Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 44 · Number 5 · May 15, 1958

*PROTEIN TURNOVER IN MICRO-ORGANISMS**

BY ERNEST BOREK,† LAURA PONTICORVO, AND D. RITTENBERG

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK CITY

Communicated March 3, 1958

The existence of the dynamic state of tissue constituents of living organisms is one of the few general patterns of biological systems which have emerged during the past few decades. It would be surprising, therefore, if the dynamic state which had been observed in the tissues of many species of Metazoa should be absent in micro-organisms. Such a tentative conclusion was suggested from studies of the synthesis and disappearance of the inducible enzyme β-D-galactosidase by Hogness, Cohn, and Monod.[1]

These authors found, in an investigation on the synthesis of the inducible enzyme β-galactosidase in *Escherichia coli*, that its synthesis did not utilize amino acids resulting from the breakdown of cellular proteins. On the basis of these experiments they suggested that the dynamic state described by Schoenheimer, Ratner, and Rittenberg[2] in mammals does not exist but that these investigators were merely observing the total breakdown and dissolution of cells with the concomitant synthesis of completely new cells. The turnover rate determined by Schoenheimer, Ratner, and Rittenberg was ascribed to the rate of synthesis of new cells to replace those worn out. In essence, Hogness, Cohn, and Monod have revived the wear-and-tear theory. This work has been reviewed by Kamin and Handler[3] and by Tarver.[4]

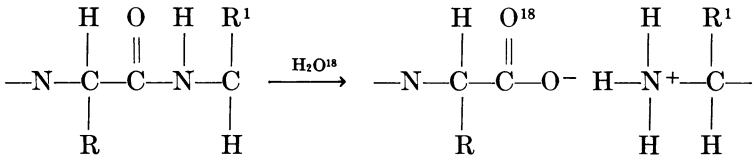
The theory of the dynamic state does not rest solely on the regeneration of proteins. Turnover has been observed with the fats, the carbohydrates, and the proteins. It has been observed in vivo and in vitro, and it applies to the amino acids, the fatty acids, and the monosaccharides, as well as to the most complex constituents of the cell. The rate is rapid in some tissues and compounds as, for example, rat liver and glutathione, and slow in others, such as human muscle.[5, 6]

It must be remembered that the theory of the dynamic state was developed to describe systems whose mass was either stationary or slowly varying. Slow growth, for example, could be explained by a decreased degradative reaction with an unaffected synthetic rate. Direct application of this theory to a bacterial system in logarithmic growth is not immediately justified. In the experiments of Hogness *et al.*, the bacterial mass was doubling each hour. It seems reasonable to deduce that there is a profound difference in the synthetic mechanisms, or their controls, between the non-growing *E. coli* or mammal and the cell in logarithmic growth. The results obtained on cells in logarithmic growth might not apply to cells in the non-growing state, let alone to the mammal. It seemed of interest to study this question

in the non-growing micro-organism preparatory to investigating it in cells in logarithmic growth.

Previous studies by other investigators have attacked this question by using labeled amino acids to label cell proteins and then determining by more or less indirect methods whether labeled amino acids are liberated from the labeled protein.[1, 7, 8] We have studied this question using a direct procedure.

We have determined the rate of protein turnover in *E. coli* K_{12} W · 6 during methionine starvation, using both O^{18} and deuterium-labeled water in the medium. The incorporation of an amino acid into a pre-existing protein requires the hydrolysis of at least one peptide link and its re-formation. The hydrolysis of a peptide link in an environment of H_2O^{18} will result in the introduction of O^{18} into the carboxyl group formed:



Because of resonance, both oxygen atoms in the carboxylate ion would be equally labeled and contain but half the isotope concentration of the medium. Re-formation of the peptide bond would remove one of the two equivalent oxygen atoms and yield a peptide bond containing half the O^{18} concentration of the water. Repetition of this process would result in the O^{18} concentration of the oxygen of the peptide bond eventually becoming equal to that of the medium.

There is no known mechanism by which deuterium from the water of the medium can be introduced into stable positions in the protein by an enzyme-catalyzed reaction. However, free amino acids, under the action of transaminase, rapidly incorporate deuterium in the α-position.[9, 10] The reincorporation of the amino acid into the protein will result in the appearance of deuterium in the protein. If the rate of the incorporation of deuterium into the α-position of the amino acid is very much more rapid than the rate of hydrolysis and re-formation of the peptide bond, then the appearance of deuterium in the protein will be a measure of the turnover of the protein. The rate at which deuterium will appear in the protein will thus offer a minimum value for the rate of protein turnover.

In these experiments no substance was used which would impose a novel condition on the micro-organisms which had been previously adapted to a specific, well-defined milieu. Nor were there diffusion barriers or problems connected with active transport to complicate the interpretation.

In order to simplify the conditions, it was desirable that there be no protein synthesis; of course, the organisms must remain viable during the course of the study. The auxotroph *E. coli* K_{12} W · 6 is known to fulfil these requirements. It is an extraordinarily stable auxotroph. Twelve years after its isolation[11] it shows no detectable tendency to back-mutation to prototrophy. Not only is the organism unable to increase the amount of its protein during the period of starvation of its essential nutrient, methionine, but even after the restoration of the amino acid there is a lag of about 90 minutes before protein synthesis is resumed.[12] The viability of the organism during several hours of starvation has been established.[12]

Experimental.—*E. coli* K_{12} W · 6 were grown on a chemically defined medium[13] and were harvested during the logarithmic growth phase. They were then incubated for 100 minutes in a similar medium lacking methionine to exhaust the amino acid pool of this amino acid and to establish an internal condition in which no net protein synthesis is possible. The organisms were then transferred to a fresh methionine-free medium containing a known concentration of D_2O^{18}—13 per cent D; 1.4 per cent O^{18}—and incubated aerobically. At hourly intervals bacteria were isolated by centrifugation at 5° C. and were immediately extracted with 5 per cent trichloroacetic acid. For the isolation of the protein fraction the procedure described by Roberts *et al.*[14] was followed, with the exception that the extraction with hot trichloroacetic acid was repeated. This precaution was essential to insure the removal of the final traces of nucleic acids. When $P^{32}O_4^{\equiv}$ was incorporated into the culture medium of the organisms, some residual radioactivity was still measurable after a single extraction with hot trichloroacetic acid. However, after the second extraction, this became negligible, indicating less than 0.2 per cent residual nucleic acids. The protein was dried at 100° *in vacuo* for 2 hours. Four to 6 mg. of the dried product were analyzed for its O^{18} content by the method of Rittenberg and Ponticorvo.[15] The proteins contained no detectable O^{18} in experiments in which normal cells were transferred to 1.4 atom per cent excess O^{18} water and were immediately killed and analyzed as above. This demonstrates that O^{18} found in proteins was not the result of adsorbed water or water of hydration.

Data are given in Table 1 to demonstrate that the cells were still viable after 2 hours. The presence of dinitrophenol had no permanent toxic effect. To demonstrate that these cells, under our conditions, are incapable of *de novo* protein synthesis, an aliquot of the bacteria without dinitrophenol was incubated for 2 hours in a medium containing lactose but no glucose or methionine. The concentration of β-D-galactosidase in these cells was assayed by determining the amount of *o*-nitrophenol produced per hour per 10^8 cells from *o*-nitrophenyl-β-D-galactoside.[16] The value, 0.3 μg., was not larger than values found in these organisms not exposed to the inducer.

TABLE 1

THE VIABILITY OF *E. coli* K_{12} W · 6 DURING STARVATION OF METHIONINE

Adjuvant	0	2 Hr.
...	9.0×10^4	8.6×10^4
Dinitrophenol 10^{-3} *M*	8.6×10^4	8.6×10^4

Discussion.—While it is reasonable to assume from general theoretical considerations that the incorporation in stable positions of both O^{18} and D into the proteins is the result of an enzymatic process in an intact cell, we are able to offer direct experimental evidence for this. In the presence of 10^{-3} *M* dinitrophenol, cells incubated in D_2O^{18} incorporate only traces of isotope (see Fig. 1). This concentration is known to uncouple oxidative phosphorylation. The inhibition is not unexpected, since the mechanism for the isotope incorporation involves the formation of peptide bonds, a process known to require chemical energy.

In the absence of dinitrophenol, both O^{18} and D are incorporated into proteins. To calculate the rate of turnover of the proteins, it is necessary to know the rate of change of the O^{18} concentration of the protein and the maximum value which could

be attained. Repeated opening and closing of the peptide bond must eventually equalize its O^{18} concentration with that of the water. However, not all of the protein oxygen is present in the peptide bond; some oxygen is found as hydroxyl groups or as the free carboxyl groups of aspartic and glutamic acid. The repeated opening and reclosing of the peptide bonds will have no effect upon the isotope concentration of the non-peptide bond oxygen. The exact composition of the mixture of bacterial proteins is not known. However, for insulin and β-corticotrophin—two proteins whose exact structure is known—the peptide oxygen accounts for 64 and 67 per cent of the total oxygen, respectively. For the purposes of this paper, we shall assume that two-thirds of the total oxygen of the bacterial proteins which we analyzed is peptide oxygen. Similarly, we estimate, using insulin and β-corticotrophin as models, that in the average protein the α-hydrogen accounts for 12.5 per cent of all the hydrogen atoms.

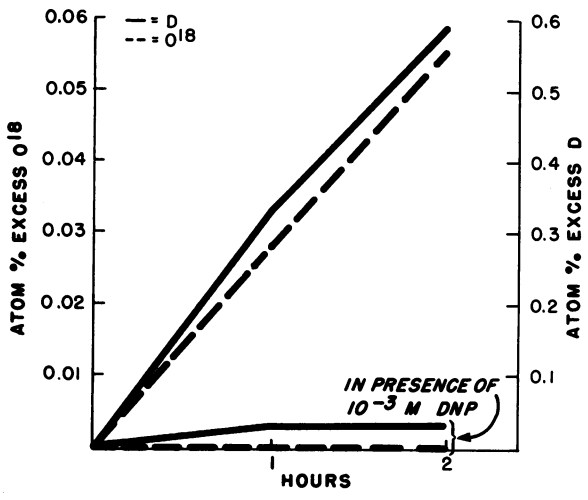


FIG. 1

The data of Figure 1 show that the uptake and therefore the turnover rate are linear during the 2 hours of methionine starvation. Using the estimate that two-thirds of the total protein oxygen is peptide-bond oxygen, the maximum O^{18} concentration that the protein of the non-growing cell could reach would be $2/3 \times 1.4 = 0.93$ atom per cent excess. The rate of increase of O^{18} in the protein corresponds to a turnover of 3 per cent per hour (Fig. 1). The opening of a peptide bond leads to the introduction of one oxygen atom from the medium, but the formation of a peptide bond can take place with either one of the two identical atoms of the carboxylate ion. The result of the opening and reclosing of a peptide bond will be, on the average, that half of the peptide bonds will contain an oxygen atom derived from the medium. Because only half of an O^{18} atom is introduced in the peptide bond as a result of the opening and reclosing of a peptide link, our estimate of the rate of the reaction should be doubled. The turnover will thus amount to 6 per cent per hour. If, however, the carboxyl group of the amino acid while free can undergo exchange under the action of the proteolytic enzyme,[17] then the turnover rate will be less. At minimum, the rate is 3 per cent per hour; at maximum, 6 per cent per hour. Even accepting the lower value, 3 per cent per hour, the rate of protein turnover is considerable.

The rate of incorporation of deuterium into the protein is about 0.33 atom per cent excess per hour. It is difficult from our present data to fix the maximum isotope concentration to be expected in the protein while in the free state. If deuterium is present only in the α-position of the amino acid residues, then the maximum value could be about one-eighth that of the medium or $13/8 = 1.62$ atom per cent

excess. This corresponds to a turnover of $0.3/1.62 \times 100$ or 20 per cent per hour. If deuterium is present in other positions of the amino acid residue as well as the α-position, then the calculated turnover rate will be lower. A minimum value can be obtained from the following data. These bacteria, when grown up from a small inoculum in a medium containing glucose as the sole source of carbon and 13 per cent D_2O, contain 5.6 atom per cent D in their protein. Using this value in the above calculations rather than 1.62 atom per cent excess as the maximum, we calculate a rate of turnover of $0.33/5.6 \times 100 = 5.9$ per cent per hour.

These data demonstrate that the proteins of the non-growing bacteria are continuously being degraded and resynthesized. The rate is faster than that found in any mammalian tissue. In the logarithmic phase of growth of a bacterial system, this turnover could be masked by the enormous rapidity of protein synthesis. The bulk of the protein in such a system is too young to permit easy measurement of the cumulative results of turnover.

Protein turnover in micro-organisms was recently demonstrated by Mandelstam.[8] Measuring the rate of incorporation of C^{14}-labeled glycine into a leucineless mutant of *E. coli*, he found the turnover rate to be $4^1/_2$ per cent per hour when the bacterial culture was not growing because of the absence of leucine. Somewhat similar results were obtained in bacteria in their growing phase.

Were the premise of Hogness *et al.* valid, that the bulk of the proteins of *E. coli* as well as β-galactosidase is in a static state, then an extrapolation to the mammalian cell might be significant. However, the results reported here, together with those of Mandelstam,[8] clearly demonstrate that, at least in the non-growing *E. coli*, the peptide bonds are being opened and reclosed. The question as to whether or not the dynamic state exists in *E. coli* in exponential growth is not directly touched by our experiments. However, it seems unnecessary, in the absence of direct experimental evidence, to abolish those mechanisms in the slowly growing cell. It is obvious that during exponential growth the rate of protein synthesis is much larger than the rate of degradation. In *E. coli* the synthetic rate seems twenty times larger than the degradative one. Evidently great care might be needed to observe this relatively slow reaction. In the absence of direct evidence to the contrary, the existence of the dynamic state should not be dismissed.

* This work was supported in part by grants from the National Institutes of Health (E-1181-C and E-1461-C), the Atomic Energy Commission (AT (30-1) 1803), and by a contract between the Office of Naval Research (ONR 26602) and Columbia University. Reproduction of this article in whole or in part is permitted for any purpose of the United States government. The H_2O^{18} was obtained on allocation from the United States Atomic Energy Commission.

† Fellow of the John Simon Guggenheim Memorial Foundation, 1957–58.

[1] D. S. Hogness, M. Cohn, and J. Monod, *Biochim. et Biophys. Acta*, **16**, 99, 1955.

[2] R. Schoenheimer, S. Ratner, and D. Rittenberg, *J. Biol. Chem.*, **130**, 703, 1939; R. Schoenheimer, and D. Rittenberg, *Physiol. Rev.*, **20**, 218, 1940.

[3] H. Kamin and P. Handler, *Ann. Rev. Biochem.*, **26**, 471, 1957.

[4] H. Tarver, in *The Proteins*, II B, ed. H. Neurath and K. Bailey (New York: Academic Press, Inc., 1954), p. 1199.

[5] D. Shemin and D. Rittenberg, *J. Biol. Chem.*, **153**, 401, 1944.

[6] A. San Pietro and D. Rittenberg, *J. Biol. Chem.*, **201**, 457, 1953.

[7] A. L. Koch and H. R. Levy, *J. Biol. Chem.*, **217**, 947, 1955.

[8] J. Mandelstam, *Nature*, **179**, 1179, 1957.

[9] M. A. Hilton, F. W. Barnes, Jr., and T. Enns, *J. Biol. Chem.*, **219**, 833, 1956.

[10] Unpublished results of this laboratory.

[11] E. L. Tatum, these PROCEEDINGS, **31**, 215, 1945.
[12] E. Borek, J. Rockenbach, and A. Ryan, *J. Bacteriol.*, **71**, 318, 1956.
[13] C. H. Gray and E. L. Tatum, these PROCEEDINGS, **30**, 404, 1944.
[14] R. B. Roberts, D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten, *Studies of Biosynthesis in Escherichia coli* ("Publications of the Carnegie Institution of Washington," No. 607 [Washington, D.C., 1955]), p. 13.
[15] D. Rittenberg and L. Ponticorvo, *J. Appl. Radiation and Isotopes*, **1**, 208, 1956.
[16] M. Cohn and J. Monod, *Biochim. et Biophys. Acta*, **7**, 153, 1951.
[17] D. B. Sprinson and D. Rittenberg, *Nature*, **167**, 484, 1951.

---

*THE TRANSFER OF IRRADIATION-ELICITED INDUCTION IN A LYSOGENIC ORGANISM**

BY ERNEST BOREK† AND ANN RYAN

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK

Communicated by D. Rittenberg, March 7, 1958

From studies on the mechanism of induction of lysogenic organisms by ultraviolet irradiation, we arrived at the tentative conclusion that the effect of the irradiation is not direct but rather that it is mediated by some radiation-sensitive metabolite.[1] Recently Northrop has offered a similar hypothesis.[2]

To explore the validity of our hypothesis, we have made numerous attempts to transfer the effects of irradiation from an irradiated lysogenic cell to a non-irradiated one. We have irradiated in the cold inducible lysogenic organisms, disintegrated them by a variety of methods, incubated intact lysogenic cells in the debris, and assayed for induction by the standard techniques. All such attempts at the transferring of induction have proved unsuccessful so far.

If such an irradiation-mediating metabolite does exist, two attributes of it might defeat any attempt at transfer: it might be too unstable; it might not penetrate the barrier of the bacterial cell wall.

It occurred to us that the optimum condition for carrying out such a transfer might be during the conjugation of an irradiated cell with an unirradiated one, provided, of course, that the irradiation does not inhibit conjugation. We report here the transfer of induction from an irradiated lysogenic donor cell to an unirradiated acceptor cell during mating.

The principle of these studies is as follows: A donor organism (with an F^+ sexual orientation), *E. coli* $K_{12} \cdot W \cdot 6 \cdot F^+ \cdot M^- \cdot L_p^+$ is deprived of its essential nutrient, methionine, and is irradiated. Without its essential amino acid, the organism is known to be incapable of producing lambda phage[3] (see Table, col. 5, Expts. 1, 2, 3). The irradiated, methionine-deprived F^+ organism is then admixed with a receptor organism (with an F^- sexual orientation) *E. coli* K_{12} $W_{1177} \cdot F^- \cdot Thr^- \cdot Leu^- \cdot B_1^- \cdot L_p^+$ in a medium adequate for the latter but not for the former, and the formation of free phage is assayed.

E. coli K_{12} $W \cdot 6 \cdot F^+$ was centrifuged during the logarithmic phase of growth and was washed with medium lacking methionine.[4] It was suspended to a concentration of 10^8 cells/ml in the medium lacking methionine and was irradiated for 180