The Influence of Temperature and pH Value on the Macromolecular Composition of Magnesium-limited and Glycerol-limited *Aerobacter aerogenes* Growing in a Chemostat

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SUMMARY

Progressive alteration of the incubation temperature of *Aerobacter aerogenes* cultures, growing in a chemostat at a fixed dilution rate, caused a progressive change in bacterial concentration and in bacterial RNA, carbohydrate and protein contents; the DNA content did not vary significantly. The change in bacterial RNA concentration was associated with a variation in bacterial ribosome content, similar to that found in organisms grown at a fixed temperature but different dilution rates. Changes in bacterial yield also resulted from alterations in the pH value of the culture, but corresponding changes in bacterial RNA, carbohydrate and protein contents were small.

INTRODUCTION

A close correlation has been found between the RNA content of organisms and the rate at which they grow (Wade & Morgan, 1957; Neidhardt & Magasanik, 1960; Dean, 1962; Rosset, Monier & Julien, 1964; Sykes & Tempest, 1965). As these variations in cellular RNA content appeared to be due largely, if not entirely, to changes in the ribosome content of the organisms (Ecker & Schaechter, 1963*a*; Kjeldgaard & Kurland, 1963), and as ribosomes seemed to be the primary sites of protein synthesis, it was suggested that in growing organisms the rate of protein synthesis per ribosome particle was constant and independent of growth rate (Schaechter, Maaløe & Kjeldgaard, 1958; Ecker & Schaechter, 1963*b*). Apparently opposed to these findings was the further observation of Schaechter *et al.* (1958) that the macromolecular composition of *Salmonella typhimurium* organisms was not a function of growth rate when this was altered by changing the temperature of cultivation. This latter investigation was done with organisms grown under conditions of unrestricted growth, in a batch culture. In the chemostat the growth rate is controlled and it is possible, therefore, to vary the temperature of cultivation without simultaneously altering the growth rate of organisms in the culture.

The present paper reports the changes in RNA, DNA, carbohydrate and protein contents of *Aerobacter aerogenes* which occurred when the incubation temperature of the cultures, growing at a constant dilution rate, was progressively altered. These changes are shown to be similar to those reported by Tempest, Hunter & Sykes (1965) to occur in cultures of this organism when the dilution rate was progressively changed but the growth temperature maintained constant. By comparison, changes

in the culture pH value are shown to have only a small influence on the gross macromolecular composition of the organisms. These results, and some of those of Schaechter *et al.* (1958), are interpreted in terms of environmental effects on ribosome synthesis and activity. A preliminary report on our findings has been published (Tempest & Hunter, 1965).

METHODS

Organism. *Aerobacter aerogenes* (NCTC 418) maintained by monthly subculture on tryptic meat digest agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0.5 l. chemostats of the design described by Herbert, Phipps & Tempest (1965). The temperature differential at each set point was less than 0.2° and the pH differential less than 0.2 unit. Fluctuations in dilution rate, due to changes in culture volume and medium flow rate, were always less than $\pm 5\%$ of the desired value.

The composition of the media, and other conditions, were the same as those used by Tempest *et al.* (1965).

Viability measurements. These were made with the slide-culture technique of Postgate, Crumpton & Hunter (1961).

Fractionation procedure. 1–2 l. volumes of effluent culture were collected overnight in ice-cooled receivers, transferred to 250 ml. centrifuge buckets and sedimented (16,000g, 20 min.) in an MSE High Speed 17 refrigerated centrifuge. Packed bacteria were resuspended in ice-cold buffer (0.01 M-tris + 0.001 M-magnesium acetate; pH 7.5) and again sedimented (25,000g, 20 min.). Bacterial pastes were transferred to a Hughes press (Hughes, 1951), pre-cooled to -20° , and the organisms crushed. The crushed pastes were diluted with buffer (4 ml./g. frozen crushed paste, plus a crystal of deoxyribonuclease) and homogenized. Homogenates were centrifuged (20,000 rev./min., 45 min.) in a no. 30 rotor of a Spinco model L ultracentrifuge and the clear supernatant fluids (labelled 'Crude extract') were collected. These fluids were again centrifuged (40,000 rev./min., 3½ hr.) in a no. 40 rotor of a Spinco model L ultracentrifuge and separated into two fractions—a second supernatant fluid (labelled 'Supernatant'), and a pellet fraction (labelled 'Ribosomes') which was resuspended in ice-cold buffer to a convenient volume (10 ml. for material from 10 g. crushed paste). The RNA and protein contents of all samples were determined and the values obtained adjusted to correspond with a protein value of 1.000 in the 'Crude extract'.

Analytical procedures. These were identical with those used previously by Tempest *et al.* (1965).

RESULTS

Effect of temperature on the steady-state macromolecular composition of organisms growing at a fixed dilution rate

There is a maximum dilution rate beyond which cultures of any organism cannot be maintained in the chemostat. This 'critical' value (D_c) corresponds with the maximum specific growth rate of that organism (μ_m) in the environment provided (Herbert, Elsworth & Telling, 1956); it varies with incubation temperature, pH value and chemical complexity of the medium. In a simple salts medium, regulated at pH 6.5 and with glycerol as the carbon source, cultures of *Aerobacter aerogenes* have a minimum doubling time of 50 min. at 35°; at 25° this time is increased to about

90 min. Therefore in order to maintain the growth rate of this organism at a constant value whilst varying the incubation temperature it was necessary to operate the chemostat at a dilution rate substantially less than the 'critical' value for cultures growing at the least favourable temperature. As the value of D_c , at 25°, was approximately 0.45 vol./hr., we chose a dilution rate of 0.2 vol./hr. for our experiments. We progressively altered the temperature of cultivation from 40° to 25°, in increments of 5°, and determined the effect of these changes on the steady-state composition of organisms. Both glycerol-limited and Mg^{2+} -limited cultures were investigated.

Table 1. *Macromolecular composition of Aerobacter aerogenes grown at a fixed dilution rate and at different temperatures*

All the analytical data contained in this table are average values obtained from at least two samples (harvested and processed on different days) grown at each steady-state temperature.

Temp. (° C.)	Dilution rate	% of dry weight				mg./ml. culture				
		RNA	DNA	Carbo- hydrate	Protein	Dry wt. organisms	RNA	DNA	Carbo- hydrate	Protein
Glycerol-limited growth										
40	0.20	8.6	2.4	2.9	74.1	4.10	0.35	0.10	0.12	3.04
35	0.20	10.1	3.3	3.3	68.4	4.86	0.49	0.16	0.16	3.32
30	0.21	11.0	2.6	3.6	67.5	4.71	0.52	0.12	0.17	3.18
25	0.22	11.8	2.7	8.9	59.4	4.91	0.58	0.13	0.43	2.91
Mg ²⁺ -limited growth										
40	0.18	9.4	2.9	2.1	69.0	4.14	0.39	0.12	0.09	2.86
35	0.18	10.7	2.9	2.2	70.0	4.12	0.44	0.12	0.09	2.88
30	0.19	12.4	2.9	2.6	67.5	3.46	0.43	0.10	0.09	2.83
25	0.19	14.9	2.8	4.3	68.9	2.82	0.42	0.08	0.12	1.94

The results obtained are shown in Table 1 and clearly differ from those reported by Schaechter *et al.* (1958) in that changing the temperature of cultivation produced marked changes in the RNA and carbohydrate content of the organisms. Similar changes were evident in both types of culture, indicating that the changes were independent of the nature of the growth-limiting component of the medium. Furthermore the stoichiometry between Mg^{2+} and RNA, previously observed in Mg^{2+} -limited cultures of this organism (Tempest *et al.* 1965), was also apparent here; the culture-RNA content varied only slightly with growth temperature and changes in cellular RNA content were matched by gross changes in the steady-state concentration of organisms in the culture. In contrast, in the glycerol-limited culture both the yield of organisms and their RNA content increased with decreasing growth temperature; the culture-RNA value increased by 66% as the incubation temperature was lowered from 40° to 25°.

The above pattern of changes in macromolecular composition and yield of organisms was very similar to that reported to follow changes in growth rate of organisms at a fixed temperature (Herbert, 1961; Neidhardt, 1963; Tempest *et al.* 1965); this was particularly true for the changes in cellular RNA content. Therefore we determined the distribution of RNA and protein in fractions from organisms grown at two different temperatures in order to establish whether these changes reflected

changes in the ribosome content of organisms similar to those reported by Ecker & Schaechter (1963*b*) to follow changes in growth rate. The data obtained are shown in Table 2, which shows that with glycerol-limited and with Mg^{2+} -limited organisms a decrease of 10° in the growth temperature produced a marked increase (about 30 %) in their ribosome contents. The amounts of RNA and protein which could be sedimented under the prescribed conditions increased, but not in an identical manner. Thus the RNA:protein ratios in the 'Ribosome' fractions changed (from 0.37 to 0.57 in the material from Mg^{2+} -limited organisms, and to a lesser extent in the material from glycerol-limited organisms). The change in composition of this fraction is similar to that observed by Tempest *et al.* (1965) in fractions from organisms grown at a fixed temperature but at different dilution rates.

Table 2. *Distribution of RNA and protein in fractions from extracts of glycerol-limited and Mg^{2+} -limited Aerobacter aerogenes organisms grown at different temperatures*

	35°		25°	
	Protein	RNA	Protein	RNA
	Glycerol-limited growth			
Crude extract	1.000	0.194	1.000	0.236
Supernatant fluid	0.685	0.044	0.585	0.040
Ribosomes	0.317	0.141	0.398	0.187
Ribosomal RNA + protein	0.458		0.585	
	Mg ²⁺ -limited growth			
Crude extract	1.000	0.175	1.000	0.262
Supernatant fluid	0.686	0.048	0.621	0.050
Ribosomes	0.318	0.118	0.369	0.211
Ribosomal RNA + protein	0.436		0.580	

Effect of pH value on the steady-state macromolecular composition of organisms growing at a fixed dilution rate

Although organisms are not able to maintain a temperature differential between themselves and their environment they may well maintain a pH differential; alteration of the culture pH value may not, therefore, effect a corresponding change in intracellular pH value. The fact that relatively small changes in culture pH value influence the enzymic constitution and content of organisms (Gale & Epps, 1942) suggests, however, that some change in intracellular pH value does follow a gross change in the environmental pH value. The optimum pH value for growth of *Aerobacter aerogenes* occurred within the range pH 6–7, though growth was not totally inhibited at values as low as pH 4.5 or as high as pH 8.5. The effect of progressively altering the culture steady-state pH value from 4.5 to 8.5, in steps of 1 pH unit, on the composition of organisms in glycerol-limited and Mg^{2+} -limited cultures (temperature, 35° ; dilution rate, 0.2 vol./hr.) is shown in Table 3. It is apparent here that the changes in macromolecular composition of organisms with pH value were much less than those observed to follow changes in the temperature of cultivation. The main effect of changing the pH value, in both types of culture, was on the bacterial yield, which was maximal at the optimum pH value for growth of this organism. In the Mg^{2+} -limited culture the RNA: Mg^{2+} ratio remained constant, though some change

Table 3. *Macromolecular composition of Aerobacter aerogenes organisms grown at different pH values (temperature and dilution rate constant)*

All the analytical data contained in this table are average values obtained from at least two samples (harvested and processed on different days) grown at each steady-state pH value.

pH value	Dilution rate	% of Dry weight			mg./ml. culture			
		RNA	Carbo-hydrate	Protein	Dry wt.	RNA	Carbo-hydrate	Protein
Glycerol-limited growth								
4.5	0.18	10.8	4.0	73.0	4.27	0.46	0.17	3.12
5.5	0.19	11.1	4.2	71.5	4.31	0.48	0.18	3.08
6.5	0.18	10.5	3.3	71.0	4.58	0.48	0.15	3.25
7.5	0.19	10.3	3.1	73.1	4.24	0.43	0.18	3.10
8.5	0.19	10.3	3.2	74.3	3.31	0.34	0.11	2.46
Mg ²⁺ -limited growth								
4.5	0.18	11.7	4.2	68.1	3.60	0.42	0.15	2.45
5.5	0.18	11.3	2.9	69.5	3.80	0.43	0.11	2.64
6.5	0.19	10.3	2.3	68.1	4.14	0.43	0.10	2.82
7.5	0.19	10.6	2.3	73.7	3.87	0.41	0.09	2.85
8.5	0.21	12.8	2.5	67.5	3.19	0.41	0.08	2.15

in the RNA content of organisms occurred. However, there was little change in the culture-RNA content of glycerol-limited cultures with changes from pH 4.5 to 7.5. In both cultures there was a significant increase in the carbohydrate content of organisms when they were grown at low pH values.

DISCUSSION

Whereas varying the temperature of cultivation of *Salmonella typhimurium* in a batch culture has no effect on their gross composition (Schaechter *et al.* 1958) it clearly does so with chemostat cultures of *Aerobacter aerogenes* where the growth rate is controlled. Though superficially these observations appear to be contradictory they are, in fact, quite compatible assuming that the following two hypotheses are correct: (1) that the rate of protein synthesis per unit ribosome is constant and independent of growth rate (Schaechter *et al.* 1958); (2) that the concentration of ribosomes within an organism cannot exceed a certain fixed amount. One may assume the first hypothesis to be correct only for a fixed temperature and pH value. Lowering the environmental temperature would decrease ribosomal activity and the resulting decrease in protein synthesis would be reflected in a lower growth rate. If, however, growth rate is maintained at a constant value (as happens in the chemostat), then clearly the ribosome content of organisms must increase to offset the decreased ribosomal activity and to maintain the overall rate of protein synthesis constant. On the other hand, if the organisms already contain their maximum ribosome content (which one assumes to be so with fast-growing organisms in a batch culture) then a decrease in growth temperature cannot be compensated by a further increase in ribosome concentration, and the rate of protein synthesis must therefore decrease. Though growth rate is correspondingly decreased, the ribosome content remains maximal and thus the RNA content of organisms does not alter.

The above two hypotheses therefore satisfactorily account both for our observations and also those of Schaechter *et al.* (1958).

Assuming that organisms maintain a pH differential between their intracellular and extracellular environments, large variations in culture pH value may produce only small fluctuations (or none) of pH value within the bacteria. In Mg^{2+} -limited cultures, organisms grown at the optimum pH value do, however, have a lower RNA content than organisms grown at a higher or lower pH suggesting that a change in ribosomal activity occurs with alterations in culture pH value. In glycerol-limited cultures, on the other hand, the RNA content of organisms is virtually independent of culture pH value, indicating little if any change in their ribosome content, and therefore in their activity.

Changes in the concentration of bacteria in the Mg^{2+} -limited *Aerobacter aerogenes* culture with temperature could be correlated with changes in the RNA content of organisms and the maintenance of a stoichiometry between culture RNA and Mg^{2+} concentrations. With the glycerol-limited culture a decrease in growth temperature effected a marked increase in yield of bacteria, indicating a more efficient utilization of the carbon source. This increased efficiency probably resulted from a lower maintenance energy requirement (Marr, Nilson & Clark, 1963) for organisms growing at the lower temperatures. The culture pH value also influenced the bacterial yield which was maximal at the optimum pH value for rapid growth of *A. aerogenes*. Again the changes in bacterial concentration in the Mg^{2+} -limited culture paralleled changes in the RNA content of organisms. In the glycerol-limited culture the lower yields at the less favourable pH values probably resulted from an increased energy expenditure which would be required to maintain a large pH difference between the intracellular and extracellular environments.

It is of interest to note that both temperature and pH influenced the carbohydrate content of *Aerobacter aerogenes* which increased at the lower values. Even in cultures where the carbon + energy source was limiting growth, organisms grown at 25° contained substantially more carbohydrate than those cultured at 35° (8.9% versus 3.3% of the bacterial dry weight). Whether this extra carbohydrate was present as a storage compound (e.g. glycogen), or whether it resulted from an increase in structural (cell wall) polysaccharide material has not been determined.

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REFERENCES

- DEAN, A. C. R. (1962). Nucleic acid and protein content of *Bacterium lactis aerogenes*. 1. Equilibrium and non-equilibrium conditions. *Proc. Roy. Soc. B*, **155**, 580.
- ECKER, R. E. & SCHAECHTER, M. (1963*a*). Bacterial growth under conditions of limited nutrition. *Ann. N.Y. Acad. Sci.* **102**, 549.
- ECKER, R. E. & SCHAECHTER, M. (1963*b*). Ribosome content and the rate of growth of *Salmonella typhimurium*. *Biochim. Biophys. Acta*, **76**, 275.
- GALE, E. F. & EPPS, H. M. R. (1942). The effect of the pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. *Biochem. J.* **36**, 600.
- HERBERT, D. (1961). The chemical composition of microorganisms as a function of their environment. In *Microbial Reaction to Environment. Symp. Soc. gen. Microbiol.* **11**, 391.

- HERBERT, D., ELSWORTH, R. & TELLING, R. C. (1956). The continuous culture of bacteria; a theoretical and experimental study. *J. gen. microbiol.* **14**, 601.
- HERBERT, D., PHIPPS, P. J. & TEMPEST, D. W. (1965). The chemostat; design and instrumentation. *Laboratory Practice* (in the Press).
- HUGHES, D. E. (1951). A press for disrupting bacteria and other micro-organisms. *Br. J. exp. Path.* **32**, 97.
- KJELDGAARD, N. O. & KURLAND, C. G. (1963). The distribution of soluble and ribosomal RNA as a function of growth rate. *J. molec. Biol.* **6**, 341.
- MARR, A. G., NILSON, E. H. & CLARK, D. J. (1963). The maintenance requirement of *Escherichia coli*. *Ann. N.Y. Acad. Sci.* **102**, 536.
- NEIDHARDT, F. C. (1963). Effects of environment on the composition of bacterial cells. *Annu. Revs. Microbiol.* **17**, 61.
- NEIDHARDT, F. C. & MAGASANIK, B. (1960). Studies on the role of ribonucleic acid in the growth of bacteria. *Biochim. Biophys. Acta*, **42**, 99.
- POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.
- ROSSET, R., MONIER, R. & JULIEN, J. (1964). RNA composition of *Escherichia coli* as a function of growth rate. *Biochem. biophys. Res. Comm.* **15**, 329.
- SCHAECHTER, M., MAALØE, O. & KJELDGAARD, N. O. (1958). Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *J. gen. Microbiol.* **19**, 592.
- SYKES, J. & TEMPEST, D. W. (1965). The effect of magnesium and of carbon limitation on the macromolecular organisation and metabolic activity of *Pseudomonas* sp., Strain c-1B. *Biochim. Biophys. Acta*, **103**, 98.
- TEMPEST, D. W. & HUNTER, J. R. (1965). Influence of temperature on the macromolecular composition of C(glycerol)-limited and Mg²⁺-limited *Aerobacter aerogenes*, growing in a chemostat. *J. gen. Microbiol.* **39**, vi.
- TEMPEST, D. W., HUNTER, J. R. & SYKES, J. (1965). Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat. *J. gen. Microbiol.* **39**, 355.
- WADE, H. E. & MORGAN, D. M. (1957). The nature of the fluctuating ribonucleic acid in *Escherichia coli*. *Biochem. J.* **65**, 321.