INACTIVATION AND REACTIVATION OF B. MEGATHERIUM PHAGE

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SUMMARY

Preparation of Reversibly Inactivated (R.I.) Phage.—

If *B. megatherium* phage (of any type, or in any stage of purification) is suspended in dilute salt solutions at pH 5–6, it is completely inactivated; *i.e.*, it does not form plaques, or give rise to more phage when mixed with a sensitive organism (Northrop, 1954).

The inactivation occurs when the phage is added to the dilute salt solution.

If a suspension of the inactive phage in pH 7 peptone is titrated to pH 5 and allowed to stand, the activity gradually returns. The inactivation is therefore reversible.

Properties of R.I. Phage.—

The R.I. phage is adsorbed by sensitive cells at about the same rate as the active phage. It kills the cells, but no active phage is produced. The R.I. phage therefore has the properties of phage "ghosts" (Herriott, 1951) or of colicines (Gratia, 1925), or phage inactivated by ultraviolet light (Luria, 1947).

The R.I. phage is sedimented in the centrifuge at the same rate as active phage. It is therefore about the same size as the active phage.

The R.I. phage is most stable in pH 7, 5 per cent peptone, and may be kept in this solution for weeks at 0°C.

The rate of digestion of R.I. phage by trypsin, chymotrypsin, or desoxyribonuclease is about the same as that of active phage (Northrop, 1955 *a*).

Effect of Various Substances on the Formation of R.I. Phage.—

There is an equilibrium between R.I. phage and active phage. The R.I. form is the stable one in dilute salt solution, pH 5 to 6.5 and at low temperature (<20°C.).

At pH >6.5, in dilute salt solution, the R.I. phage changes to the active

form. The cycle, active $\rightleftharpoons$ inactive phage, may be repeated many times at 0°C. by changing the pH of the solution back and forth between pH 7 and pH 6.

Irreversible inactivation is caused by distilled water, some heavy metals, concentrated urea or quanidine solutions, and by *l*-arginine.

Reversible inactivation is prevented by all salts tested (except those causing irreversible inactivation, above). The concentration required to prevent R.I. is lower, the higher the valency of either the anion or cation. There are great differences, however, between salts of the same valency, so that the chemical nature as well as the valency is important.

Peptone, urea, and the amino acids, tryptophan, leucine, isoleucine, methionine, asparagine, *dl*-cystine, valine, and phenylalanine, stabilize the system at pH 7, so that no change occurs if a mixture of R.I. and active phage is added to such solutions. The active phage remains active and the R.I. phage remains inactive.

The R.I. phage in pH 7 peptone becomes active if the pH is changed to 5.0. This does not occur in solutions of urea or the amino acids which stabilize at pH 7.0.

Kinetics of Reversible Inactivation.—

The inactivation is too rapid, even at 0° to allow the determination of an accurate time-inactivation curve. The rate is independent of the phage concentration and is complete in a few seconds, even in very dilute suspensions containing $<1 \times 10^4$ particles/ml. This result rules out any type of bimolecular reaction, or any precipitation or agglutination mechanism, since the minimum theoretical time for precipitation (or agglutination) of a suspension of particles in a concentration of only 1×10^4 per ml. would be about 300 days even though every collision were effective.

Mechanism of Salt Reactivation.—

Addition of varying concentrations of $MgSO_4$ (or many other salts) to a suspension of either active or R.I. phage in 0.01 M, pH 6 acetate buffer results in the establishment of an equilibrium ratio for active/R.I. phage. The higher the concentration of salt, the larger proportion of the phage is active.

The results, with $MgSO_4$, are in quantitative agreement with the following reaction:

$$\text{R.I. phage} + n\text{Mg}^{++} \rightleftharpoons \underset{\text{active}}{[\text{Mg}_n\text{—phage}]}$$

Effect of Temperature.—

The rate of inactivation is too rapid to be measured with any accuracy, even at 0°C.

The rate of reactivation in pH 5 peptone, at 0 and 10°, was measured and

found to have a temperature coefficient $Q_{10} = 1.5$ corresponding to a value of E (Arrhenius' constant) of 6500 cal. mole^{-1}. This agrees very well with the temperature coefficient for the reactivation of denatured soy bean trypsin inhibitor (Kunitz, 1948).

The equilibrium between R.I. and active phage is shifted toward the active side by lowering the temperature. The ratio R.I.P./AP is 4.7 at 15° and 2.8 at 2°. This corresponds to a change in free energy of -600 cal. mole^{-1} and a heat of reaction of 11,000. These values are much lower than the comparative one for trypsin (Anson and Mirsky, 1934 *a*) or soy bean trypsin inhibitor (Kunitz, 1948).

Neither the inactivation nor the reactivation reactions are affected by light.

The results in general indicate that there is an equilibrium between active and R.I. phage. The R.I. phage is probably an intermediate step in the formation of inactive phage. The equilibrium is shifted to the active side by lowering the temperature, adjusting the pH to 7–8 (except in the presence of high concentrations of peptone), raising the salt concentration, or increasing the valency of the ions present. The reaction may be represented by the following:

High salt (>0.01 M)	Low salt	Very low salt concentration
pH 7–9	pH 6	pH < 5
Cold (0–10°)	Heat (10–40°)	>40°
Active $\rightleftharpoons$	reversibly inactivated	$\longrightarrow$ inactive

The assumption that the active/R.I. phage equilibrium represents an example of native/denatured protein equilibrium predicts all the results qualitatively. Quantitatively, however, it fails to predict the relative rate of digestion of the two forms by trypsin or chymotrypsin, and also the effect of temperature on the equilibrium.

EXPERIMENTAL RESULTS

Preparation of Reversibly Inactivated (R.I.) Phage.—

The results of an experiment, in which a stock phage preparation in 5 per cent, pH 7 peptone was diluted in peptone, or in 0.01 M, pH 6 acetate buffer, are shown in Table I A. The phage diluted in 5 per cent, pH 7 peptone formed 320 plaques. The phage which was diluted first in 0.01 M, pH 6 acetate buffer and then in 5 per cent peptone formed no plaques. If, however, a sample of this inactive phage in pH 7 peptone is titrated to pH 5 and allowed to stand an hour or so, it again forms about 300 plaques.[1, 2]

[1] Puck (1949) has described somewhat similar results with T 1 coli-phage. In this case, however, reactivation occurred in pH 7.0 peptone. Puck concluded that the inactivation occurred when the phage became attached to the sensitive cell.

[2] It may be noted that these results differ qualitatively from the "photoreactivation" experiments with bacteria (Kelner, 1949) or phages (Dulbecco, 1950). In the

The inactivation is prevented by 1×10^{-3} M $MgSO_4$ (but not by 1×10^{-5} M) (*cf.* page 243) and this fact may be used to determine in which solution the inactivation occurs. In the experiment shown in Table I B, 6 dilution series were made up, each consisting of 4 dilution steps. The first series contained 1×10^{-3} M $MgSO_4$ in each dilution step. No inactivation occurred in this series. The second series contained no $MgSO_4$, and complete inactivation occurred. Addition of the $MgSO_4$ to the second tube of the series, in which the phage was diluted from 10^{-2} to 10^{-4} in 0.01 M, pH 6 acetate buffer, prevents

TABLE I A

Inactivation and Reactivation of megatherium Phage T

Stock phage in 5 per cent, pH 7 peptone diluted as below:	Plaques/plate
(*a*) 0.05/5 × 0.05/5 × 0.05/5 in 5 per cent, pH 7 peptone, 1 hr. at 20°, × 0.1/5 in plating suspension. Plate	320
(*b*) 0.05/5 × 0.05/5 in 0.01 M, pH 6 acetate buffer—0°—5 min. × 0.05/5 in 5 per cent, pH 7 peptone (tube B1) × 0.1/5 in plating suspension. Plate	0
1 ml. solution B1 above—titrate to pH 5 with HCl. Stand 20°, 1 hr., dilute 0.1/5 in plating suspension. Plate	310

inactivation. Addition of the $MgSO_4$ to any other solution does not prevent inactivation. The inactivation therefore must occur when the phage preparation is diluted 1×10^{-4} in the acetate buffer.

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present experiments, the phage is tested and found to be inactive (in the pH 7.0 peptone solution), before the reactivation takes place. In the "photoreactivation" experiments, on the other hand, the sample is divided before the reactivation procedure is carried out. One part of the sample is exposed to the light and one part left in the dark. The sample in the light is found to be more "active" than the one in the dark, but it is not possible to tell whether this is due to the loss of activity in the dark sample ("dark inactivation") or to gain in activity in the light sample, after the samples were separated (*cf.* Nash and Hirch, 1954). In order to make the photoreactivation results analogous to the present experiments, it would be necessary to keep the sample in the dark until no further activity (growth?) could be detected. The material is now inactive (dead). If the exposure of this inactive material to the light then results in the recovery of activity after the sample is returned to the dark, there would be evidence for photoreactivation. This apparently cannot be done.

There is a further complication in the photoreactivation of phage, due to the fact that illuminating the phage alone has no effect; it is necessary to illuminate the infected bacteria, after the phage has become adsorbed. It is not possible to say, therefore, whether the reactivating effect of the light is on the phage particle, the bacteria, or both, or whether the system becomes active in the light, or inactive in the dark.

Inactivation does not occur in the first dilution step of $1/10^2$ in 0.01 M, pH 6 acetate buffer because the concentration of peptone (0.05 per cent) from the stock phage preparation is high enough to prevent the reaction.

TABLE I B

Effect of Addition of 0.01 M $MgSO_4$ to Various Steps in the Dilution Series

Stock phage diluted as below—6 series, 4 tubes each. 1×10^{-3} M $MgSO_4$ added to tube noted in each series before phage dilution made.

Solvent	0.01 M, pH 6 acetate—0°, 5 min.		5 per cent, pH 7 peptone		
Dilute	0.05/5 ×	0.05/5	0.05/5 ×	0.1/5	
Dilution step	1	2	3	4	
Dilution series	1×10^{-3} M $MgSO_4$ in. tubes as noted below				Plaques/plate
1	+	+	+	+	330
2	−	−	−	−	0
3	−	−	−	+	5
4	−	−	+	−	0
5	−	+	−	−	300
6	+	−	−	−	0

TABLE II

Effect of Time of Standing in Various Steps of the Dilution Series on Active Phage

Stock phage diluted as below. Kept in tubes noted for 12 seconds or 1 hour as shown before continuing the dilution.

Solvent	0.01 M, pH 6 acetate, 0°		pH 7, 5 per cent peptone		Add sensitive cells and plate. Phage/plate
Dilution in each tube	0.05/5	0.05/5	0.05/5	0.1/5	
Dilution step	1	2	3	4	Average 4 separate dilution series
Dilution series	Time in each tube, *min.*				
1	0.2	0.2	0.2	0.2	100 ± 30
2	60	0.2	0.2	0.2	80 ± 25
3	0.2	60	0.2	0.2	3 ± 1
4	0.2	0.2	60	0.2	120 ± 30
5	0.2	0.2	0.2	60	150 ± 40
C	All dilutions in pH 7, 5 per cent peptone				300 ± 20

The solution in which the inactivation occurs may also be identified by the change in activity with the time of standing in the various solutions. The results of such an experiment are shown in Table II.

Partial inactivation occurred when the phage stood in each solution for 0.2 minute and there was no change when it was allowed to stand for 60 minutes in the first (0.01 M, pH 6 acetate, 0.05 per cent peptone), third (5 per cent peptone), or fourth (5 per cent peptone). Complete inactivation occurred, however, when the phage was left 60 minutes in the second solution (0.01 M, pH 6 acetate). This confirms the result of the previous experiment.

TABLE III

Reversible Inactivation of Various Phage Solutions in 10^{-3} M pH 6 Acetate Buffer 25°—3 Minutes

Phage type	P/ml.	Method of preparation	Dilution in 10^{-3}M acetate buffer	P/ml. in acetate	
				A.P.	R.I.P.
T	5×10^8	Lysis of sensitive *megatherium* in peptone	$\frac{0.1}{10} \times \frac{0.1}{10}$	0	200×10^2
T		Stock phage dialyzed 5 days *vs.* 0.1 M sodium acetate-0°	$\frac{0.1}{10}$	0	400×10^4
T	5×10^{10}	Lysis of sensitive *megatherium* in peptone	$\frac{0.1}{10} \times \frac{0.1}{10} \times \frac{0.1}{10}$	0	150×10^2
T	5×10^8	From lysogenic *megatherium* in peptone	$\frac{0.1}{10} \times \frac{0.1}{10}$	0	400×10^2
T	1×10^{11}	Precipitated with $(NH_4)_2 SO_4$ Precipitated three times with $MgSO_4$ 4×10^{10} plaques/γ protein N (Northrop, 1955 *b*)	$\frac{0.1}{10} \times \frac{0.1}{10} \times \frac{0.05}{10}$	0	100×10^2
C	1×10^{10}	Lysis of sensitive *megatherium* in peptone	$\frac{0.1}{10} \times \frac{0.1}{10} \times \frac{0.1}{10}$	0	80×10^2
T, S, C, VBR	1×10^8	Lysogenic *megatherium* growing in $(NH_4)_2SO_4$ culture medium	$\frac{0.1}{10} \times \frac{0.1}{10}$	0	60×10^2

Reversilbe Inactivation of Various Phage Preparations.—

The results of an experiment in which various phage preparations containing different types of phage (Murphy, 1953) or in different stages of purification were inactivated, are shown in Table III.

All types of phage tested are inactivated by dilution in 0.001 M, pH 6.0 acetate buffer and all are reactivated by standing in pH 5.0 peptone. It is very unlikely, therefore, that the inactivation is due to combination of the active phage with some inhibiting substance in the phage preparation (*cf.* page 242).

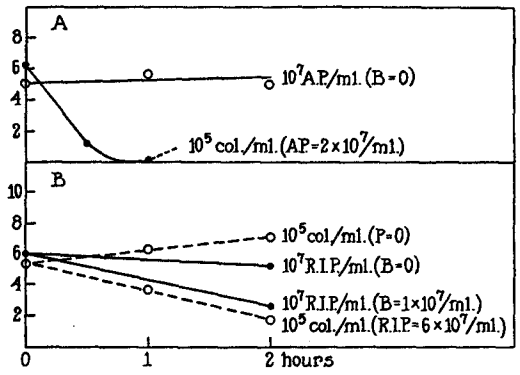

FIG. 1 A. Decrease in colony count in mixture of *megatherium*-sensitive and active T phage.

FIG. 1 B. Decrease in colony count and R.I. phage in mixtures of *megatherium*-sensitive and R.I. phage

TABLE IV

Adsorption of Active and R.I. Phage by Megatherium-Sensitive

Active, stock phage diluted 10^{-6} in 5 per cent peptone—5 ml. in 12 tubes—noted concentration of *megatherium*-sensitive added, 25°, centrifuged after 30 minutes. Plate supernatant $\frac{1}{100}$.

R.I., stock phage diluted 10^{-5} in 0.01 M pH 6 acetate buffer—0°—5 minutes. Then dilute 5/50 in 5 per cent peptone. *Megatherium*-sensitive added as below 25°, 30 minutes centrifuge. Supernatant plated for active and R.I. phage 1/100.

Megatherium-sensitive/*ml.*	0	1×10^7	3×10^7	1×10^8
10^2 active P/ml. supernatant	90	34	3	0
10^2 R.I. P/ml.	70	25	2	0

Adsorption by Sensitive Cells.—

The R.I. phage is adsorbed by cells of *megatherium*-sensitive, at about the same rate as active phage (Table IV).

The sensitive cells are killed by the R.I. phage although not as rapidly as by the active phage (Fig. 1). It is not possible to determine whether lysis occurs, since there are too many uninfected cells present. The infected cells do not produce more phage, however, since R.I. phage added to a growing suspension of sensitive cells does not cause lysis of the culture, nor does the suspension contain any active phage after growth (Table V). The R.I. phage, therefore,

has lost its ability to cause sensitive cells to form more phage, but still kills them. It has therefore the properties of phage ghosts (Herriott, 1951) or of colicines (Gratia, 1925), or phage inactivated by ultraviolet light (Luria, 1947). This may mean that the inactivation is due to a reversible change in the nucleic acid part of the molecule, rather than in the protein, since Hershey's and Chase's (1952) beautiful experiments have shown that (in all probability) only the nucleic acid is needed to cause the infected cell to produce more phage.

TABLE V

Activity Determination by Lysis of Growing Culture

T phage diluted 1×10^{-6} in H_2O—25°—5 minutes = S
1 ml. S + 10 ml. 5 per cent peptone—25°—1 hour = P
1 ml. S + 10 ml. 1 M Na acetate—25°—1 hour = A

Plaque count	Plaques/plate
0.5 ml. P + 3.5 ml. 5 per cent peptone + *megatherium*-sensitive + 1 ml. agar. Plate	0
0.5 ml. A + 3.5 ml. 5 per cent peptone + *megatherium*-sensitive + 1 ml. agar. Plate	100
Lysis test	**Lysis time**
1 ml. P + 9 ml. *megatherium*-sensitive suspension in 5 per cent peptone—shake 35°	No lysis
1 ml. A + 9 ml. *megatherium*-sensitive suspension in 5 per cent peptone—shake 35°	4 hrs.
Test suspensions plated 0.5/5 for phage: P suspension	Plaques/plate: 0
A suspension	$>10^4$

This confirms Herriott's observation that phage particles which have lost their nucleic acid no longer cause sensitive cells to produce more phage.

Rate of Sedimentation.—

The R.I. phage sediments in the high speed centrifuge at almost the same rate as active phage (Table VI).

The ratio of the phage/milliliter in the supernatant to the phage/milliliter in the residue was 3.8 ± 0.6 for the R.I. phage and 3.4 ± 0.55 for the active phage. There is no appreciable difference in size, therefore, and hence, the inactivation cannot be ascribed to agglutination or precipitation, since the loss in activity is so great that it would be necessary to assume at least 1000 particles in each aggregate. This conclusion is borne out by the fact that stirring does not affect the result (*cf.* page 241) and that the rate of inactivation is many times faster than the theoretical limit for a precipitation reaction (*cf.* page 242).

Stability of the R.I. Phage.—

There is an equilibrium in salt solutions between the R.I. and the active phage (see below) and hence, it is impossible to measure the rate of inactivation of either one alone in such solutions. In the presence of peptone, urea, or certain amino acids, however, at pH 7.0, this equilibrium does not exist (or if it does,

TABLE VI

Rate of Sedimentation of Active and R.I. Phage in High Speed Centrifuge, 1 Hour, 14,000 G

Purified phage (Northrop, 1955 *b*) dialyzed *versus* 0.1 M sodium acetate 3 days, 0°, 3×10^9 P/ml.

0.2 ml. P added to 20 ml. distilled H_2O—0°—15 minutes—diluted 20/200 in pH 7, 5 per cent peptone = R.I. phage.

0.2 ml. P added to 20 ml. pH 7, 5 per cent peptone—0°—15 minutes—diluted 20/200 in pH 7, 5 per cent peptone = active phage.

Four 20 ml. samples, active or R.I., centrifuged, etc., as below:

Assay, dilute 0.1/5 in pH 7, 5 per cent peptone—2 hours 25°, dilute 0.2/5 in plating suspension = active. 1 ml. × 1 ml. 0.016 M HCl (⇨pH 5.0)—2 hours 25°, dilute 0.2/5 in pH 7, 5 per cent peptone × 0.2/5 in plating suspension = active + R.I.

	Inactive phage		Active phage
	(Average of four separate tubes) (Plaques/plate)		
	Active	R.I.	Active
20 ml.—stand 2°—3 hrs.	0	490	700
20 ml. in 4 tubes, centrifuge 1 hr.—0°—14,000 g 15 ml. supernatant pipetted off, assay separately—average—supernatant	0	370	470
5 ml. residue in tube—stir up and assay residue	0	1400 ± 120	1600 ± 120
Ratio $\frac{\text{P/ml. residue}}{\text{P/ml. supernatant}}$		3.8 ± .6	3.4 ± .55

the equilibrium state is reached very slowly). Under these conditions, the active phage is much more stable than the R.I. phage (Fig. 2).

The R.I. phage may be kept for several days in 0.01 M, pH 6.0 acetate buffer at 0°C.

Inactivation by Trypsin, Chymotrypsin, and Desoxyribonuclease.—

Active and R.I. phage are both digested at about the same rate by trypsin, chymotrypsin, or desoxyribonuclease (Northrop, 1955 *a*).

Effect of Various Substances on the Formation of R.I. Phage.—

R.I. phage can exist only in a narrow range of salt concentration (10^{-2} to 10^{-5} M) pH 5 to 6 and 0 to 25°C.

The effect of the pH of 10^{-3} molar buffer on the relation between R.I. and active phage is shown in Fig. 3. It is evident that, at this concentration of

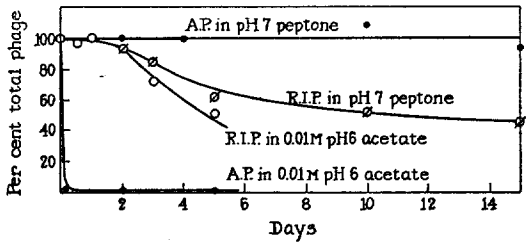

FIG. 2. Inactivation of active and R.I. phage in 0.01 M, pH 6 acetate buffer, or 5 per cent, pH 7 peptone at 0°.

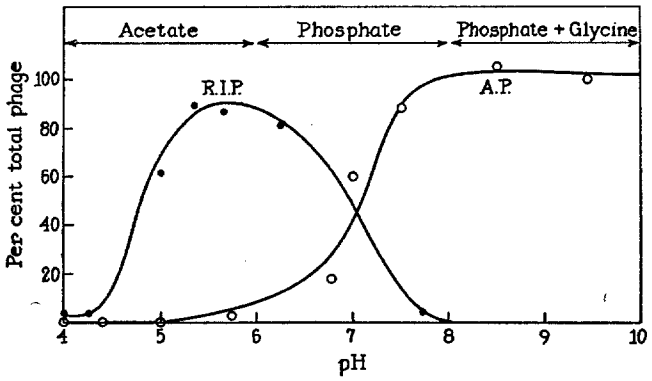

FIG. 3. Relative amounts of active and R.I. phage in 10^{-3} M buffer and at various pH's after 5 minutes at 0°. Stock phage diluted 1 × 10^{-6} in buffer noted. Assay for active and R.I. phage.

salt, the R.I. phage is the stable form from about pH 5 to 6, while the active phage is stable from 7 to 9. On the acid side of pH 5, both forms are irreversibly inactivated.

The curve resembles the titration curve of an acid with pK of 7. The R.I. phage is the un-ionized form; the ionized (salt) is the active phage. On the acid side of pH 5, an entirely different (irreversible) inactivation occurs.

The effect of the buffer concentration, pH 6.0, air-free or saturated with air, is shown in Fig. 4. It is evident that, in air-free buffer, the salt concentration

range of stability of the R.I. phage is narrow, whereas in water saturated with air, it is wide. This difference is due to the fact that the dissolved CO_2 from the air acts like many other electrolytes to stabilize the R.I. phage.

If active phage is diluted in cold distilled water (or $<10^{-3}$ M, pH 6 acetate), saturated with air, therefore, it is transformed to R.I. phage and may be reactivated. If *air-free* distilled water (or $<10^{-3}$ M, pH 6 acetate) is used, the phage is rapidly and irreversibly inactivated. For this reason, the effect of dilution in distilled water is very irregular, depending upon how much CO_2 happens to be present.

Control experiments showed that CO_2-free air, oxygen, nitrogen, or hydrogen had no effect on the stability of R.I. phage.

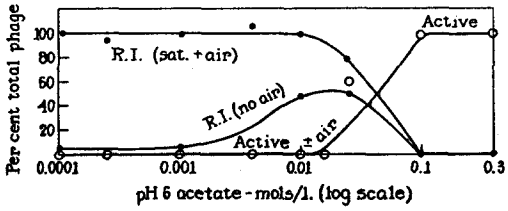

FIG. 4. Active and R.I. phage present in various concentrations of pH 6, acetate buffer, air-free, or saturated with air, after 10 minutes at 0°. Stock phage diluted 1 $\times$ 10^{-6} in solution noted -0° – 10 minutes. Assay for active and R.I. phage.

Reversible Inactivation and Reactivation at pH 6 and 7.—

Figs. 3 and 4 show that the change from active to R.I. phage is very sensitive to small changes in pH and salt concentration. If the effect of pH is reversible, then titrating a solution of active phage from pH 7 to 6 should result in the formation of the R.I. phage and this reaction should then be reversed by titrating back to 7 again. This is true, but if the solution is titrated in the usual way by adding acid and then alkali, the reaction soon stops owing to the increase in the salt concentration. This difficulty may be avoided by regulating the pH of the solution by means of O_2 (or other neutral gas) containing more or less CO_2. If the CO_2 concentration is high, the pH will drift rapidly to 6, while if no CO_2 is in the gas, the pH will drift back to 7.0. The results of an experiment, in which the pH of a solution of phage in 2×10^{-5} M $NaHCO_3$, 10^{-3} M NaCl was changed back and forth from pH 7 to 5.8, are shown in Fig. 5. The solution was sampled for active phage as shown. The phage is active when the pH of the solution is $\geqq$ 7.0, and completely inactive at pH 5.8. There is a gradual decrease in the total active phage, indicating the formation of irreversibly inactivated phage. The cycle was repeated seven times and many more cycles may be carried out if they are of shorter duration.

Effect of Salts, Amino Acids, etc., on Active and R.I. Phage.—

The preceding experiments have shown that active phage, under certain conditions at least, is in equilibrium with a reversibly inactive form and that this R.I. phage is probably an intermediate step in the formation of inactive

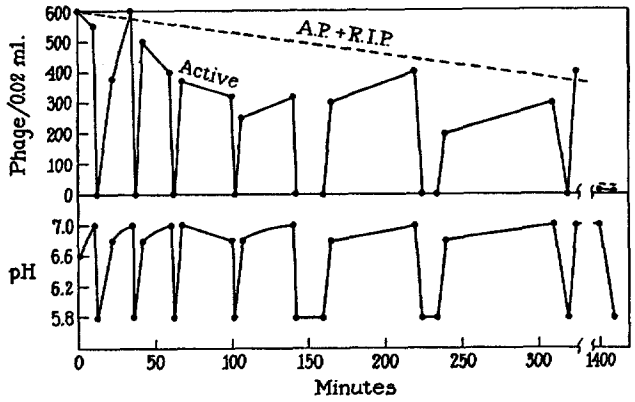

FIG. 5. Inactivation and reactivation of T phage in 2×10^{-5} M $NaHCO_3$ and 10^- M NaCl and 1 γ brom cresol purple/ml. by changes in pH. Stock phage diluted 1×10^{-6} in above solution at 0°. O_2 bubbled through M/10 $NaHCO_3$ and then through solution until pH = 5.8. O_2 then bubbled through water and then through the solution until pH = 7. Repeat as noted. Assay for active phage as noted. pH determined colorometrically.

phage. Changes in concentration or in the components of the solutions, therefore, can affect the phage as follows:—

1. Cause irreversible inactivation.
2. Cause reversible inactivation.
3. Prevent reversible inactivation and reactivate R.I. phage.
4. Prevent reversible inactivation (but not reactivate R.I. phage).

The conditions which prevent the formation of R.I. phage also prevent irreversible inactivation and cause reactivation of R.I. phage in most cases.

A summary of the effect of a number of salts and amino acids tested in this way is shown in Table VII.

1. Substances Which Cause Irreversible Inactivation

Some heavy metal salts, distilled water ($<10^{-6}$ M salts—any pH), *l*-arginine, urea solutions (>3 M) cause irreversible inactivation.[3] The only amino acid

[3] Krueger and Elberg (1934) and Krueger and Baldwin (1933) have shown that inactivation of staphylococcus phage by $HgCl_2$ or KCN is also reversible, provided

TABLE VII

Effect of Various Substances on Inactivation and Reactivation of Phage

	Effective concentration
1. Substances which cause irreversible inactivation: Distilled water all salts below 10^{-7} M, $FeCl_3$, Ce^{+++}, Ce^{++++}, Hg^{++}, $AuCl_3$, $LaCl_3$, $Th(NO_3)_2$, $KMnO_4$, $PbNO_3$, $Na_2Cr_2O_7$, $AgNO_3$, I_2, 3 M urea, saturated guanidine HCl, >0.2 M Na salicylate, 5×10^{-4} M *l*-arginine	
2. Substances which cause reversibly inactivated phage formation: All salts, etc., in group 3, below their effective stabilizing concentration, and in the pH range of 5–6 at low temperatures	
3. Substances which prevent reversible inactivation of active phage in pH 6, 0.01 M acetate buffer—0° (reactivate R.I. phage in 0.01 M, pH 6 acetate):	
Na_2SO_4, NaCl, $NaNO_3$, KCl, KI, $(NH_4)_2SO_4$, Na acetate, Na salicylate	0.1–0.01 M/liter
$CaCl_2$, $MgCl_2$, $ZnCl_2$, $BaCl_2$, $SrCl_2$, $MgSO_4$, $MnSO_4$, Na_2SO_3, Na oxalate, Na_2S, NaF, Co^{++} acetate	1×10^{-3} to 1×10^{-5} M/liter
$CuSO_4$, $SnCl_4$, $AlCl_3$	10^{-2} M/liter, 10^{-5} to 10^{-8} M/liter
Peptone	20 γ/ml.
4. Substances which prevent reactivation in 0.1 M sodium acetate:	
dl-Tryptophane, *dl*-leucine, isoleucine, methionine, asparagine, *dl*-cystine, valine, phenylalanine (other amino acids do not)	Saturated solution in 0.1 M sodium acetate
Peptone	10 mg./ml.
Urea	1 to 2 M/liter
Guanidine	0.5 saturated
Labtone	1 γ/ml.

to cause inactivation is *l*-arginine. *d*-Arginine has no effect on the system, even in 1 M concentration. This result is unexpected, and, in view of the great sensitivity of the system, may be due to traces of impurity. This experiment was repeated with two different samples of *l*-arginine prepared in different labora-

---

the inactivating reagent is removed from the solution. This would be the expected result if the phage-ion complex is inactive, instead of active, as is the case with other, non-inactivating ions (*cf.* page 244).

tories; the result was the same with either sample. *B. coli* phage is inactivated by serine, threonine, homoserine, and aspartic or glutamic acids (Amos, 1954).

2. Substances Which Cause Reversible Inactivation

All salts tested, except those under section 1, in low concentration, between pH 5.5 and 6.5 cause reversible inactivation.

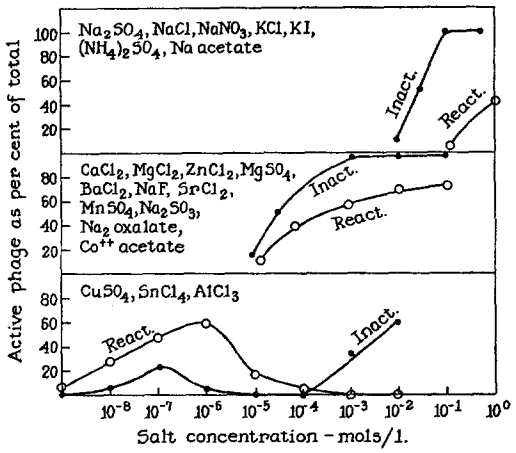

FIG. 6. Effect of various salts on inactivation and reactivation of T phage. All salts made up in 0.01 M, pH 6 acetate buffer, 0°. *Inactivation curves*, active phage diluted 1×10^{-6} in salt solution noted. Stand 0°, 20 minutes. Assay for active phage. *Reactivation curves*, active phage diluted 1×10^{-6} in 0.01 M, pH 6 acetate buffer, 0°, 5 minutes. Salt noted added to make the required final concentration. Solutions stand 0°, 20 minutes. Assay for active phage.

The effect of the various salts was determined separately. The results which did not differ from each other by more than the experimental error were then averaged, and the average figure for the group used to plot the curves shown in the figure.

3. Substances Which Prevent Reversible Inactivation (and Reactivate)

All salts tested (except for those in section 1) prevent reversible inactivation when added to 0.01 M, pH 6 acetate buffer in sufficient concentration.

The concentration required to prevent inactivation is lower, in general, the higher the valency of the ion, either cation or anion. There are, however, striking exceptions to this rule, so that the chemical nature as well as valency is of importance. Na_2SO_4 and $(NH_4)_2SO_4$, for instance, are much less efficient than Na_2SO_3, Na_2 oxalate, or Na_2S. The efficiency of these latter salts suggests the possibility of an oxidation-reduction step in the reaction, but preliminary experiments designed to test this possibility gave negative results. The most efficient stabilizing (reactivating) salts are $CuSO_4$, $SnCl_4$, and $AlCl_3$. These

salts show two zones of concentration effects (Fig. 6), one from 10^{-2} M to 10^{-4} M and a second around 10^{-6} to 10^{-7} M. These salts exist as metal ions in concentrated solutions, but in very dilute solutions probably are present as (possibly colloidal) metal hydroxides. This may account for the second effective range. The stabilizing effects of various cations, on the whole, are very similar to those described by Adams (1949) in connection with the irreversible inactivation of *coli* phage. Adams' experiments were carried out at 30–40°C. At this temperature the inactivation of *megatherium* phage is also irreversible, because of the instability of the R.I. phage.

The effect of salts in general is very similar indeed to that found by Crewther (1953) in a study of the inactivation of dilute trypsin solutions in aqueous salt solutions.

4. Substances Which Prevent Reactivation of R.I. Phage (in 0.1 M Na Acetate)

No electrolytes tested (except those which cause irreversible inactivation) prevent the reactivation of R.I. phage in 0.1 M Na acetate (pH 7.0). Peptone, urea (1 to 2 M/liter), guanidine, and some amino acids (Table VII) do prevent the reaction. The other amino acids do not. The stabilizing effect of urea and guanadine solutions can be predicted from their effect on the native/denatured protein equilibrium. Sodium salicylate, which stabilizes the native/denatured hemoglobin equilibrium (Anson and Mirsky, 1934 *b*), however, does not stabilize the phage system.

The Effect of Peptone on the R.I. Phage/Active Phage System.—

Peptone has a very striking effect on the R.I.P./A.P. equilibrium, different from that of any other substance tested. The effect is entirely different at different pH ranges (Fig. 7).

In the range of pH 7 to 9, a few milligrams of peptone per milliliter stabilize both R.I. and active phage so that, no matter which form is added, no change occurs. Were it not for this fact, the existence of the R.I.P./A.P. equilibrium could not be detected, since if equilibrium existed under the conditions of the activity test (*i.e.*, a mixture of phage with susceptible cells in pH 7 peptone), the results would be the same no matter which form of phage was added.[4]

In 0.001 M, pH 6 acetate buffer, a few gamma of peptone per milliliter prevent the formation of R.I.P., (stabilize A.P.), but do not cause rapid activation of R.I.P.

At pH 5.0 to 5.5 a few milligrams of peptone per milliliter cause the R.I. phage to become active. The other substances which act like peptone at pH 7.0 do not cause this reactivation at pH 5.0 to 5.5.

[4] It is a curious fact that peptone is also a very efficient stabilizing agent for trypsin (Northrop, 1922) or desoxyribonuclease (McCarty, 1946).

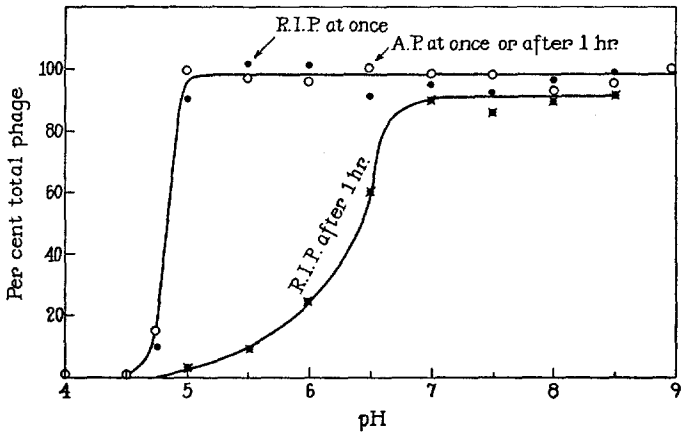

FIG. 7 Active and R.I. phage in 5 per cent peptone at various pH's. Active phage or R.I. phage added to series of tubes of 5 per cent peptone of various pH's. Assay for active or R.I phage at once, and after 1 hour at 20°.

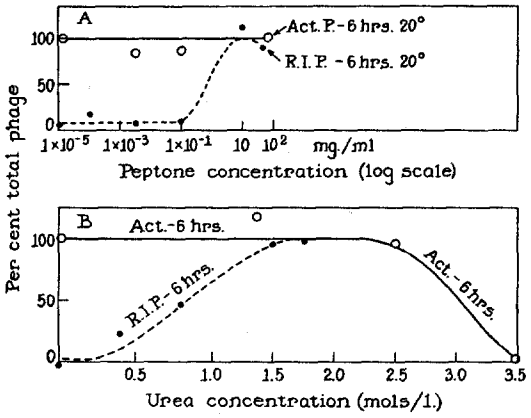

FIG. 8 A. Effect of various concentrations of peptone (pH 7.4, made up in 0.1 M sodium acetate) on active and R.I. phage. 1×10^5 active phage added to a series of tubes containing different concentrations of peptone in 0.1 M sodium acetate. Stand 6 hours, 20°. Assay for active phage. 1×10^5 R.I. phage added to a second series. Assay for R.I. phage after 6 hours.

FIG. 8 B. Effect of various concentrations of urea (pH 7 in 0.1 M Na-acetate) on active and R.I. phage. The experiment was carried out as described under Fig. 8 A, except that various concentrations of urea were used instead of peptone.

The effect of various concentrations of peptone on active and R.I. phage in 0.1 M sodium acetate is shown in Fig. 8 A, and that of urea in Fig. 8 B. There is a range of concentration for both substances in which no change occurs in

either R.I. or active phage. Guanidine, and presumably other substituted ureas, act similarly. These substances (but not peptone) also stabilize the native/denatured protein equilibrium (Greenstein, 1938). Tobacco mosaic virus also is inactivated by urea and guanidine (Stanley and Lauffer, 1939; Bawden and Pirie, 1940).

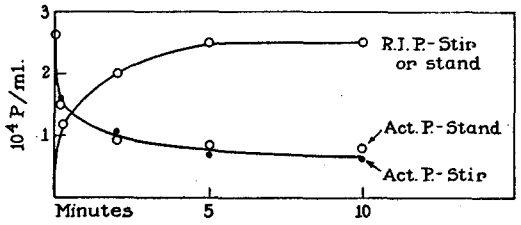

FIG. 9. Effect of stirring on reversible inactivation of phage in 0.01 M, pH 6 acetate buffer at 0°.

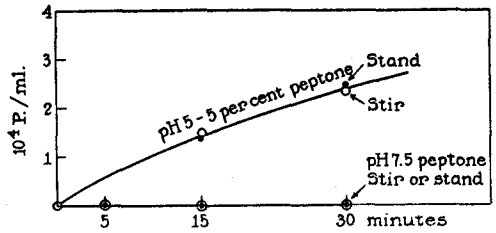

FIG. 10. Effect of stirring on rate of reactivation of R.I. phage in 5 per cent, pH 5 and 7.5 peptone at 0°.

Kinetics of the Inactivation Reaction.—

Accurate data for the rate of reversible inactivation in dilute salt solution could not be obtained, owing to the fact that the reaction is extremely rapid and also extremely sensitive to the most minute quantities of various substances. For instance (Table VII), 5 γ peptone per ml. or 1 γ "labtone,"[5] or 10^{-7} molar Cu^{++}, Sn^{++}, or Al^{+++} stop the reaction completely.

The following observations were made: (1) The rate of inactivation or reactivation is not affected by stirring, (unless there is foaming) (Figs. 9 and 10). (The stirring in these experiments was done with a large glass rod in a test tube (Northrop, 1930).) Violent stirring, as in a Waring blendor, inactivates rapidly and irreversibly. (2) The rate of inactivation (in 0.01 M, pH 6.0 acetate at 0°C.) is too fast to measure accurately, no matter whether the phage concentration is 1×10^8 or 1×10^6/ml. (3) The fact that the rate appears to be independent of the concentration, and that it is so fast in a solution as dilute

[5] An organic detergent obtained from Braun-Knecht-Neimann, San Francisco.

as 1×10^8 particles/ml. (1×10^{-17} molar) rules out any mechanism which depends upon the interaction of two or more molecules, since the rate is far in excess of that predicted by molecular theory, even though every collision is efficient.

The theoretical minimum time for the agglutination (precipitation) of particles is given by Smoluchowski's (1916) classical equation:

$$t = \frac{1}{4\pi D\, 2r\, V_o} \tag{1}$$

r = radius of particle

V_o = particles/ml.

D = diffusion coefficient $= \frac{RT}{N} \cdot \frac{1}{6\pi\eta r}$ (Einstein, 1905) (2)

η = viscosity of solution.

or $t_{0.0.} = \frac{3 \times 10^{11}}{V_o}$ (approximately) at 0° and in aqueous solution.

In the present experiments, $V_0 = 1 \times 10^8$. The minimum time for agglutination therefore is 3×10^8 seconds or about 3000 days.

The actual time is less than 10 seconds.

The only variable in the equation which could be changed enough to predict this rapid rate is V_0, the number of particles. If it be assumed that the reaction consists of the formation of a complex between the active phage particles, and some other (unknown) molecule, which is present in very high concentration compared to the phage particles ($>10^8$ times as many), then the high rate can be predicted. The number of such reacting (inhibiting) molecules per phage molecule is so large, however, that it is hardly possible to suppose that they are present in the phage solution, since some of the purified phage preparations used in these experiments were analyzed as 75 per cent or more pure phage (Northrop, 1955 *b*).

The reaction must therefore be monomolecular (a rearrangement in the phage molecule) or (more likely) a reaction with some of the ions in the inactivating solution (*cf.* Adams, 1949).

This latter conclusion is supported by the results of experiments in which the effect of various concentrations of $MgSO_4$ on the inactivation or reactivation of phage in 0.005 M, pH 6.0 acetate at 0°C. was determined (Fig. 11).

In this experiment active phage was added to 0.005 M, pH 6.0 acetate containing 0, 2×10^{-5}, or 8×10^{-5} M $MgSO_4$. In one series of tubes, the $MgSO_4$ was added before the phage. This series shows the inactivation rate. In the second series, the phage was added first and was reversibly inactivated. The $MgSO_4$ was then added and the rate of reactivation followed. The curves show that an equilibrium ratio, active/R.I. phage, exists, since approximately the same value for the activity is found no matter whether the initial phage was active or R.I. The curves do not actually coincide, even after long standing, due to the slow irreversible inactivation of the phage.

The ratio, active/R.I. phage, is independent of the total phage concentration, at least within a range of 10 to 1 (Fig. 12).

The results of a series of experiments in which the effect of various concentrations of $MgSO_4$ on the ratio of R.I./active phage present in 0.005 M, pH 6.0

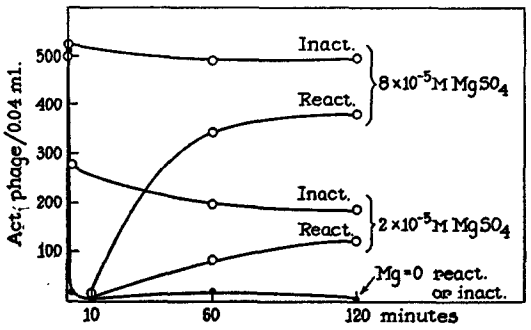

FIG. 11. Effect of various concentrations of $MgSO_4$ on active and R.I. phage at 0°, in 0.005 M, pH 6 acetate buffer *Inactivation curves*, active phage added to $MgSO_4$ solution and assayed for active phage as noted. *Reactivation curves*, active phage added to 0 005 M, pH 6 acetate buffer, 10 minutes, 0°. $MgSO_4$ added to give required final concentration. Assay for active phage as noted.

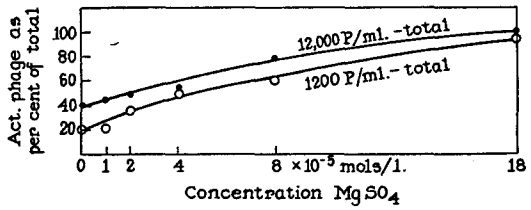

FIG. 12. Effect of $MgSO_4$ added to 0.005 M, pH 6 acetate buffer on the reversible inactivation of phage solutions of different concentrations. 1200 active P/ml. added to a series of tubes containing various concentrations of $MgSO_4$ (made up in 0.005 M, pH 6 acetate buffer). Assay for active phage after 1 hour, 0°. 12,000 active P/ml. added to a second series and analyzed as above.

acetate at 0°C. was determined are shown in Fig. 13. Each point is the average of 10 to 12 separate experiments. The average deviation of the mean $\left(\frac{\Sigma\Delta}{n\sqrt{n}}\right)$ is indicated by the size of the circles. The solid line is calculated with the aid of the following assumptions:

(1) Inactive phage (P) reacts with Mg^{++} to form an active complex Mg P. (2) The complex dissociates according to the law of mass action; *i.e.*, $\frac{P \times Mg^{++}}{[P\,Mg]} = K = 4 \times 10^{-5}$ mol/liter. (3) The concentration of Mg^{++} is very high com-

pared to that of the complex (P Mg). The per cent of the phage combined is therefore independent of the total phage concentration.[6]

It does not follow, from the above experiments, that only 1 Mg^{++} combines with 1 phage particle since any number of Mg^{++} combining with 1 phage molecule will cause the same quantitative result providing any one of the following assumptions is correct:

1. The first Mg^{++} which combines is the effective one.
2. The reaction of the phage with the first Mg^{++} is the slowest and therefore determines the rate.

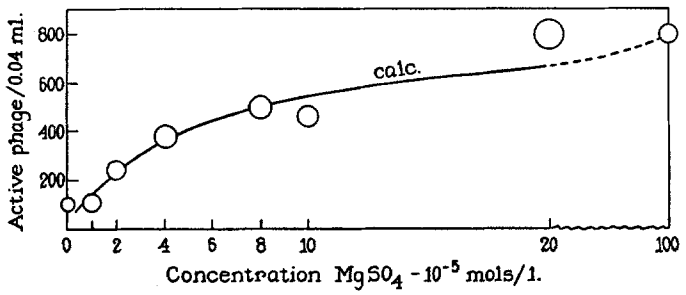

FIG. 13. Effect of $MgSO_4$ concentration on the activity of phage on 0.005 M, pH 6 acetate buffer at 0°. 2×10^4 active phage/ml. added to 0.005 M pH 6 acetate buffer containing $MgSO_4$ noted, 0°, 1 hour. Sample for active phage. Circles are the average results from 14 tubes (7 experiments, 2 tubes each). The size of the circles represents (approximately) the average deviation of the mean. The solid line is calculated on the assumption: inactive phage + Mg⇌phage Mg^{++} (Active) and that

$$\frac{\text{Inactive phage} \times [Mg^{++}]}{[\text{Phage Mg}]} = 5 \times 10^{-5} \text{ mole/liter.}$$

3. Each group in the phage particle reacts *independently* with Mg^{++}, probably because they are so far apart. This is the most probable explanation. The inactivation of enzymes by mustard gas (Herriott, Anson, and Northrop, 1946) is an example of such a reaction in which it is known that many molecules of the reagent combine with one molecule of the active protein, and yet the kinetics of the reaction agrees with the assumption that only one molecule combines.

It cannot be concluded either, with any certainty, that the only active form of the phage is an ionic complex. If an equilibrium exists between active and R.I. phage (as in the case of some reversibly denatured proteins), then A.P. = $K \cdot$R.I.P. If the Mg ion combines only (or more) with the A.P., then the addition of Mg will cause the equilibrium to shift in favor of the A.P. (with A.P. and [Mg A.P.] considered as active). Since A.P. = $K \cdot$R.I.P., any equation con-

[6] A similar relationship in the case of phage-antiphage has been described by Andrewes and Elford (1933) and is very likely due to the same condition.

taining either of them will be unaffected (except for the numerical value of the constants) by substituting $K \cdot$R.I.P. for A.P. or *vice versa.*

Such an equilibrium will also account for the irreversible (by dilution) inactivation by heavy metals, except that in this case the metal-phage complex must be considered inactive (or the heavy metal combines with the denatured form).

The Effect of Temperature on the Reaction.—

The results of the inactivation experiments are in general agreement with the assumption that the formation of the R.I. phage is a protein denaturation reaction, which is reversed when the activity is restored. The reactivation of heat-inactivated staphylococcus phage was reported by Krueger and Mundell (1936).

Reversible denaturation, as Anson and Mirsky showed (Anson, 1945), is a property of most proteins, but the ease with which the reaction may be carried

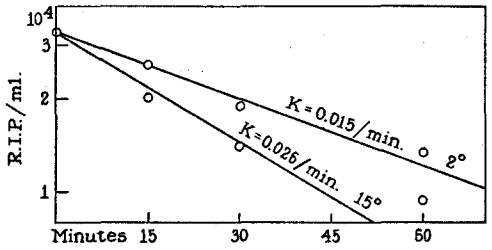

FIG. 14. Rate of reactivation of R.I. phage in 5 per cent, pH 5 peptone at 2 and 15°. Each point is the average of nine determinations (3 experiments of 3 tubes each).

out varies enormously. In at least two instances, trypsin (Northop, 1932; Anson and Mirsky, 1934 *a*) and trypsin soy bean inhibitor (Kunitz, 1948), there is a true equilibrium between the denatured and native form of the protein, just as in the phage experiments described in the preceding sections. In both the trypsin and trypsin inhibitor systems, the equilibrium is shifted toward the inactive side by increasing the temperature. The trypsin inactivation reaction is too fast to follow (as is the phage inactivation) but the rate of denaturation of trypsin inhibitor may be accurately determined. The results show that the inactivation reaction has a high temperature coefficient (*i.e.*, Q_{10} = about 10; $\Delta H^{\ddagger}$ = 55,000) although still smaller than that for irreversible denaturation. The temperature coefficient for the reversal of denaturation, on the other hand, is very low (Q_{10} = 1.8, $\Delta H^{\ddagger}$ = 15,000) and E = 9600 in the range of 10–20°C. (In some other temperature ranges E is even negative.)

The rate of inactivation of the *megatherium* phage is so great that no satisfactory time curves for the reaction could be obtained, even at 0°.

The rate of reactivation, in pH 5, 2 per cent peptone is readily determined, however. The results of a series of such measurements are shown in Fig. 14.

R.I. phage was formed by diluting in 0.01 M, pH 6 acetate at 0°C. as usual and added to 2 per cent, pH 5 peptone at the temperature noted. Samples were taken and plated for active phage as noted. The curves show a low temperature coefficient (Table VIII), $Q_{10} = 1.5$, $E = 6{,}500$. These values are very similar to those for trypsin inhibitor (Kunitz, 1948).

Effect of Temperature on the A.P./R.I.P. Equilibrium.—

The equilibrium between A.P. and R.I.P. is shifted in favor of the active from by lowering the temperature from 15 to 2°.

The results of a series of experiments of this type are shown in Table IX. The value of K (the ratio of R.I.P./A.P.) decreases as the temperature changes from 15 to 2°C. and the ratio $K_{15}/K_2 = 1.75 \pm 0.1$. This corresponds to a value of ΔH of about 6,600 calories/mol (van't Hoff's constant), ΔF varies from -500 at 2° to -900 at 15°. The effect of temperature on the phage equilibrium is,

TABLE VIII

Effect of Temperature on Rate of Reactivation of R.I. Phage in 2 Per Cent, pH 5 Peptone

Temperature, °C.	2	15
Reactivation rate ($min.^{-1}$)	0.015	0.026
Arrhenius constant $E = \dfrac{-4.6 \log \frac{K_1}{K_2}}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)} = 6500$ cal./mol.		

E for soy bean trypsin inhibitor 0–15° = 9,600 cal./mol. (Kunitz, 1948 and personal communication).

therefore, much less than on the native/denatured equilibrium of trypsin or trypsin inhibitor. Actually the value for the heat of reaction varies with the solution and is therefore not comparable in different experiments.

These temperature effects are in qualitative, but not quantitative, agreement with the assumption that the primary reaction is reversible denaturation of a protein. The results of the digestion experiments are also unexpected from this point of view, since (with the exception of chymotrypsinogen and soy bean trypsin inhibitor, which are peculiar proteins) native proteins are attacked more slowly than denatured proteins by trypsin and chymotrypsin.

The salt precipitation test, which is the most sensitive test for denaturation, cannot be used with the R.I. phage, since the R.I. phage is changed to active phage by the salt. The test for S-S groups is not practical either, owing to the difficulty of obtaining enough R.I. phage.

The question as to whether the reaction is a case of reversible denaturation, must therefore be left undecided.

TABLE IX

Effect of Temperature on the Ratio of RI/Active Phage (in 0.03 M, pH 6 Acetate Buffer)

Stock phage diluted 1×10^{-4} in 1 M, pH 6 acetate buffer $\times$ 0.05/5 in 0.02 M, pH 6 acetate buffer at 15°C. Stand 2 minutes, sample 0.05/5 in pH 7 peptone and plate = active phage at 15°. Acetate tubes transferred to 2°. Stand 10 minutes, plate = active phage at 2°. Control experiments showed no further change on standing for 30 minutes longer.

Twelve experiments with 12 tubes each. The equilibrium constant $K = \frac{\text{RIP}}{\text{Active P}} = \frac{\text{Total P—active P}}{\text{Active P}}$ was calculated for each tube at each temperature and the ratio of $\frac{K_{15}}{K_2}$ found. (This method compensates as much as possible for variations in the individual tubes, which may be large (*cf.* Experimental Procedure).) These 144 ratios were then averaged.

	Phage		Trypsin inhibitor (Kunitz, 1948)	Trypsin (Anson and Mirsky, 1934)
Temperature, °C.	2	15*		
K (average 144 tubes)	2.8 ± .2	4.7 ± .2		
$\frac{K_{15}}{K_2}$		1.7 ± .1		
Change in free energy $\Delta F = -4.6T \log K$.	−500	−900	−500 to 3000	
van't Hoff's constant $\Delta H = \frac{-4.6\,\Delta \log K}{\Delta\,(1/T)}$ (Heat of reaction)		6,600	57,000	67,000

Effect of Light.—

The inactivation and reactivation proceed in the same way in the dark as they do in bright daylight.

Experimental Procedure

General Procedure. (Northrop, 1951).—

Stock Phage.—Prepared by lysis of sensitive *megatherium* (KM) in 5 per cent peptone with T phage (Northrop, 1955 *b*).

Determination of Active Phage.—The samples were diluted in 5 per cent, pH 7 peptone, 2×10^8 sensitive cells/ml. added, and 1 ml. plated by Gratia's double layer technique.

Active + R.I. Phage.—(1) An aliquot of the pH 7 peptone (above) titrated to pH 5 with HCl, allowed to stand at 25° 1 hour, and plated. (2) The sample was diluted in either 5 per cent, pH 5 peptone or 1 M sodium acetate, and allowed to stand at 20° for 1 to 2 hours. It was then diluted in 5 per cent, pH 7 peptone and plated as above.

These plates give the active and the R.I. phage present. The count is the same by either method. The R.I. phage therefore is this value minus the active phage.

Preparation of R.I. Phage.—The R.I. reaction is affected by very minute traces of many substances and the results may be extremely irregular unless special precautions are taken.

The test tubes used for the dilution in 0.01 M, pH 6 acetate (in which the reaction occurs) were not used for any other purpose, nor were they washed, or cleaned with cleaning solution. When not in use, they were filled with distilled water. Before use, they were rinsed 4 times with the acetate solution.

The distilled water used must be of "conductivity water" quality and even so, the results are more reproducible if the same sample of water is used. The pH 6 acetate buffer also varies somewhat and results obtained with different lots of buffer, or different lots of distilled water cannot be compared.

Even though the same reagents are used, there is still considerable variation, when the ratio of R.I./A.P. in different tubes is compared. The variation among different tubes is far greater than the variation in different samples of the same tube, as is usually the case in any type of kinetic experiment.[7]

In the present experiments the average deviation of the mean of ten values for the ratio of R.I.P./A.P. taken from 10 separate tubes (1 sample from each tube) was ±20 per cent. The average deviation of the mean of ten samples, all from the same tube, was ±2.5 per cent.

This result simply shows that the inactivation reaction is the most sensitive and irregular step. The 20 per cent variation represents the variation in this reaction, while the 2 per cent represents the variation in the remaining steps of the experiment.

In view of this fact, it is evident that a large number of separate tubes must be set up in order to obtain any accurate result. The contents of the series of tubes is then mixed, before the sample is analyzed. This corresponds to making an "experimental average," and results in the same figure as though each tube were analyzed separately and the results averaged arithmetically. The experimental average method has the advantage of avoiding a large number of separate analyses. It has the disadvantage that the accuracy of the result cannot be determined.

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[7] Luria and Delbrück (1943) have suggested that the occurrence of much greater variation between samples taken from a series of different tubes compared to a series of samples from the same tube is an indication of the occurrence of a mutation. The phenomenon is common to kinetic experiments in general (as Dean and Hinshelwood have remarked, 1952) and to enzyme reactions in particular.

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