Synthesis of Protein and Ribonucleic Acid by Starved *Streptococcus lactis* in Relation to Survival

By T. D. THOMAS* AND R. D. BATT

*New Zealand Dairy Research Institute, Palmerston North, New Zealand, and
Chemistry and Biochemistry Department, Massey University, Palmerston North,
New Zealand*

(Accepted for publication 7 July 1969)

SUMMARY

Protein synthesis, as estimated from incorporation studies with [¹⁴C]valine, was barely detectable when organisms were starved in phosphate buffer containing Mg²⁺. The addition of an energy source promoted limited protein synthesis. In this respect glucose produced a much higher rate of [¹⁴C]valine uptake and incorporation than arginine. Although arginine prolonged survival, experiments with inhibitors showed that this was unlikely to be due to the protein synthesis which had been promoted. The ability of starved organisms to assimilate [¹⁴C]valine and to incorporate the isotope into protein in the presence of glucose appeared to be correlated with survival.

Synthesis of RNA in starved organisms, as determined from [¹⁴C]uracil incorporation experiments, depended on exogenous glucose. During brief exposures to [¹⁴C]uracil plus glucose at intervals during starvation, organisms incorporated decreasing amounts of isotope into RNA although incorporation by non-viable organisms occurred at approximately half the initial rate. Although resynthesis of both protein and RNA was demonstrated with added energy sources, no evidence was obtained to suggest that this favoured survival.

INTRODUCTION

When bacteria are starved of a nitrogen source, net protein and RNA synthesis stops but protein and RNA turnover may continue for several hours. Degradation of pre-existing protein and RNA provides amino acids and bases for resynthesis (Mandelstam, 1960, 1963). Although the rates of protein degradation may be similar in both nitrogen- and carbon-limited media (Willetts, 1967), the rate of protein synthesis depends on an energy source (Schlessinger & Ben-Hamida, 1966). These authors reported declining, although significant protein turnover in nitrogen-starved *Escherichia coli* for at least 20 hr. Ben-Hamida & Schlessinger (1966) recorded a much lower rate for RNA turnover in nitrogen-starved *E. coli* and concluded that ribosomes were not resynthesized and that the net effect of the turnover process was to transfer amino acids and nucleotides from a surplus of ribosomes to the soluble proteins, energy supply and reserves required for subsequent adaptations.

Macromolecule resynthesis may represent a mechanism whereby cell constituents, whose loss is particularly likely to result in death, may be selectively reformed from dispensable material (Burleigh & Dawes, 1967). The present paper describes experiments undertaken to determine the role of polymer resynthesis in the survival of starved *Streptococcus lactis*.

* Present address: Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

METHODS

General procedures. The organism (*Streptococcus lactis* ML₃), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969*a*).

Measurement of protein synthesis. Protein synthesis in starved organisms was estimated by following the incorporation of [¹⁴C]valine into cell protein (Willetts, 1967). Culture samples (2 ml.) were added to an equal volume of trichloroacetic acid (TCA, 10 %, w/v) and, after heating 30 min. at 90° (see Marchesi & Kennell, 1967), the precipitates were filtered off on membrane filters (25 mm., pore size 0.45 μ ; Millipore Filter Corp., U.S.A.). Each filter was washed successively with three 1 ml. volumes of TCA (5 %, w/v) containing unlabelled DL-valine (150 μ g./ml.), TCA (5 %, w/v) alone and acetic acid (1 %). Material isolated by this procedure may include cell wall substance as well as 'true' protein. However, lactic acid bacteria are reported to contain little valine in the cell walls (Ikawa & Snell, 1960).

For the measurement of [¹⁴C]valine uptake by whole cells, culture samples (2 ml.) were filtered on membrane filters, washed with ten 2 ml. volumes of 0.075 M-phosphate buffer containing unlabelled L-valine (200 μ g./ml.) and dried. The washing procedures used for incorporation and uptake measurements removed all radioactivity from the membrane filters in control systems to which cells had not been added. [¹⁴C]Valine incorporation into protein was estimated with a standard deviation of ± 4 % (16 determinations); for [¹⁴C]valine uptake by cells it was ± 6 % (8 determinations).

Measurement of RNA synthesis. Estimates of RNA synthesis were made by analogous procedures with [¹⁴C]uracil. Trichloroacetic acid at 0° was added to culture samples giving a final concentration of 5 %. After 30 to 60 min. at 0°, the acid-insoluble material was collected on a membrane filter, washed at 0° with 5 % TCA (10 $\times$ 2 ml.) and finally with 0.1 N-HCl (2 $\times$ 2 ml.); the filters were then dried. For the measurement of [¹⁴C]uracil uptake by whole cells, the organisms were collected on a membrane filter and washed with ten 2 ml. volumes of 0.075 M-phosphate buffer containing uracil (200 μ g./ml.). [¹⁴C]Uracil incorporation into RNA was estimated with a standard deviation of ± 2 % (16 determinations); for [¹⁴C]uracil uptake by cells it was ± 4 % (8 determinations).

The radioactivity on the dried membrane filter discs was measured with a Packard Tri-Carb 2000 series liquid scintillation spectrometer (gain settings 10 %, window settings 50–1000) with an efficiency of 70.5 % as determined by channels ratio measurements. The scintillation solution consisted of 2,5-diphenyloxazole (5.0 g.) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.3 g.) per litre of toluene. [¹⁴C]-DL-valine and [¹⁴C]uracil were obtained from the Radiochemical Centre, Amersham.

RESULTS

Protein synthesis. Protein synthesis, determined by the measurement of [¹⁴C]valine incorporation into hot TCA-insoluble material, required an exogenous energy source (Table 1); there appeared to be no endogenous energy source capable of supporting protein synthesis. Exogenous glucose produced approximately four times as much protein synthesis as arginine.

Uptake of [¹⁴C]valine by starved organisms occurred without an exogenous energy

source but to a much lesser extent (Table 1). This initial uptake of a small amount of [^{14}C]valine and the subsequent loss of activity (Table 1) may indicate the presence of a small ATP pool at the onset of starvation. Retention of the accumulated TCA-soluble [^{14}C]valine by starved organisms required an exogenous energy source. At the bacterial density used in Table 1, 10 mM-glucose would be completely fermented in approx. 2 hr while 10 mM-arginine would require about 7 hr for complete metabolism (Thomas & Batt, 1969*b*).

Table 1. [^{14}C]Valine uptake and incorporation by starved *Streptococcus lactis*

Washed organisms, 0.64 mg. dry wt./ml., were starved at 30° in 0.075 M-phosphate buffer (pH 7.0) containing 10 μM -EDTA, 1 mM-MgSO₄, [1- ^{14}C]-DL-valine (25 μC ./ml., 6 μg ./ml.) and L-valine (200 μg ./ml.). Additions or omissions from this buffer are given in the first column. Samples (2 ml.) were removed at intervals and treated as described in Methods. Counts of total [^{14}C]valine uptake are given in parentheses.

Additions to above buffer	Time (min.)							
	7	17	30	45	60	90	120	190
	[^{14}C]valine incorporation and uptake (c.p.m./mg. dry wt bacteria [*]).							
None	9 (619)	11 (659)	11 (446)	—	14 (318)	—	20 (185)	11 (154)
+ Glucose (10 mM)	247 (1513)	471 (1981)	626 (2227)	—	801 (3144)	—	1080 (3090)	1137 (3180) [†]
+ L-arginine (10 mM)	44 (705)	91 (898)	167 (934)	—	243 (1364)	—	242 (1381)	265 (1378)
+ L-arginine (10 mM) + casamino acids (2.78 mg./ml.) + L-valine	61 (499)	115 (703)	228 (845)	—	319 (904)	—	331 (912)	340 (856)
+ Glucose (10 mM) + chloramphenicol (200 μg ./ml.)	—	—	—	152 (1934)	—	214 (2224)	—	—
L-arginine (10 mM) + chloramphenicol (200 μg ./ml.)	—	—	—	28 (981)	—	34 (1209)	—	—

* Based on initial bacterial mass.

[†] Equivalent to approx. 0.5 % total [^{14}C]valine.

Net protein synthesis appeared to stop when the glucose was exhausted. By contrast, net synthesis stopped in the presence of excess arginine, even with casamino acids present (Table 1). *Streptococcus lactis* ML₃ organisms contained 23 μg . valine/mg. dry wt (Thomas & Batt, 1969*a*). Assuming that all the valine is present in cell protein and that the protein synthesized in starved organisms contained the above proportion of valine, then the amount of protein synthesis in starved organisms could be calculated from the rates of [^{14}C]valine incorporation. On this basis, with exogenous glucose and arginine, protein synthesis would amount to about 2 % and 0.5 % of the total cell protein in the first hour of starvation. The apparent cessation of protein synthesis after a period of starvation in the presence of arginine may have resulted from a balance being reached between protein synthesis and degradation. However, no relevant data were available to estimate the rate of protein degradation in the presence of exogenous arginine. Any TCA-insoluble protein released from starved organisms into the external medium during these experiments would be measured as cell protein. The concentration of free valine in *S. lactis* accounted for only 1 % of the total amino

acid pool (Thomas & Batt, 1969*a*). On incubation with glucose and radioactive valine, the concentration of free intracellular valine was calculated to increase by about three-fold so that the over-all change in the free amino acid pool during incorporation experiments was probably small and the differences in the rates of protein synthesis are not likely to result from differences in the levels of free amino acids.

Chloramphenicol caused 78 % and 86 % inhibitions of protein synthesis in the presence of glucose and arginine respectively (Table 1) while little or no inhibition of [^{14}C]valine uptake into the TCA-soluble pool was detected. (For these calculations, 45 and 90 min. values were obtained by expressing the data graphically.) This finding is consistent with the reports that chloramphenicol does not inhibit the transport or accumulation of other amino acids by micro-organisms (Gale & Paine, 1951; Holden, 1965).

Fig. 1. Ability of starved *Streptococcus lactis* to assimilate and incorporate [^{14}C]valine. Washed organisms were resuspended at a density of 0.93 mg. dry wt./ml. in 0.075 M-phosphate buffer (pH 7.0, + 10 μM -EDTA + 1 mM- MgSO_4) containing: L-arginine (10 mM), (O, ●); no addition (□, ■). The cultures were starved at 30°, samples (2 ml.) were withdrawn at intervals and added to 2 ml. 0.075 M-phosphate buffer containing MgSO_4 (1 mM), D-glucose (20 mM), [1- ^{14}C]-DL-valine (0.5 μC ./ml., 12 μg ./ml.) and L-valine (400 μg ./ml.). After incubation for 1 hr at 30°, the incorporation of [^{14}C]valine into cells (closed symbols) and protein (open symbols) was determined (see Methods). Results are based on the initial bacterial mass. The dashed lines indicate slide-culture viabilities.

The ability of starved organisms to assimilate [^{14}C]valine and to synthesize protein appeared to be correlated with survival (Fig. 1). With the bacterial concentration used in this experiment, 10 mM-arginine would be metabolized in approx. 4 hr (Thomas & Batt, 1969*b*) and consequently survival was not enhanced to the same extent as at lower bacterial densities. Although chloramphenicol inhibited protein synthesis, it did not increase the death rate and, in fact, there appeared to be a reduction in the death rate in some systems (Table 2).

RNA synthesis. RNA synthesis in starved organisms, as determined by the incorporation of [^{14}C]uracil into cold TCA-insoluble material, required exogenous

glucose (Table 3). Exogenous arginine gave only trace [^{14}C]uracil incorporation in contrast to its effect on [^{14}C]valine incorporation (Table 1). Net RNA synthesis stopped in the presence of excess glucose and uracil after a short starvation period (Table 3). This may have been due to either a shortage of other intermediates or to a balance being reached between synthesis and breakdown.

Fig. 2. Ability of starved *Streptococcus lactis* to incorporate [^{14}C]uracil. Washed organisms were resuspended at a density of 0.96 mg. dry wt/ml. in 0.075 M-phosphate buffer (pH 7.0, 30°) containing 10 μM -EDTA and 1 mM-MgSO₄. Samples (2 ml.) were removed at intervals and added to 20 μl . buffer containing [^{14}C]uracil (0.5 μC ., 60 μg .) and glucose (20 μmoles). After incubation for 10 min. at 30°, the incorporation of [^{14}C]uracil into RNA was determined (○) (see Methods). Results are based on the initial bacterial mass and viability (●) was determined by slide-culture.

Table 2. Effect of chloramphenicol on the survival of *Streptococcus lactis*

Washed organisms, 30 μg . dry wt/ml., were starved at 30° in phosphate buffer (0.075 M, pH 7.0, + 10 μM -EDTA + 1 mM-MgSO₄) containing the additions given in the first column. Samples were removed at intervals and the organisms washed (except where indicated). Viability was determined by slide-culture.

Additions to phosphate buffer	Time (hr)							
	0	0*	16	20	24	28	40	45
	Viability (%)							
None	99	99	96	81	79	37	1	0
+ Chloramphenicol (200 μg ./ml.)	99	0	98	91	92	80	6	1
+ Glucose (10 mM)	99	99	69	43	26	14	15	3
+ Glucose (10 mM) + chloramphenicol (200 μg ./ml.)	99	0	97	87	78	82	26	1
+ Arginine (10 mM)	99	99	99	96	98	94	85	52
+ Arginine (10 mM) + chloramphenicol (200 μg ./ml.)	99	0	98	99	99	87	76	31

* Slide-cultures prepared with unwashed organisms.

The rate of RNA synthesis rose to a reproducible maximum soon after the onset of starvation and then fell steadily (Fig. 2). Non-viable organisms still incorporated [^{14}C]uracil into RNA at an appreciable rate.

When a brief incubation of organisms with [^{14}C]valine plus glucose or with [^{14}C]uracil plus glucose was followed by a 100-fold excess of unlabelled valine or uracil, most of the isotope incorporated was retained in the starved organisms. Therefore exchange of the incorporated isotope does not occur readily with the external medium. Since *Streptococcus lactis* does not metabolize either valine or uracil, conversion of the label would not have affected the interpretation of the present results.

Table 3. [^{14}C]Uracyl uptake and incorporation by starved *Streptococcus lactis*

Washed organisms, 0.36 mg. dry wt./ml., were starved at 30° in 0.075 M-phosphate buffer (pH 7.0) containing 10 μM -EDTA, 1 mM-MgSO₄, [^{14}C]uracil (0.25 μC ./ml., 200 μg ./ml.) and the substrates given below. Samples (2 ml.) were removed at intervals and treated as described in Methods. Results are given as c.p.m./mg. initial dry wt bacteria.

Starvation period (min.)	Additions to buffer					
	None		Glucose (10 mM)		Arginine (10 mM)	
	Uptake	Incorp.	Uptake	Incorp.	Uptake	Incorp.
7	16	0	2242	875	0	0
18	174	11	4322	1230	168	9
30	568	3	5639	1277	364	13
60	477	14	7002	1273	547	31
120	116	0	7650	1112	502	14
180	160	6	7201*	1003	566	29

* Equivalent to approx. 1 % total [^{14}C]uracil.

DISCUSSION

Since macromolecule synthesis is an energy-consuming process, protein and RNA synthesis in starved *Streptococcus lactis* was not expected as no substantial endogenous energy source has been detected (Thomas & Batt, 1969*a*). The present investigation demonstrated that the accumulation and incorporation of [^{14}C]valine and [^{14}C]uracil required an exogenous energy source. Exogenous glucose promoted greater isotope accumulation and subsequent incorporation than arginine, perhaps because glucose metabolism produced a higher rate of ATP production (Thomas & Batt, 1969*b*). The acceleration of [^{14}C]valine uptake in the presence of an added energy source and the energy requirement for the retention of accumulated amino acid was consistent with mechanisms for the accumulation of amino acids in other bacteria (see Holden, 1962).

The survival capacity of starved organisms could be correlated with the ability to synthesize protein which may in turn be influenced by RNA stability. In contrast, non-viable organisms retained the ability to synthesize RNA at a substantial rate. Schlessinger & Ben-Hamida (1966) and Ben-Hamida & Schlessinger (1966) reported rapidly declining protein and RNA synthesis in nitrogen-starved *Escherichia coli* from the onset of starvation although it is not clear whether or not the energy source was exhausted after a period giving rise to the reduced isotope incorporation.

No evidence has been obtained in the present investigation to suggest that resynthesis of protein or RNA is important for survival. In fact, exogenous glucose, which gave maximum rates of resynthesis, increased the death rate and the rate of RNA hydrolysis (Thomas & Batt, 1969*a*). Experiments with chloramphenicol indicated that the pro-

longed survival with added arginine is unlikely to be a result of the limited protein synthesis which took place. In some starvation environments chloramphenicol was shown to reduce the death rate. As well as inhibiting protein synthesis, chloramphenicol may indirectly inhibit protein degradation (Schlessinger & Ben-Hamida, 1966; Willetts, 1967) and hence may exert some sparing action on specific cellular proteins which are essential for survival of *Streptococcus lactis*. All reported studies involving the starvation of vegetative bacteria indicate that, while macromolecule degradation may be balanced by synthesis during the initial starvation period if an adequate energy source is present, diminishing synthesis and a net increase in catabolism eventually results.

We are grateful to Dr R. C. Lawrence for many helpful discussions.

REFERENCES

- BEN-HAMIDA, F. & SCHLESSINGER, D. (1966). Synthesis and breakdown of ribonucleic acid in *Escherichia coli* starving for nitrogen. *Biochim. biophys. Acta* **119**, 183.
- BURLEIGH, I. G. & DAWES, E. A. (1967) Studies on the endogenous metabolism and senescence of starved *Sarcina lutea*. *Biochem. J.* **102**, 236.
- GALE, E. F. & PAINE, T. F. (1951). Assimilation of amino acids by bacteria. XII. Action of inhibitors and antibiotics on the accumulation of free glutamic acid and the formation of combined glutamate in *Staphylococcus aureus*. *Biochem. J.* **48**, 298.
- HOLDEN, J. T. (1962). Transport and accumulation of amino acids by micro-organisms. In *Amino Acid Pools*. Ed. by J. T. Holden. p. 566. Amsterdam: Elsevier.
- HOLDEN, J. T. (1965). Restoration of normal glutamic acid transport in vitamin B₆-deficient *Lactobacillus plantarum* by acetate, ammonium and vitamin B₆. *Biochim. biophys. Acta* **104**, 121.
- IKAWA, M. & SNELL, E. E. (1960). Cell wall composition of lactic acid bacteria. *J. biol. Chem.* **235**, 1376.
- MANDELSTAM, J. (1960). The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. *Bact. Rev.* **24**, 289.
- MANDELSTAM, J. (1963). Protein turnover and its function in the economy of the cell. *Ann. N.Y. Acad. Sci.* **102**, 621.
- MARCHESI, S. L. & KENNEL, D. (1967). Magnesium starvation of *Aerobacter aerogenes*. III. Protein metabolism. *J. Bact.* **93**, 357.
- SCHLESSINGER, D. & BEN-HAMIDA, F. (1966). Turnover of protein in *Escherichia coli* starving for nitrogen. *Biochim. biophys. Acta* **119**, 171.
- THOMAS, T. D. & BATT, R. D. (1968). Survival of *Streptococcus lactis* in starvation conditions. *J. gen. Microbiol.* **50**, 367.
- THOMAS, T. D. & BATT, R. D. (1969*a*). Degradation of cell constituents by starved *Streptococcus lactis* in relation to survival. *J. gen. Microbiol.* **58**, 347.
- THOMAS, T. D. & BATT, R. D. (1969*b*). Metabolism of exogenous arginine and glucose by starved *Streptococcus lactis* in relation to survival. *J. gen. Microbiol.* **58**, 371.
- WILLETTS, N. S. (1967). Intracellular protein breakdown in non-growing cells of *Escherichia coli*. *Biochem. J.* **103**, 453.