*ANOMALOUS GROWTH OF MICROORGANISMS PRODUCED BY CHANGES IN ISOTOPES IN THEIR ENVIRONMENT**

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A variety of effects on growth have been observed in the presence of excess deuterium in living cells. The effects reported include: extremely slow, sporadic, and unpredictable growth in chlorella,[1] morphological abnormalities in algae[2] and in bacilli,[3] and complete inhibition of germination in the tobacco seedling.[4]

These effects have been invariably attributed to the toxicity of deuterium.

We offer here evidence that the rare isotopes of hydrogen and of oxygen may be no more toxic than the more abundant isotopes. The anomalous growth effects are caused not by the rare isotope *per se* but rather by the unusual mixtures of isotopes within the organism when they are transferred from the environment of one isotope to another.

The conclusions of other investigators were based on studies of events occurring when organisms grown in H_2O were transferred to D_2O. We have adventitiously observed growth phenomena during the reverse situation: the fate of microorganisms grown in 92% H_2O^{18} or 100% D_2O when they are transferred to H_2O^{16}.

We found that *Escherichia coli* can be grown either in 100% D_2O or in 92% H_2O^{18}. The organisms in logarithmic growth phase in either medium do not differ in shape, size, or morphological features from organisms grown in H_2O. However, if the labeled organisms are transferred into media containing normal H_2O, they undergo similar erratic growth phenomena as do organisms which are subjected to the reverse change in their milieu: from the more abundant to the less abundant isotope.

The experiments were carried out as follows. *E. coli* B and *E. coli* K_{12} (ATCC No. 10798) were grown in H_2O in synthetic medium.[5] They were centrifuged when in logarithmic growth phase and were suspended in media containing either 100% D_2O or in water containing 92% O^{18} and 8% deuterium.† The carbon source was ordinary glucose. Enough organisms were added in each case to yield a concentration of 10^7 viable cells per ml and the cultures were incubated aerobically at 37°.

Experiments with O^{18}.—The organisms grown in normal water when suspended in H_2O^{18} showed a growth lag of about 2 to 3 hr and then grew at a somewhat slower rate than they do in H_2O^{16}. The generation time of *E. coli* B under our conditions of culture was found to be 1 hr in H_2O^{16} and 1.5 hr in 92% H_2O^{18}.

The organisms which were fully labeled with O^{18} when viewed under a microscope could not be distinguished from a culture grown in H_2O^{16} in the same growth phase.

The heavily labeled organisms were harvested by centrifugation and were inoculated to a population of 10^8 cells per ml into a synthetic medium prepared with normal H_2O.

The culture was incubated and the organisms were observed under a phase

contrast microscope at intervals. Growth was measured turbidimetrically and by plating for viable cell counts on both nutrient and minimal agar.

The two strains of organisms *E. coli* B and *E. coli* K_{12} (ATCC No. 10798) exhibited different morphological changes when transferred from H_2O^{18} to H_2O^{16}. The cells of *E. coli* B became slightly thicker and appeared more densely granulated. However, *E. coli* K_{12} labeled with O^{18} exhibited a whole spectrum of morphological changes during 4 hr of incubation in H_2O^{16}. At the start they appeared as a normal culture of these organisms. Within an hour the cells thickened and lengthened to twice the dimensions of the normal cell. Numerous biseptate cells appeared.

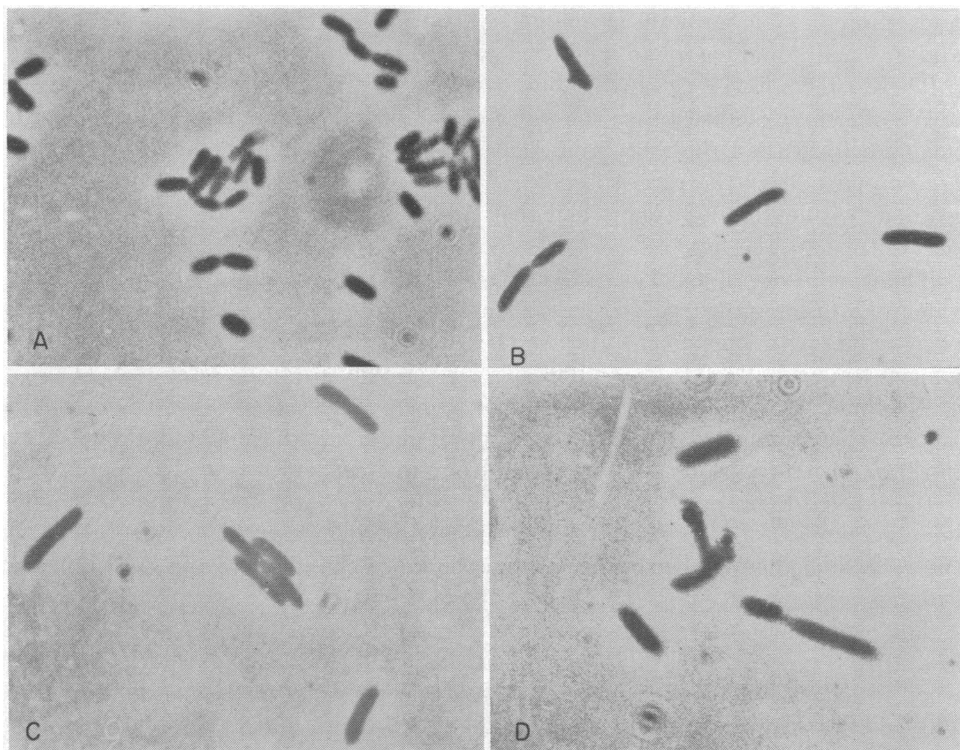

FIG. 1.—Morphological changes in *E. coli* K_{12} after transfer from H_2O^{18} to H_2O^{16}. (*A*) The cells fully labeled with O^{18}. (*B*) After 1 hr in H_2O^{16} medium. (*C*) After 2 hr in H_2O^{16} medium. (*D*) After 3 hr in H_2O^{16} medium. Magnification by the microscope was $\times 1280$. Enlargements through photography are the same.

After 2 hr of incubation the cells were monsters in size. Multiseptate and chain forms were frequent. Within 3 hr the culture was a mixture of normal cells and monsters. After 4 hr the culture reverted to its normal appearance. Typical cells from this sequence are shown in Figure 1.

The tally of the viable cells on nutrient and minimal agar revealed another anomalous condition of the O^{18} labeled cells during the first 3 hr of adaptation to the new environment of H_2O^{16}. The number of viable cells of *E. coli* B on minimal agar was 40 to 50% greater than the number of enriched agar (Fig. 2). (It should be emphasized that the random deviation in plating for viable cells is 5 per cent or

less in our hands.) It appears that the transfer of cells grown in 92% H_2O^{18} to H_2O^{16} interferes with some process involved in cell duplication or division. In the synthetic liquid medium and on the minimal agar the slowly dividing cells are able to survive, yielding eventually, after 3 hr of adaptation, a normal population. However, many of the labeled cells are unable to cope with the accelerated rate of growth when transferred too early to the nutrient agar in which their division rate is trebled over that in minimal medium.

Obviously, the effects of some unbalanced growth would be minimized on minimal agar. For example, cells which are made fragile by interference with their cell-

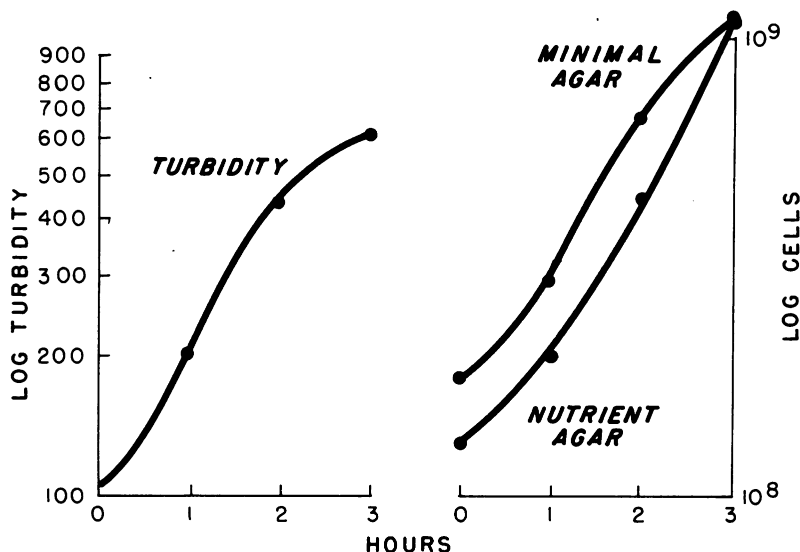

Fig. 2.—Growth of *E. coli* B in H_2O^{16} after harvesting from an H_2O^{18} medium. The curve on the left represents turbidity expressed in "nephelos" units. On the right, the upper curve represents the viable cells tallied on minimal agar, the lower curve on nutrient agar. The data for these two curves were obtained by plating identical aliquots from the same dilution tube on the different agar plates.

wall synthesizing capacity by 5-Fluorouracil can be viable on minimal but not on nutrient agar.[6] The elucidation of the question whether the transfer from an environment of O^{18} to O^{16} water involves unbalanced growth or some other disturbances with cellular synchrony awaits further study.

The differential count on nutrient agar and on minimal agar showed even greater discrepancies during the period of adaptation of O^{18} labeled *E. coli* K_{12} cells to H_2O^{16}.‡

Experiments with D_2O.—When bacteria grown in ordinary water were transferred into 100% D_2O to a concentration of 10^7 cells/ml, it was found that there was a lag of 24 to 30 hours during which no growth was measurable turbidimetrically. Once the bacteria started growth, their generation time proved to be about 6 hr for both strains of *E. coli*. These organisms in H_2O under the same conditions have a generation time of 1 hr. The organisms grown in 100% D_2O when viewed under a microscope could not be distinguished from a normal culture grown in H_2O in logarithmic growth phase.

The course of growth of the deuterium-enriched *E. coli* K_{12} when they were transferred to synthetic medium in ordinary water to a concentration of 10^8 cells/ml is represented in Figure 3. These cells gave no evidence of failure to give rise to viable clones when they were transferred to enriched media. The cell counts on minimal and on nutrient agar were identical. There was a lag of 2 hr during which there was a 30 per cent increase in turbidity but no change in viable cell count. During the third hr there was nearly a doubling of turbidity but only a 30 per cent increase in viable cell count. Examination of the culture under a phase contrast microscope revealed the reason for the discrepancy. There were many dead cells and multiseptate forms were frequent. After 4 hr the culture appeared normal.

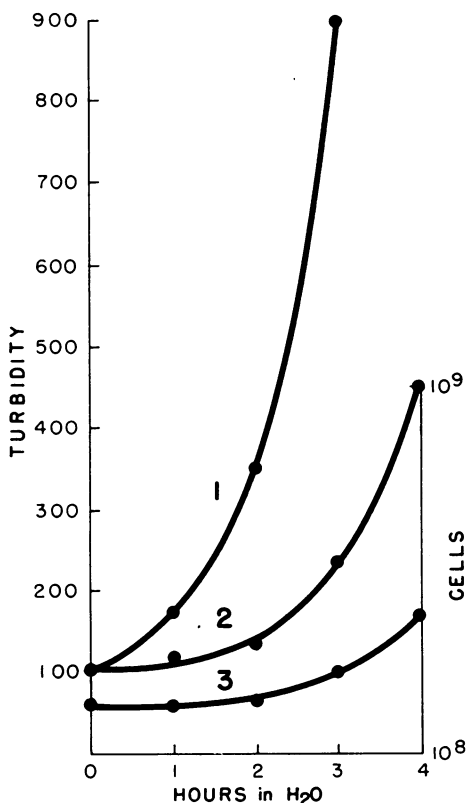

FIG. 3.—Growth of *E. coli* K_{12} labeled with D_2O when transferred into H_2O. Curve 2 represents the turbidity, curve 3 the cell count on both minimal and on nutrient agar. Curve 1 represents the growth of this organism, measured by turbidity, when the organism grown in H_2O is transferred into fresh H_2O medium.

Apparently it takes 4 hr for these deuterium-enriched organisms to adapt to the novel environment of H_2O. It is to be noted that mere transfer from one medium to an identical isotopic medium does not result in a lag or inhibition of growth rate. (See curve 1, Fig. 3.)

In Figure 4 the course of growth of deuterium-enriched *E. coli* B when they were transferred to synthetic medium in ordinary water to a concentration of 10^8 cells per ml is presented.

With this organism there appeared to be no tendency toward the formation of multiseptate forms but there was a lag of 4 hr during which the cell count remained stationary. (A culture of these organisms when transferred from H_2O to H_2O nearly doubles within the first hr. See curve, 1, Fig. 4.)

It is apparent from these data that the phenomena observed when organisms are transferred from H_2O to D_2O should not be ascribed merely to the toxicity of the rare isotope, for they occur under the reversed conditions as well: H_2O is apparently "toxic" to organisms grown in D_2O or in H_2O^{18}.

Discussion.—The anomalous growth pattern in altered isotopic environments is not a generalized phenomenon. That *E. coli* B can be shifted from high concentrations of N^{15} to N^{14} without change in their pattern of growth is obvious from the experiments of Meselson and Stahl.[7] We also found that both *E. coli* B and *E. coli* K_{12} fully labeled with either of the isotopes of nitrogen can be

transferred to the environment of the other isotope without any discernible effect.

The anomalous growth effects with the elements of H_2O are probably merely the visible summation of disturbances in the synchrony of reactions within the cell. A variety of mechanisms at the molecular level could account for the phenomena observed. The organic constituents of the *E. coli* grown in a medium of 92%-H_2O^{18} and normal glucose and phosphate will be heavily labeled with O^{18}. For example, the proteins will contain about 60% O^{18}.§ O^{18} atoms will be found in the oxygen of the peptide bonds and in the oxygen atoms of the purines and pyrimidines. Substitution of the O^{18} for O^{16} will affect the strength of the hydrogen bond between C=O and N-H groups in the proteins and the nucleic acids and could affect the geometry of these molecules. Transfer of these labeled cells to a medium containing normal H_2O will not lead to the loss of the O^{18} atoms in the above compounds.

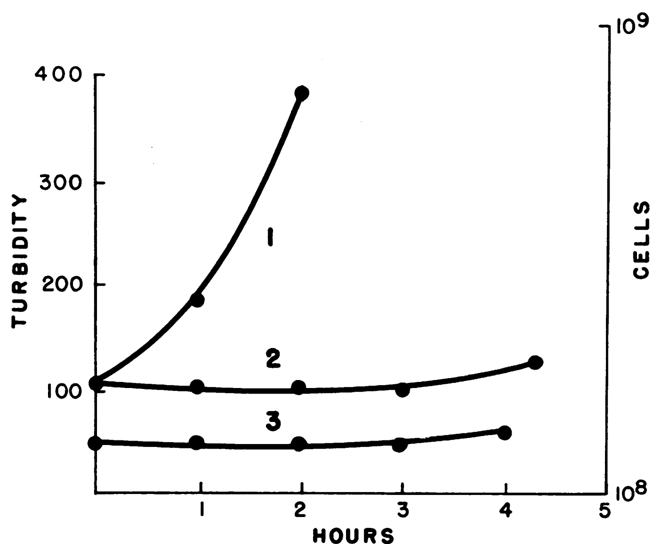

FIG. 4.—Growth of *E. coli* B labeled with D_2O when transferred into H_2O. Curve 2 represents the turbidity, curve 3 the cell count on both minimal and on nutrient agar. Curve 1 represents the growth of this organism measured by turbidity when the organism grown in H_2O is transferred into fresh H_2O medium.

Any changes in the three-dimensional configuration of the structural elements of the cell which arose from the substitution of O^{18} for O^{16} will be retained on transfer of the cells from the H_2O^{18} medium to the normal water.

Similarly, the growth of *E. coli* in D_2O leads to the incorporation of D into the newly synthesized constituents of the multiplying cells.

On transfer of these cells to a medium containing normal water the deuterium atoms bound to O, N, and S atoms will exchange. However, this exchange is not instantaneous for all the deuterium atoms of a protein. Linderstrøm-Lang has shown that some of these atoms take hours to exchange and has suggested that these slowly exchangeable atoms are involved in hydrogen bonding of proteins.[8] The strength of the hydrogen bond when either O^{18} replaces O^{16} or D replaces H should change because of the difference in the zero point energy resulting from the isotopic

substitution. Calvin *et al.*[9] have shown that replacement of the hydrogen atom of the peptide bond in poly-γ-benzyl-L-glutamate changes the strength of the hydrogen bond in the α helix of this molecule by about 100 calories per bond. Since the three-dimensional structure of most of the structural elements of the cell—the proteins, the nucleic acids, and water itself—is dependent on the strength of the hydrogen bond, isotopic substitution must affect the geometry of these components. Interference with the self-duplicating mechanisms of the cells could arise from even small energy changes of inter- and intramolecular forces.

Of course, the organic constituents of the cell will contain D atoms in stable linkage, but these have little effect on van der Waals forces or hydrogen bonding.

In addition to structural effects the admixture of cell components of different isotopic species may produce dynamic effects as well. This may be expected when cell components heavily labeled with one isotope interact with newly formed components from another species of isotopes.

The study of reactions between enzymes and substrates synthesized from dissimilar isotopic species of hydrogen and oxygen should, therefore, be of considerable interest.

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† The O^{18} water as obtained from the Weizmann Institute of Science, Rehovoth, Israel, contained over 95 atom per cent deuterium. The deuterium was removed from the water by exchange in the gas phase against normal hydrogen using a platinum catalyst. Since biological effects of deuterium have not been observed in water containing less than 20 per cent deuterium, no attempt was made to reduce the deuterium concentration below 8 per cent. In addition to the O^{18} the water contains 1.7% O^{17}.

‡ This particular strain of *E. coli* K_{12} is unusually sensitive during the early phase of its growth to changes in its nutrient environment even when its isotopic milieu is not altered. But the effects of such changes are negligible compared to those produced by the shift from H_2O^{18} to H_2O^{16}. These observations are being extended and will be presented elsewhere.

§ Unpublished data of the authors.

[1] Moses, V., O. Holm-Hansen, and M. Calvin, *Biochim. Biophys. Acta*, **28**, 62 (1958).

[2] Crespi, H. L., S. M. Archer, and J. J. Katz, *Nature*, **184**, 730 (1959).

[3] Chance, H. L. and W. C. Allen, *J. Bact.*, **51**, 547 (1946.)

[4] Lewis. G. N., *J. Am. Chem. Soc.*, **55**, 3503 (1933).

[5] Gray, C. H., and E. L. Tatum, these PROCEEDINGS, **30**, 404 (1944).

[6] Tomasz, A., and E. Borek, these PROCEEDINGS, **46**, 324 (1960).

[7] Meselson, M., and F. W. Stahl, these PROCEEDINGS, **44**, 671 (1958).

[8] Linderstrøm-Lang, K. U., in *Symposium on Potein Structure*, Int. Union of Pure and Applied Chemistry, (London, Methuen, 1958), p. 23.

[9] Calvin, M., J. Hermans, Jr., and H. A. Scheraga, *J.A.C.S.*, **81**, 5048 (1959).