PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

VII. PARTIAL PURIFICATION OF THE PROTEIN FACTOR NECESSARY FOR VIRUS SYNTHESIS

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It has been previously reported from this laboratory that a substance1 present in certain acid-hydrolyzed proteins was necessary for S. muscae cells to liberate phage in Fildes' synthetic medium (1). This substance had no effect on the multiplication rate when added to uninfected S. muscae cells in Fildes' medium.

In this paper, a partial purification of this substance will be reported. An increase of about 500-fold has been obtained in the specific activity.

A method of assay for the AHPF will also be described.

EXPERIMENTAL RESULTS

The Effect of the AHPF on the Phage Yield

Table I illustrates that the phage liberated per cell is dependent on the concentration of AHPF present within certain limits. This fact makes it possible to assay various fractions for the AHPF.

Partial Purification of the AHPF

As a source of AHPF, 1:10,000 pepsin (Cudahy) was chosen. Step 1—1000 gm. of pepsin in 5 liters of water was adjusted to pH 2.0 with 5 N HCl and incubated 18 hours at 65°C. This solution was now practically protein-free. Step 2—The autolyzed pepsin was adjusted to pH 8.2 with 5 N NaOH and 15,000 ml. of 15 per cent basis lead acetate was slowly added to the solution with constant stirring. The mixture was put at 5°C. overnight. Step 3—The precipitate was filtered off with suction and the filtrate concentrated to 3000 ml. in vacuum. The concentrated filtrate was treated with H₂S until free of lead and then aerated until free of H₂S. Step 4—To the lead-free filtrate was added 6000 ml. of 10 per cent sodium carbonate and 6000 ml. of 25 per cent mercuric acetate and 40,000 ml. of 95 per cent alcohol. This mixture was stored overnight at 5°C. Step 5—The precipitate was filtered off with suction, and the alcohol removed from the filtrate under vacuum. The alcohol-free solution was treated with H₂S until free of mercury, and then concentrated to 5000 ml. and put at 5°C. for 2

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1 This factor will be called AHPF throughout the three papers in this journal.

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days. **Step 6**—The precipitate was filtered off and the filtrate concentrated to 1500 ml. and stored at 5°C. for 2 days. The precipitate which formed was filtered off with suction. **Step 7**—The filtrate was concentrated to a syrup and extracted for 1 hour with 10 volumes of butyl alcohol at 80°C. This mixture was filtered with suction while hot, and the residue reextracted as above. The two butyl alcohol extractions were combined. **Step 8**—The butyl alcohol solution was shaken with 2000 ml. of water for 1 hour. The activity passed into the aqueous phase. **Step 9**—The aqueous solution was treated with norit for 1 hour, using 10 gm. of the charcoal for every 100 ml. of solution. This adsorption process was repeated once more. The material not adsorbed contained the purified AHPF.

From the original starting material, 300 γ of nitrogen per ml. was required to give 50 per cent stimulation, whereas only about 0.5 γ N per ml. of the purified material was needed to give the same stimulation of virus synthesis. The purified factor was destroyed by ashing.

**Further Attempts at Purification of the AHPF.**—Many attempts were made at further purifying the AHPF. The following reagents were used in order to precipitate the AHPF: Reinecke salt, phosphotungstic acid, phosphomolybdic acid, flavianic acid, and picric acid. None of these substances markedly increased the specific activity of the purified AHPF. Precipitation with calcium and alcohol and silver also yielded negative results. Adsorption on resins, permutit, calcium hydroxide, and fuller’s earth at various pH’s was also unsuccessful.

**Attempt to Substitute Known Compounds for the AHPF.**—Many substances have been tried as a substitute for the AHPF in this system. These include, besides the 16 amino acids and vitamins in the medium itself, cysteine, serine, isoleucine, norleucine, glutathione, threonine, all the B vitamins, the various purines, pyrimidines, nucleosides, and nucleotides of ribonucleic and thymonucleic acid, choline, strepogenin, acid digests of 21 amino acids listed above, vitamin C, oleic acid, inositol, β-aminobenzoic acid, β-alanine, and cozymase.

No substance has as yet been found which can replace the AHPF in this system. The effect of the AHPF on the host-virus relationship is therefore very specific.

**Experimental Methods**

**Preparation and Determination of Bacteria and Virus.**—Bacteria and phage were prepared, determined, and grown as described in the following paper (2).

**AHPF Unit.**—One unit of AHPF activity is defined as that quantity which will increase the virus yield to approximately 4 particles per cell when added to 2 × 10⁸ cells. This is half the maximum yield in the presence of excess AHPF.

**Assay for AHPF.**—The following outline gives the method used in assaying various fractions for the AHPF. All assays were carried out with strain I (2). All assay tubes contained 0.03 ml. of 0.1 m CaCl₂ per 10.0 ml. of medium.

10:30 a.m. 50 ml. of Fildes’ synthetic medium (3) was inoculated with 1.5 × 10⁸
cells per ml. prepared as described in Methods. Five samples containing 10.0 ml. each were pipetted into 2.0 X 15 cm. test tubes and shaken at 37°C.

2:10 p.m. The cell count had risen to approximately 2.0 X 10⁶ cells per ml. Tube 1 received 0.1 ml. of H₂O and the other 4 tubes received varying amounts of the AHFP in a volume of 0.1 ml.

2:30 p.m. 0.1 ml. of virus solution was added to each tube to give 2.0 X 10⁵ particles per ml. The tubes were put back into the shaker.

2:36 p.m. Suspension centrifuged 5 minutes at 1100 × g and the precipitates washed with 10.0 ml. of warmed Fildes' synthetic medium and recentrifuged.

2:50 p.m. Washed cells were suspended in 10.0 ml. of Fildes' synthetic medium and then diluted about 10⁻³ in the appropriate synthetic medium, which contained the same amount of AHFP as the original parent tube. The tubes containing the last dilution of the washed suspensions were assayed for virus and put back in the shaker.

### TABLE I

The Effect of Varying Concentrations of the AHFP on Virus Synthesis

<table>
<thead>
<tr>
<th>Tube</th>
<th>N crude AHFP</th>
<th>Average burst size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>600</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>9</td>
</tr>
</tbody>
</table>

These tubes were again assayed for phage 10, 15, 20, 30, 40, 50, 60, and 70 minutes later. The results of such an experiment on the phage yield are shown in Table I. The average burst size of each infected sample as shown in Table I is obtained from the maximum increase in plaque count during the one-step growth curve (cf. Fig. 1) (4) as described below.

In doing one-step growth experiments in the above manner in the presence of the AHFP or when cells were multiply infected, all the phage remained in the supernatant fluid after centrifuging at the end of the one-step experiment. In calculating the yield of phage liberated per cell (average burst size) under these conditions the following formula was used:

\[
\frac{FP}{Bi}
\]

One-step growth curves were carried out on the washed infected suspensions rather than the usual method because the adsorption in some instances was very low, the phage yield small, and no phage antiserum was available.
FP is equal to the maximum plaque count at the end of a one-step growth curve, and Bi is equal to the initial plaque count of the infected suspension, determined by plating the suspension immediately after centrifuging.

When cells are singly infected in the absence of the AHFP in Fildes' medium, the plaque count remains fairly constant (cf. Fig. 1). Under these conditions the phage remains in the cell. No matter at what time the sample is centrifuged, there is no phage found in the supernatant fluid, since the virus is liberated from these cells only on the tryptose phosphate assay plate (2). Therefore, virus yields from cells singly infected in Fildes' medium in the absence of the AHFP, where the titer remained constant, were calculated by the formula

\[ \frac{FP - Bi}{Bi} \]

**SUMMARY**

1. A substance is present in autolyzed pepsin solutions which stimulates the release of phage by some strains of *S. muscae* when added to Fildes' synthetic medium.

2. The substance is assayed by determining the quantity necessary to increase the phage yield to one-half the maximum value, using the one-step growth curve technique.

3. The substance has been concentrated and partially purified (500-fold) by heavy metal precipitation, butyl alcohol extraction, and absorption on norit.

4. No known amino acid or accessory growth substance tested could replace this substance.
The writer wishes to thank Mr. M. Litovchick for excellent technical assistance.

*Note Added in Proof.*—The following substances have recently been tried as substitutes for the AHPF: sodium acetate, protogen, ascorbic acid, Tween 80, oleic acid, indoleacetic acid, and the acetate factor of Snell. None of these substances could replace the AHPF. The protogen was kindly supplied by Dr. E. L. R. Stokstad, and the acetate factor by Dr. L. J. Reed.

**BIBLIOGRAPHY**