Effects of Nitrogenous Components of the Medium on the Carbohydrate and Nucleic Acid Content of *Mycobacterium tuberculosis* BCG

By F. G. WINDER AND S. A. ROONEY

*Department of Biochemistry and Laboratories of the
Medical Research Council of Ireland, Trinity College, Dublin*

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SUMMARY

The effects of varying the concentrations of the nitrogen sources in glycerol+asparagine, glycerol+asparagine+casein hydrolysate and glycerol+ammonium sulphate media on the composition and growth rate of *Mycobacterium tuberculosis* BCG grown in shaken culture for various periods were investigated. The bacteria were fractionated and the soluble, alkali-extractable, hot acid-soluble and residual fractions analysed for carbohydrate, RNA, DNA, free lipid, soluble amino compounds and soluble phosphates.

The results indicate that several forms of growth restriction operated in these experiments: a progressive reduction in growth rate even in conditions when nutrients were not limiting, due to clumping of cells; a reduction in growth rate following any reduction in the amino acid content of the medium; a marked reduction in growth rate when the amino acid content of the medium fell below a critical level, or was replaced by ammonium ions. When the growth rate was reduced because of nutrient limitation, the carbohydrate content of the bacteria increased, but the pattern of accumulation varied with the experimental conditions. The RNA: protein ratio of the cells was little affected by growth rate.

INTRODUCTION

Although much is known about the chemistry of mycobacteria, including *Mycobacterium tuberculosis*, quantitative studies on how their composition varies with environmental conditions have been few. Until very recently, only in the case of *Mycobacterium phlei* had studies been made of the effects of environment on carbohydrate content (Tepper, 1965; Antoine & Tepper, 1969*a*). The studies outlined in this paper were carried out primarily to obtain background information against which to interpret the effects of isoniazid on the carbohydrates of *M. tuberculosis* (Winder, Brennan & McDonnell, 1967; Winder & Rooney, 1970). Since this work was done, Antoine & Tepper (1969*b*) have given a short report on the environmental control of the carbohydrate and lipid content of *M. tuberculosis* R1Rv, and their results are essentially in agreement with some of those in this paper.

As far as variations in DNA, RNA and soluble phosphate contents of mycobacteria are concerned, only in the case of *Mycobacterium smegmatis* and *M. phlei* have detailed studies been made (Winder & Denneny, 1956; Winder & O'Hara, 1962; Tepper, 1965; F. G. Winder & M. P. Coughlan, unpublished observations). Consequently, observations on the effects of environment on the amounts of these materials in *M. tuberculosis* are included in this paper.

METHODS

Media. The glycerol+asparagine medium usually had the composition of Sauton medium, as described by Soltys (1952), except that the concentration of glycerol was 75.5 g./l. The glycerol+asparagine+casein hydrolysate medium usually had the same composition, with the addition of 3 g. pancreatic casein hydrolysate/l. Where required, variations in the composition of these two media are noted in the text. All of these media contained ammonium ions (about 400 mg. N/l.) in addition to the organic nitrogen. In the glycerol+ammonium sulphate media, the amino acids were replaced by ammonium sulphate, normally 8 g./l.

Growth of bacteria. *Mycobacterium tuberculosis*, var. *bovis*, strain BCG (Glaxo Laboratories Ltd) was grown as described previously (Winder *et al.* 1967) under stationary conditions for 4 days and then with shaking for further periods.

Extraction procedures. The 'soluble', 'free lipid' and 'alkali-extractable fractions' were obtained as described by Winder & Rooney (1970), and the remaining insoluble fraction was used to obtain the 'hot acid-soluble' and 'residual' fractions in the manner described by Winder *et al.* (1967).

Assays. Except where otherwise stated, carbohydrates were determined by an anthrone procedure (Winder *et al.* 1967). The phenol method was that of Dubois *et al.* (1956). In both cases the results are given in terms of glucose as the standard. 'Combined phosphate' is the difference between total phosphate and inorganic phosphate, which were determined as described by Winder & O'Hara (1962), as were total nitrogen (determined in the insoluble fraction), RNA and DNA. Amino acids were determined by the method of Moore & Stein (1954), using leucine as the standard.

The organic nitrogen content of the medium at the time of harvesting was estimated by subtracting the insoluble nitrogen content of the bacteria from the initial organic nitrogen content of the medium.

RESULTS

The effects of age on the composition of bacteria from relatively young batch cultures of *Mycobacterium tuberculosis* BCG grown in our normal glycerol+asparagine+casein hydrolysate medium are shown in Tables 1 and 2. The data on yields in the tables showed that, although the cultures were young and far from exhaustion of any component in the medium, growth was not truly exponential: the generation time increased from rather less than 24 h. to rather more than 48 h. over the 6 days of the experiment. These cultures showed a small increase in total carbohydrate content per unit of nitrogen with age over the period studied; this increase was due to an upward tendency in the soluble and alkali-extractable fractions partly balanced by a downwards tendency in the carbohydrate content of the residue. There was no trend in the RNA content per unit of nitrogen of such cultures, but there were marked upward trends in the DNA and lipid contents and perhaps in the content of soluble amino compounds, and a marked downward trend in their soluble combined phosphate content.

When the casein hydrolysate was omitted from the medium, the cultures of *Mycobacterium tuberculosis* BCG grew more slowly (Tables 1 and 2, Expt. *b*). Their carbohydrate content at low yields was higher than in the richer medium, largely due to a much higher content of hot acid-soluble carbohydrate. The carbohydrate content of

Table 1. *Effect of age of culture and medium constituents on the carbohydrate composition of Mycobacterium tuberculosis BCG*

All the media contained glycerol together with (a) 4 g. asparagine + 3 g. casein hydrolysate/l., (b) 4 g. asparagine/l. and (c) reduced asparagine + casein hydrolysate (respectively 1 g. + 0.75 g/l.). Data in (a), (b) and (c) from different experiments. Results are expressed per g. insoluble nitrogen.

Age (days)	Yield of cells (mg. insoluble N/l.)	Organic nitrogen in medium (mg. N/l.)	Carbohydrate fractions				Total carbo- hydrate
			Soluble	Alkali- extractable	Hot acid- soluble	Residue	
(a) Medium with 4 g. asparagine + 3 g. casein hydrolysate/l.							
6	7.9	1310	2.67	1.91	2.39	2.28	9.24
7	17.1	1300	2.74	2.14	2.64	2.45	9.96
8	32.0	1290	3.20	2.01	2.87	2.35	10.42
9	51.3	1270	3.64	2.36	2.31	2.19	10.50
10	72.5	1250	3.43	2.26	2.67	2.13	10.49
12	135.2	1180	3.43	3.03	2.15	1.57	10.17
(b) Medium with 4 g. asparagine/l.							
9	4.3	840	2.35	2.62	10.10	2.86	17.93
11	15.4	825	—	—	—	—	16.63
13*	42.2	798	2.48	3.20	8.21	2.37	16.26
15*	55.8	784	3.52	2.56	6.55	1.74	14.37
(c) Medium with 1 g. asparagine + 0.75 g. casein hydrolysate/l.							
8*	32.5	297	3.18	2.29	9.65	3.71	18.83
12*	141.5	188	3.29	4.27	11.56	6.73	25.83
17†	318.0	12	8.82	9.03	26.06	5.87	49.53
20†	354.0	—	7.84	11.03	27.52	8.76	55.15

* Mean values from two experiments.

† Mean values from four experiments.

Table 2. *Effect of age of culture and medium constituents on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis BCG*

Data in (a) and (b) refer respectively to same experiments as (a) and (b) in Table 1. Results are expressed per g. insoluble nitrogen.

Age (days)	Soluble fraction					Lipids (g.)
	Nucleotides (as μ moles AMP)	Amino compounds (as μ moles leucine)	Combined phosphate (μ g. atoms P)	RNA (μ g. atoms P)	DNA (μ g. atoms P)	
	(a) Medium with 4 g. asparagine + 3 g. casein hydrolysate/l.					
6	220	1140	1650	1350	670	2.40
7	235	1230	986	1440	783	2.78
8	225	1090	900	1290	716	3.08
9	234	1750	976	1460	784	3.28
10	204	1270	728	1310	857	3.40
12	208	2670	483	1450	1460	4.41
(b) Medium with 4 g. asparagine/l.						
9	466	1610	1210	1340	935	3.95
11	495	3210	1097	1300	1030	5.23
13*	299	6450	1054	1260	1013	3.85

* Mean values from two experiments.

this fraction and of the residue decreased with age, leading, in spite of an increase in the combined soluble and alkali-extractable fractions, to a smaller total carbohydrate content. The content of soluble amino compounds increased markedly with age. Initially the bacteria had higher DNA, soluble nucleotide and lipid contents per unit of nitrogen than the bacteria from the richer medium.

Cultures were also grown in a medium containing only one-quarter of the normal concentration of asparagine and casein hydrolysate (Table 1, Expt. *c*), so that the initial concentration of amino acid nitrogen in the medium was only 330 mg./l.; this fell to below 200 mg./l. after 12 days and practically to zero after 17 days. Such

Table 3. *Effect of concentration of asparagine + casein hydrolysate in the glycerol + asparagine + casein hydrolysate medium on the carbohydrate composition of Mycobacterium tuberculosis BCG*

Bacteria harvested after (a) 9 days growth and (b) 14 days growth. (a) and (b) are different experiments. Results expressed per g. insoluble nitrogen.

Medium composition		Yield of cells (mg. insoluble N/l.)	Organic N in medium (mg. N/l.)	Carbohydrate fractions				
Asparagine (g./l.)	Casein hydrolysate (g./l.)			Soluble	Alkali- extractable	Hot acid- soluble (as mmoles 'glucose')	Residue	Total carbohydrates
(a)								
8	6	43.0	2600	2.37	1.54	2.06	0.922	6.89
4	3	39.0	1280	3.26	2.28	3.36	1.90	10.74
2	1.5	28.0	630	3.06	1.93	3.81	2.38	11.18
1	0.75	25.0	305	3.16	1.83	5.25	3.73	13.97
(b)								
8	6	250	2390	1.55	2.30	2.09	0.593	6.54
4	3	250	1070	1.98	3.07	2.38	0.918	8.34
2	1.5	204	456	3.20	4.07	5.43	3.12	15.82
1	0.75	177	153	4.08	3.69	8.07	5.23	21.07
0.5	0.375	33.8	131	6.45	5.98	10.32	5.03	28.04

Table 4. *Effect of concentration of asparagine + casein hydrolysate in the glycerol + asparagine + casein hydrolysate medium on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis BCG*

Expts (a) and (b) are the same as (a) and (b) in Table 3. Results are expressed per g. insoluble nitrogen.

Medium composition		Soluble fraction					
Asparagine (g./l.)	Casein hydrolysate (g./l.)	Nucleotides (as μ moles AMP)	Amino compounds (as μ moles leucine)	Combined phosphate (μ g. atoms P)	RNA (μ g. atoms P)	DNA (μ g. atoms P)	Lipids (g.)
(a)							
8	6	210	—	1200	1300	608	—
4	3	259	—	1350	1620	750	—
2	1.5	267	—	1410	1280	663	—
1	0.75	259	—	1470	1070	568	—
(b)							
8	6	139	2340	533	1020	764	4.25
4	3	161	2500	610	1250	681	4.58
2	1.5	216	1120	890	1440	785	4.52
1	0.75	255	890	1130	1150	570	—

cultures grew initially more slowly than normal cultures. This can be seen from the results in Table 3 but is not apparent from those in Table 1 since the picture is confused by the fact that in different experiments (e.g. *a*, *c* in Table 1) the length of the lag period frequently varied. This slower growth was accompanied by a higher content of carbohydrate, even after 8 days, than in the richer cultures, largely due to an increase in hot acid-soluble carbohydrate and, to a smaller extent, in residual carbohydrate. As the growth rate declined after about 12 days, due to the falling amino acid content of the medium, the carbohydrate content of all fractions increased.

In order to confirm the effects on carbohydrate content of changing the concentrations of nitrogen sources, and to avoid complications due to interexperimental variation, cultures containing different initial concentrations of asparagine and casein hydrolysate were incubated together and all were harvested after a fixed time. Tables 3 and 4 show the results of two experiments employing different growth periods. In the early stages of growth of such cultures (e.g. up to 9 days), even though the amino acid nitrogen content of the medium in all the cultures was still more than 300 mg./l., the growth rate diminished slightly but progressively with decreasing concentrations of asparagine and casein hydrolysate. Accompanying this diminished growth rate there was a marked increase in the hot acid-soluble and residual carbohydrate, while the carbohydrate content of the other two fractions remained relatively unaffected. There were no consistent changes in nucleic acid content with the nitrogen content of the medium. When the organic nitrogen content of the medium was reduced to half of the lowest concentration of 0.5 g. asparagine and 0.375 g. casein hydrolysate/l., growth was extremely slow and insufficient material was available for analysis after 9 days growth.

After longer incubation periods there was a tendency for the differences in growth rates to become reduced, probably due to the growth-retarding effect of the formation of cell clumps (see below) which would operate sooner in the more rapidly growing cultures. After 14 days growth the cultures with 2 g. asparagine + 1.5 g. casein/l. showed signs of a general increase of carbohydrate content in all fractions, a decrease in soluble amino compounds and an increase in soluble combined phosphate. All of these results are characteristic of limitation of amino acids in the medium, even though there was still about 450 mg. amino acid nitrogen/l. of medium. These effects were decidedly more marked in the cultures containing 1 g. asparagine + 0.75 g. casein hydrolysate/l., in which the organic nitrogen content of the medium had fallen to 150 mg./l. after 14 days growth. Cultures which initially had a still lower concentration of amino acids in the medium grew very slowly and the accumulation of carbohydrate in all the fractions (except the residue) was more marked.

The results indicate that growth rate and carbohydrate content of the bacteria, particularly in the hot acid-soluble and residual fractions, was affected by the amino acid content of the medium within a wide range of concentrations, but that the effects became marked when the organic nitrogen content of the medium dropped below about 500 mg/l., and particularly when it dropped to 200 mg/l. or below.

Results in general conformity with this were obtained in glycerol + asparagine media (Tables 5, 6). Reduction in the asparagine content of the medium in the range 16 to 4 g./l. reduced the growth rate, though the results for the carbohydrate content of the cells were somewhat erratic. At the lower asparagine concentrations, corresponding to about 400 and 200 mg. amino acid nitrogen/l. much more marked changes occurred. The growth rate was strikingly reduced. The cells had a high carbohydrate content and,

particularly at the lowest nitrogen level (1 g. asparagine/l.), a low content of soluble amino compounds and some increase in soluble combined phosphate. In one respect, however, the effects of reduction in the amino acid content of the glycerol + asparagine media differed from the effects of reduction in nitrogen content of the glycerol +

Table 5. *Effect of concentration of asparagine in glycerol + asparagine medium and of ammonium sulphate in glycerol + ammonium sulphate medium on the carbohydrate composition of Mycobacterium tuberculosis BCG*

Bacteria harvested after 15 days growth. Results are expressed per g. insoluble nitrogen. Data in (a) and (b) from different experiments.

			Carbohydrate fractions				Total carbohydrate
Nitrogen source (g./l.)	Yield (mg. insoluble N/l.)	Organic N (a) or NH ₄ -N (b) in medium (mg. N/l.)	Soluble	Alkali-extractable	Hot acid-soluble	Residue	
(a) Asparagine							
16	104.7	3260	3.42	4.70	4.94	1.61	14.67
8	85.7	1590	3.26	4.06	8.56	2.73	18.61
4	58.7	780	3.04	6.40	5.20	1.95	16.59
2	11.3	409	2.74	3.46	15.35	4.10	25.65
1	1.8	208	1.10	7.55	25.80	8.69	43.14
(b) Ammonium sulphate							
8	16.9	1680	9.95	4.55	23.25	4.77	42.52
2	17.8	407	7.43	4.44	23.10	5.58	40.55

Table 6. *Effect of concentration of asparagine in glycerol + asparagine medium and of ammonium sulphate in glycerol + ammonium sulphate medium on the phosphorus components, amino compounds and lipids of Mycobacterium tuberculosis BCG*

Bacteria harvested after 15 days growth. Results are expressed per g. insoluble N. Expts (a) and (b) are the same as (a) and (b) in Table. 5.

Nitrogen source (g./l.)	Soluble fractions					
	Nucleotides (as μ moles AMP)	Amino compounds (as μ moles leucine)	Combined phosphates (μ g. atoms P)	RNA (μ g. atoms P)	DNA (μ g. atoms P)	Lipids (g.)
(a) Asparagine						
16	230	7470	995	1808	1045	3.82
8	227	7500	—	1509	1165	3.84
4	227	6850	1260	1260	1013	3.83
2	258	1750	1323	1528	1044	—
(b) Ammonium sulphate						
8	528	7620	1880	1559	1408	3.67
2	428	2500	1670	1715	1545	—

asparagine + casein hydrolysate media: in the former case the reduction led to a reduced content of soluble carbohydrate in the cells, while in the latter case it led to an increase in this material.

The use of an ammonium salt as the sole nitrogen source led to slow growth and a high carbohydrate content of all fractions (Tables 5, 6). It also led to a high soluble pool of nucleotides and combined phosphates, and to a rather high DNA content.

Reducing the concentration of $(\text{NH}_4)_2\text{SO}_4$ from 1700 mg. $\text{NH}_4\text{-N/l.}$ to 400 mg. $\text{NH}_4\text{-N/l.}$ of medium had little effect on the composition of the cells, apart from the concentration of soluble amino compounds, which showed a substantial decrease (Tables 5, 6). It should be noted, however, that the ninhydrin method, which was used to measure the amino compounds, also responds to ammonium ions, and it seems likely that it was the concentration of these which changed in response to the external ammonium ion concentration.

Table 7. *Changes in the composition of Mycobacterium tuberculosis BCG, grown in glycerol + asparagine + casein hydrolysate medium and then transferred to other media for 6 h.*

Figures for insoluble nitrogen were obtained by dividing final values by initial values. Other figures were obtained by dividing final values per g. of insoluble nitrogen by initial values per g. of initial insoluble nitrogen. The results are the means from two experiments.

Transfer medium	Insoluble nitrogen	Soluble carbohydrate	Alkali-extractable carbohydrate	Hot acid-soluble carbohydrate	Residual carbohydrate	Total carbohydrate
Glycerol	1.00	1.35	1.27	1.33	1.24	1.32
Glycerol + ammonium sulphate	1.06	1.82	1.15	0.96	0.76	1.18
Glycerol + asparagine + casein hydrolysate	1.18	1.11	1.11	1.25	1.03	1.15
Soluble fraction						
	Nucleotides	Amino compounds	Combined phosphate	RNA	DNA	
Glycerol	1.19	1.01	1.29	1.16	1.05	
Glycerol + ammonium sulphate	1.22	1.17	1.05	1.06	1.03	
Glycerol + asparagine + casein hydrolysate	1.24	1.60	1.09	1.02	1.02	

In order to study the speed with which the composition of *Mycobacterium tuberculosis* BCG responded to altered growth conditions, bacteria which had been grown in glycerol + asparagine + casein hydrolysate medium were washed and resuspended either in the same medium or in glycerol + ammonium sulphate medium, or in glycerol medium with no nitrogen, and incubated for 6 h. (Table 7). As judged by total nitrogen content, organisms resuspended in the original medium resumed growth at something like the previous rate, those in the ammonium sulphate medium grew slightly, while those in the nitrogen-free medium did not change in nitrogen content. Even in the short period of the experiment, the carbohydrate per unit of nitrogen increased markedly in all the fractions obtained from the bacteria incubated in the nitrogen-free medium, while only a slight increase took place in the original medium. In the ammonium sulphate medium there was an unexpected fall in the carbohydrate content of the residue and a marked increase in the soluble fraction. An increase in soluble nucleotide content took place in all media, there was a marked increase in the soluble amino acid content in the original medium and in the soluble phosphate in the nitrogen-free medium. Other

changes in the composition of the bacteria during the experimental period were marginal.

Since the glycerol concentration in the media employed in this work was high (75.5 g./l.), experiments were carried out to compare the carbohydrate content of *Mycobacterium tuberculosis* BCG grown in such media with its content in media with one-tenth the amount of glycerol. The lower concentration was still sufficient to provide a substantial excess of glycerol, since the concentration in the medium had not been appreciably reduced at the time of harvesting. It was found both with asparagine +

Table 8. *The effect of reducing the glycerol concentration in the medium on the carbohydrate content of Mycobacterium tuberculosis BCG*

Carbohydrate was assayed by the phenol method. Results are the mean values from two experiments.

Medium		Carbohydrate content of fractions			
Nitrogen source (g./l.)	Glycerol (g./l.)	Soluble	Alkali-extractable	Hot acid-soluble plus residue	Total carbohydrate
			(as mmoles 'glucose'/g. insoluble nitrogen)		
Asparagine (4) + casein hydrolysate (3)	75.5	2.88	3.56	8.15	14.59
	7.55	2.78	2.99	5.89	11.66
Ammonium sulphate (8)	75.5	6.87	4.73	15.17	26.80
	7.55	6.61	4.55	9.65	20.82

Table 9. *Carbohydrate compounds of the soluble fraction from Mycobacterium tuberculosis BCG grown with different amounts of glycerol in asparagine + casein hydrolysate medium and in ammonium sulphate medium*

The fraction was concentrated, de-ionized with Amberlite MB-1, chromatographed in ethyl acetate + pyridine + water (10:4:3, by vol.) and the bands eluted and assayed by the phenol method, all as described by Winder & Rooney (1970). The results are given as μ moles 'glucose'/g. insoluble nitrogen.

Medium		Total soluble carbohydrate	Recovery after de-ionization (%)						Total recovery from paper (%)
Nitrogen source (g./l.)	Glycerol (g./l.)			Trehalose	Polysaccharide	Glucose	Mannose + arabinose	Oligo-saccharide	
Asparagine (4) + casein hydrolysate (3)	75.5	3140	84	1070	642	65	186	16	75
	7.55	3040	56	850	350	55	69	14	79
Ammonium sulphate (8)	75.5	6415	91	2570	1415	274	174	251	81
	7.55	6645	63	2000	978	94	56	415	85

casein hydrolysate medium and ammonium sulphate medium that this reduction in glycerol concentration slightly reduced the carbohydrate content of the bacteria, the reduction taking place mainly in the insoluble fraction (Table 8). No experiments in glycerol-limited media were carried out.

In one such experiment the carbohydrates of the soluble fraction were subjected to further fractionation by paper chromatography, followed by elution and assay (Table 9). It was found that the change from the asparagine + casein hydrolysate medium

to the ammonium sulphate medium increased the amounts of trehalose and polysaccharide in about the same proportion and also caused a large increase in the oligosaccharide content of the fraction. Reduction in the glycerol content in both media reduced the trehalose and polysaccharide content of the fraction to a greater extent than the total carbohydrate content. It was noticed that when material from bacteria grown in the low glycerol medium was de-ionized by a mixed-bed resin treatment, the recovery of total carbohydrate was poor, suggesting that the soluble fraction of these bacteria had a high content of ionic derivatives of carbohydrates, presumably phosphorylated compounds.

DISCUSSION

The results obtained suggest that complex combinations of environmental effects operate even in the apparently simple experimental systems employed. Even in young cultures, in which all the known nutrients are far from exhaustion, the growth rate of *Mycobacterium tuberculosis* BCG declines progressively with age of culture. This has been observed also in shaken cultures of *M. smegmatis* when dispersing agents are not employed (F. G. Winder & M. P. Coughlan, unpublished observations). The reduction in growth rate is probably due to growth in the form of clumps, which increase in average size during the life of the culture, and within which the ill-defined phenomenon of 'cellular crowding' may take place. Effects of crowding have been demonstrated in *M. smegmatis* (Maruyama, Ono & Sato, 1963), and in *Escherichia coli* crowding appears to operate by leading to a shortage of energy sources which can be utilized under the redox conditions prevailing (Freter & Ozawa, 1963): such conditions may exist within the clumps. Decline in growth rate in *M. tuberculosis* BCG under these conditions is characterized by constant carbohydrate content and an increased DNA:protein ratio.

A second form of growth rate limitation is observed when the concentration or variety of amino acids in the medium is reduced. A progressive impoverishment of the medium in this fashion leads to a progressive decline in growth rate, even though the total pool of soluble amino compounds in the bacteria is relatively constant. This decline in growth rate is accompanied by a relatively small increase in total carbohydrate, confined to the hot acid-soluble and residual fractions. It is suggested that the richness of the medium in amino acids affects the steady state concentration of a limited number of amino acids within the cell, presumably amino acids whose carbon skeleton can only be synthesized slowly from glycerol, and that this affects the rate of protein synthesis. The cellular carbohydrate is largely derived from glycerol (F. G. Winder, P. J. Brennan & S. A. Rooney, unpublished observations) and hence its synthesis tends to outrun that of protein when the synthesis of the latter is thus slowed.

A third form of growth limitation appears when the amino acid concentration in the medium drops below a certain critical value, or when amino acids are replaced by ammonium salts. The critical organic nitrogen concentration in the medium varies with circumstances, but lies in the region of 200 to 500 mg. N/l. Calculations based on the figures of Antoine & Tepper (1969*b*) suggest that a similar range of organic nitrogen concentrations is also critical for *Mycobacterium tuberculosis* RIRv. When the amino acid concentration is below the critical level, the growth rate is very markedly reduced and high levels of all carbohydrate fractions of the bacteria are reached. It is suggested that at concentrations of amino acids below the critical one the organisms have

difficulty in abstracting them from the medium, leading to the observed reduction in the intracellular pool of soluble amino compounds and to a reduction in the rate of protein synthesis with consequent carbohydrate accumulation. These conditions operate even when ammonium ions are present in high concentration, leading to a large intracellular soluble nitrogen pool, as can occur in the glycerol + ammonium sulphate medium; in this case it must be assumed that the provision of at least some of the organism's amino acid requirements from ammonium salts and glycerol is a slow process. Bowles & Segal (1965) have reported that glutamate represses glycerol utilization of *M. tuberculosis* H37 Rv, so that de-repression of glycerol utilization might reinforce the tendency of the bacteria to accumulate carbohydrate at low concentrations of amino acids. However, investigations into the uptake of labelled glycerol, asparagine and glutamate in each other's presence do not suggest that this process plays a major role in regulating the carbohydrate:protein ratio (Winder & Rooney, 1970; F. G. Winder, S. A. Rooney, & P. J. Collins, unpublished observations).

Characterization of the carbohydrates present in the various fractions of *Mycobacterium tuberculosis* BCG grown in glycerol + asparagine + casein hydrolysate medium is reported elsewhere (Winder & Rooney, 1970). In the present studies, only in the case of the soluble fraction was an attempt made to identify the individual carbohydrates whose concentration changed in response to growth conditions. In this case, trehalose and a glucan were found to be mainly involved. The carbohydrates which were usually measured in the other fractions contained hexose, since the anthrone method responds only slightly to pentose. Antoine & Tepper (1969*a, b*) reported that glycogen is the main carbohydrate which accumulates in *Mycobacterium phlei* and *M. tuberculosis* under their accumulation conditions.

The relative constancy of the RNA:protein ratio in spite of large changes in growth rate is somewhat surprising in view of the fact that in several bacteria it has been found that the amount of RNA (mainly ribosomal RNA) per unit of nitrogen varies approximately linearly with growth rate over a range of conditions (Magasanik, Magasanik & Neidhardt, 1959; Neidhardt, 1963). However, at lower growth rates bacteria appear to approach a constant minimal RNA:protein ratio (Magasanik *et al.* 1959; Maaløe & Kjeldgaard, 1966) and even the maximal growth rate of *Mycobacterium tuberculosis* is far below the range studied with other organisms, so that presumably its whole range of growth rates can be dealt with by a minimal concentration of ribosomes, though the constancy of this value still requires explanation. In the more rapidly growing *M. smegmatis*, variation in this ratio can be observed (Winder & O'Hara, 1962; F. G. Winder & M. P. Coughlan, unpublished observations), even though its maximal growth rate still lies below the rates which have been studied with other bacteria.

On the other hand, mycobacteria appear to have a rather variable DNA:protein ratio. This ratio is generally constant in growing bacteria, although a limited increase can occur during starvation (Neidhardt, 1963; Maaløe & Kjeldgaard, 1966). In *Mycobacterium smegmatis* substantial variations in this ratio can occur (Winder & O'Hara, 1962; F. G. Winder & M. P. Coughlan, unpublished observations), while in *M. tuberculosis* there is clear evidence for an increase in the ratio as the growth rate in a rich medium declines through clump formation.

The maximal RNA:DNA ratio achieved by *Mycobacterium tuberculosis* BCG is very low at 2:1, compared with 4:1 achieved by *M. smegmatis* and the higher values

reached by other micro-organisms. This is presumably related to its low maximal growth rate. The values reported here for this ratio for BCG are similar to those found for a number of other strains of *M. tuberculosis* (Winder & Denneny, 1956). Interference by inorganic polyphosphate in their assay probably contributed to the somewhat high value of 5:1 reported for *M. tuberculosis* BCG by Tsumita & Chargaff (1958), while the very high ratios reported by Youmans & Youmans (1968) for H37 Ra were probably due to interference by arabinose-containing polysaccharides in the orcinol method used for measuring RNA.

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