IONIC RELATIONS AS A FUNCTION OF METABOLISM IN *AEROBACTER AEROGENES*

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Summary

A review is given of the irreversible thermodynamic interpretation of the transport of ions against electrochemical gradients. Measurements were made of the ratio of potassium inside and outside the cell to that of sodium (K^+/Na^+ specificity ratio) as a function of the growth rate of cells of *Aerobacter aerogenes*. Simultaneous measurements of the fluxes of glucose, oxygen, carbon dioxide, ethanol, and acids were also made. The results show that the logarithm of the K^+/Na^+ specificity ratio is a linear function of the various metabolite fluxes measured, as predicted by the thermodynamic model.

Separate experiments were performed with culture solutions in which the glucose concentration and the Mg^{2+} concentration were limiting growth rate. In the glucose-limiting system the K^+/Na^+ specificity ratio *increases* with metabolic rate while for the Mg^{2+}-limiting system it *decreases* with metabolic rate.

It is concluded that the present results can be explained more readily in terms of the coupling of fluxes than in terms of a sodium pump.

The role of selective adsorption of ions is also briefly discussed in relation to the ion specificity at zero growth rate.

I. Introduction

A large number of living cells maintain internal concentrations of inorganic ions which cannot be accounted for by the concentration of these ions in the external environment and the difference of electrical potential across the membrane surrounding the cell. Three alternative hypotheses are currently considered as possible explanations of this situation: (1) the sorption-type theories associated with Troschin (1960) and Ling (1952); (2) pumps located in the membrane, first proposed by Dean (1941) and supported by Ussing (1949), Keynes (1954), and many others; (3) entrainment of ions suggested by Hearon (1949) and developed by Kedem and Katchalsky (1958), Nims (1961), and Coster and George (1968). In order to distinguish between these theories, further observations are required, particularly under situations in which different results are predicted by the three theories enumerated above.

In this paper we describe a series of observations designed to test hypothesis (3) by using *Aerobacter aerogenes*. This was done by varying the growth rate of this microorganism by changing the rate of supply of substrate. Other parameters such as pH, temperature, oxygen tension, etc. were maintained constant in a

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continuous culture apparatus similar to that described by Monod (1950) and Novick and Szilard (1950).

The advantages of using a continuous culture system in the present experiments are twofold. Firstly, it is possible to grow cells in constant environmental conditions for a large number of generations and, secondly, the method provides a ready means of studying a wide range of growth rates.

Variation of the growth rate would cause corresponding variation in metabolite flux across the outer cell membrane which should cause a predictable change of the sodium and potassium ion concentration ratio. The expected theoretical changes in ion concentration are first considered and then compared with the experimental observations.

II. Theory

The force usually considered responsible for the transport of molecules through a cell membrane is the gradient of the electrochemical potential for that species. The essential feature of the model presented below is that there is a second force to be taken into account, namely the frictional drag between the various species moving in and out of cells.

In a membrane in a steady state, the net force, F_j, per mole on any species j is zero since there is no net acceleration. Thus:

$$F_j = \sum_i F_{ij} = 0, \qquad i,j = 1, 2, \ldots, n \tag{1}$$

where the F_{ij} represents the interaction forces between species i and j. In equation (1) two forces are included: (i) a conservative force F_{jj},

$$F_{jj} = -\text{grad } \bar{\mu}_j, \tag{2}$$

where $\bar{\mu}_j$ is the electrochemical potential of j, and (ii) the dissipative force

$$F_{ij} = -c_i r_{ij} v_{ij}, \tag{3}$$

where c_i is the concentration of i and r_{ij} is a constant of proportionality (resistance coefficient). This force is due to the relative velocity v_{ji} between j and i and which, for the very low drift velocities in membranes, is proportional to the first power of velocity. Equation (1) expresses the fact that the conservative force is balanced by the sum of all the frictional forces in the steady state. From equations (1), (2), and (3):

$$\text{grad } \bar{\mu}_j = \sum_{i \neq j} -c_i r_{ij} v_{ij}. \tag{4}$$

If velocities are measured with respect to the membrane (subscript m), then

$$-v_{ij} = v_{ji} = v_{jm} - v_{im}, \tag{5}$$

and (4) becomes

$$\text{grad } \bar{\mu}_j = \sum_{i \neq j} c_i r_{ij} (v_{jm} - v_{im}). \tag{6}$$

But the flux ϕ_j is given by

$$\phi_j = c_j v_{jm},$$

and

$$\phi_i = c_i v_{im}.$$

Therefore

$$\text{grad } \bar{\mu}_j = (\phi_j/c_j)(\sum_{i\neq j} c_i r_{ij}) - \sum_{i\neq j} r_{ij}\phi_i, \tag{7}$$

or defining

$$r_{jj} = -\sum_{i\neq j} c_i r_{ij}/c_j, \tag{8}$$

and considering variation to take place only in the x direction, equation (7) becomes with equation (8)

$$\mathrm{d}\bar{\mu}_j/\mathrm{d}x = -\sum_{\text{all } i} r_{ij}\phi_i. \tag{9}$$

In the steady state, all ϕ_i's are constant and integration of equation (9) across the membrane gives

$$\Delta\bar{\mu}_j = -\sum R_{ij}\phi_i, \tag{10}$$

where $\Delta\bar{\mu}_j$ is the difference in electrochemical potential of species j and

$$R_{ij} = \int_0^\delta r_{ij}\mathrm{d}x \tag{11}$$

is the integral or total coupling resistance for the membrane of thickness δ. Equation (10) is the fundamental equation whose validity is being tested in the present experiments. It states that the difference in electrochemical potential of any species is a linear function of the net fluxes in the steady state.

The difference in electrochemical potential across the membrane for the species j is given by

$$\Delta\bar{\mu}_j = \boldsymbol{RT}\ln(\gamma_j^i c_j^i/\gamma_j^o c_j^o) + z_j \boldsymbol{F} V, \tag{12}$$

where z_j = valence of species j, c_j^i, c_j^o are internal and external concentrations of j, γ_j^i, and γ_j^o are activity coefficients, V is the membrane potential, and $\boldsymbol{R}$, T, and $\boldsymbol{F}$ have their usual meanings.

In order to test equation (10) using equation (12) it would be necessary to determine the value of V, which is not possible with microorganisms such as *A. aerogenes*. However, taking the difference of equation (12) for a pair of ions such as K^+ and Na^+, and assuming that

$$\gamma_{K+}^i = \gamma_{Na+}^i \text{ and } \gamma_{K+}^o = \gamma_{Na+}^o,$$

we obtain from equations (10) and (12)

$$\boldsymbol{RT}\ln\left[\frac{(c_K^i/c_K^o)}{(c_{Na}^i/c_{Na}^o)}\right] = \sum_i (R_{Na,i} - R_{K,i})\phi_i. \tag{13}$$

The ratio

$$\frac{c_K^i/c_K^o}{c_{Na}^i/c_{Na}^o}$$

is the specificity ratio of potassium over sodium ions. Equation (13) is amenable to experimental verification and is the form tested in the present paper. It demonstrates that selective accumulation of potassium or sodium is possible provided $R_{Na,i} \neq R_{K,i}$ for one or more species i for which the corresponding flux ϕ_i is not zero.

The fluxes ϕ_i in equation (13) will themselves be approximately proportional to the metabolic turnover rate of the cells and thus one would expect the ionic

specificity ratio to be linearly related to some index of metabolic rate. For example, under steady-state conditions

$$\phi_i = -(1/A)(\mathrm{d}n_i/\mathrm{d}t),$$

where A is the area of cell membrane and $\mathrm{d}n_i/\mathrm{d}t$ is either rate of production (positive) or rate of destruction (negative) of species i. Thus equation (13) may be written:

$$\boldsymbol{RT} \ln\left[\frac{(c^i_{\mathrm{K}}/c^o_{\mathrm{K}})}{(c^i_{\mathrm{Na}}/c^o_{\mathrm{Na}})}\right] = -(1/A)\sum_i (R_{\mathrm{Na},i} - R_{\mathrm{K},i})(\mathrm{d}n_i/\mathrm{d}t). \tag{14}$$

The various $\mathrm{d}n_i/\mathrm{d}t$ in equation (14) are related to each other through a set of stoichiometric coefficients. These coefficients will be constant provided the relative magnitudes of the rates of reaction, through the various metabolic pathways, remain constant. Thus we may put, for example,

$$(\mathrm{d}n_i/\mathrm{d}t) = a_i(\mathrm{d}n/\mathrm{d}t)_{\mathrm{glucose}}, \tag{15}$$

where a_i is the ith stoichiometric coefficient relative to glucose. Substitution of equation (15) into equation (14) yields

$$\boldsymbol{RT} \ln\left[\frac{(c^i_{\mathrm{K}}/c^o_{\mathrm{K}})}{(c^i_{\mathrm{Na}}/c^o_{\mathrm{Na}})}\right] = -(1/A)\sum_i a_i(R_{\mathrm{Na},i} - R_{\mathrm{K},i})(\mathrm{d}n/\mathrm{d}t)_{\mathrm{glucose}}. \tag{16}$$

Equation (16) is applicable to a cell in the steady state. In the continuous culture experiments described in this paper individual cells do not achieve a steady state, but the culture as a whole does (see Appendix I). Hence the specificity ratio in equation (16) now refers to the average for the whole culture. Provided that the a_i do not change with metabolic turnover rate (i.e. provided there is no shift in metabolic pathways with the growth rate) equation (16) can be reduced to

$$\boldsymbol{RT} \ln\left[\frac{(c^i_{\mathrm{K}}/c^o_{\mathrm{K}})}{(c^i_{\mathrm{Na}}/c^o_{\mathrm{Na}})}\right] = \text{constant} \times M, \tag{17}$$

where M is the metabolic turnover rate per unit cell area, this quantity itself being proportional to the growth rate per unit cell area.

The growth rate, μ, is defined as

$$\mu = (1/N)(\mathrm{d}N/\mathrm{d}t), \tag{18}$$

where N is the number of cells in the culture and $\mathrm{d}N/\mathrm{d}t$ is the rate of production of cells.

Since increased cell material is built up by total flux inputs and outputs to the cells, μ can be expressed as

$$\mu \propto (1/N)\sum_i \left[\sum_{p=1}^{N} (\mathrm{d}n_i/\mathrm{d}t)_p\right], \tag{19}$$

where the rate of production or consumption of each species i is summed over all the cells in the culture, i.e.

$$\sum_{p=1}^{N} (\mathrm{d}n_i/\mathrm{d}t)_p = (\mathrm{d}n/\mathrm{d}t)_i \tag{20}$$

$$= \text{total production or consumption of the species } i \text{ in the whole culture.}$$

Thus $(1/N)\sum_i (dn_i/dt)$ is proportional to the growth rate μ. Further,

$$(1/N)\sum_i (dn/dt)_i = (1/N)\sum_i \phi_i . A$$
$$= \sum_i \phi_i \text{ (average per cell)} . A \qquad (21)$$

Hence it can be seen that the sum of all the fluxes, $\sum_i \phi_i$, is directly proportional to $(1/A)\mu$. Thus equation (13) can also be written in terms of the growth rate μ, i.e.

$$\boldsymbol{RT} \ln\left[\frac{(c^i_K/c^o_K)}{(c^i_{Na}/c^o_{Na})}\right] \propto (1/A)\mu. \qquad (22)$$

The validity of equation (22), again, is dependent on a constant relationship between the various metabolic pathways in the cell. The validity of this can be tested by noting that for this case the rate of production of each species i per cell, i.e.

$$(dn_i/dt) = (1/N)(dn/dt)_i$$

should be proportional to the growth rate μ. This was verified experimentally in the present work.

III. Experimental Methods

(*a*) *Organism*

The organism used in the experiments was *Aerobacter aerogenes* (NCTC 418). An inoculum was prepared from a nutrient agar slope subcultured into 10 ml of standard glucose–salts media (Dean 1962), and thence into 1·8 litres of medium in the continuous culture vessel.

(*b*) *Medium for Continuous Culture*

In a continuous culture system of the type described in Appendix II, the rate of growth of the microorganisms is limited by the rate of supply of one particular nutrient. For the first series of experiments, in which the medium given in the following tabulation was used, the concentration of glucose was rate-limiting:

Nutrient	Concn. in Medium (g/l)	Concn. in Medium (m-equiv/l)	No. of Cells Produced per Litre
Glucose	5·0		$1·9\times10^{12}$
NH_4^+	0·68	37·8	$>10^{14}$*
Na^+	0·79	34·5	†
K^+	0·33	8·25	$3·1\times10^{13}$
Mg^{2+}	0·038	3·17	$2·5\times10^{13}$
Fe^{3+}	0·004	0·21	‡
SO_4^{2-}	1·82	38·0	$2·2\times10^{14}$
PO_4^{3-}	2·42	104	$4·8\times10^{13}$

* It is difficult to estimate the number of cells produced by NH_4^+ because 2N NH_4OH was added to the culture to maintain a pH of 7·1. In any event the concentration of NH_4^+ added to the medium together with that added as alkali gave values in excess of that for other nutrients.

† Na^+ not required for growth.

‡ Trace Fe^{3+} required for growth.

The observations were repeated using a different medium in which increased glucose concentrations and decreased Mg^{2+} concentration ensured that Mg^{2+} was now the factor limiting growth rate:

Nutrient	Concn. in Medium (g/l)	(m-equiv/l)	No. of Cells Produced per Litre
Glucose	10·0		$3·8 \times 10^{12}$
NH_4^+	0·26	14·5	$> 10^{14}$*
Na^+	0·79	34·5	†
K^+	0·33	8·25	$3·1 \times 10^{13}$
Mg^{2+}	0·0038	0·32	$2·5 \times 10^{12}$
Fe^{3+}	0·0004	0·21	‡
SO_4^{2-}	0·70	14·5	$8·5 \times 10^{13}$
PO_4^{3-}	2·42	104	$4·8 \times 10^{13}$

* See footnote to first tabulation.

† Na^+ not required for growth.

‡ Trace Fe^{3+} required for growth.

Modifications in the medium composition from that of the standard glucose–salts medium were based on results gained with *A. aerogenes* in continuous culture by Dean and Rogers (1967). The above tabulations set out the concentration of each nutrient in the medium, and give the maximum number of cells per litre that each particular concentration could support with all of the other nutrients in excess. The rate-limiting nutrient is the one which will support the lowest number of cells.

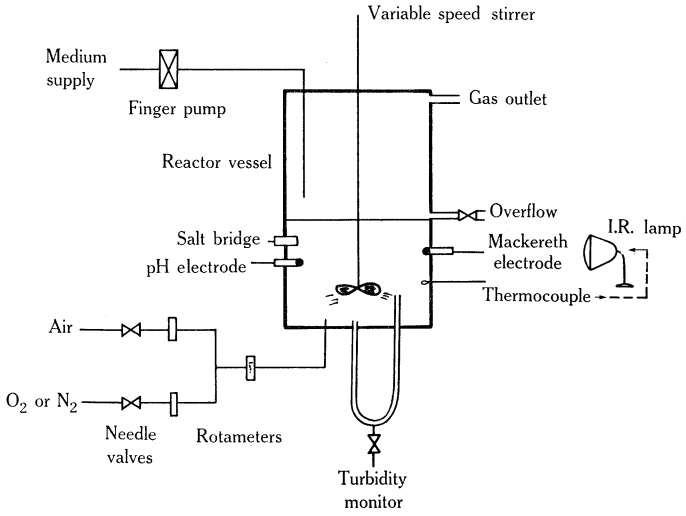

Fig. 1.—Schematic diagram of the continuous culture apparatus. The full details of the operation are given in the text. The pH, temperature, and oxygen tension of the culture medium is kept constant with the aid of an external feed-back system and appropriate sensing electrodes.

(*d*) *Experimental Techniques*

The culture vessel has been previously described by Herbert, Elsworth, and Telling (1956) and although the present vessel has been designed to be sterilized *in situ* by steam, it is similar in general design to that described by Herbert and his co-workers.

The vessel is a cylinder of Pyrex glass, 12 in. long and 6 in. in diameter, sealed at each end with a stainless steel plate and a neoprene rubber gasket. The top plate carries the housing of the impeller shaft bearings, two large ports to hold oxygen and pH electrodes, and six smaller ports to hold the inlet lines for medium, acid, alkali, etc. The lower plate carries the inlet and outlet ports for the turbidometer circulation arm (allowing continuous measurement of the turbidity of the culture), the cooling coil inlet and outlet, the overflow tube, and the sampling ports.

The level of the overflow tube was set such that at a stirrer speed of 400 r.p.m. the culture volume was 1·8 litres. Medium was pumped at a controlled rate into the culture vessel using a peristaltic pump. Effective aeration was achieved by an air flow at a point below the impeller with the fine air bubbles being widely dispersed throughout the culture. The influence of foam production was reduced by means of a "foam breaker" set at the top of the impeller shaft.

The oxygen tension in the culture was measured with a "Mackareth" oxygen electrode A15A (Mackareth 1964). It was maintained relatively constant at 100 mmHg at different growth rates by varying the air and oxygen flow rates between 100–300 ml/min and 0–150 ml/min, respectively. Using an HRL (Horiba) pH-meter, the pH was measured and the output fed to a recorder controller which kept the pH constant at $7 \cdot 1 \pm 0 \cdot 05$ by the addition of 2N NH_4OH or HCl.

Temperature was controlled at 40°C by means of a simple relay which could operate either an infrared heating lamp or a solenoid valve on the cooling water line. The temperature sensor itself was a small thermister fitted into a stainless steel pocket projecting into the culture.

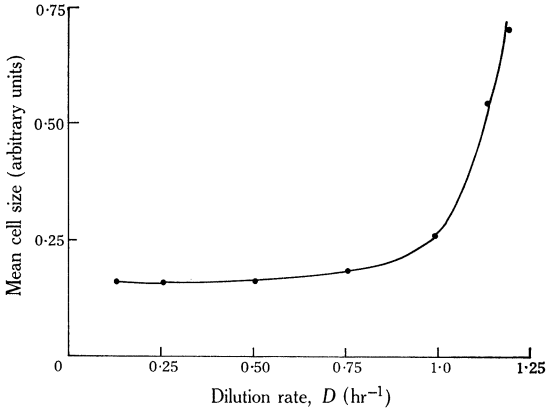

Fig. 2.—Cell size (i.e. cell volume) as a function of the growth rate (= dilution rate) for cells of *Aerobacter aerogenes*.

(e) Quantities Determined at each Growth Rate

(i) *Gaseous Metabolites.*—An analysis of both the inlet and outlet gas, for percentage oxygen, nitrogen, and carbon dioxide, was carried out using an Orsat gas analysis apparatus in which carbon dioxide and oxygen are selectively absorbed by suitable reagents.

(ii) *Dry Weight.*—Samples for dry weight determination were taken from the cold point.* Samples (10 ml) were centrifuged for 30 min at 18,000 r.p.m., the supernatant poured off, and the cells resuspended in 10 ml of distilled water. The 10-ml suspension was then placed into a weighed crucible and dried at 105°C for 48 hr and reweighed.

(iii) *Cell Size.*—Direct microscopic counts of cells were made using a Helber counting chamber—a microscope slide with a chamber of known depth having a measured grid ruled on its surface. Using this method, an estimation of the number of cells per millilitre was gained. The mass per cell is then the dry weight per millilitre divided by the total number of cells per millilitre. Assuming uniform density of cellular material, the mass per cell can be correlated with the volume of the cell, and therefore its surface area. The variation of cell size with growth rate is shown in Figure 2.

* i.e. Sample point not steam-jacketed.

(iv) *Glucose.*—The sample for glucose measurement was taken from a steam-jacketed sample point at 120°C. The sample was cooled and centrifuged for 30 min at 18,000 r.p.m., and the glucose in the supernatant was estimated by the glucose oxidase method of Huggett and Nixon (1957).

(v) *Ethanol.*—Samples were taken from the cold point and centrifuged. Alcohol was determined on a Beckman G.C.4 gas chromatograph.

(vi) *Sodium and Potassium Content.*—Duplicate 10-ml samples were taken from the cold point and centrifuged. The supernatant was removed and the cells resuspended in 10 ml distilled water. Both the supernatant and the resuspended cell samples were analysed for Na^+ and K^+ using an EEL flame-photometer. For the resuspended cell samples this method of analysis yields the concentration of the cell contents dispersed in 10 ml of water. Since we are interested only in the ratios of K^+ to Na^+, this is all that is required.

IV. Results

(*a*) *Achievement of Steady-state Conditions*

It was first necessary to establish the length of time required to reach steady-state conditions following a step change in the dilution rate, D (the dilution rate equals the growth rate in the steady state—see Appendix II). Earlier studies with *A. aerogenes* showed that the culture had reached a steady state with respect to population density, growth rate, and cell size after 10 generations (Dean and Rogers 1967). For these experiments, each growth rate was maintained for a time sufficient to give the requisite number of cell divisions.

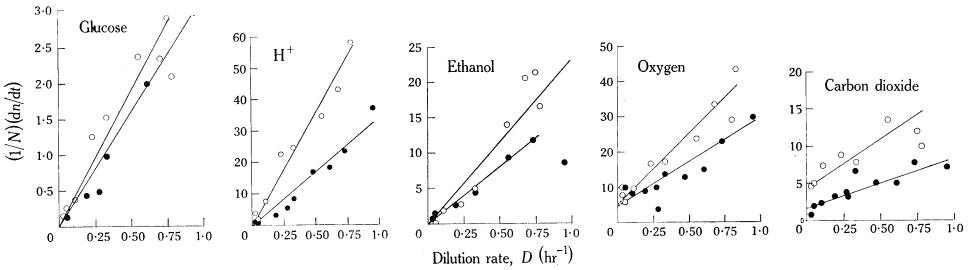

Fig. 3.—Rate of uptake or production, per cell, as a function of the dilution rate (= growth rate). Open circles refer to results obtained when the cells were grown in a medium in which the glucose concentration was limiting the growth rate, while the closed circles refer to the corresponding Mg^{2+}-limiting system. Although some scatter is evident it appears that there is a linear relationship between $(1/N)(dn/dt)$ and the dilution rate for both systems.

A preliminary experiment on the transient response of the K^+/Na^+ specificity ratio to small changes in dilution rate established that within two to three generations a steady value had been achieved.

(*b*) *Fluxes v. Growth Rate*

The experimental results are shown in Figure 3 for both the glucose-limiting and the Mg^{2+}-limiting systems. The rate of glucose uptake, ethanol production, and acid production appear to be linear with changes in growth rate although some scattering of points is evident.

The rate of oxygen uptake and the rate of carbon dioxide production are also linear with growth rate but do not extrapolate to pass through the origin. This latter phenomenon has been reported by Herbert (1958) and attributed to an endogenous metabolism, or a maintenance energy, which the cells require to remain viable even though not in a state of active growth. It is this energy which is required to maintain concentration gradients between the cell and the external environment.

(*c*) *Ion Specificity Ratio*

In Section II the ion specificity ratio has been shown theoretically to be linearly proportional to the fluxes (equation 13) and for a constant division between the metabolic pathways, to the flow of any one metabolite (equation 16) and hence also to the metabolic turnover rate or growth rate per unit cell area (equation 22). These predictions have been tested experimentally with the following results:

(1) Glucose-limiting system—the results for the K^+/Na^+ specificity ratio *v.* growth rate for this system are shown in Figure 4(*a*). It is seen that the logarithm of the K^+/Na^+ specificity ratio is proportional to the growth rate per unit area as predicted by equation (22).

(2) Magnesium-limiting system—the K^+/Na^+ specificity ratio as a function of growth rate per unit area in this system is shown in Figure 4(*b*). These results display two remarkable features. The first is that the K^+/Na^+ specificity ratio *decreases* as the metabolic turnover increases. This is in the reverse direction to that found for the corresponding factors in erythrocytes by Thurber (1965). The second remarkable feature is the discontinuity in the otherwise linear regression which occurs at $\mu/A \simeq 0{\cdot}35$–$0{\cdot}4$. This discontinuity was confirmed on three separate trials and appears to be genuine. No corresponding discontinuity was found in the fluxes of metabolites at this growth rate.

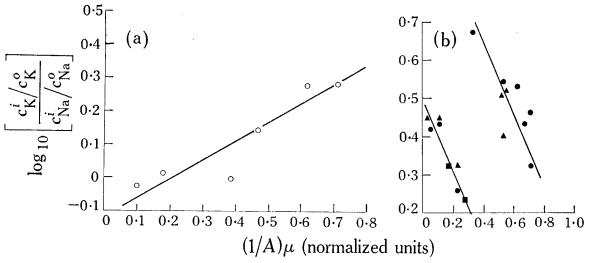

Fig. 4.—Logarithm of the K^+/Na^+ specificity ratio as a function of the growth rate per unit cell area for the glucose-limiting system (*a*) and for the Mg^{2+}-limiting system (*b*). The different symbols in (*b*) represent results for three separate experiments. For further explanation, see text.

V. Discussion

(*a*) *Ion Specificity Ratios*

According to sorption-type theories, ion accumulation is not a membrane phenomenon but depends on the electrical configuration of the molecules forming the cytoplasm. It is not clear how this would depend, if at all, on the membrane fluxes ϕ_i. According to the sodium pump theory, sodium should be pumped out of the cells at a rate which increases with metabolic activity although no specific functional dependence has been proposed. It is clear from the results with the

glucose-limiting system [Fig. 4(*a*)] that both the entrainment theory and the sodium pump theory are consistent with observation. On the other hand, the results for the Mg^{2+}-limiting system [Fig. 4(*b*)] are inconsistent with the sodium pump theory as it is normally considered, reversal of the slope of the ion specificity curves being difficult to envisage by this theory. Indeed one is confronted with the paradox of the pump apparently reversing because of this change in the medium.

The results may, however, be simply understood in terms of the entrainment theory. Here, all that is required is that, for the glucose-limiting system,

$$\sum a_i(R_{\mathrm{Na},i}-R_{\mathrm{K},i}) > 0,$$

and for the Mg^{2+}-limiting system

$$\sum a_i(R_{\mathrm{Na},i}-R_{\mathrm{K},i}) < 0.$$

This result could be brought about either by a change in the relative values of the a_i (i.e. a change in the relative rates of the various metabolic pathways), or by changes in $R_{\mathrm{Na},i}$ or $R_{\mathrm{K},i}$ due to modifications of the membrane structure, or both. To which of these possibilities we should attribute the slope change cannot be decided upon at this juncture, but clearly neither of them is excluded.

Ion specificity results for model systems have been reported by Salminen (1963) and Nims and Thurber (1966). These workers both obtain a linear relation between ion specificity ratio and the rate of chemical reaction as required by equation (14). Nims and Thurber were the first to show that a similar relation occurred in living systems. Working with erythrocytes they varied the metabolic rate over a wide range by varying the temperature from 40 to 34°C. They found that the logarithm of the ion specificity ratio increased linearly with metabolic turnover rate, as required by equation (17). The objection could be raised that the temperature variation could have caused a significant variation in the R_{ij} values.

It was largely for this reason that the present experiment was designed so that the metabolic turnover rate could be varied while maintaining the temperature constant. As far as we know this is the first time the experiment has been performed under these conditions.

(*b*) *Discontinuity in the K^+/Na^+ Specificity Ratio*

The discontinuity [Fig. 4(*b*)] suggests either (1) a switch in the relative rates of metabolism in the various pathways for the Mg^{2+}-limiting condition at a growth rate of about $0{\cdot}35\ \mathrm{hr}^{-1}$, or (2) a change in the resistance coefficients $R_{\mathrm{Na},i}$ and $R_{\mathrm{K},i}$ (in the membrane) at this growth rate in the Mg^{2+}-limiting system, or both. A change in the relative rates of the metabolic pathways is not supported by the influx and efflux results shown in Figure 3. A change in the resistance coefficients could perhaps therefore be implied although more direct evidence of this would require flux experiments with potassium and sodium ions. However, in an Mg^{2+}-limiting system membrane properties are affected as is indicated by the observation that under these conditions cells sometimes elongate abnormally (Dean and Rogers 1967). A deficiency of Mg^{2+} in the culture medium of *Escherichia coli* cells was found to cause marked filamentation and loss of viability (Brock 1962), while Webb (1949) reported that low concentrations of Mg^{2+} partially inhibited transverse cell wall formation.

(*c*) *Zero Growth Rate*

It can be seen from Figures 4(*a*) and 4(*b*) that if the experimental results are extrapolated back to zero growth rate, then for both of the two K^+/Na^+ specificity curves there is K^+/Na^+ specificity at zero growth rate when the fluxes are zero. This cannot be accounted for by the theory outlined before unless a substantial endogenous metabolism is present at zero growth rate. It seems more likely that other terms have to be included in equation (3) which take into account the absorption energy of potassium and sodium ions into the cytoplasm. A theory which describes this process has been proposed by Ling (1952, 1965). He considers the cytoplasm as a bulk fixed-charge system and calculation shows that potassium ions are preferentially adsorbed into such a system. The exchange of sodium ions for potassium ions in this case can be described by

$$K^+_{cyt.} + Na^+_{aq.} \rightleftharpoons K^+_{aq.} + Na^+_{cyt.} + \Delta\mathscr{F}, \tag{23}$$

where $\Delta\mathscr{F}$ is the free energy for the reaction. The K^+/Na^+ specificity ratio due to this process can then be described in terms of an equilibrium constant K:

$$\boldsymbol{RT} \ln\left[\frac{(c^i_K/c^o_K)}{(c^i_{Na}/c^o_{Na})}\right] = \boldsymbol{RT} \ln K, \tag{24}$$

where

$$K = \exp(\Delta\mathscr{F}/\boldsymbol{RT}).$$

The expression for the overall K^+/Na^+ specificity ratio due to both effects (i.e. coupled fluxes and selective adsorption) is then given by

$$\boldsymbol{RT} \ln\left[\frac{(c^i_K/c^o_K)}{(c^i_{Na}/c^o_{Na})}\right] = \boldsymbol{RT} \ln K - (1/A)(\mathrm{d}n/\mathrm{d}t)_{glucose} \sum a_i(R_{Na,i} - R_{K,i}). \tag{25}$$

VI. Conclusions

From the work reported in this paper the following conclusions may be drawn:

(1) The logarithm of the K^+/Na^+ specificity ratio varies in a linear manner with growth rate in *Aerobacter aerogenes*.

(2) This dependence is consistent with the theory based on irreversible thermodynamics.

(3) For the glucose-limiting system there is no discontinuity in the logarithm of the K^+/Na^+ specificity ratio versus growth rate curve in the range of growth rates investigated, namely $\mu = 0{\cdot}1\text{–}1{\cdot}0\ \mathrm{hr}^{-1}$.

(4) In the Mg^{2+}-limiting system there is a discontinuity at about $\mu \simeq 0{\cdot}35\ \mathrm{hr}^{-1}$.

(5) The slopes of the logarithm of K^+/Na^+ specificity ratio with growth rate is positive for the glucose-limiting system, and negative for the Mg^{2+}-limiting system.

(6) The negative slope of the logarithm K^+/Na^+ specificity ratio with growth rate for the Mg^{2+}-limiting conditions is simply explained in terms of ion entrainment and is difficult to explain in terms of pumps or sorption.

(7) The K^+/Na^+ specificity ratio at zero growth rate is not unity, suggesting that sorption plays a role which is significant at low metabolic turnover rates.

VII. References

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Appendix I

For the individual cells in the continuous culture when a steady state has been attained

$$\text{grad } \bar{\mu}_j = -\sum_i r_{ij}\phi_i.$$

The fluxes ϕ_i may have different magnitudes for different cells. In this case it is possible to define an average value of grad $\bar{\mu}_j$, viz;

$$\langle \text{grad } \bar{\mu}_j \rangle = (1/N) \sum_1^N \text{grad } \bar{\mu}_j,$$

where N is the number of cells in the culture. Therefore

$$\langle \text{grad } \bar{\mu}_j \rangle = -(1/N) \sum_{p=1}^N \sum_i (r_{ij}\phi_i)_p,$$

and, since r_{ij} are constant,

$$\langle \text{grad } \bar{\mu}_j \rangle = -\sum_i r_{ij}(1/N) \sum_{p=1}^N (\phi_i)_p,$$

where $(1/N) \sum (\phi_i)_p$ are the values of the fluxes ϕ_i for the N cells.

Now, since for the whole culture in the steady state the rate of production and consumption of all metabolites is constant, and since N is also kept constant,

$$A\langle\phi_i\rangle = (1/N)\sum_{p=1}^{N}(\mathrm{d}n_i/\mathrm{d}t)_p,$$

therefore

$$\langle\mathrm{grad}\ \bar{\mu}_i\rangle = -(1/A)\sum_i r_{ij}(1/N)(\mathrm{d}n_i/\mathrm{d}t)_{\mathrm{total}}.$$

Then, using equations (10) and (11),

$$\boldsymbol{RT}\ \ln\left[\frac{(c^i_{\mathrm{K}}/c^o_{\mathrm{K}})}{(c^i_{\mathrm{Na}}/c^o_{\mathrm{Na}})}\right]_{\mathrm{av.}} = (1/A).\sum_i (R_{\mathrm{K},i}-R_{\mathrm{Na},i})(1/N)(\mathrm{d}n_i/\mathrm{d}t)_{\mathrm{total}},$$

and hence, by equations (15) and (19),

$$\boldsymbol{RT}\ \ln\left[\frac{(c^i_{\mathrm{K}}/c^o_{\mathrm{K}})}{(c^i_{\mathrm{Na}}/c^o_{\mathrm{Na}})}\right] = \text{constant}\times\text{growth rate}/A.$$

Appendix II

The basic theoretical principles of continuous culture are well developed (Monod 1950; Novick and Szilard 1950; Herbert, Elsworth, and Telling 1956).

Fresh, sterile medium is added continuously at a given rate f, and the product is removed at the same rate from a culture vessel of fixed volume V. The dilution rate D, is defined as $D = f/V$. If the total concentration of bacteria is x cells per unit volume, then the rate of removal of cells $= Dx$.

For exponentially growing cells, the rate of increase due to growth can be expressed:

$$\mathrm{d}x/\mathrm{d}t = \mu x,$$

where μ = specific growth rate. For steady-state conditions, there is no change in the concentration of cells, i.e.

$$\mu = D. \qquad (1')$$

In a medium in which all of the essential materials are in excess, except one, μ varies with the concentration, S, of this substrate in the culture vessel. The relationship was shown by Monod (1942) to be fitted reasonably well by the following equation:

$$\mu = \mu_m[S/(K_s+S)], \qquad (2')$$

where μ_m = the maximum specific growth rate, and K_s = the saturation constant for the substrate (analogous to the Michaelis–Menten constant) and numerically equal to the substrate concentration in the culture vessel when $\mu = \frac{1}{2}\mu_m$.

Considering now the changes in the substrate concentration over the whole system:

Rate of increase = rate of input—rate of output—(rate of growth/yield constant).

Therefore

$$ds/dt = DS_R - DS - (\mu x/Y), \tag{3'}$$

S_R = input substrate concentration,
S = output substrate concentration,
Y = the yield (weight of cells produced per unit weight of substrate),
x = cell concentration, and
D = dilution rate.

At steady state,

$$dS/dt = 0 \text{ and } \mu = D.$$

Substituting for S from (2')

$$x = Y\{S_R - [K_s D/(\mu_m - D)]\}.$$

This is the general equation relating the cell concentration to the dilution rate, when one of the essential substrates is reduced in concentration to growth-limiting values.

In the experiments, the dilution rate could be set at any value between $D = 0{\cdot}03$ hr^{-1} and $D = 1{\cdot}20$ hr^{-1}. At low values of D, the viability of the culture decreases significantly (Postgate and Hunter 1962) and for D greater than $1{\cdot}2$ hr^{-1}, the dilution rate exceeds the maximum growth rate for *Aerobacter aerogenes* and "washout" occurs. By reducing the concentration of either glucose or magnesium in the input medium (S_R), its concentration in the culture vessel (S) was sufficiently low to reduce the growth rate. For a given dilution rate, the value of S adjusts itself to give $\mu = D$ at steady state.

The continuous culture method therefore provides the means of studying a set of different growth rates, at environmental conditions which are stabilized.