Agar Layer Method for Production of High Titer Phage Stocks.* (19076)

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Bacteriophage stocks prepared by the lysis of fluid bacterial cultures usually average 10^{10} infectious particles per ml and rarely exceeded 10^{11}/ml. A method of achieving higher virus concentrations than this is desirable. Luria(1) suggested a modification of a method originally used by Hershey(2) which under proper conditions gives a virus stock about 10 times as concentrated as those obtainable in fluid cultures. This method has been briefly described(3) and has been used successfully by Cohen(4) and by Marshak(5) for the preparation of purified phage suspensions. The purpose of the present paper is to describe the method in general terms and to point out those significant variables which should be investigated and controlled by anyone wishing to use the method with a new phage-host cell system.

Materials and methods. In essence the method consists in the lysis of a dense suspension of the host bacterium by the phage in a thin agar layer which has been poured over a much thicker base layer of nutrient agar. When bacterial lysis is complete the upper agar layer is scraped from the plates and the phage is extracted with minimum amounts of broth. A sample experiment using coli phage T2r+ will illustrate the method. The basal agar contains 0.8% Difco nutrient broth, 0.5% NaCl and 1.5% agar. About 40 ml of melted agar is poured into each petri dish and allowed to harden with the dishes resting on a leveled sheet of plate glass. The soft agar for the agar layer differs only in the agar content which is 0.7%. The melted soft agar is distributed in 2.5 ml amounts in test tubes and cooled to 46°C. The bacterial inoculum is prepared by washing the overnight growth from an agar slant with 2 ml of broth giving a concentration of about 5 x 10^9 bacteria/ml. Each tube of agar is inoculated with 5 x 10^8 bacteria and 7 x 10^5 T2r+ phage particles, and then is poured over the base agar in a petri dish resting on leveled plate glass. After the agar layer has hardened the plates are incubated at 37°C overnight, and on examination the bacteria are found to be completely lysed. Five ml of broth are added to each plate and the agar layer is scraped off with a sterile glass rod into a beaker. The broth and agar from 10 plates are pooled, transferred by pipette to centrifuge tubes and shaken vigorously for a few minutes to break up the agar and extract the phage. The agar is sedimented in a horizontal centrifuge at 2000 RPM and the slightly opalescent supernatant fluid decanted and assayed for virus content. The yield in this experiment was 36 ml assaying 10^{11} infectious particles per ml. For comparison Hook *et al.*(6) prepared T2r+ phage on a large scale in fluid media, obtaining from 5 x 10^9 to 2 x 10^{10} infectious particles/ml. The yield per ml of medium used is about the same in the two methods but the concentration achieved is from 5 to 20 times greater in the agar layer method.

Typical yields obtained by this method for the various coli phages of the T series are given in Table I, together with the phage inoculum used per plate. In each case about 40 ml of virus stock were recovered from the

* Aided by a grant from the National Foundation for Infantile Paralysis. This paper is the essence of a thesis submitted by M. Swanstrom in partial fulfillment of the requirements for the degree of M.S. in the Graduate School of Arts and Sciences of New York University.

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TABLE I. Yields of Phage Obtained by Extraction of Soft Agar Layers of Lysed Plates. Pooled agar layers from 10 plates were shaken with 50 ml of broth and centrifuged to remove agar. The average recovery was 40 ml.

Phage used	Inoculum per plate	Phage assay per ml	Yield per plate
T1	4×10^3	7×10^{11}	2.8×10^{12}
T2r+	7×10^5	1.1×10^{11}	4.5×10^{11}
T3	2×10^3	1.5×10^{11}	6×10^{11}
T4r	2×10^5	1.4×10^{11}	5×10^{11}
T5	9×10^5	1×10^{12}	4×10^{12}
T6r+	4×10^5	4.7×10^{11}	1.8×10^{12}
T7	4×10^3	1×10^{11}	4×10^{11}

pooled extracts of 10 plates. The yields with phage T1 and T5 have been consistently greater than with other viruses. The T4r strain used in these experiments was a variant of T4 obtained from A. H. Doermann. In earlier experiments yields with this strain were very poor being of the order of 3×10^{10}/ml, but even this was much better than the yields in broth lysates, about 4×10^9/ml. Therefore, this strain of T4 phage was used in a systematic study of those variables which might affect the concentration and yield of phage obtainable by this method. It should be noted that different strains of the same phage type may differ considerably in the yields obtained.

Experimental. Since a limited number of virus particles are liberated from each infected bacterium (burst size), it is evident that the yield obtainable from each inoculated petri dish is dependent on the number of bacteria which can be produced before all are lysed by the virus, while the concentration of virus obtainable is dependent on the volume in which the bacterial population can be induced to grow. The nutritional state of the host bacterium is another factor affecting the yield of virus. Bacterial overpopulation results in reduced virus yield per infected cell. Bacterial starvation ultimately results in failure of lysis which is the principal reason why single plaques do not increase indefinitely in size. Another factor limiting the virus yield is the readsorption of excessive amounts of virus to unlysed bacteria. All such particles adsorbed above the one needed to infect the bacterium are lost and contribute nothing to the final yield(7). This loss by readsorption increases in proportion to both bacterial and virus concentrations. From these observations one might anticipate that the optimal phage inoculum would consist of barely enough virus particles to give confluent plaques when the plate has been incubated long enough to give maximum plaque size. This would permit maximum growth of the bacterial population before all were overwhelmed by the virus. With virus T4r confluent lysis is barely attained with 2×10^4 particles per plate. The effect of varying phage inocula on the phage yield was determined while keeping all other experimental conditions constant. Over the range of inocula from 3×10^4 to 3×10^5 phage particles per plate the phage yield remained constant within a factor of 2 averaging 4×10^{10} particles per plate. With inocula below 2×10^4 per plate bacterial lysis was incomplete and the phage yield was markedly decreased. With inocula above 6×10^5 per plate the phage yield decreased presumably because all bacteria were lysed before the optimum bacterial population was reached. Needless to say, the optimal phage inoculum is different with different phage strains, being only 4×10^3 per plate for a large plaque phage such as T1, and should be determined for each new phage-host cell combination.

The size of the bacterial inoculum also affects the phage yield, the yield increasing with the inoculum to an optimum at about 2×10^9 cells per plate. The physiological state of the bacterial inoculum seems to have little influence on the phage yield, since the yields obtained using bacteria washed from an agar slant after overnight incubation did not differ from those obtained using broth grown bacteria in the logarithmic growth phase, providing the inocula contained equal numbers of cells. However, the yields obtained by using slants stored in the refrigerator for some days were decreased.

Variation in the incubation period of the inoculated plates also affected the phage yield, the optimum being about 10 hours at 37°C. Plates harvested after 4 hours' incubation contained large numbers of unlysed bacteria with a consequent reduced phage

7. Delbruck, M., *J. Gen. Physiol.*, 1940, v23, 643.

yield. The yield was reduced to ¼ the maximum in plates harvested after 24 hours of incubation and further reduced to 1/10 the maximum after 48 hours. This loss is probably due mainly to diffusion of phage into the basal agar layer from which it is not recovered, although there may be some inactivation of the phage on prolonged incubation. The best time to harvest the plates is immediately after all host bacteria are lysed as judged by complete clearing of the plates.

Variation in the volume of base agar used per plate did not have an appreciable effect on phage yield over a range from 25 to 40 ml per plate, but a further reduction to 15 ml per plate did result in a decrease in yield. Omission of the base agar resulted in a virus yield no better than that obtained in fluid media. The layer of base agar serves as a source of nutrient for the dense population of bacteria growing in the soft agar layer.

It was thought that an increase in volume of soft agar in the agar layer might produce a larger yield per plate and thus make for a more efficient utilization of material. Consequently 2 sets of 5 plates were inoculated using 2.5 ml and 5 ml of soft agar per plate. The inocula for the first set were 1.1 x 10^5 T4r and 2 x 10^8 B per plate and for the second set twice these amounts. After incubation the agar layers were washed off using 5 ml of broth per plate for the first set and 10 ml for the second set. The recovered yields were 16 ml at 6 x 10^{10}/ml or 9.6 x 10^{11} total for the first set and 36 ml of 2 x 10^{10}/ml or 7.2 x 10^{11} total for the second set. Hence doubling the agar layer resulted not only in a decrease in the virus concentration but in a decrease in total yield per plate as well. This may have been due to a poorer nutritional state of the bacteria in the deeper soft agar layer.

The volume of broth used in extraction of phage from the agar layer is an obviously important factor in determining both the concentration and yield of phage obtainable. A number of plates were inoculated with 9 x 10^4 T4r and 1.5 x 10^9 B in 2.5 ml of soft agar. After overnight incubation groups of 3 plates were washed with varying amounts of broth and the pooled washings were assayed for virus after removal of the agar by centrifugation with the results seen in Table II.

TABLE II. Yield and Concentration of Recovered Virus as a Function of Volume of Broth Used for Washing Each Plate. Yields are based on pooled washings from 3 plates.

Broth vol, ml/plate	Phage yield per ml	Broth recovered, ml	Phage recovered, total
2.5	1.3×10^{11}	7	9.1×10^{11}
5	9.6×10^{10}	15	1.4×10^{12}
10	4.6×10^{10}	29	1.3×10^{12}

Increasing the volume of broth from 5 to 10 ml per plate reduced the concentration of phage obtained but did not affect the total yield. Reducing the volume to 2.5 ml increased the concentration of phage obtained in the extract but decreased the yield of phage since a larger proportion of the total phage is lost in the sedimented agar. It would appear from this experiment that the maximum concentration of phage obtainable in the broth extract is limited by the phage concentration in the agar layer itself, which in this case appears to lie between 2 and 3 x 10^{11}/ml. The true phage concentration may be higher than this if there is any tendency for the phage to adsorb preferentially to the agar. The maximum recoverable phage was determined by repeatedly extracting the pooled agar layers from 5 plates using 25 ml of broth each time. The titers obtained in 7 successive extractions were 9.1 x 10^{10}, 2.8 x 10^{10}, 1.2 x 10^{10}, 6 x 10^9, 4.1 x 10^9, 2.6 x 10^9 and 1.8 x 10^9/ml. The total phage recovered thus amounts to 2.9 x 10^{11} particles per ml of agar layer, most of which was in the first two washings.

As a result of these studies it was possible to increase the concentration of phage T4r in plate washings from about 3 x 10^{10}/ml to 10^{11}/ml. However, this is still far less than the 10^{12}/ml readily obtainable with phage T5. This discrepancy in phage yields may in part be explained by the difference in burst sizes, that of T5 being 400 while that of T4r is only 125. If equal numbers of bacteria undergo lysis in the two cases and other factors are equivalent, the yield of T4r should be 1/3 that of T5 rather than 1/10.

Another factor which may affect the virus yield is the rate of adsorption of virus to host cell. Delbruck(7) concluded that the yield of virus particles in broth cultures would be increased by a decrease in adsorption rate for two reasons: the loss of liberated virus by multiple adsorption onto unlysed bacteria would be lessened, and the bacterial population would reach a higher total figure because delayed adsorption would give more time for bacterial growth. Since the adsorption rate of T4 is about 20 times that of T5, this difference may well contribute to the better yields obtainable with phage T5. The rate of adsorption of T4 is in part determined by the salt concentration in the medium(8). An attempt to further increase the yield of phage T4 by reducing the salt concentration to decrease the adsorption rate did not result in a significant gain in phage production.

Summary. Some of the variables involved in the production of high titer phage stocks by the agar layer method have been investigated using coli phage T4r as an example. The significant factors have been shown to be the number of virus particles and the number of bacteria inoculated per plate, the length of incubation, the volume of soft agar in the agar layer and the volume of broth used for extraction of the virus from the agar. By an appropriate adjustment of these variable factors it is possible to obtain stocks of the various coli phages of the T series ranging from 10^{11} to 10^{12} infectious particles/ml.

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Received April 27, 1951. P.S.E.B.M., 1951, v78.

Therapeutic Activity of Xanthopterin Added to p-Aminobenzoylglutamic Acid in Experimental Macrocytic Anemia of Rat.* (19077)

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We have reported(1) the results of experiments on rats in which macrocytic anemia was induced by a folic acid-deficient diet containing sulfaguanidine as an inhibitor of the intestinal flora. These animals were injected with the following substances separately: Folic acid, vitamin B_{12}, xanthopterin, cobalt ions and a mixture of xanthopterin and p-aminobenzoylglutamic acid. It was found that simultaneous administration of the last 2 drugs did not prevent the death of rats showing a severe vitamin deficiency. However, rats receiving streptomycin, instead of sulfaguanidine, and given the xanthopterin-paraminobenzoylglutamic acid mixture, showed a clear improvement of the blood picture.

In the present experiments we attempted to obtain a chronic folic acid deficiency in the rat by adding streptomycin, as an inhibitor of intestinal flora, to the usual synthetic diets. In addition the activity of xanthopterin, of p-aminobenzoylglutamic acid, and of both substances administered together was tested.

Experimental procedure. A total of 50 male albino rats, from this Institute strain, weighing 40 g each, were used. The animals were fed *ad libitum* with a standard folic acid-deficient diet(2). One percent of streptomycin was added to the diet as inhibitor of intestinal flora, instead of sulfasuxidine. The symptoms of deficiency were studied according to the technic previously described(1).

* We are indebted to Lepetit Laboratories-Milano for supplying p-aminobenzoylglutamic acid, and to F. Hoffmann La Roche-Basel for xanthopterin used in these studies.

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