STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

IX. Evidence of Hydrolysis of Bacterial Protein during Lysis.

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(Received for publication, May 22, 1928.)

The disappearance of bacteria and the coincident accumulation of an agent capable of bringing about this disappearance in serial subcultures are the characteristics which originally drew the attention of Twort to the phenomenon which later became known under the name of bacteriophagy, or the phenomenon of d'Hérelle. From the outset, Twort saw three distinct possibilities in interpreting his observations: He thought that the disappearance of the bacteria might be due to their passage into a subvisible state as a stage in a peculiar life cycle initiated under the conditions of the experiment. He saw as an alternate possibility that the disappearance of bacteria was due to rapid autolysis of the cells. Finally, he deemed it possible that bacteria underwent disintegration as the result of an acute infectious disease caused by an extraneous, ultramicroscopic virus.

Each one of these tentative explanations found a number of supporters among those subsequently studying the phenomenon. Our investigations, however, have suggested still another explanation of the appearances, namely, that they are due to the rupture of the bacterial cell wall which is unable to withstand the rise in internal pressure caused by the imbibition of water. The fact that the cytoplasm is quickly dissolved in the surrounding medium after the bursting of the bacteria indicates that the intracellular contents have been rendered soluble through some process of digestion prior to the bursting. Granting this to be true, the lysed cultures should show the presence of the products of digestion of the bacterial cytoplasm. Attempts have been made unsuccessfully in the past to find the products of hydrolysis of bacterial protein in lysed cultures. It seems possible that the failure was due to the fact that lysis was

carried out in beef infusion broth—a medium so rich in various protein-split products that a small increase caused by the hydrolysis of bacterial protein might have been masked. In our experiments here to be reported the lysis was made to take place in synthetic media, free from organic nitrogen.

EXPERIMENTAL.

Media.—Since the lysis of bacteria is conditioned by their free multiplication, it was first necessary to find synthetic media in which the bacteria which we intended to use, namely, *B. coli*, *B. pestis caviæ*, and a thermophilic bacillus respectively, would grow freely, and in which typical lysis would take place upon the addition of phage. After several trials we came upon two such media. The first, which represents a slight modification of the synthetic medium of Pozerski, and which was found to be suitable for *B. coli* and *B. pestis caviæ*, is prepared as follows:

Saccharose	20.0 gm.
Magnesium sulfate	1.0 "
Potassium sulfate	1.0 "
Disodium phosphate (anh.)	1.0 "
Ammonium succinate	6.0 "
Distilled water	1000.0 cc.

The pH is adjusted to 7.3 before sterilization and the medium is autoclaved at 15 pounds pressure for 15 minutes. After sterilization the pH varied from 7.0 to 7.1.

The thermophilic bacillus was grown in a medium similar to the one proposed by Frankel, Barber, and Pyle,[1] the composition of which is:

Ferric chloride	0.001 gm.
Magnesium sulfate	0.001 "
Calcium chloride	0.001 "
Dextrose	10.000 "
Disodium phosphate (anh.)	7.000 "
Dipotassium " "	9.000 "
Ammonium acetate	1.400 "
Distilled water	1000.0 cc.

The pH is adjusted to 7.0 with 10 per cent phosphoric acid. Sterilization is by autoclave at 15 pounds pressure for 15 minutes.

Chemical Methods.—After a suitable period of growth, all cultures were subjected to the same treatment. 20 gm. of hydrated barium hydroxide were added to each liter of medium. The volume of the liquid was reduced to 100 cc. by distillation under diminished pressure from a water bath kept at a temperature

[1] Frankel, Barber, and Pyle, *J. Infect. Dis.*, 1921, xxiv, 9.

between 60° and 70°C. At this point the material was tested for ammonia by Nessler's reagent. If ammonia was still present, sufficient 5.0 per cent barium hydroxide solution to give a slight excess of alkali together with 500 cc. of distilled water was added, and the distillation was continued until the Nessler's reagent gave a negative test. The precipitate of barium salts was then removed by filtration, and the precipitate thoroughly washed. The barium remaining in the filtrate and washings was removed by carefully adding dilute sulfuric acid. The reaction was made slightly alkaline to litmus by dilute sodium hydroxide, and again distilled to a volume of 100 cc. The precipitate of barium sulfate was removed by filtration or by centrifuging and then washed. The filtrate and washings were distilled as before and washed into a 50 cc. volumetric flask, to

TABLE I.

	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of $\frac{NH_2}{N_2}$ ratio in the presence of phage
	Control	Phage	Control	Phage	Control	Phage	
Column	1	2	3	4	5	6	7
	gm.	*gm.*	*gm.*	*gm.*			*per cent*
B. coli	0.00248	0.00206	0.000842	0.000842	0.339	0.408	20.3
" "	0.00700	0.00707	0.00155	0.00183	0.221	0.259	17.2
" "	0.00686	0.00700	0.00140	0.00168	0.205	0.241	17.5
" *pestis caviæ*	0.00945	0.00875	0.00325	0.00553	0.344	0.632	83.7
" " "	0.00794	0.00770	0.00232	0.00383	0.293	0.497	69.7
" " "	0.00472	0.00490	0.00155	0.00291	0.329	0.595	80.8
Thermophile	0.00805	0.00770	0.001725	0.001725	0.214	0.224	4.58
"	0.00630	0.00630	0.001390	0.001668	0.221	0.265	20.2
"	0.005425	0.005775	0.000973	0.001390	0.180	0.241	39.6

which sufficient acetic acid had been added to make the solution acid. The total nitrogen in this filtrate was determined on 10 or 20 cc. aliquots by the Kjeldahl method. Amino nitrogen was determined by the method of Van Slyke

General Procedure.

All cultures used in the experiments, as well as the lytic filtrates employed, were carried through a number of passages on appropriate synthetic media previous to their use in the tests described below.

The first series of tests was made with the idea of determining any difference in the free amino acid content between cultures containing phage and those without phage.

TABLE

	18 hr. period. Before addition of phage					
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$	
	Control culture	Culture to which phage is to be added	Control culture	Culture to which phage is to be added	Control culture	Culture to which phage is to be added
Column	1	2	3	4	5	6
	gm.	*gm.*	*gm.*	*gm.*		
B. pestis caviæ	0.002975	0.002975	0.0006356	0.0006356	0.214	0.214
" " "	0.002800	0.002800	0.0005520	0.0005520	0.197	0.197
" *coli*	0.001750	0.001750	0.000349	0.0003490	0.199	0.199
" "	0.002275	0.002275	0.0004147	0.0004147	0.182	0.182

TABLE

	18 hr. period. Before the second addition of bacteria						
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of the $\frac{NH_2}{N_2}$-ratio in the culture with phage $\left(\frac{\text{Column 6}-\text{Column 5}}{\text{Column 5}}\right)$
	Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage	
Column	1	2	3	4	5	6	7
	gm.	*gm.*	*gm.*	*gm.*			*per cent*
Experiment I	0.00350	0.00315	0.000281	0.000280	0.0804	0.0889	10.57
" II	0.00263	0.00245	0.000281	0.000351	0.107	0.1430	33.70

II.

24 hrs. after addition of phage. Total of 42 hrs.								
Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of the $\frac{NH_2}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 12–Column 11}}{\text{Column 11}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the control during second period of incubation $\left(\frac{\text{Column 11–Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 12–Column 6}}{\text{Column 6}}\right)$
Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
7	8	9	10	11	12	13	14	15
gm.	*gm.*	*gm.*	*gm.*			*per cent*	*per cent*	*per cent*
0.004725	0.00560	0.001400	0.00203	0.2963	0.3625	22.3	38.4	69.4
0.005950	0.00577	0.001242	0.001656	0.2087	0.2867	37.3	10.6	45.2
0.005770	0.00245	0.001576	0.000685	0.2729	0.2797	2.4	37.1	40.5
0.005075	0.00245	0.001380	0.000828	0.2719	0.3379	24.3	49.4	85.6

III.

6 hrs. after the second addition of bacteria. (Total period 24 hrs.)								
Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino } N_2}{\text{Total } N_2}$		Increase of the $\frac{NH_2}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 13–Column 12}}{\text{Column 12}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the control during the second period of incubation $\left(\frac{\text{Column 12–Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_2}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 13–Column 6}}{\text{Column 6}}\right)$
Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
8	9	10	11	12	13	14	15	16
gm.	*gm.*	*gm.*	*gm.*			*per cent*	*per cent*	*per cent*
0.00455	0.00420	0.000557	0.000697	0.1224	0.1658	35.4	52.1	86.5
0.00560	0.00612	0.000826	0.001380	0.147	0.202	37.4	37.4	41.2

In each experiment two flasks of 1 liter each, containing precisely the same medium, were used, and each was inoculated with 1 cc. of an 18 hour culture of bacteria grown in the synthetic medium. To one of the flasks was added 0.5 cc. of phage, also grown on synthetic medium—the phage titer varying between 10^{-7} and 10^{-9}. The other flask served as a control.

The cultures of *B. coli* and *B. pestis caviæ* were incubated for 40 to 48 hours at 37°C., while the thermophilic bacillus was grown for 24 hours at 48°C. After the period of growth the cultures were treated as previously described.

Table I gives the results of these experiments. A comparison of Columns 5 and 6 shows that the amount of amino acids in the cultures containing phage is much larger than that in the control cultures.

The direct source of the increase in amino nitrogen in the presence of phage is not immediately apparent. The accumulation of amino nitrogen may be the result of increased hydrolysis of bacterial protein. It is possible, however, that, owing to a marked diminution in the numbers of live bacteria resulting from active lysis, the utilization of amino nitrogen present in the medium as the result of normal autolysis, is much slower in the flask containing phage than in the control. Such a condition would also lead to the accumulation of amino nitrogen in the flask with phage.

To determine the point thus brought up, the following experiment was performed:

Two flasks, each containing 2 liters of medium, were inoculated with 2 cc. of an 18 hour culture of bacteria and incubated for 18 hours. At this time 1 liter of the culture was removed from each flask for analysis (Table II, Columns 1 to 6), 1 liter of culture being retained in each flask. To one of the flasks 10 cc. of purified (amino nitrogen-free) bacteriophage were added; the other flask received 10 cc. of the same phage inactivated by heat and served as a control. After a further period of 24 hours of incubation at 37°C., the contents of both flasks were analyzed (Table II, Columns 7 to 12).

From a comparison of the data recorded in Table II (Columns 5 and 11), it appears that in the absence of bacteriophage, the concentration of amino nitrogen continues to increase on prolonged incubation of cultures. Thus (Column 14), if the effect of phage was merely to bring about destruction of bacteria, the result would have been to lower the rate of accumulation of amino nitrogen. The actual observation (see Table I, Columns 5 and 6, and Table II, Columns

11 and 12) shows, however, that in the presence of active lysis, amino nitrogen accumulates in the culture containing the phage considerably faster than in the control culture (compare Columns 14 and 5). These findings indicate that phage increases the rate of hydrolysis of bacterial protein.

It was thought that this point could be further elucidated by adding quantities of young, living bacteria to test cultures, and determining the effect upon the amino nitrogen content.

2 cc. of an 18 hour culture of *B. pestis caviæ* were added to each of two flasks containing 2 liters of the synthetic medium. In addition, 0.5 cc. phage (titer 10^{-8}) was added to one of them. After incubation for 18 hours, 1 liter was taken from each and analyzed as before (Table III, Columns 1 to 6). Equal amounts of a heavy suspension of young bacteria in synthetic medium, obtained by centrifuging the organisms from 2 liters of an 18 hour culture, were added to the remaining portions of the test cultures. 6 hours later these cultures were analyzed (Table III, Columns 8 to 13).

By comparing Columns 14 and 7, it is readily seen that after the addition of large numbers of living bacteria, the free amino acid content increases, and the increase is greater in those cultures containing phage. These results strengthen the conclusion drawn from preceding tests, namely, that lysis of bacteria by phage is accompanied by hydrolysis of bacterial protein.

CONCLUSIONS.

1. During the process of lysis by bacteriophage, there is an appreciable increase in the amount of free amino acid present in the culture.

2. The increase of free amino acid is due to hydrolysis of bacterial protein.