CONSTANCY OF UPTAKE DURING THE CELL CYCLE IN *ESCHERICHIA COLI*

H. E. KUBITSCHEK

From the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT Rates of uptake of several labeled compounds were measured during the cell cycle for three strains of *Escherichia coli* in balanced growth. Uptake rates were constant during more than the first two-thirds of the cycle, or reasonably so, for all of these compounds: glycine, leucine, glucose, acetate, phosphate, sulfate, and thymidine. When added *de novo*, uptake of glycine and leucine were not constant, but appeared to be proportional to mean cell volume. These results are in agreement with the finding that cell sizes increase linearly during most of the cell cycle for *E. coli*. They support the hypothesis, for cultures in balanced growth, that linear growth during the cell cycle is due to constant rates of uptake of all major growth factors. They also support the interpretation that uptake is limited by the presence of a constant number of functional binding or accumulation sites for these growth factors.

INTRODUCTION

In the preceding paper (1) evidence was presented for linear cell growth during the major part of the cell cycle for cultures of *Escherichia coli* in balanced growth. Linear growth, which must represent a constant rate of accumulation of nutrients (2) may imply a constant rate of uptake of those compounds upon which the culture is grown. Furthermore, this interpretation (1) provides a direct conceptual basis for linear growth: uptake would be constant if it is limited by a constant number of binding or accumulation sites for the low molecular weight compounds taken in by the cell. Since several different classes of compounds are involved in growth, we may correspondingly anticipate the presence of several, or perhaps many, kinds of these sites. As a working hypothesis, then, we may expect constant numbers of each of the different kinds of such sites needed for the uptake of each major growth factor.

The hypothesis that each major growth factor is taken up at a constant rate during most of the cycle in steady-state cultures can be tested by examining uptake of specific compounds as a function of cell size. The method of cell size fractionation developed to measure bacterial DNA synthesis during the cell cycle (3) was used to

examine uptake of two amino acids, two carbon sources, two inorganic ions, and a nucleoside in steady state cultures of *E. coli.*

MATERIALS AND METHODS

Bacterial Cultures

Cultures of *Escherichia coli*, strains B_{s-1}, WP2-HCR$^-$, and THU, were grown for about 18 hr at 37°C in 50ml volumes of minimal media containing M9 salts, glucose or acetate as an energy source, and sucrose (2%) to increase osmotic pressure (1). Required growth factors were added when needed: tryptophan (5 μg/ml) for strain WP2-HCR$^-$, and thymidine (2 μg/ml), histidine (50 μg/ml), and uracil (10 μg/ml) for strain THU. In addition, in order that balanced growth would be maintained at the time when isotope was added, parent cultures were grown in the presence of an abundance of the corresponding unlabeled growth factor.

Isotope Uptake and Assay

Isotopes were added to cultures in the exponential growth phase or to concentrated cultures produced by rapid filtration through a membrane filter (Bac-T-Flex, Schleicher and Schuell Co., Keene, N. H.) and resuspension into a part of the original growth medium. To maintain balanced growth, the concentration of the labeled compound added to the culture usually was not permitted to exceed about 10%. After an exposure period of about 3–5% of the doubling time, the cells were fixed with formaldehyde at a final concentration of 3.7%. One minute later they were concentrated by filtration, and washed repeatedly on the filter (six or seven times) with 5ml volumes of unlabeled medium. To resuspend the filtrate, 0.2 ml of unlabeled medium was dropped upon the filter and the cells were scraped off with a glass rod, into a drop. The cells were then layered upon a sucrose density gradient (linear, 5–15% sucrose in M9 salt solution) and banded by centrifugation (1500 × *g*, 2–4 min). From these bands, 8 to 12 samples (0.1 ml) were removed at equal intervals. Each sample was diluted in 1 ml of M9 salt solution. Equal portions (0.5 ml) of each diluted sample were used to determine sample radioactivity in a scintillation counter (CPM-100 Liquid Scintillation System, Beckman Instruments, Inc., Fullerton, Calif.) and to obtain cell counts and size distributions with a Coulter counter-multichannel analyzer system. Size distributions were measured after further dilution in 0.1 N HCl and were the same as in saline, but electronic noise appeared to be reduced. An earlier paper gives further details of techniques and instrumentation (3).

Mean Cell Age

For each sample, mean cell age was estimated from the mean cell volume $\bar{V}_s$ by comparison with the mean cell volume at birth $\bar{V}_b$ of the parent culture. The mean birth volume can be calculated from the relationship:

$$\bar{V}_b = \bar{V} \ln 2 = \bar{V}_d/2$$

where $\bar{V}_d$ is the mean cell volume for dividing cells, and is $\bar{V}$ the mean cell volume of the parent culture. The assumptions involved in this relationship have been discussed (3). The ratio $\bar{V}_s/\bar{V}_b$ is a linear measure of the sample interdivision age, with a range of values of 1 to 2 from birth to division in the parent culture.

RESULTS

Isotope Uptake Experiments

Culture conditions and details of the procedures used in the standard experiments involving isotopes are given in Table I. The measured values for the average uptake per cell are shown as a function of mean cell volume ($\bar{V}_s/\bar{V}_b$) for each experiment in Fig. 1 and 2. Sample activities were usually within the range of 500 to 2000 counts per min; extreme ranges were 30 and 20,000 counts per min; and the counter background was 12.5 counts per min. The largest statistical errors were those for the experiments on acetate uptake, and these standard errors are shown in Fig. 2 *A*.

In some of the experiments samples were taken to the bottom of the band to be certain that the largest cells were included. The average uptake per cell did increase

TABLE I

PROCEDURAL DETAILS FOR ISOTOPE INCORPORATION EXPERIMENTS

Strain	Culture			Compound				Figure	
	Carbon source	Cells/ml	Volume	Label	Activity	% Increase in concentration	Exposure	Number	Difference values
			(*ml*)		(*μC/ml*)		(*min*)		
B_{s-1}	acetate	5×10^7	50	glycine-2-^{14}C	0.5	14	5	1*A*	3
		5×10^9	2.0		5	8.2	6	1*B*	0
		7×10^7	50	L-leucine-UL-^{14}C	10	12	5	1*C*	0
WP2-HCR$^-$	glucose	2×10^9	1.1	glucose-UL-^{14}C	90	1.5	2	1*D*	3
B_{s-1}	acetate	1×10^9	2.8	acetate-1-^{14}C	14	2.4	5	2*A*	0
		9×10^6	50		20	0.3	5	2*A*	0
		7×10^7	45	$Na_2{}^{35}SO_4$	4	2.2	4	2*B*	4
	glucose	3×10^7	50	$Na_3{}^{32}PO_4$	12	0.9	2	2*C*	4
THU	acetate	4×10^7	70	thymidine-5-methyl-^{3}H	1.4	4	5	2*D*	2

for these samples from the deepest levels, but the results could not be analyzed with resolution comparable to the data presented, for two reasons. First, the dispersion of these samples was extremely broad with a range of cell sizes approaching that of the parent culture. Characteristic distributions are shown in an earlier paper (3), and as is discussed there, the presence of small cells at the deepest levels of the band is probably due to cell aggregation upon the centrifuge tube wall followed by later release into suspension at a deeper level. Second, although clumps of cells were not found in samples from higher levels, clumping of cells into twos and threes could be seen in the deepest samples. Because of these factors, broad dispersion and clumping, only the data for samples with mean volumes less than about 1.7 $\bar{V}_b$ were analyzed.

In every experiment the average uptake per cell remained relatively independent of cell size during the first two-thirds of the cycle, or showed a slight depression for

cells of intermediate volumes. However, when steady-state conditions were abandoned by *de novo* addition of the compound for which uptake was to be measured, as discussed in the section on control experiments, uptake was greater for larger cells.

Resolution

From experiment to experiment, the dispersion of cell sizes was unpredictable for samples obtained from sucrose gradients. Both the poorest and the best resolution

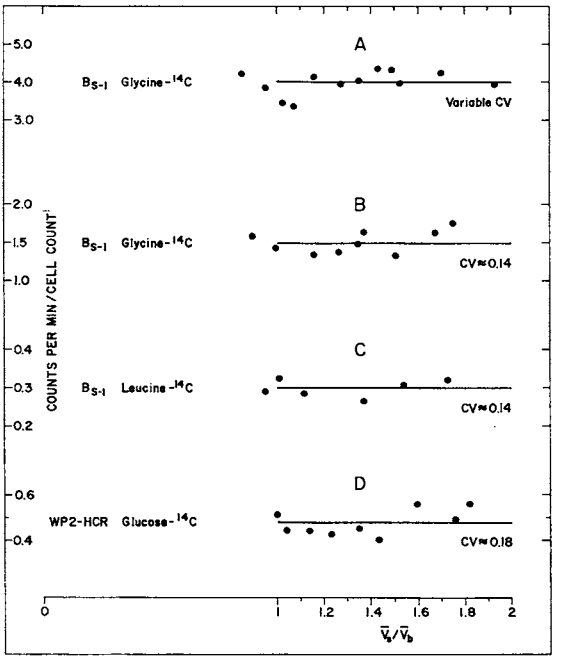

FIGURE 1 Uptake of glycine, leucine, and glucose as a function of mean cell volume in samples removed after banding steady state cultures of *E. coli*. $\bar{V}_b$ is the mean cell volume at birth, $\bar{V}_s$ is the sample mean cell volume, and CV is the average coefficient of variation for all samples. Cell concentrations, per milliliter, were about $10^4 \times$ cell count in all experiments.

were obtained in the experiments with glycine, as shown in Figs. 3 and 4, respectively. In both experiments, nevertheless, the relative activity per cell remained constant during the cycle. In order to increase resolution in some experiments, a procedure of the kind illustrated in Fig. 3 was adopted when the leading edges of two distributions could be made to coincide by a linear expansion of the ordinate of one of them. Differences were taken between these pairs of distributions, thereby subtracting out the contribution of the smallest cells. These difference distributions were compared with the corresponding differences in counts per minute. Activities per cell calculated by difference were indistinguishable from the values for the un-

corrected fractions. The number of final points calculated by difference for each of the experiments is shown in the last column of Table I.

Release of Activity During Banding

In some experiments samples above the visible band contained no detectable cells but had, nevertheless, appreciable activities. With acetate, for example, a plot of the

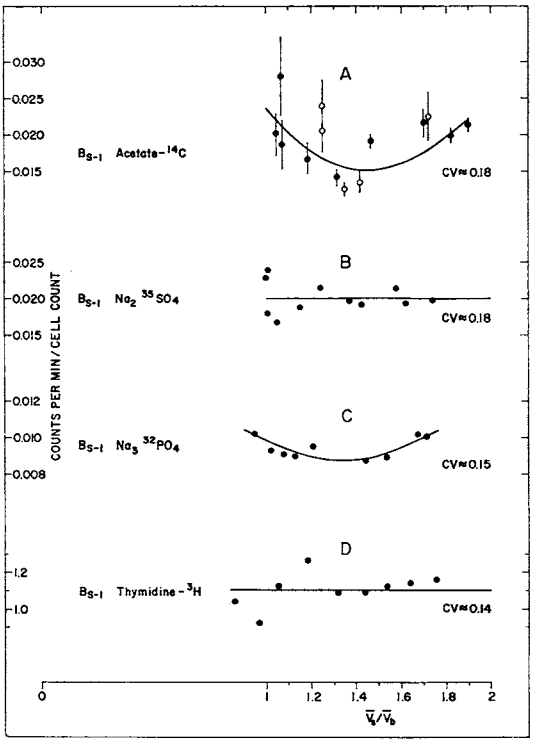

FIGURE 2 Uptake of acetate, sulfate, phosphate, and thymidine as a function of cell volume in cell volume in samples removed after banding steady state cultures of *E. coli*. In Fig. 2 *A* points are shown for two experiments: closed circle, concentrated culture, usual 5–15% gradient; open circle, cells not concentrated, 5–25% gradient, mean value of cell activity corrected to that of first experiment, for comparison. The vertical bars above and below the points in Fig. 2 *A* indicate calculated standard errors arising statistically during counting.

activity per sample (Fig. 5) shows that the first two samples, which contained no detectable cells, had greater activities than the next three samples, which did contain cells. In control experiments with and without cells, these cell-free sample activities diminished at an exponential rate in successive samples. Clearly, radioactive particles or fibers from the filters were transported along the gradient, or ^{14}C was carried in some other manner as the cells were centrifuged. Extrapolation of the values from the cell-free samples gave an estimate of this additional background, shown by the

dashed line in this figure, which was subtracted from the values of those samples containing cells. The values indicated by the filled circles in Fig. 2 *A* were obtained with this correction. It was also necessary to apply this kind of correction to the activities in the experiments with the inorganic ions, Figs. 2 *B* and 2 *C*.

Fig. 2 *A* also gives values (open circles) for a second experiment in which the culture was banded in a steeper gradient, 5–25 % sucrose. In order to show the values for this experiment in Fig. 2 *A*, the mean cell activity was corrected to that of the

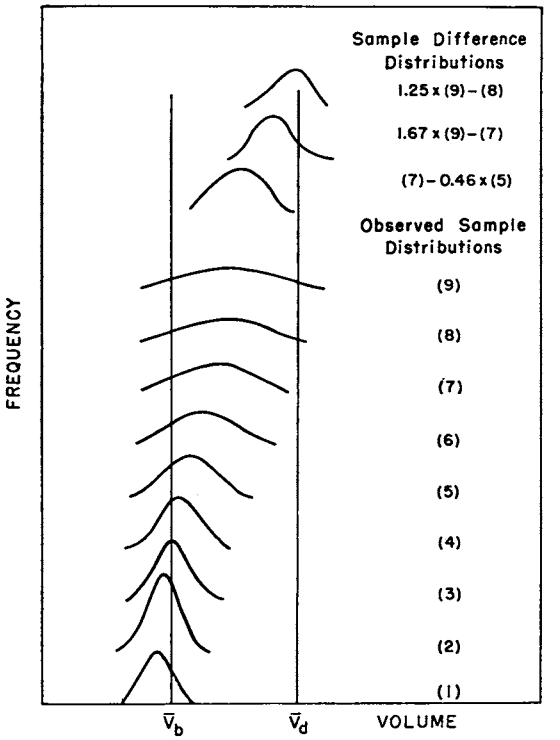

FIGURE 3 Cell size distributions for Fig. 1 *A*. V_b and V_d are the mean cell volume at birth and division, respectively. Numbers in parentheses represent sample numbers.

first experiment. For both experiments cell activities appeared to be depressed during the middle of the cycle ($\bar{V}_s \approx 1.5\ \bar{V}_b$).

Differences in Cell Concentration

Cell concentrations differed greatly in the two acetate experiments and also in the two glycine experiments (see Table I). The more concentrated cultures were far less efficient in uptake of the labeled compound, probably due to depletion of available acetate or to insufficient oxygen. Nevertheless, the dependence of uptake upon cell size was unaffected by cell concentration (Figs. 1*A* and 1 *B*, and 2 *A*).

Control Experiments

The effect of upsetting steady-state conditions was examined by adding labeled glycine or leucine to an acetate culture of strain B_{s-1} that previously contained neither of these amino acids. In these experiments uptake increased with cell size (Fig. 6). Uptake also increased with cell size in other experiments (glycine, sulfate)

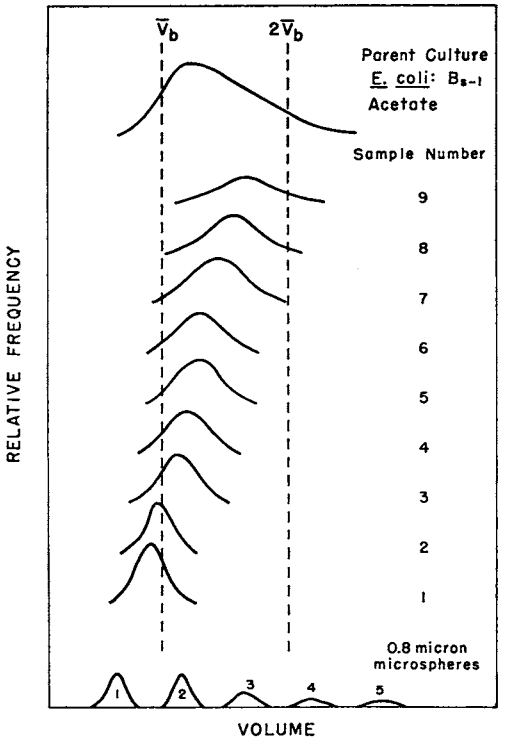

FIGURE 4 Cell size distributions for Fig. 1 *B*. Calibration is provided by the volume distributions for microspheres 0.8μ in diameter, and for aggregates containing 2, 3, 4, and 5 microspheres.

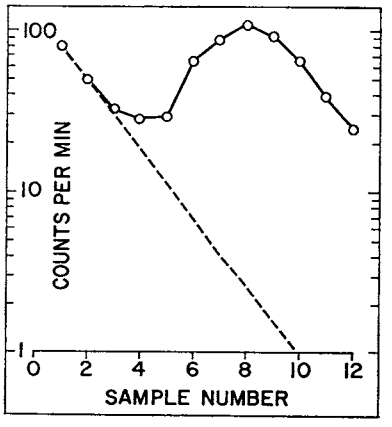

FIGURE 5 Sample activities for Fig. 2 *A*. The point corresponding to sample number 3 was omitted in Fig. 2 *A* because of the unduly large correction for the cell-free background that led to a standard error of the same order of magnitude as the value determined.

in which the addition of the label increased compound concentration by a factor of two or more. These experiments demonstrated the necessity for maintaining steady-state conditions during labeling.

Two kinds of control experiment were also performed to investigate possible effects of formaldehyde upon uptake measurements. In the first, three acetate cultures of strain B_{s-1} were exposed to leucine-^{14}C for 5 min. The first culture was fixed with formaldehyde after exposure to the label, the second was fixed prior to exposure to the label, and the third culture was not exposed to formaldehyde. Cells fixed with formaldehyde after exposure to the label were about 10% less radioactive than cells that were not fixed. Furthermore, when cells were exposed to formaldehyde before

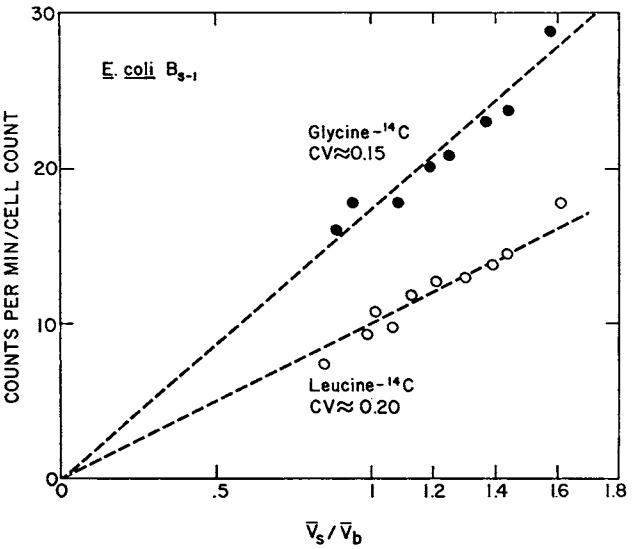

FIGURE 6 Uptake of glycine and leucine in acetate cultures of *E. coli* B_{s-1} grown in the absence of these amino acids.

exposing them to label, activities per cell were greatly reduced, to about 6% of the value of unexposed cells. Thus formaldehyde reduced leucine transport effectively.

As a second kind of control, leucine uptake was measured in an acetate culture without adding formaldehyde. This experiment was complicated by the residual cell division that took place in the band during the time required to remove samples (approximately 45 min), even though no exogenous energy source was present. Cell division led to the bimodal distributions shown in Fig. 7. These distributions permit an approximate calculation of the number of cells initially present in each sample, as well as their initial mean cell volumes. The corresponding corrected activities per cell are shown in Fig. 8. Although errors are necessarily larger here than in the standard experiments with formaldehyde-treated cells, values of uptake per cell are again relatively constant.

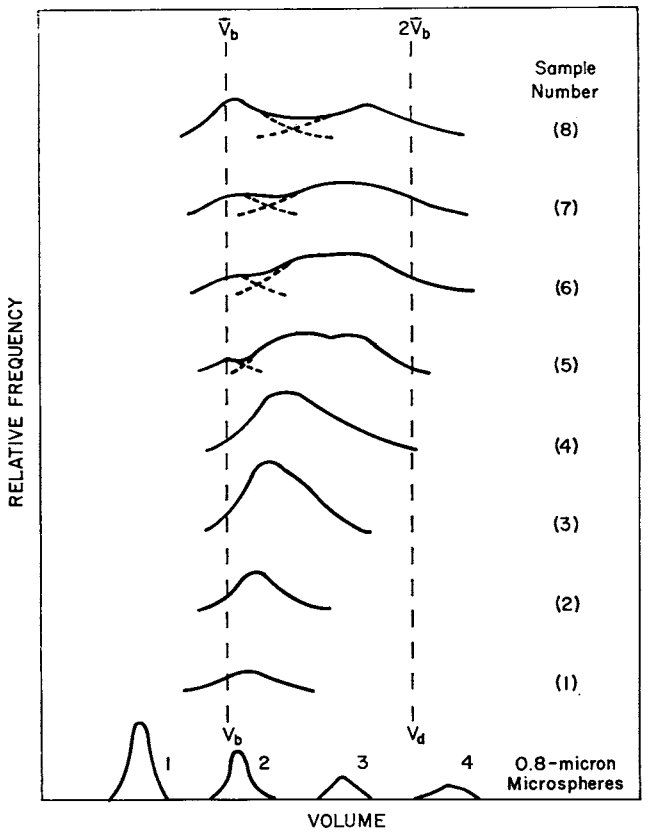

FIGURE 7 Cell size distributions for samples removed after banding an acetate culture of *E. coli* B_{s-1} that was not exposed to formaldehyde.

DISCUSSION

Uptake Dependence Upon Binding

In most of the steady-state experiments there was no apparent dependence of the rate of uptake of a compound upon cell volume, supporting linear cell growth. Furthermore, uptake did not increase with cell volume in any of these experiments in the manner that would have been expected if cell volumes increased exponentially during the cycle. However, values of uptake for acetate and phosphate, and perhaps also for glucose, decreased during the middle of the cell cycle. In order to understand these results, we must first consider the possible effects of the degree of binding of labeled compounds in precursor pools.

Measured values of isotope activity per cell will depend upon the degree of binding at pool sites in the cell. If rates of uptake are constant there are two extreme possibilities when a culture is pulse-labeled: (1) If the isotope is bound so tightly that none of it is extracted by washing then the activity per cell should be inde-

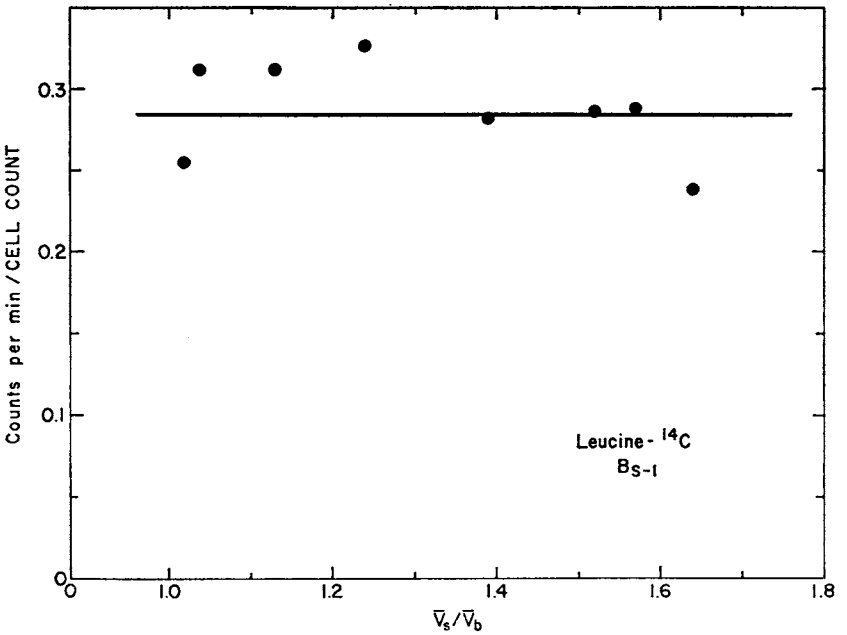

FIGURE 8 Leucine uptake by the same culture as for Fig. 7.

pendent of cell size and age. (2) If the isotope is bound so loosely that all of it is extracted during washing then cells will have their activities reduced by the amount of isotope lost from their pools. Therefore, uptake values will depend upon the manner in which pool size varies during the cycle. But linear growth and exponential macromolecular synthesis would require the existence of regularly varying pools that first increase and then decrease during the cycle as Mitchison demonstrated for yeast (2). Thus, the amount of isotope extracted by washing should increase to a maximum value during the middle of the cycle, depressing measured cell activities for this part of the cycle.

Constancy of Uptake

Some of the experiments reported here gave results corresponding to the dependence expected for an extractible pool: values for the uptake of acetate and phosphate (Figs. 2 *A* and 2 *C*) and possibly glucose (Fig. 1 *D*) were slightly depressed during the middle of the cell cycle. It should be noted that these results also provide indirect evidence supporting the same kind of regular variation of pool size during the cell cycle in *E. coli* that Mitchison found for yeast; his cautionary remarks about the interpretation of labeling experiments are appropriate for this bacterium.

For glycine, leucine, sulfate, and thymidine (Figs. 1 *A*, 1 *B*, 1 *C*, 2 *B*, and 2 *D*), there is no evidence for an extractible pool; within experimental errors, uptake was constant for each. The results for acetate, phosphate, and glucose also support the

hypothesis of constant uptake during the cycle for these compounds. Thus these experiments, all taken together, provide consistent evidence for constant uptake during more than the first two-thirds of the cell cycle of *E. coli* in balanced growth, and in turn support linear cell growth for this period.

Constant uptake is not necessarily in contradiction with the results obtained by Abbo and Pardee (4) who found that protein and ribonucleic acid increased essentially exponentially during the growth of synchronized bacterial cultures. Their results could be ascribed to the fact that these authors measured macromolecular incorporation only, while total uptake must also include the additional contribution of precursor pool material.

The present observations of constant uptake and the earlier observations of constant growth rate (1) during most of the growth-duplication cycle are, however, in disagreement with the results obtained by Harvey, et al. (5). They derived values for the average cellular rate of volume increase during the cell cycle by an analysis of steady-state volume distributions. Their method is one of differences of integrals of distributions, and therefore requires that the distributions be known with great accuracy if even moderately accurate growth rates are to be determined. The values that the authors obtained may be questioned because precise distributions for the volumes of dividing and new-born cells were not measured but assumed. Also, the resolution of their apparatus (differentiating-integrating technique) was inferior to that obtainable by the usual instrumentation and their measured distribution curves have not been corrected for the pronounced shape changes that result from lack of resolution.

It must be emphasized that uptake was not constant, but was increased in larger cells when a compound was added in its labeled form to a growth medium that previously did not contain the compound, Fig. 6. It would appear that control of the limiting number of active binding or accumulation sites requires that steady-state conditions be maintained.

Although the nature of these sites is unknown, it seems likely that growth is limited at some step prior to the residence of compounds in precursor pools. Formally, we can consider the following rather general steps in the uptake of a factor F. First, it is bound by a binding site B, and then transferred to an intermediate site of accumulation A, before being stored at a precursor pool site P:

$$F \rightleftharpoons (F - B) \rightleftharpoons (F - A) \rightleftharpoons (F - P) \rightarrow \text{metabolic product.}$$

Clearly, if the number of binding sites or the number of accumulation sites were constant and served to limit uptake, linear growth could occur if the reaction rates in the reverse direction were small compared to those in the forward direction. On the other hand, controls would have to be far more complex if they were applied at the level of the precursor pool, since pools vary during the cell cycle. Such a control system would be required to maintain a relatively constant number of *un-*

occupied pool sites during the cycle. In this case, the *total* number of sites, both occupied and unoccupied, would have to vary during the cycle in a manner similar to that described for pools. But there is persuasive evidence against this kind of control for methionine uptake: measurements of maximum pool size (equivalent to the number of pool sites) in a synchronous culture of bacteria (*A. faecalis*) gave a constant value during the growth cycle (7).

The manner in which the number of active binding or accumulation sites is established is also one of speculation at this early stage. The number of such sites must double with each division. Possibly, the number of functional sites is fixed at or near the beginning of each cycle (with changes in their number occuring only at the very end or beginning of the cycle) or alternatively, functional constancy might be regulated by some feedback mechanism operating upon a much larger number of potential sites.

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