RADIOACTIVE STUDIES OF THE PHOSPHORUS METABOLISM OF ESCHERICHIA COLI

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This investigation of the uptake of radioactive phosphorus, P^{32} , by the B strain of *Escherichia coli* was undertaken as a necessary preliminary to work being conducted on the phosphorus metabolism of bacteriophages having *E. coli* as host. It has previously been shown that *E. coli* may be made radioactive by culturing the bacteria in a medium containing P^{32} as inorganic $Na_2HP^{32}O_4$ (Williams, Sandholzer, and Van Voorhis, 1940; Ely, 1942; Cohen, 1948; Morse and Carter, 1949), but quantitative studies of radioactive uptake have not been published.

Standard chemical methods have established the amount and distribution of phosphorus in bacteria of the B strain of *E. coli*. Thus, analyses (Taylor, 1946) have shown that ca. 2.72 per cent of the dry weight of organisms from an 8-hour broth culture is phosphorus; of this, 66 per cent is in the form of ribose and 19 per cent of desoxyribose nucleic acid. Of the remaining 15 per cent, 12 per cent occurs in phospholipids and 3 per cent in unidentified compounds.

Other, less orthodox, methods are needed when one seeks to determine how the synthesis of the nucleic acids of bacteria is related to their over-all growth and multiplication. Most of this information to date has been gained from measurements of the light absorption of single bacterial cells using the ultra-violet microscope (Malmgren and Hyden, 1947; Malmgren and Hedén, 1947). The fact that 85 per cent of the bacterial phosphorus is found in the nucleic acids, however, makes it possible to employ radioactive phosphorus to follow the synthesis of these acids during bacterial growth. Results thus obtained differ from the ultraviolet data in being averages over all bacteria in a culture and in giving no indication of the distribution of nucleic acids within the bacterial cells. The radioactive tracer method can, however, be applied to microorganisms too small for accurate measurements of cellular absorption.

Many novel experiments bearing on phosphorus uptake could be made using radioactive material, but the present studies have been limited to correlations of this uptake with the numbers of viable bacteria at points on the growth curve ranging from the lag phase through the later stationary phases of bacterial growth.

METHODS

The *E. coli* cultures for these experiments have been taken from the B strain employed in this laboratory for other analytical studies (Polson, 1948) and for work with bacteriophage (Polson and Wyckoff, 1948). During the period of these

experiments the organisms were transferred daily in broth, and fresh cultures were started at weekly intervals from carefully selected single colonies developing from dilute platings on agar.

All cultures were made at 37 C employing tryptose phosphate broth or agar buffered at a pH of 7.2 to 7.3, unless it is specified otherwise. The number of viable organisms has been established by the usual dilution plate counts, care being taken to count enough colonies (ca. 300) in each case to obtain reasonable accuracy. For all experiments the radiophosphorus, P^{32} , was introduced as Na_2HPO_4 at concentrations sufficient to give the conveniently measurable count of 10^5 disintegrations per minute per milliliter of final culture medium. In order that all results should be directly comparable, data have been normalized to this radioactive concentration whenever it was not exactly realized in setting up an experiment. The radioactive phosphorus content of the bacteria in a culture has been measured by the following procedure: Samples of 8-ml volume have been removed at specified time intervals. These were fixed immediately upon removal by the addition of enough strong formalin to make them 2 per cent with respect to formaldehyde. At the end of an experiment bacteria in the samples have been sedimented by centrifugation from the radioactive broth and washed twice in 0.85 per cent saline to remove traces of active medium. Radioactivity in the resulting pellets, dried under a heat lamp, has been measured by the usual counter methods employing an end-window Geiger counter.

These washing and fixing procedures have been carefully checked to make certain that neither influences the final count. Thus it was established that unfixed *E. coli* samples that had been chilled and washed immediately after withdrawal from the culture gave the same uptake curve as a parallel set of samples fixed and washed as indicated above. It was also shown that the amount of radiophosphorus remaining in the supernatant of the final salt wash was not enough to give 20 counts per minute per ml. This means that activity was not being washed from the organisms and that, since the maximum volume of saline remaining with the bacterial pellet before drying was 0.2 ml, measurable amounts were not being left behind from the radioactive culture medium. It was realized that some of the radioactivity of the pellet might be due to Na_2HPO_4 absorbed to the bacterial surfaces. In the attempt to see if this was true, test experiments were made using 0.001 N H_2PO_4 as washing liquid. The radioactive count from these samples also gave a curve identical with the one made from concurrent samples washed with physiological saline.

RESULTS

In a first series of experiments phosphorus-labeled tryptose phosphate broth was inoculated with *E. coli* from a 1-hour culture in nonlabeled broth. As the results plotted in figure 1 indicate, growth from such a young inoculum proceeds without a lag phase. Both the number of viable bacteria and the radioactivity of the bacteria from 8 ml of the culture were determined at 10-minute intervals. A and B are smooth curves drawn through these experimental points. Measurements of the pH of the culture every 20 minutes gave curve C. Curve D, which

represents the number of counts per minute per viable bacterium *formed* in the radioactive medium, was computed from curves A and B. This was done by subtracting the number of inoculated viable bacteria from subsequent bacterial counts and dividing this bacterial count into one-eighth the radioactive count shown in curve A (since this applied to an 8-ml sample). The initial horizontal part of D brings out the fact that during the early part of the logarithmic growth phase the phosphorus content per organism is nearly constant. During this interval the individual colon bacilli are especially large and rich in protoplasm as seen in the electron microscope; further studies are planned to see how close a correlation there is between this curve and electron microscopic measurements

Figure 1. Uptake of P^{32} in a growing culture of *E. coli* in tryptose phosphate broth containing 10^5 counts per min per ml when inoculated from a 1-hour-old culture. Curve A shows the radioactivity in bacteria from 8 ml of the culture; curve B, the number of viable bacteria; curve C, the pH of the culture; and curve D, the computed radioactive content per viable bacterium.

of average bacterial size. The pH curve C serves the dual purpose of giving a secondary indication of the culture growth and of supplying information needed to determine the role that may be played by Na_2HPO_4 in buffering the bacteria against pH change.

The second experiment was designed to throw light on this possible buffering action. In it the tryptose broth was buffered with sodium citrate (1.5 per cent $Na_3C_6H_5O_7 \cdot 2H_2O$) instead of phosphate (0.25 per cent Na_2HPO_4). Chemical analysis showed that this increased by 4.8 the ratio of P^{32} to P^{31} in the tryptose broth compared to its value in the previous experiment. If an appreciable fraction of the phosphorus uptake shown in figure 1 had been used to buffer the organisms against pH changes rather than to make nucleic acids, the radioactive phosphorus

content per bacterium grown in tryptose citrate broth would be expected to be less than 4.8 times that of the bacteria grown in tryptose phosphate broth. The results of this experiment in which tryptose citrate broth was inoculated with 1-hour bacteria are shown by curves *A* to *D* of figure 2. Abscissae of the initial horizontal parts of curves *D* of figures 2 and 1 have a ratio of about five. Evidently, then, the phosphorus uptake measured in these experiments is not primarily for buffering.

The next experiment was a repetition of the first, made with an initial volume of culture great enough to permit carrying it through into the early part of the

Figure 2. Uptake of P^{32} in a growing culture of *E. coli* in tryptose citrate broth containing 10^5 counts per min per ml when inoculated from a 1-hour-old culture. Curve *A* shows the radioactivity in bacteria from 8 ml of the culture; curve *B*, the number of viable bacteria; curve *C*, the pH of the culture; and curve *D*, the computed radioactive content per viable bacterium.

stationary phase of the growth curve. The results are shown in figure 3. Here again the labeled tryptose phosphate broth was inoculated from a 1-hour culture to suppress the lag phase. In this and subsequent experiments to be reported, a different batch of broth was used from that in the first experiment. The phosphorus content per bacterium during the initial logarithmic phase was 4.3×10^{-6} counts per minute per bacterium. The difference in value between this and 8×10^{-6} (the intercept of curve *D* in figure 1) is probably, in part at least, to be attributed to the different mean bacterial size resulting from the use of a different batch of broth. Figure 3 demonstrates that during the early part of the stationary phase the number of bacteria per unit volume and the radioactive content of these bacteria run sensibly parallel; that is, the activity per bacterium during this time is constant within the limits of error of the experiment. The observed rise in the pH curve at the end of the logarithmic growth phase without

Figure 3. Results of an experiment similar to the one summarized in figure 1 except that the duration of the experiment was increased to 5½ hours.

Figure 4. Summary of an experiment differing from the one giving the results shown in figure 1 by an adjustment of the pH after 130 minutes.

a corresponding change in the measured radioactivity also points to the fact that the buffer role of utilized P^{32} is negligible.

Figure 4 shows the results of a further experiment made to check whether any of the P^{32} taken up could be attributed to pH changes in the culture. Here the experiment of figure 1 was repeated except that the pH of the tryptose phosphate broth was abruptly changed from 6.8 to 7.6 by adding NaOH when the culture was 130 minutes old. As the figure shows, this does not bring about the discontinuity in radioactive uptake that might be expected if phosphorus were being diverted from nucleic acid formation; instead this uptake and the rate of bacterial multiplication proceeded unaltered by the pH change. The figures show high initial values of curves *A* in this and the next experiment. This was found to be due to the presence of a centrifugally sedimentable material in the stock radioactive solution. As a consequence, about 10^{-4} of the original activity put into the broth could be sedimented by centrifugation before its inoculation with bacteria. The amount of this insoluble radioactive impurity, as found in the sample taken at zero time, was therefore subtracted from all the subsequent radioactive values for curve *A* when computing curve *D* of figure 4.

The next two experiments were designed to show the effect of using an inoculum of bacteria old enough to introduce an appreciable lag phase into the growth curves. The results of inoculating from an 18-hour broth culture are shown in figure 5. The number of viable bacteria remained constant in the 20-minute lag phase and then increased logarithmically as in the previous cultures. In this case as in the one immediately preceding, the insoluble impurity in the radioactive $Na_2HP^{32}O_4$ produced a curve *A* with a high initial value and a considerable scatter in the value of following points.

Another experiment was therefore conducted using a new shipment of radioactive phosphorus from which the sedimentable activity was absent. As can be seen from figure 6, this eliminated the high count of the zero sample. Here the inoculation was made directly from an older 25-hour culture, which increased the lag phase to between 30 and 40 minutes. Inspection of curves *A* and *B* makes it clear that, whereas there is no measurable increase in the number of viable bacteria during the first half-hour of growth of the inoculated culture, the uptake of phosphorus starts immediately. This indicates that there is no delay in the synthesis of nucleic acids by the introduced bacteria. During the lag phase they must therefore become richer in these substances unless the acids they already contain are steadily disintegrating. That they are actually increasing in nucleic acid content, and presumably also in size, agrees with ultraviolet (Malmgren and Hyden, 1947) and electron microscopic observations and with the work of Hershey (1939), who found that the turbidity caused by the *E. coli* increased at the same rate in a freshly seeded culture whether or not there was a lag in the bacterial count. The regular uptake of phosphorus during as well as after the lag phase is further shown by the fact that the radioactive content of the bacteria increased as if they were multiplying logarithmically from zero time (even though the viable bacterial count was initially constant). This is shown by the flatness of curve *D*, which was computed from the dashed prolongation of the loga-

Figure 5. Results of an experiment similar to the ones summarized in figures 1 and 3 except that the culture was inoculated from an 18-hour-old culture. There was radioactivity in the pellets not associated with the bacteria in curve A.

Figure 6. A repetition of the experiment summarized in figure 5 except that here the inoculation was from a 25-hour-old culture. There was no sedimentable radioactivity except that in the bacteria.

rithmic part of curve *B* backwards toward an initial hypothetical count of 1.6×10^6 bacteria per ml. A similarly flat curve *D* for the experiment of figure 5 was obtained by subtracting from curve *A* the insoluble radioactive impurity and extrapolating curve *B* back to a hypothetical initial bacterial count of 1.6×10^6 organisms per ml.

It is a definite limitation of the radioactive tracer method used here compared with the ultraviolet absorption technique (Malmgren and Hyden, 1947; Malmgren and Hedén, 1947) that it can only measure the increase in bacterial phosphorus and not the amount in the initial inoculum drawn from any but very young, frequently transferred cultures. It might be thought that this could be accomplished by growing the inoculum in radioactive broth, but the presence in

Figure 7. The radioactive phosphorus content per bacterium from figure 6 assuming an initial content in the inoculum of 2.0×10^{-6} counts per minute per bacterium and using the actual number of viable organisms.

an old culture of dead bacteria that would still contain labeled phosphorus makes correlation of the radioactive count with the number of viable bacteria impossible.

These results do, however, check qualitatively with the results of the ultraviolet absorption technique and with those of a chemical determination of nucleic acids (Morse and Carter, 1949) if a reasonable value is assumed for the phosphorus content in the inoculum. In the last experiment (figure 6) such a reasonable value is 2.0×10^{-6} counts per minute per bacterium. If this amount is added to curve *A* during the lag phase (the first 30 minutes) and curve *D* computed by combining this corrected *A* curve with the solid curve *B* (approximately the number of viable organisms), the resultant radioactivity per bacterium gives the curve of figure 7 when plotted on a linear rather than a semi-logarithmic scale. From the end of the lag phase until a couple of generations have been produced, the amount of the original 2.0×10^{-6} counts per minute

per bacterium that should be added is indefinite and so is this portion of the growth curve. In figure 7 this is indicated by a broken line. Figure 7 closely resembles the corresponding ones deduced by both ultraviolet absorption and chemical analysis.

The next group of experiments has dealt with the problems of whether phosphorus is taken up by bacteria in a culture that has passed from the logarithmic to the stationary stage and whether there is an exchange of phosphorus between the formed nucleic acids of bacteria and the medium in which they are

Figure 8. The uptake of P^{32} in an 11-hour-old culture. Curve A shows the radioactive uptake in bacteria from 8 ml of culture. Curve B shows the number of viable bacteria. The total age of the culture is given by the time shown on the abscissa.

living. Figures 3 and 5 show that after a period of about three hours the curves of both activity (A) and cell counts (B) quite abruptly level off, run parallel, and approach the horizontal for some time. It is important to determine what is happening in this region where diminished cell increase is being so compensated by the beginnings of cell death as to give a population nearly in equilibrium. Evidence bearing on this is given by the experiment of figure 8. In this case labeled disodium phosphate was added to a culture after 11 hours of growth. Samples were withdrawn thereafter at half-hourly intervals for 7 hours and irregularly for the next week. As curve A indicates, there was a steady but slow uptake of phosphorus during the 7-hour period with very little thereafter. At this same time the curve B marks the end of the stationary stage and the beginning of population decline. The rise of curve A is the probable consequence of a

continuing slow cell multiplication throughout the stationary phase; this stops only when the culture is about 18 hours old. The fact that the activity count does not decline after this point means that the bound phosphorus is not released into the medium as cell death proceeds. This type of phosphorus-retention curve is similar to that obtained by Hevesy and Zerahn (1946) for yeast.

Still better evidence that phosphorus does not exchange with the medium once it has been incorporated into the nucleic acids of a bacterium is provided by the following experiments. In the experiment the results of which are plotted in figure 9, parallel cultures were grown: one in tryptose broth, the other in broth

Figure 9. These results are from an experiment in which two parallel cultures of *E. coli*, one of which contained labeled disodium phosphate, were interchanged at 2 hours after initial inoculation. The time axis represents the time after transferring the cultures. Curve B shows the viable bacteria count for the culture grown 2 hours in radioactive broth, then transferred to the inactive medium. The radioactivity contained in bacteria from 8 ml of the culture after the transfer is curve A'. Curve A shows the radioactive uptake in the non-radioactive bacteria transferred to the radioactive broth.

to which radiophosphorus had been added. Both flasks of medium were inoculated at the same time with *E. coli* from a 1-hour culture and allowed to grow at 37 C for 2 hours. The bacteria from the two cultures were then interchanged and incubation continued. The usual 8-ml samples were taken at half-hourly intervals for 7 hours and counts made of viable bacteria and radioactivity. Curve B records the bacterial count in the culture for which the radioactive bacteria had been transferred to inactive medium. Counts for the other culture were in close agreement. Curve A' shows the phosphorus uptake by the previously nonradioactive bacteria after they had been transferred to the labeled broth; it continues parallel to the bacterial increase. The activity of the labeled bacteria in the inert medium remained constant (curve A). That there was no detectable loss of radioactive phosphorus points to the fact that there was no appreciable

return of phosphorus once utilized by the bacteria to the medium either through metabolic exchange or by complete disintegration of dead organisms.

A similar experiment involving the interchange of older cultures has yielded confirming results. In this case parallel cultures were grown for 23 hours, one in ordinary, the other in radiophosphorus-labeled, broth. At the end of this period their bacteria were interchanged, as in the preceding experiment, and their bacterial and radioactive counts were followed for the next 7 hours on half-hourly samples. The results are plotted in figure 10. The two curves of bacterial count (B and B') are close together and have the flatness to be expected of stationary parts of the growth curves. The equal flatness of curve A means again that the

Figure 10. The same as for figure 8 except here the parallel cultures were interchanged after 23 hours. Curve B is the bacterial count and curve A the radioactive count on the bacteria grown in radioactive medium for 23 hours then transferred. Curve B' is the bacterial count and curve A' the radioactive uptake for the nonradioactive bacteria put into the radioactive broth.

radioactive bacteria have not lost appreciable activity to the medium when transferred to ordinary broth. The slow rise of curve A' over the 7-hour period to a final value considerably below that reached in the preceding experiments reflects the greatly retarded metabolic activity and growth in so old a culture. The end of growth, which evidently occurred when the total age of this culture was about 30 hours, corresponds roughly to the onset of the logarithmic decay phase for this particular culture medium as shown in figure 8. This type of experiment involving the exchange of bacteria between parallel cultures, one of which is radioactive, evidently provides an unusually sensitive way of following the decline of metabolic activity in old bacterial cultures.

Throughout the entire series of experiments recorded in this paper the phosphorus activity per bacterium has not shown large variations. Thus, in the experiments of 3, 4, 5, and 6, for which the same batch of broth was used, the activities

per bacterium at the beginning of the logarithmic phase have been 4.3, 5.6, 5.5, and 6.6×10^{-6} counts per minute, respectively. As indicated by curves *D* the phosphorus content per bacterium has always declined toward the end of the logarithmic phase from its initial high value to a steady value from $\frac{1}{2}$ to $\frac{1}{4}$ as great. This decline roughly parallels the diminution in bacterial size noted in electron microscopic observations. The foregoing experiments have given another opportunity to measure with considerable accuracy the interval between cell divisions for *E. coli*. For ten experiments conducted in tryptose phosphate broth the mean generation time during the phase of logarithmic growth has been 23 ± 2 minutes.

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SUMMARY

The synthesis of nucleic acids in a growing *Escherichia coli* culture has been followed quantitatively using radioactive phosphorus. To do this the phosphorus uptake has been correlated with the number of viable bacteria during the several stages of a typical growth curve. The average phosphorus contents are in general agreement with the results of ultraviolet absorption and chemical methods. Experiments indicate that the measured uptake of phosphorus is not primarily used for buffering, and that phosphorus once taken up by the bacteria does not exchange with the nutrient medium. It is further shown that uptake and a corresponding cell multiplication continue through the stationary phase until the onset of logarithmic decay.

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