

## **Growth of *Mycobacterium smegmatis* in minimal and complete media**

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**Abstract.** The growth patterns of *Mycobacterium smegmatis* SN2 in a minimal medium and in nutrient broth have been compared. The growth was monitored by absorbancy (Klett readings), colony forming units, wet weight and content of DNA, RNA and protein. During the early part of the growth cycle, the bacteria had higher wet weight and macromolecular content in nutrient broth than in minimal media. During the latter half of the growth cycle however, biosynthesis stopped much earlier in nutrient broth and the bacteria had a much lower content of macromolecules than in the minimal medium. In both the media, a general pattern of completing biosynthesis rapidly in the initial phase and a certain amount of cell division at a later time involving the distribution of preformed macromolecules was seen. The possible adaptive significance of this observation has been discussed.

**Keywords.** *Mycobacterium smegmatis*; growth; macromolecular content.

### **Introduction**

The growth of bacteria under different nutritional conditions has been studied in considerable detail (Doelle, 1969; Nierlich, 1979; Oginski and Umbreit, 1959; Payne and Weibe, 1978; Sokatch, 1969). However, comparatively little is known regarding mycobacterial nutrition and physiology (Barksdale and Kim, 1977; Ramakrishnan *et al.*, 1972; Ratledge, 1976). Our interest in this field was aroused by the observation that the burst size of mycobacteriophage 13 (Gadagkar, 1979) depends on the medium in which the host bacteria are being grown. The phage grows well on *Mycobacterium smegmatis* growing in a complete medium (nutrient broth) but very poorly if the bacteria are growing in a synthetic minimal medium. With the hope of gaining an insight into the reasons for this difference in phage growth, we have compared the growth of the host bacterium *M. smegmatis* in complete (nutrient broth) and that in minimal media. It is evident from the results described in this paper that such a comparison not only suggested a possible clue to the effect of the medium on phage growth but also revealed a number of interesting factors concerning bacterial growth *per se*.

### **Materials and methods**

#### *Growth Media*

- a) Minimal medium (Youmans and Karlson, 1947). L-asparagine, 5g; potassium dihydrogen phosphate, 5.9g; potassium sulphate, 0.5g; citric acid, 1.5g;

magnesium carbonate, 0.6g; glycerol, 20ml and distilled water to make 1000 ml; pH was adjusted to 7.2 and the medium was supplemented with Tween-80, (0.2% v/v).

- b) Nutrient broth. peptone, 10g; sodium chloride, 5g; beef extract, 5g; distilled water to make 1000 ml; pH was adjusted to 7.4 and the medium was supplemented with Tween-80, (0.2% v/v).
- c) Nutrient agar. Nutrient broth with 1.5% agar-agar.

#### *Buffer*

10 mM potassium phosphate buffer with 150 mM NaCl and 1 mM EDTA, pH 7.4 is referred to as the standard buffer in this paper.

#### *Growth curves*

For all experiments described in this paper, *Mycobacterium smegmatis* SN2 was grown in 100 ml of minimal medium or nutrient broth taken in a 250 ml Erlenmeyer flask with a side arm. A 1 % inoculum of bacteria, freshly grown upto 24 h in minimal medium was added and incubated in a rotary shaker at 37°C. At 2 h intervals, the absorbance of the culture was monitored in a Klett-Summerson colorimeter (using filter no. 54). At the same time a small portion of the culture was removed and plated on nutrient agar for the determination of colony forming units.

#### *Determination of wet weights and dry weights*

At different time intervals, a sample of the culture was centrifuged at 12000 g for 30 min, washed with 0.5% NaCl and the pellet weighed to get the wet weight. For the determination of dry weights, cells were dried in an oven at 90°C cooled, weighed and the process repeated, until a constant weight was reached.

#### *Determination of protein, DNA, and RNA contents*

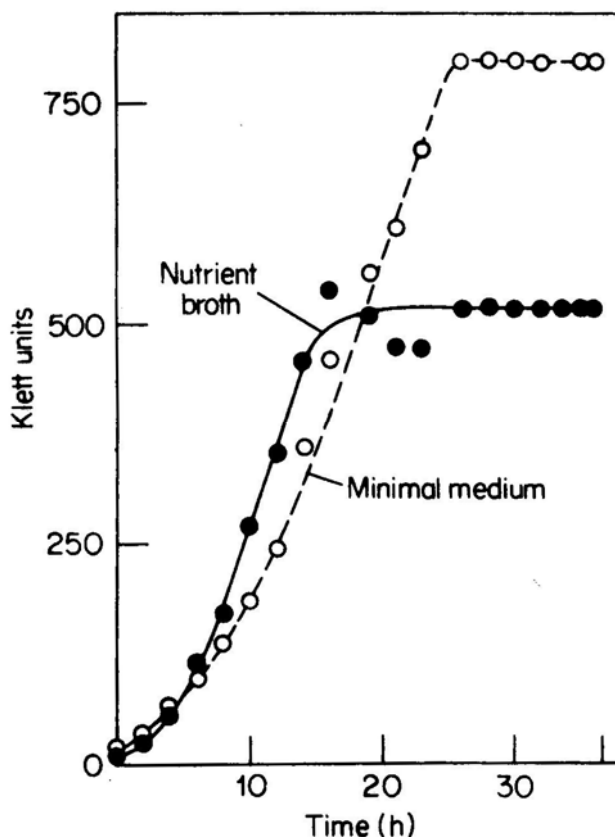
Protein, DNA and RNA contents from packed wet cells were determined according to the method of Schneider (1957). Washed cells were suspended at about 1g/10 ml standard buffer and 2 vol. ice-cold 10% trichloroacetic acid were added. After 30 min at 4°C, the mixture was centrifuged at 12000 g for 20 min. The pellet, containing acid-insoluble macromolecules was washed twice with ethanol, suspended in 10 ml of ethanol-ether (3:1) and heated in a water bath at 60°C for 30 min. This was chilled, centrifuged at 12000 g for 15 min at 4°C and the supernatant discarded. The precipitate was suspended in 1N KOH and incubated in a water bath at 37°C for 20 h to hydrolyse the RNA. To this hydrolysate, 0.4 ml of 6 N HCl and 2 ml of 5% trichloroacetic acid were added and it was incubated for 30 min at 4°C and then centrifuged at 12000 g for 20 min. The supernatant was used for RNA estimation by the Orcinol procedure (Ceriotti, 1955).

The precipitate was suspended in 10 ml of 5% trichloroacetic acid and heated in a water bath at 90°C with occasional stirring for 20 min. It was then cooled to 4°C, retained at that temperature for 30 min and centrifuged at 12000 g for 20 min. This supernatant was used for DNA estimation by the diphenylamine reaction (Burton, 1956) and the pellet was used for protein estimation (Lowry *et al.*, 1951).

## Results

### Growth curve monitored as Klett units

Figure 1 shows typical growth curves of *M. smegmatis* in minimal medium and in nutrient broth. Several observations can be made from the figure:



**Figure 1.** Growth curve of *M. smegmatis* in minimal medium and nutrient broth monitored as Klett Units.

- 1) There was no significant lag period, as this has been deliberately avoided by using a fresh inoculum and a pre-warmed medium.
- 2) Initial growth rates were higher in nutrient broth. This was expected because nutrient broth would contain ready made precursors for macromolecular synthesis unlike the minimal medium.
- 3) Growth stops at about 16-18 h (at a Klett reading of about 500) in nutrient broth, whereas, in the minimal medium growth stops only at about 25 h (at a Klett reading of 800). The reasons for this are not immediately obvious (see below).

## Growth curve monitored by colony forming units

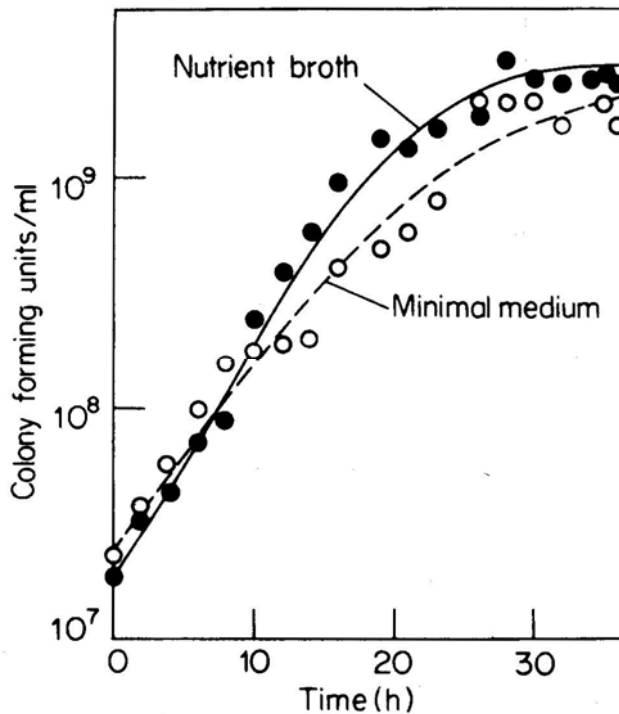
Figure 2 shows typical growth curves monitored by colony forming units in nutrient broth and in minimal medium. The actual data points are shown and the smooth curves (solid and broken lines) have been fitted using the logistic equation, where,  $N$  is the number of bacteria at any given time,  $K$ , the maximum population reached and  $r$ , the intrinsic growth rate. The intrinsic growth rates

$$\frac{dN}{dt} = rN \left[ \frac{K-N}{K} \right]$$

$$r = \frac{\ln \left( \frac{K-N_0}{N_0} \right) - \ln \left( \frac{K-N}{N} \right)}{t}$$

where,  $N_0$  is the initial number of bacteria and  $t$ , the time in h were calculated (Odum, 1973) for pairs of adjacent points. Using the mean intrinsic growth rate, the expected population numbers

$$N = \frac{K}{1 + \exp \left[ \ln \left( \frac{K-N_0}{N_0} \right) - rt \right]}$$
 were generated.



**Figure 2.** Growth curve of *M. smegmatis* in minimal medium and nutrient broth monitored by colony forming units.

The smooth curves so generated fitted reasonably well to the data points. Besides, mean intrinsic growth rates calculated by the logistic equations agreed well with the realized growth rates at the beginning of the growth curves (table 1) as calculated by the exponential equation

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

**Table 1.** Growth rates of *Mycobacterium smegmatis* monitored by colony forming units in minimal medium and nutrient broth.

	Minimal medium	Nutrient broth
<b>Mean intrinsic growth rate</b>	0.19±0.03	0.24±0.03
<b>Mean intrinsic doubling time (h)</b>	3.6 ±0.5	2.9 ±0.3
<b>Realized or specific growth rate at the beginning 0-2 h.</b>	0.19	0.24
<b>Realized or specific doubling time at the beginning 0-2 h.</b>	3.7	2.9

Intrinsic growth rate and doubling time were calculated using the logistic equation while realised or specific growth rate and doubling time were calculated using the exponential equation (see text for details).

In terms of colony forming units the difference between nutrient broth and minimal medium seen in figure 1 was no longer evident. In fact, the cell density was slightly higher in nutrient broth. Thus, there was a discrepancy between Klett readings and colony forming units. A higher Klett reading during the stationary phase in minimal medium inspite of similar numbers of colony forming units (these are confirmed by repeated determinations, table 2) may possibly be due to the secretion of some coloured compound or due to a large excess of dead cells in the minimal medium that are unable to form colonies. Both these hypotheses have been ruled out because the total cells counted under a phase contrast microscope were not significantly different from colony forming units and the absorbance (Klett units) of the spent medium in either case did not contribute significantly to the Klett readings of the culture (table 2).

**Table 2.** Characteristics of stationary phase cultures of *Mycobacterium smegmatis*

Parameter	Growth medium	
	Minimal medium	Nutrient broth
Absorbance of the culture (Klett units)	828 ± 87 (10) <sup>a</sup>	466 ± 25(10)
Wet wt. (mg/ml)	21.25 ± 2.39 (8)	9.82 ± 0.6(6)
Dry wt. (mg/ml)	3.61 ± 0.51 (8)	1.64 ± 0.2(6)
Water content of cells	83%	83%
Colony forming units/ml	1.5 ± 0.5×10 <sup>9</sup> (9)	3.8 ± 1.7× 10 <sup>9</sup> (9)
Total cells/ml <sup>b</sup>	1.2 ± 0.7×10 <sup>9</sup> (10)	3.2 ± 1.6×10 <sup>9</sup> (10)
Absorbance (Klett units) of spent medium	30	3
pH of spent medium	7.2	7.6

*a* The numbers in paranthesis refer to the number of experiments from which the mean and standard deviation have been calculated.

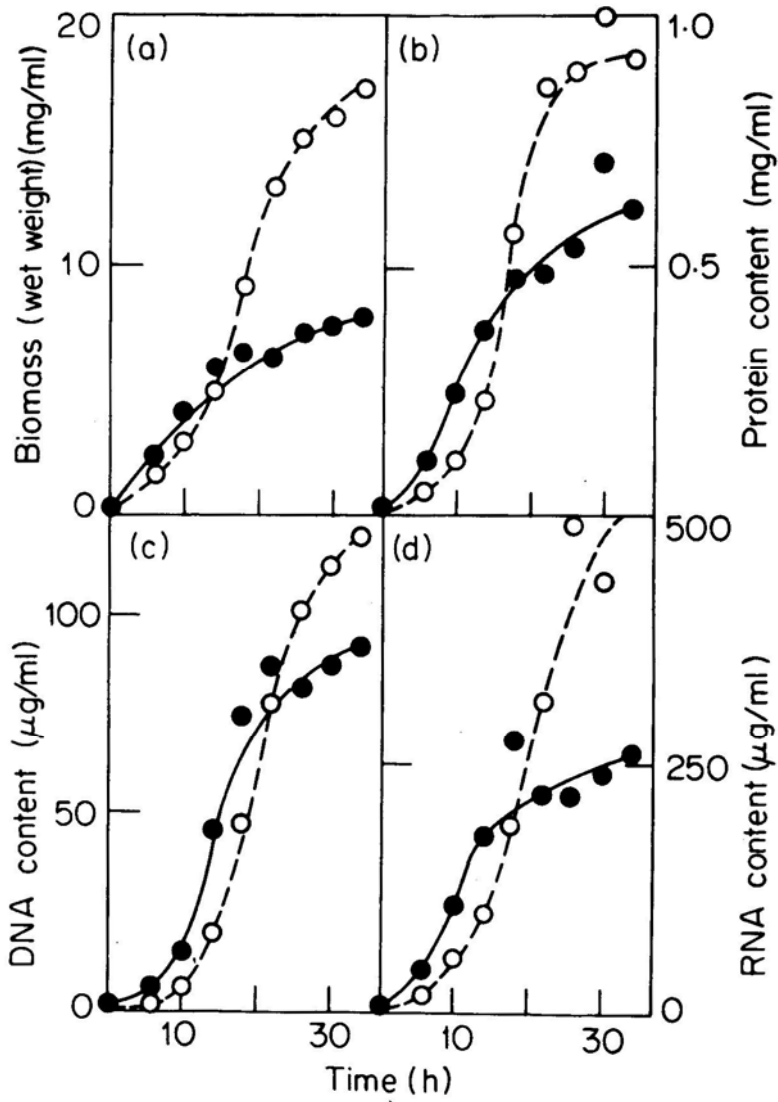
*b* Cell numbers counted in a haemocytometer.

#### *Growth curve monitored by wet weight, protein, DNA and RNA contents*

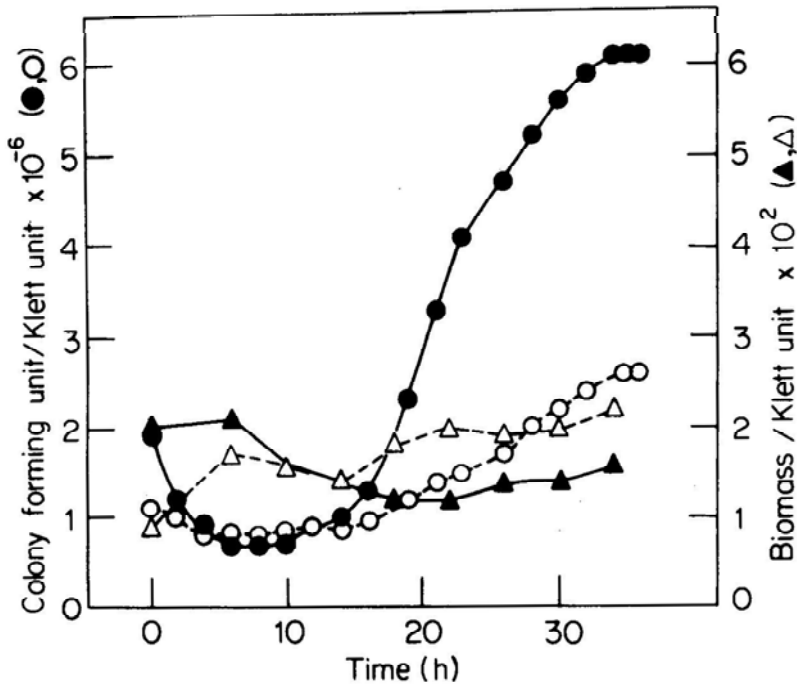
Growth curves in minimal medium and nutrient broth monitored by wet weight (mg/ml), protein content (mg/ml), DNA content (µg/ml) and RNA content (µg/ml) are shown in panels A, B, C and D respectively of figure 3. In all the cases growth stopped earlier in time and at a lower density in nutrient broth as compared to that in minimal medium. During the late exponential or early stationary phase, at which time the cultures are generally harvested, the yield in minimal medium was nearly twice that in nutrient broth. Therefore it is better to use minimal medium when large quantities of cells are required, especially since this difference in yield was seen not only in terms of wet weight but also in terms of DNA, RNA and protein contents.

The ratio between colony forming units and Klett readings was reasonably constant in minimal medium. When one Klett reading corresponds to 1–2×10<sup>6</sup> cells. However, in nutrient broth, one Klett unit corresponds to about 1×10<sup>6</sup> cells in the first half of the growth curve, but more than 6×10<sup>6</sup> cells during the latter half (figure 4).

The wet weight and protein, DNA and RNA content per cell in the two media are shown in panels, A, B, C and D respectively of figure 5. As suggested earlier, these were much lower in nutrient broth as compared to minimal medium during the latter half of the growth curve. During the first half of the growth curve however, the situation was the reverse. The cells in nutrient broth had more wet weight, protein, DNA and RNA content than cells in minimal medium (figure 5).



**Figure 3.** Growth curves of *M. smegmatis* in minimal medium and nutrient broth monitored by wet weight, protein, DNA and RNA contents. Minimal medium, (O); nutrient broth, (●).



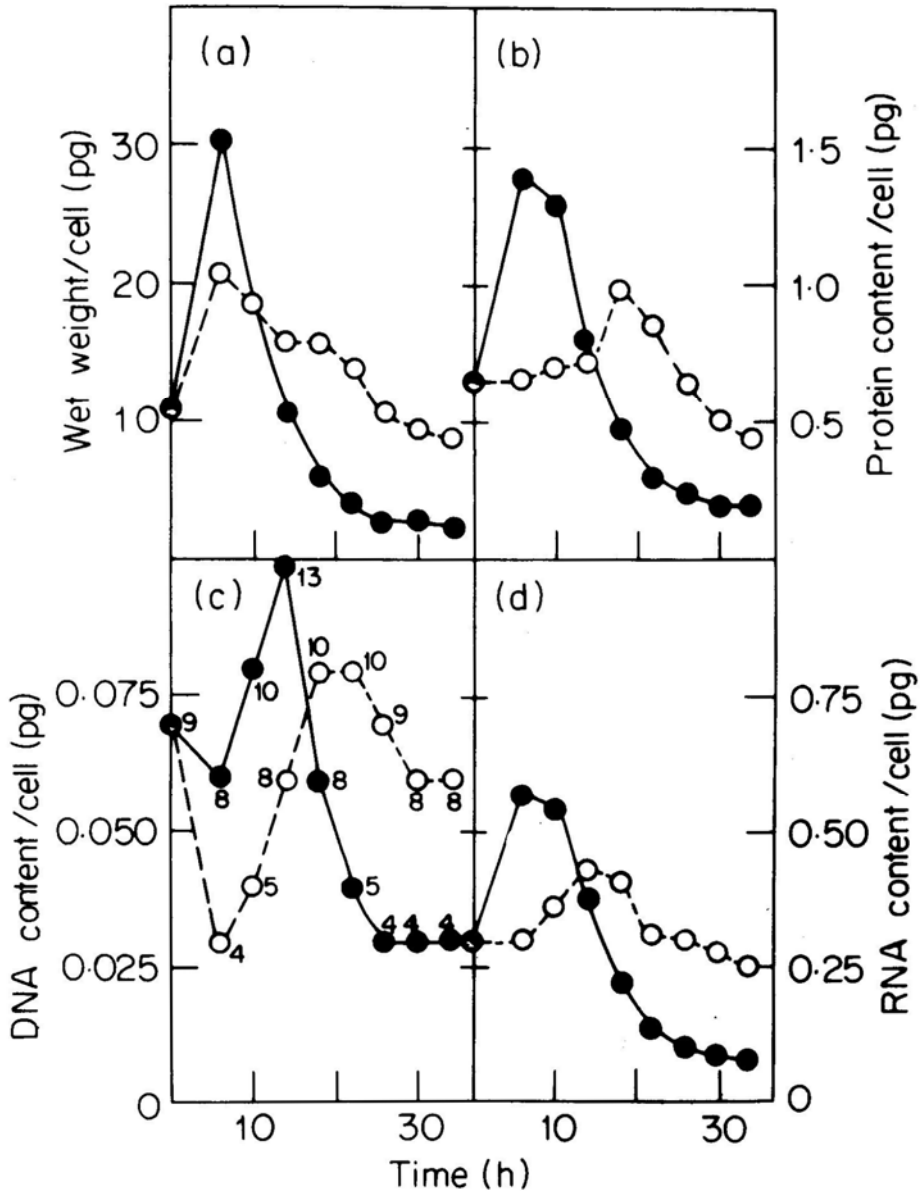
**Figure 4.** Absorbance (Klett Units) as a function of cell numbers and biomass in cultures of *M. smegmatis* in minimal medium and nutrient broth.

Colour forming unit/Klett Unit –minimal medium, (O); Colour forming unit/Klett unit – nutrient broth, (●); Biomass/Klett unit–minimal broth, (Δ); Biomass/Klett unit–nutrient broth, (▲).

An inspection of figure 5 reveals other interesting features. The fact that the weights and macromolecular contents per cell vary over such a wide range of values suggested that cell division and the biosynthetic machinery were somewhat loosely coupled. In general, two or sometimes three distinct phases can be seen in the life of a cell in a batch culture growth curve (see figure 5):

- a) an early phase during which synthesis proceeds more rapidly than cell division,
- b) a later phase where, cell division occurs more rapidly than biosynthesis of cellular components and
- (c) sometimes, a very early phase that shows constant or decreasing macromolecular content. Such a very early phase is prominent in minimal medium because no preformed growth factors (amino acids, nucleotides etc.) would be present.

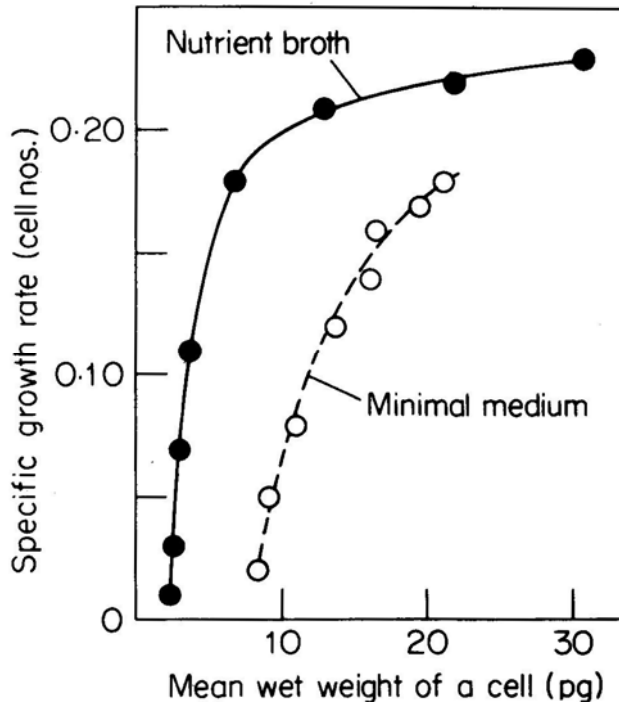




**Figure 5.** Mean wet weight (a), protein (b), DNA (c), and RNA (d) contents per cell in cultures of *M. smegmatis* in minimal medium and nutrient broth.

The wet weight, protein, DNA and RNA contents per ml (figure 3) were divided by the numbers of cells per ml (figure 2) to give the mean properties of single cells. In the case of DNA content per cell, the approximate numbers of DNA molecules per cell calculated using Bradley's (1972, 1973) estimate of the molecular weight of *M. smegmatis* DNA ( $4.5 \times 10^9$  daltons) have been shown adjacent to the data points.

Minimal medium, O; Nutrient broth ●.



**Figure 6.** Correlation between mean wet weight per cell and the specific growth rate. The specific growth rate was calculated by using the data in figure 2 and the equation  $N_t = N_0 e^{kt}$ , where  $N_t$  is the final population,  $N_0$  is the initial population,  $t$ , the time interval and  $k$ , the specific growth rate.

## Discussion

The onset of the stationary phase in bacterial growth curves is generally attributed to the exhaustion of nutrients and/or the accumulation of toxic substances (Stainer *et al.*, 1970). Experiments with *Escherchia coli* have shown that the accumulation of toxic materials is more important in reducing bacterial growth (Landwall and Holme, 1977). Comparison of the yields as dry weight per liter in the two media (table 2) and the nutrient contents in these media shows that only about 10% of the constituents of the media are converted into biomass. Therefore there does not appear to be exhaustion or even a significant depletion of nutrition in either of the media. It is possible that toxic materials would accumulate more rapidly in nutrient broth and hence the early cessation of growth.

The difference between growth in nutrient broth and that in minimal medium as seen by Klett readings may indeed be real and the similarity in the numbers of colony forming units only apparent for the following reasons:

Firstly, growth is the conversion of non-living matter into biomass and therefore cell number taken alone is not likely to be a sufficient index of growth. For example, a cell weighing say,  $Xg$  can divide into two cells of  $0.5 Xg$  each without any growth. Secondly Klett readings are more likely to be a function of biomass rather than cell

numbers for, it is total absorbance that is measured. If these assumptions are valid, it then follows that, a) growth curves monitored by biomass should reflect the pattern of the growth curve as judged by Klett readings rather than colony forming units, (b) the ratio between colony forming units and Klett units should be constant in minimal medium but not in nutrient broth, whereas the ratio between biomass and Klett units should be constant for both the media, and (c) cells in the latter half of the growth curve should be smaller in nutrient broth as compared to those in minimal medium. All these predictions are borne out by the data shown in figures 3-5.

It seems somewhat surprising that even the DNA content per cell shows wide fluctuations, but this may be explained if mycobacterial cells can harbour more than one copy of the chromosome as observed in the case of *E. coli* and *Bacillus subtilis* (Lark, 1966; Watson, 1975). In the latter cases, during the early exponential phase, as many as six copies of the chromosome are known to accumulate which are then distributed to the daughter cells during the late exponential phase. Using the highest estimate of the molecular weight of *M. smegmatis* DNA ( $4.5 \times 10^9$  daltons) reported by Bradley (1972,1973), the number of chromosome copies can be roughly calculated and is shown with the data points in panel C of the figure 5. If we are justified in using Bradley's estimate of the molecular weight for *M. smegmatis* SN2, then relatively large numbers of chromosome copies seem to accumulate under our experimental conditions. Moreover, the number of chromosome copies (or simply DNA content) does not deplete sufficiently to give only one copy per cell during the late exponential phase. Thus it is possible to start the growth curve with an initial drop in the DNA content per cell. It must be pointed out that since methods employed for the determination of nucleic acids are based on reactions of pentoses, there may be some error due to interference by arabinose from the mycobacterial cell wall. However, the overall pattern of the changes in nucleic acid content is not likely to have been qualitatively altered.

In general, although cell division and the bio-synthetic machinery are coupled, a certain amount of flexibility present in this coupling seems to be exploited to complete biosynthesis as quickly as possible and convert the environmental nutrition into biomass before conditions become unfavourable. As a result, a few cell divisions involving only the distribution of preformed molecules may take place at later times. It may be easier to complete biosynthesis rapidly before cell division because cell division depends on a series of biochemical and morphological changes that would have to take place in a definite sequence. The strategy outlined above is manifest in both the media but much more so in nutrient broth. Here, biosynthesis is completed by 16-18 h but cell division goes on at least until 28 h giving rise to smaller cells.

The differences in the weight or macromolecular contents per cell between the two media at any given time are not due to lack of separation of the daughter cells. An examination of the cultures under a phase contrast microscope did not reveal any significant numbers of unseparated chains of cells in either medium. It is also unlikely that the above results are due to differences in the rates of cell wall synthesis between the two media. The cells do not weigh consistently more in any one medium; during the early growth phase, cells in nutrient broth weigh more while during the later phase, cells in minimal medium weigh more.

In spite of the apparent loose coupling between biosynthesis and cell division, and inspite of the different phases of growth seen in this system, it is reassuring to note that the general correlation between weight per cell and rate of cell division seen in other systems (Williams, 1971) also holds good in this system (figure 6).

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