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Reactivation of Thermally Inactivated Bacteriophage.

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It has been shown by Anson and Mirsky[1] in a long series of studies that the denaturation of many proteins is a reversible process. The isolation of crystalline trypsin[2] and pepsin[3] has furnished proof that at least some enzymes are protein compounds. In them the denaturative reaction is paralleled by loss of enzymatic activity and conversely, as the denatured protein is changed back to native protein this activity is regained.[4]

Krueger[5] found that the thermal inactivation of phage follows the course of a unimolecular reaction and that the critical thermal increment for inactivation is about 100,000. Since critical thermal increments of this order of magnitude are uniquely characteristic of protein-denaturation he concluded that the inactivation of phage by heat involved protein-denaturation as a significant reaction. Additional experimental support for this concept is available in other observations:

1. Vedder[6] has noted that the loss of activity on heating dried phage is much less than with phage in aqueous suspension; dried proteins are typically more thermostable than proteins in solution.

2. While immunological experiments are not theoretically conclusive they are most readily interpreted by considering phage to be a protein capable of functioning as an antigen.[7]

3. Purified phage gives the usual chemical tests for proteins and on elementary analysis has been found to contain carbon, hydrogen, nitrogen and phosphorus[8, 9] in proportions empirically compatible with a protein structure.

Taken together these data suggest that phage is composed of

1 Anson, M. L., and Mirsky, A., Publications in *J. Gen. Physiol.*, beginning with 1925, **9**, 169.

2 Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1932, **16**, 267.

3 Northrop, J. H., *J. Gen. Physiol.*, 1930, **13**, 739.

4 Northrop, J. H., *J. Gen. Physiol.*, 1932, **16**, 323.

5 Krueger, A. P., *J. Gen. Physiol.*, 1932, **15**, 363.

6 Vedder, A., *Centralbl. f. Bakt.*, I, Orig., 1932, **125**, 111.

7 Krueger, A. P., *Physiol. Rev.*, 1936, **16**, 129.

8 Schlesinger, M., *Biochem. Z.*, 1934, **273**, 254.

9 Meyer, Karl, Thompson, R., Khorazo, D., and Palmer, J. W., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 129.

one or more proteins and that its lytic activity is intimately linked with maintenance of the native protein structure. This would be entirely in keeping with the view of some workers that phage is an enzyme and it must be admitted that certain analogies exist between the properties of phage and those of enzymes. For example, phage can be quantitatively reactivated after complete inactivation by concentrated $HgCl_2$,[10] or by HCN[11] if the toxic Hg or CN ions are removed by chemical means. The action of phage on its bacterial substrate is accelerated by the presence of certain cations in very minute concentrations.[12] Such phenomena, among others, are common both to phage and to enzymes. However, as one of us has pointed out[7] they fail to afford formal proof of identity simply because the characteristics of living cells in general are largely determined by the enzymes they elaborate and as a consequence many analogous properties are shared by cells and their enzymes. It has not been possible, therefore, definitely to disprove the theory that phage is a special sort of living ultramicrobe and to relegate it to the class of enzymes by reasoning based on the analogies between the reactions of phage and enzymes.

Since it is known that the heat-inactivation of phage is closely connected with protein-denaturation it should be possible to reverse the reaction. If then phagic activity is dependent upon the native state of protein present there should be an accompanying regeneration of this activity. In the present paper we wish to present experimental evidence indicating that the thermal inactivation of phage actually is reversible.

To 24 ml. of standard phage, containing 1×10^{10} activity units,[13] in beef-infusion broth at pH 7.4 was added 5.4 ml. of HCl-phthalate buffer of pH 3.2. The mixture had a final pH of 5.75 and was heated at 54°C. for 20 minutes. At the end of this time a sample (A) was removed and cooled as rapidly as possible in a salt-ice mixture. Another 9.8 ml. sample (B) was added to 9.0 ml. of chilled broth containing enough NaOH to bring the mixture to a final pH of 8.1. Both samples were kept on ice for 16 hours and then tested for phage by the activity-titration method.[13] As controls, aliquots of unheated phage were adjusted to pH 5.75 (C) and to pH 8.1 (D) as noted above. A third control (E) consisted of unheated phage adjusted to pH 5.75 for 20 minutes and then brought to

[10] Krueger, A. P., and Baldwin, D. M., *J. Infect. Dis.*, 1935, **57**, 207.

[11] Krueger, A. P., and Elberg, S. S., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 483.

[12] Krueger, A. P., and West, N. S., *J. Gen. Physiol.*, 1935, **19**, 75.

[13] Krueger, A. P., *J. Gen. Physiol.*, 1930, **13**, 557.

pH 8.1. All controls were prepared at 10°C. and were kept at ice-box temperature for 16 hours before titration.

In all, 24 successful experiments were performed with the following average results (after correcting for dilution):

Sample A contained 20% of the original phage present.

Sample B contained between 70% and 85% of the original phage.

Controls C, D and E showed no effect from exposure to pH 5.75 and pH 8.1 at 10°C. for 16 hours.

From the above data it is evident that alkalinization and rapid cooling combine to increase the activity of phage suspensions partially inactivated by heat. In view of the fact that the thermal inactivation of phage has been shown to involve protein-denaturation our results are probably best interpreted by assuming that the denaturation and concomitant inactivation occurring when phage is heated at pH 5.75 are in part reversed at pH 8.1. It may be objected that the apparent increase in activity is due to the effect of an increased [OH^-] on residual active phage remaining after the heating and has nothing to do with the inactivated fraction. Our controls prove that this is not the case for in E no change in [phage] could be demonstrated. Similarly, the possibility that the regeneration of activity may involve a stimulating effect on the titrated mixtures by ions introduced with the buffers, must be considered. If this were the case the time of lysis in the titration sample would be less and the solution would have an apparently increased titre. Again, the controls, C, D and E indicate that no such effect is obtained with either buffer singly or when mixed as is done in reversing the denaturation.

The most logical conclusion is that the thermal inactivation of phage is quite analogous to that of enzymes. In the case of pure trypsin in solution the enzyme can be heated almost to boiling without significant loss of activity as long as the pH is maintained well on the acid side.[2] Northrop[4] has shown that this remarkable thermoresistance is due to the influence of the H ion in favoring the reversal of denaturation. Krueger and Scribner[14] have described the catalyzing effect of H and OH ions on the thermal inactivation of phage. Their data together with those reported here, indicate that when phage is inactivated by heat (denaturation) the pH of the solution during the period of heat treatment and during cooling has a most important bearing on the final titre obtained. Under some circumstances a considerable proportion of the phage so in-

[14] Krueger, A. P., and Scribner, E. J., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 21.

activated may again be activated. This the authors interpret as contributing further to the analogies already existing between phage and enzymes. While this still does not prove that phage is an enzyme, it should be emphasized that there no longer exist valid philosophical objections to entertaining such a consideration. Stanley's[15] isolation of a crystalline protein possessing all the properties of tobacco-mosaic virus is proof that there exist inanimate substances which though lethal for certain cells may yet possess the capacity for being propagated by contact with these cells. It is not altogether illogical to speak of phage in such terms.

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Comparative Effects of Pancreas and Choline on Blood Cholesterol of Depancreatized Dog Maintained with Insulin.*

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It was shown[1] that the completely depancreatized dog can survive for well over 4 years when maintained with insulin and a diet containing meat, sucrose, bone ash and vitamin supplements, B (in the form of a rice bran concentrate), and A and D (as cod liver oil). No other accessory substances were found essential. Despite their survival, however, such dogs are not normal in all respects. The appearance of cataracts was first observed in this laboratory as early as one year after pancreatectomy.[2] A striking change also takes place in the lipid metabolism. When the animals are maintained on the dietary constituents listed above, there occurs a fall in all blood lipid constituents and a rise in the lipid content of the liver.[3, 4] It was then shown that the addition of raw pancreas to the diet not only decreased the lipid content of the

15 Stanley, W. M., *Science*, 1935, **81**, 644.

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1 Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 211.

2 Chaikoff, I. L., and Lachman, G. S., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 237.

3 Chaikoff, I. L., and Kaplan, A., *J. Biol. Chem.*, 1934, **106**, 267.

4 Kaplan, A., and Chaikoff, I. L., *J. Biol. Chem.*, 1935, **108**, 201.