation and activation by this residual tryptophane concentration. The equilibrium degree of activity is proportional to the fifth power of the tryptophane concentration at limiting concentration at all temperatures tested (5° C to 45° C). The activation rate at limiting concentrations of tryptophane rises a factor of ten thousand when the temperature is raised from 5° C to 37° C. Reducing the ionic strength of the activation mixture greatly facilitates the ability of tryptophane to activate the phage. In raising the pH from 4 to 8, the activity imparted to T4 phage increases by a factor of more than ten. Cofactor requiring mutants of T4 exist with qualitatively and quantitatively different cofactor requirements. Amino acids other than tryptophane possess cofactor activity.

Stent and Wollman also found that cofactor requiring phage, upon being liberated from lysing bacteria, possess activity which is lost much more slowly in the absence of external cofactor than tryptophane induced activity (34). Phage newly liberated from bacteria are called nascent phage, and their long-lasting activity is called nascent activity. Phage which have lost this nascent activity are

designated quiescent phage. Nascent activity is responsible for the ability of bacteria infected with cofactor requiring phage to form plaques on F agar.

This extensive list of experimental facts are all consistent with the model proposed by Stent and Wollman. According to the model, phage particles contain sites which can exist in an active or inactive state. Upon addition of tryptophane to inactive phage, the tryptophane molecules rapidly adsorb and desorb from these sites. If due to fluctuation, a site has five or more tryptophane molecules adsorbed to it, these tryptophane molecules may undergo some slow, rate limiting interaction, resulting in their being more tightly bound to the site, and resulting in activity of the site. It should be emphasized here that this model envisages a surface of constant structure to which cofactor molecules adsorb, and interact to form a surface complementary to the bacterial membrane. The rate limiting nature of this interaction accounts for the first order kinetics of activation, while the necessity for five molecules of tryptophane accounts for the fifth power dependence of the activation rate on the tryptophane concentration. Active sites deactivate spontaneously at the rate found when the tryptophane is removed, and the equilibrium degree of activity is then the outcome of simultaneous activation and deactivation. To account for the effect of residual tryptophane in retarding deactivation, Stent and

Wollman postulated that an active site having a sixth subsite occupied by tryptophane does not deactivate. The enormous effect of temperature on the degree of activity at limiting concentrations was explained by postulating a moderate dependence on the temperature of the affinity of cofactor for the subsites. The effect of pH and ionic strength, the existence of mutants possessing different cofactor requirements, the cofactor activity of compounds other than tryptophane, etc. could all be explained by postulating a difference in one or more of the parameters of the model, such as number of cofactor molecules required, affinity of subsites for cofactor, rate of deactivation of active sites, etc.

The indole findings are more difficult to interpret in terms of the Stent and Wollman model. The degree of activity attained in mixtures of tryptophane and various concentrations of indole is proportional to the inverse first power of the indole concentration, and the degree of activity remains constant when the ratio of tryptophane to indole is held constant providing that the concentration of tryptophane is sufficiently high to impart maximum activity to the phage in the absence of the indole. The structural similarity of indole and tryptophane makes it highly probable that tryptophane and indole would compete for the same subsites. When the degree of activity in indole and tryptophane mixtures is calculated on this assumption and

on the further assumption that a key site having one or more of its subsites occupied by indole cannot become active, an entirely different relation is derived. McKee was able to show that the inverse first power relation was due to the fact that indole reacts only with active phage, and that when one molecule of indole combines with an active phage, it quickly deactivates.

We now turn to the interpretation of the tryptophane and indole findings in terms of the denaturation hypothesis of cofactor action. According to this concept, the protein of the phage attachment organ exists in two forms, an active form, and an inactive form. These two forms are in equilibrium with each other, but in the absence of cofactor the equilibrium is overwhelmingly in favor of the inactive form. Activation consists in denaturation of this inactive protein. The cooperation of five or more tryptophane molecules is then envisaged as necessary for opening the structure of this inactive protein. After the opening of this structure, it is conceivable that relatively few and perhaps only one tryptophane molecule can prevent the refolding of the protein by combining with the groups exposed by the unfolding process. In this way we can account for the great efficiency of small concentrations of tryptophane to retard the deactivation process, and also if we postulate that indole competes with tryptophane for these newly exposed groups, and that indole is somehow efficient in promoting

the refolding process, we can also account for the efficiency of indole in deactivating active phage. It is not possible to say how tryptophane effects this denaturation. Perhaps it acts by lowering the thermal energy necessary for the breaking of the requisite number of bonds. The very large temperature coefficient of the tryptophane activation process could be attributed to the necessity of breaking a large number of bonds, simultaneously. The effect of pH and ionic strength on the tryptophane activation process could, on this view, be attributed to their effect on the charge on the protein, if it is assumed that this charge effects its sensitivity to denaturation. At any rate, denaturation of protein is known to depend on pH and ionic strength in a similar manner. The existence of nascent phage, and of cofactor requiring phage having different cofactor requirements, could be attributed to a difference in configuration of their attachment organ protein. In the case of nascent phage, we would say that when they are released from bacteria, a number of the secondary and tertiary bonds present in quiescent phage are yet unformed. According to the denaturation hypothesis, the deactivation rate can be either attributed to the rate at which cofactor desorbs from active phage, or to a rate limiting refolding after all cofactor has desorbed. Either point of view is consistent with the different deactivation rates observed for tryptophane, phenylalanine, and urea-activated phage. According to the

first view, different cofactors have different affinities for active phage, and according to the second view, different cofactors denature to different extents.

Further support for the denaturation hypothesis comes from the work of N. K. Jerne (35). Jerne found that if cofactor requiring phage are activated with tryptophane, and these active phage are treated with the early serum of a horse immunized with T4, these phage become stably cofactor independent. That is, these phage do not lose their ability to adsorb to bacteria in cofactor-free medium when the tryptophane is removed by dilution. Jerne has also shown that the fraction of the serum responsible for this phenomenon is the gamma globulin fraction. If the tryptophane acted by adsorbing to the static surface of the phage protein and thereby made it complementary to the bacterial surface, it would follow that the role of the gamma globulin would be to prevent the desorption of the tryptophane when the external concentration of tryptophane is lowered by dilution. Under this picture, it seems certain that the gamma globulin molecules would sterically hinder the adsorption of phage to bacteria. If, on the other hand, the tryptophane were to cause a widespread unfolding of the phage protein resulting in adsorbability, then the gamma globulin could be pictured as stabilizing this unfolded configuration by adsorbing to it and thus preventing the refolding of the protein when external cofactor is

removed. The gamma globulin could then perform its function by adsorbing to the protein at a point distant from the actual point of attachment of phage to bacteria.

It is tempting to generalize the hypothesis of the denaturing activity of tryptophane. The remarkable efficiency and specificity of small molecules in regulating the physiological activity of cells could be explained if it were assumed that the effect of the small molecules is a specific and reversible denaturation of proteins. The extreme sensitivity of denaturation to concentration of denaturant, temperature, ionic environment and pH would make this mechanism extremely useful in regulating cellular physiology.

Figure 1a

Activation in various concentrations of urea at 0° C, pH 6.5

Curve A: 3.0 M urea

Curve B: 2.5 M urea

Curve C: 2.0 M urea

FIGURE 1a

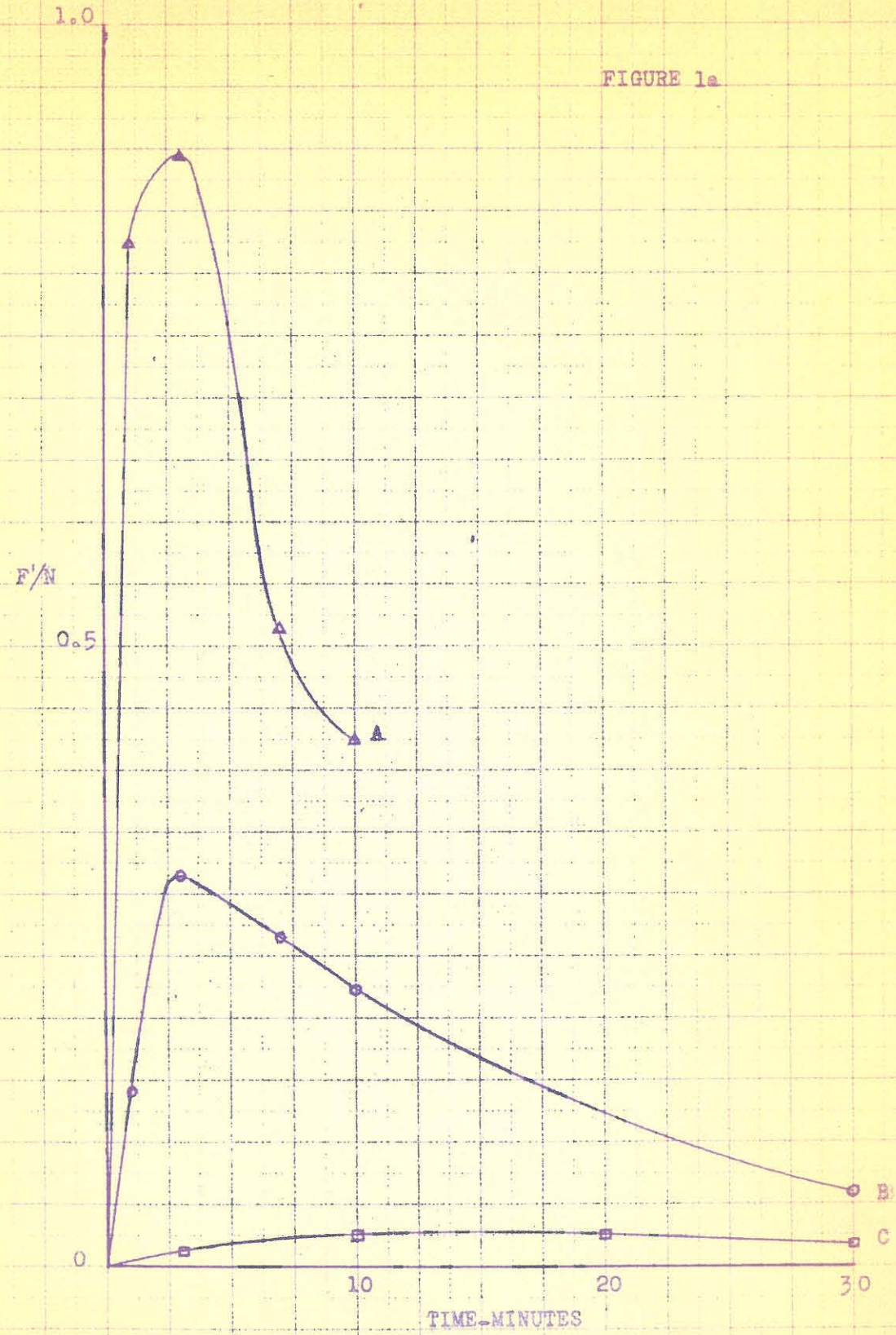


Figure 1b

Killing by various concentrations of urea at 0° C, pH 6.5

Curve A: 3.0 M urea

Curve B: 2.5 M urea

Curve C: 2.0 M urea

FIGURE 1b

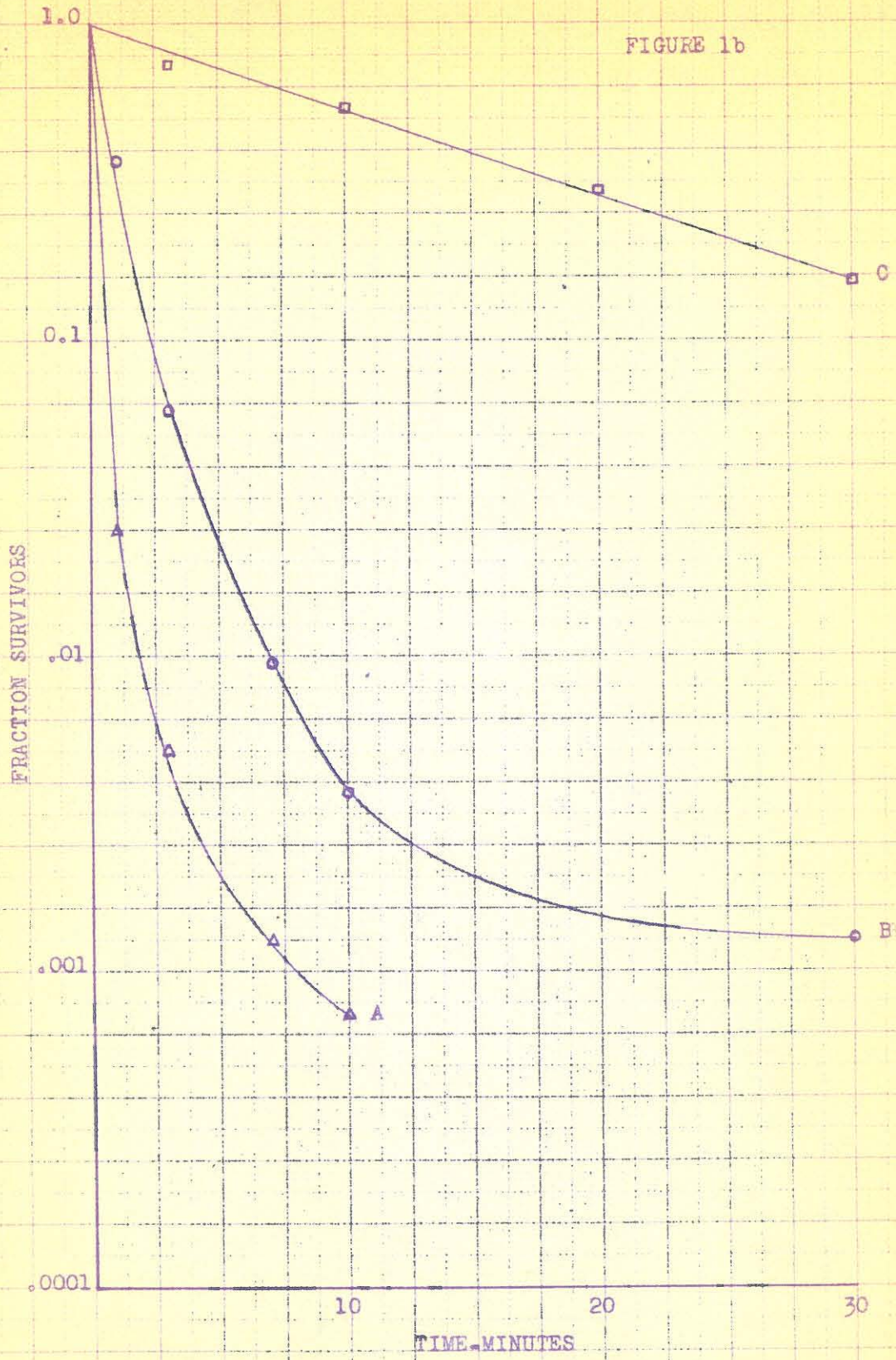

1.0
0.1
.01
.001
.0001
FRACTION SURVIVORS
10
20
30
TIME-MINUTES
A
B
C

Figure 1c

Log activation rate constant, k, versus log concentration of urea

$$F/N = kt.$$

$$k = K\ (u)^{14}$$

FIGURE 1c

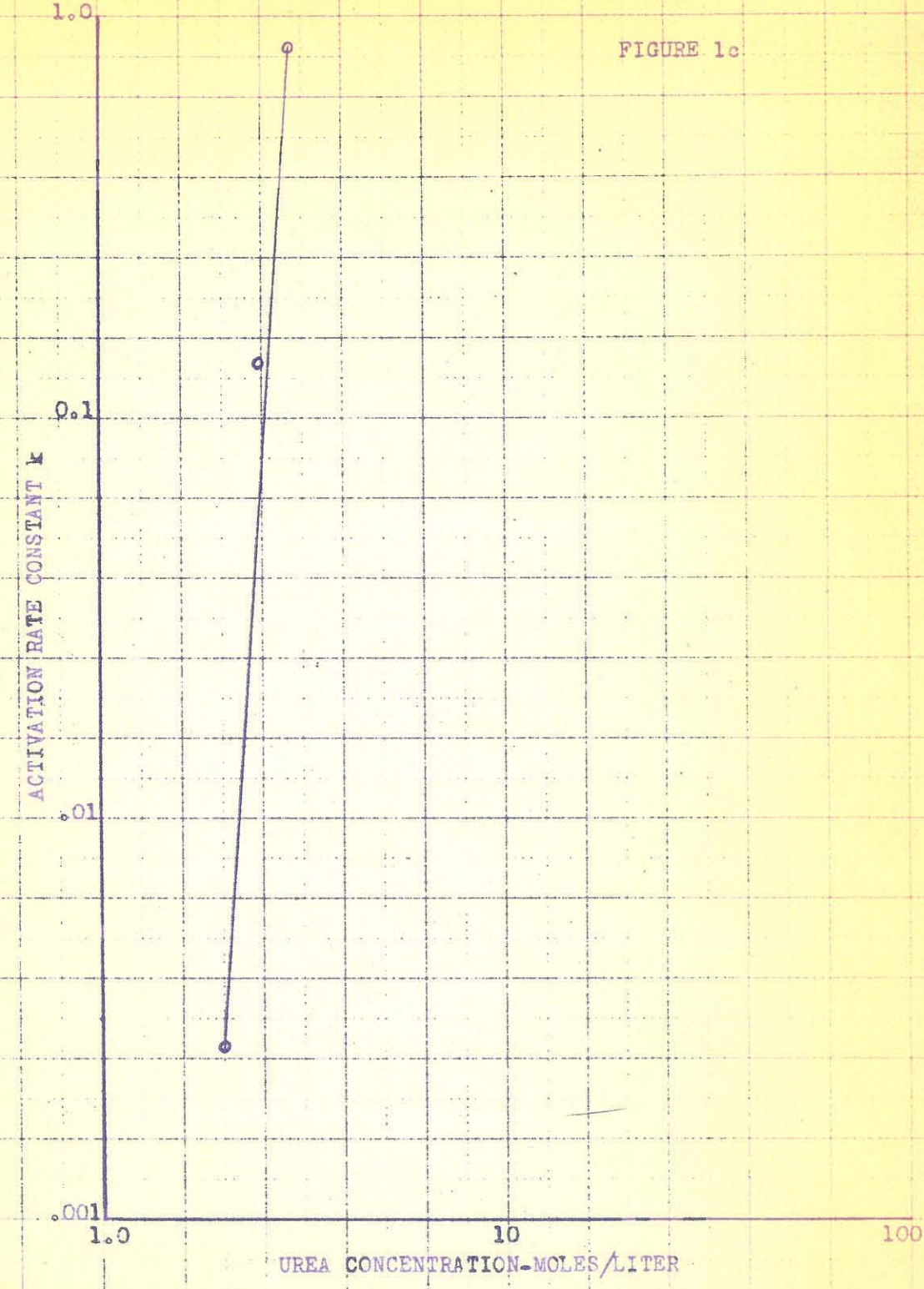

1.0
0.1
.01
.001
ACTIVATION RATE CONSTANT k
1.0
10
100
UREA CONCENTRATION-MOLES/LITER

Figure 1d

Killing rate constant, c, versus concentration of urea

$$N/N_o = e^{-ct}$$

$$c = C(u)^{10.5}$$

FIGURE 1d

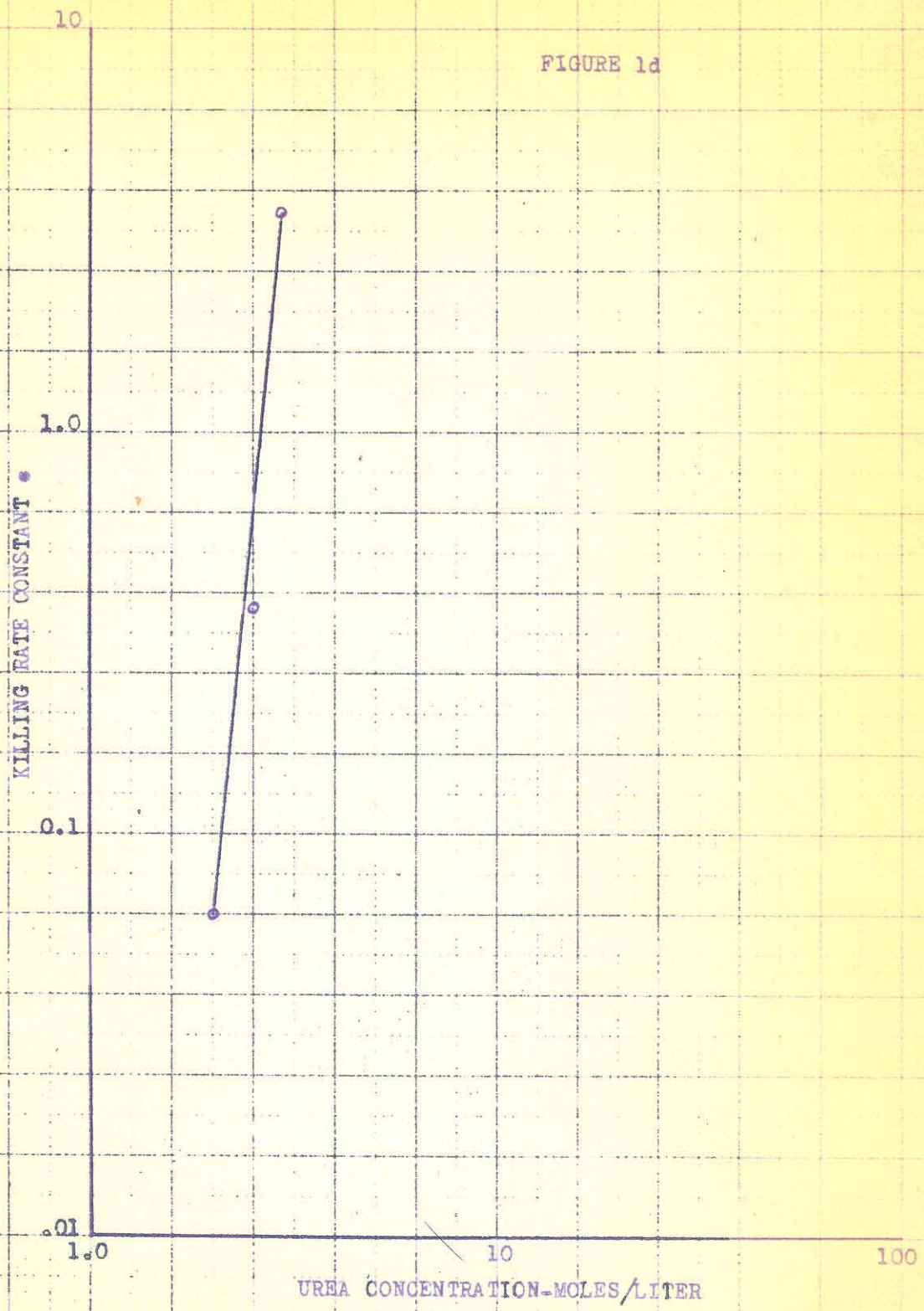

10
1.0
0.1
.01
KILLING RATE CONSTANT
1.0
10
100
UREA CONCENTRATION-MOLES/LITER

Figure 2a

Activation of S35 labelled T4.38 in a 2.5 M solution of urea in distilled water at 0° C, and unspecified pH

Curve A: Fraction of S35 adsorbed versus time of exposure to urea

Curve B: F/N versus time of exposure to urea

Curve C: Fraction of S35 adsorbed to B/4 versus time of exposure to urea

FIGURE 2a

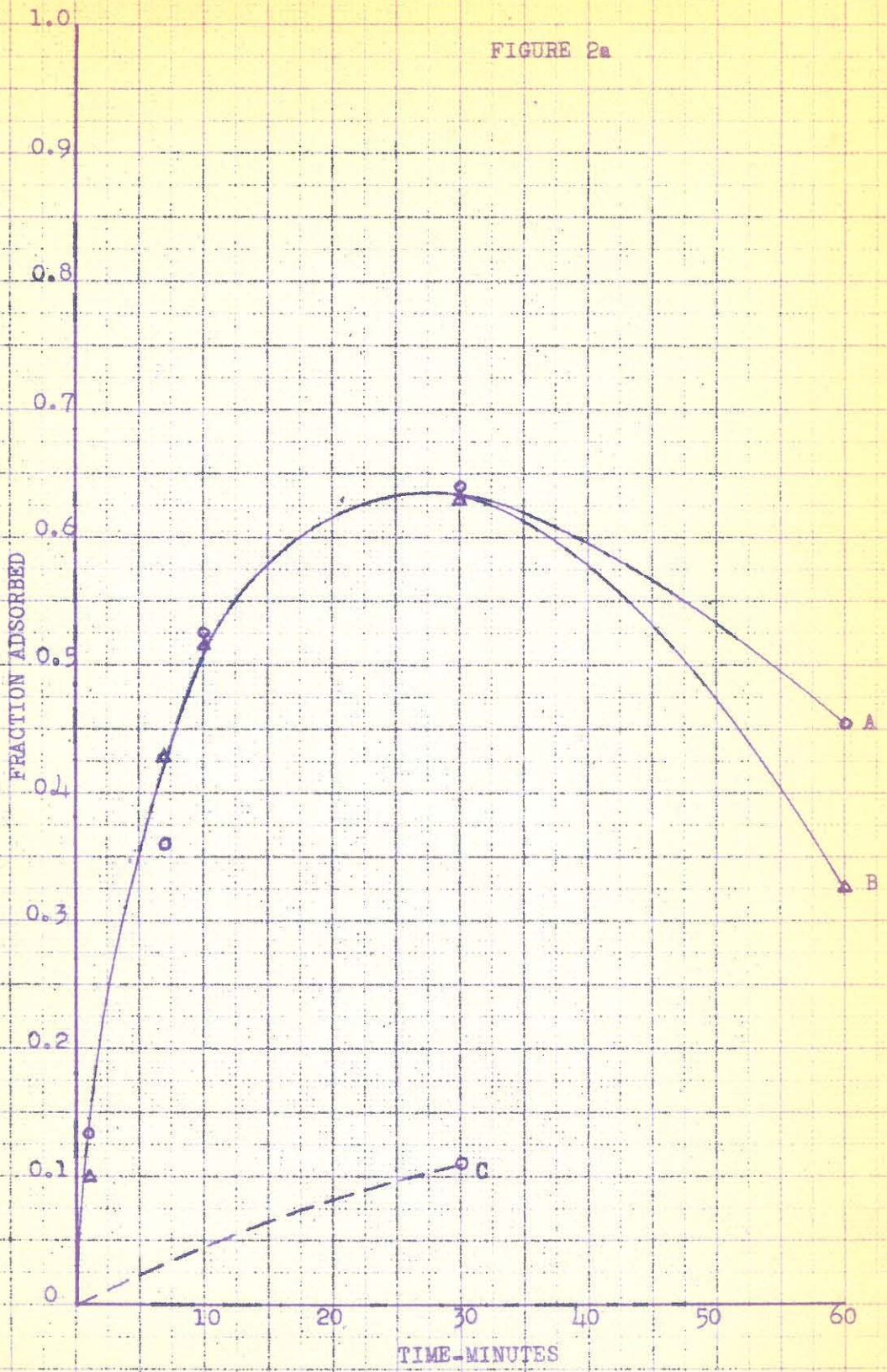

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
FRACTION ADSORBED
10
20
30
40
50
60
TIME-MINUTES
A
B
C

Figure 2b

Killing of S35 labelled T4.38 by 2.5 M solutions of urea in distilled water at 0° C and unspecified pH

FIGURE 2b

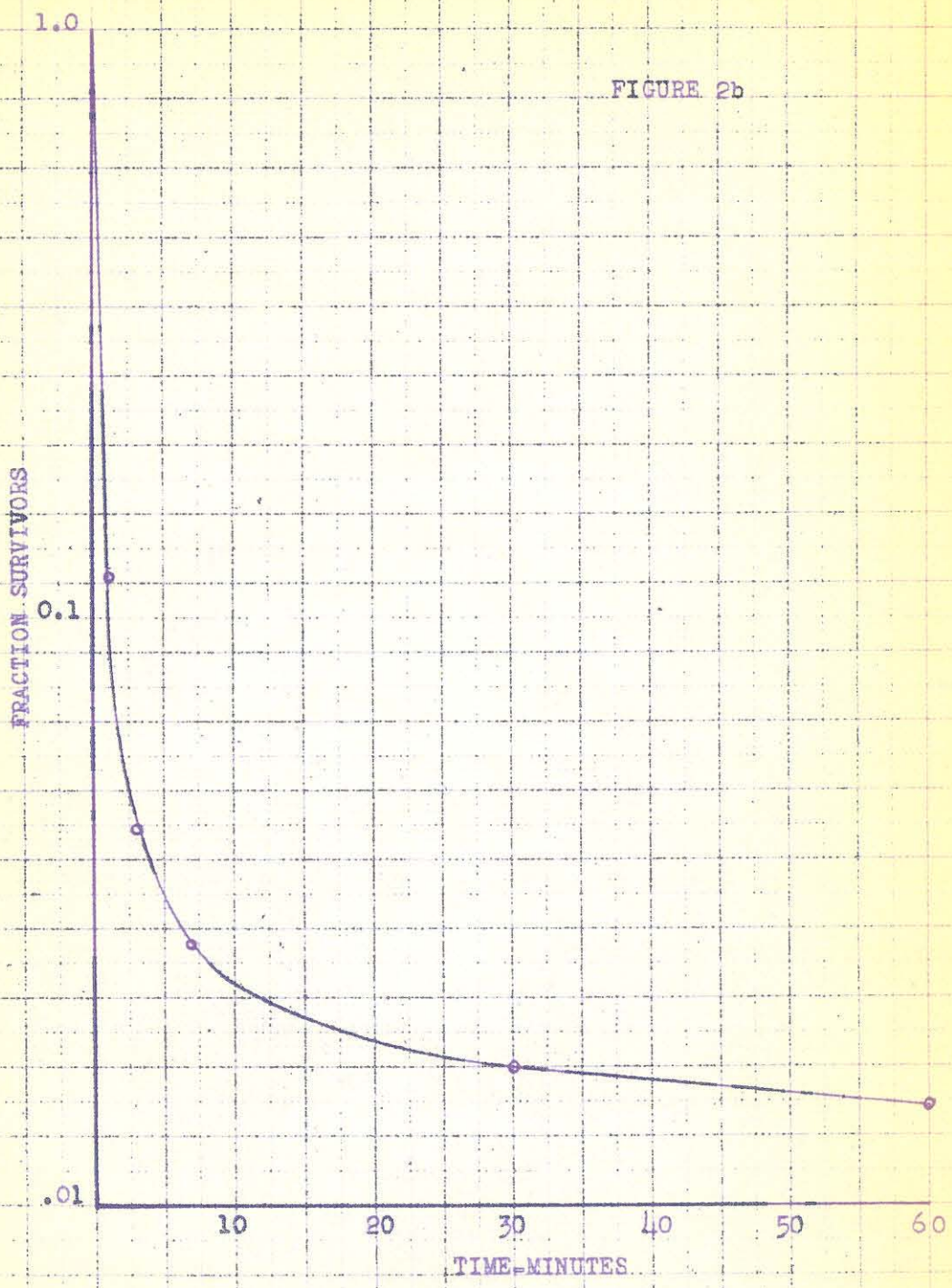


Figure 3

Deactivation in 15° C F' medium

Curve A: Activation in 3.0 M urea at 0° C and pH 6.5 for two minutes

Curve B: Activation in 2.5 M urea at 0° C and pH 6.5 for two minutes

Curve C: Activation in 2.5 M urea at 0° C and pH 6.5 for ten minutes

Curve D: Activation in 2.0 M urea at 0° C and pH 6.5 for twenty minutes

FIGURE 3

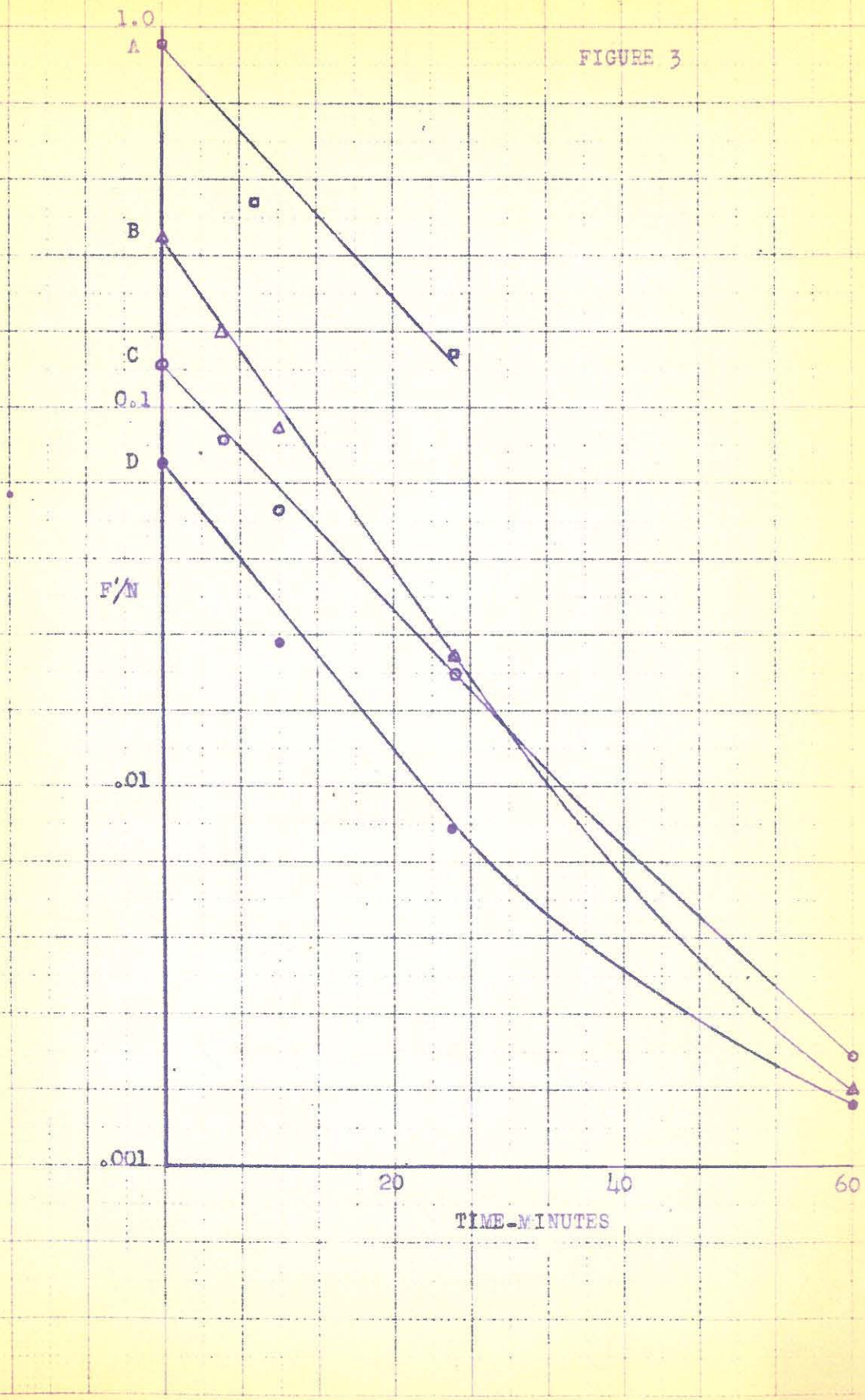


Figure 4

Adsorption of T4.38, which has been activated by urea at 0° C, and pH 6.5, in the standard bacterial suspension

o 2.0 M for twenty minutes

. 2.5 M for ten minutes

x 2.5 M for two minutes

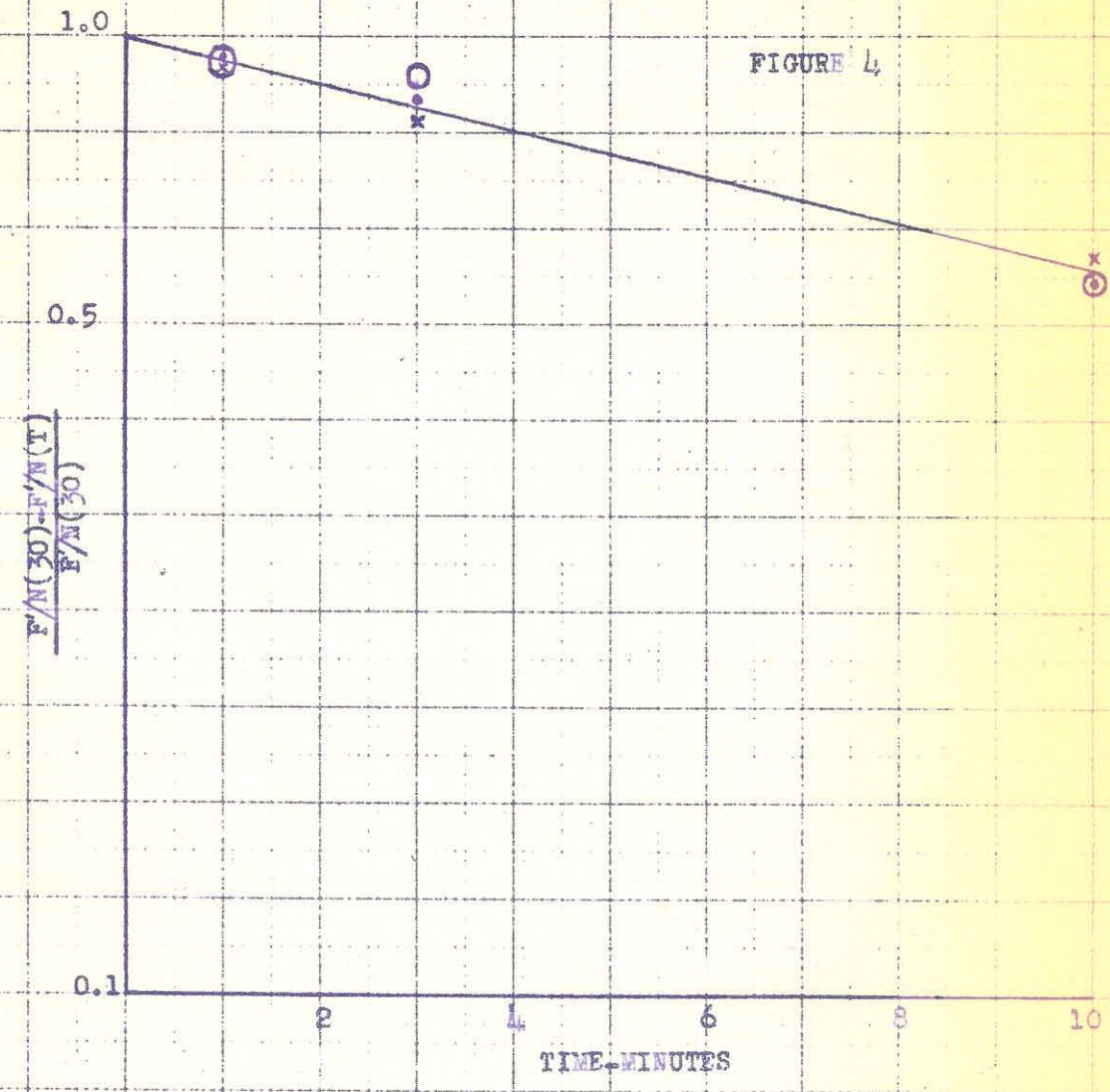


FIGURE 4

Figure 5a

Activation in 2.5 M urea at pH 6.5, and at various temperatures

Curve A: 0° C

Curve B: 47° C

Curve C: 37° C

Curve D: 4.5° C

Curve E: 15° C

FIGURE 5a

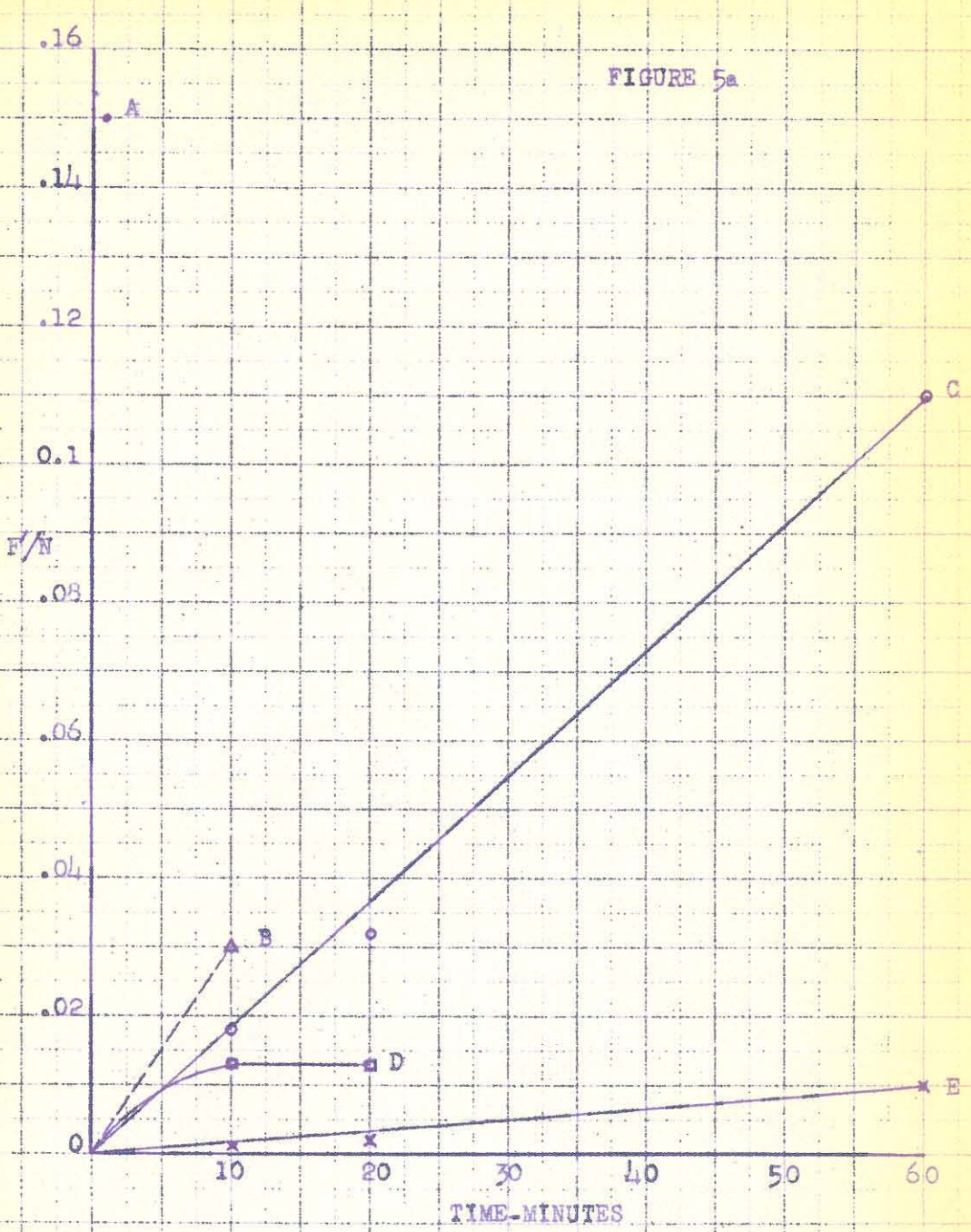


Figure 5b

Killing at various temperatures by 2.5 M urea at pH 6.5

Curve A: 15° C

Curve B: 37° C

Curve C: 4.5° C

Curve D: 0° C

Curve E: 47° C

FIGURE 5b

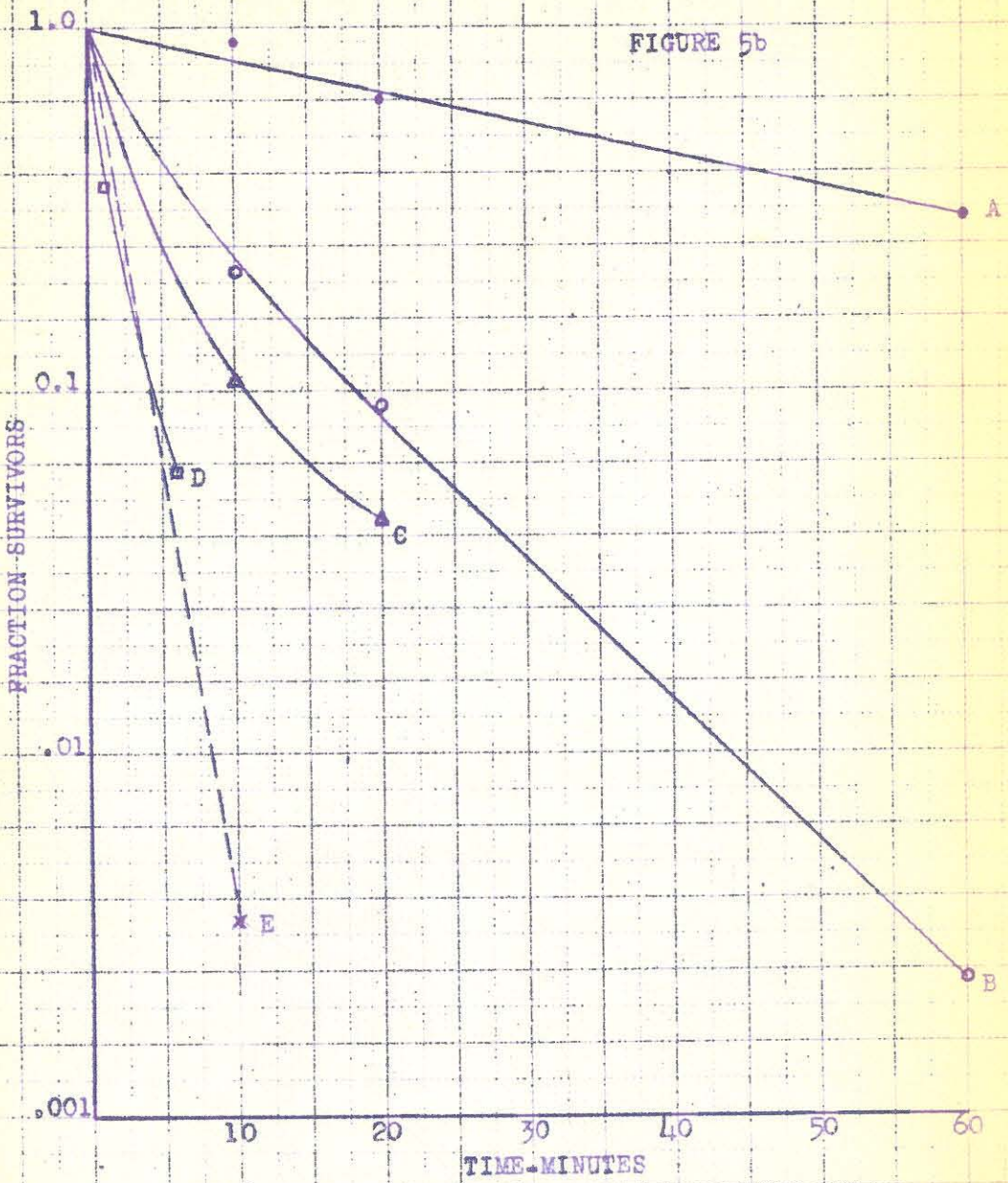

1.0
0.1
.01
.001
FRACTION SURVIVORS
10
20
30
40
50
60
TIME-MINUTES
A
B
C
D
E

Figure 5c

Activation rate constant, k, in 2.5 M urea at pH 6.5 versus temperature.

FIGURE 5c

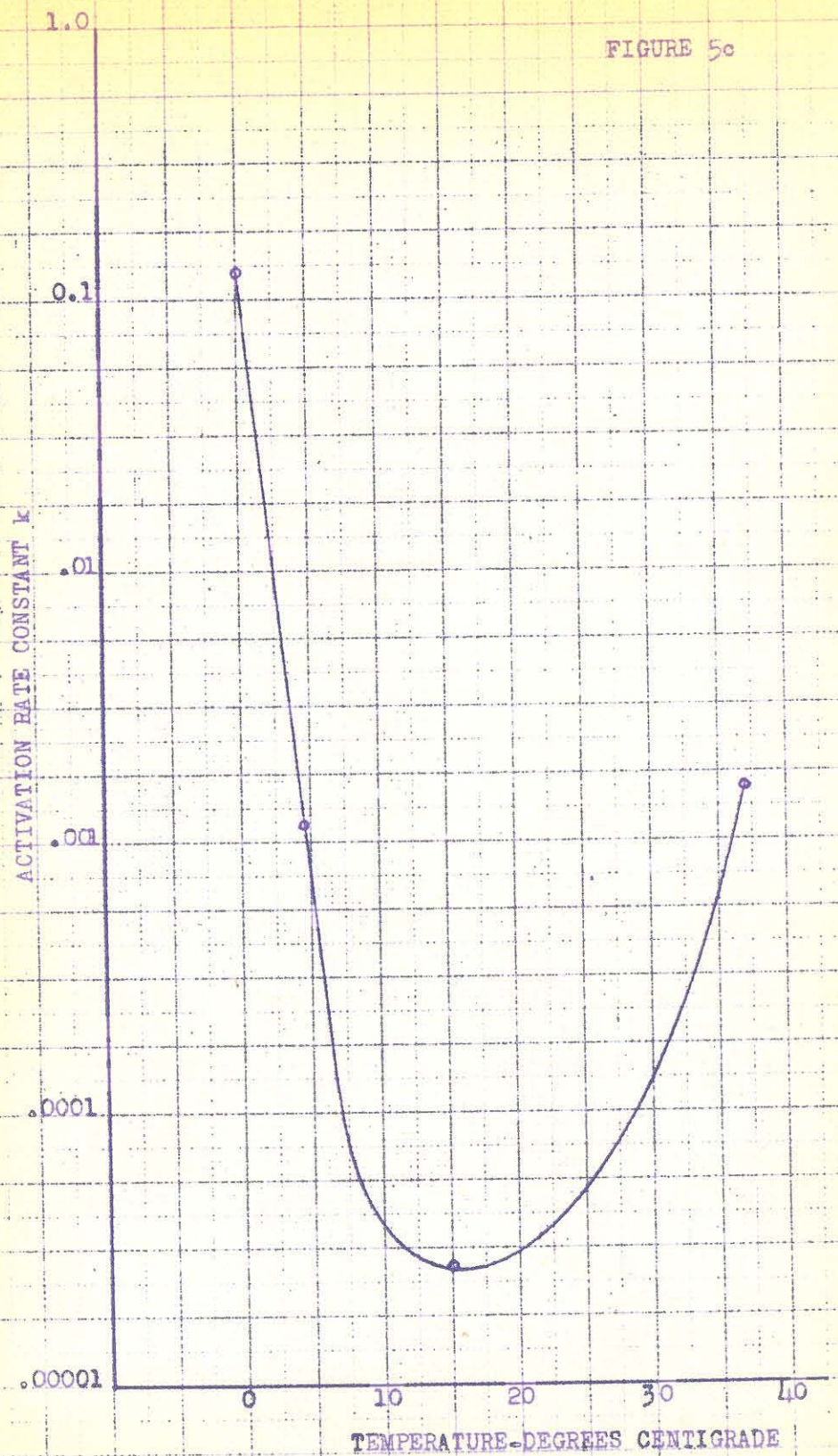

1.0
0.1
.01
.001
.0001
.00001
ACTIVATION RATE CONSTANT k
0
10
20
30
40
TEMPERATURE-DEGREES CENTIGRADE

Figure 6a

Activation in 2.0 M urea at 0° C, and at various pHs

Curve A: pH 8.0

Curve B: pH 7.0

Curve C: pH 6.5

Curve D: pH 6.0

FIGURE 6a

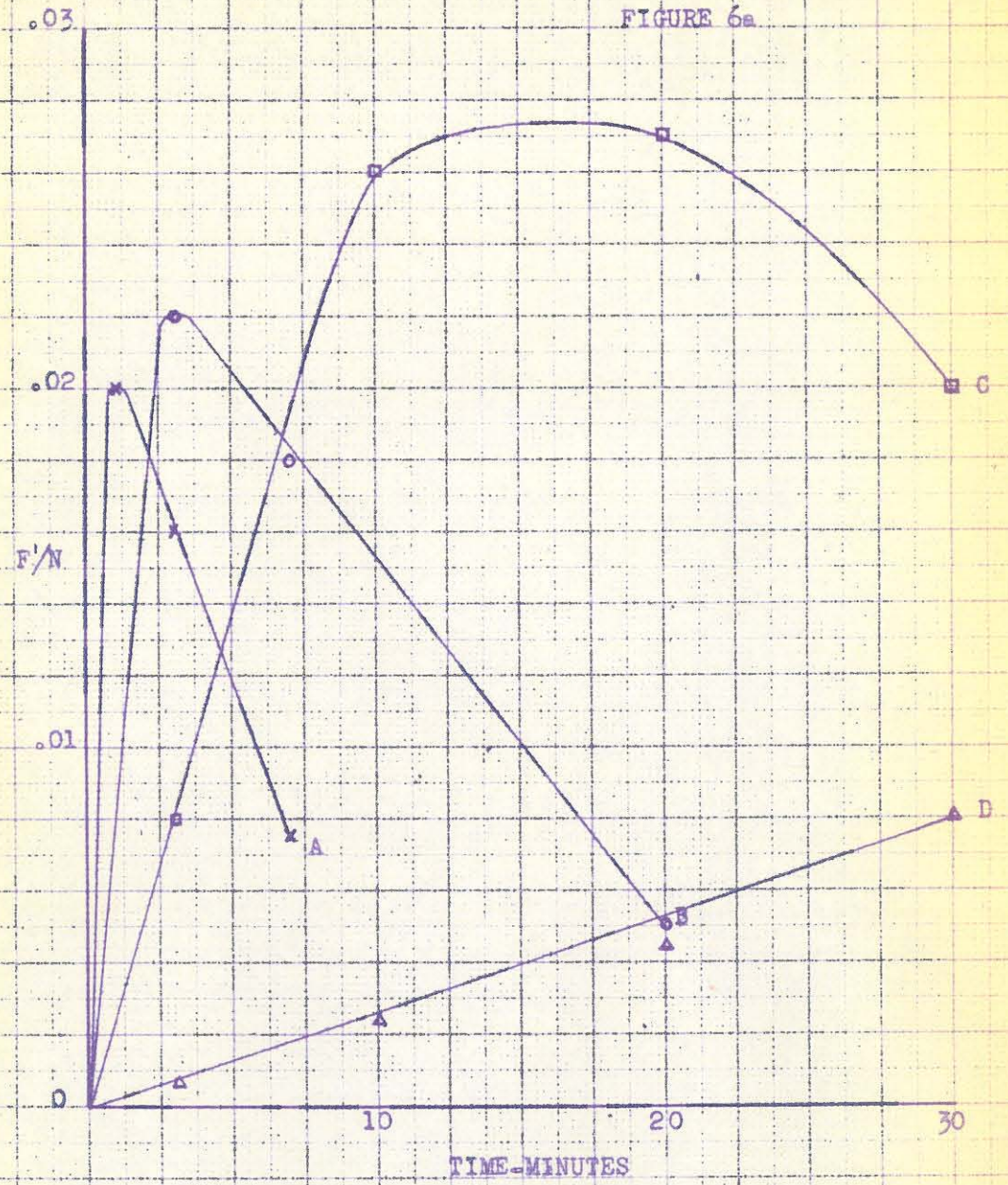


Figure 6b

Killing by 2.0 M urea at 0° C and various pHs

Curve A: pH 8.0

Curve B: pH 7.0

Curve C: pH 6.5

Curve D: pH 6.0

FIGURE 6b

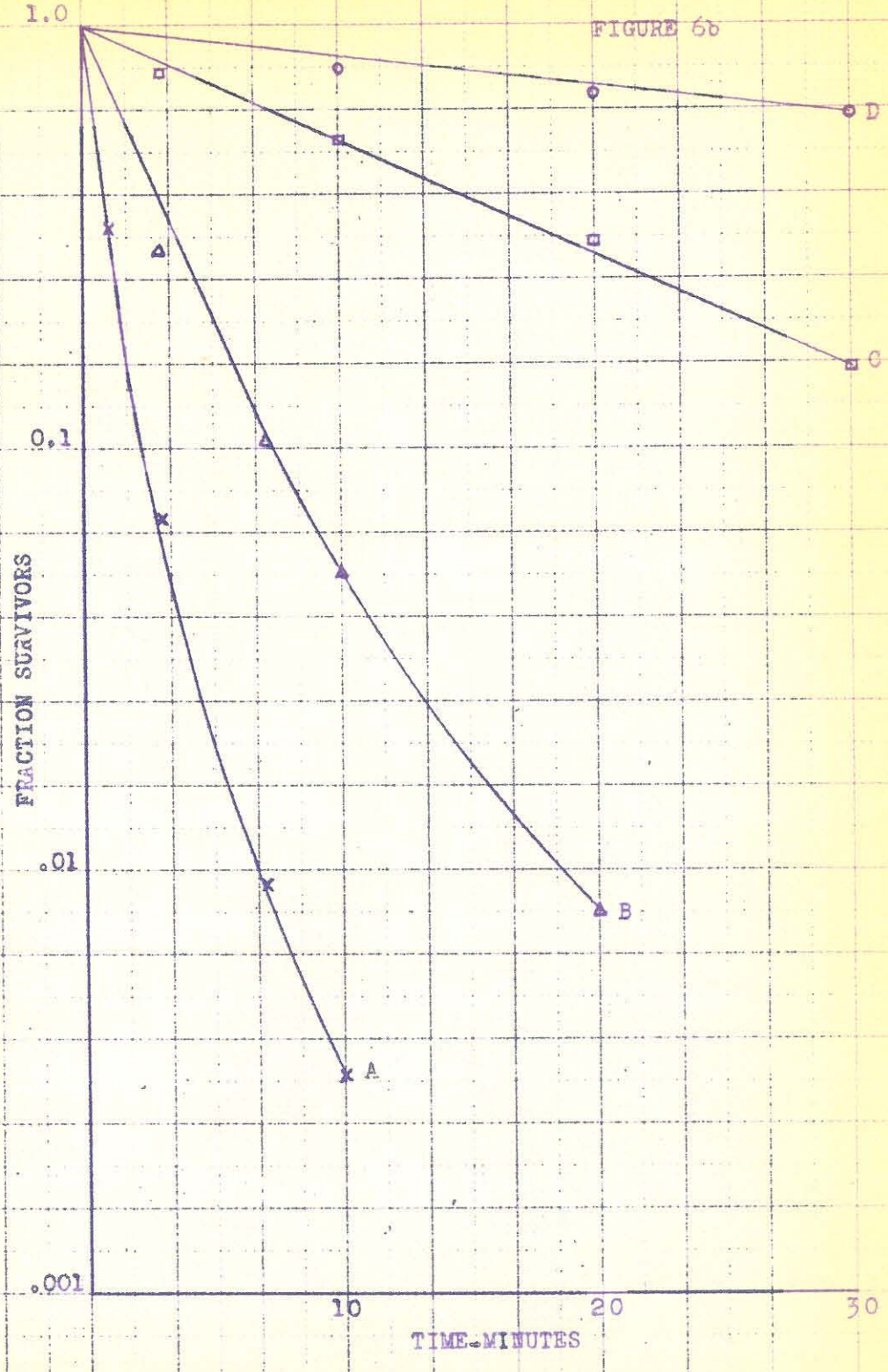


Figure 7a

Activation at 0° C and pH 6.5

Curve A: 2.5 M urea

Curve B: 2.5 M urea plus 20 gamma/ml L-tryptophane

Curve C: 20 gamma/ml tryptophane

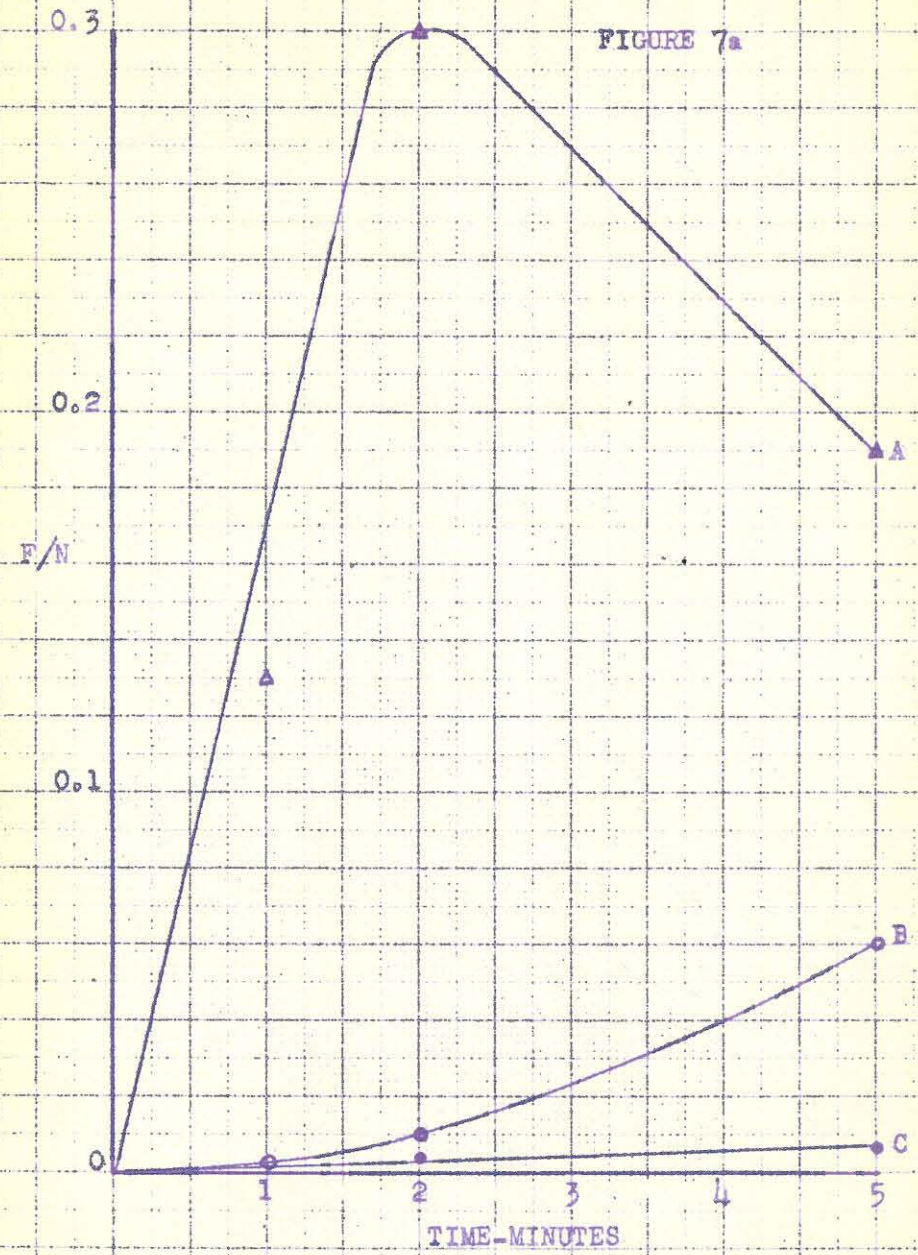


FIGURE 7a

Figure 7b

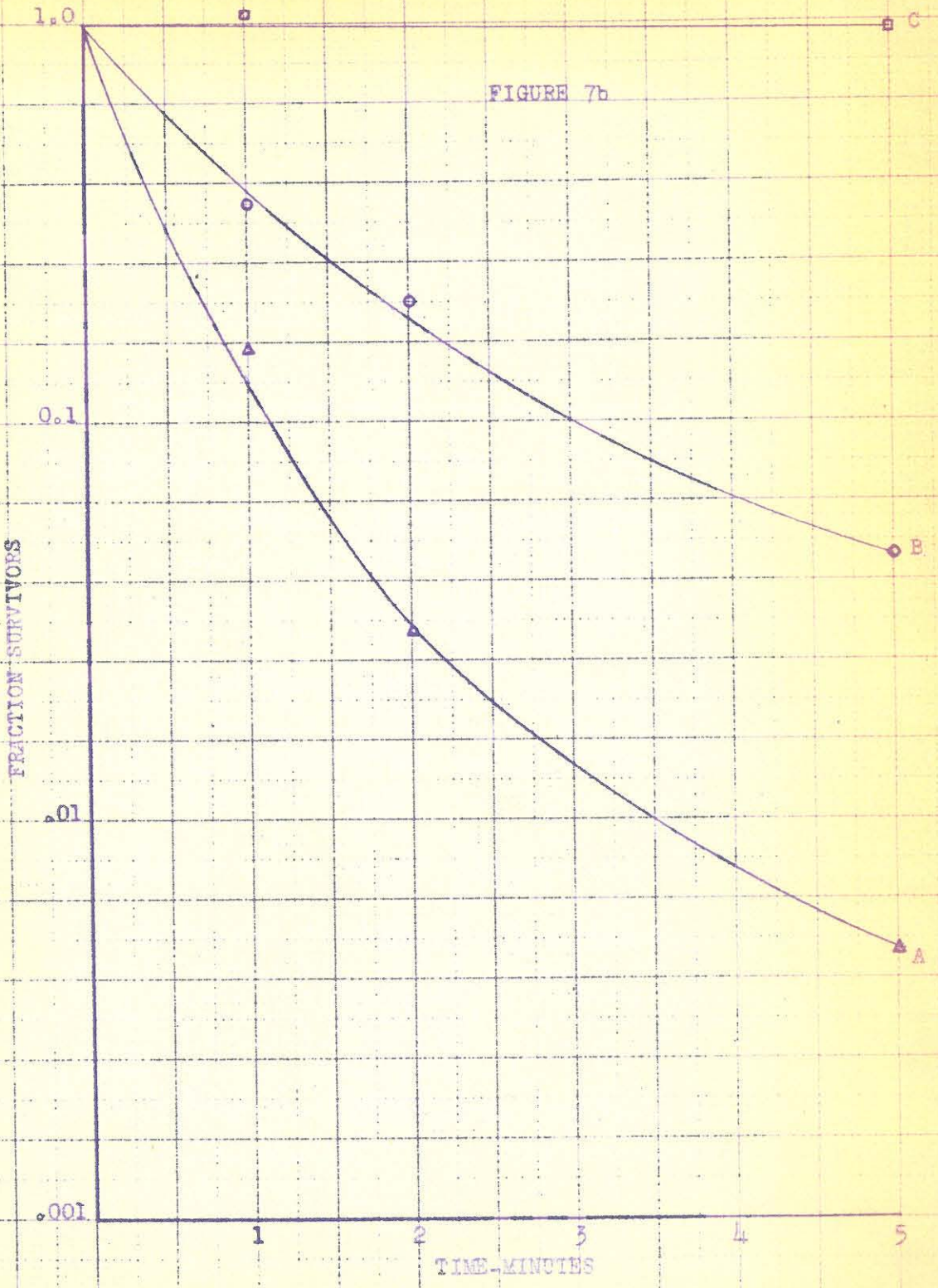
Killing at 0° C and pH 6.5

Curve A: 2.5 M urea

Curve B: 2.5 M urea plus 20 gamma/ml of L-tryptophane

Curve C: 20 gamma/ml L-tryptophane

FIGURE 7b



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