COMPARISON OF THE EFFECTS OF CARBON, NITROGEN AND IRON LIMITATION ON THE GROWTH AND ON THE RNA AND DNA CONTENT OF *MYCOBACTERIUM SMEGMATIS*

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Summary

MYCOBACTERIUM SMEGMATIS was grown in shaken culture in carbon-, nitrogen- and iron-limited media, adjusted to decelerate at about the same time. The exponential phase was brief; the initiation of a more prolonged deceleration phase being probably due to the granular form of growth. Iron-limited cultures showed prolonged slow growth. In all three cultures the RNA/nitrogen ratio was high at the beginning of the period of maximal growth and declined thereafter. The DNA/nitrogen ratio also declined in the iron-limited cultures, but increased somewhat in the nitrogen-limited cultures and, after a brief decline, increased markedly in the carbon-limited cultures. In all cultures the soluble deoxyribonucleotide pool stayed within the range of 10-50 μmoles/g. of nitrogen, while the total soluble nucleotide pool fell from about 1000 to about 250 μmoles/g. of nitrogen as the growth rate declined.

Introduction

The effects of environmental conditions on the content of nucleic acids in mycobacteria have not been extensively investigated, and some of the available reports have been affected by failure to recognise some special problems which arise in the assay of nucleic acids in these organisms. For example, several reports on the RNA content of mycobacteria (e.g. Tsukamura, 1961 a, b; Youmans and Youmans, 1968) are based on the use of a pentose assay for the measurement of RNA, in spite of the high arabinose content of these organisms. Several methods for the measurement of nucleic acids in mycobacteria have been devised to avoid these problems (Winder & Denneny, 1956; Winder & O'Hara, 1962; Winder & Coughlan, 1969).

Tepper (1965) investigated changes in DNA content during the growth of cultures of *Mycobacterium phlei* in several media. Youmans & Youmans (1968) reported values for the DNA content of *Mycobacterium tuberculosis* H37Ra from cultures of various ages, while Winder & Rooney (1970a) investigated the RNA and DNA content of *M. tuberculosis* BCG under a variety of growth conditions. *Mycobacterium smegmatis* has been investigated in most detail. The behaviour of some components of the soluble nucleotide pool of this organism in response to environmental conditions

was reported by Winder & O'Hara (1962), and the components of this pool in this organism were studied in detail by Reutgen & Iwainsky (1967), as has been done for this pool in *M. tuberculosis* H37Ra by Iwainsky & Reutgen (1967). Carlberg & Jann (1963) reported on the effect of phage infection on the DNA content of *M. smegmatis*. Winder & O'Hara (1962) reported on the changes in DNA and RNA relative to protein in this organism during growth on iron-limited, zinc-limited and 'metal-sufficient' media. Harris (1967, 1969) repeated this work, using shaken in place of stationary cultures. However, there were a number of flaws in his reports. In particular, he failed to perceive that cultures which he described as being in exponential growth were not, in fact, in a steady state of such growth for most of his period of observation. Further, in a number of respects the results of the published experiments did not agree with other, unpublished, experiments by the same author.

We now report on the changes which take place in the content of DNA and RNA relative to protein in shaken cultures of *M. smegmatis* in iron-limited, carbon-limited and nitrogen-limited media. Results for zinc-limited media are not included because, at least in shaken culture, zinc deficiency leads to successive phases of autolysis and re-growth on the zinc thus released (Winder & O'Hara, 1966). A more sophisticated experimental approach would be required before such phases of re-growth could be accurately correlated with changes in nucleic acid content. Harris (1969) presented his results for zinc-limited cultures in terms of a single re-growth phase, but examination of his published and unpublished data leaves considerable uncertainty as to whether one, two, or even conceivably three phases of re-growth were involved during his experimental periods.

Materials and Methods

Organism. The strain of *M. smegmatis* is one which is maintained in this Department and which has been used in previous studies (Winder & O'Hara, 1962).

Media. All three media contained 5.0 g. of KH_2PO_4, 0.5 g. of K_2SO_4 and 0.5 g. of $MgSO_4.7H_2O$ per l. The media also contained glycerol and asparagine in the following concentrations (g. per l) : 3.07 and 5.0 respectively, in the carbon-limited medium; 24.6 and 1.25, in the nitrogen-limited medium; and 24.6 and 5.0, in the iron-limited medium. The pH was adjusted to 6.8 with NaOH and the media were depleted in trace metals by autoclaving with alumina as described by Winder & O'Hara (1962), except that 2 per cent of Merck alumina (pre-washed to remove fines) was used. Zn^{2+} (0.4 μg./ml.) and Fe^{2+} (2μg./ml. in the cases of the carbon- and nitrogen-limited media, and 0.06 μg./ml. in the iron-limited medium) as the sulphate salts were then added to the media. The media were then transferred as 100 ml. lots to 250 ml. conical flasks, which had been cleaned as described by Ratledge & Winder (1962), and were sterilized by autoclaving.

Growth and harvesting. Inocula were prepared from 3-4 day old stationary cultures in Proskauer & Beck medium (American Trudeau Society, 1950). The pellicle was lightly ground in 0.9 per cent NaCl, and about 0.7 μg. of bacterial nitrogen/ml. of medium was used. The cultures were

incubated at 37° on a rotary shaker. After the appropriate period the cells were harvested and washed with 0.9 per cent NaCl by centrifugation at about 2°.

Extraction procedure. Freshly harvested cells were extracted by the 'general extraction procedure' described by Winder, Brennan & McDonnell (1967), employing the volumes given there for an amount of cells equivalent to about 20 mg. of nitrogen. Most of the trichloracetic acid was removed from the acid-soluble fraction by extraction with ether before it was combined with the 30 per cent ethanol extract to give the 'soluble fraction'.

Assay procedures. DNA, RNA and total nitrogen were determined as described by Winder & O'Hara (1962). The total nucleotide content of the soluble fraction was estimated from the extinction at 260 nm., and the results calculated as AMP-equivalents by using the molar extinction co-

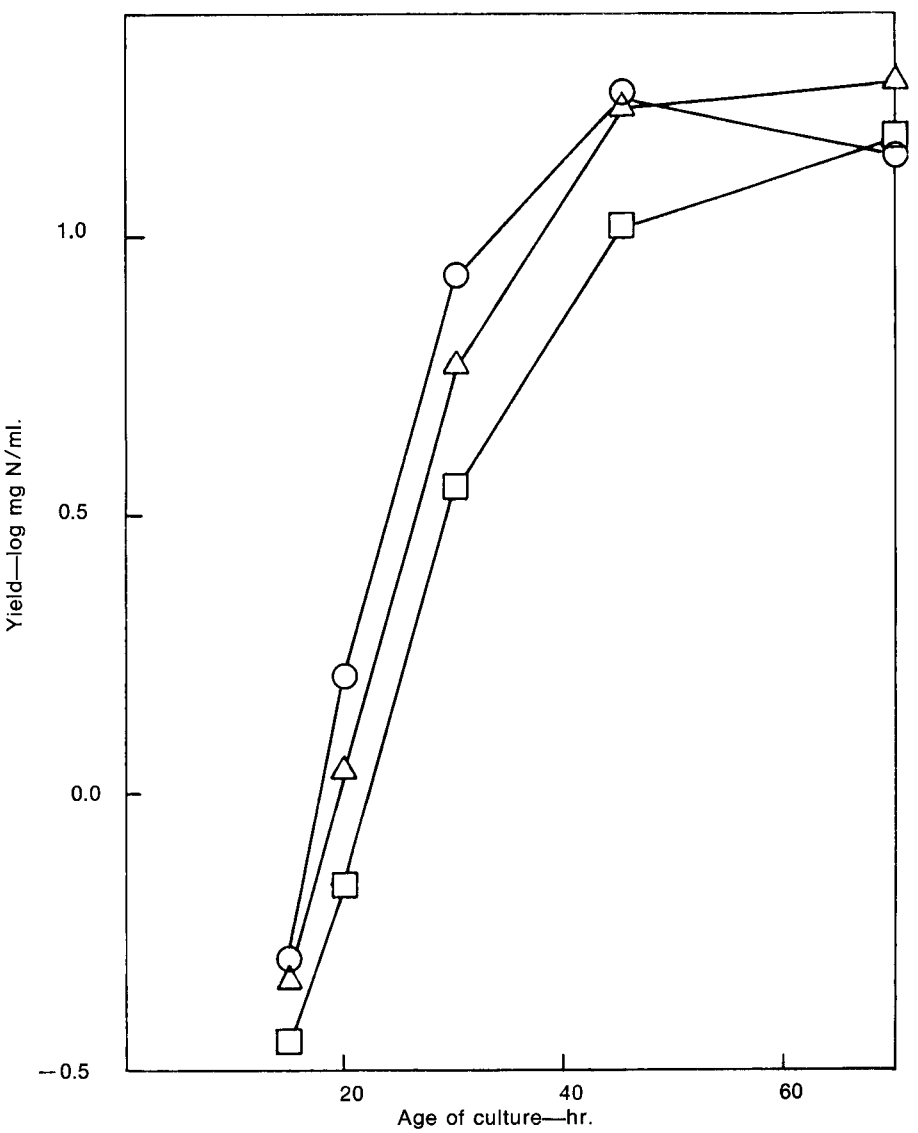

Fig. 1—Growth in terms of insoluble nitrogen of *Mycobacterium smegmatis* in carbon-limited (O), nitrogen-limited (Δ) and iron-limited (□) media.

efficient of AMP. The total deoxyribonucleotide content of the soluble fraction was measured by the diphenylamine method and by microbiological assay, both as described by Winder & Coughlan (1969).

Reagents. The chemicals were as described by Winder & Coughlan (1969).

Results

Growth

Attempts were made to discover conditions under which *M. smegmatis* would grow in shaken culture in dispersed form throughout the full growth cycle of a culture. However, although incorporation of Tween 80 into the medium resulted in dispersed growth during the early part of the growth cycle, clumping took place subsequently unless very high concentrations of Tween 80 were used. Hence, no dispersing agent was added during these investigations. Under these conditions, growth was obtained in the form of small granules which increased somewhat in size as the culture aged but did not grow beyond about 0.5 mm. Consequences of this form of growth will be referred to later.

The concentration of the limiting nutrient in each of the three types of culture was adjusted so that deceleration of growth occurred at about the same time in the three cultures. However, since the composition of the medium affected other phases of the growth cycle, the three cultures could not be kept in phase with each other throughout the growth cycle. The growth curves of the three cultures are shown in fig. 1. Each point represents a mean value derived from five experiments. Since the lag phase varied slightly from experiment to experiment, cultures from successive experiments were slightly out of phase with each other and, consequently, curves based on mean values are slightly distorted in certain respects referred to below.

Acceleration of the cultures to the maximal growth rate required several generations. When our observations began, 15 hr. after inoculation, there had been a 5-10 fold increase in yield over the inoculum, yet not all cultures had fully achieved maximal growth rate. The curves illustrated suggest an exponential growth period extending from the ages of 15 to 30 hr. and having a generation time of about 4 hr. This is slightly misleading due to the combining experiments which were slightly out of phase with each other. Examination of the curves for individual experiments showed that in some experiments acceleration had not finished by 15 hr., so that in these cases the most rapid growth took place during the period 20 to 30 hr., while in other experiments acceleration had been completed by 15 hr. and the most rapid growth took place from 15 to 20 hr. Hence the true situation was that the organisms in their most rapid phase of growth had a generation time, as measured by cell nitrogen, of rather less than 4 hr. and that this phase extended over a period of at most two generations.

The carbon-limited cultures had a slightly shorter combined lag and acceleration phase than the other cultures, and there was a more marked evidence of deceleration after 20 hr., even in the mean curve illustrated,

than in the cases of the other cultures. The iron-limited cultures were last to reach their maximal growth rate.

The carbon-limited cultures showed distinct evidence of loss of nitrogen after 45 hr., no true stationary phase being observed, in contrast to what was found with the nitrogen-limited cultures. The iron-limited cultures, on the other hand, showed prolonged slow growth.

RNA content

The RNA content per unit of insoluble nitrogen of nitrogen-limited and iron-limited cells reached a peak at about 20 hr. (fig. 2) which was the beginning of the period of maximal growth rate. In carbon-limited cultures, which reached their maximal growth rate slightly sooner, a decline in RNA content in the cells had begun by 20 hr. Comparison of the growth curves for each individual experiment with the corresponding curves for RNA content brought this point out more clearly than does a comparison of the composite curves in fig. 1 and 2.

As the growth rates subsequently declined progressively the RNA content of the cells in all of the cultures fell more or less smoothly until

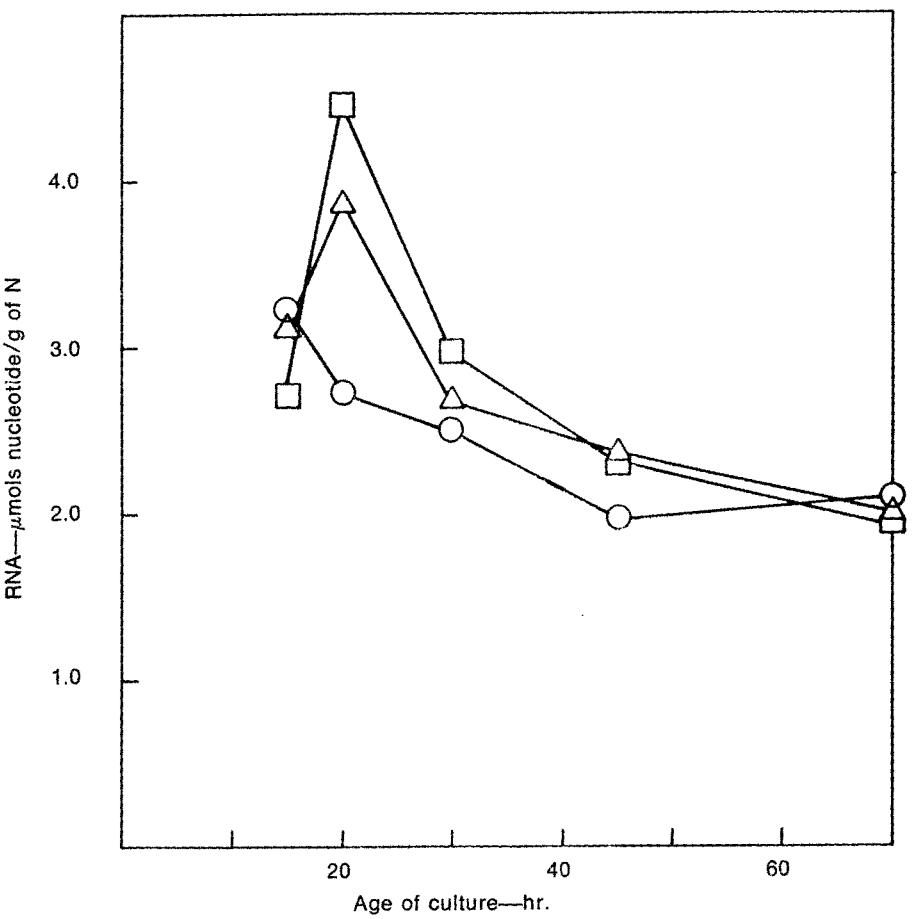

Fig. 2—RNA per g of insoluble nitrogen in *Mycobacterium smegmatis* from carbon-limited (O), nitrogen-limited (Δ) and iron-limited (□) media.

a value about half of the maximum was reached. No clear differences between the behaviour of the three types of culture was observed throughout this period.

DNA content

Like the RNA content, the DNA content (per unit of insoluble nitrogen) of nitrogen-limited and iron-limited cells increased between 15 and 20 hr., while again a peak value in the carbon-limited cultures appeared to have been reached somewhat earlier (fig. 3). Again, comparison of growth and DNA content curves for each experiment brought this point out more clearly.

The subsequent behaviour of the DNA content of the cells varied with the medium. The DNA content of the iron-limited cultures declined in a similar fashion to the RNA content. The DNA content of the nitrogen-

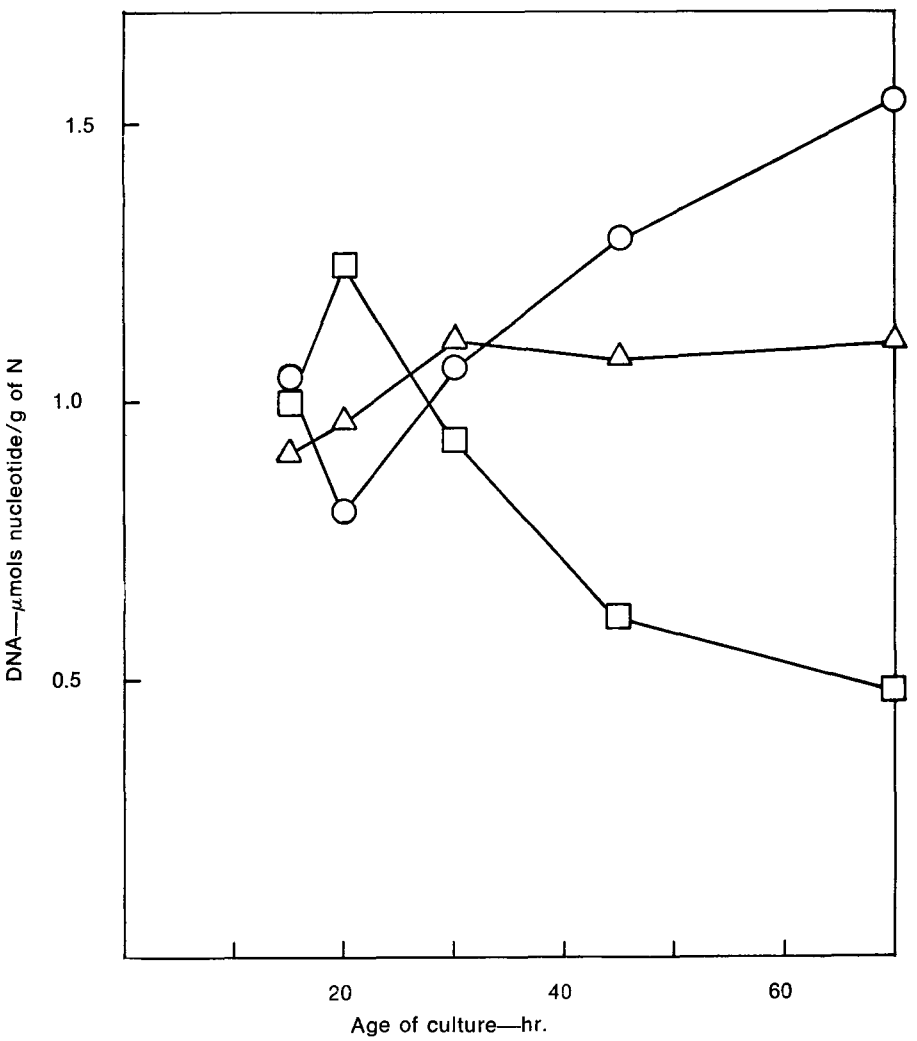

Fig. 3—DNA per g of insoluble nitrogen in *Mycobacterium smegmatis* from carbon-limited (O), nitrogen-limited (Δ) and iron-limited (□) media.

limited cultures increased slightly further and then settled to a constant value for about the last two generations of growth. In the carbon-limited cultures the downward trend was replaced after 20 hr. (about three generations before cessation of growth) by an upward trend for the remainder of the observation period. The continuation of this upward trend over the last period of observation was due to a constant amount of DNA and a declining amount of protein.

Soluble nucleotide pool

The total soluble nucleotide pool, as measured by the extinction at 260 nm., represented about 1000 μmoles of AMP-equivalent/g of insoluble nitrogen at 15 hr. with no distinct difference being shown between the cultures. This declined until it reached a value in the vicinity of 250 μmoles at 30 hr. and remained constant thereafter, all the cultures again behaving similarly.

The soluble deoxyribonucleotide pool was measured by microbiological assay and by chemical means. Values obtained by the two methods were in good agreement. All values obtained fell within the range 10-15μmoles/g of nitrogen. However, there was too much variation between experiments for detailed conclusions to be drawn about the behaviour of this pool during the growth of the cultures. On the whole, the soluble deoxyribonucleotide content of cultures moved in the same direction as the DNA content. For example, there was no evidence for an accumulation of soluble deoxyribonucleotides when the DNA level fell in iron deficiency; if anything, the deoxyribonucleotide content was low under these conditions.

Discussion

The employment of high concentrations of dispersing agents is necessary to obtain truly dispersed growth of this organism throughout the entire growth cycle of the cultures. Since, for several reasons, we did not wish to use such concentrations of these agents, it was not possible to follow the growth cycle by turbidity measurement and, as a consequence, cultures could not be harvested at exactly fixed physiological ages. Hence, interpretation of our results is complicated by the fact that cultures in different experiments were slightly out of phase with each other.

The fact that, at most, only a small portion of the growth cycle of the cultures was truly exponential was also probably due to the granular form of growth. These granules increase in average size during the growth of cultures and some form of progressive growth limitation appears to operate within them. This phenomenon has also been observed in the case of shaken cultures of *M. tuberculosis*, and it is suggested that some form of 'cellular crowding' is involved (Winder & Rooney, 1970 b). This form of growth is probably responsible for the fact that the bacteria do not maintain a steady state with respect to composition for any appreciable proportion of the growth cycle of the cultures.

The fact that a decline in the growth rate, with accompanying decline

in the RNA/protein ratio, occurred earlier in the carbon-limited cultures than in the other two was probably due to early exhaustion of the small amount of glycerol in the carbon-limited medium, the remainder of the growth of that culture involving the use of asparagine as the sole source of carbon as well as of nitrogen. This explanation involves the assumption that glycerol is more rapidly taken up than is asparagine: this has been shown to be true in the case of *M. tuberculosis* BCG (Winder & Rooney, 1970 a).

The rapid initiation of a decline in cell nitrogen following termination of growth in the carbon-limited cultures was presumably due to the utilization of protein as a source of energy for the maintenance of the cells. In contrast, prolonged slow growth was observed in the iron-limited cultures, during which the iron content of the cells can drop to about one quarter of normal (Winder & O'Hara, 1966). This prolonged slow growth has also been observed with this organism in surface culture on iron-limited medium (Winder & O'Hara, 1962), while prolonged arithmetic growth has been observed in iron-limited cultures of *Escherichia coli* (Ratledge & Winder, 1964). These findings are in keeping with a catalytic rather than a structural role for the bulk of iron in these organisms, as would be expected.

The RNA/protein in the cells behaved in a relatively simple and constant fashion in the three media, reaching a maximum at about the start of the period of maximal growth rate and declining thereafter in each medium. This is in keeping with the well-documented positive correlation between the content of RNA, particularly ribosomal RNA, and the rate of protein synthesis in bacteria, irrespective of the limiting nutrient, usually interpreted as indicating that the rate of protein synthesis is partly determined by the concentration of ribosomes (Neidhardt, 1963; Maaloe & Kjeldgaard, 1966). It suggests that the maximal growth rate achieved by this organism also is limited by the concentration of ribosomes, and that growth limitation under the three conditions studied is mediated by an effect on the rate of ribosome synthesis. Less evidence for a correlation between RNA/protein ratio and growth rate has emerged in the case of *M. tuberculosis* BCG (Winder & Rooney, 1970 b), which suggests that the very low maximal growth rate of that organism is largely determined by factors other than the concentration of ribosomes.

The increase in DNA per unit of nitrogen in the later stages of carbon-limited cultures is similar to that in late stages of surface cultures of metal-sufficient Proskauer & Beck medium (Winder & O'Hara, 1962) and to what occurs in *M. tuberculosis* BCG in a medium rich in nitrogen (Winder & Rooney, 1970 b). This diminution in the ratio of cytoplasm to nuclear material may be related to survival under certain types of unfavourable conditions. The fall in DNA in iron limitation suggests a requirement for iron for DNA synthesis. The fact that the soluble deoxyribonucleotide pool tended to fall under these circumstances suggests that polymerization is not the stage in DNA synthesis which requires iron. A requirement for iron for ribonucleotide reductase activity, as has been shown to apply in *Escherichia coli* (Brown, Eliasson, Reichard & Thelander, 1968), may be responsible. The small, but highly reproducible, fall in DNA/protein ratio which occurred in an early stage of the carbon-limited cultures may be related to the switch from glycerol to asparagine discussed earlier.

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