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21: Dry weight, packed cell volume and optical density

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CONTENTS

I. INDICES OF GROWTH

Physiological and biochemical investigations of microscopic algae are generally conducted on cultures of algal cells as experimental objects. The obtained data reflect the activities of a multitude of cells. Quantitatively, they are usually referred to a unit of cell mass. Cell mass can be expressed as dry weight of cells or as packed volume of cells in a volume unit of a given microbial culture. In a growing culture, dry weight and packed volume of cells per volume of cell suspension (of medium) increase with time, and this increase, termed growth, can be the specific subject of observation. Another characteristic, widely used as a measurement of growth, is an increase in optical density (turbidity) of a suspension of algal cells. Other indices of growth, such as increase in cell number, accumulation of carbon, nitrogen, protein, or some products of cell metabolism (starch, acids, etc.), are used in growth measurements (see Chps. 19, 20).

II. YIELD AS A GROWTH INDICATOR

Growth can be expressed as yield or as growth rate. Yield, as an expression of organic production, is usually given in terms of dry or fresh weight of the organic mass produced over a period of time per unit of volume or unit of area occupied by a given organism. Thus,

$$Y = \frac{X_1 - X_0}{A \text{ (or } V)} \qquad (1)$$

where X_0 and X_1 are quantitative expressions of the mass of cells given usually in terms of dry or fresh weight of cells at the beginning (X_0) and at end (X_1) of the growth period under observation and A (or V) are correspondingly the area or volume occupied by a given population of microbial cells. Since illumination is one of the most critical factors determining the productivity of photoautotrophic organisms, the yield for these organisms is often expressed in terms of production per unit of illuminated area.

Yield, as defined in the preceding paragraph, is equal to what has been previously designated as total growth, G (Monod 1949). Since the term

'growth' generally applies not only to the product of the process but also, and even more frequently, to the process itself, it is expedient to use the term 'yield' for the designation of the product and to reserve the term 'growth' only for the process of the accumulation of the product.

The adjective 'total', in the term 'total growth', is also misleading, because total growth, or what is here called yield, does not represent total production as the outcome of all growth processes, but only the balance between the anabolic and catabolic processes. In addition to losses of organic mass due to catabolism, there may be losses caused by other factors – such as death of some cells. These losses may or may not have any relation to productivity of the organism. If an attempt is made to estimate these losses, the distinction can be made between net yield (designating the actually-harvested cell mass) and the corrected or potential yield, which would also include losses due to external factors and, depending on the purposes of the investigation, also losses due to catabolic processes.

The limitations, in using 'yield' as an indicator of growth, must be clearly understood. In estimations of yield, time period is either chosen arbitrarily or is determined by the cessation of growth. Cessation of growth is a valid consideration for determinations of yield of organisms with limited growth. Cessation of growth comes naturally, for instance, in annual and biennial plants at the end of the vegetative period, and thus indicates the moment of harvesting when the yield must be determined.

In populations of microorganisms, growth is generally unlimited unless external factors change to preclude further growth. By manipulating external factors, the moment of growth cessation can be moved or eliminated altogether. The decision to interrupt growth and to determine the yield or to wait until the complete cessation of growth is, therefore, arbitrary and may contradict the basic purposes of the investigation.

A serious limitation of yield, as an indicator of growth, is that the course of growth, within the period for which yield is estimated, remains unknown. During some portions of that period, growth rate may be lower, net growth may be absent, or it may even give place to loss of organic substance due to a predominance of catabolic over anabolic processes. For all of the above reasons, in all possible cases, estimations of yield must be accompanied by evaluations of the kinetics of growth as a process within the overall period for which yield is determined. The kinetics of growth are described by examination of the rate of growth.

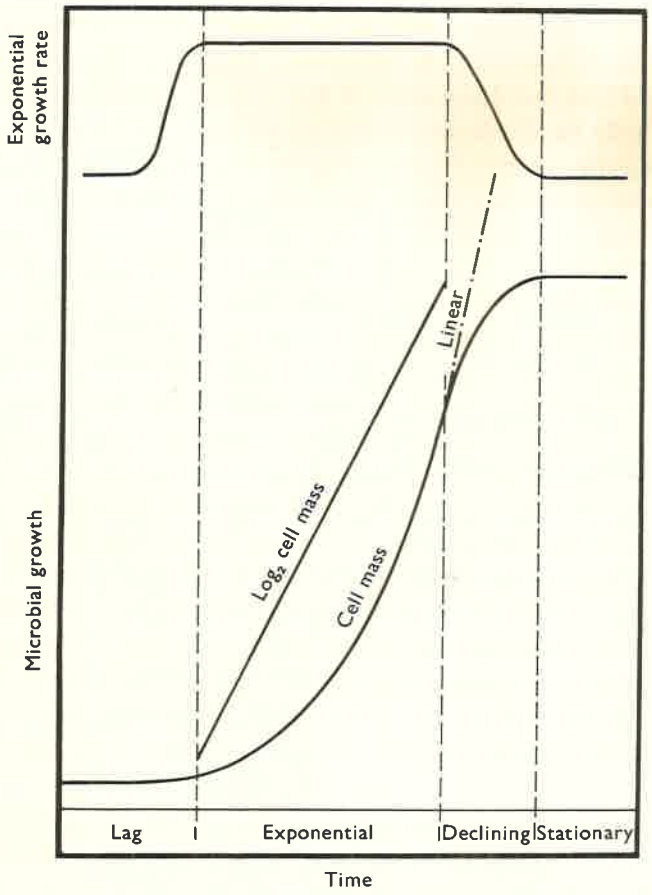

Fig. 21-1. Growth phases in batch cultures of microbial organisms.

III. GROWTH RATES, PHASES OF GROWTH CYCLE

As a measure of velocity of growth, growth rate is an index relating the process of multiplication of organic mass to time. The concept of growth rate and the techniques of its measurements, as applied to populations of microbial organisms, has been based on observations originally made on the so-called batch cultures. Growth of a microbial culture, confined to a constant volume of unchanged medium, is usually described by a sigmoid growth curve (Fig. 21-1). Special significance has been attached to the universality of the sigmoid curve and the whole period of growth of a batch culture, for which the sigmoid curve stands, has been denoted as the 'growth cycle' (Monod 1949). Historically, the term 'growth cycle' is an

adaptation to microbial cultures of an earlier term, 'grand period of growth', introduced by Sachs (1873).

Based largely on the way in which the growth rate behaves, that is on whether it is increasing, constant or falling, the growth cycle has been divided into phases of growth (Fig. 21-1). For the purposes of the following discussion, the phases of growth to be mentioned here are:

lag phase
exponential phase
declining phase, a portion of which may become a linear phase
stationary phase.

The lag phase of growth, or its subdivisions, has also been labeled by different authors as the increased logarithmic phase (Buchanan 1918), the phase of adjustment (Winslow and Walker 1939) or the acceleration phase (Monod 1942). During the lag phase, growth rate is changing – usually increasing with time. However, during a portion of the lag phase, net growth may be absent or there may even be a decline in cell mass (dry weight of cells, turbidity of cell suspension, etc.) per unit volume of cell suspension.

During the exponential (Monod 1942) or logarithmic (Buchanan 1918) growth phase, the mass of microbial cells doubles over each of the successive and equal time intervals. The doubling time and, therefore, the growth rate during the entire exponential phase is thus constant. Inasmuch as the stability of the rate, within the time period for which it is measured, is a basic condition for meaningful determinations of growth rate, the exponential phase of growth is uniquely suitable for growth rate determinations. In fact, when growth rate is mentioned without further specifications, it generally means an exponential growth rate.

The declining phase of growth has also been called the retardation phase (Monod 1942) and the decreased logarithmic growth phase (Buchanan 1918). The doubling time for the cell mass is increasing during the declining phase of growth and this phase is generally unfit for growth rate measurements. However, under certain conditions, the decline in the exponential growth rate with time may assume such a pace that increments in cell mass over the successive time intervals may, in absolute figures, be uniform. The mass of cells in a given and unchanged volume of cell suspension increases linearly with time though the (exponential) rate related to the increasing mass of cells is falling. Under these specific conditions, growth phase and growth rate are called linear phase of growth and linear growth rate respectively.

During the stationary growth phase, a stable concentration of cell

mass per unit volume of cell suspension is maintained. This is, of course, possible only if losses due to catabolism are exactly compensated by anabolic processes. If a microbial culture is maintained beyond the stationary phase, then the preponderance of catabolic processes causes a decline in cell mass and eventual death of the culture. This phase of decline can hardly be considered as growth.

IV. EXPONENTIAL GROWTH RATE

The most comprehensive way to introduce the technique and significance of determinations of exponential growth rate is to present it as the number of doublings of cell material over a specified time interval – commonly over a one day (24 h) period. The mathematical expression for the exponential growth rate is:

$$R_E = \frac{\log_2 X_2 - \log_2 X_1}{t_2 - t_1} \qquad (2)$$

where

R_E = exponential growth rate,

X_1, X_2 = the numerical values for cell mass (given as dry weight, packed cell volume, optical density, etc.) at the beginning and end of the period during which exponential growth rate is constant,

t_1, t_2 = corresponding times, at which X_1 and X_2 values are determined, in terms of the unit (or its fractions) of time (day = 24 h) used in calculations.

Despite the convincing reasoning advanced more than twenty years ago (Monod 1949) in favor of usage of logarithms to the base 2, the practice persists to express exponential growth rate in terms of common or natural logarithms. The usefulness of the exponential growth rate in biological research lies in its direct application as an index of the number of doublings of cell material per specified period of time. From this, the generation (doubling) time is easily obtainable by dividing time period, for which the rate has been determined, by R_E – the exponential growth rate. References to the exponential growth rate based on logarithms other than those to the base 2 involve, in practical applications, the usage of conversion factors ($\log_{10} \times 3.32 = \log_2$ and $\log_e \times 1.44 = \log_2$) and thus additional efforts and possible confusion.

The ambiguity of growth rate calculations using natural logarithms as well as of attaching to the exponential growth rate the term 'specific growth rate' with the symbol 'k' or 'μ' designated as a 'first-order reaction rate constant' involves implications that 'k' is an intrinsic characteristic of a given organism with a theoretical significance well above that of other cellular characteristics. Actually, growth rate is not characteristic of any specific reaction and may not be characteristic of any individual cell.

For a particular cell, growth (as an increase in cell mass) is an outcome of a multitude of anabolic and catabolic reactions. For an organism of a given genetic constitution, the direction and rate of each of these reactions depends on the intracellular environment which, in turn, is affected by extracellular conditions operating during and prior to observations. An important factor affecting the growth rate is the developmental stage of the cell. A multitude of factors affects the exponential growth rate as they do any other cellular characteristic.

One outstanding feature of the exponential growth rate is its easily obtainable reproducibility. However, the reproducibility of the exponential growth rate for a given organism depends on the reproducibility of external conditions under which the rate is observed. Individual cells in a microbial culture are at any given moment in different developmental stages characterized by different rates of metabolic activities and different growth rates. The exponential growth rate, as any other cellular characteristic, is actually a statistical average which may not be characteristic of any particular cell. The stability and reproducibility of the exponential growth rate depends more than anything else on the stability and reproducibility of the age composition of individual cells comprising a particular population of cells.

An understanding of the concept of the growth rate and of factors affecting its reproducibility is essential in an evaluation of the significance and limitations of growth rate measurements. By using the abbreviation 'R' (some authors prefer a small r) for the rate, by attaching the subscript 'E' to distinguish the exponential rate from the linear rate, R_L, and by expressing the exponential rate as number of doublings of cell material per day, the concept and usefulness of the exponential growth rate are defined in simple and unequivocal terms.

V. DRY WEIGHT

Measurements of growth as increase in dry weight of cells involve taking aliquots (samples) of an algal suspension, drying samples to a constant weight, and expressing the dry weight of cells per unit of volume or of illuminated area of the algal culture. Taking a sample, which is representative of a given culture, is one of the crucial conditions for reliable estimation of dry weight of cells. Adequate stirring of the algal suspension and

fast pipetting, to prevent cell settling in the process of sampling, are routine requirements for proper sampling. Each measurement of dry weight of cells is usually done on at least two parallel samples (in duplicate). Size of the sample generally depends on the population density of the culture – the aliquot increasing with the decrease in population density.

After taking the aliquot of algal suspension, cells are separated from culture medium by centrifugation. The deposit of cells is then resuspended in distilled water and centrifuged again. This washing of cells in distilled water removes salts (present in the nutrient medium) which can affect measurements of dry weight. After being washed once in distilled water, cells are transferred with a pipette in a small volume of distilled water into a tared weighing dish. Washing in distilled water must be omitted for marine algae if transferring cells from high salinity medium into distilled water causes plasmolysis resulting in bursting of cells and release of their contents. The techniques employed to circumvent this handicap have not been subjected to a rigorous verification to enjoy a general acceptance.

The weighing dishes may be of disposable or reusable type, glass or metal, open or covered with a lid. The aluminum, light-weight, open, disposable dishes are much in use for quick determinations. For precise measurements on small samples, glass covered dishes are preferable. In our laboratory, drying at 100 °C is continued until two successive weighings of the dish, done at intervals, give a constant weight. It is essential to avoid excessive temperatures and the extension of drying time beyond that necessary to achieve constant weight, since an excessive drying causes changes (oxidation) in dry weight of cells other than loss of water. Weighing is done after the dish is cooled in a desiccator (in our laboratory for 15 min) to room temperature. Dry weight of cells is determined for each of the duplicates and the average weight from these duplicates is used to calculate dry weight per unit of volume, or of surface, of the original algal culture. The sample protocol of dry weight determinations is given in Table 21-1.

Instead of centrifugation, cells can be separated from the medium by filtration on a membrane filter, washed on the filter with distilled water and dried on the filter to a constant weight. The dry weight of cells is determined by subtraction of the dry weight of the filter.

Among factors most commonly affecting the accuracy of dry weight determinations the following are the most important.

1. Poor sampling resulting in an aliquot of cells which is not representative of the original culture.

2. Loss of cells during separation of cells from the medium or after washing the cells in distilled water.

TABLE 21-1. *Dry weight measurements. Sample protocol*

Weighing dish, no.	Suspension volume, ml	Dish weight, g	Successive weighings			Net weight, g	Dry weight, g/l
			1	2	3		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
19	25	22.6989	22.7417	22.7417	—	0.0428	1.712
95	25	23.6019	23.6448	23.6445	23.6445	0.0426	1.704
						Average	1.708

3. Improper drying – either insufficient drying to bring the sample to constant weight or excessive drying resulting in changes other than removal of water.

4. Improper cooling of the sample before weighing it.

VI. PACKED CELL VOLUME

Determination of growth, as increase in packed volume of cells, involves sampling, centrifugation of the sample until the deposit of cells is compressed to a constant volume, and calculations of packed volume of cells per volume or surface unit of algal culture. Sampling does not generally differ from that involved in dry weight measurements. However, depending on the population density of an algal suspension, it may become necessary to condense (or to dilute) the original suspension before using it for packed volume measurements. To avoid changes in volume of individual cells, due to osmotic effects, measurements of packed cell volume are done in the same nutrient medium in which cells have been grown.

Centrifugation to a constant packed volume is done in calibrated capillary tubes. The tube consists of an enlarged upper portion, the receiver, and a lower calibrated portion which is a capillary tube. The capacity of the calibrated section is usually 0.05 ml. It is calibrated to 0.001 ml and, with an approximation, the volume of packed cells can be read to 0.0001 ml. The capacity of the upper section, in two different makes employed in our laboratory, is either 4 or 7 ml. A sample of the suspension of algal cells pipetted into the receiver must contain an amount of cells which, when compressed to a constant volume, will occupy not more than 0.05 ml (the capacity of the calibrated section). On the other hand, to achieve higher accuracy, the packed volume of cells should be large enough to occupy at least half of the calibrated section of the packed cell volume tube.

The volume of the algal suspension placed into the receiver, and/or

TABLE 21-2. *Packed cell volume determinations. Sample protocol*

Cell volume tube, no.	Volume suspension, ml	Volume packed cells, cm^3	Volume packed cells, cm^3/l
(1)	(2)	(3)	(4)
1	20	0.0425	2.13
2	20	0.0430	2.15
		Average	2.14

the amount (concentration) of cells in this suspension, can be manipulated by dilution or condensation. Thus, in case of an algal suspension of low population density, it may become necessary to take a large sample (e.g., 100 ml), to condense it by centrifugation, and to transfer (with a pipette) this condensed suspension into the receiver. The dilutions (or condensations) must be, of course, taken into consideration in final calculations of the packed volume of cells in a given algal suspension.

The conditions for centrifugation of a particular organism must be found by experimentation. For *Chlorella*, centrifugation at 1500 g for 30 min is sufficient to pack cells to a constant volume. This can be proven by reading the packed volume after 30 min of centrifugation and then again after additional centrifugation for extended periods of time. The packed volume of cells in the capillary tubes must be read immediately after centrifugation, since the postponement of readings results in swelling of cells and thus in exaggerated values for packed cell volume. Determinations of packed cell volume are usually done in duplicate and the average from two measurements is expressed as packed volume of cells in cm^3 per volume (liter) or surface unit of the original algal culture. The sample protocol of packed cell volume determinations is given in Table 21-2.

In the given example (Table 21-2), volume of the sample of the suspension of *Chlorella* cells was 20 ml. Inasmuch as this volume was too large to be placed in the receiver section (4 ml capacity) of the packed cell volume tube, it was first condensed by centrifugation, then resuspended in a small volume of medium and transferred with a pipette into the receiver. After centrifugation for 30 min at 1500 g, the volume of packed cells in the calibrated section of the tube was read (column 3 of Table 21-2) and then expressed in terms of a liter of cell suspension by dividing the figures in column 3 by 20 and multiplying them by 1000.

The sources of errors during packed cell volume determinations are:

1. Poor sampling producing a sample which is not representative of the original culture.

2. Inadequate centrifugation (insufficient time and/or insufficient speed).

3. The delay in readings of packed cell volume causing swelling of cells and thus inflated values of packed cell volume.

VII. OPTICAL DENSITY

Of all indices of growth, measurements of optical density (turbidity technique) are particularly suitable for determinations of growth rate. The basic advantage of the turbidity technique in growth rate measurements, in addition to its efficiency, is the possibility of taking repeated readings on the increase in turbidity of the same batch of the suspension of microbial cells. The same growth vessel, such as a test tube, containing a given volume of cell suspension, can be repeatedly taken out of the constant temperature bath (at specified intervals), subjected to turbidity measurements, and then returned to the bath. The time during which the growth vessel is out of the bath and, therefore, the interruption in conditions under which growth is observed, can be made minimal, and its effect on growth observations can be considered negligible.

In estimations of other indices of growth, the cells subjected to observations are either killed, as in dry weight determinations, or affected unfavorably as to their capacity for subsequent growth – as in packed cell volume measurements. The destruction or damage to cells, used for measurements, makes it imperative either to limit observations on growth to just one determination of yield; or, if measurements of the growth rate are intended, to rely in subsequent determinations on samples repeatedly withdrawn at intervals from the growth vessel. Sampling is an integral and a crucial element of statistical investigations. The possibility of avoiding sampling by the turbidity technique saves time and increases the accuracy of growth measurements.

To assure dependability of the turbidity measurements, they can be checked against some other index of growth – such as the increase in dry weight or in packed cell volume. However, a technique is available which makes such comparison superfluous. This technique, described in a later section, makes turbidity measurements self-sufficient and as dependable as the determination of growth rate by any other index of growth.

In measurements of exponential rate as increase in optical density (O.D.), percent transmission for a growing cell suspension is determined at intervals with a measuring device (spectrophotometer, colorimeter, or

TABLE 21-3. *Measurements of the exponential growth rate. Sample protocol*

Time, h	T	O.D.	log O.D.	$\log_2$ O.D. (log O.D. × 3.32)	$\log_2$ O.D. + 10	Δ
(1)	(2)	(3)	(4)	(5)	(6)	(7)
0	70.00	0.155	−0.810	−2.689	7.311	0
1	63.75	0.196	−0.709	−2.353	7.647	0.336
2	55.25	0.258	−0.589	−1.955	8.045	0.734
3	45.50	0.342	−0.466	−1.547	8.453	1.142
4	35.25	0.453	−0.344	−1.142	8.858	1.547
5	24.75	0.606	−0.217	−0.721	9.279	1.968
6	15.25	0.817	−0.088	−0.292	9.708	2.397
7	9.00	1.046	0.020	0.065	10.065	2.754

similar instrument suitable for measurements of the increase in turbidity of microbial suspensions) as:

$$T = I/I_0, \tag{3}$$

where

I = the transmission of the sample in percent of the transmission of the blank (similar vessel, same medium, no cells),

I_0 = latter adjusted to read 100%.

Transmission, T, is then converted into optical density:

$$\text{O.D.} = \log (I_0/I). \tag{4}$$

Since tables of logarithms to the base two are not readily available, the $\log_2$ O.D. can be obtained by using tables of common logarithms and the conversion factor ($\log_{10} \times 3.32 = \log_2$).

These steps are illustrated in detail in a sample protocol of a growth experiment given in Table 21-3. Suspensions of the high-temperature green alga, *Chlorella sorokiniana* Shihira and Krauss (Shihira and Krauss 1965) strain 7-11-05 (IUCC 1230) were grown in a culture medium (Sorokin and Krauss 1958), in 18 × 150 mm test tubes placed in an illuminated constant temperature bath. The test tubes were fitted with cotton plugs through which cotton-plugged bubbling tubes were passed to supply the cultures with a 5% CO_2/air mixture. The bubbling of gas served also to stir the cell suspensions. Test tubes, in groups of four at a time, were taken from the bath at regular (1 h) intervals (column 1 in Table 21-3) and wiped dry with clean dry cheese cloth. The cotton plugs (together with the bubbling tubes) were removed. The tubes were stoppered with rubber stoppers and shaken to insure a uniform distribution of cells throughout the volume of the medium prior to turbidity measurement.

It may be noticed that turbidity measurements of the exponential growth rate are generally conducted under nonsterile conditions. In our practice, sterile conditions are maintained in stock agar cultures. From these agar cultures, inoculations are made into sterile culture medium several days in advance of actual growth measurements. This preculture of algae in sterile liquid medium serves the purpose of bringing algal cells into a good physiological condition, thus eliminating or at least shortening the lag phase during subsequent growth measurements. Sterile conditions are maintained during preculture until the morning of the growth experiment. The abandonment of sterile conditions during growth measurement is justified on two grounds (but see following):

1. During the course of a growth experiment, some algal cells may settle to the bottom and/or stick to the walls of the growth vessel despite the stirring by gas bubbling. By vigorous shaking of the growth vessel before it is inserted into colorimeter, these cells are returned into the suspension and the accuracy of measurements of optical density is greatly improved.

2. Under conditions of adequate illumination necessary for good algal growth in inorganic media, bacterial contamination is usually at a low level and generally does not affect the algal growth or measurements of optical density of the algal cultures. If observations are conducted in organic media and particularly in darkness (heterotrophic growth), the maintenance of sterile conditions during growth measurements becomes imperative. Under these conditions, bubbling tubes are lifted (but not removed) enough to permit light during turbidity measurements to pass through the algal suspension unimpeded by the bubbling tube. Shaking of the growth vessel before inserting it into the colorimeter is omitted with the consequence that the growth measurement may be expected to be less precise.

In the sample experiment (conducted under nonsterile conditions) given in Table 21-3, the transmission of the algal suspension in each culture tube was read on a Coleman Junior II Spectrophotometer (Model 6/35; Coleman Instruments Division, Perkin Elmer Corp., 42 Madison St., Maywood, Illinois 60153; 8250 Mountain Sights Ave., Montreal 308, Quebec) at 678 nm by placing each tube directly into the spectrophotometer fitted with a 19 × 150 mm, Coleman no. 6-102 adapter. The tubes were then fitted again with the corresponding cotton plugs and returned into the bath. With some practice, it takes about 3–4 min for a group of 4 test tubes to be taken from the growth bath, read on the spectrophotometer, and returned to the bath.

The transmission, as it changed in the course of the growth experiment and read with an approximation to the nearest 0.25%, is given for one of

TABLE 21-4. $\log_2$ *O.D.* + 10 *against transmission* (*T*)

T	0	0.25	0.50	0.75	*T*	0	0.25	0.50	0.75
1	10.999	10.928	10.867	10.813	26	9.227	9.217	9.206	9.196
2	10.764	10.720	10.680	10.642	27	9.186	9.176	9.166	9.156
3	10.606	10.573	10.542	10.512	28	9.145	9.135	9.125	9.115
4	10.483	10.456	10.429	10.404	29	9.105	9.095	9.085	9.075
5	10.379	10.356	10.333	10.311	30	9.065	9.055	9.045	9.035
6	10.289	10.268	10.247	10.227	31	9.025	9.015	9.005	8.996
7	10.208	10.188	10.170	10.151	32	8.986	8.976	8.966	8.956
8	10.133	10.116	10.098	10.081	33	8.946	8.936	8.926	8.917
9	10.065	10.048	10.032	10.016	34	8.907	8.897	8.887	8.877
10	10.000	9.984	9.969	9.954	35	8.868	8.858	8.848	8.838
11	9.939	9.924	9.910	9.895	36	8.828	8.819	8.809	8.799
12	9.881	9.867	9.853	9.839	37	8.789	8.779	8.770	8.760
13	9.826	9.812	9.799	9.785	38	8.750	8.740	8.730	8.721
14	9.772	9.759	9.746	9.733	39	8.711	8.701	8.691	8.681
15	9.721	9.708	9.696	9.683	40	8.671	8.662	8.652	8.642
16	9.671	9.659	9.646	9.634	41	8.632	8.622	8.612	8.602
17	9.622	9.610	9.599	9.587	42	8.592	8.583	8.573	8.563
18	9.575	9.563	9.552	9.540	43	8.553	8.543	8.533	8.523
19	9.529	9.517	9.506	9.495	44	8.513	8.503	8.493	8.483
20	9.484	9.472	9.461	9.450	45	8.473	8.463	8.453	8.443
21	9.439	9.428	9.417	9.406	46	8.433	8.423	8.413	8.402
22	9.396	9.385	9.374	9.363	47	8.392	8.382	8.372	8.362
23	9.353	9.342	9.331	9.321	48	8.351	8.341	8.331	8.321
24	9.310	9.300	9.289	9.279	49	8.310	8.300	8.290	8.279
25	9.268	9.258	9.248	9.237	50	8.269	8.259	8.248	8.238

TABLE 21-4. $\log_2$ *O.D.* + 10 *against transmission* (*T*) (*cont.*)

T	0	0.25	0.50	0.75	*T*	0	0.25	0.50	0.75
51	8.227	8.217	8.206	8.196	76	6.933	6.916	6.898	6.881
52	8.185	8.174	8.164	8.153	77	6.863	6.845	6.827	6.808
53	8.142	8.132	8.121	8.110	78	6.790	6.771	6.752	6.733
54	8.099	8.088	8.078	8.067	79	6.714	6.694	6.675	6.655
55	8.056	8.045	8.034	8.023	80	6.635	6.614	6.594	6.573
56	8.012	8.000	7.989	7.978	81	6.552	6.531	6.509	6.488
57	7.967	7.956	7.944	7.933	82	6.466	6.443	6.421	6.398
58	7.922	7.910	7.899	7.887	83	6.375	6.351	6.328	6.303
59	7.876	7.864	7.852	7.841	84	6.279	6.254	6.229	6.204
60	7.829	7.817	7.805	7.793	85	6.178	6.151	6.125	6.098
61	7.781	7.770	7.757	7.745	86	6.070	6.042	6.013	5.984
62	7.733	7.721	7.709	7.697	87	5.955	5.925	5.894	5.863
63	7.684	7.672	7.659	7.647	88	5.832	5.799	5.766	5.732
64	7.634	7.622	7.609	7.596	89	5.698	5.663	5.627	5.590
65	7.583	7.570	7.557	7.544	90	5.553	5.514	5.475	5.434
66	7.531	7.518	7.505	7.491	91	5.393	5.350	5.307	5.262
67	7.478	7.465	7.451	7.437	92	5.215	5.168	5,118	5.068
68	7.424	7.410	7.396	7.382	93	5.015	4.961	4.904	4.846
69	7.368	7.354	7.340	7.325	94	4.785	4.722	4.656	4.587
70	7.311	7.297	7.282	7.267	95	4.515	4.439	4.359	4.275
71	7.252	7.238	7.223	7.208	96	4.186	4.091	3.989	3.881
72	7.192	7.177	7.162	7.146	97	3.763	3.636	3.497	3.343
73	7.131	7.115	7.099	7.083	98	3.171	2.977	2.753	2.488
74	7.067	7.051	7.034	7.018	99	2.165	1.748	1.162	0.160
75	7.001	6.984	6.967	6.950					

the test tubes in column 2 (Table 21-3). The O.D. (column 3) for the corresponding readings was obtained by using Eq. (4). The $\log_{10}$ O.D. (column 4) was obtained by using tables of common logarithms. To transform values of $\log_{10}$ O.D. into $\log_2$ (column 5), figures in column 4 were multiplied by the conversion factor, 3.32. To avoid possible errors connected with the usage of negative logarithms, an arbitrary figure 10 was added to all values of $\log_2$ O.D. This transformed all negative logarithms into positive ones (column 6). To facilitate plotting of the data, the initial optical density of the cell suspension and, therefore, the $\log_2$ O.D. + 10 at zero time was treated as equal to zero and the increases in $\log_2$ O.D. + 10 at the subsequent readings were obtained by subtracting from the values of $\log_2$ O.D. + 10 at each of the readings (column 6) the values of $\log_2$ O.D. + 10 at zero time. The Δ values (column 7) actually represent the number of doublings of O.D. at indicated time intervals from the beginning of observations (zero time).

To expedite calculations illustrated in great detail in Table 21-3, Table 21-4 was constructed in which the values of $\log_2$ O.D. + 10 (column 6 in Table 21-3) are directly related to transmission readings (column 2 in Table 21-3). By using Table 21-4, the intermediate steps (columns 3, 4, 5 in Table 21-3) are eliminated and the protocol of a growth experiment is shortened to contain the columns:

1. time, hours
2. transmission, T (read on colorimeter)
3. $\log_2$ O.D. + 10 (taken from Table 21-4)
4. accumulated increments of $\log_2$ O.D. + 10, Δ (obtained by subtracting the value of $\log_2$ O.D. + 10 at the initial zero time from each of the values of $\log_2$ O.D. + 10 obtained at subsequent readings).

In Table 21-4, values of $\log_2$ O.D. + 10 are given for transmission readings expressed as integrals and as fractions of the integrals. On most instruments, the transmission can be directly read to one unit (one percent transmission). Between the units of the transmission (integrals), transmission can be estimated with some approximation to one quarter (0.25%) of a unit. The transmission readings, as integrals, are given in Table 21-4 in the column 'T' and the corresponding values of $\log_2$ O.D. + 10 for these integrals under the heading 'O'. For the fractions between integrals, the $\log_2$ O.D. + 10 values are given in Table 21-4 in columns 0.25%, 0.50% and 0.75%. Thus, the example, for readings of the transmission:

51.0% the $\log_2$ O.D. + 10 is 8.227
51.25% the $\log_2$ O.D. + 10 is 8.217
51.50% the $\log_2$ O.D. + 10 is 8.206

51.75% the $\log_2$ O.D. + 10 is 8.196
52.0% the $\log_2$ O.D. + 10 is 8.185

To obtain the exponential growth rate, increments of $\log_2$ O.D. + 10 (column 7 in Table 21-3) are plotted against time as illustrated in Fig. 21-2 and a straight line is drawn to fit as many points as possible. Plotting of data and drawing of a straight line through a number of points, as illustrated in Fig. 21-2 serves several purposes.

First, it permits a judgment as to whether the exponential growth was observed during the growth experiment as well as to provide an indication of the duration of the exponential growth phase.

Second, it allows an improvement in the accuracy of measurements by disregarding small deviations from the expected strictly exponential increase. By drawing a straight line, a judgment can be made as to whether these deviations, caused by imperfections of the technique, are small enough to be disregarded in calculations of the exponential growth rate.

Third, larger one-directional deviations, which cause the curve at both ends to deflect from a straight line, indicate the lag phase (at the beginning of measurements) and the declining phase (at the end of the observations) of growth which must be excluded from the measurements of the exponential growth rate. By proper care of the culture used for inoculation and by using a heavier inoculum to start with a reasonably dense cell suspension, an attempt can be made to shorten or even to eliminate the lag phase of growth. Bending of the curve at the upper end, indicating a transition from the exponential phase to declining phase of growth, denote time (population density of the cell suspension) at which measurements must be discontinued.

The exponential growth rate is determined from the slope of the curve in Fig. 21-2. In this example, the exponential growth was observed from the first hour reading to the sixth hour reading. By using equation (2) and values as read in Fig. 21-2, the exponential rate is calculated as:

$$R_E = \frac{2.4 - 0.3}{\frac{6}{24} - \frac{1}{24}} = \frac{2.1}{\frac{5}{24}} = 10.1. \qquad (5)$$

A growth experiment usually consists of two or more replications with each represented by a cell suspension in a separate growth vessel. The change in transmission with time is recorded for all replications and the transmission readings for each of the replications are treated separately in the protocol of the growth experiment. It is recommended to plot data and to calculate values of R_E separately for each replication. Then, at a final stage, and only if the difference in values of R_E obtained for each

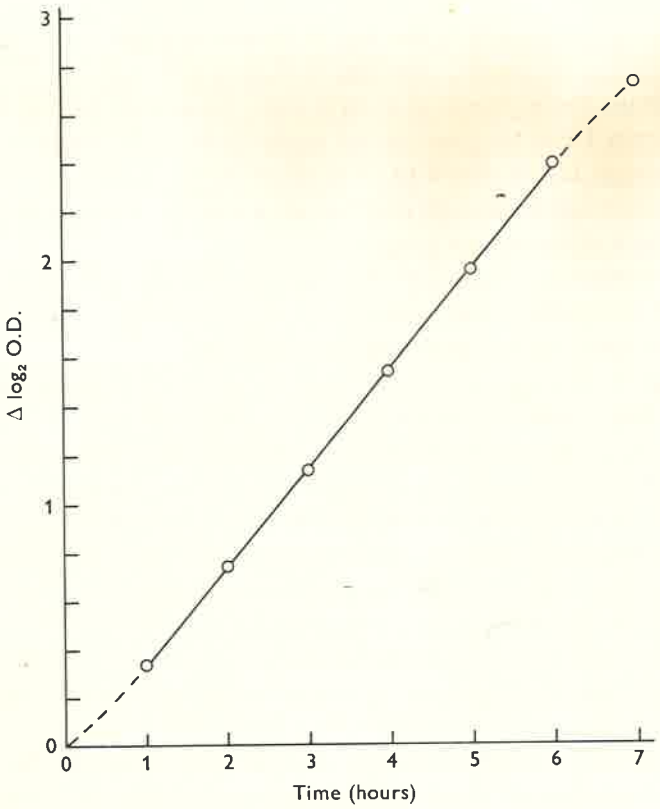

Fig. 21-2. Plotting of the growth experiment presented in Table 21-3.

replication is small, a value of R_E is calculated as an average of R_Es for individual replications.

The precision of measurements of optical density can be adversely affected by a number of factors listed below:

1. An inadequate mixing of the sample before it is used for O.D. measurements or sedimentation of cells in the process of measurements.

2. Poor optical properties of the vessels (test tubes) in which measurements are made. Optical unevenness of the glass can be checked by filling a number of vessels with water and determining variations in transmission.

3. Inadequate wavelength of light used for optical density measurements.

4. Poor adjustment of the instrument. The standardization must be done after a warming period and must be checked frequently during measurements.

The exclusion of all of these factors can be summarily ascertained by the technique described as a dilution curve.

VIII. DILUTION CURVE

In addition to the linearity of the exponential growth curve over an extended period of time, another major condition for using the increase in optical density in growth rate measurements is a proof that the increase in the O.D. (slope of the curve in Fig. 21-2) actually represents the increase in population density of a given cell suspension. Several factors affect the strict correspondence of growth measurements as increase in optical density to actual increase in cell mass. Of these factors the most important are: the make of the measuring device and its proper functioning (adjustment); the wavelength of the light source during measurements; properties of the organism subjected to measurements; and, the population density of the cell suspension used for measurements.

Each of these factors is complex. Without entering into a detailed discussion on the nature of these factors, a technique is offered to obtain a demonstration of the degree of the correspondence of the measurements of the exponential growth rate as increase in optical density to the actual (known in the given example in advance) increase in the population density of the suspension of the given organism.

A reasonably dense suspension of the organism is repeatedly diluted by half by adding at each dilution to the measured volume of the well-stirred cell suspension an equal volume of the medium. The dilutions obtained are: 1 (no dilution), $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$ and so on. With every dilution, the cell mass per volume unit of the suspension is reduced by half and, by moving in the opposite direction (from a more dilute to a denser suspension) the cell mass doubles. It remains to be proven that the optical density of the suspension increases correspondingly.

The transmission of each of the dilutions is determined with the available instrument. In the example given in Table 21-5 the transmissions of the suspensions of *Chlorella* were read on a Coleman Junior II Spectrophotometer at 438, 540, and 678 nm (columns 2, 5, and 8 in the Table 21-5). The $\log_2$ O.D. + 10 for each of the transmissions was obtained from Table 21-4 (columns 3, 6, and 9 in Table 21-5). The accumulated increments of the $\log_2$ O.D. + 10 (columns 4, 7, and 10 in Table 21-5 read in reverse beginning with the most diluted suspension) were then plotted against doublings of the cell mass (dilutions in reverse) in Fig. 21-3.

The slopes of the straight lines in Fig. 21-3 can be quantitatively evaluated by dividing the number of doublings in optical density by the corresponding number of dilutions in cell material. Thus, for example, the straight line drawn through points obtained by reading O.D. at 678 nm

TABLE 21-5. *Number of doublings in optical density for successive dilutions of the suspension of Chlorella*

	438 nm			540 nm			678 nm		
Dilutions	T	$\log_2$ O.D. + 10	Δ	T	$\log_2$ O.D. + 10	Δ	T	$\log_2$ O.D. + 10	Δ
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1	—	—	—	8.75	10.081	6.738	1.50	10.867	7.696
$\frac{1}{2}$	2.50	10.680	6.405	27.25	9.176	5.833	10.00	10.000	6.829
$\frac{1}{4}$	14.75	9.733	5.458	52.00	8.185	4.842	30.00	9.065	5.894
$\frac{1}{8}$	37.75	8.760	4.485	72.25	7.177	3.834	54.75	8.067	4.896
$\frac{1}{16}$	60.75	7.793	3.518	85.00	6.178	2.835	73.75	7.083	3.912
$\frac{1}{32}$	77.00	6.863	2.588	91.50	5.307	1.964	85.50	6.125	2.954
$\frac{1}{64}$	87.00	5.955	1.680	95.25	4.439	1.096	92.50	5.118	1.947
$\frac{1}{128}$	92.50	5.118	0.843	97.00	3.763	0.420	96.00	4.186	1.015
$\frac{1}{256}$	95.75	4.275	0	97.75	3.343	0	98.00	3.171	0

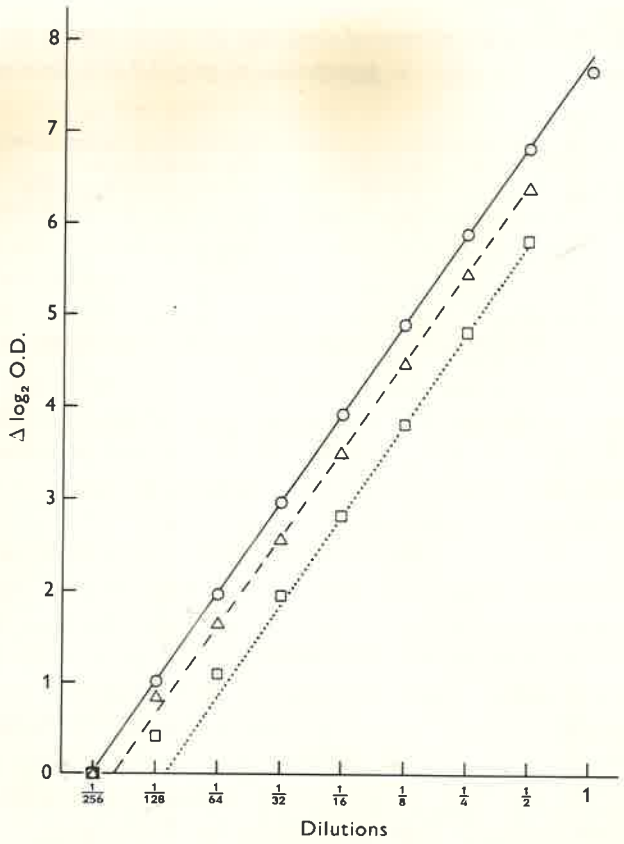

Fig. 21-3. Plotting of the experiment in which optical densities of successive dilutions of *Chlorella* were determined at different wavelengths (data of Table 21-5). Measurements: Δ – at 438 nm; □ – at 540 nm; ○ – at 678 nm.

extends from 0 to 7.84. This corresponds to the actual number of doublings of cell material (number of dilutions) equal to 8. By dividing 7.84 by 8, a figure, 0.98, is obtained, indicating that for each actual doubling of cell material the number of doublings, as determined by the increase in O.D. read at 678 nm is 0.98.

Evaluation of the curves of Fig. 21-3 offered in Table 21-6 permits some comparisons. The slope of the line obtained for readings of O.D. at 438 nm referred to number of dilutions of cell material, that is the ratio Δ O.D./Δ P.D. (population density), equals 0.956. This means that the obtained value of the increase in O.D. is 4.4% lower than the actual increase in cell material. Compared to this, the ratios of Δ O.D. to Δ P.D. at 540 nm and 678 nm are correspondingly 1.0 and 0.98. Thus, for the

TABLE 21-6. *Ratio of the number of doublings in optical density (O.D.) to the known number of doublings in population density (P.D.) determined from Fig. 21-3*

Wavelength, nm	$\frac{\Delta \text{ O.D.}}{\Delta \text{ P.D.}}$	Deviation from theoretically expected
(1)	(2)	(3)
438	0.956	−4.4%
540	1.000	0.0
678	0.982	−1.8%

organism and conditions employed in the experiment illustrated in Fig. 21-3 and Table 21-6, measurements at 540 nm gave a perfect correspondence between the increase in O.D. and the known increase in population density of the cell suspension.

If 540 nm is chosen for measurements of O.D. as an indication of growth, attention must be drawn to the fact that the line drawn through the $\log_2$ O.D. obtained at 540 nm (Fig. 21-3) is straight and, therefore, the increase in optical density is strictly proportional to the increase in population density only at higher population densities. As seen in Fig. 21-3, the line connecting $\log_2$ O.D. at 540 nm is perfectly straight between readings obtained for dilutions $\frac{1}{16}$ to $\frac{1}{2}$. For population densities below that of the dilution $\frac{1}{16}$, the $\log_2$ O.D. curve begins to deviate from the straight line; the deviations are much more pronounced for dilutions $\frac{1}{64}$, $\frac{1}{128}$ and $\frac{1}{256}$. From Table 21-5 we find that the transmission for the dilution $\frac{1}{16}$ at 540 nm (column 5 in Table 21-5) is 85.0. Therefore, the initial population density during growth measurements, determined as increase in O.D. at 540 nm, must be at least equal or higher than that corresponding to the transmission 85.0. Otherwise, the bending of the $\log_2$ O.D. growth curve, often observable during the earlier portion of the growth experiments which we denote as a lag phase (Fig. 21-1), will actually be due not only to biological factors operating during and prior to observations, but also to the lack of proportionality of the O.D. measurements to the actual increase in population density.

Measurements of O.D. at 678 nm produce a $\log_2$ O.D. curve with a slope giving the ratio Δ O.D./Δ P.D. equal to 0.982. Thus, the exponential growth rate, as determined from O.D. measurements, is expected to be lower than the actual increase in population density by 1.8%. This error for biological observations cannot be considered large. If desirable, the exponential growth rate, obtained as the increase in O.D. determined at 678 nm, can be corrected by adding 1.8% of its value. The advantage of determinations of exponential growth rate, as increase in O.D. measured at 678 nm, lies in the perfect linearity of the obtained $\log_2$ O.D. curve over a wide range of population densities, extending, in the example given in Fig. 21-3 and Table 21-5, to the extreme dilutions (1/256) characterized by such a high transmission (98.0) as indicated in column 8 of Table 21-5.

Factors which affect measurements of O.D. may change with a change in the organism under observation and in measuring device. Therefore, the dilution curve, as an evidence of applicability of optical density to measurement of population density, must be obtained every time when new factors affecting measurement of O.D. are introduced, and also from time to time even under seemingly unchanged conditions to ascertain that the strict proportionality of O.D. and P.D. continues to exist.

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