Propagation of Mammalian Viruses in Protista. IV. Experimental Infection of *C. albicans* and *S. Cerevisiae* with Polyoma Virus[1] (34348)

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The infection of yeast with viruses of warm-blooded animals has been a controversial subject since the claims of Silber and Wastruchowa (1) on the growth of vaccinia in *Torula* were not confirmed by Amies (2). Recently, however, the successful propagation of certain mammalian enteroviruses in yeasts and *Tetrahymena* was reported by Kovács *et al.* (3, 4), both with isolated viral genome and complete infectious particles. Transfer assays with the oncogenic DNA virus, polyoma (PyV), are described in the present communication. Similar results with Protozoa will be published separately. Brief mention of these findings has been made in an abstract (5) and a report (6).

Experimental Methods. PyV was the large plaque variant of the T strain passaged on secondary mouse embryo cells (MEC) grown in medium CMRL-1066 (7) or yeast-extract-lactalbumin hydrolyzate supplemented with 5–10% horse serum. The virus was harvested and purified on a column according to Sheinin (7) and others (8) or batchwise on Dowex 1-X2 (50–100 mesh). Infectivity was titrated on monolayers of secondary MEC in roller tubes (7); the end points were rechecked also by hemagglutination (HA) and repeated by plaque assays. The MEC were seeded with yeast–lactalbumin hydrolyzate containing 5% horse and 5% fetal calf serum on 60 × 15-mm plastic petri dishes (Falcon) as recommended by Crowford *et al.* (9) and Sheinin (10). The appearance of plaques took about 6 days, neutral red staining about 1 or 2 days. Heat inactivation of the PyV was carried out by boiling in sealed ampules for 1 hr. For HA, 0.2 ml of guinea pig RBC, 0.8% in phosphate buffered saline (PBS) was added to 0.2 ml of test material in 6 × 100-mm glass tubes and the results read after overnight incubation at 4°; the reciprocal of the last geometrical dilution giving partial agglutination was taken as 1 unit (HAU). *Saccharomyces cerevisiae* H and *Candida albicans* isolated from a patient were grown axenically on a fluid medium containing salts, bactopeptone, yeast extract, lactate, and glucose (3, 4, 11). Occasionally a chemically defined tissue culture medium (12) alone or with 0.15 *M* sucrose was also used.

Unpurified or highly purified PyV of known potency, 0.1–1 ml in medium or PBS, was equilibrated with an enumerated log phase yeast population, suspended in a 250-ml screwcapped Erlenmeyer flask with 0.5 ml of medium. After 30-min interaction at 24°, with or without intermittent agitation on a Clinical Rotator (Fisher Sci. Co.) at 120 rpm, 10–50 ml of medium were added and the cultures were reincubated at 28°. The cells were counted after sampling at zero, 24, and 72 hr, with or without medium replacement, under constant agitation at 120 rpm, on a conventional hemocytometer and/or Coulter counter, model B. Volumetric pipettes, flasks and micropipettes were used for sampling and in the preparation of 200–500-fold dilution, the former for microscopic, the latter for the electronic counting. Hand mixing by overturning the containers 5 times was made before sampling the original or taking 10 individual aliquots with a micropipette (20 μl) delivering approx 10 μl of the dilution on each side of the cytometer. All the cells covering both measured surfaces were counted and recorded by a Clay-Adams

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8 digit Laboratory counter. For the Coulter counter with 80 μ apertures, 10 individual samples were taken from the 400–500-fold dilution which remained under agitation during the whole procedure (13). The geometrical mean of 10 individual determinations was calculated and the results were expressed as the number of cells per milliliter ± standard error of the mean (SE) percent (4) or per mil (‰) according to the formula SE = $SD/n^{1/2}$. When cytometric and electronic counts were made simultaneously the average of 20 individual counts was recorded. Dye exclusion with trypan blue was a criterion of viability and the number of dead cells calculated in ‰ ± SE of the population. Budding cells were also counted and the rates were recorded as ‰ ± SE. Bud and mother cells were considered one single unit for the population counts. Controls were handled similarly, but with extract of normal MEC culture or heat-inactivated PyV, as sham inoculum. Recently the yeasts were homogenized by the zeolite techniques (14, 13) following centrifugation at 20.000 rpm for 45 min at 0–4°. The supernatant was removed and zeolite (Union Carbide, Type 4A 1/16 particles or Type 4A powder) 3.3 times wet weight of the yeast was added. Grinding was done in a glass mortar by mixing the cells with small portions of zeolite and water, or the original medium. After 3–5 min the homogenate was extracted with chloroform, Genetron, and ethyl ether, flushed by N_2 and dialyzed

TABLE I. Experimental Infection of Yeasts with Unadapted Polyoma Virus.

Cells (count ± SE; %) Incubation (28°)	Inoculum per system	Mortality (‰ ± SE)	Budding (‰ ± SE)	PyV yield per system	Serol. tests remarks
C. albicans[a]					
Infected: 72 hr	1360 HAU	105 ± 0.53	92 ± 0.69	82,000 HAU	HI: +(1:256)
1.58×10^7/ml ± 0.16	$10^{2.4}$ PFU			$10^{6.2}$ PFU	Neut.: +(1:128)
Normal:					
1.86×10^7/ml ± 0.25	MEC extract	59 ± 0.10	69 ± 0.63	Nil	Negative
C. albicans[b]					
Infected: 120 hr	2048 HAU	74 ± 1.10	57 ± 0.65	86,000 HAU	HI: +(1:512)
1.56×10^7/ml ± 0.20	$10^{3.2}$ PFU			$10^{6.5}$ PFU	Neut.: +(1:374)
Control:	Inact. PyV	43 ± 0.50	64 ± 0.18	Nil	Negative
1.98×10^7/ml ± 0.16	HAUϕ, PFUϕ				
S. cerevisiae[c]					
Infected: 72 hr	2048 HAU	97 ± 0.37	76 ± 0.21	141,000 HAU	HI: +(1:2048)
1.4×10^7/ml	$10^{3.2}$			10^7 PFU	Neut.: +(1:768)
Control:	Inact. PyV	54 ± 0.40	79 ± 0.80	Nil	Negative
1.54×10^7/ml ± 0.40	HAUϕ, PFUϕ				

[a] Expt. 30: 24-hr yeast culture, 0.5 ml + 0.2 ml of unpurified PyV, both in PBS; 0.5-hr interaction at 24° with 1-min agitation every 5 min; brought to 50 ml with growth medium; reincubated after sampling 4 vol % at zero-, and 10% at 24 and 48 hr with medium replacement. At zero-time: 284,000 cells/ml, 34‰ dead, 96‰ budding; ratio of budding/dead (B/D) = 2.85, dead/budding (D/B) = 0.35. Control: Same original culture, identical treatment, but 0.2 ml of MEC extract as sham inoculum. At zero-time: 277,000 cells/ml, 29‰ dead, 97‰ budding; B/D–3.27, D/B = 0.31. At 72 hr the mean of 10 cytometer counts, at zero-hr mean of 10 cytometer and 10 automatic counts are recorded. Titers as HAU/system and PFU/system; inhibition of HA (HI) per 0.2 ml, neutralization per 0.1 ml of 48-hr infected cell homogenate.

[b] Expt. 30/D: Similar to *a* but higher cell and virus input and longer incubation. No medium change after a single sample (1 vol %) at zero-time: 624,000 cells/ml, 32‰ dead, 73‰ budding: B/D = 2.28, D/B = 0.43. Control: 0.2 ml of heat-inactivated PyV. At zero-time: 624,000 cells/ml, 22.8‰ dead, 78‰ budding; B/D = 3.42, D/B = 0.29.

[c] Expt. 30/A: Similar to *b* but *S. cerevisiae* and shorter incubation without medium change. At zero-time: 230,000 cells/ml; 43‰ dead, 78‰ budding; B/D = 1.80, D/B = 0.55. Control: 240,000 cells/ml; 33‰ dead, 66‰ budding; B/D = 2.00, D/B = 0.50. Counts in Expt. 30/D and 30/A are the mean of 10 cytometer countings.

against PBS and water. Sterility tests were made and the preparation was stored at —25 or —90° (13). More recently, instead of centrifugation, Millipore membrane, 0.45-μ pore size, was used to separate cells from medium; the filter was dissolved with 3 drops of acetone and the cells homogenized with zeolite. Further refinement was the omission of organosolvents, other than ether, to prevent loss of titers. Immune sera were prepared by subcutaneous, intramuscular, and intravenous administration of column purified PyV (1.5×10^8 PFU/ml) in increasing doses to Belgian rabbits. The sera were used for HA inhibition (HI) and neutralization tests (15).

Results. Infection of the yeasts was carried out in over 60 individual experiments. Characteristic findings are presented in Table I. The first example, Expt. 30, illustrates an assay with medium replacement at samplings assuring by this way constancy of volume and fresh nutrient but also causing virus loss, which has to be taken into account at the calculation of the titers. At the end of the incubation with PyV the normal yeast culture exhibited significantly higher cell counts than the infected one, perhaps because the mortality rate rose 44%, the budding 25% in the experimental system, relative to the normal control. Simultaneously, a 60-fold increase in HA and 3.8 log in infectivity was noted over the inoculum, suggesting the multiplication of the PyV in yeast cells. The consequence of this virus propagation was the increase of the mortality over the budding rate. The opposite was true with the uninfected culture. The progeny was identified as PyV by HI and neutralization tests. When the experimental design was altered some variation was observed as illustrated with the findings in Expt. 30/D. Here the incubation lasted 48 hr longer without changing medium and the control received inactivated PyV as a token inoculum. The titers recorded as HAU/system increased 42-fold, the infectivity increased 3.3 logs over the input, thus the yield was negatively affected by the prolonged incubation without renewal of the medium. The mortality rate conformed to the established pattern. The budding was 13% lower than that of the control and it was significantly lower than the mortality rate, resulting in a drop in the density of the infected cell population. Assays with a different species, but 48-hr shorter incubation, revealed a great increase in HAU (68-fold) and infectivity (3.8 log) over the inoculum, as illustrated with the findings of Expt. 30/A. Although the mortality was 45% higher in the infected culture and the budding 4.7% lower, a relatively small difference was found in cell counts, at the end of the experimental period. Additional parameters are presented in Table II. The decrease in population den-

TABLE II. Additional Parameters of PyV-Infected Yeast Population.

Cells, incubation (28°), difference in cell counts	Distribution of PyV as HAU	Reduplication times cell input	Budding/mortality	Mortality/budding
Candida[a]	Med.: 26.2%			
Infected: 72 hr	Cells: 73.8%	59.70	0.87	1.14
Normal:	Nil	77.80	1.16	0.86
Infect./norm. = 18.2%				
Candida[b]	Med.: 4.6%			
Infected: 120 hr	Cells: 95.4%	25.10	0.79	1.29
Control:	Nil	37.40	1.48	0.67
Infect./contr. = 21.3%				
S. cerevisiae[c]	Med.: 12.50%			
Infected: 72 hr	Cells: 87.50%	59.40	0.78	1.20
Control:	Nil	64.10	1.40	0.67
Infect./contr. = 12%				

[a] Expt. 30: cell population density, as mean of 10 cytometer and 10 electronic counts.

[b] Expt. 30/D: control with heat-inactivated virus.

[c] Expt. 30/A: assay with a different species. Viability and budding as mean of 10 cytometer counts; for details see Table I.

sity of the infected culture, tabulated as percentage difference between the cell counts of the system and control, is due to the increased mortality and, in some assays, the depressed budding rate as well. At the end of the experiments the virus yield was mainly cell associated, ranging from 76 to 95%. The variations are maybe due to the different designs and techniques used. The great decline of the factor of reduplication of the cell input in the infected culture is also noteworthy. For instance the uninfected population doubled its original size 18.1 times more than the infected one in Expt. 30. The adverse effect of long incubation may explain the findings in Expt. 30/D and the species difference in Expt. 30/A. The ratio of budding/dead cells is another useful index of the effect of PyV infection on yeast populations. In the controls this B/D ratio was over unity (1.16–1.48), but well under 1.0 in the infected population (0.78–0.87). However

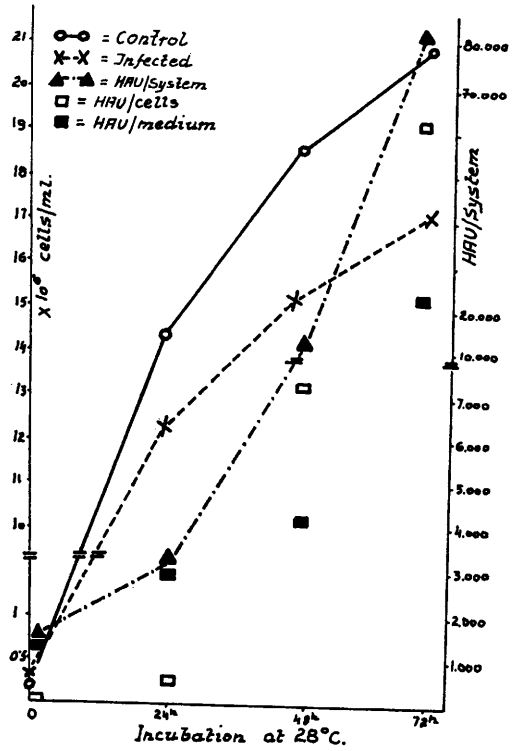

FIG. 1. Change in cell population density of PyV-infected *C. albicans*; Expt. 30, average of 10 cytometer and 10 automatic (Coulter) counts; SE ranging ± 1% (for methods see Table I).

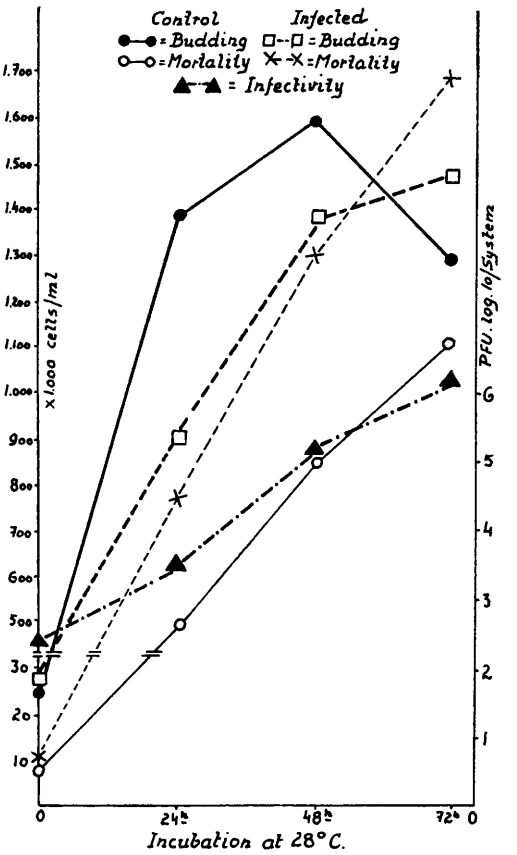

FIG. 2. Viability and budding in *C. albicans* infected with PyV; Expt. 30. Average of 10 cytometer counts (see Table I and Fig. 1).

from the zero-time values listed under Table I it is evident that similar changes may start with the first virus–cell interaction as will be further discussed. The sequence of events will be analyzed in Figs. 1–4. The change in cell population density is illustrated in Fig. 1, the mean of 10 automatic and 10 microscopic cell counts being plotted against time of incubation. A small but progressive difference between system and control is evident, ranging from 14% at 24 hr to 18.2% at 72 hr. The increase in HAU (and infectivity, v.i.) went parallel with the population changes, exhibiting a steep rise—a 3-fold increase at 24 hr to 10-fold at 48 hr and 60.2-fold at 72 hr. At zero-time approx 98% of the inoculum was free, but after incubation this distribution changes gradually, at 24 hr almost 11% being intracellular, at 28 hr approx 67% and

at the end about 76% was cell associated. Similar was the distribution of infectivity (not plotted). The cell-biological aspects are illustrated in Fig. 2, demonstrating the increase in mortality of the yeast cells during incubation with virus and simultaneously with a 3.8 log rise in infectivity. The number of budding cells increased substantially, the experimental system exhibiting lower values up to 48 hr, then an abrupt drop in the mitotic activity of the controls and a steady rise in that of infected cells, thus a crossover of the curves occurred. The next section deals with comparison of individual assays repeated under similar experimental conditions. Figure 3 illustrates the factor of reduplication of the original cell input during incubation. Expt. 39 received a somewhat larger inoculum, but adsorption and further treatment were identical with that of Expt. 30. The similarity in behavior is striking, as regards the increase in HAU/system (59.5-fold for Expt. 39 versus 60.2 of Expt. 30) and cell population changes. After infection the cell seed increased in size 37.7-times during 24 hr, in Expt. 39, but 47.8-times in the control. This compares well with Expt. 30 where a similar deficit was observed, in spite of the fact that in both systems the cell counts at zero-time were larger than in the controls. The pattern did not change with further incubation and there was much similarity between the systems which remained significantly below the reduplication capacity of the controls. These data are further substantiated in Fig. 4, where the mortality and budding rate as percentage of the cell population, assessed by the usual techniques, is plotted against increase in HA of the system. 96.7% of the yield was cell associated at 72 hr. The corresponding final infectivity titer is also recorded in the Fig. 4 legend, measured in PFU and checked by neutralization assays in tube cultures of MEC. The results are very similar to those illustrated before, as regards the increased mortality or the final crossover of the budding and mortality curves at 72 hr. Virus distribution also conformed with the general observation. Thus Figs. 3 and 4 illustrate the reproducibility of the findings, the difference in HA and infectivity may be due to the organosolvent extraction in Expt. 30, which was replaced by the zeolite techniques in Expt. 39, allowing higher virus recovery in the latter.

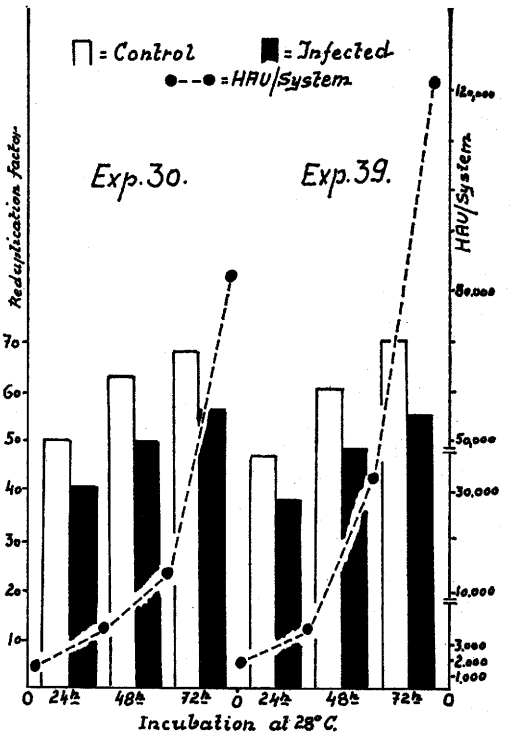

FIG. 3. Reduplication of PyV-infected yeast cultures; comparison of two experiments; Expt. 39, input 3.26 × 10^5 cells/ml + 0.2 ml of PyV (2048 HAU, $10^{8.2}$ PFU); infectivity titer at 72 hr = $10^{6.9}$; neutralization: +(1:1024) in 0.1 ml, HI: +(1:1024) in 0.2 ml of yeast cell homogenate. Control, 2.94 × 10^5 cells/ml, further treatment same as Expt. 30 (Table I and Figs. 1, 2).

Discussion. Unadapted PyV was successfully propagated in two different species of yeast, *S. cerevisiae* and *C. albicans* isolated from a female patient. The present paper reports this primary experimental infection of Protista with phylogenetically distant mammalian virus, carried out in over 60 individual assays, using various techniques. Thus the findings are reproducible, although the yields were relatively low. In many instances the estimate is rather conservative, because the extensive mechanical homogenization and organosolvent extractions (chloroform, Genetron, ether) caused considerable virus loss. The filtration and zeolite homogenization,

combined with washings facilitates the quantitative recovery of the progeny. The low efficiency of virus production however, may depend on many other factors. First of all on the defective mechanism of virus release, the majority of the yield remaining firmly associated to the yeast cells, precluding superimposition of infective cycles. The spontaneous sedimentation of the yeast cells in stationary cultures does not promote virus–host interaction. Further the virus input was of low multiplicity to facilitate quantitation, the adsorption period was short, and for this reason the unattached inoculum was not eliminated to allow further uptake by the cells. Unpublished assays underway demonstrated that adaptation, supraoptimum temperatures, aeration, and a defined medium (12) may enhance the virus yield considerably and shorten the incubation period. Similarly the adsorption is more efficient when carried out longer and under agitation although the mechanism of uptake has to be clarified. The species difference may be another factor, which needs further investigation. The cell biological and pathological changes occurred simultaneously with the propagation of PyV in yeast. Since extreme accuracy was realized in automatic enumeration of the cells, rechecked in double-blind experiments, the standard error ranged with the 1% limit claimed by the manufacturers (Coulter Co.). The microscopic counting in our hand had a comparable margin of error, thus the changes in mortality and budding rate are significant. In the cells inoculated with live virus the mortality rate starts to rise during the adsorption relative to the prezero counts. There was a slight, perhaps not significant change with heat-inactivated virus, as will be reported separately, thus the PyV particles, adsorbing to, or viral macromolecules penetrating into the cells, may initiate these early events. The parallelism between virus growth and cell mortality suggest a causal relationship. The adverse effect of heterologous macromolecules on yeast was described recently (16), proteins, enzymes, and nucleic acids causing gross changes in permeability and viability of these microorganisms, generally thought to be extremely resistant to environmental influences. Particulate nucleoproteins, such as viruses or viral genomes may exert similar effects. From the early onset of viability changes, we may assume that the virus particles adsorb to, and interfere with, the activity of some important enzyme systems. Cytological, biochemical, and fine-structural work is needed for the elucidation of the pathomorphological substrate of the alterations described in PyV-infected yeast. There is no doubt about the specificity of the infection, because various serological tests were carried out in every important experiment with polyoma-antiserum. Infectivity and HA titers were measured simultaneously, with fair agreement, indicating the production of complete virus. This may be of special interest, because others used isolated DNA of PyV to infect Protista (17) but we succeeded with the virion. Further we confirmed (6) in a different system, the results of Bayreuther and Romig (17), thus we assume that similarly to bacteriophage, nucleic acid penetration may be the final operative mechanism. However even after infection with virion, the production of new progeny occurs within a few hours, as observed in unpublished "one step" experiments. This rapid biosynthesis may be due to the rich biochemical setup of the yeast cells. The nutritional factors have decisive influence, as illustrated by examples from assays with medium replacement and others with prolonged incubation, without renewal of the nutrient fluid. This question is under further study with help of a defined medium (12), the apparent stimulation of budding by PyV having been confirmed in unpublished experiments. The biological significance of the observations described lies in the demonstration of the universality of virus infection as a cellular event based on the general validity of the DNA code (18). We assume therefore that practically any cell should be able to reproduce any type of virus, under certain experimental or ecological conditions (19). The medical significance seems to be that a human parasitic microorganism, living in an organ of high cancer incidence, is able to take up a mammalian oncogenic virus, carry, multiply, and perhaps liberate it slowly. This

new vista in the genesis of infection and/or cancer may be worthy of further consideration. The possibility of spheroplast formation, due to the use and misuse of antibiotics (20), underlines the above speculations and the need for further experimental and clinical work.

Summary. Experimental infection of yeasts, *C. albicans* and *S. cerevisiae* with unadapted polyoma virus was carried out reproducibly under various experimental conditions, measured by increase in hemagglutination and infectivity during incubation. All important assays were repeated several times with similar positive results. Approximately 68-fold increase in HAU and approx 10^4 increase in PFU/system over the inoculum was observed with unadapted PyV, but this yield may be increased with adaptation and further technical refinements as will be published separately. The virus propagation brought about cell biological and population changes which were illustrated and discussed together with the significance and bearing of the primary infection of two different species of yeasts with an oncogenic virus.

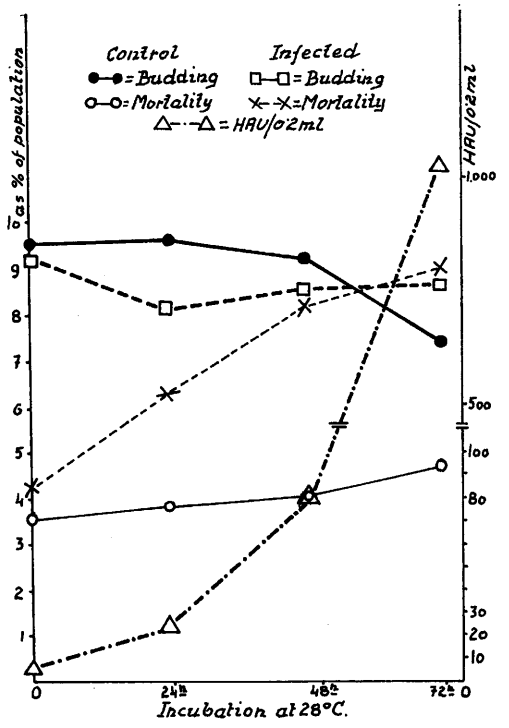

FIG. 4. Reproducibility of the changes in mortality and budding rate of PyV-infected *Candida*; Expt. 39. techniques as Fig. 3; rates as percentage of population; actual uncorrected HA values/0.2 ml of system; for further details see Fig. 3.

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