tests. Positive reactions were obtained in all patients with mycetoma due to *N. brasiliensis* but not in patients with other diseases or in healthy individuals. It is concluded that these tests may be of value in diagnosis of mycetoma due to *N. brasiliensis*.

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Quality and Yield of Vaccinia Virus from L Cell Cultures.* (28271)

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Technical difficulties have delayed progress in the measurement of the quality of viruses other than those of the influenza group for which the I/HA ratio has been so successfully applied, but direct particle counting has now advanced to fill this need. The quality of the vaccinia virus reported here will be expressed as the ratio of plaque forming units (pfu) to the number of particles as seen and counted in the electron microscope. Certain changes in virus quality have been observed, particularly in the early hours of rapid new virus production in infected L cells. These may be related to the von Magnus effect(1) with influenza viruses but there are essential differences.

Materials and methods. The WR (mouse neurotropic) strain of vaccinia virus, used in this work, has been kept in continuous passage in screw-capped bottle cultures of L cells. The last 280 passages have been made with 48- to 72-hour virus at multiplicity M = 20. Earle's L cells, in screw-capped tube cultures, were used for experiments on virus growth. These were incubated slantwise at 37°C until time for titration and virus particle count. Monolayers of L cells were used for plaque titration. Growth medium and complete details of the growth experiment have been published(2).

We wanted the increase in virus to be an index of particle production in all cells rather than of the fraction of cells producing. One requirement for this is synchrony in inoculation. This was provided by the sedimentation inoculation procedure(3) which accomplishes this purpose in less than 7 minutes. This short time has been shown to be adequate(4). The multiplicity of inoculation was 10 in all growth experiments. This number seems to have been adequate to infect all the cells.

Plaque titrations were made at the times when the samples were ready. None were frozen or otherwise stored to await titration. Samples were thoroughly dispersed with 9 kc sonic waves just prior to titration. Aggregation analyses, made on the electron micrographs used for particle counting, have shown the suspensions to contain over 80% active units(5).

Counting of virus particles was done by the agar sedimentation method of Sharp(6). Re-

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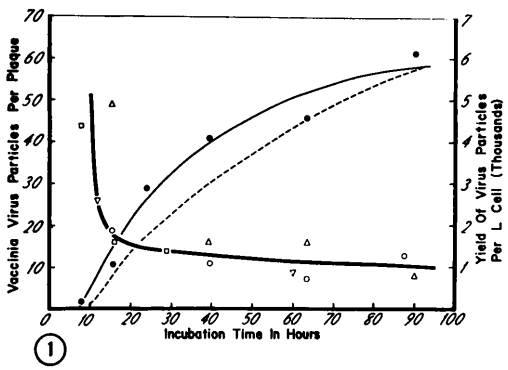


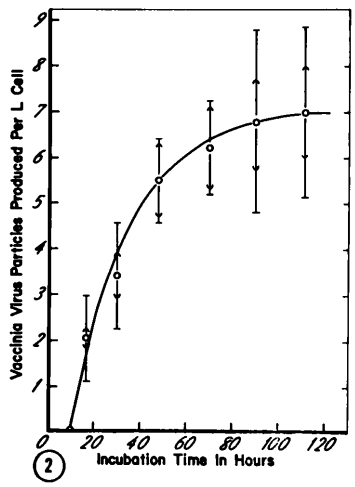


FIG. 1. Quality and growth curves for vaccinia virus produced in closed tube cultures of L cells. Heavy solid line shows virus particles per pfu, light solid line shows number of virus particles produced, and dotted line shows corresponding curve for pfu adjusted, for comparison, to match the particle curve at 90 hr.

FIG. 2. Growth curve of vaccinia virus particles in L cells. Composite of 16 experiments. Maximum limits plotted are the observed standard deviations. Calculated standard errors of particle counting lie within, as shown.

duction of debris in electron micrographs of some infected cell lysates was accomplished through the use of enzymes. Trypsin and chymotrypsin were employed for this purpose as previously described(7). Pronase (Calbiochem) was tried and found useful as well but all data in the following presentation were obtained with the trypsin-chymotrypsin treatment. All lysates of cells containing an average of less than 1000 virus particles each required enzyme treatment in the counting process. None of the cell cultures or materials for titration were enzyme treated.

Experiments and results. Several million L cells from 3-day-old cultures were scraped from the glass bottles and suspended in fresh growth medium. They were inoculated, by sedimentation, with fresh virus at M = 10, then they were divided into several screw-capped test tubes and incubated at 37°C. At appropriate times the cells from one tube were removed, lysed by sonic waves and the virus counted and titrated. Four such experiments were performed and the results are presented in Fig. 1. In all 4 cases the quality of the early virus was 3 to 5 times poorer than that found after further incubation. The improvement was very rapid at first, continuing more slowly through 90 hours to reach a value of about 100 pfu per 1000 particles or 10 particles per plaque.

The corresponding growth data are shown also in Fig. 1. The points show average virus particle yields from all experiments. The solid line is of the type $N = N_{max}\ [1 - e^{-k(t-to)}]$ where N_{max} and N are numbers of virus particles at maximum and at time t respectively and t_o is the time when new virus first appears. The values of the constants k, N_{max} and t_o used in the construction of this curve were .0330, 6200 and 7.5 respectively. The fit is quite good. A more complete particle growth curve was calculated from a series of experiments performed for other purposes. From these, a set of 16 were chosen in which the input particle multiplicity was 10 for all and incubation times were about the same as those of the quality experiments. Virus of passage 150 to 285 was used. The average number of particles produced and counted was calculated for each incubation time and these were plotted (Fig. 2) along with the standard errors for each series and the estimated particle counting error(8). The curve is again of the type found in Fig. 1. It was drawn with constants $k = .0391$, $N_{max} = 7100$ and $t_o = 9$ hours. The virus particle growth curve may thus be closely approximated by the formula which predicts maximum slope or rate of particle production at the beginning of growth. This provides evidence that all the cells began producing particles at essentially the same time. Some evidence of a short lag may be seen in particle

growth curves of earlier passage virus(7). In that case it is possible that the input multiplicity of *infectious* particles was too low.

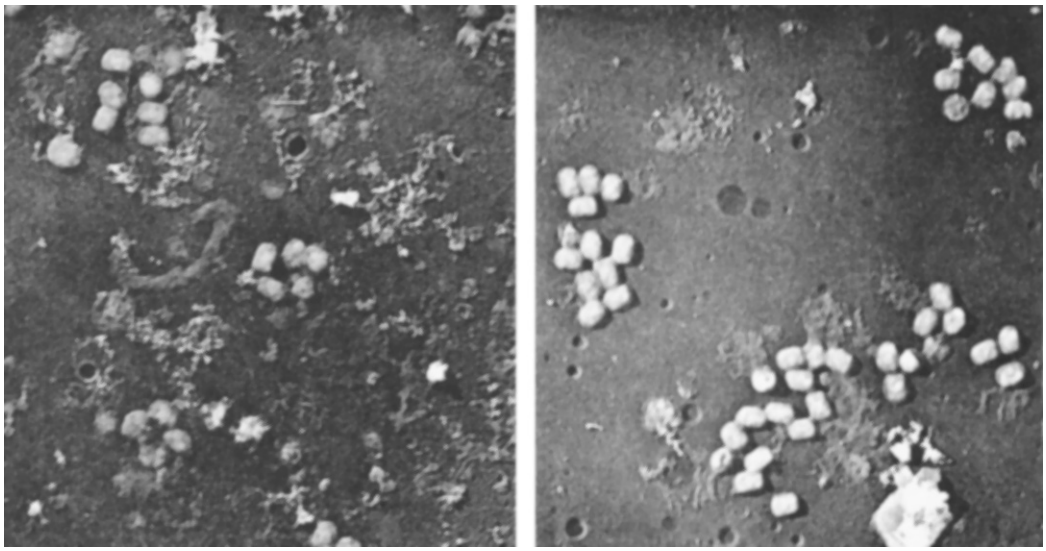
Growth experiments with $M_a = 10$ inoculum and frequent particle counts during the early period of incubation have shown a variability between 8 and 12 hours before the appearance of new virus but once growth was detected the increase was rapid.

The dotted curve drawn on Fig. 1 indicates virus growth in terms of pfu. The ordinate of this curve has been adjusted to match the particle curve at maximum incubation time in order that the curve *shapes* can be better compared. They are quite similar except for the short transient period at the beginning of growth when the pfu curve rises very slowly because of poor virus quality but swings steeply upward with the rapid increase in particle number and simultaneous improvement in quality. While little can yet be said of the shape of the curve in the interval of 1 to 100 particles per cell because of particle counting difficulties which reduce the precision, the observed lag in the pfu curve extends well into the countable range.

Sonic lysates of all cells containing less than 1000 virus particles each require treatment with enzyme prior to counting in the electron microscope. Pronase was found useful for this purpose in preliminary tests. In the growth experiments, however, we have used the same mixture of trypsin and chymotrypsin that we have used in past work(7). This has no effect on the appearance or number of mature virus particles as seen in the electron microscope. In Fig. 3 are samples of virus taken at 12-hour and at 60-hour incubation from the same growth experiment. Both were enzyme treated. These are fair samples of the degree of the heterogeneity generally seen and it is quite difficult to say with any assurance that early virus looks different from mature virus although the former usually appears slightly more flattened in this type of preparation.

Discussion. Quality measurements on vaccinia virus have been made by pock titration, on the CAM, of virus grown in L cell cultures (9). The ratio of pocks to particles was minimum 6 to 8 hours after inoculation and it increased quite smoothly and steadily during 40 hours of further incubation. While the present findings with plaque titration are essentially similar they show the quality improvement as very rapid at the start of particle production with continued improvement at a much reduced rate after about 20 hours of incubation. Although prominent in both, it is particularly noteworthy in the present work that virus particle quality *continues* to improve through 90 hours of incubation at 37°C. Hahon and Kozikowski(10) have shown the rate of degradation in infectivity for vaccinia virus in cell-free media at 37°C to be 100- to 300-fold in 10 days. Under ordinary circumstances, one could account for a continuously rising titer in terms of particle production more rapid than decay but this route is not available here. The rate of accumulation of particles is known and it is not great enough to maintain a constantly rising titer for 90 hours in the presence of degradation at the rate reported for cell-free media. It seems necessary, therefore, to assume that the particles, most of which remain within the cell, are not exposed to the full rigors of destruction which they encounter when separated from it.

It is not possible to decide, at this point, whether early virus is incomplete according to the concepts of von Magnus, Henle and others for influenza virus or whether it is unfinished and needs only further incubation and maturation. There may be immature forms among the particles shaken, by sonic waves, from cells which have not yet normally released their progeny. Our electron micrographs do not produce an unequivocal solution to this problem (Fig. 3). They do not show the clear morphological difference between low and high quality vaccinia virus that has been seen with incomplete and fully active influenza virus. In order to see and count the vaccinia virus from cells lysed during the early hours of incubation it is necessary to treat the lysate with enzymes. It is quite difficult to show that these have no effect upon the new virus; it cannot be counted by other methods. However, if the enzymes do destroy substantial numbers of the early virus particles before count, the true quality



a b

FIG. 3. Enzyme treated sonic lysates of L cells infected with vaccinia virus, (a) from a 12-hr culture and (b) from a 60-hr culture. Both were lightly shadowcast with chromium. Magnification 16,000.

must be even poorer than our data indicate.

All virus particle growth data reported here are from cells inoculated within a time span of 7 minutes. Whatever asynchrony they display in beginning production of new virus is not due to uncertainty regarding time of initial contact with infecting virus. The multiplicity of particles capable of infecting cells was probably high enough to infect all the cells initially. Evidence for this is seen in the shape of the particle growth curve, Fig. 1, and in the composite curve, Fig. 2. These curves rise rapidly, beginning at maximum slope. Experiments involving frequent counts during the early rise period have shown only the briefest upswing before maximum slope is attained and this may be due to asynchrony in phase of growth of the cells. The general shape of the pfu curve, Fig. 1, is the same except for the short lag imposed by the poor quality of the early virus.

Extended periods of "exponential" growth of infectious virus have been reported even for cultures carefully prepared for one-step growth studies (Review by Isaacs, 11). As we have seen, such a transient upswinging curve may be pseudo-exponential because of changes in virus particle quality. The particle growth curve seen here is similar to that of a chemical reaction that proceeds at ever decreasing speed as one of the reactants becomes depleted. The proposed empirical curve, which fits the data well, is of this type.

Summary. The first vaccinia virus particles produced in cultures of L cells are low in quality; poor in plaque forming ability. The quality of the total virus product of these cultures improves rapidly at first, then more slowly throughout the 90-hour incubation period. Electron micrographs have not shown clear distinguishing morphological features of the early virus such as those of incomplete influenza virus. Growth curves of vaccinia virus particles in our cultures are single phase. They rise abruptly and approach a maximum in the manner of a chemical reaction which is limited by shortage of one reactant.

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Lipid Composition and Metabolism of *Trichomonas foetus*. (28272)

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Little is known of the metabolism of *Trichomonas foetus*, especially in the lipid field(1). The present paper investigates its lipid composition and metabolism.

Material and methods. *Trichomonas foetus* (TF) strains BR and 3P were used in this study. They were grown in modified Diamond's medium(2) containing proteose peptone, yeast extract (Difco) and 10% inactive horse serum. Agar was omitted. The parasites were propagated in 4 oz screw cap bottles containing 40 ml of medium. Incubation proceeded at 37°C for about 40 hours. At the end of the experiment, cells were harvested by centrifugation, and washed several times.

Extraction of the samples, column chromatography and radioactivity determination have been described(3). Lipid components of the various chromatographic fractions were detected by the Liberman-Burchard method (LB)(4) for sterols, and by methods used by Carlson *et al.*(5) for glycerol and by Knight *et al.*(6) for phosphorus. Phospholipids were calculated by multiplying the amount of phosphorus by 25.

Sterols were identified by co-chromatography using thin layer chromatographic method (TLC)(7). Chromatograms were developed with hexane - ethyl ether - acetic acid (50:50:2). Color was detected by spraying with $SbCl_3$ in ethanol. In some experiments sodium acetate-1-C^{14} specific activity 1 millicurie 12.6 mg (Volk Radiochemical Co.) was added to furnish 0.1 mc/100 ml medium. Experiments were repeated at least 3 times. The average results are presented.

TABLE I. Lipid Composition of *T. foetus*.

Strains	Sterol esters	Sterols	Glycerol	Phospholipids
		(μg per mg protein)		
BR	2.8	38.6	.37	77.5
3P	2.4	37.5	.36	77.5

Results and discussion. As seen from Table I, there was no appreciable difference in lipid composition between the 2 strains examined. Most of the sterols present were in a free form. The LB method distinguishes sterols on the rate of color development. The rate of color produced was that of a "slow acting" sterol, and was calculated as cholesterol. The amount of sterol present was about one hundred-fold more than glycerol, and about half the amount of phospholipids.

To elucidate further the nature of the present sterol, eluate from silicic acid column containing the free sterol was rechromatographed using the TLC method. After spraying with $SbCl_3$, a spot appeared on the chromatoplate. The color of the spot, its relative R_f value, and the rate of color developed with LB reagent, were similar to, or identical with, cholesterol. Lipid composition of the medium was also examined. The findings, mg per 100 ml medium, were: cholesterol esters 11.00, cholesterol 5.38, ergosterol 0.06, glycerol 0.37, phospholipids 11.00.