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Radiometric Estimation of the Replication Time of Bacteria in Culture: An Objective and Precise Approach to Quantitative Microbiology

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In a recently developed, two-compartment liquid scintillation vial, the evolution of $^{14}CO_2$ resulting from bacterial metabolism of uniformly labeled d-glucose was measured sensitively, cumulatively, and automatically in a liquid-scintillation counter. In each of eight species tested, a period of log-linear expansion of cumulative counting rate with time was observed. The exponential increase in cumulative counting rate was related to cell replication time by the integral of a first-order differential equation. Within a given species, the replication time measured by radioassay was found to be remarkably constant, unaffected by a fourfold variation in the activity of added labeled d-glucose, insensitive to the presence of carrier dextrose, and independent of the number of bacteria in the initial inoculum over a range of five orders of magnitude. These experiments demonstrate that the replication rate of an organism in culture is a highly reproducible characteristic that is susceptible to precise radiometric measurement in fundamental units of time under a variety of experimental conditions.

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The manifest utility of automation in microbiology has prompted the development of a number of automatic growth-detecting and -recording systems. Methods reported to date have been obliged to report their results in arbitrary, system-dependent units—e.g., "growth index" (*1*), "light scattering index" (*2*), "differential light scattering" (*3*)—which must be empirically related to some function of bacterial growth. The established empirical relationships have been valid only in a very restricted set of experimental conditions.

In this paper we describe a radiometric procedure that sensitively, cumulatively, and automatically tracks the cell population through its early phase of exponential expansion. The technique utilizes a two-compartment scintillation vial, carbon-14-labeled d-glucose and a liquid-scintillation counter. From the measurements so obtained, a mathematical expression is derived for the determination of cell replication rate in basic units of time. The equations indicate that the replication times determined by this technique should be independent of counting sensitivity and the concentration of the inoculum. This hypothesis is tested under a variety of conditions in the experiments that follow.

MATERIALS AND METHODS

The scintillation vial. These components have been described previously (*4,5*). Briefly, they consist of a sterile glass metabolism vial surrounded by a cylinder of specially treated scintillating filter paper, the whole contained within a wide-mouthed, plastic liquid-scintillation vial assembled as shown at the

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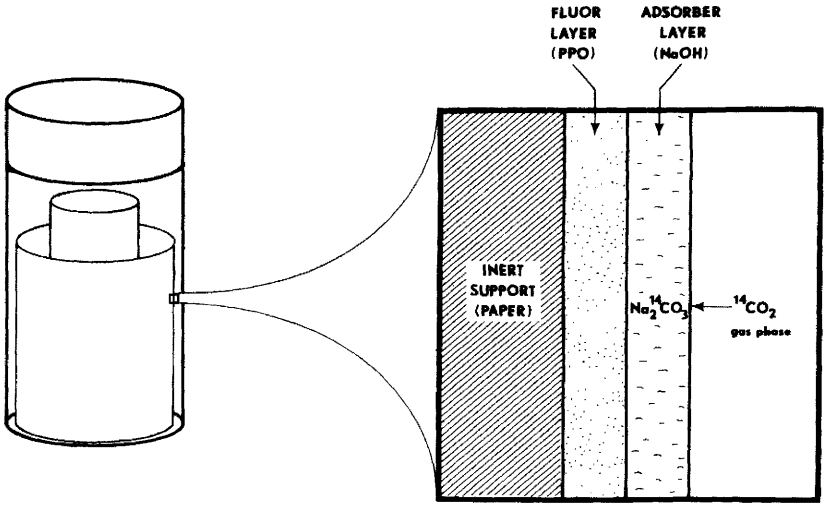

FIG. 1. Components of the two-compartment scintillation vial.

left of Fig. 1. The paper is prepared by dipping 38 mm-wide strips of convenient length through a concentrated toluene solution of fluors after which the solvent is evaporated (in a hood) leaving an adherent layer of fluors as in the idealized enlargement at the right of Fig. 1. The dried strips are then cut to such a length as to form a closely fitting cylindrical shell within the plastic scintillation vial. After they are in place, the strips are moistened with 100 μl of 2 N NaOH distributed around the circumference of the cylinder. The vials are then tightly capped to prevent neutralization of the alkali by atmospheric CO_2, and stored in the dark to preclude photoactivation of the alkalinized fluor.

On the day of the test, the inner vials are aseptically inoculated with (typically) 3 ml dextrose-free trypicase soy broth and two milliliter quantities of dextrose-free TSB, the first containing suspended bacteria, the second 1 μCi uniformly labeled [^{14}C] d-glucose. The loaded inner vials are placed within the previously prepared scintillation vials under subdued light, and the assembled units are counted automatically and repetitively in a liquid-scintillation counter modified to maintain sample temperature at 37°C.

When glycolytic metabolism in the inner vial produces $^{14}CO_2$, this gas is absorbed by the treated paper, which then produces detectable scintillations. The sampling method is cumulative and continuous, so that the observed counting rate either increases or remains constant according to the rate at which metabolism is or is not proceeding within the inner vial.

The biphasic, aqueous/organic scintillation system is similar to one described by Sternberg (*6*) in that the beta particles interact with the fluor by direct incidence so that there is little opportunity for the solute quenching that is troublesome in conventional liquid scintillation counting. The principal determinant of counting efficiency is the ratio of aqueous $Na^{14}CO_2$ to organic fluor. Excess water results in increased sample self-absorption in the aqueous phase, hence the need to limit the alkaline solution to a volume (100 μl) that is manifestly insufficient to wet the paper evenly.

The determination of replication time. For so long as the substrate activity is not significantly depleted, the rate of evolution of labeled CO_2 will be proportional to the numbers of bacteria present. When the bacterial population is experiencing exponential growth, the counting rate, exhibited by an integrative sampling system sensitive to the evolved, labeled CO_2, will increase at the same exponential rate as the population of cells.

The replication time of the organisms was found by plotting the observed cumulative activity (log scale) against incubation time (linear scale) on semi-log paper. The curves were inspected to discover the interval during which log-linear expansion had taken place. A transparent straight-edge laid over the curve was used to select the three most nearly log-linear data points. The cumulative counting rate at the first of these was established as "$\sum R_1$" and at the third, as "$\sum R_2$" in the equation below. Log-linear expansion usually began at the time when the counting rate had reached several hundred cpm. From sequential observations of cumulative activity during log phase, an exponential growth constant (to the base 10) was found by substitution into equation (1);

$$\log R_2 - \log R_1 = K(t_2 - t_1), \qquad (1)$$

where:

R_1 = indicated activity (CPM) accumulated at time t_1,

R_2 = indicated activity (CPM) accumulated at time t_2,

K = exponential growth constant, min^{-1}, and

$t_2 - t_1$ = the time interval, min.

The replication time is found from the exponential growth constant by equation (2):

$$t_{2x} = \frac{0.3010}{K}, \qquad (2)$$

where

t_{2x} = replication time, min, and

0.3010 = log 2.

Note that the exponential growth constant, K, and hence the derived replication time, t_{2x}, depend exclusively on the slope of the line and is independent of the absolute values of $\sum R_1$ and $\sum R_2$. The derivation of these expressions has been reported (*5*) and is repeated as Appendix A.

The replication times of eight organisms were determined. These were *E. coli* (ATCC #25922), *S. faecalis* (clinical isolate), *Klebsiella* (resistant clinical isolate), *Pseudomonas* (ATCC #27853), group B β streptococcus (stock culture), *S. aureus* (ATCC #25923), *S. epidermitus* (resistant clinical isolate), and *Candida Albicans* (clinical isolate). The organisms were cultured overnight in standard TSB broth. On the morning of the test, the suspensions were diluted with dextrose-free TSB broth to a concentration of 10^4 organisms/ml. The determinations were made in quadruplicate with a 1 ml inoculate of 10^4 organisms per vial. The TSB broth was enriched with carrier dextrose to a final concentration of 50 mg/dL. The vials were counted automatically at intervals of 45 min.

The effect of inoculum concentration was determined in an experiment in which the vials were prepared and analyzed as described above, except that the determinations were run in quintuplicate with 10^6, 10^5, 10^4, 10^3, and 10^2 *E. coli* per vial.

The effect of substrate activity and/or counting sensitivity was measured in another experiment in quintuplicate using 10^4 *E. coli* per vial. The total activity of the added labeled d-glucose was 1.0, 0.5, and 0.25 μCi in the three sets of five in the series. Varying the substrate activity is the exact equivalent of varying the sensitivity of detection, and the results may be taken to be representative of either effect.

The effect of carrier dextrose on the measured replication time of *E. coli* was determined by comparison of the results obtained when the broth was enriched with 50, 25, 12.5, and 6.25 mg/dl dextrose with those found when no carrier dextrose was added. In the latter case, the broth was not entirely carrier-free since a small amount of dextrose (10^{-4} mg/dl) remained from the original dextrose-containing TSB in which the organisms were initially cultured even after a 10^9–10^4 dilution in dextrose-free TSB.

The within-species reproducibility of observed replication time was tested with six clinical isolates of *E. coli*. Each isolate was run in quintuplicate.

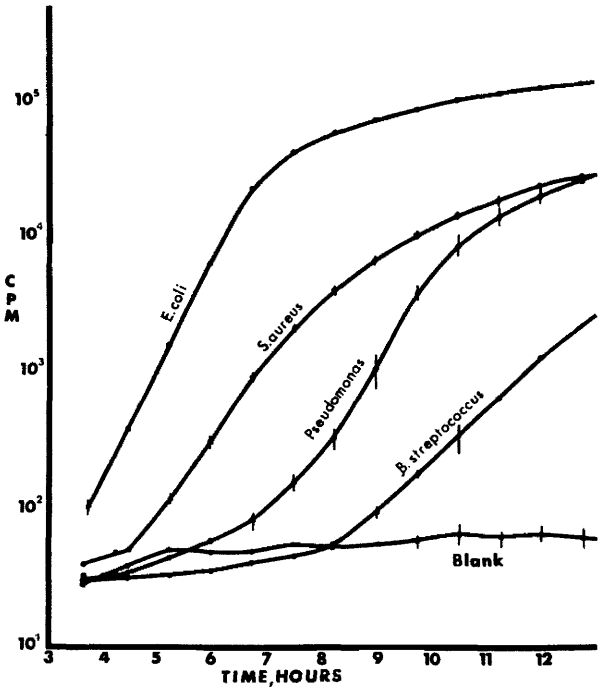

FIG. 2. Representative semi-log growth curves for several species.

RESULTS

Measured replication times of several species. Representative results with several of the species tested are shown in Fig. 2 (semilog plotting). The standard deviations at each point are shown by the vertical

TABLE 1. OBSERVED REPLICATION TIMES, VARIOUS ORGANISMS

Organism	n	$t_{2x} \pm$ s.d. (min)	% s.d.
E. coli	4	21.89 ± 0.38	1.74
S. faecalis	4	22.65 ± 0.14	0.62
Pseudomonas	4	25.20 ± 2.31	9.17
S. aureus	4	30.10 ± 0.90	2.99
Klebsiella	4	30.19 ± 0.85	2.82
β streptococcus	4	42.34 ± 2.37	5.60
S. epidermidis	4	48.19 ± 2.56	5.31
Candida	4	55.69 ± 2.11	3.78

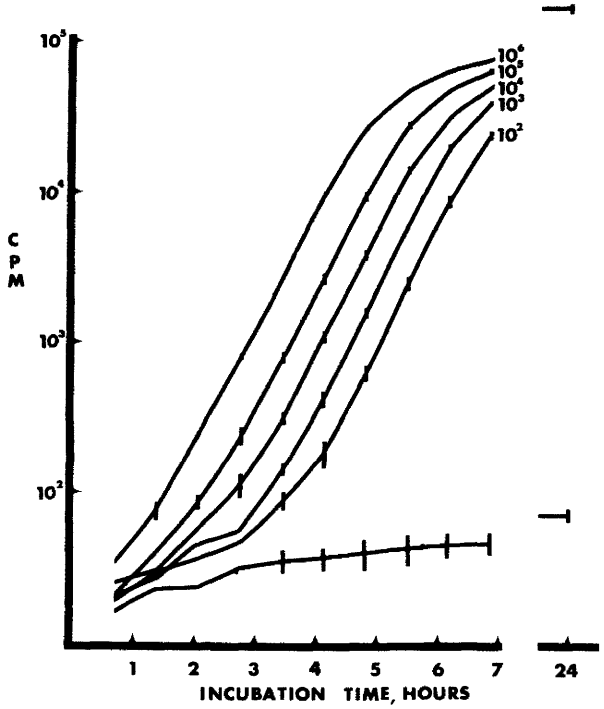

FIG. 3. Effect of bacterial concentration in inoculum.

bars, a convention followed in subsequent figures, except as noted.

In general, there was a lag phase of varying length, then an exponential phase of several hours, and then an asymptotic approach to a plateau value. The replication times obtained during exponential phase of all eight organisms are summarized in Table 1. The measured replication times have a worst-case relative s.d. of ±9.2% (*Pseudomonas*) and are generally ±5% or less.

Within-species errors. The effect of bacterial concentration is illustrated in Fig. 3. The larger the number of organisms, the sooner the result is obtained. Apart from that, the inoculum concentration has little or no effect upon the measured replication time of *E. coli*. Table 2 summarizes these results. Analogous experiments (*5*) with several other organisms (*S. aureus, S. typhosa, β* streptococcus) have indicated similar independence from the effect of bacterial concentration.

TABLE 2. BACTERIAL CONCENTRATION VS. MEASURED REPLICATION TIME, t_{2x}

Inoculum	n	t_{2x} (min)	Grand mean
10^6	5	23.00 ± 0.31	
10^5	5	23.06 ± 0.31	
10^4	5	22.34 ± 0.35	(n = 25)
10^3	5	22.59 ± 0.35	22.51 ± 0.57
10^2	5	21.77 ± 0.39	

The effect of substrate activity and/or counting efficiency is illustrated in Fig. 4 and tabulated in Table 3. The indicated cumulative activity at any time (conveniently, at 24 hr) is perfectly and linearly correlated (r = 1.00) with substrate activity, as would be expected of a precise and linear counting system. Measured replication time, however, is constant to within ±2% (relative standard deviation of the grand mean, n = 15) over this fourfold variation in activity/sensitivity. The organism evidently reproduces as rapidly as its nature and the conditions of incubation will permit, whether or not it is observed to do so. Provided only that it be sufficient for the purpose, the sensitivity of the surveillance system has no effect at all on spontaneous replication time.

The effect of carrier dextrose is illustrated in Fig. 5, which omits the s.d. bars to avoid overcrowding. Competitition from the presence of unlabeled carrier delayed radiometric detection by several hours, although all samples approached the same end-point activity. The log-linear portions of the growth curves have essentially the same slopes and hence yield nearly identical replication times as presented in Table 4. This result suggests that, for *E. coli*, the replication time as determined radiometrically is independent of the dextrose concentration. This particular finding is not likely to be universally true, however, since the growth rate of less versatile organisms may be more dependent upon the concentration of dextrose in the broth. Nevertheless, it is reasonable to infer from these data that this radiometric method is no more dependent on the presence of carriers than are the organisms themselves.

The within-species reproducibility is illustrated in Fig. 6 (s.d. bars again omitted). The slopes of the growth curves are virtually identical, as are the derived replication times. The grand mean of all thirty determinations (five of each of six isolates) was 22.14 ± 0.85 min (mean ± s.d.). This within-species variation is equivalent to a relative s.d. of ±3.84%.

DISCUSSION

Methods that specify, a priori, any particular incubation time or time interval necessarily become dependent upon the time or interval selected as well as the status of the culture at that time (lag phase, log phase, plateau phase), the concentration of bacteria in the inoculum, the sensitivity of detection, and a number of other variables. When the chosen method is radiometric, it may also be dependent upon counting sensitivity and the specific activity of the labeled substrate. The response of such systems cannot be related to bacterial growth except by em-

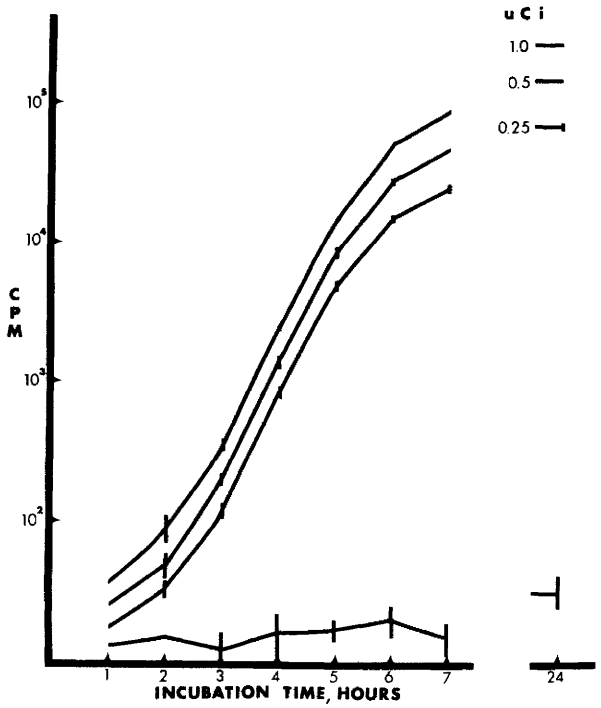

FIG. 4. Effect of subtrate activity.

perical methods, with arbitrary units that must be empirically redefined each time a system parameter is changed.

With the cumulative scintillation system described herein, there is no need to preselect any particular inoculum size or incubation period. Whatever the size of the inoculum, the system automatically tracks the cell population to and through its exponential growth phase and the measurements are made during that interval, whenever it occurs. During uninhibited exponential growth, the rate of change in the number of cells in the population at any time is exactly described by a first-order differential equation. This equation cannot be solved unless the number of cells initially present and the absolute sensitivity of detection are both known. In its integrated form, however, the equation yields an estimate of cell replication rate in basic units of time from any two consecutive observations of counting rate, and neither counting sensitivity nor bacterial concentration need be known. The cumulative-sampling scintillation system automatically provides direct measurements of counting rate.

The nonlinear behavior of replicating cell populations confounds linear analysis even when objective methods of measurement (optical, radiometric, etc.) are applied, hence the proliferation of empirical methods in microbiology. By taking advantage of the observation that linearity can be found in the exponential domain, much of the empiricism can be removed. Just as the half-life of a radionuclide can be rigorously deduced from serial measurements of exponential decay, even with an uncalibrated counting system, so can replication time be derived from a series of measurements of exponential growth, which bear a constant but unknown relationship to the number of cells in an exponentially expanding population.

In a system that measures the evolution of a metabolite of a labeled substrate, first-order kinetics are closely approximated only for so long as the concentration of that substrate is not significantly reduced. Only systems that can complete their measurements before, say, less than 10% of the substrate is consumed, will exhibit a first-order response to growth rate that can be related directly to replica-

TABLE 3. REPLICATION TIME, t_{2x} VS. SUBSTRATE ACTIVITY

μCi [^{14}C] d-glucose	n	t_{2x} ± s.d. (min)	Grand mean
1.00	5	22.08 ± 0.47	
0.50	5	22.10 ± 0.45	(n = 15)
0.25	5	22.34 ± 0.43	22.17 ± 0.43

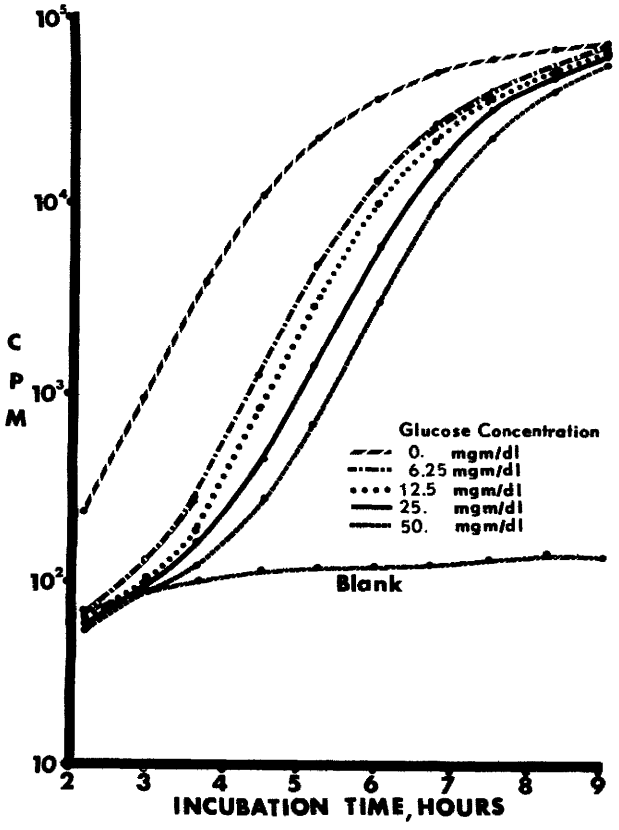

FIG. 5. Effect of carrier dextrose.

TABLE 4. EFFECT OF CARRIER DEXTROSE

Stable dextrose concentration	n	Replication time (min)	Grand mean
50	5	23.31 ± 0.41	
25	5	23.18 ± 0.63	
12.5	5	22.28 ± 1.21	(n = 25)
6.25	5	21.94 ± 0.34	22.72 ± 0.82
0	5	22.74 ± 0.39	

tion time independently of sensitivity and the number of bacteria initially present in the inoculate. The scintillation method is more than sufficiently sensitive to provide three or more measurements during the early, log-linear phase of exponential growth. Moreover, the counting rate of the vials may be sampled as often as desired (even continuously) without compromising the sensitivity of detection.

The experiments reported here demonstrate that this replication time determined radiometrically is unaffected by a fourfold variation in the activity of added labeled d-glucose, insensitive to the presence of carrier dextrose and independent of the number of bacteria in the inoculum, over a range of five orders of magnitude. The within-run, within-species, and between-run errors are of the order of ±5% or less, even in the face of these large and deliberate variations in technique.

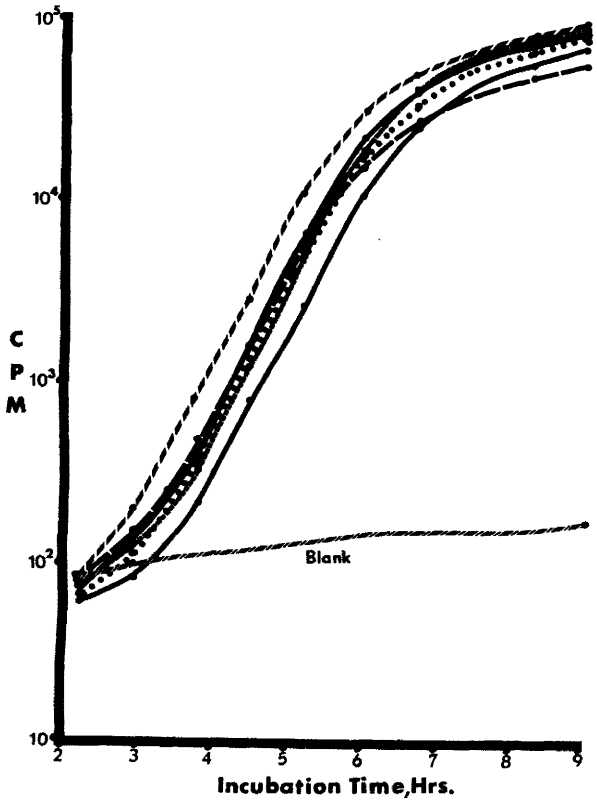

FIG. 6. Within-species reproducibility (6 isolates of *E. coli*).

These results indicate that the replication time of an organism in culture is a highly reproducible characteristic that is susceptible to precise measurement in basic units of time under a variety of experimental conditions. The effects of subtle influences on growth rate can therefore be determined to a high degree of confidence and expressed in rational units without the need to adhere to arbitrarily defined procedures and units. The method provides the opportunity to obtain objective measurements of bacterial growth and inhibition to a precision equal to that of the more reproducible methods of clinical chemistry.

APPENDIX

It has been shown that the number of cells in a population replicating at a constant rate may be described by the following equation:

$$N_t = N_0 e^{kt},$$

where N_t = no. of cells at time t; N_0 = no. of cells at time zero; and k = exponential growth constant, in reciprocal units of time.

If the cell population is producing CO^2, the rate of CO_2 production will be proportional to the number of cells present at the time:

$$\frac{d}{dt}(CO_2) \propto N_0 e^{kt}.$$

When the CO_2 is radioactive and when it is being continuously evolved, cumulatively collected, and counted with some overall efficiency, ϵ, the counting rate, R, will increase with time at the rate:

$$dR/dt = \epsilon N_0 e^{kt}.$$

The counting rate due to accumulated CO_2 at any time, t, is the integral of the above expression, viz:

$$\int_0^t dR/dt = \epsilon N_0 \int_0^t e^{kt}.$$

By definition, the integral of dR/dt from t = 0 to t = t is the counting rate from CO_2 accumulated to time t, R_t. Then

$$R_t = \epsilon N_0 \left(\frac{e^{kt}}{K}\right)_0^t,$$

$$R_t = \epsilon N_0 \left(\frac{e^{kt}}{K} - \frac{1}{K}\right),$$

$$R_t = \frac{\epsilon N_0}{K}(e^{kt} - 1).$$

After a period of time sufficient for several replications, e^{kt} becomes much larger than 1, so that the expression reduces to

$$R_t = \frac{\epsilon N_0}{K} e^{kt} \text{ (when } e^{kt} >> 1).$$

The value of k can be found conveniently from two consecutive measurements of R_t taken over an interval during which the logarithm of R_t is increasing linearly with time (i.e., when $e^{kt} >> 1$) as follows:

$$\frac{R_{t_1}}{R_{t_2}} = \frac{\frac{\epsilon N_0}{K} e^{kt_2}}{\frac{\epsilon N_0}{K} e^{kt_1}},$$

$$\frac{R_{t_2}}{R_{t_1}} = \frac{e^{kt_2}}{e^{kt_1}} = e^{k(t_2 - t_1)},$$

$$\ln R_{t_2} - \ln R_{t_1} = k(t_2 - t_1).$$

In common logarithms where k is an exponent of 10 rather than of e, the equivalent expression is

$$\log R_{t_2} - \log R_{t_1} = k(t_2 - t_1). \quad (1)$$

The characteristic replication time of the organisms, t_{2x}, may be found by using the value of k so obtained to solve for the time at which $R_{t_2} = 2R_{t_1}$, viz:

$$\log \frac{2R_{t_1}}{R_{t_1}} = kt_{2x},$$

$$\log 2 = kt_{2x},$$

$$0.3010 = kt_{2x},$$

$$t_{2x} = \frac{0.3010}{k}. \quad (2)$$

One could of course chose to work with logarithms to other bases, the base "e" or, most logically for replicating populations, the base "2." We have chosen to work in common logarithms chiefly because of the ready availability of semilog graph paper with a y-axis vertical scale in those units. We can thus present results in a graphical form directly comparable with equations 1 and 2, as in the figures in the text.

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