APPLIED MICROBIOLOGY, Nov., 1966
Vol. 14, No. 6

Printed in U.S.A.

# Virustat, a Device for Continuous Production of Viruses

HOMER JACOBSON AND LESLIE S. JACOBSON[1]

*Department of Chemistry, Brooklyn College of the City University of New York, Brooklyn, New York, and Division of Biology, California Institute of Technology, Pasadena, California*

Received for publication 31 May 1966

ABSTRACT

JACOBSON, HOMER (Brooklyn College of the City University of New York, Brooklyn, N.Y.), AND LESLIE S. JACOBSON. Virustat, a device for continuous production of viruses. Appl. Microbiol. **14**:940–952. 1966.—Methods for continuous production of viruses, and operation of the virustat, an apparatus in which such production was accomplished, were studied. Continuous production requires a separate continuous host growth chamber, such as the chemostat, and a multiunit virus growth chamber into which the virus-inoculated host cells are led. Successful continuous output of MS-2 and $\phi$X174 viruses, the latter in lysates, over periods of several days and at titers approximating those of batch lysates, was observed. Design problems include chamber sizes and flow rates, growth of resistant mutants within both virus and host growth chambers, clogging by lysis debris, and the phenomenon of self-inoculation. The latter represents virus growth in the first section of the chamber in excess of the washout rate, leading to lack of need for virus inoculation after an initial period. Use of the virustat for production and research purposes will require some attention to the formation of resistant bacterial colonies at pockets and surface sites of limited washout. With the virustat as a continuous virus production device, continuous purification methods are desirable. Research use of the virustat in continuous mutagenic population studies would require suppression of self-inoculation by use of many sections in the chamber, and improved servo control of host populations at low concentrations.

This paper presents methods for continuous production of viruses, particularly bacteriophages, and the first results with the virustat, an apparatus for accomplishing this end. Virus culture methods published prior to this report have been batch methods, in which a host organism or tissue, or a batch of host cells (if they are culturable in single-cell suspension), is treated with a small inoculum of virus particles, and a larger quantity of virus is harvested after some required time in a batch of product. Using appropriate hosts and media, we have constructed and operated a relatively simple device giving continuous output of bacterial viruses $\phi$X174 and MS-2. Suitable for production of any virus whose host cells can be cultured in infectible suspension, we have named it "virustat" in analogy with the bacteria-producing chemostat of Szilard and Novick (10). Over run periods of 1 to 6 days, the apparatus continuously produced virus lysates at flow rates of 20 to 170 ml/hr, and at titers ranging nearly to those given by optimal batch cultures.

*General design considerations.* Continuous production of virus requires (i) a continuous supply of host cells from a host cell growth tube (the method is inapplicable to cultures which require host tissues or whole multicellular organisms), (ii) a virus growth chamber in which host cells and virus inoculum are continuously mixed and kept together long enough to produce a continuous supply of new virus, and (iii) a continuous inoculum, either from an available virus supply or by return of a fraction of the production (under certain conditions given below, this may be omitted). Bacterial viruses are especially convenient for continuous culture, as the host cells are easily grown in continuous pure culture by several methods (3, 7, 10).

Plant and animal viruses present greater problems, in that they require single-cell or suspended cell-clump cultures; however, with methods under current study (2, 6), these problems should be-

[1] Present address: Department of Biology, Long Island University, Brooklyn, N.Y.

come amenable to the methods described here. The simple method of Monod (7), comprising continuous medium flow and simultaneous withdrawal of the well-mixed cell culture at the same rate, produces a continuous supply of host cells. The equilibrium concentration reached depends upon the flow rate. As medium flow rate increases from zero up to a critical rate, the concentration decreases from near-saturation to the concentration at which the exponential growth rate is maximal, i.e., the shoulder of the growth curve. Above the critical flow rate, the medium washes out the bacteria or other cells faster than they reproduce, and they disappear, except for surface growth and growth in unmixed pockets. Virus cultures with bacteriophage are generally carried out at concentrations of host bacteria near the shoulder of growth curves for maximal virus production, and the virustat was designed to operate in this range.

Due to the need for turbulence for aeration and suspension of particles, a simple tube-type virus growth chamber with host and inoculum coming in at one end and production coming out at the other will not work well. Nor will a single well-mixed and aerated chamber, in which new cells and virus are being continuously added with continuous product removal. Such a chamber mixes the entire contents, and would have an excessively wide distribution of "ages" of infected cells; the production would be heavily contaminated with both uninfected and unlysed cells, and would also retain considerable virus (titers of which are frequently labile) in a warm and heavily absorbing culture long after it was produced. The method used practically must provide aeration of the stream of infected material along a path which does not excessively mix cells of different ages.

The continuous supply of bacteria, together with enough virus particles to inoculate them, must be held together in some kind of chamber long enough for the cells to lyse and then run off as production. The virustat uses a virus growth chamber containing many Cells in series, with separate volumes for individual mixing and aeration. (We capitalize "Cell" when referring to the units of the virus growth chamber, so as not to confuse the term with the biological one.) Mixing is rapid in each Cell, aeration is present in each, and the infected cells pass through each Cell in turn. This system causes a peaking of the distribution of the "ages" of the cells in the production, as described in detail below, the distribution becoming more peaked as the number of Cells is increased. Sufficient Cells allow uniformity of ages to approach that attained in a batch of virus growth.

## MATERIALS AND METHODS

Cultures of two bacteriophages, $\phi$X174 and MS-2, and their *Escherichia coli* hosts, strains C and K-12 3000 Hfr, respectively, kindly supplied by R. L. Sinsheimer's laboratory, were used in the experiments. Batch cultures of $\phi$X174 gave us 45-min lysates with titers of up to $4 \times 10^{11}$/ml; cultures of MS-2 yielded up to $4 \times 10^{12}$/ml, but required 3 to 5 hr for often somewhat cloudy lysates. Media used for growth were Fraser's glycerol-Casamino Acids and Zinder's tryptone-yeast extract, respectively, as described by Sinsheimer (9) and Davis and Sinsheimer (1). Plating methods used were also as there described. All growth was carried out in a room held at $37 \pm 0.3$ C. Medium, held at room temperature in an adjoining room, was pumped into the room and into the bacterial growth tube at the desired rate with a Bowman (peristaltic) infusion pump (Process and Instruments, Brooklyn, N. Y.). When the tube had filled to the desired level, it was inoculated with a few per cent by volume of fresh overnight culture of host cells, aerated, and allowed to reach upper log phase ($2 \times 10^8$ to $5 \times 10^8$/ml). Inflow of medium, usually interrupted during growth of host cells, was then resumed; inflow was balanced by gravity-controlled outflow into the virus growth chamber so as to preserve the level of volume in the bacterial growth tube. With both hosts and the media used, setting the medium input (and cell culture output) flow rates at from 0.5 to 0.8/hr times the volume in the bacterial growth tube (i.e., 2 to 1.25 hr average turnover time) resulted in maintaining the bacterial count in the $2 \times 10^8$ to $5 \times 10^8$/ml region. In practice, since the desired flow rate was set first from consideration of desired average time in the virus growth chamber, this means choosing a volume of culture in the growth tube to give the proper steady-state at the selected flow rate.

Virus inoculum, held in a refrigerator, was then pumped into the effluent stream of host bacteria before it entered the first Cell. Valve-type pumps proved unsatisfactory owing to debris clogging. The same peristaltic pump used for the medium, with flow reduced by the use of narrow-bore tubing, carried the virus inoculum into the stream with a flow rate set to give the approximate multiplicity of infection desired, at the existent bacterial and inoculum virus titers. Further movement of culture was accomplished by gravity flow through the Cells of the virus growth chamber in order, and thence back into the collecting vessel in the refrigerator.

Figure 1 shows the arrangement of the entire apparatus schematically. The dotted boxes enclose the refrigerated area (RA) and the room temperature area (RTA). Shown in these areas are the medium reservoir bottle (MB), peristaltic pump (PP), inoculum reservoir bottle (IB), and production reservoir bottle (PB). The remainder of the apparatus depicted, within the warm room, includes the bacterial growth tube (Y), backflow traps (B), blowover bottle (BB), air intake (AI) and output (AO) manifolds, three Cells (first and last are labeled 1 and 10, with a schematic hiatus after the second), and tees for host-cell inoculation (I) and for sampling (T). Arrows indicate path of air, and of medium (M), host cells (H), virus inoculum

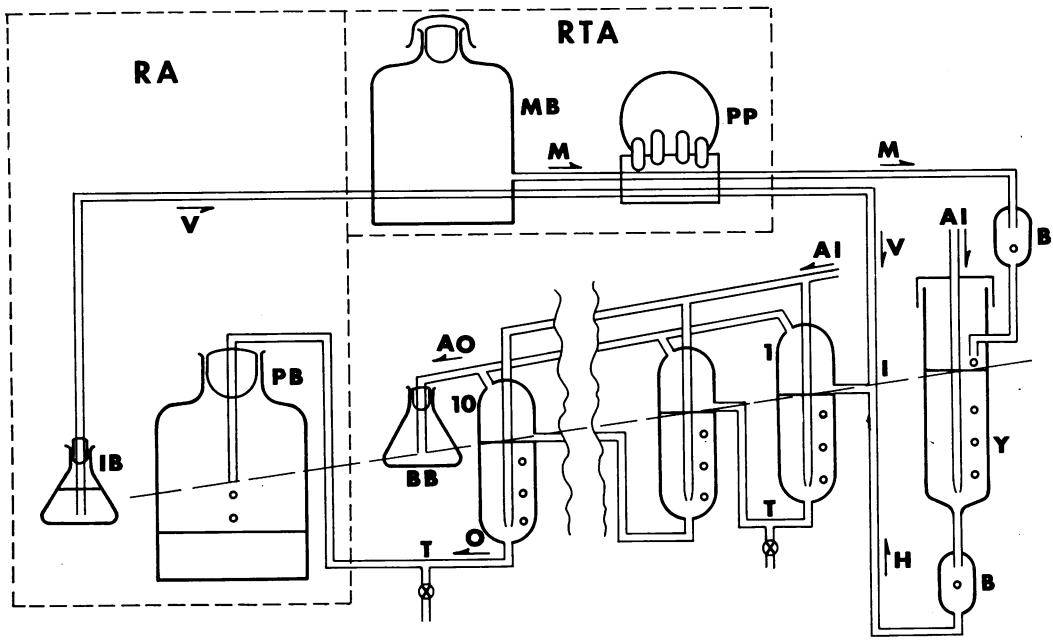


FIG. 1. *Schematic diagram of virustat.*

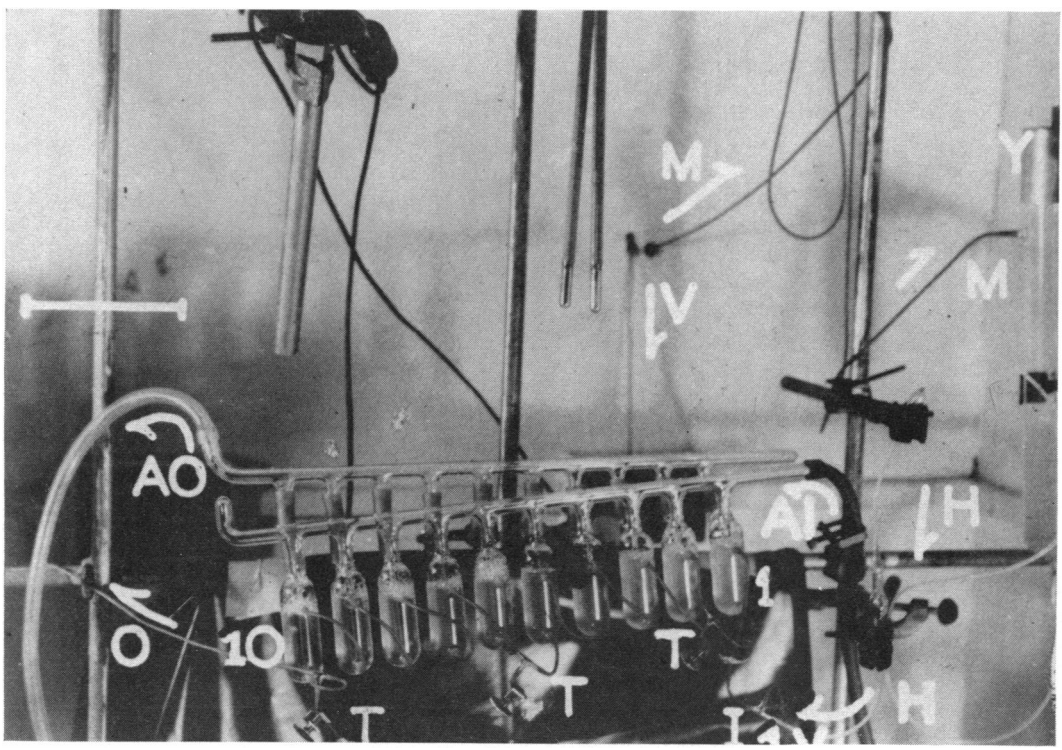


FIG. 2. *Virustat in operation during run φ3. Symbols as in text.*

(V), and production output (O). The long dashed line indicates the gradual liquid level drop, somewhat exaggerated, driving the gravity flow from bacterial growth tube to production reservoir bottle inlet.

Figures 2–5 show the actual apparatus under run conditions. Figure 2 shows a run with a ten-Cell chamber; note the striking change in culture turbidity from Cell 1 (right) to Cell 10 (left). It is labeled with symbols identical to those of Fig. 1.

Figure 3 shows the overall setup during a run with a 20-Cell chamber. The flask at the far left is a blow-over vessel connected to the air outlet to catch any foaming-over culture. Backflow traps, a necessary precaution to avoid contamination of medium and bacterial growth tube, are visible in Fig. 2 and 3; the Fenwal Thermoswitch controlling room temperature is likewise prominent. Figures 4 and 5 show the details of the virus growth chamber. The section comprising Cells 1 to 10 (seen right-to-left) is shown in Fig. 4; Cells 11 to 20 are shown in Fig. 5. Increased foaminess and some clearing is visible in Cells 11 to 20, although not as dramatically as in Fig. 2. The scale in Fig. 3–5 can be estimated from the approximately 3-cm-wide pinch clamps, and in Fig. 2 from the 10-cm scale line.

Normal volume complement of each Cell in the earlier runs (Fig. 2) was 10 ml; in the 20 and 25 Cell setups (Fig. 3–5), 5 to 6 ml. A 20-Cell chamber would have a holdup total of about 120 ml; therefore, a total average duration of 1 hr in the chamber from inoculation to collection would require a flow rate of 120 ml/hr. Flow rates varied between 20 and 170 ml/hr, in experiments performed. Samples for bacterial and virus counts were taken, as required, from the sampling tees and from the side arm in the bacterial growth tube; they were diluted in cold broth and titered within 12 hr. Resistant mutants were measured in virus-containing production samples by direct normal plating of sequential dilutions, and in bacterial growth tube samples containing no virus, by adding approximately $10^7$ phage particles from lysates filtered through a type HA membrane filter (Millipore Filter Corp., Bedford, Mass.) with each 0.1-ml sample of bacterial dilution plated.

## RESULTS

Production of host cells continuously at the desired concentration was found possible in preliminary runs, by use of Monod's method (7), and the hosts and media specified above. When small batches of the continuously produced cells were inoculated with virus, even after several days of running in a bacterial growth chamber, they were found to give lysates.

Results of the first set of 13 significant virustat runs are summarized in Table 1, which gives the number of Cells, culture flow rate, size of growth

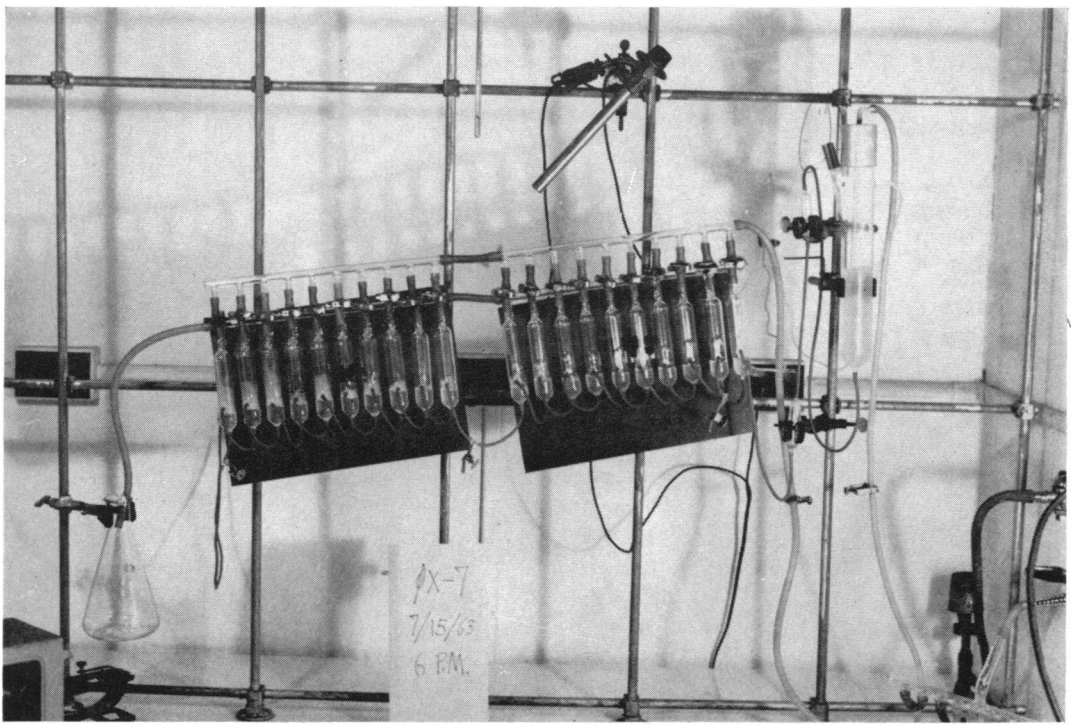


FIG. 3. *Virustat in operation during run φ7. Complete system at 37 C.*

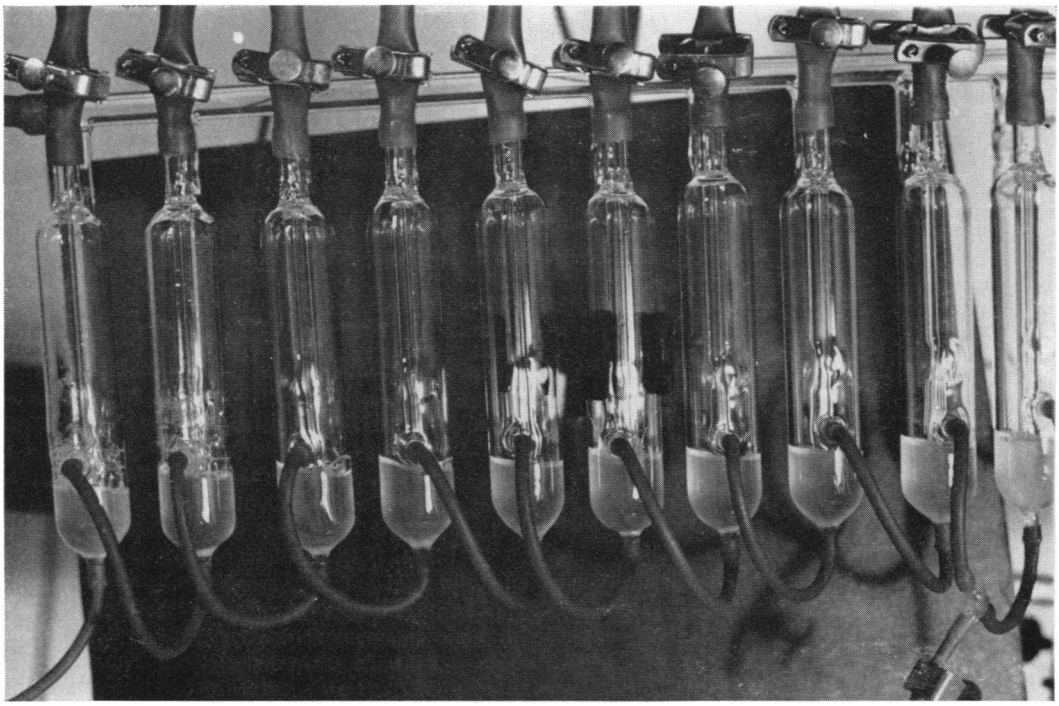

FIG. 4. *Virustat in operation during run φ7. First ten tubes of chamber.*

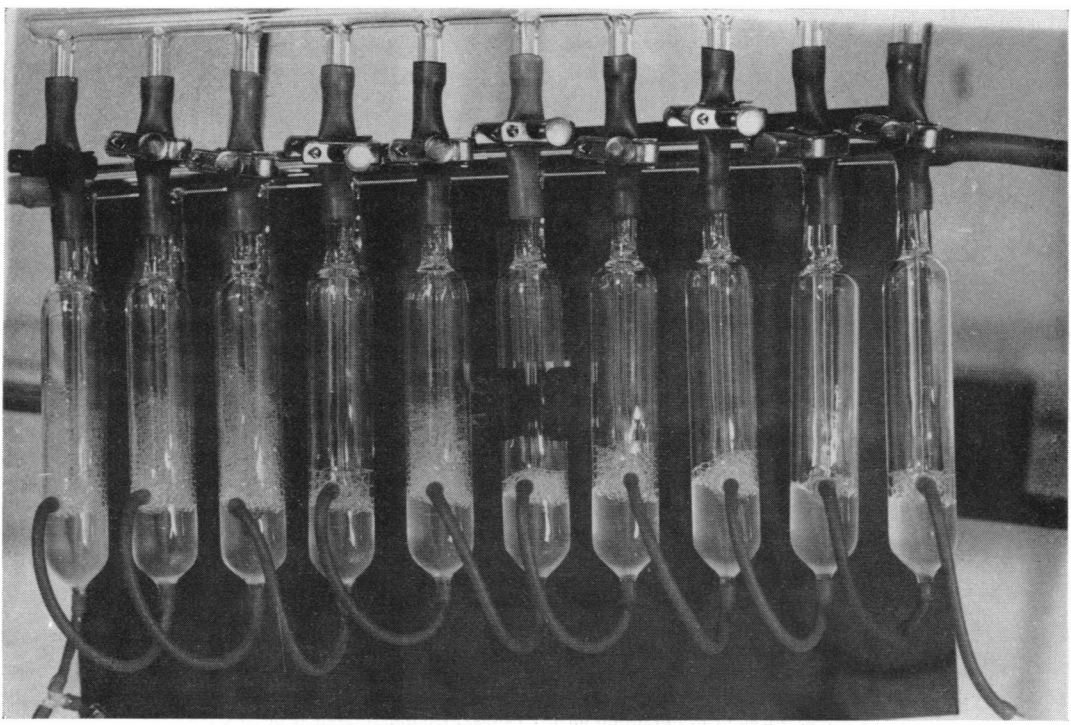

FIG. 5. *Virustat in operation during run φ7. Last ten tubes of chamber.*

TABLE 1. *Summary of results of virustat runs*[a]

| Run | Flow rate | Growth tube vol | Growth tube cell count ($\times 10^8$) | Virus count in first cell ($\times 10^8$) | Virus count in last cell ($\times 10^{10}$) | Duration of run |
|---|---|---|---|---|---|---|
| | *ml/hr* | *ml* | | | | *hr* |
| M1 | 35 | 50 | 2.5 | 3 | 10–30 | 64 |
| M2 | 20 | 55 | 2 | 100 | 20–40 | 20 |
| $\phi$1 | 44 | 67 | 4 | 150–350 | 3–10 | 24 |
| $\phi$2 | 50 | 70 | 2 | 40–50 | 2–4 | 45 |
| $\phi$3 | 50 | 75 | 2.5 | 90–150 | 10–20 | 40 |
| $\phi$4[b] | 54 | 84 | 3 | — | 0.7–1.7 | 96 |
| $\phi$5[c] | 50 | 120 | 3.6 | 13 | 1–2.5 | 74 |
| $\phi$6[c] | 80 | 170 | 3 | 200–600 | 1 | 46 |
| $\phi$7[b] | 52 | 120 | 4 | 16–23 | 0.3–0.7 | 73 |
| $\phi$8[b] | 52 | 105 | 3 | 15–22 | 2.5–4 | 93 |
| $\phi$9[c] | 20 | 100 | 5.5 | 300 | 3 | 45 |
| | 52 | 128 | 4.5 | 70 | 10 | |
| | 170 | 200 | 3 | 2[d] | 12 | |
| $\phi$10[b] | 142 | 170 | 0.6 | 9[d(?)] | 2–6 | 25 |
| $\phi$11[b] | 54 | 85 | 3.2 | 14–90 | 1–3 | 125 |

[a] Data on the flow rate, growth tube volume, and cell count are generally averaged over the fluctuations of the run. 10 Cells; average volume, 10 ml each (except for M1 and M2, with 11 Cells, the 11th having 10 ml in M1 and 20 ml in M2).

[b] 20 Cells; average volume, 5.2 ml, except for run $\phi$4 (average volume, 7.5 ml).

[c] 25 Cells; average volume, 5.2 ml.

[d] Run is not self-inoculating.

tube culture, representative bacteria count in growth tube, and virus titer in first and last ("production") Cells. Run duration and self-inoculation (below) is also recorded.

Runs M1 and M2, made with MS-2 virus and host, showed no lysis along the growth chamber at the flow rates employed, even though the culture spent 3 to 6 hr in it, a time long enough for lysis of batch cultures. Continuing the experiments in the absence of visible indications of lysis, phage titers revealed, nevertheless, a continuous production in the $10^{11}$/ml range over the entire duration of the runs (20 to 64 hr). Turning off the virus inoculum pump early in the first run led to no appreciable drop in output titer, verifying the self-inoculation concept treated in the discussion. As *E. coli* 3000 has an exceptionally large fraction (order of $10^{-4}$) of MS-2-resistant bacteria, presumably $F^-$ species, even in freshly cloned cultures, and as these would be expected to increase with time in the virustat, lysis must have been hidden by the large population of resistant bacteria.

The total production of M1, about 2,000 ml at a titer of $>10^{11}$/ml, contained $>2 \times 10^{14}$ phage particles, compared to a maximal inoculum of 20 ml containing $<2 \times 10^{12}$/ml, or a total of $<4 \times 10^{13}$ particles, a minimal phage increase of fivefold in the apparatus. For M2, the 400-ml production at $>2 \times 10^{11}$/ml, compared with <10 ml of inoculum containing $<5 \times 10^{10}$/ml, represents a minimum of 160-fold phage increase, leaving no doubt of actual phage production. Subsequent runs (below) gave similarly large increases over the rather variable inoculum phage content.

Further work with the virustat was resumed with the $\phi$X174 system, which gives clearer lysates and a lower fraction of resistant host cells. Immediate results were obtained with ten-Cell and larger chambers. Figure 2 shows run $\phi$3, and Fig. 3–5 show $\phi$7. The following were the primary observations of interest.

(i) Continuous production of virus over at least several days is possible. Titers of $\phi$X174 ran from $10^{10}$ to upwards of $10^{11}$/ml, a substantial fraction of the (also variable) batch yield, and to a relatively higher maximum fraction than with MS-2 in the virustat, at least as far as here observed.

(ii) After about 1 day, the initially clear lysates turned cloudy, and the foaming in the later Cells diminished to imperceptibility, although substantial production, usually $10^{10}$ or more, proved to be still continuing. Content of resistant mutants in the bacterial growth tube and in the output of the virus growth chamber during run $\phi$11, with a 20-Cell chamber, is shown in Fig. 6. Growth of resistant mutant content in both is apparent; the

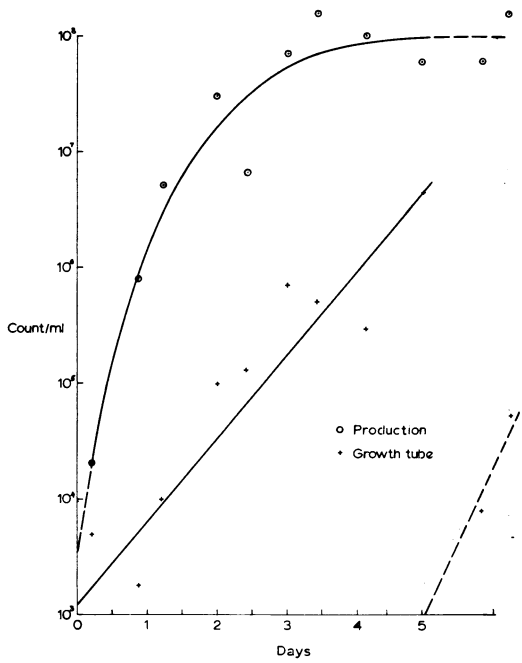


FIG. 6. *Growth of resistant mutants in virustat bacterial growth tube and virus growth chamber output, during run $\phi$11.*

content of the output stream rose to $10^8$/ml within 3 days of the run, and stayed there. (The irregularity in count is probably experimental variance.) Virus titer in the output following this nevertheless remained at $10^{10}$ to $2 \times 10^{10}$, even at the highest levels of contamination with mutants, for the duration of the 6-day experiment. An interesting approximately exponential growth of mutants was noted in the bacterial growth tube. Changing of the bacterial growth tube on the fifth day for one with a culture containing many fewer mutants (dotted line in Fig. 6) did not appreciably decrease the high mutant concentration in the production of lysate.

(iii) Sampling along the virus growth chamber was generally done at the first and last Cells, and generally every fifth Cell in between. A monotonic increase in virus content was generally observed, except at slow flow rates in which some drop could be noted toward the end of the path. A careful monitoring of the "titer profile" of a 25-Cell virus growth chamber at three different flow rates in run $\phi 9$ is shown in Fig. 7. Salient features are the increases with Cell numbers at high flow rates, a maximum at the slow rate, and the sharp decrease in titer in the first Cell as the flow rate increased.

(iv) Self-inoculation proved more prominent than expected. All runs made in the 10-Cell chamber showed self-inoculation (and also the slower runs with 20 to 25 Cells), which required flow rates of at least 120 to 150 ml/hr to abolish the process. The criterion for loss of self-inoculation was rapid fall in virus titer in the first Cell on cutting off the external inoculum stream; for presence of the process, the criterion was maintenance of the same virus titers on cutoff of inoculum.

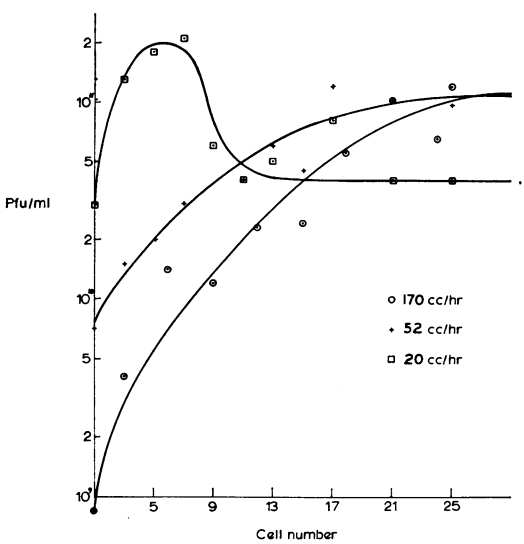


FIG. 7. *Virus "titer profile" along Cells of the virus growth chamber in the virustat, for various flow rates, during run $\phi 9$.*

(v) Clogging of tubes with lysis debris was a problem complicating the longer runs. A certain amount of stringy substance (obviously containing proteins and nucleic acids, judging from some browning and partial insolubility in strong $HNO_3$) eventually appeared in all of the Cells, particularly the later ones.

## DISCUSSION

While the workability of the virustat might be considered inevitable, once its design is examined, many interesting problems and possibilities appeared during its planning and reduction to an operating device. Originally conceived and still of primary interest as a research tool to aid in mutagen studies in prospect and in progress, it was early clear that interest might also attend its use as a means for continuous production of viruses. No truly continuous methods of virus production have been published to our knowledge, and the principles of the virustat represent an original set of methods for accomplishing this. The following aspects of the design and operation of the virustat merit some detailed comment.

*Host cell production.* As pointed out above, continuous virus culture requires radically different, and more complex, apparatus from that of the well-established continuous pure-culture of complete microorganisms. Nor can viruses be merely cultured similarly to products of the microorganisms (e.g., penicillin, colicin), since they must by definition destroy, at least eventually, their host cells. (We exclude lysogenic viruses from this statement.) A *separate* host-cell culture, continuously operating, is thus the first requisite of our system.

For both bacterial hosts, *E. coli* strains C and 3000, the medium flow rates required to keep the steady-state concentration of bacteria in the shoulder-of-the-growth-curve region, i.e., $2 \times 10^8$ to $5 \times 10^8$/ml, were between 0.5 and 0.8 $V$ per hour, $V$ being the growth tube volume. The resultant corresponding average turnover times (i.e., lifetime during which each cell can multiply) of 2.0 to 1.25 hr thus lead to bacterial concentrations corresponding to current practice in batch lysates. At these concentrations, the doubling time has increased from the minimum, log-phase, time of about 40 min (in these media) to a figure 25 to 100% larger, owing to the approach to saturation. With constant inflow and efflux, rather than a single total turnover, it is easy to show that the *effective* time for multiplication is the system time, i.e., the doubling time divided by

$\log_e 2$. When this time equals the turnover time, the count in the tube remains at equilibrium. Thus, a 40-min doubling time corresponds to a system time of close to 1 hr, which, if increased by 25 to 100%, will give the system time equal to the turnover times used.

For sheer production purposes, the highest lysable concentration of host cells is desirable, both because of linear improvement in yield and because of an occasional supralinear increase due to lysis-inhibition effects. For experiments at much lower host-cell concentrations, however (e.g. those in which bacteria in strict log phase are needed), the present system for generating host cells would not have worked. At strict log phase, the maintenance of host-cell concentration at a constant level would require precise delivery of enough medium to dilute out the bacteria as fast as they grow; i.e., the turnover time would equal the log-phase system time. Slight variations in the turnover time, or in the system time constant, due to changes in flow rate or temperature, would cause change in the equality, after which the cell concentration would change over a wide range. Either the concentration would run down to zero, if the turnover time were less than the system time, or, if the opposite held, the concentration would run up to the nonlinear part of the growth curve. Thus, control of the output cell concentration, easily held within rather narrow limits under the near-saturation conditions of our experiment by the slowing-down of bacterial multiplication as the cell concentration increases and vice versa, would be highly erratic at the lower cell concentrations of the log-phase area. At such concentrations, a servomechanism, e.g., the "breeder" of Fox and Szilard (3), would be necessary to maintain accurate constancy of output, raising and lowering medium input rate as the host cell concentration went up or down.

For virus production and many research purposes, the chemostat (10), relying as it does on a nutrient-limited state of near-saturation, is unsuitable. The limiting nutrient may slow up virus production, or the bacteria may be rather less susceptible to successful virus infection. Moreover, the rate of output for a given volume of culture medium is slowed down substantially when close to saturation, thereby requiring a larger apparatus for a given amount of desired cell product.

*Virus chamber Cells.* The heart of the virustat method, of course, lies in the multi-Cell virus growth chamber. Although one can imagine devices which carry the inoculated cells continuously through their life cycle, rather than through the set of stirred discrete Cells comprising the virustat chamber, use of these would multiply the problems of stirring, aeration, and surface accumulations of mutants and debris. On the experimental scale used in our work, a continuous chamber would have been well-nigh impossible; on a larger scale, such would offer no advantage over the multi-Cell chamber. Decision as to the number and design of the Cells, however, rests with the system requirements. A cell-virus system has a cycle complexity, with need for "harvesting" at the right time. Hence, batch methods, properly used for organisms with any morphologically distinct life stages, have also been the methods of choice for viruses (and for replicating factor, tails, early enzymes, etc). The narrowing of the harvesting times is the major factor dictating the optimal number of Cells and the permissible distribution of "ages" in the product. For a cell-lysing system, the product "age" must exceed the lysis time for a period sufficient to allow the large majority of all infected cells to have spent at least this amount of time in the chamber. Nor can this time be extended too long without both decreasing the production capabilities of the apparatus and allowing some fraction of the possibly labile virus to adsorb onto debris or otherwise be inactivated before emerging.

The use of a number of Cells can be seen to reduce the spread in average "ages" of emerging cells in the product, simply by an intuitive statistical argument that a cell from a population spending variable time in many Cells will have less variability of average total time than will one from a population spending the same average total time, but doing so in only one or a few Cells. For any single Cell, assuming good mixing, the incremental "age" distribution between input and output is a simple exponential, with decay constant (base $e$) equalling the reciprocal of the average turnover time of the Cell. Expressing "age" in units of this turnover time leads to the dimensionless variable $T$, defined equal to $rt/V_0$, where $r$ is the flow rate in milliliters per second, $V_0$ the Cell volume, and $t$ the actual "age" in seconds. $T$, simplifying the resulting distributions, may be regarded as the average "age," expressed as number of Cells worth of turnover. Use of the "age" variable $T$ reduces the distribution function in the first cell simply to $W(T) = e^{-T}$.

For the second and subsequent (labeled $n$th) Cells, it can readily be shown (9) that the "age" distribution for the population in the Cells (always assuming rapid and complete intra-Cell mixing) is, simply:

$$W(n, T) = T^{n-1}e^{-T}/(n-1)! \qquad (1)$$

$W(n, T)$ is recognized as the Poisson distribution function $P_{n-1}(T)$, with $T$ the "age" variable, a

continuous argument, and $n$, the number of Cells, a discrete index number, taking on values from 1 through $N$. $W(n, T)$ is, of course, normalized to unity when integrated over all $T$ for any value of $n$. It is also normalized to unity for a summation over all $n - 1$, from 0 to infinity, with a fixed value of $T$.

The second, spatial, normalization has important consequences. It means that the cells with a particular "age" $T$ are distributed among the various Cells in the manner given by equation 1, the fractions summed over all possible Cells totalling unity. If any terms in this summation have non-negligible value for values of $n$ larger than $N$, the total number of Cells, such cells would be in hypothetical Cells numbered $N + 1$ and higher. Physically, this means the fractions would be in the product emerging from the virus growth chamber. This distribution, giving, as it does, the positions of the cells of one particular "age," can now be used to establish the distributions of location at which an event occurring at a fixed time, such as (to a first approximation) lysis, takes place. If we define the dimensionless $T_l$ equal to $rt_l/V_0$, where $t_l$ is the physical lysis time, and ask where the cell is at this "age," i.e., at lysis, equation 1 with $T_l$ substituted for $T$ gives an answer for the $N$ Cells of the chamber and for the infinity of succeeding Cells representing the production stream.

In general, the summation

$$\sum_{n-1}^{N} P_{n-1}(T_l) = 1 - \sum_{n-N}^{\infty} P_n(T_l) \qquad (2)$$

gives the fraction of the cells which still find themselves in the growth chamber Cells when their "age" equals $T_l$. This is, of course, the lysed fraction, and the unlysed fraction is the summation on the left in equation 2 subtracted from unity, recognizable as the summed Poisson distribution with continuous argument $T_l$ and discrete index number $N$, generally written as $P_n(N, T_l)$.

Parenthetically, equation 2 can also be derived by the more physically appealing approach of integrating equation 1 over $T$ from $T_l$ to $\infty$ to get the fraction of cells in the $N$th Cell which are older than $T_l$, i.e., lysed. The summed distribution (equation 2) appears as a result of the iterated integration by parts of $P_{n-1}(T)$.

From the properties of $P_n(N, T_l)$, we can now closely predict the performance of a chamber with known $N$, Cell volume, flow rate, and containing a host-virus system with a known lysis time. Computing $T_l$ and substituting in equation 2 gives the lysed fraction of cells in the product. Table 2 illustrates the efficiency of several multiplicities ($N$) of chambers in carrying most of the infected cells through to "ages" of at least $T_l$. $T_l$ should not exceed $N$, or the cell will spend an average total time in the chamber less than its actual lysis time, and the majority of cells will not be lysed on emergence. This can be seen from the fact that the average total time equals $NV_0/r$, while the actual lysis time equals $T_lV_0/r$, from the definition of $T_l$, and the fact that the average total time equals $N$ times the turnover time of $V_0/r$. For values of $N$ equal to 1, 10, and 20, and of $T_l$ equalling 0.5, 0.8, 0.9, and 1.0 $N$, we read the lysed fraction of product from Table 2. Table 2 also presents the necessary value of $T_l$ for those sizes of chamber which give 99% lysis.

For a single-Cell chamber, the calculations are very simple, and can be made from either equation 1 or 2. If the average time in the Cell equals the lysis time $t_l$, then $T_l = 1$, and only $1/e$, or 36.8%, of the cells lyse before emerging, a poor performance. Setting $T_l = 0.5$ $N$, or 0.5, is accomplished by halving the flow rate. One might expect a striking increase in the lysed fraction, but it only rises to 60.6%. In fact, to assure 99% lysis, it is necessary to slow down the flow rate 100-fold, to a $T_l$ of 0.01. This simply reflects the substantial number of fresh cells which can wash out of a single Cell chamber, unless the rate is made impractically slow. On the other hand, for a 10-Cell chamber, when the rate is slowed down to half of the (average time = lysis time) figure, only 3.2% of the cells are unlysed; for a 20-Cell chamber, only 0.02% of the cells escape lysis under these conditions. For production purposes, the value of a *very* minute unlysed fraction is small, and a 20-Cell chamber would simply be run somewhat faster, say at $T_l = 0.7$ to 0.8 $N$, or at average total times of 1.2 to 1.3 times lysis time.

For lysis which does not occur with absolute synchrony, the above calculations are in error on the optimistic side. Spread in actual lysis times will increase unlysed fraction at any flow rate, although in many cases by imperceptibly small amounts. In general, such asynchrony will limit

TABLE 2. *Lysed fraction of the product with an n-Cell chamber and various T values*

| $T_l$[a] | Value of $N$ | | |
|---|---|---|---|
| | 1 | 10 | 20 |
| 0.5 $N$ | 0.607 | 0.968 | 0.993 |
| 0.8 $N$ | 0.449 | 0.717 | 0.812 |
| 0.9 $N$ | 0.407 | 0.587 | 0.651 |
| 1.0 $N$ | 0.368 | 0.458 | 0.470 |

[a] Values of $T_l$ for 99% lysis: N = 1, 0.01 N; N = 10, 0.42 N; N = 20, 0.56 N.

the wisdom of using more Cells than necessary to get an "age" distribution with the same order of narrowness of time spread as that of the lysis times.

The number of Cells in the chamber is thus seen as a quantity whose increase gives better uniformity of "age" of product. Economics militates against the encumbrance of too many Cells; indeed, little advantage accrues when the "age" peaking is sharper than the natural peaking of lysis time. The Cell minimum number is dictated by the desirability of a culture which is nearly completely lysed, the natural loss of titer in an overaged culture, and the decrease in production when the flow is slowed up (and by factors discussed below, namely, self-inoculation and sweep-out of resistant mutants). For large-scale production, we would estimate that something like ten Cells would prove optimal, with a self-inoculating system.

*Self-inoculation.* This phenomenon, a semi-expected one, appeared in the first run (M1 of Table 1) with the breakdown of an inoculum pump shortly after the start of the run. On the assumption that sufficient infected cells could survive in the first Cell so that the burst of virus produced would at least replace the inoculum swept out by the flow of medium, the experiment was continued without external inoculation. Production of virus continued for several days without external inoculation, indicating that self-inoculation as described was indeed occurring. Especially noteworthy as a criterion of self-inoculation is the titer in the first Cell. If no self-inoculation is taking place, this will drop precipitously with interruption of external inoculum; if external inoculum is present, the first Cell titer will equal that of the inoculum times the flow ratio of inoculum to host cell streams. Substantially higher titer, with or without inoculum flow, shows self-inoculation building up to levels of multiplicity of infection which depend on the flow rate.

One can, however, easily predict fulfilment of the critical condition for self-inoculation. Over the dimensionless time $T_l$, the fraction of cells remaining in the first Cell will be, by equation 1, simply $e^{-T_l}$. This unwashed-out fraction applies also to any cell type present, in particular to infected cells. If the average burst size is $B$ (a reasonable distribution of burst sizes will affect matters but slightly), then $R = Be^{-T_l}$ defines a multiplication constant giving the fraction of the inoculum remaining in the first Cell after washout for time $T_l$ and subsequent bursting of the fraction remaining. Values of $R$ below unity can be seen to allow washout of inoculum, if not supplied; at the critical value of unity or higher, virus derived from bursting of infected cells in the first Cell will replace inoculum as fast or faster than it can be washed out. If $R$ is less than 1, the inoculum multiplies in this cell by the overall factor $\sum_0^\infty R^i = 1/1 - R$ ($i = 0$) prior to washout. At $R = 1$, the inoculum is exactly replaced as fast as it washes out; at $R > 1$, the titer in the first cell grows larger even without added inoculum. The increase of titer is, however, self-limiting. The effective value of $B$, i.e., the number of released virus per adsorbed virus, must decrease as the multiplicity of infection, which we designate $m$, increases. Assuming a constant burst size independent of $m$, and complete absorption within the first Cell, $B$ will be decreased by the factor $(1 - e^{-m})/m$, a number approaching $1/m$ at high $m$, and unity at low $m$. This decrease in the effective value of $B$ will limit the value of $R$ to unity, when steady-state conditions have been established: $R = 1 = B_0[(1 - e^{-m})/m]e^{-T_l}$ ($B_0$ being $B$ at low $m$). For values of $m$ higher than four or five, it is accurate to write: $1 = [B_0/m]e^{-T_l}$, hence,

$$m = B_0 e^{-T_l} \tag{3}$$

From the burst size and multiplicity of infection, if known, one could thus calculate $T_l$, and, from this and the turnover time, the true (average) lysis time prevailing in the system, $t_l$. As here calculated, and as generally observed, $t_l$ includes the adsorption time.

The first two MS-2 runs show this dependence of self-inoculation on the flow rate (which alters $T_l$, and thereby $m$ and $R$) strongly. The observed ratios of virus to bacteria in the first Cell were 1.5 and 50, respectively, the difference being due to the two flow rates giving 18- and 30-min turnover times ($V_0/r$) in the first Cell, respectively. Assuming that the first run was just beyond the borderline of self-inoculation (a condition certainly not far from the truth), and assuming also a burst size of $10^4$ (also close to the truth and not critical), we can calculate the average lysis time in this Cell to be 18 $ln$ ($10^4$) = 166 min, a figure in line with visual experience with this virus strain. By use of this lysis time with the turnover rate in the second run, in one lysis time there should be 166/30 = 5.5 turnovers in the Cell. The overall multiplication of virus here would then be expected to give $10^4 \times e^{-5.5} = 40$, a figure in agreement with the observed first-Cell virus-bacteria ratio of 50.

Similar calculations with the $\phi$X 174 runs suggest an average lysis time of 25 to 30 min, a range somewhat lower than that visually observed. Uncertainty of burst size and the threshold of self-inoculation, and complex weighting of both burst size and lysis time, however, along with some

possible effects of washout (see below), have made this calculation uncertain.

Figure 7 shows, among other things, the effect of flow rate on self-inoculation. At the two slower rates, the first Cell showed high titers indicative of self-inoculation; moreover, the inoculum flow could be discontinued without appreciable change in virus titer in the first Cell or in the production. At 170 ml/hr, however, inflow of inoculum was necessary, and the first-Cell virus content was approximately twice that calculated from inoculum flow, indicating a value of $R$ of about 0.5. A faster rate would decrease $R$ rather precipitously, but would also result in incomplete lysis in the product, unless more Cells than the 25 present were used, as one can extrapolate from inspection of the curves of Fig. 7.

For simple production purposes, self-inoculation is a great convenience, as it eliminates necessity for a portion of the system. Run thus, the virustat, once set up and inoculated, has a single (medium) liquid inflow, and a single (lysate) liquid outflow. For certain purposes, however, running in a non-self-inoculating condition is desirable. Under such conditions, a relatively large number of Cells in the chamber is necessary. All runs with 10 Cells giving reasonably clear lysate required slow enough flow rate to self-inoculate. Even with 20 to 25 Cells, some part of the virus produced was generated within the first Cell, at fairly high flow rates.

Inoculation, made in these runs where necessary with prepared filtered lysate (sometimes diluted to control the $m$), can also be accomplished by recycling a small fraction of the product into the system. For control of resistant host mutants, this requires filtration, centrifugation, or possibly temporary bactericidal treatment of the recycled product.

*Mutants and the washout process.* A problem considered early in the design of the virustat was that of formation of resistant host-cell mutants. A spontaneous mutant rate, generally small, puts new mutants into the system each time the bacteria double. In the presence of the virus inoculum, such mutants can multiply. For a self-inoculating system, or one whose inoculum is rendered free of bacteria, this multiplication is at first glance seen as unimportant. In the slowest runs considered, the host bacteria spend no more than a half-dozen generations in the virus growth chamber, and cannot therefore multiply more than 100-fold or so (even if not destroyed by virus) without being washed out of the chamber by the flow of medium. In the faster, non-self-inoculating runs, the multiplication of resistant cells in the chamber is only about fourfold. Thus, use of a culture with less than $10^{-3}$ or so resistant organisms should result in the overwhelmingly largest portion of the cells lysing. Expectation with the *E. coli* C culture is for a mutation rate to $\phi$X174-resistant forms of the order of $10^{-6}$ per generation. The experience of Szilard and Novick (10) with the chemostat bears out the postulate of a linear increase in mutants with time. The number of generations required to reach a mutant level of $10^{-3}$, about 1,000, would require several weeks of growth in the bacterial growth tube. Experience with the virustat, however, showed visible clouding of the originally clear production within 1 day, and complete loss of normal lysate appearance within 2 days, although production still continued for substantially longer periods. The results cited for run $\phi$11 (Fig. 6) show that the picture is more unfavorable than that predicted from idealized considerations, both in the bacterial growth tube and in the virus growth chamber. The remarkable slow exponential increase of resistant mutants in the bacterial growth tube, in the certain absence of any virus challenge (which would forthwith terminate the run), means that the mutants must have some slight and unpredictable a priori growth advantage, even in the absence of virus.

Recent reports (4, 8) suggest that, with complex media not relying on a single growth-limiting nutrient, a continuous bacterial culture may show a better-than-constant rate of emergence of auxotrophic mutants. Its applicability here is not certain, but such effects in the growth tube may also account for the unexpected rise in the mutant population.

Replacing the growth tube is a way of cutting down the number of mutants entering the virus growth chamber; the last 2 days of the run show the (new) growth tube with relatively few, but increasing, mutants. The curve giving the resistant concentration in the production, however, shows that resistant mutants, at least under conditions prevailing in the virustat, arise primarily in the virus growth chamber, and not in the bacterial growth tube. If the latter were the case, the resistant concentration in the production would be expected to run reasonably close to, and not more than fourfold larger than, the lower line. This is clearly not the case, except conceivably at the outset. The rise of resistant mutants is faster in the virus growth chamber, and when they have reached the surprising level of about $10^8$/ml, even replacement of the growth tube and depression of mutant input by a factor of at least $10^3$ has no significant effect on reducing their output concentration. We have therefore reluctantly concluded that there is some source of resistant mutants of unexpectedly high degree within the virus growth chamber. This source must reflect a

partial failure of washout and some growth of resistant bacteria in slow-moving "pockets," or on inner surfaces of the apparatus. It is likely that technical improvements in this situation can be made. Coating the walls of the chamber with silicone should reduce the tendency of bacteria to grow on the walls; reasonable amounts of a compatible detergent will keep some bacteria from clumping and standing still. Simple increase in the size of the apparatus, i.e., increasing the volume-surface ratio, will decrease such effects. Above all, stagnant pockets, such as those which can form at sampling tees and glass-rubber tubing junctions, should be avoided. Although we are as yet not reporting on solutions to these problems, some preliminary observations with combinations of nonionic detergents and antifoams have indicated that it is quite possible to culture bacteria to the point of lysis with relatively low foaming, and with improvement in the washout of resistant mutants, especially from siliconized glass surfaces, if used in the virustat.

A further limitation, related to washout, is the debris accumulation observed. This, too, may be ameliorated if the medium has sufficient detergent properties (and the vessel walls nonadhesive ones) to carry most of it off. It must be recognized that any substantial amount of adherent debris will by itself create conditions in which flow rates are extremely slow at some portion of the chamber surfaces, which thereby furnish shelter for culture of mutants. The debris formed is directly proportional to the total number of cells lysed. Lower host cell concentrations will give longer debris-free runs. For production purposes, this is not much help, but, in certain experimental situations where running at low bacteria concentrations can give the desired data, such would indeed lengthen the useful life of the experiment.

*Continuous production of pure virus.* For most virus applications, purification is desirable. One of the most appealing possibilities for use of the virustat in production is as a supplier of raw lysate to some continuous purification process. Processing involving ordinary centrifugation, liquid-liquid partitioning, treatment with reagents or absorbents, and some two-dimensional separations (e.g., curtain electrophoresis) are readily adapted to continuous operation. Operations on solid residues, including collection and dispersion sediments and pellets and filtration, settling, or centrifugation with processing of the solids, are difficult to make continuous, as is simple column or supporting medium separation of any sort, which generally needs a batch charge. Thus, the usual techniques lead to pellet collection and redispersion, or columnlike separation, and cannot be directly used in a method with a continuous lysis inflow. Large initial reductions of handling of volumes into pellets, or slurries of high virus content, however, are quite feasible addenda to the virustat. For instance, the two-phase separation of virus at the liquid-liquid interface can take a raw lysate input, add and mix streams of the soluble but incompatible polymers (e.g., Dextran and Carbowax), pass into an ordinary continuous centrifuge, drawing off clear top and bottom fractions, and building up the interface yield. Some servomechanism would be needed to prevent the interface from leaving the inside of the centrifuge. When a maximal amount of material had accumulated in the interface, the contents could then be emptied in batch for further ordinary processing, with the remainder of the material requiring only small-scale manipulations. Alternatively, a continuous low-speed cycle of centrifugation, followed by a high-speed one, could give an increasing pellet containing virus-sized particles. The requirements of ultracentrifuge-range velocities for putting virus into a density gradient band demands a special kind of equipment, now reaching availability in the commercial zonal ultracentrifuge.

Completely continuous production-scale purification of virus would require some modification of present methods. Continuous production of an interface slurry, followed by a stage of curtain electrophoresis and a final continuous density-gradient in a zonal ultracentrifuge, could perhaps accomplish this in a satisfactory way. Virus-derived substances, including intracellular by-products of the viral attack, such as early enzymes, replicating factor, partially complete particles, or, with mammalian host cells, interferon, are all continuously producible and perhaps purifiable by running the infections in the virustat so as to produce viruses, or unlysed cells of the proper age, and subsequent treatment as the product requires.

The *raison d'être* of the virustat, like that of the chemostat and family (3, 7, 10), lies not only in its ability to manufacture virus efficiently, but also in its use as a research tool for population studies in virus-host systems, particularly accelerated mutation and evolution. It was originally envisioned as a simpler solution to the problem of "exhaustive mutagenesis" (H. Jacobson and L. M. Blatt, Meeting Abstr., 6th Intern. Congr. Biochem., 1964), i.e., complete attack on all possible nonlethal mutable sites by a specific mutagen. This would be incredibly laborious by any kind of batch method, but feasible with a few months of continuous treatment in the virustat. Sending the virus output into a mutagen treatment or selection chamber, or both, and recycling survivors (which would include a large mutant

population, also possibly selected for resistance to a given set of conditions), accomplishes a continuous culture change far greater than that obtainable in any other way. For instance, acid-resistant varieties of virus could be efficiently selected by acid treatment of the lysate, recycling the survivors of this challenge so as to reinfect the host-cell stream. In vitro mutagens can be used at this point to stimulate variability. In vivo mutagens, of course, must be introduced into the virus growth Cells.

For such purposes, the virustat should be run at low host cell concentrations, to minimize clogging and to keep growth in strict log-phase. Moreover, to receive the effect of the change in the species, the self-inoculation factor would have to be eliminated, or unchanged viruses would dilute out the changed ones. Thus, research runs will typically require upwards of 25 Cells and improved servo host-cell concentration controls. Samples withdrawn continuously during this procedure should show continuous, if slow, population alterations in host range, survival, and immunological and, probably, chemical properties.

## ACKNOWLEDGMENTS

We gratefully acknowledge fellowship support from the John Simon Guggenheim Foundation and the National Institutes of Health for one of us (H.J.) during the conception and design of the virustat, and research grant support from the National Institutes of Health, project GMS-09023. We thank the National Science Foundation for support of Richard Sohn, whose data on runs $\phi$7–$\phi$11 are reproduced here, as NSFURPP student.

We also thank R. L. Sinsheimer for hospitality in his laboratory, basic cultures, and critical discussions. The method and apparatus described are the subject of a patent application owned by the California Institute Research Foundation.

## LITERATURE CITED

1. DAVIS, J., AND R. L. SINSHEIMER. 1963. The replication of bacteriophage MS-2. Transfer of parental nucleic acid to progeny phage. J. Mol. Biol. **6**:203–207.
2. FILNER, P. 1965. Semiconservative replication of DNA in a higher plant cell. Exptl. Cell Res. **39**:33–41.
3. FOX, M. S., AND L. SZILARD. 1955. A device for growing bacterial populations under steady-state conditions. J. Gen. Physiol. **39**:261–266.
4. IERUSALIMSKY, N. 1964. Use of continuous culture method for analysing cell functions, p. 89–92. *In* Continuous cultivation of microorganisms, 2nd Intern. Symp., Prague, 1962. Academic Press, Inc., New York.
5. KUBITCHEK, H. C., AND H. C. BENDIGKEIT. 1964. Mutation in continuous cultures. I. Dependence of mutational response upon growth-limiting factors. Mutation Res. **1**:113–120.
6. MCLIMANS, W. F., F. G. GIARDINELLO, E. V. DAVIS, C. J. KUCERA, AND G. W. RAKE. 1957. Submerged culture of mammalian cells: the five liter fermenter. J. Bacteriol. **74**:768–774.
7. MONOD, J. 1950. La technique de culture continué. Theorie et applications. Ann. Inst. Pasteur **79**:390–410.
8. NORTHROP, J. H. 1964. The proportion of streptomycin-resistant mutants in B. Megatherium growing in continuous cultures, p. 141–44. *In* Continuous cultivation of microorganisms, 2nd Intern. Symp., Prague, 1962. Academic Press, Inc., New York.
9. SINSHEIMER, R. L. 1959. Purification and properties of bacteriophage $\phi$X 174. J. Mol. Biol. **1**:37–42.
10. SZILARD, L., AND A. NOVICK. 1950. Description of the Chemostat. Science **112**:715–716.