THE INFLUENCE OF HYDROSTATIC PRESSURE AND URETHANE ON THE THERMAL INACTIVATION OF BACTERIOPHAGE*

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Kinetic analyses of the action of urethane on intracellular oxidations, including bacterial luminescence (Johnson, Eyring, Steblay, Chaplin, Huber, and Gherardi, 1945), methylene blue reduction and oxygen consumption (Koffler, Johnson, and Wilson, 1947), as well as on extracted, crude invertase (Johnson, Kauzmann, and Gensler, 1948) have indicated that this drug catalyzes a reversible and/or irreversible denaturation of the enzyme protein. It also accelerates the disinfection of bacterial spores at high temperatures (Johnson and ZoBell, 1949 *b*). Direct evidence that urethane catalyzes the denaturation of proteins has been obtained in spectroscopic studies with methemoglobin (Schlegel and Johnson, 1949) and in the precipitation of pure tobacco mosaic virus (Fraser, Johnson, and Baker, 1949).

With the exception of certain denaturations occurring at 25–30°C., *e.g.*, methemoglobin in the presence of 4.5 M urethane (Schlegel and Johnson, 1949) or antistaphylococcus toxin in the presence of 6 M urea (Wright and Schomaker, 1948), the protein-denaturing action of urethane and similar drugs is to a large extent opposed or reversed by hydrostatic pressures of some 600 atmospheres. The thermal denaturation of various protein systems, both in living cells and in extracts without added drugs, is likewise opposed by pressure (Brown, Johnson, and Marsland, 1942; Eyring and Magee, 1942; Johnson, Eyring, Steblay, Chaplin, Huber, and Gherardi, 1945; Johnson and Campbell, 1945, 1946; Johnson and Wright, 1946; Johnson and Lewin, 1946 *a*, *b*; Eyring, Johnson, and Gensler, 1946; Johnson, Baylor, and Fraser, 1948; Johnson and ZoBell, 1949 *a*).

The present study was undertaken primarily to determine the extent to which the thermal inactivation of bacteriophage, like that of tobacco mosaic virus (TMV) and other proteins, would be catalyzed by urethane and opposed by hydrostatic pressure. Moreover, quantitative data concerning the sensitivity of the free phage to these factors are of obvious importance in interpreting

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the action of the same factors on the multiplication of the phage in living cells, which will be the subject of a later report. Four phages[1] with the common host *Escherichia coli* B, have been included; *viz.*, T1, T2, T5, and T7. They are distinguished by differences in plaque size, morphology of the virus itself, serological groupings, characteristics of latent period, burst size, absorption rate, and certain other properties (Delbrück, 1946). We have found some major differences also in regard to the influence of pressure, although in each case the thermal inactivation is accelerated by small concentrations of urethane.

Methods

The phage stocks were prepared as filtrates from cultures of the infected organisms in Difco nutrient broth containing 0.5 per cent NaCl, pH 6.6. For exposing to heat, suitable dilutions of the stock preparations were made in sterile medium, or medium containing a desired concentration of urethane. The latter was resterilized, after addition of the drug, and before adding the phage, by filtration through a Pyrex ultrafilter. Aliquot portions of the diluted phage were placed in small Pyrex glass tubes, capacity about 0.8 ml., and closed by small rubber stoppers. Each tube was placed in a small stainless steel "bomb" filled with water, and then connected either to a manifold from a hydraulic pressure pump, or to a "dummy" connection for heating at atmospheric pressure, according to the method previously described (Johnson and ZoBell, 1949 *a*). The bombs at both normal and increased pressure were placed on an automatic tilting rack in a water bath maintained at a constant temperature ±0.04°C. Continual agitation of the phage specimens was accomplished by means of a stainless steel ball, ⅛ of an inch diameter, which, with change in position of the rack, made excursions at about 1 second intervals throughout the length of the tube. The bombs were withdrawn from the hot bath, after successively longer periods of time, placed in cold water, and the specimens assayed, at the end of the experiment, by the usual agar layer method for number of infective centers. Temperature equilibration in both the hot and cold water baths required approximately 1 minute.

RESULTS

Preliminary results showed that, in a corresponding medium at 66°C., T2 and T5 were inactivated at nearly the same rate, and T1 slightly faster, while T7 was much more rapidly destroyed. In fact, only 3 per cent of the initial number of T7 infective centers remained after 1 hour at 60°C. Furthermore, although the rate of destruction of the first three was retarded under a pressure of 10,000 pounds per square inch, that of T7 was considerably increased. At 30°C., over a period of a few hours, this pressure had no measurable effect on the number of infective centers of T1, T5, or T7 but caused a 35 per cent decrease in T2 within 60 minutes.

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The kinetics of destruction at the higher temperatures for each of the phages, at both normal and increased pressure, is illustrated in Figs. 1 and 2. The shape

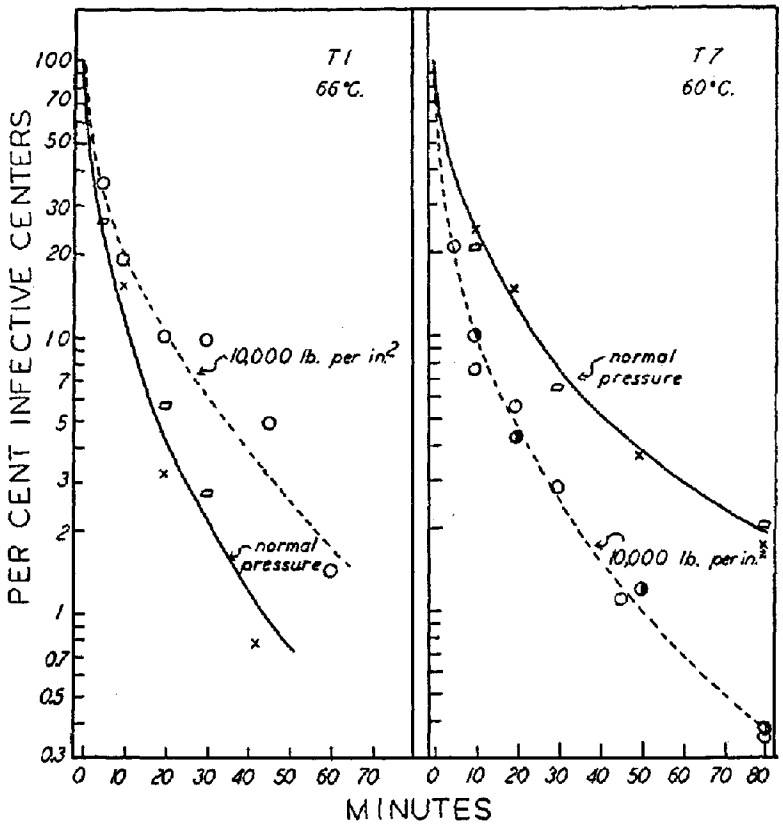

FIG. 1. Rate of loss of infective centers of T1 (left) at 66° and of T7 (right) at 60°C., at normal (solid line) and at 10,000 pounds per square inch hydrostatic pressure (broken line). Different types of points along the same curve represent data obtained in different experiments.

of the curves with T1 and T7 (Fig. 1) at 66° and 60° C. respectively is similar, but the influence of pressure is in opposite directions. At either normal or increased pressure, there is a progressive decrease in rate of destruction with time of heating,[2] as previously observed with another coli phage (Nanavutty

[2] Under certain conditions, an apparent decrease in rate of ultraviolet inactivation of phage occurs through recombination of active units from different phage particles (Luria, 1947). Demonstration of this phenomenon involves multiple infection in order to bring together the active elements. In the present experiments, adsorption was usually carried out with 2×10^8 cells per cc., with not more than 10^7 whole phage

1930). A similar tendency is apparent with T2 under pressure, and with T5 under both normal and increased pressure (Fig. 2), to the extent that the initial rate *i.e.*, during the first 10 to 30 minutes, is somewhat faster than subsequently. With T2 at normal pressure there was a small but consistent rise in the assay, amounting to from 20 to 50 per cent after heating for several minutes at 64–66°

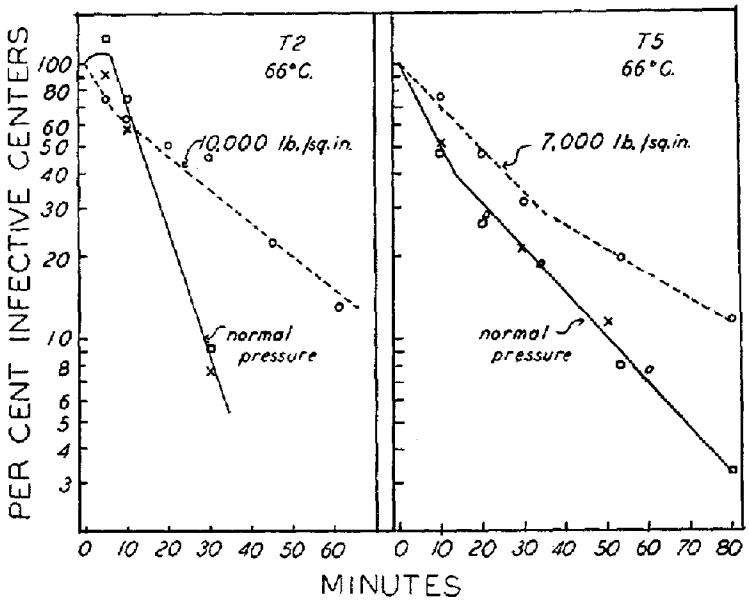

FIG. 2. Rate of loss of infective centers of T2 (left) and of T5 (right) at 66°C., at normal (solid lines) and at increased hydrostatic pressure (broken lines) as indicated in the figure. Different types of points along the same curve represent data obtained in different experiments.

C. A few preparations of T5 indicated a similar phenomenon. While these changes in rate during the course of heating show that, under the stated conditions, the process is not limited throughout by a single reaction, their interpretation is not clear. Among other things, the method of assay does not distinguish between single phage particles and clumps containing one or more active particles. Thus, either the formation or the breaking up of clumps during heat treatment constitute a possible source of error. On the other hand, it is known that brief subjection to high temperatures sometimes results in an increase in activity of enzymes (Bodine and Allen, 1938) as well as in germination of

particles per cc., making multiple infection very unlikely. Thus, the decreasing slopes of the lines in Figs. 1 and 2 could scarcely result from a recombination phenomenon. The present data provide no evidence in regard to the possibility of reactivation of thermally inactivated phage by recombination of active units.

spores of certain molds and bacteria (Dodge, 1912; Shear and Dodge, 1927; Goddard, 1935; Curran and Evans, 1945, 1947). Consequently, a true heat activation of T2 would not be a unique phenomenon. Similarly, the decreasing rate of inactivation with length of time heated is not without parallel in the denaturation of pure proteins, *e.g.* human serum globulin (Johnson and Campbell, 1946) as well as specific antibody activity (Gerlough and White, 1934; Johnson and Wright, 1946).

Preparations of heat-inactivated T5 had no influence on the viability or growth of normal host cells, showing that either there was no adsorption of the heat-treated phage, or that, if adsorbed, the cells were unaffected. Adsorption of ultraviolet-inactivated phage T2 apparently causes the cell to lose its ability to divide (Luria and Delbrück, 1942; Luria, 1947).

In apparent contrast to the results reported by Krueger (1932) for the thermal inactivation of staphylococcus bacteriophage in broth, and for the inactivation of *coli* phages T1 to T7 inclusive, under certain conditions of shaking at 26° according to Adams (1948), the curves shown in Figs. 1 and 2 do not conform to the kinetics of a first order reaction. The characteristics of these curves, however, were found to be essentially independent of the initial number of infective centers with T2, T5, or T7, respectively, between 1×10^4 and 1×10^7 per cc., and hence, within this range, independent also of the concentration of substances present in the bacterial lysates. Moreover, they were very much the same with ten separate preparations of T5 from single plaque isolations and it seems unlikely, therefore, that the multiplicity of reaction rates indicated by the changing slopes of the curves resulted from heterogeneity of the phage stocks; *i.e.*, a distribution of sensitivity to heat of the active particles of the phage population. Further experiments in regard to the influence of other factors showed that the observed rates are affected by salt concentration, specific ions, and differences in pH, as might be expected. Although extensive studies of these factors were not undertaken, it was evident that the addition of 0.005 M phosphate somewhat increased the rate of inactivation of all four phages. With T5, the addition of phosphate also appeared to decrease the effect of pressure. With T7, NaCl retarded the rate with increasing concentration up to about 3 per cent, beyond which the rate was accelerated. The addition of small concentrations of the divalent cations, Mg or Ca, retarded the rate in all except T7 which was not appreciably affected. Furthermore, after addition of these ions the rate with T5 became typically first order at both normal and increased pressure and in the presence of urethane. Under these conditions, therefore, the data are susceptible to simple analysis in terms of a single limiting reaction. The remaining experiments were done with T5, with an optimum concentration of 0.005 M $MgCl_2$ added to the medium.

Typical experiments on the influence of pressure and urethane are illustrated in Figs. 3 and 4. Small concentrations of urethane accelerate the rate of inacti-

vation, while pressure retards it, both with and without urethane. The magnitude of the pressure effect is the same as that with TMV precipitation at 68.8° C. (Johnson, Baylor, and Fraser, 1948) and likewise the effect of 0.1 M urethane

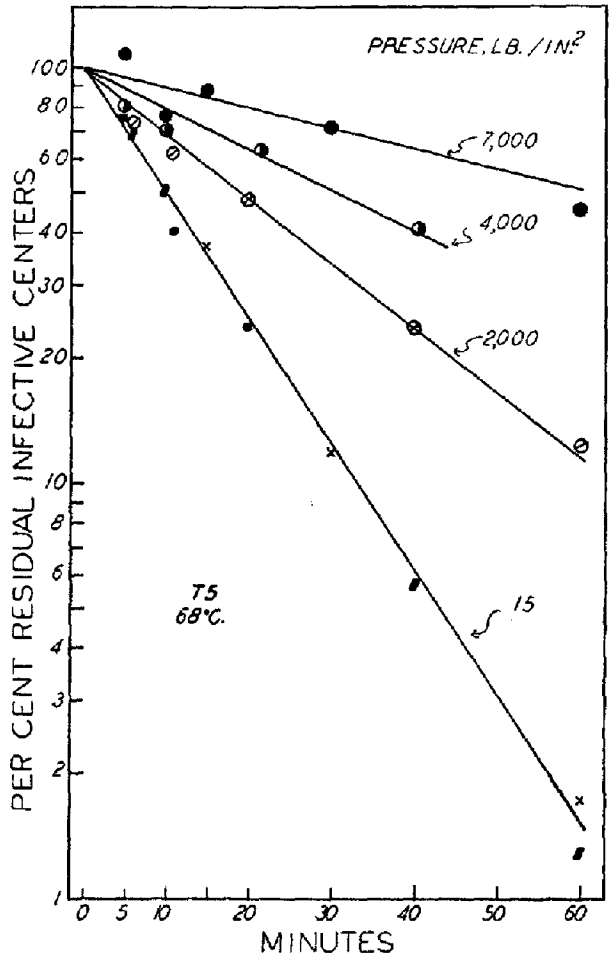

FIG. 3. Influence of hydrostatic pressure on the rate of destruction of T5 at 68°C. in broth to which 0.005 M $MgCl_2$ had been added.

(Fraser, Johnson, and Baker, 1949). Moreover, the volume change of activation $\Delta V^{\ddagger}$, within the limitations of experimental error at different pressures up to at least 7,000 pounds per square inch, is constant, as shown by the straightness of the lines relating the logarithm of the first order rate constant, obtained from data such as shown in Figs. 3 and 4, to the pressure (Fig. 5). The numerical

value of $\Delta V^{\ddagger}$, calculated from the slope of the control line in Fig. 5, according to the relation (Glasstone, Laidler, and Eyring, 1941):

$$\Delta V^{\ddagger} = \left(\frac{\partial \Delta F^{\ddagger}}{\partial p}\right)_T = -RT\left(\frac{\partial \ln k}{\partial p}\right)_T \qquad (1)$$

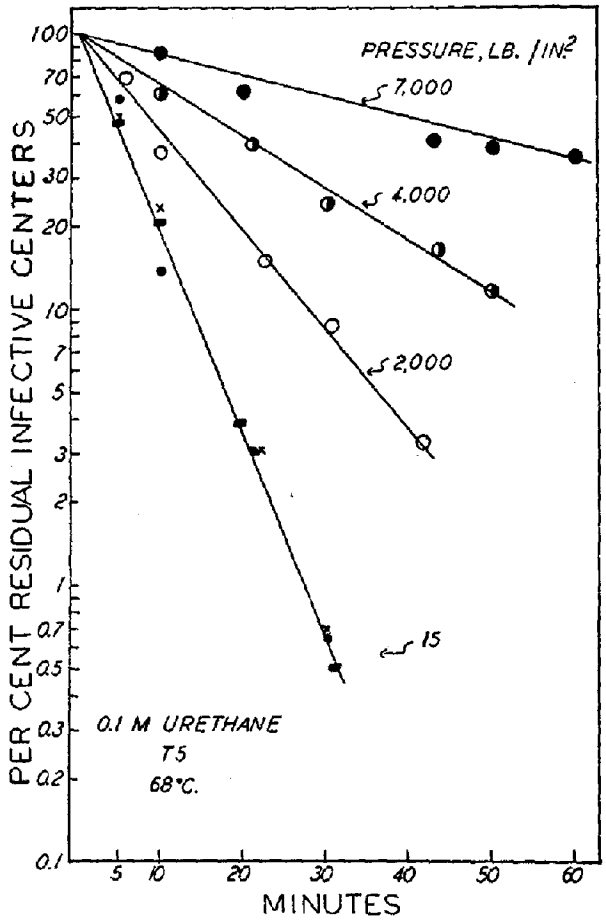

FIG. 4. Influence of hydrostatic pressure on rate of destruction of T5 at 68°C., in broth containing 0.1 M urethane, with addition of 0.005 M $MgCl_2$.

amounts to 113 cc. per mol, very nearly the same as the 100 cc. per mol found for TMV. The slope of the line for specimens containing 0.1 M urethane is apparently somewhat steeper and would indicate a value of 129 cc. per mol.

The relation between concentration of urethane and the rate of inactivation of T5, at normal pressure and at a constant temperature of 66°C., is shown

in Fig. 6. The simplest quantitative interpretation, consistent with the data given in Figs. 3 to 6, is that destruction of the phage under these conditions takes place through two distinct first order reactions, (1), the thermal inactivation alone with rate constant k_0 and (2), a urethane-catalyzed reaction, with constant k_u. In the presence of urethane, as well as without it, the kinetics of

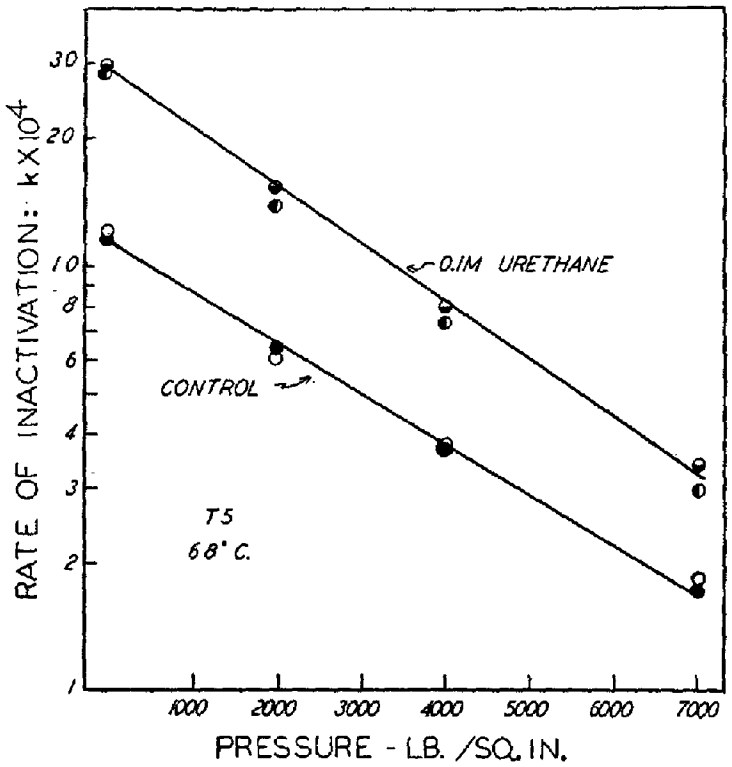

FIG. 5. Relation between pressure and rate of inactivation of T5 at 68°C., with and without 0.1 M urethane, respectively. The double set of points along each line represents data from repeated experiments. The rate constants were calculated in reciprocal seconds from data as illustrated in Figs. 3 and 4.

the total rate will still be first order with respect to the amount of phage remaining, as noted in Figs. 3 and 4, since the total rate will be determined by the algebraic sum of the individual rates which, at a given temperature and pressure, will be a constant. The urethane is present in such excess that even if the urethane were destroyed in the reaction with phage, which is unlikely, the concentration of the drug in solution would remain virtually unchanged. Thus, assuming only the two, independent first order reactions for the destruction of active phage, the total rate may be expressed by the following equation,

in which (P) represents the amount of active phage, (U) the molar concentration of urethane, and x the average number of urethane molecules combining

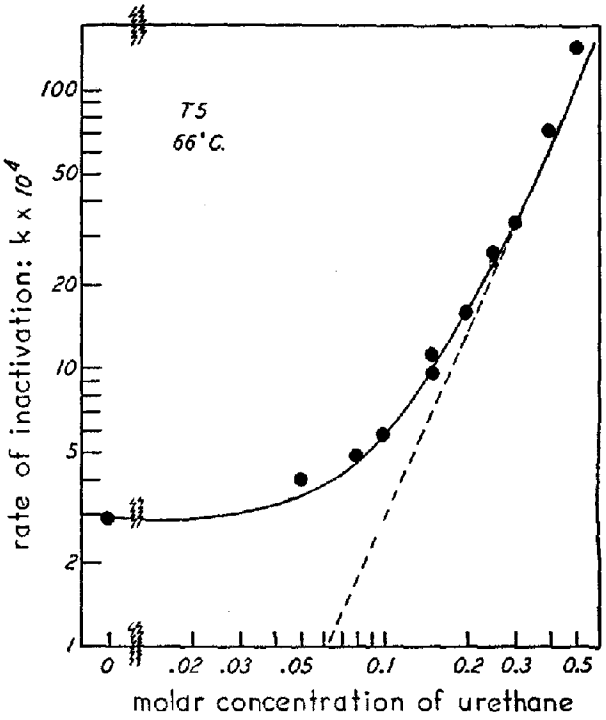

FIG. 6. Relation between the rate of inactivation of T5 at 66°C., and concentration of urethane. The first order rate constants on the logarithmic scale of the ordinate were calculated in reciprocal seconds from the slopes of curves such as illustrated in Figs. 3 and 4. The smooth curve was calculated according to equation (2) in the text for the total rate of the thermal inactivation plus the urethane-catalyzed reac.tion The broken line represents the calculated rate of the urethane-catalyzed reaction alone. All points are from the data obtained in experiments.

in the activated state with the phage molecule whose destruction results in loss of activity:

$$-\frac{dP}{dt} = k_0(P) + k_u(P)(U)^x = (k_0 + k_u(U)^x)(P) \qquad (2)$$

In Fig. 6 the numerical value of k_0 may be obtained from the rate with no added urethane, *viz.* 2.9×10^{-4} sec. $^{-1}$, and the value of x may be estimated as 2.3 from the slope of the line at relatively high concentrations of the drug; *e.g.*, between 0.2 and 0.4 M. Similarly, after estimating the value of $k_u(U)^x$

from the observed rate at any high concentration of the drug where k_0 is relatively small, k_u appears to be about 6×10^{-2}. When the constants thus obtained are substituted in equation (2) for the various concentrations of urethane, the smooth curve of Fig. 6 results. The calculated curve indicates a close approximation to the points determined in experiments covering a range in urethane concentrations over which fairly accurate data could be obtained. At less than 0.05 M urethane the rate is too nearly that of the control, while above 0.5 M urethane the rate is too fast to be determined satisfactorily.

Although the simple hypothesis of the two reactions (Equation 2) is sufficient for a fairly close quantitative agreement with the data in Fig. 6, the fact that the value of x is 2.3 rather than an integer probably means that more than one urethane-catalyzed reaction takes place. In the denaturation of TMV there is evidence that at least two urethane-catalyzed reactions occur, the first involving one molecule of urethane per TMV and a large volume increase of activation, the second involving three molecules of urethane and essentially no volume change (Fraser, Johnson, and Baker, 1949). The curve in Fig. 6 is remarkably similar to that for TMV at normal pressure. Unfortunately, it has not been possible in this study to include experiments at high pressure corresponding to those of Fig. 6 at normal pressure. It is reasonable to believe, however, that such experiments will reveal evidence for additional reactions catalyzed by urethane in the inactivation of bacteriophage as in the denaturation of TMV, and will account more precisely for the fractional value of $x = 2.3$.

At different temperatures between 64 and 70°C., the log of the rate of inactivation at normal and increased pressure, with or without 0.1 M urethane, appears to be a linear function of the reciprocal of the absolute temperature (Fig. 7). The apparent heat of activation, $\Delta H^{\ddagger}$, at normal pressure, calculated from these data in accordance with the theory of absolute reaction rates (Glasstone, Laidler, and Eyring, 1941), amounts to 170,000 calories, with an entropy of activation, $\Delta S^{\ddagger}$, of 425 E.U. At 7,000 pounds per square inch the apparent energy of activation, $\Delta E^{\ddagger}$, appears to be somewhat less, the slope of the line drawn by inspection in Fig. 7 indicating 145,000 calories, or a difference of some 25,000 calories. From the relation, $\Delta H^{\ddagger} = \Delta E^{\ddagger} + p\Delta V^{\ddagger}$, it may be shown that for a single reaction with a volume change of + 113 cc., $\Delta E^{\ddagger}$ at 7,000 pounds per square inch would be less than $\Delta H^{\ddagger}$ by only about 1,000 calories. Consequently, the difference of 25,000 calories, if it is real, indicates that more than a single reaction influences the slope of the line. The apparent straightness of the line, within the limits of experimental error, is not an adequate basis for concluding that a single limiting reaction determines its slope over this range of temperature. In the presence of 0.1 M urethane, the slopes of the lines are nearly the same at normal and 7,000 pounds per square inch, but are somewhat steeper than those without urethane, thus indicating a higher activation energy for the reaction or reactions catalyzed by urethane. Conclusive evidence concern-

ing these points, however, must await more exhaustive data with regard to the influence of pressure, temperature, and concentration of the drug.

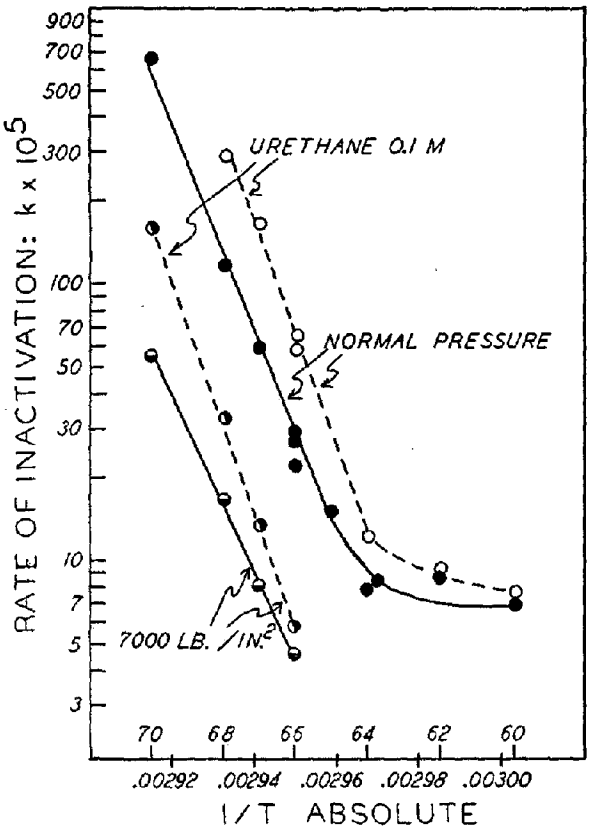

FIG. 7. Relation between temperature and the rate of inactivation of T5, with (broken lines) and without (solid lines) 0.1 M urethane, at normal pressure and 7,000 pounds per square inch hydrostatic pressure, respectively. The first order rate constants on the logarithmic scale of the ordinate under given conditions were calculated in reciprocal seconds. Degrees centigrade are indicated on the abscissa for corresponding values of the reciprocal of the absolute temperature.

At temperatures below 64°C., the loss of activity of the phage is slow, but is much faster than would be predicted on the basis of the above reactions alone, as shown by the sharp change in slopes of the (normal pressure) lines in Fig. 7. The activation energy for the reactions responsible for the loss of phage at the lower temperatures could scarcely be greater than 5,000 calories to be consistent with the observed changes in slope.

DISCUSSION

The foregoing results provide data concerning the sensitivity of the several phages to temperature, pressure, and urethane, in a medium free of host cells but otherwise favorable for propagation of the virus, which should aid in the interpretation of subsequent studies in regard to the influence of these factors on phage growth. In addition, they reveal that among related bacteriophages produced in the same host cell, the chemical architecture may be fundamentally different. Otherwise, one could not readily account for the difference in susceptibility to heat inactivation, and more especially for the opposite effects of hydrostatic pressure on T7 and the other phages studied. These differences, of course, do not necessarily imply that the chemical components of the phages are greatly different, since they might be accounted for by differences in the way the amino acids and other components are joined together to give a final specific configuration. The final configuration presumably depends upon the specific templet mechanisms provided by and associated with the production of a given virus. Phage T7, which exhibits such remarkable differences in characteristics of thermal inactivation in relation to pressure, is also distinguished from T1, T2, and T5 by its morphology as seen in the electron microscope. In contrast to the others, it is round and tailless, with a somewhat smaller body. Although phage T3 is morphologically the same as T7, it was not available for the present study.

With regard to the action of pressure, in particular, T7 is the only protein system studied thus far whose denaturation or inactivation at high temperatures is not fairly strongly opposed by moderate increases in hydrostatic pressure. The possibility is not excluded, of course, that the apparent increase in rate of destruction under pressure results from some totally different action than on the denaturation of phage protein. For example, if pressure caused a clumping or aggregation of the phage particles, so that each infective center revealed by the assay consisted initially of a number of active phage particles, a retardation by pressure of the destruction of individual particles might be completely obscured. On the other hand, unless some such phenomenon is actually involved, the difference in the influence of pressure on the thermal destruction of T7 and on that of the others indicates a marked difference in the mechanism of the limiting reactions which in all cases appear to be protein denaturation, as evidenced by the very high temperature coefficient. The full extent to which the pressure effect and hence the mechanism of destruction, may be altered by various factors, such as pH, salt concentration, etc., remains to be established, but it is evident that the pressure acceleration of T7 destruction may be reduced or abolished by increasing the concentration of NaCl in the medium, and likewise the pressure retardation of T5 destruction by the addition of phosphate. Thus, it is reasonable to expect that conditions may exist under which the thermal inactivation of T7, like that of the other

phages studied, proceeds with a volume increase opposable by pressure. There is no evidence that it does, however, under essentially the same conditions of pH, salts, etc., in the nutrient broth medium used for the four phages in the present study.

The volume increase of reaction or of activation, accompanying the reversible or irreversible denaturation of proteins, respectively, has been interpreted as a drastic change in configuration of the native molecule, as by unfolding from a somewhat globular to a more linear form, and similar changes have been postulated as essential in the multiplication of a virus (Eyring, Johnson, and Gensler, 1946; Johnson, 1947). Evidence in regard to volume changes, obtained through studies of the influence of pressure, is not yet available with regard to virus growth, but it has been shown that relatively small pressures readily oppose growth of many bacterial species under otherwise optimal conditions (Johnson and Lewin, 1946; ZoBell and Johnson, 1949). While the influence of pressure on virus multiplication involves more complex phenomena than on thermal denaturation, and is therefore more difficult to interpret clearly, it should be of particular interest to investigate this factor in relation to the propagation of T7 and the other *coli* phages.

Apart from the difference in the influence of pressure, the action of urethane on all four of the phages in similar in that small concentrations accelerate their thermal destruction. Under conditions where the rate of destruction follows simple kinetics, as with T5 in the presence of added $MgCl_2$, a partial analysis of the action of this drug has revealed a fundamental similarity to its effect on diverse other systems. Thus, the reaction it catalyzes evidently has a high heat and entropy of activation, and involves the combination of more than one urethane molecule with the protein, possibly involving more than one distinct reaction. The actual ratio of the combining molecules, as estimated from the quantitative relation between concentration of the drug and its effect on the rate, is about the same as the average number combining in the denaturation of TMV (Fraser, Johnson, and Baker, 1949), and the reversible reaction with the enzymes limiting the rate of oxygen consumption and methylene blue reduction in *Rhizobium* (Koffler, Johnson, and Wilson, 1947), as well as the intensity of bacterial luminescence (Johnson, Eyring, Steblay, Chaplin Huber, and Gherardi, 1945). The actual temperature at which the drug acts in corresponding concentrations differs widely among these several processes, ranging from below room temperature with luminescence, to 68.8°C. with TMV or as high as 95°C. with the disinfection of bacterial spores (Johnson and ZoBell, 1949 *b*). Yet the fundamental mechanism of action may be the same in each case. Current studies concerning the influence of urethane on phage multiplication should provide further evidence in regard to the action of the drug in diverse phenomena.

SUMMARY

In Difco nutrient broth, containing 0.5 per cent NaCl, pH 6.6, *Escherichia coli* phages T1, T2, and T5 were inactivated at 66°C., and T7 at 60°C., at nearly the same rate. In each case the rate of destruction was not uniform but more or less decreased with time of heating. With T2 there was an initial increase in number of infective centers after heating for several minutes at 66°C.

Hydrostatic pressures up to 10,000 pounds per square inch retarded the thermal destruction of T1, T2, and T5, but accelerated that of T7, while small concentrations of urethane accelerated the rate of each.

The rate of inactivation was increased by the addition of 0.005 M phosphate, and was decreased by 0.005 M $MgCl_2$ in all but T7, whose rate was unaffected by this amount of Mg. The influence of Ca was similar to that of Mg.

The addition of 0.005 M $MgCl_2$ to the broth medium resulted in a first order rate of destruction of T5 at either normal or increased pressure, and with as well as without urethane. Analysis of data obtained under these conditions indicated that the thermal inactivation proceeds with a volume increase of activation of 113 cc. per mol, and with a heat and entropy of 170,000 calories and 425 E. U., respectively, in the rate-limiting reaction. In the presence of 0.1 M urethane the heat and volume change of activation are apparently slightly greater. The relation between concentration of urethane and the amount of acceleration in rate of destruction at normal pressure and 66°C. indicated that the total rate involves at least two first order rate processes: the thermal inactivation itself and a urethane-catalyzed reaction, the latter involving the combination of an average of 2.3 molecules of urethane in the activated state of the bacteriophage molecule whose destruction results in loss of phage activity.

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