Effect of R Plasmid RP1 on the Nutritional Requirements of *Escherichia coli* in Batch Culture

By RUTH M. M. KLEMPERER, NAHLA T. A. J. ISMAIL*
AND M. R. W. BROWN

*Microbiology Research Group, Department of Pharmacy,
University of Aston in Birmingham, Birmingham B4 7ET*

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The minimal nutritional requirements of *Escherichia coli* have been quantitatively determined in batch culture for cells with (R⁺) and without (R⁻) the R plasmid RP1. In these conditions R⁺ cells have a greater requirement than R⁻ for several nutrients, particularly Mg²⁺, K⁺, Fe²⁺ and PO₄³⁻. The maximum growth rate in a simple salts medium was the same for R⁺ and R⁻ cells. At low concentrations of phosphate, the specific growth rate of R⁺ cells differed from that found for R⁻ cells. The R plasmid was stable in simple salts medium, irrespective of the nutrient ultimately depleted by growth, but, on storage, R⁺ cells survived for a shorter time than R⁻ cells.

INTRODUCTION

Escherichia coli grows readily in simple salts media, for which there are a number of widely used formulations (e.g. Davis & Mingioli, 1950; Vogel & Bonner, 1956). These media consist essentially of glucose, NH₄⁺, Mg²⁺ and SO₄²⁻ buffered with phosphates. Neidhardt *et al.* (1974), using a potassium morpholinopropanesulphonate buffer, defined the relation between growth yield and the concentration of several added nutrients for *Salmonella typhimurium* and formulated a medium based on this information for *S. typhimurium* and *E. coli*. However, complete nutritional requirements were not determined.

Although phenotypic variation of bacteria is a long established phenomenon, it is only recently that the critical role of the medium has been clarified, particularly with respect to changes in the cell envelope (Brown & Melling, 1969; Ellwood & Tempest, 1972). Depletion of different nutrients may often result in changes in bacterial sensitivity to antibiotics (Melling & Brown, 1975; Brown, 1977) and to disinfectants (Ismail *et al.*, 1977; Gilbert & Brown, 1978). Although chemically defined media have been introduced to improve reproducibility in testing disinfectants (Kelsey & Maurer, 1974), no specification has been made for the nutrient(s) depleting growth of the test cultures.

Since most naturally occurring bacteria are probably nutrient-depleted (Brown, 1977), there is a need to study their properties under such conditions. For this purpose, the nutritional requirements were quantified in batch culture for *E. coli* W3110 wild-type and for the same cells containing the R plasmid RP1; appropriate media were then formulated. Plasmid RP1 originates from a clinical isolate and confers resistance to ampicillin, carbenicillin, kanamycin, neomycin and tetracycline (Grinsted *et al.*, 1972). Results presented below indicate that the presence of RP1 affects the nutritional requirements of *E. coli*. It was therefore of interest to study the influence of nutrient depletion on the stability and survival of plasmid-containing cells.

A preliminary report of some of these results has been published (Ismail *et al.*, 1977).

* Present address: P.O. Box 3614, Dubai-Deira, United Arab Emirates.

METHODS

Organisms. *Escherichia coli* W3110 [a mutant of *E. coli* K12 (Bachmann, 1972)] was obtained from Dr P. A. Meacock, University of Leicester. R plasmid RP1 was transferred to it (Meynell & Meynell, 1970) from *Proteus mirabilis* 2885(RP1) obtained from Dr E. J. L. Lowbury, The Accident Hospital, Birmingham. Cultures were stored at 4 °C on slopes prepared from simple salts sufficient for 'unlimited' growth (see Table 2) solidified with Lab M agar (1.5%, w/v). Fresh slopes were made every month. R⁺ slopes were made from cultures plated on nutrient agar (NA) containing ampicillin (20 µg ml⁻¹), kanamycin (20 µg ml⁻¹) and tetracycline (10 µg ml⁻¹). These concentrations were completely inhibitory to R⁻ cells.

Chemicals. Ampicillin was a gift from Beecham Research Laboratories, Brentford, London, and tetracycline from Lederle Laboratories, Gosport, Hants.; kanamycin was from Sigma; Lab M agar from London Analytical and Bacteriological Media, London; agar CM3 and nutrient broth CM1 from Oxoid. All other chemicals were from BDH and were Analar grade, except 3-(N-morpholino)propanesulphonic acid (MOPS), which was Laboratory Reagent grade. The pH of MOPS was adjusted with NaOH (1 M-MOPS contains 0.5 M-Na⁺ at pH 7.2) and solutions were prepared fresh each week. Water was deionized and then glass-distilled. Glassware was washed in Decon 90 (Decon Laboratories, Brighton). All solutions were sterilized by autoclaving, except antibiotics, glucose and buffers, which were filtered through membranes (0.2 µm pore diam.) that had previously been boiled in three changes of distilled water (Brown *et al.*, 1969).

Growth in simple salts media. Baffled 100 ml Erlenmeyer flasks containing 25 ml medium were inoculated from overnight cultures that had been grown in similar medium and then washed three times in medium lacking the nutrient under study. Other ingredients, including Fe²⁺, were present in excess, as far as possible sufficient for the culture to reach a theoretical absorbance of 10. However, to check the requirements for various ions, a number of different salts had to be used and their concentrations in the basal medium varied. Checks on the complete formulation indicated that these variations had no significant effects on requirements for other ingredients. Flasks were incubated at 37 °C in a water bath shaking at 120 rev. min⁻¹ and growth was assessed turbidimetrically at 30 min intervals at 420 nm. The initial reading was about 0.01, and samples with an absorbance (*A*) above 0.25 were diluted appropriately. Maximum exponential growth was defined as the absorbance at which the logarithmic plot of growth deviated from linearity. Plots were made by linear regression analysis. Maximum exponential growth was then plotted against the concentration of added depleting nutrient, using linear regression analysis, and the concentration of depleting nutrient derived from contamination of other chemicals present was calculated by extrapolating the regression line back to the abscissa.

Nutrient-depleted cultures. These were grown in a basal medium sufficient to allow growth to an *A* of 2.0. Glucose-depleted cultures had sufficient glucose to grow to an *A* of 0.2 and were harvested at once. Other depleted cultures had sufficient of the appropriate ingredient to grow exponentially to an *A* of 0.1 and were harvested when *A* reached 0.2. Harvested cultures were centrifuged and resuspended in fresh medium lacking both the depleted ingredient and glucose, to prevent further growth.

Total counts, viable counts and dry weights. These were determined according to Meynell & Meynell (1970). Plates for colony counts were surface-seeded following dilution of samples in broth. For dry weight determinations, pellets were dried to constant weight at room temperature *in vacuo* over P₂O₅.

Oxidase and catalase tests. These were carried out according to Cowan & Steele (1974).

RESULTS

Choice of buffer and pH

To permit the determination of PO₄³⁻ requirements, MOPS neutralized with NaOH was used as buffer. MOPS at 75 mM did not affect the yields of glucose-limited cells but inhibited the growth rate by 10%; at 50 mM and below, MOPS had no detectable effect on either. A concentration of 25 mM was chosen and used at pH 7.2 (pK_a MOPS = 7.2). In the media described, the pH fell to about 7.0 when cultures had reached *A* = 0.9. The absorbance reached in the absence of added SO₄²⁻ or NH₄⁺ was not altered by the addition of MOPS, and no diauxic growth was detected following SO₄²⁻ or NH₄⁺ depletion. This suggested that MOPS was not metabolized by the cells.

Nutrient requirements

Absorbance, as a measure of growth, varies with the environment (Avi-dor *et al.*, 1956). However, growth was assessed for cells growing exponentially with medium components in

Table 1. Concentrations of added nutrients which permit exponential growth to an A_{420} of 1.0*

Cells were grown in medium as in Table 2, adjusted to permit depletion of individual nutrients. Data were derived from regression of maximum exponential growth on concentration of added nutrient.

Nutrient	Concn added (mM)		Contamination (mM)	Concn required (mM)		$P^\dagger$
	R ⁻	R ⁺		R ⁻	R ⁺	
Glucose	2.5	2.8	0	2.5	2.8	> 0.1
NH ₄ ⁺	1.85	1.75	0.1	1.95	1.85	> 0.1
Mg ²⁺	0.013	0.025	0.0015	0.015	0.027	< 0.001
K ⁺	0.026	0.042	0.01	0.036	0.052	< 0.001
Fe ²⁺	0	0.0001	0.0004	ND	0.0005	ND
SO ₄ ²⁻	0.026	0.019	0.009	0.035	0.028	> 0.1
Cl ⁻	0	0	ND	ND	ND	ND
PO ₄ ³⁻	0.17	0.56	0.02	0.19	0.58	< 0.001

ND, Not determined.

* An A_{420} of 1.0 is equivalent to $5.58 \pm 0.19 \times 10^8$ cells ml⁻¹ and 0.24 ± 0.005 mg dry wt ml⁻¹ for R⁻ and $5.62 \pm 0.90 \times 10^8$ cells ml⁻¹ and 0.17 ± 0.015 mg dry wt ml⁻¹ for R⁺.

† Comparison of regression coefficients.

Table 2. Simple salts medium permitting maximum growth of *E. coli*

Formulation based on information in Table 1.

Nutrient	Concn added (mM)		Theoretical A obtainable		
	R ⁻	R ⁺	Nutrient	R ⁻	R ⁺
Glucose	27	27	Glucose	10	10
MgSO ₄	0.2	0.3	Mg ²⁺	13	11
			SO ₄ ²⁻	6	10
KCl	0.3	0.5	K ⁺	8	10
NH ₄ Cl	10	10	Cl ⁻	—	—
NH ₄ H ₂ PO ₄ /(NH ₄) ₂ HPO ₄ *	2.5	5.5	NH ₄ ⁺	8	11
			PO ₄ ³⁻	15	10
NH ₄ FeSO ₄	0	0.005	Fe ²⁺	—	10
MOPS†	25	25	—	—	—

* pH 7.2. † pH 7.2; total Na⁺ added 12.5 mM.

known excess, so that variations in the ratio absorbance:dry weight:total count were likely to be very small in comparison with other differences. For such cultures, the total count of R⁺ and R⁻ cells at $A = 1.0$ was the same, but the dry weight of R⁻ cells was about 1.4 times greater (Table 1).

R⁺ cells required significantly higher concentrations of Mg²⁺, PO₄³⁻ and K⁺ than did R⁻ cells to reach the same absorbance with exponentially growing cells (Fig. 1 *a, b, c*). R⁺ cells grew exponentially to $A = 0.8$ without added Fe²⁺, but to reach a higher absorbance added Fe²⁺ was required (Fig. 1 *d*). Iron contamination of the medium from other ingredients and glassware was calculated from Fig. 1 (*d*) as 0.4 μ M. This was sufficient for the growth of R⁻ cells up to the maximum obtainable absorbance. It seemed possible that the additional Fe²⁺ requirement of R⁺ cells was due to a change in their catalase or cytochrome content. However, the semi-quantitative catalase test showed no change and the oxidase test remained negative.

No differences were found in the requirements for glucose, NH₄⁺ and SO₄²⁻ for cultures to reach the same absorbance (Table 1). No Cl⁻ requirement was detectable, but as chlorides had to be used when studying the requirements for other anions, some Cl⁻ was always included to reduce the fluctuations in its concentration. A number of trace elements – Ca²⁺, Co²⁺, Mn²⁺, Mo²⁺ and Zn²⁺ – were tested together, each at 5 μ M; no effect was observed on

Fig. 1. Maximum exponential growth as a function of depleting nutrients for R^- ($\circ$) and R^+ ($\bullet$) cells. Cells were grown in medium as in Table 2 adjusted to permit depletion of nutrients: (a) Mg^{2+} ; (b) PO_4^{3-} ; (c) K^+ ; (d) Fe^{2+} .

either culture. At 25 μM , the mixture reduced the yield by 75%, although it had no effect on the growth rate.

From these observations, a medium was formulated which permits maximum growth; the ingredients are present in known excess, sufficient in most cases for a theoretical absorbance of about 10 (Table 2). Using Table 1 it was then possible to devise media that would ultimately deplete cells in any desired manner. In the maximum growth medium, R^+ cells consistently grew exponentially to a higher A (4.50 ± 0.11) than did R^- cells (3.58 ± 0.08) (Fig. 1). This corresponded to dry weights of 0.765 mg ml^{-1} (R^+) and 0.859 mg ml^{-1} (R^-) (Table 1). These maximum population densities were a function of aeration and were lower when shaking rates were below $100 \text{ rev. min}^{-1}$ or when larger culture volumes relative to the flask were used (McDaniel *et al.*, 1965).

Effect of PO_4^{3-} and Mg^{2+} concentrations on growth rate

The average doubling time in the maximum growth medium was 65 min for both R^+ and R^- cells. With lower concentrations of PO_4^{3-} the relationship between the reciprocal of the specific growth rate and the reciprocal of the PO_4^{3-} concentration was linear for R^+ cells over the range tested, but not for R^- cells (Fig. 2). For R^- cells, the relationship was linear only at low concentrations of PO_4^{3-} . The change in growth rate with PO_4^{3-} concentration was the same for both cultures at low concentrations, but R^+ cells had a shorter doubling time than comparable R^- ones. When Mg^{2+} was the nutrient limiting growth rate, no difference was found between R^+ and R^- cells.

Effect of medium composition on the stability and survival of R^+ cultures

Media were formulated to facilitate the study of the effects of nutrient depletion, but it seemed possible that the R plasmid might not be stable in all circumstances. However, R^+ cultures 1 h after depletion of glucose, NH_4^+ , K^+ , Mg^{2+} , SO_4^{2-} or PO_4^{3-} showed no signifi-

Fig. 2

Fig. 3

Fig. 2. Effect of PO_4^{3-} concentration on specific growth rate for R^- (○) and R^+ (●) cells. Cells were grown in simple salts medium, with each ingredient sufficient to allow growth to an A of 2, adjusted to permit different PO_4^{3-} concentrations. Each point is from a single growth curve, except those for the highest PO_4^{3-} concentration which were determined in triplicate.

Fig. 3. Survival of glucose-depleted R^- (○) and R^+ (●, ▲) cells at 37 °C. Cells were grown in simple salts medium with each ingredient, apart from glucose, sufficient to allow growth to an A of 2.0. Counts were done in triplicate on NA (○, ●) or NA containing ampicillin (20 $\mu\text{g ml}^{-1}$), kanamycin (20 $\mu\text{g ml}^{-1}$) and tetracycline (10 $\mu\text{g ml}^{-1}$) (▲).

cant loss in the R plasmid when viable counts on NA were compared with counts on NA containing ampicillin (20 $\mu\text{g ml}^{-1}$), kanamycin (20 $\mu\text{g ml}^{-1}$) and tetracycline (10 $\mu\text{g ml}^{-1}$).

Cultures were routinely stored on agar slopes prepared from the simple salts medium. When stored on NA, the rate of loss of the R plasmid from R^+ cells was significantly higher (e.g. the percentages of R^- cells in R^+ cultures from NA or simple salts slopes after 12 weeks storage were 34 and 8, respectively). Stability of the plasmid shortly after depletion of the cells suggested that these differences might reflect more efficient survival of a few R^- cells in the initial R^+ cultures. Following glucose depletion and storage in liquid medium at 37 °C, survival of R^- cells was greater than of R^+ cells (Fig. 3). Similar results were obtained following Mg^{2+} depletion.

DISCUSSION

The major difference between the media described here and previous formulations is that, with the possible exception of micronutrients, presumably present as contaminants, all essential nutrients have been defined quantitatively and each may be included either in concentrations leading to cell depletion at a predetermined absorbance or in known excess. The majority of previous formulations contain a large excess of phosphate. The MOPS-buffered medium of Neidhardt *et al.* (1974) has a high K^+ concentration, owing to the use of KOH for neutralizing the buffer. Since the K^+ requirement of cells is quantifiable, NaOH was used to neutralize MOPS in this study, with a resultant high Na^+ concentration (12.5 mM) in the medium. Whilst this was considered expedient, since the quantification of Na^+ requirements was made impossible by the Na^+ contamination of the other chemicals in the medium, the consequences of its high concentration cannot be ignored. Na^+ displaces loosely bound Mg^{2+} on the surface of *Aerobacter aerogenes* (Strange & Shon, 1964). Tempest & Meers (1968) observed that Na^+ interfered with Mg^{2+} uptake and that whole cell K^+ content and medium NaCl concentration were interrelated. Buffers may also be utilized by micro-organisms as a source of nutrient. Unlike *E. coli* B (Neidhardt *et al.*, 1974) and *Pseudomonas aeruginosa* NCTC 6750 (unpublished results), the cells used here did not use MOPS as a source of nitrogen or sulphur.

The minimum doubling time (65 min) was greater than that of *S. typhimurium* and *E. coli* B

in a number of minimal media (Neidhardt *et al.*, 1974), although comparable to the result for the Tris-buffered medium of Kaempfer & Magasanik (1967). However, Neidhardt *et al.* (1974) found that the growth rate in minimal media could be increased by adding micro-nutrients, NaCl, amino acids and growth factors. It seems likely that a medium designed to have only a small excess of constituents will not necessarily support a high growth rate.

Maintaining excess O_2 in batch culture is a problem (McDaniel *et al.*, 1965; Meynell & Meynell, 1970), but there was no evidence that the batch cultures described here were O_2 -limited. With shaking rates from 100 to 140 rev. min⁻¹ there was no difference in the onset of the stationary phase or of doubling time (24 min) in nutrient broth cultures. From the relationship of absorbance to dry weight for exponentially growing cells, the approximate yields of cells at maximum exponential growth as determined by the glucose concentration were calculated. The results, 90.6 g mol⁻¹ (R⁻) and 64.2 g mol⁻¹ (R⁺), are comparable to the Y_{glucose} values of 67.8, 94.0 and 90.0 g mol⁻¹ reported for different strains of *E. coli* (a wild-type, W and ML30, respectively) grown aerobically in batch culture and greatly in excess of the Y_{glucose} value of 24.0 g mol⁻¹ for *E. coli* K12 grown anaerobically (Payne, 1970).

A direct relationship between amount of growth and amount of added substrate in batch culture was first shown by Monod (1942). Although it would be necessary to grow cells in the constant conditions of a chemostat to compare small differences in yield, the large differences recorded here indicate major effects of plasmid RP1 on the cell. RP1 affects both the structure and composition of the cell envelope (Richmond & Curtis, 1975; Gilbert & Brown, 1978; Kenward *et al.*, 1978), which might account for some differences. However, when RP1 is inserted into *P. aeruginosa*, there is no change in wall phosphorus and a slight decrease in wall magnesium content (Kenward *et al.*, 1978).

Differences in nutritional requirements may be of considerable significance *in vivo*. In a number of organisms, the acquisition of a plasmid has been associated with a decrease in virulence (Lacey, 1975). The high Fe requirement associated with RP1 is particularly relevant, as there is much evidence that tissue Fe concentrations may determine the outcome of an infection (Weinberg, 1978). Weinberg (1974) noted a fall in plasma phosphate in response to Gram-negative infections. It is therefore of interest that although R⁻ cells appeared to have a dual uptake system for phosphate of low and high affinity, as found by Shehata & Marr (1971) for *E. coli* ML30, only the high affinity mechanism was detected in R⁺ cells.

The stability of RP1 appeared to be unaffected by nutrient depletion in batch culture, a result similar to the findings of Melling *et al.* (1977) for glucose-, Mg²⁺- and PO₄³⁻-limited cells in continuous culture. However, long-term survival appears to be affected by the presence of RP1. This result is similar to that of Anderson (1973), using the R plasmid R1 in *E. coli* stored in saline or water.

REFERENCES

- ANDERSON, J. D. (1973). The effect of resistance-transfer (R) factor carriage upon the survival of *Escherichia coli in vitro* and experimentally in man. *Journal of Medical Microbiology* **6**, Pxi-Pxx.
- AVI-DOR, Y., KUCZYNSKI, M., SCHATZBERG, G. & MAGER, I. (1956). Turbidity changes in bacterial suspensions: kinetics and relation to metabolic state. *Journal of General Microbiology* **14**, 76-83.
- BACHMANN, B. J. (1972). Pedigree of some mutant strains of *Escherichia coli* K12. *Bacteriological Reviews* **36**, 525-557.
- BROWN, M. R. W. (1977). Nutrient depletion and antibiotic susceptibility. *Journal of Antimicrobial Chemotherapy* **3**, 198-201.
- BROWN, M. R. W. & MELLING, J. (1969). The role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. *Journal of General Microbiology* **59**, 263-274.
- BROWN, M. R. W., FARWELL, J. A. & ROSENBLUTH, S. A. (1969). Use of membrane filters for measurement of 260 mμ-absorbing substances from bacterial cells. *Analytical Biochemistry* **27**, 484-491.
- COWAN, S. T. & STEELE, K. J. (1974). *Manual for the Identification of Medical Bacteria*, 2nd edn. Cambridge: The University Press.
- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *Journal of Bacteriology* **60**, 17-28.
- ELLWOOD, D. C. & TEMPEST, D. W. (1972). Effects of environment on bacterial wall content and composition. *Advances in Microbial Physiology* **7**, 83-117.
- GILBERT, P. & BROWN, M. R. W. (1978). Effect of R-plasmid RP1 and nutrient depletion on the gross cellular composition of *Escherichia coli* and

- its resistance to some uncoupling phenols. *Journal of Bacteriology* **133**, 1062–1065.
- GRINSTED, J., SAUNDERS, J. R., INGRAM, L. C., SYKES, R. B. & RICHMOND, M. H. (1972). Properties of an R-factor which originated in *Pseudomonas aeruginosa* 1822. *Journal of Bacteriology* **110**, 529–537.
- ISMAIL, N. T. A. J., KLEMPERER, R. M. M. & BROWN, M. R. W. (1977). Resistance of *Escherichia coli* to disinfectants: influence of R-plasmid RPl and nutrient depletion. *Journal of Applied Bacteriology* **43**, xvi–xvii.
- KAEMPFER, R. O. R. & MAGASANIK, B. (1967). Effect of infection with T-even phage on the inducible synthesis of beta-galactosidase in *Escherichia coli*. *Journal of Molecular Biology* **27**, 453–468.
- KELSEY, J. C. & MAURER, I. M. (1974). An improved (1974) Kelsey–Sykes test for disinfectants. *Pharmaceutical Journal* **213**, 528–530.
- KENWARD, M. A., BROWN, M. R. W., HESSEWOOD, S. R. & DILLON, C. (1978). Influence of R-plasmid RPl of *Pseudomonas aeruginosa* on cell wall composition, drug resistance, and sensitivity to cold shock. *Antimicrobial Agents and Chemotherapy* **13**, 446–453.
- LACEY, R. W. (1975). A critical appraisal of the importance of R-factors in the enterobacteriaceae *in vivo*. *Journal of Antimicrobial Chemotherapy* **1**, 25–37.
- MCDANIEL, L. E., BAILEY, E. G. & ZIMMERLI, A. (1965). Effect of oxygen supply rates on growth of *Escherichia coli*. I. Studies in unbaffled and baffled shake flasks. *Applied Microbiology* **13**, 109–114.
- MELLING, J. & BROWN, M. R. W. (1975). The effect of the bacterial environment on resistance. In *Resistance of Pseudomonas aeruginosa*, pp. 35–70. Edited by M. R. W. Brown. London: John Wiley.
- MELLING, J., ELLWOOD, D. C. & ROBINSON, A. (1977). Survival of R-factor carrying *Escherichia coli* in mixed cultures in the chemostat. *FEMS Microbiology Letters* **2**, 87–89.
- MEYNELL, G. G. & MEYNELL, E. (1970). *Theory and Practice in Experimental Bacteriology*, 2nd edn. Cambridge: The University Press.
- MONOD, J. (1942). *Recherches sur la croissance des cultures bactériennes*. Paris: Hermann & Cie.
- NEIDHARDT, F. C., BLOCH, P. L. & SMITH, D. F. (1974). Culture medium for Enterobacteria. *Journal of Bacteriology* **119**, 736–747.
- PAYNE, W. J. (1970). Energy yields and growth of heterotrophs. *Annual Review of Microbiology* **24**, 17–52.
- RICHMOND, M. H. & CURTIS, N. A. C. (1975). R-factors and intrinsic resistance to penicillins. In *Drug-inactivating Enzymes and Antibiotic Resistance*, pp. 35–41. Edited by S. M. Mitsuhashi, L. Rosival & V. Krčmery. Berlin: Springer-Verlag.
- SHEHATA, T. E. & MARR, A. G. (1971). Effect of nutrient concentration on the growth of *Escherichia coli*. *Journal of Bacteriology* **107**, 210–216.
- STRANGE, R. E. & SHON, M. (1964). Effects of thermal stress on viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension. *Journal of General Microbiology* **34**, 99–114.
- TEMPEST, D. W. & MEERS, J. L. (1968). The influence of NaCl concentration of the medium on the potassium content of *Aerobacter aerogenes* and on the interrelationship between potassium, magnesium and ribonucleic acid in the growing bacteria. *Journal of General Microbiology* **54**, 319–325.
- VOGEL, H. J. & BONNER, D. M. (1956). Acetyl-ornithinase of *Escherichia coli*: partial purification and some properties. *Journal of Biological Chemistry* **218**, 97–106.
- WEINBERG, E. D. (1974). Iron and susceptibility to infectious disease. *Science* **184**, 952–956.
- WEINBERG, E. D. (1978). Iron and infection. *Microbiological Reviews* **42**, 45–66.