Protein Turnover in *Escherichia coli* as Measured with an Equilibration Apparatus

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ABSTRACT

LEVINE, ELLIOT M. (National Institutes of Health, Bethesda, Md.). Protein turnover in *Escherichia coli* as measured with an equilibration apparatus. *J. Bacteriol.* 90:1578-1588. 1965.—Intercellular protein turnover (the reutilization by one cell of amino acids derived from the protein of another cell) occurs at a rate of 0.16 to 0.18% per hour in nongrowing cultures of *Escherichia coli*, as determined in an apparatus that rapidly equilibrates the culture fluids of two separated bacterial suspensions.

Protein turnover may be defined as a dual process: (i) degradation of protein molecules to peptides or amino acids and (ii) their subsequent reincorporation into protein molecules. The nature of protein turnover in microbes has been widely discussed (Rotman and Spiegelman, 1954; Weinbaum and Mallette, 1959; Cohn, 1957; Borek, Ponticorvo, and Rittenberg, 1958; Markovitz and Klein, 1958a, b; Mandelstam, 1960). As will be seen, a critical evaluation of protein turnover measurements requires that any description of protein turnover in a cell population should distinguish among three possibilities: (i) that each cell degrades and reforms protein exclusively within itself (hereafter termed *intracellular* turnover), (ii) that amino acid residues derived from damaged or secreting cells are reutilized by other cells (*intercellular* turnover), and (iii) that both intracellular and intercellular turnover are operative under physiological conditions.

The usual procedures employed for measuring protein turnover in microorganisms (Borek et al., 1958; Halvorson, 1958; Mandelstam, 1958, 1960) cannot distinguish intracellular turnover from intercellular turnover. Generally, cells whose protein has been prelabeled with a radioactive amino acid are incubated in the presence of an exogenous "trap" of unlabeled amino acid, and the appearance of radioactive amino acid in the medium is taken as a measure of protein turnover. This procedure does not measure intracellular turnover exclusively, because labeled amino acid found in the medium may be derived

from sources other than the endogenous pool of viable cells (e.g., from lysed, damaged, or secreting cells). Thus, the usual determinations reflect both intercellular and intracellular turnover, a value which may be termed *total* turnover.

Determining intercellular turnover, therefore, is important not only because the phenomenon itself is of interest, but because intracellular turnover cannot be determined unless intercellular turnover has been accurately measured. Furthermore, because experimental manipulation can damage cells (Koch, 1959), a given value for total turnover may be greatly increased by certain experimental procedures. Only an accurate determination of intercellular turnover can detect such an artifact. The object of the present study has been to determine the contribution of intercellular turnover to total turnover in *Escherichia coli*.

Previous studies have attempted to evaluate intercellular turnover in several ways. Cellular lysis, damage, and amino acid secretion were assessed by measuring cell viability and appearance of endogenous cellular constituents or amino acids in the medium. Such estimates may be misleading. For example, viable counts are considered neither precise nor sensitive, and do not reflect cell lysis in a nongrowing culture if new cells grow by utilizing dead cells as nutrients (Koch, 1959; Mandelstam, 1960). The method of assaying for appearance of endogenous enzymes in the medium (Mandelstam, 1958; Halvorson, 1958; Urbá, 1959), has also been subject to reservations (Koch, 1959). As shown with *Bacillus subtilis* and *E. coli*, some endogenous enzymes are not totally released into the medium after cell damage, and others are inhibited by

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extracellular bacterial products (Pollock, 1961; Gershanovitch, Avdeeva, and Goldfarb, 1963; Neu and Heppel, 1964a, b). As far as detection of amino acids in the medium is concerned, this is difficult if the uptake rate into the cell is comparable to the exit rate; the consequent steady-state level of amino acid in the medium can be very small despite a high rate of protein turnover. Finally, the rate of amino acid appearance in a medium initially devoid of amino acid only reflects the degradative phase of intercellular turnover. Overall intercellular turnover can be determined only by measuring the rate of amino acid reincorporation into cells.

Clearly, for all the above reasons, intercellular protein turnover in bacteria has not been adequately studied, and, thus, the true magnitude of intracellular turnover still remains in question. However, a successful experimental approach has been devised to study intercellular turnover in mammalian cells (Eagle et al., 1959). By coupling two culture vessels together, a monolayer of labeled mammalian cells adhering to a glass surface of one vessel was allowed to share the same culture fluid as a monolayer of unlabeled cells in a second vessel. In the absence of an exogenous amino acid "trap" in such a system, incorporation by the unlabeled cells of the labeled amino acid released into the medium proceeds almost as efficiently as reincorporation by the initially labeled cells themselves. The rate of incorporation reflects the rate of intercellular protein turnover.

To utilize this principle for studies with bacterial suspensions, certain modifications were necessary. This report describes experiments with *E. coli* performed in a new apparatus designed for rapid equilibration of the culture fluids of two bacterial suspensions separated by a Millipore filter. Cells of an *E. coli* threonine-tryptophan auxotroph were prelabeled with threonine- C^{14} and were then suspended in medium devoid of threonine and tryptophan; the transfer of threonine- C^{14} to an unlabeled culture of the same auxotroph was measured. The results show that a definite intercellular turnover does occur in nongrowing *E. coli*, but at a slow rate.

MATERIALS AND METHODS

Amino acids. All unlabeled amino acids were "A" grade reagents purchased from Calbiochem. L-Threonine- C^{14} , uniformly labeled (specific activity, $165 \mu\text{C}/\mu\text{mole} \pm 5\%$), was purchased from New England Nuclear Corp., Boston, Mass.

Other materials. Cab-O-Sil was purchased from Packard Instrument Co., LaGrange, Ill. *E. coli* K-12 strain PA209 was kindly furnished by F. Jacob, Pasteur Institute, Paris, France. This

strain requires L-histidine, L-leucine, L-tryptophan, L-threonine, and thiamine for growth, and cannot ferment lactose, galactose, and several other sugars (Jacob and Wollman, 1961).

Preparation of cells. *E. coli* K-12 strain PA 209 was grown in a medium containing, in a total volume of 1 liter: K_2HPO_4 , 1.5 g; KH_2PO_4 , 0.5 g; $MgSO_4$, 1.0 g; D-glucose, 5.0 g; $(NH_4)_2SO_4$, 2.5 g; thiamine, 10 mg; L-threonine, 100 mg; L-tryptophan, 100 mg; L-histidine, 100 mg; L-leucine, 100 mg; L-isoleucine, 100 mg; and glycine, 100 mg; final pH was 7.0. Cells grown in the above medium with L-threonine- C^{14} contained 95% of their radioactivity in protein-bound threonine. Unless unlabeled glycine and isoleucine were included in the growth medium, radioactivity appeared in the glycine and isoleucine residues of the cell protein.

Media were filtered through PH (pore size, 0.30μ) Millipore filters for sterilization; this also removed particulate matter which could clog the equilibration apparatus. The culture was grown to a density of approximately 150 Klett units (no. 540 filter, Klett-Summerson photometer, equivalent to an absorbancy of 0.450 at $540 m\mu$ in a Beckman DU spectrophotometer, 1-cm light path; the dry weight of cells was 0.46 mg/ml and the weight of bacterial protein was 0.22 mg/ml). The following procedure was used to wash cells free from growth medium. Cells were centrifuged at room temperature, the supernatant fluid was decanted, and cells were suspended in filtered medium devoid of $(NH_4)_2SO_4$, L-threonine, L-tryptophan, L-isoleucine, and glycine. Resuspended cells were incubated with shaking for approximately 10 min in a water bath at 37 C. Cells were centrifuged, suspended, and incubated with shaking twice more, and were then suspended to a density of 50 Klett units. This washing procedure diluted threonine carried over from the original supernatant fluid to less than 5% of the lowest concentration ($0.12 \mu\text{mole}/\text{ml}$) added in any experiment.

Equilibration apparatus. Several simple apparatuses have been described for the equilibration of two bacterial cultures. In these, cultures were separated by cellophane membranes (Nurmikko, 1955, 1956, 1957) which allowed only a slow equilibration (a matter of days) or ultrafine sintered-glass filters (Davis, 1950) which clogged after several transfers of culture medium. In the new apparatus, Millipore filters were used to separate the bacterial suspensions, and alternate applications of air pressure and vacuum were employed to flush the culture fluid between the two bacterial suspensions. The culture fluids of the two bacterial suspensions were equilibrated quite rapidly: 57% after 20 sec, 92% after 40 sec. The details of a typical experiment, including a complete description of the apparatus, its assembly, and operation, follow.

As shown in Fig. 1, culture flasks I and II (modified 125-ml suction flasks) were joined by a filtering unit consisting of two Millipore filters mounted between two fritted-glass filter bases. The filter

FIG. 1. *Equilibration apparatus.* The apparatus itself is shown in the center of the diagram; the upper left insert is a wiring diagram, and the lower right insert an enlarged view of a filter holder (one-half of the filtering unit). In the diagram of the apparatus, "A" and "B" represent electric hosecocks; "A" is shown open, and "B" is closed. Air entered the apparatus through the shaded tubing; vacuum was applied through the plain tubing. See text for details. The following materials were used in the construction and assembly of the apparatus: vacuum gauge, pressure gauge, glass joint clamps no. 28 and no. 18, Pyseal cement, and electric hosecocks, purchased from Fisher Scientific Co., Pittsburgh, Pa.; MF cement (formulation no. 1), Millipore filters (25 mm, types PH and HA), and Pyrex microanalysis filter holders purchased from the Millipore Filter Corp., Bedford, Mass.; Siliclad (a siliconizing agent) obtained from Clay-Adams, Inc., New York, N.Y.; Café-au-lait rubber stoppers (no. 2) obtained from Emil Greiner Co., New York, N.Y. (used because they are more pliable than conventional stoppers); an interval timer purchased from Harco Products, Philadelphia, Pa.; and a single-pole-double-throw relay obtained from Potter and Brumfield, Princeton, Inc.

bases (shown in the lower insert of Fig. 1) were similar to those available from the Millipore Filter Corp., except that the ordinary stem was replaced with thick-walled capillary tubing (2-mm bore). A coarse-grade fritted-glass disc (19 to 20 mm in diameter) was sealed into the supporting glass tube with its top surface about 0.5 mm below the tubing; the end of the protruding tubing was ground flat, and its inner edge was beveled smooth with a carborundum stick (Fig. 1, lower insert). The internal volume of the entire filtering unit was approximately 2 ml. To prepare the filtering unit for use, MF cement was placed on the ground ends of the filter bases, and a 25-mm type PH Millipore filter (pore size, 0.30μ) was mounted on each holder. The holders then were butted together and secured by two ball-and-socket joint clamps (no. 18 and no. 28). The junction of the two filter holders was sealed with Pyseal cement. The filter holder stems, previously inserted into café-au-lait rubber stoppers (no. 2), were placed in the culture flasks through openings made for that purpose.

After the filtering unit was in place, the apparatus could then be employed to equilibrate the media of two bacterial suspensions, one in unit I and the other in unit II, by alternate application of pressure and vacuum to each unit. Air pressure (shaded tubing) was supplied to the equilibration apparatus at 20 psi and vacuum (unshaded tubing) at 15 inches (38.1 cm) of mercury ("house line") through heavy-walled rubber tubing. Those portions of the lines which pass through electric hosecocks A and B were of thin-walled small-diameter tubing. Air first passed through a central saturator (a hot water bath at 70 C) and a central trap (neither shown in Fig. 1) located outside the incubator containing the culture flasks. Inside the 37 C incubator were two additional small traps, one for each symmetrical half of the apparatus. These latter traps contained several layers of water-soaked absorbant cotton. The air was saturated with water vapor to reduce evaporation of culture fluid during the vacuum cycle. The two small traps were connected to the two culture flasks in the shaking incubator at 37 C. Air pressure was applied to each culture flask through its side arm from the adjoining trap; vacuum was applied through the top opening of the culture flask.

The mechanism for controlling the application of pressure and vacuum consisted of two electric hosecocks, A and B, regulated by an interval timer and an electric relay connection as indicated in Fig. 1 (upper left insert). There were two separate circuits, one for hosecock A and the other for B. The interval timer in circuit B closed hosecock B for a certain portion of a given cycling time; the closing of circuit B also activated a relay which broke circuit A, opening hosecock A. Thus, when hosecock B was closed, hosecock A was opened, and vice versa; the pressure and vacuum lines were arranged so that pressure for unit I and

vacuum for unit II were controlled by hosecock A; hosecock B controlled pressure for unit II and vacuum for unit I. At the stage of operation depicted in Fig. 1, air pressure was being applied to unit I and vacuum to unit II; i.e., culture fluid was flowing from unit I into unit II.

A typical experiment was preceded by testing the apparatus with sterile medium as follows. After the central saturator reached 70 C and units I and II were at 37 C, 20 ml of washing medium (see *Preparation of cells*) were added to each culture flask. The electric current was turned on, and the interval timer was set so that hosecocks A and B opened and closed every 10 sec. Air pressure and vacuum were applied gradually to the apparatus by means of valves in increments per cycle of 2 psi and 3 inches (7.6 cm), respectively, until operating pressure and vacuum were reached. If no leaks appeared at the junction of the filter holders and filtration rates were equal in both directions, the washing medium was removed from the flasks and filtering unit.

Immediately after testing the apparatus, 10 ml of washed cells of strain PA 209 (absorbancy, 50 Klett units) were added to each culture flask. The experiment was begun as above by gradually increasing air pressure and vacuum until the operating levels were reached. The culture fluids of the separated bacterial suspensions were equilibrated for 1 hr at a filtration rate of approximately 7 ml per 10 sec. The filtration rate was determined by measuring the amount of fluid delivered in 10 sec into one unit when the other unit contained 14 ml of cell suspension.

After each experiment, Millipore filters in the filtering unit were tested as follows for microscopic leaks. At 10 min before the end of an experiment, 0.1 to 0.2 ml of a suspension (ca. 35 Klett units, equivalent to 0.07% of the total cells in unit I) of a prototrophic strain of *E. coli* (lactose-positive) was added to the culture flask in unit I. In contrast to the auxotrophic strain PA 209, the prototroph grew on acid casein hydrolysate and formed characteristic galactose-positive colonies on eosin-methylene blue-agar plates. At the end of the experiment, small portions of the cultures in both units were streaked onto three types of agar plates: nutrient broth, eosin-methylene blue, and acid casein hydrolysate. The agar plates were incubated for 24 hr at 37 C. If the filters were intact, no prototrophic cells entered unit II, no growth was apparent on casein hydrolysate, and no galactose-positive colonies appeared on the eosin-methylene blue plates. [Preliminary experiments of this type indicated the necessity of using type PH filters (pore size, 0.30μ); type HA filters (pore size 0.45μ) did not prevent the passage of some prototrophic *E. coli* cells from one unit to the other.] Several experiments were performed in which all the cells in unit I were prototrophs: no cells were transferred to unit II after 1 or 2 hr of incubation. In many experiments, additional evidence that cells were not transferred from unit I

to unit II was provided. By careful removal of the Pyseal coating with a razor blade, the two holders with their filters were separated without disturbing the cement bond between each holder and its filter. The exposed filter faces were then pressed onto nutrient agar plates. If the filters were intact, no bacteria were present on the outer surfaces of the filters (i.e., the surfaces not in direct contact with bacteria), and no growth occurred on nutrient agar plates.

Sampling. After 1 hr, the equilibration was stopped, the cell suspensions were removed from each unit, and their absorbancies were determined. Then, the cells from each unit were collected on separate Millipore filters for analysis of their radioactivity. Several procedures were adopted which assured better than a 90% recovery of cells in the apparatus. Treatment of the glass parts of the apparatus with Siliclad aided in the removal of fluid from the culture flasks. The cells remaining in the capillary stems of each unit were recovered by flushing with washing medium and were also collected on two separate Millipore filters. The only significant amount of cells still remaining in the apparatus (about 10% of the cells added) was found on the filters within the filtering unit. These filters were removed from their filter holders with the aid of a razor blade. In each experiment, three pairs of filters were analyzed for radioactivity: the two filters used to collect the cells in the initial samples from units I and II, the two filters used for collecting cells obtained by flushing units I and II, and the two filters from the filtering unit.

Analysis of bacterial protein. Cellular protein was obtained by extracting cell samples with hot 5% trichloroacetic acid. The total radioactivity of this fraction was measured, and in some cases the sample was hydrolyzed overnight in 6 N HCl at 107 C, subjected to paper chromatography, and analyzed for radioactivity in individual amino acid fractions.

Radioactive analysis of chromatograms. Standard solutions of threonine- C^{14} , and acid hydrolysates of labeled bacteria, were chromatographed in a phenol-borate buffer (93:7) solvent (Wade, Matheson, and Hanes, 1961). The R_F values of threonine, serine, glycine, and alanine were: 0.35, 0.18, 0.25, and 0.46, respectively. Chromatograms were developed overnight and dried, and radioactive spots were located by use of a Vanguard model 880 auto-scanner.

Radioactivity determination with a gas-flow counter. Cells collected by filtration were deposited within an area of 2.5 cm² in the center of Millipore filters. All radioactive solutions were placed within an area of 2.5 cm² in the center of stainless-steel planchets. Samples were counted in a thin end-window gas-flow counter (efficiency for C^{14} , 35%; background, 20 count/min) to a standard error of less than 5%.

The activity of samples collected on filters was multiplied by 1.18 to correct for reduced backscatter in these samples (Weatherford and Larson, 1959). A correction factor for self-absorption was

applied to samples containing cells: for example, the factor for the cells from 10 ml of a culture having a reading of 50 Klett units was 1.21. A self-absorption correction factor was applied to all samples of culture fluid: for example, the factor for 2 ml of culture fluid was 3.5.

Radioactivity determination with a scintillation counter. Samples were analyzed in a liquid scintillation counter by use of a water-miscible dioxane phosphor (Davidson and Feigelson, 1957). The sample (3 ml) and phosphor (15 ml) were mixed in a scintillation vial filled with Cab-O-Sil (Ott et al., 1959), a gelling agent that prevents salt-phosphor precipitates from settling. The counting efficiency was approximately 45%. Addition of Cab-O-Sil or culture medium did not decrease the efficiency.

RESULTS

Threonine- C^{14} uptake. Amino acid uptake is an obligatory step in intercellular turnover and is essential to the measurement of that process in an equilibration apparatus. If the amino acid uptake rate is very fast, then C^{14} -threonine released into the medium of unit I may be reincorporated by unit I cells before the medium can be transferred to unit II. The uptake of L-threonine was examined at added concentrations of 0.12 to 280 μ moles/ml. As shown in later experiments, the threonine concentration in media from turnover experiments was 6 to 8 μ moles/ml.

Two types of uptake experiments were performed: threonine- C^{14} uptake by a culture was studied under normal conditions in an Erlenmeyer flask, and the relative uptake of threonine- C^{14} by unlabeled unit I cells as compared with unlabeled unit II cells was studied in the equilibration apparatus.

Uptake under normal conditions. The initial rate of threonine- C^{14} uptake was a linear function of the exogenous threonine concentration over a range of 5 to 250 μ moles/ml of added threonine- C^{14} (Fig. 2). Approximately 8% of the amino acid in the medium was removed by the cells in the first minute of incubation. Such a rapid uptake rate would vitiate results obtained under conditions where equilibration of the two cultures was slow (no flushing of the culture fluid); however, the apparatus described here, which equilibrated the two culture fluids in 40 sec, theoretically was able to serve its purpose.

Uptake under equilibration conditions. The effectiveness of the apparatus also was tested directly. Initially unlabeled cultures were placed in both unit I and unit II, and trace amounts of labeled threonine were added to unit I while the two cultures were equilibrating. After 1 hr, the cells from both sides were harvested and counted. Figure 3 shows that unit I cells accumulated an

FIG. 2. Uptake of threonine- C^{14} by strain PA 209 under normal conditions. Cells were prepared as indicated for turnover experiments, but were incubated with shaking in Erlenmeyer flasks at 37 C. Threonine- C^{14} was added at various concentrations to different batches of cells. Samples were taken at zero-time and at 1-min intervals for at least the first 5 min. Some cultures also were sampled at 10, 15, 30, and 60 min. Cells were collected on Millipore filters, but were not washed, since adhering medium did not alter rate calculations. The radioactivity in collected cells was measured in a Geiger counter. For all threonine concentrations tested, a linear uptake rate was obtained for the first 5 min. These initial rates are plotted in the figure as a function of external threonine concentration. The percentage of added threonine- C^{14} taken up in the first minute of incubation was obtained from the slope of the uptake curve:

$$\frac{\text{rate of threonine-}C^{14} \text{ uptake into cells (counts per min per ml of culture/min)}}{\text{concentration of threonine-}C^{14} \text{ added (counts per min per ml of culture)}} \times \frac{1}{\text{min}} \times 100.$$

An average uptake in the first minute of 8% of the added threonine occurred within the concentration range tested.

average of 54% of the total isotope incorporated by both units. Such a small deviation from a theoretical 50/50 distribution was not of quantitative significance in these studies. The apparatus, then, transferred culture fluid between units I and II so rapidly that both units incorporated amino acid added to unit I to the same extent.

Turnover as measured in the equilibration apparatus. The above experiments demonstrated the feasibility of using the equilibration apparatus in studies of intercellular turnover. As detailed in Materials and Methods, intercellular turnover was determined by measuring the transfer of threonine- C^{14} from protein of prelabeled cells to protein of initially unlabeled cells. After placing

labeled cells in unit I and unlabeled cells in unit II, the culture fluids were equilibrated in the apparatus for 1 hr. The cells of each unit were harvested and analyzed for radioactivity as indicated previously.

Table 1 presents data from three equilibration experiments with labeled cells in unit I and an unlabeled "trap" culture in unit II. The amount of turnover per hour was calculated by dividing the activity of unit II by that of unit I and multiplying by a factor of 2 to allow for equal reincorporation of released threonine by both units (Eagle et al., 1959). The values for intercellular turnover ranged from 0.16 to 0.18% per hour, whether data from whole cells or extracted cells were used in the calculations. (Cells in both units apparently have the same minor percentage of counts in the hot water- and hot trichloroacetic acid-soluble fractions.) This intercellular turnover rate compares with a total turnover rate for non-growing *E. coli* of 3 to 4% per hour, as determined by Mandelstam (1958) and verified for strain PA 209 in this laboratory.

Control experiments. As shown in Table 2, only about 20% of all the isotopic material released by initially labeled cells in unit I was subsequently detected in unit II cells; the remaining 80% was found in the medium and on the filters in the

FIG. 3. Uptake of threonine by strain PA 209 under equilibration conditions. Unlabeled cells were prepared as for turnover experiments (see text) and added to both units of the equilibration apparatus. While the culture fluids were equilibrating, trace amounts of L-threonine- C^{14} were added to unit I. Cells were harvested after 1 hr and assayed for radioactivity as in turnover experiments. In the figure, the abscissa is the total amount of threonine incorporated into all cells (units I and II); the ordinate is the percentage of total counts detected in unit I cells. The dashed line represents the theoretical level of incorporation (50%) into unit I cells, if both units trapped added isotope with equal efficiency.

TABLE 1. *Transfer of threonine-C¹⁴ from prelabeled unit I cells to unlabeled unit II cells*

Expt	Unit I initially labeled (counts per min per ml) ^a	Unit II initially unlabeled (counts per min per ml) ^a	Transfer ^b (%/hr)	Intercellular turnover rate ^b (%/hr)
1. Whole cells	24,300	21.1	0.087	0.17
2. Cell residue after hot water extraction ^c	192,000	150 ^d	0.078	0.16
3. Cell residue after hot water and hot trichloroacetic acid extraction ^c	1,080,000	962 ^e	0.089	0.18

^a Refers to counts per minute present after 1 hr in the cells in 1 ml of a culture having a Klett reading of 50. Some cultures exhibited slight absorbancy changes (less than 10%); these were corrected for in calculating counts per minute per milliliter of culture. All results were normalized to counts per minute which would have been detected if samples were plated at infinite thinness and counted in a Geiger counter. The specific activities of the L-threonine-C¹⁴ used to prepare labeled cells for unit I were: experiment 1, 794,000 counts per min per μ mole; experiment 2, 6,260,000 counts per min per μ mole; and experiment 3, 52,400,000 counts per min per μ mole.

^b Per cent per hour transfer was calculated as follows:

$$\frac{\text{counts per minute/unit II cells in 1 ml of culture}}{\text{counts per minute/unit I cells in 1 ml of culture}} \times 100.$$

Intercellular turnover rate was obtained by multiplying the per cent per hour transfer by 2, to compensate for the equal reincorporation of isotope by units I and II.

^c Cells were extracted for 20 min with water (90 C). In experiment 3, this was followed by a 20-min extraction with 5% trichloroacetic acid (90 C). Samples of extracts were plated and counted in a Geiger counter. Trichloroacetic acid extracts were washed with ether before plating; none of the isotopic material was found in the ether phase. The counts per minute found in the extracts were subtracted from the counts per minute found in unextracted whole cells to give the values for extracted cells.

^d Represented 88% of the total radioactivity in unextracted whole cells.

^e Represented 77% of the total radioactivity in unextracted whole cells. After extraction with hot water, only 2% of the total radioactivity in unextracted whole cells was soluble in hot trichloroacetic acid.

center filtering unit. In the light of these data, a series of control experiments was performed to eliminate the following possible artifacts as causes of intercellular turnover: (i) labeled cells, lysed or damaged by equilibration, may have contributed large amounts of isotopic material to the medium which subsequently was incorporated by unit II cells; (ii) the isotopic material in the medium may have been derived from healthy labeled cells, but may have been adsorbed (rather than incorporated) by unit II cells; extraction with trichloroacetic acid may not have removed adsorbed material;

TABLE 2. *Radioactivity released by unit I cells and detected in the cells, medium, and center filter of unit II*

Expt	Radioactivity (counts per min per ml)*			Percentage of total released detected in unit II cells ($\frac{A}{A+B+C} \times 100$)
	Unit II cells (A)	Medium† (B)	Center filter‡ (C)	
2	150	499	141	19
3	962	2,170	1,270	22
4	354	739	686	20

* The cells, medium, and center filter were all from unit II; the sum of their isotopic content was taken as the total radioactivity released by 1 ml of unit I cells (normalized as in footnote a, Table 1). The counts per minute in unit II cells were normalized as in Table 1. The counts per minute in the medium were normalized to the equivalent of 1 ml of culture with a Klett reading of 50. The total radioactivity on a center filter was assumed to be derived from 10 ml of culture; the values in the table are given per milliliter of culture.

† When medium harvested at the end of turnover experiments was chromatographed in buffered phenol solvent, about 10% of the total radioactivity was localized in a peak having the R_F of threonine; the remainder was found in a sharp peak at the origin (14%), a peak of fluorescent material (18%; R_F , 0.19), and a large, diffuse peak at the solvent front (47%). The threonine peak was increased to 40% when hydrolyzed medium (6 N HCl, 18 hr, 107 C) was chromatographed.

‡ Most of the radioactivity in center filters did not represent adhering labeled cells, but rather material adsorbed from the medium, since it was present on a unit II center filter when labeled cells in unit I were equilibrated with only medium in unit II. The exact nature of this material is not clear, but the following characteristics indicate it is not free threonine-C¹⁴: it could not be eluted from the filter by washing with 10 ml of unlabeled L-threonine (1 mg/ml), 1 M NaCl, 5% trichloroacetic acid (0 or 90 C), nor displaced with serum albumin or redissolved trichloroacetic acid-precipitable bacterial protein.

or (iii) at the start of a turnover experiment, the medium in unit I may have contained enough labeled material to account for all the isotope incorporated by unit II cells in the following hour of equilibration.

Evidence that cells incubated under equilibration conditions did not suffer gross damage was provided by experiments in which absorbancy readings of experimental and control cultures were compared. In a control Erlenmeyer flask in which a culture in deficient medium was incubated with shaking for 1 hr, the Klett reading decreased from 50 to 48. The Klett readings of a culture equilibrated in the apparatus under the same conditions were identical with those of the control flask. More convincing evidence that cells were not damaged by the experimental procedure comes from measurements of the labeled intracellular pool before and after 1 hr of equilibration. If cell membranes were damaged, the intracellular pool size should decrease, since metabolites normally concentrated within the cell would leak out to the medium. In experiment 2, a hot water extract of 1 ml of unit I cells contained 3,710 counts/min before and 3,400 counts/min after 1 hr of equilibration; in experiment 4, the values were 10,000 counts/min before and 10,900 counts/min after equilibration. No significant change in the size of the labeled pool was apparent, and, thus, no membrane damage was indicated by this criterion.

Because it affects the initial size of the intracellular threonine- C^{14} pool, the method used in washing cells before equilibration (see *Preparation of cells*) should be mentioned here. Labeled cultures were not incubated, prior to experiments, in media containing unlabeled threonine to remove the intracellular pool of labeled threonine. This procedure was omitted to eliminate relabeling of the intracellular pool as a complicating factor in the transfer of labeled protein-bound threonine from unit I cells to unit II cells. At the start of the equilibration, the amount of labeled threonine in the intracellular pool of unit I cells should equal exactly the amount of unlabeled threonine in the intracellular pool of unit II cells. Whatever additional labeling of unit II cell protein is derived from the intracellular pool of labeled unit I cells should be counterbalanced by the initial lag in labeling caused by the pre-existing unlabeled intracellular pool in unit II cells.

Further evidence that little, if any, cell damage occurred as a result of equilibration was provided by measurements of total and trichloroacetic acid-soluble radioactivity in media from cultures equilibrated in the apparatus and from cultures incubated in Erlenmeyer flasks with shaking (control flasks). If appreciable cell damage occurred during equilibration, there should have been

more radioactivity in media from the apparatus than in media from control flasks. Furthermore, any severe physiological insult to the cells might have resulted in leakage of macromolecules (Koch, 1959; Pollock, 1961) precipitable by trichloroacetic acid. However, similar total amounts of labeled material were found in media from the apparatus and from control flasks (499 and 533 counts per min per ml in experiment 2, and 2,170 and 1,689 counts per min per ml in experiment 3), and in both cases most of the material was soluble in trichloroacetic acid (406 and 518 counts per min per ml in experiment 2, and 1,750 and 1,594 counts per min per ml in experiment 3). No attempt was made to evaluate the relative amounts of material found on center filters from the apparatus as compared with filters used to harvest media from labeled control cultures. Finally, when chromatographed, media from both sources exhibited similar distributions of radioactive and ninhydrin-positive material (see second footnote, Table 2).

The second series of control experiments (Table 3) was designed to show that the amount of isotopic material adsorbed (rather than incorporated) by unit II cells was negligible, and that the radioactivity in the medium at zero-time could not account for the level of incorporation by unit II cells after 1 hr of equilibration. In these control experiments (lines 2 and 4), labeled medium was harvested from the apparatus at the beginning or end of turnover experiments (lines 1 and 3) and added to unlabeled "acceptor" cells in an Erlenmeyer flask. The resultant cell suspension was incubated for 1 hr at either 37 or 0 C, and the radioactivity detected in "acceptor cells" (lines 2 and 4) was compared with that in unit II cells at the end of turnover experiments (B as % A).

That the level of adsorbed labeled material is low is shown by a comparison of the turnover experiment on line 1 and its control experiment on line 2. In these experiments, the culture fluid at the end of the turnover experiment contained only enough radioactive material to subsequently label "acceptor" cells 13% as much as unit II cells. This low level of adsorption was not due to a lack of adsorbable material in the medium, since adsorption could be demonstrated again with medium already treated with "acceptor" cells. Furthermore, "acceptor" cells incubated with media from control flasks exhibited a similar low level of adsorbed material.

That the level of "zero-time" incorporation is also low is shown by a comparison of the turnover experiment on line 1 and its control experiment on line 4. Clearly, the culture fluid from the turnover experiment at zero-time contained only enough radioactive material to label "acceptor"

TABLE 3. Levels of adsorption and zero-time incorporation in turnover measurements

Determination	Turnover incubation	Medium source for controls ^b	Control incubation	Radioactivity (counts per min per ml) ^a		B as % A
				(A) Unit II ^c	(B) "Acceptor" cells ^d	
I. Level of adsorption						
1. Turnover expt 3	Apparatus, 1 hr, 37 C	—	—	962	—	—
2. Control ^d	—	Medium from end of turnover expt 3	Erlenmeyer flask, 1 hr, 0 C	—	128	13.0
II. Level of zero-time incorporation						
3. Turnover expt 2	Apparatus, 1 hr, 37 C	—	—	150	—	—
4. Control ^d	—	Medium at zero-time of turnover expt 2	Erlenmeyer flask, 1 hr, 37 C	—	14	9.3

^a See first footnotes to Tables 1 and 2.

^b The medium harvested after 1 hr contained approximately 10% free threonine. The total radioactivity in the medium was: lines 1 and 2, 2,170 counts per min per ml; line 3, 499 counts per min per ml; and line 4, 117 counts per min per ml. Other characteristics of the medium are given in Table 2, second footnote.

^c Unit I cells contained 1,080,000 counts per min per ml for line 1 and 192,000 counts per min per ml for line 3.

^d For the adsorption control experiment on line 2, unlabeled "acceptor" cells were prepared as for turnover experiments, and were resuspended to a Klett reading of 50 in medium harvested from unit II (see Medium Source for Controls) at the end of the turnover experiment (line 1). The resulting control cultures were incubated in an Erlenmeyer flask with shaking at 0 C, since adsorption should not be temperature-dependent. After 1 hr, "acceptor" cells were collected on Millipore filters and counted as usual. The zero-time incorporation control experiment on line 4 was performed as follows. Immediately before the start of the turnover experiment (line 3), medium was harvested from a portion of a labeled culture prepared for unit I (see Medium Source for Controls) and was added to an equal volume of an unlabeled "acceptor" cell suspension (prepared as in turnover experiments but having a Klett reading of 100). The resulting control culture (Klett reading 50) was incubated in an Erlenmeyer flask with shaking at 37 C. After 1 hr, "acceptor" cells were collected on Millipore filters and counted as usual.

cells 9.3% as much as unit II cells were labeled after 1 hr in the apparatus.

One further observation is also of interest. If the control experiment on line 2 is performed at 37 C instead of 0 C, the level of incorporation is still only 31% of that which occurred in the turnover experiment on line 1. This indicates that continued equilibration with labeled unit I cells is required for maximal isotope incorporation by unit II cells.

All the above experiments support a scheme of intercellular turnover in which cells constantly release amino acids derived from proteins into the medium and continually reincorporate amino acids from the medium into proteins.

DISCUSSION

As determined with the equilibration apparatus, a definite intercellular turnover of proteins

occurs in nongrowing *E. coli* at a rate between 0.16 and 0.18% per hour. The reliability of this value depends upon: (i) the ability of "trap" cultures (unit II) to take up released threonine-¹⁴C from the medium as rapidly as initially labeled cultures (unit I); (ii) the absence of abnormal cell lysis or damage in the apparatus; (iii) the lack of nonspecific adsorption of extraneous labeled material by "trap" cultures (unit II).

By inference from the threonine uptake rate for strain PA 209 (Fig. 2) and by direct test in the apparatus (Fig. 3), one may conclude that threonine-¹⁴C released by unit I cells was "trapped" effectively by unit II cells. As judged by Klett readings, intracellular pool measurements, and culture fluid characteristics (see *Control experiments*), cultures equilibrated in the apparatus showed no evidence of cell damage over that which may occur in cultures incubated in control

Ehrlenmeyer flasks. Finally, the radioactivity "trapped" by unit II cells was not nonspecifically adsorbed material, but rather was incorporated threonine- C^{14} derived from unit I cells and taken up via the medium (Table 3).

In addition to the above considerations, the possibility that labeled threonine is being degraded before it can be incorporated by the "trap" culture should be noted. However, in an experiment using cells prelabeled with L-leucine- C^{14} approximately the same turnover rate (0.2% per hour) as that obtained with cells prelabeled with L-threonine- C^{14} was obtained. It seems unlikely that both threonine and leucine are degraded appreciably and to the same extent.

In nongrowing *E. coli*, then, intracellular turnover accounts for almost all of the observed total protein turnover of 3 to 4% per hour (Mandelstam, 1958) because intercellular turnover is only 0.16 to 0.18% per hour. Although the magnitude of intercellular turnover in *E. coli* now has been determined, the exact nature and function of this process still remain to be elucidated. The experiments reported here do not provide evidence for distinguishing between minimal cell lysis or damage, or some form of secretion, as the source of amino acids transferred between cells. A major unanswered question is whether intercellular turnover is merely the fortuitous result of leakage and uptake of amino acids produced during intracellular turnover, or whether intercellular and intracellular turnover are separate processes. Clearly, however, leakage of nutrients from cells may "condition" the medium for subsequent culture growth and account for population effects observed in bacterial cultures (Jannasch, 1962) in the same way as it does for population effects found in mammalian cell cultures (Eagle and Piez, 1962).

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