EFFECTS OF RADIOPHOSPHORUS DECAY ON SOME SYNTHETIC CAPACITIES OF BACTERIA*

ELIZABETH McFALL, ARTHUR B. PARDEE AND GUNTHER S. STENT
Virus Laboratory, University of California, Berkeley, Calif. (U.S.A.)

INTRODUCTION

It appears likely that nucleic acids play some important role in the biosynthesis of specific proteins and enzymes[1,2]. In the case of bacteria, it has been possible to show that there exists a connection between the synthesis of ribonucleic acid (RNA) and the formation of enzymes, since interference with RNA synthesis also arrests enzyme formation[3,4]. Synthesis of deoxyribonucleic acid (DNA), on the other hand, does not appear to be required for enzyme formation, since enzymes are produced by bacterial populations in which the synthesis of DNA has been specifically blocked[3,5]. In view of the presumed genetic character of DNA, and hence of its ultimate role in governing cellular syntheses, one may ask whether it is not the *integrity* of the bacterial DNA rather than its *synthesis* which is a precondition for the formation of enzymes. The answer to this question has been sought previously, but apparently not obtained unequivocally, through study of enzyme synthesis in bacterial *fragments* from which the DNA has been removed[6,7].

In the present work, an attempt has likewise been made to determine whether there is a necessary connection between the integrity of the bacterial DNA, and hence of the bacterial "nucleus", and the capacity of *intact* bacterial cells to synthesize enzymes, by use of a method based on the lethal effects of the decay of radiophosphorus ^{32}P atoms. *Escherichia coli* bacteria which have incorporated ^{32}P atoms into phosphorylated constituents lose their viability (*i.e.* colony-forming capacity) as the radioactive atoms decay, the rate of loss of viability being proportional to the specific radioactivity of such cells[8]. By means of differential labeling experiments, in which it was found that bacteria are inactivated at a rate dependent on the number of ^{32}P atoms incorporated into their DNA rather than into their RNA, it could be shown that it must be the destruction of the bacterial DNA by decay of its own ^{32}P atoms which is principally responsible for the observed loss of viability[8]. In order to examine, therefore, whether and how such radiochemical destruction of the nucleus affects the synthetic capacity of bacteria, a series of experiments have been carried out in which the synthesis of inducible and constitutive enzymes, as well as of RNA and protein, was measured in highly ^{32}P-labeled *E. coli* cultures at various stages of radioactive decay. It was found in these experiments that the capacity for enzyme formation is as sensitive to suppression by ^{32}P decay as the viability of the cell. The capacity

* Supported by grants from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service and The Rockefeller Foundation.

References p. 297.

for RNA and protein synthesis is likewise eliminated rapidly by ^{32}P decay, though apparently at a rate somewhat less than viability.

MATERIALS AND METHODS

Bacterial strains

Three strains of *Escherichia coli* were used in this study: strain 15T⁻, a thymine-requiring mutant[5] of strain 15; strain B/r, a radiation resistant mutant[9] of strain B; and strain W1317, a lysogenic derivative of K12, capable of constitutive β-galactosidase synthesis[10].

Media

H is the glycerol-lactate medium described previously[11], buffered, however, in the present experiments with 0.05 *M* tris (hydroxymethyl) aminomethane at pH 7.6. *H* contains 5 mg/l phosphorus, supplied entirely by hydrolyzed casein (casamino acids DIFCO). In the "low-phosphate" *H*, half of the casein hydrolysate is replaced by a phosphorus-free casamino acid preparation from which the phosphorus has been removed by precipitation as the magnesium ammonium phosphate. The casein hydrolysate was doubled in amount in *H* medium in the experiments concerned with the study of total RNA and protein synthesis. Four mg/ml thymine was always added to *H* medium for experiments with the thymine-requiring 15T⁻, except when specific suppression of DNA synthesis was desired. *Minimal medium*, used as a storage medium for frozen cultures, contains per liter of distilled water: 7 g K_2HPO_4, 0.5 g sodium citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, and 30 g/l glycerol. GCA is a glycerolcasamino acid medium[12]. *Nutrient broth* contains per liter of distilled water: 8 g nutrient broth (DIFCO), 5 g NaCl, and 1 g dextrose.

Chemical fractionation

The specific radioactivity of various phosphorylated bacterial constituents was measured by the SCHMIDT-THANNHAUSER technique[13] after trichloracetic acid (TCA) precipitation in the cold of the radioactive bacteria in the presence of sufficient non-radioactive carrier bacteria to provide a pellet on centrifugation. The samples were washed with cold 5% TCA to remove low-molecular weight materials and phospholipid extracted with alcohol and an alcohol–ether mixture prior to hydrolysis with alkali. Measurements of radioactivity contained in the various fractions were made on dry samples by means of an end-window Geiger Müller tube, whose counting efficiency for ^{32}P had been established by reference to a standard solution of radiophosphorus supplied by the National Bureau of Standards.

Bacterial assays

The number of bacteria capable of colony formation, *i.e.* "viable", was assayed by spreading aliquots of the cultures after appropriate dilutions on nutrient agar plates. The plates were allowed to dry at room temperature and then incubated for 24 hours at 37° C for colony development. The *total* bacterial concentration was also estimated in some cultures by direct cell counts under the microscope by means of a calibrated Petroff-Hausser chamber.

Biochemical microassays

In order to avoid excessive levels of total radioactivity in these experiments, it was desirable to use the minimum possible number of highly labeled bacteria per individual biochemical assay. The colorimetric tests for enzyme activity were carried out on samples of about $4 \cdot 10^6$ bacteria, while approximately $8 \cdot 10^7$ bacteria were employed in each assay for RNA and protein synthesis. Except where noted otherwise, the temperature of incubation was always 37° C.

β-Galactosidase[14]

Triplicate 0.1 ml samples of frozen bacteria were thawed and induced to synthesize β-galactosidase by addition of 0.01 ml of a 10% solution of lactose. The cultures were incubated for various times before 0.01 ml of toluene was added to halt the induced enzyme synthesis and disrupt the bacterial membranes. The amount of enzyme formed was then determined by addition of 0.1 ml of a 1% solution of *o*-nitrophenyl-β-D-galactopyranoside (NPG), followed by incubation for 32 minutes until the reaction was stopped by addition of 0.2 ml acetone. The intensity of the color of *o*-nitrophenol liberated by action of the β-galactosidase was measured at a wave length of 420 mμ. β-galactosidase synthesis by the *constitutive* mutant culture was determined in the same way, except that no lactose inducer was added.

D-*Serine deaminase and* L-*threonine deaminase*[15]

Triplicate 0.1 ml samples of frozen bacteria were thawed and induced to synthesize D-serine deaminase by addition of 0.01 ml of a 0.1 *M* solution of DL-serine (the presence of any L-serine

References p. 297.

deaminase which might also be formed does not interfere with the subsequent assay). The cultures were incubated for various times before 0.01 ml of toluene was added to halt enzyme synthesis and disrupt the bacterial membranes. The amount of enzyme formed was then determined by addition of 0.03 ml of a mixture of 0.02 *M* D-serine in 0.04 *M* phosphate buffer at pH 8.0, and further incubation for 45 minutes. To the reaction mixture was then added 0.07 ml of a 0.1% solution of dinitrophenylhydrazine in 1.2 *N* HCl, which mixture was allowed to stand for another 30 minutes at room temperature before the reaction was stopped by addition of 0.2 ml of 2.5 *N* NaOH. The intensity of the orange color produced by the dinitrophenylhydrazone of the pyruvic acid formed by deamination of the D-serine by the enzyme was measured at the wave length of 450 mμ. (A larger differential between the absorption of the samples and a blank is obtained at this wave length than at 525 mμ where the orange color is ordinarily measured.) For assay of the ability to synthesize the constitutive enzyme L-threonine deaminase, triplicate 0.2 ml samples of the frozen bacteria were thawed and incubated for various times without addition of any inducer. The samples were then treated identically to those in the D-serine deaminase test except that L-threonine was substituted for D-serine in the enzyme-substrate assay mixture.

Protein and ribonucleic acid (RNA)

Triplicate 1.0 ml samples of frozen bacteria, stored in acid-cleaned test tubes, were thawed and incubated for various lengths of time before being added to 0.1 ml of 100% TCA at 0°. The "TCA-insoluble" precipitate formed upon overnight storage at 0°, was collected by centrifugation and, after removal of the supernatant fluid, suspended in 0.7 ml of 5% TCA and heated for 15 min at 100°. The sample was then centrifuged and the precipitate containing the insoluble protein separated from the supernatant fluid containing the solubilized nucleic acids. 0.7 ml of a 1% solution of $FeCl_3$ in concentrated HCl, containing 10 mg/ml orcinol[17] was then added to the *supernatant* fluid and the mixture heated for 30 minutes in a boiling water bath. The intensity of the green color—dependent on the amount of ribose in the sample—was measured at a wave length of 660 mμ. The total amount of protein in the *precipitate* was determined according to the modified Folin-Ciocalteau procedure of LOWRY *et al.*[16].

EXPERIMENTAL

Inactivation of phosphorus-starved bacteria by ^{32}P decay

Upon disintegration of their incorporated radioactive atoms *Escherichia coli* bacteria which harbor radioactive phosphorus ^{32}P within their phosphorylated constituents lose their viability, *i.e.* the ability to give rise to a colony when plated on nutrient agar. If the logarithm of the fraction of radioactive bacteria surviving (*i.e.* still capable of colony formation) is plotted against the fraction of ^{32}P atoms which have decayed by the time of assay, a "multiple hit" survival curve of the type

$$s_n = 1 - (1 - s)^n \qquad (1)$$

is found, as if each cell contained n "sensitive units", whose surviving fraction s decreased with the fraction of ^{32}P atoms decayed, f, according to

$$\log s = -K f \qquad (2)$$

(in which K is a constant), and as if the colony-forming ability of the cell was preserved as long as one of these units remained intact[8]. The final slope K of the survival curve represents the rate of inactivation of the last "sensitive unit" per cell and the extrapolation of the final asymptote to zero decay the number, n, of sensitive units per cell. The fraction of ^{32}P atoms decayed, f, is related to the number of days, t, for which radioactive decay has been allowed to proceed by

$$f = 1 - e^{-\lambda t} \qquad (3)$$

where λ is the fractional rate of ^{32}P decay per day.

It has been shown previously that the bactericidal effect of radioactive decay

References p. 297.

arises principally from the decay of ^{32}P atoms incorporated into the bacterial deoxyribonucleic acid (DNA). For the rate of ^{32}P-decay-inactivation of cells of the thymine-requiring strain of *E. coli*, 15_T-, which had been allowed to assimilate their radiophosphorus in the *presence* of thymine, *i.e.* had incorporated the ^{32}P uniformly into all of their phosphorylated constituents, is much greater than the rate of inactivation of similar cells which had assimilated the same total amount of ^{32}P in the *absence* of thymine, *i.e.* contained little or no ^{32}P in their DNA[8]. The conclusion that the radioactive disintegrations within the bacterial DNA are responsible for the suppression of colony-forming ability suggests further that the "sensitive units" of equation (1) are the *bacterial nuclei*, the seat of localization of the bacterial DNA[18]. The kinetics of inactivation of ^{32}P-labeled cells of *E. coli* strain B/r, known to possess an average of *three* such nuclei per cell in their logarithmic phase of growth[9], agree with this notion, since these bacteria are inactivated as if they possessed an average of *three* "sensitive units" per cell. On the basis of the rate of ^{32}P inactivation of *E. coli* bacteria at various specific activities of incorporated ^{32}P, it could be estimated that at $-196°$ on the average one in every 50 ^{32}P disintegrations kills that nucleus in which it occurs, compared to an efficiency of killing of one in every 20 to 25 ^{32}P decays in various bacteriophage strains[11].

These observations on the location of bactericidal action of the decay of incorporated ^{32}P atoms have been extended in the present work by a study of the kinetics of inactivation of radioactive populations of strain B/r which have been starved for phosphorus to various extents. A growing culture of B/r bacteria is capable of undergoing some further cell divisions after having exhausted the phosphorus supply of its growth medium. Under these conditions, the cells become progressively smaller until they appear in the microscope to be about one-fourth of the size of cells of a rapidly growing culture. This size reduction during phosphorus starvation is attended by important changes in total and relative content per cell of the two nucleic acids RNA and DNA, two principal phosphorylated constituents of *E. coli* bacteria. In particular, we observed that in the course of such phosphorus starvation the average content of RNA and DNA per cell may decrease by factors of six and three respectively, so that the ratio of the amounts of RNA to DNA in the culture is reduced by a factor of about two. That these changes represent differences in the composition of *viable* bacteria, and not artifacts resulting from the analytical averaging of heterogeneous cell mixtures of living cells of unchanged composition and dead cells from which variable amounts of nucleic acid have escaped into the medium, is indicated by the fact that total cell counts in the microscope and viable colony assays of the cultures agree closely at all of these stages of phosphorus starvation. An experiment has been carried out to examine how such changes in nucleic acid composition of B/r cells affect their sensitivity to inactivation by ^{32}P decay.

Procedure

A culture of B/r bacteria growing in *H* medium was inoculated to a density of 10^7 cell/ml into "low phosphate" *H* medium, containing ^{32}P at a specific activity of 25 mc/mg. This radioactive culture was allowed to grow at 37° and pairs of samples withdrawn at various times. One of the two samples was diluted 13-fold into cold GCA medium and 0.1 ml aliquots frozen and stored at $-196°$ in liquid nitrogen. From day to day, one of the frozen aliquots was thawed and assayed for viable count.

References p. 297.

The other of the two samples was diluted into an ice-cold 10 % solution of trichloracetic acid (TCA) and its phosphorylated constituents fractionated according to the Schmidt-Thannhauser technique. The amount of radioactivity contained in the RNA and in the DNA fractions of the various samples was then counted.

TABLE I

NUCLEIC ACID CONTENT OF PHOSPHORUS-STARVED BACTERIA

Hours of growth	*Total cell number per ml*	*Viable cell number per ml*	*RNA*		*DNA*		*RNA/DNA*
			c.p.m. per ml	*c.p.m. per bacterium*	*c.p.m. per ml*	*c.p.m. per bacterium*	
A. Viability experiment							
0	10^7	$1.1 \cdot 10^7$	0	0	0	0	
2.5	10^8	$9.7 \cdot 10^7$	$1.6 \cdot 10^7$	0.16	$0.47 \cdot 10^7$	0.048	3.4
3.5	$2 \cdot 10^8$	$2.5 \cdot 10^8$	$1.5 \cdot 10^7$	0.062	$1.0 \cdot 10^7$	0.026	2.4
6	$4 \cdot 10^8$	$5.0 \cdot 10^8$	$1.3 \cdot 10^7$	0.026	$0.82 \cdot 10^7$	0.016	1.6
B. Enzyme-synthesizing capacity experiment							
2.5	$8 \cdot 10^7$	$7.3 \cdot 10^7$	$1.6 \cdot 10^7$	0.23	$0.38 \cdot 10^7$	0.051	4.4
3.5	$5 \cdot 10^8$	$3.7 \cdot 10^8$	$2.1 \cdot 10^7$	0.057	$0.96 \cdot 10^7$	0.026	2.2

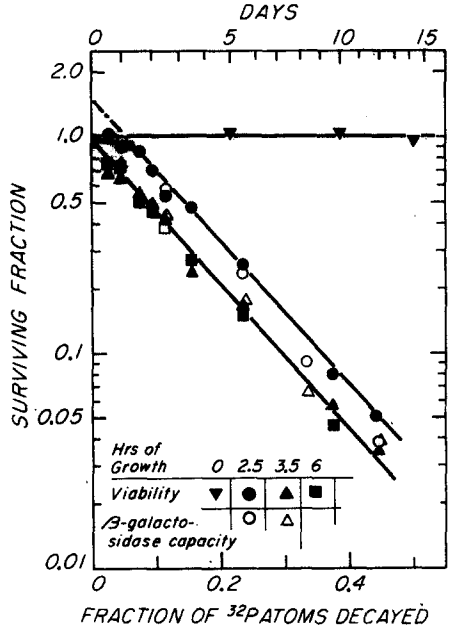

Fig. 1. Inactivation by radioactive decay of viability and enzyme-forming capacity in B/r bacteria grown in ^{32}P-labeled "low phosphate" medium for various times. Ordinate: fraction of the bacterial population still capable of forming a colony or ratio of the differential rates of enzyme synthesis manifested by the culture after and before decay.

The results of this experiment are presented in part A of Table I and in Fig. 1. It is seen in Table I that the bacteria had already depleted most of the phosphorus of their growth medium by the time the second, or 2.5 hour sample, had been taken, since subsequent to this time very little additional ^{32}P appeared in the nucleic acid

References p. 297.

fractions. The number of cells in the culture, however, can be seen to have increased by more than a factor of 5 during the next 3.5 hours, so that the amount per cell of each nucleic acid had decreased considerably. If, as seems reasonable, the decreased DNA content per bacterium induced by phosphorus starvation reflects a decreased number of nuclei per cell and if the prolonged starvation has finally rendered the bacteria of the 6-hour culture uninucleate, then one would conclude that the bacteria of the 2.5-hour culture, which contain exactly three times as much DNA per cell, harbor 3 nuclei each, in agreement with previous cytological, genetic, and radiobiological estimates of the nuclear number of B/r bacteria growing under similar conditions[8,9]. The ratio of the number of radioactive atoms in RNA to that in DNA, is seen to have changed by more than a factor of two. The kinetics of ^{32}P inactivation of the four samples, presented in Fig. 1, show that:

(1) the bacteria of the 0 hour sample, withdrawn immediately after inoculation into the radioactive growth medium and containing no appreciable ^{32}P in their nucleic acids, are stable under the conditions of frozen storage;

(2) the bacteria of the 2.5-hour sample lose their viability according to a "multiple-hit" survival curve whose form suggests that the average number n of "sensitive units" per cell is about 1.5;

(3) the bacteria of the 3.5- and 6-hour samples lose their viability according to a "single-hit" survival curve, whose form suggests that the number of "sensitive units" has been reduced to the neighborhood of one by further phosphorus starvation;

(4) the final slope K of the survival curves of the last three cultures is roughly the same.

These observations fit with the previous conclusions concerning the mechanism of ^{32}P-decay-inactivation of bacteria. The fact that the number of "sensitive units" per cell is reduced when the amount of DNA per cell is diminished supports the idea that these sensitive units are the bacterial nuclei, although, on the basis of our chemical estimate of three nuclei per cell, the 2.5-hour culture should really have been inactivated according to a curve indicating 3 instead of 1.5 sensitive units per cell. It is possible that for some reason the radioactive culture techniques employed in this experiment caused half of the nuclei to be already inactive before ^{32}P decay was allowed to take place during frozen storage, or that the 2.5-hour culture may actually have possessed fewer than 3 nuclei per bacterium, and that the changes in nucleic acid content inferred from the Schmidt-Thannhauser analysis—against which certain criticism can be made[19]—have led to a fortuitous agreement with the estimated value. In similar experiments, in which no starvation nor chemical fractionation of the assimilated ^{32}P had been attempted, survival curves corresponding to three sensitive units per cell in logarithmically growing B/r cells could usually be found. The fact that the final slope of all three survival curves is the same, supports the idea that it is principally the disintegrations of ^{32}P atoms incorporated into the DNA fraction which suppress the reproductive ability of the cell. For if disintegrations of atoms incorporated into the RNA fraction played a major role in the inactivation processes, the cells of the 6-hour culture which contained relatively less of their total radioactivity as RNA, should have been inactivated at a slower rate than the bacteria of the 2.5-hour culture, relatively richer in RNA ^{32}P atoms.

References p. 297.

Effects of ^{32}P decay on the formation of inducible enzymes

Uniformly labeled bacteria. In the experiments to be described in the following paragraphs, the decay of ^{32}P atoms incorporated into *E. coli* has been investigated with regard to its lethal effects on the ability of bacteria to carry out certain syntheses. The first of such studies to be considered here is the effect of ^{32}P on the capacity of *E. coli* to respond to an enzyme inducer[20] and synthesize the inducible enzymes β-galactosidase and D-serine deaminase. For this purpose, a culture of the thymine-requiring strain 15_T^- was inoculated into thymine-supplemented *H* medium containing ^{32}P at the specific activity of 25 mc/mg and permitted to grow through several generations to a density of 10^8 cells/ml. The radioactive culture was then

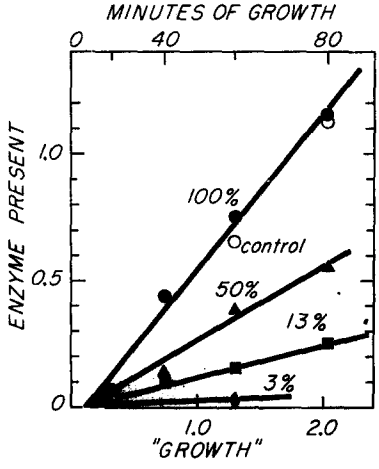

Fig. 2. Induced β-galactosidase synthesis by radioactive 15_T^- bacteria after ^{32}P decay and by a non-radioactive control culture. The percentage of cells remaining viable is indicated on each curve. Ordinate: optical density of color in enzyme activity test. Abscissa: "growth" calculated according to formula (4), where $D = 50$ minutes.

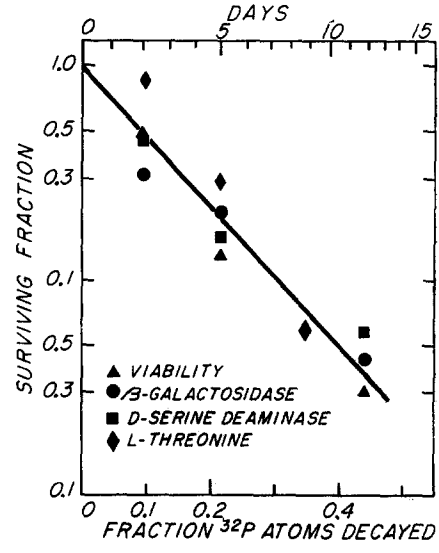

Fig. 3. Inactivation by radioactive decay of viability and enzyme forming capacity of radioactive 15_T^- bacteria. Ordinate: as in Fig. 1.

diluted tenfold into storage medium and 0.1 ml aliquots were frozen and stored at −196°. From day to day, at various stages of ^{32}P decay, aliquots of the frozen culture were thawed and assayed for their viability (*i.e.* colony formation) and for their capacity to synthesize β-galactosidase and D-serine deaminase.

The results of this experiment are presented in Figs. 2 and 3. Shown on Fig. 2 are the kinetics of β-galactosidase formation of four aliquots of the radioactive culture in which ^{32}P decay has been allowed to progress for various times before thawing and in which 1.0, 0.65, 0.13 and 0.03 respectively of the original population of bacteria remained viable. Also shown in Fig. 2 are the kinetics of β-galactosidase formation manifested by an equivalent concentration of *H*-medium grown, non-radioactive 15_T^- bacteria which have not been frozen and thawed prior to the addition of the lactose inducer. The ordinate of Fig. 2 shows the amount of enzyme present (or intensity of color developed in the enzyme assay) and the abscissa the "growth" which has occurred (or amount by which the total bacterial mass has

References p. 297.

increased) during the incubation period of length t minutes. The "growth" is not measured directly but estimated from the relation

$$\text{growth} = 2^{t/D} - 1 \qquad (4)$$

where D is the time required for doubling of the bacterial mass. The slope of any curve on such a plot of amount of enzyme present *vs.* growth represents the *differential* rate of synthesis of the enzyme[21]. It may be seen from the results presented in Fig. 2 that neither freezing and thawing nor growth in the highly radioactive medium had any serious effect on the capacity of the culture to synthesize this enzyme, since the differential rate of β-galactosidase formation by the first aliquot in which so little ^{32}P decay has been permitted to occur that all the cells remained viable, is very similar to the rate of enzyme synthesis by the non-radioactive, non-frozen control culture. (The brief lag before onset of enzyme synthesis indicated in Fig. 2 is commonly observed when lactose is used as inducer.) The kinetics of β-galactosidase formation by the three remaining aliquots in which more and more ^{32}P decay has been allowed to occur show that the differential rate at which the enzyme is formed subsequent to the addition of inducer becomes progressively lower the fewer of the radioactive bacteria remain viable.

The differential rates of induced enzyme synthesis have been estimated on the basis of the slopes of the curves of Fig. 2 and a surviving fractional capacity for synthesis calculated by dividing the rate observed after a given amount of ^{32}P decay by the rate of enzyme synthesis manifested by the totally viable aliquot in which no radioactive decay at all had been allowed to take place. The logarithm of the surviving fractional capacity for β-galactosidase formation has been plotted on Fig. 3 as a function of the fraction of the ^{32}P atoms which have decayed by the time of the enzyme test. Also plotted on Fig. 3 is the logarithm of the fraction of viable bacteria in the same culture as a function of ^{32}P decay, and it may be seen that loss of β-galactosidase forming capacity and loss of colony-forming ability of the radioactive culture of 15_T- bacteria decrease hand in hand.

The results of an analysis of the capacity of an equally ^{32}P-labeled culture of 15_T- bacteria to synthesize the inducible enzyme D-serine deaminase at various stages of radioactive decay were essentially similar to those described above for β-galactosidase. The logarithm of the surviving fractional capacity to synthesize this second enzyme has likewise been plotted as a function of radioactive decay in Fig. 3, where it may be seen that also in this case loss of enzyme forming capacity and viability of the population decreased in a very similar manner.

The interpretation of the results presented in Figs. 2 and 3 is complicated by the fact that, whereas loss of viability is an "all-or-none" phenomenon in that a bacterium either is or is not capable of giving rise to a colony, the gradual loss of enzyme forming capacity of a bacterial culture could reflect either an "all-or-none" phenomenon in that an ever increasing *fraction* of the bacterial population is completely incapable of *any* enzyme synthesis while the "surviving" remainder still preserves its capacity to form the enzyme at the normal rate, or it could be an "intermediate-state" phenomenon in that radioactive decay results in progressively decreasing rates of enzyme synthesis by *individual* cells. On the basis of the experiments presented here it does not seem possible to decide between these two alternatives. The coincidence of loss of viability and loss of enzyme-forming capacity

References p. 297.

survival curves suggests, however, that the lethal ^{32}P disintegrations destroy enzyme forming capacity, like viability, in an all-or-none manner in each cell.

Bacteria ^{32}P-labeled preferentially in their RNA. The similarity in the rate at which viability and enzyme forming capacity are suppressed by ^{32}P decay suggests also that perhaps for induced enzyme synthesis, as for colony formation, only the disintegrations of radiophosphorus atoms incorporated into bacterial DNA, but not into RNA, may be lethal. In order to examine this possibility, an experiment has been carried out similar to that previously realized for identifying the seat of the disintegrations responsible for loss of viability. In this experiment the suppression of enzyme forming capacity has been followed in two bacterial cultures, one of which was uniformly labeled with ^{32}P and the other of which harbored a higher specific radioactivity in its RNA than in its DNA. Two aliquots of a culture of the thymine-requiring strain 15_T^- grown in thymine-supplemented *H* medium, were washed free of thymine by centrifugation and inoculated to a density of 10^8 cells/ml into two separate volumes of *H* medium labeled with ^{32}P at a specific activity of 125 mc/mg, one of these volumes containing thymine and the other being thymine-free. The bacteria were allowed to assimilate ^{32}P in these two culture fluids for 30 minutes before being diluted tenfold into thymine-supplemented minimal medium and frozen and stored at $-196°$. Samples of the two frozen cultures were thawed from day to day and tested for survival of colony-forming ability and capacity to synthesize the two inducible enzymes β-galactosidase and D-serine deaminase. The total amount of ^{32}P assimilated by the bacteria during the 30 minute incubation was determined by precipitating aliquots of the two cultures with cold TCA and assaying the amount of radioactivity precipitated after washing the precipitates with non-radioactive minimal medium.

The measurements of total ^{32}P uptake and survival of viability of the 15_T^- bacteria in this experiment showed, in agreement with previous experience, that although nearly the same *total* amount of ^{32}P had been assimilated by both cultures, the viability of the bacteria which had taken up their phosphorus in the *presence* of thymine was lost much more rapidly with radioactive decay than the viability of the bacteria which had acquired their radioactivity in the *absence* of thymine under conditions of greatly reduced DNA synthesis[8]. The results of the assays of the capacity to synthesize β-galactosidase and D-serine deaminase (expressed here, as in Fig. 3, as the ratio of the differential rate of enzyme synthesis manifested by bacteria which have sustained a certain amount of ^{32}P decay to the differential rate manifested on the first day of the experiment by bacteria which had as yet sustained no decay) are presented in Fig. 4. It may be seen there that in both cultures the capacity to synthesize both of these induced enzymes is lost in the same way as viability, *i.e.* lost rapidly in the culture which had assimilated its ^{32}P in the *presence* of thymine and is lost slowly in the culture which had acquired its radioactivity in the *absence* of thymine. This result supports the idea expressed above that as for viability, the disintegrations of ^{32}P atoms incorporated into the bacterial DNA are principally responsible for loss of enzyme-forming capacity. Both cultures of this experiment contained similar amounts of ^{32}P in their RNA fractions, and should have lost their enzyme-forming capacity at the same rate if RNA disintegrations were chiefly responsible for the lethal action.

Phosphorus-starved bacteria. In order to obtain further evidence concerning the localization within the cell of the ^{32}P disintegrations lethal to the capacity for syn-

References p. 297.

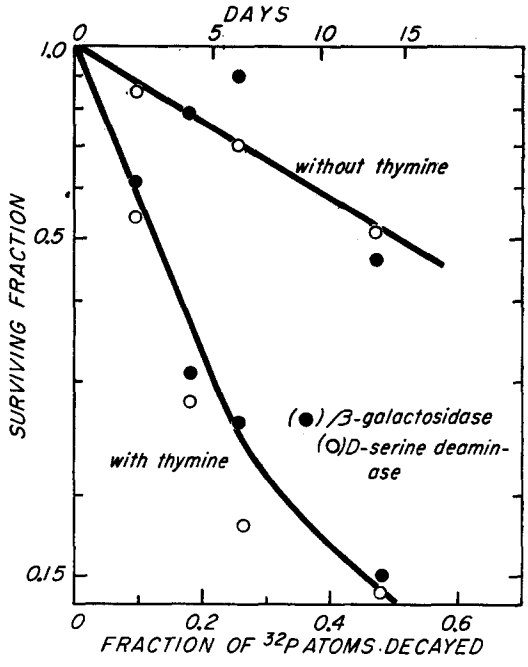

Fig. 4. Inactivation by radioactive decay of the enzyme-forming capacity of two cultures of 15T⁻, which had assimilated their ^{32}P either in the presence or in the absence of thymine. Ordinate: as in Fig. 1.

thesis of induced enzymes, the inactivation by radioactive decay of the enzyme-forming capacity of phosphorus starved ^{32}P-labeled bacteria was examined. For this purpose, a culture of strain B/r was first grown and then phosphorus-starved in "low-phosphate" *H* medium containing ^{32}P at a specific activity of 25 mc/mg, as before. Aliquots of two samples of this culture, one taken 2.5 hours and the other 3.5 hours after inoculation of the culture were frozen and stored at —196°. Two other aliquots of these samples were diluted into cold TCA and phosphorylated constituents of the bacteria fractionated by the Schmidt-Thannhauser technique as before in order to determine the amount of ^{32}P in each fraction. Aliquots of the frozen samples were thawed from day to day and assayed for viability and capacity to synthesize β-galactosidase. Control experiments showed that the kinetics of induced β-galactosidase synthesis by non-radioactive phosphorus-starved bacteria, returned to a phosphorus-rich medium at the time of induction, are essentially similar to those exhibited by non-starved cultures.

The results of this experiment are presented in part B of Table I and in Fig. 1. It can be seen in Table I that both the amount of radioactive DNA per cell as well as the ratio of the amounts of radioactive RNA to DNA decreased by a factor of two during phosphorus starvation. Fig. 1 shows that the kinetics of loss of enzyme-forming capacity, as those of viability, are not seriously affected by these changes in total and relative amounts of the two nucleic acids per cell. This finding then adds additional weight to the previous inference that the decays of ^{32}P atoms in the bacterial RNA cannot be the principal cause of loss of enzyme-forming capacity, for in that case the RNA-poor, phosphorus-starved cells should have lost that capacity more slowly than the non-starved cells which contained four times as many RNA-^{32}P atoms per cell and twice as many such atoms per bacterial nucleus.

References p. 297.

Effect of ^{32}P decay on the formation of constitutive enzymes

It has been shown in the preceding that disintegrations of incorporated ^{32}P atoms suppress the capacity of bacteria to synthesize certain enzymes as rapidly as they suppress the viability of the cells. It is conceivable that this high sensitivity of the enzyme-forming capacity to radioactive decay is related to the fact that the enzymes tested were *induced* enzymes. It is known that before the externally added inducer can reach the site of induction in a previously non-induced bacterium, a certain transport mechanism, or "permease", has to be formed by the cell[22,23]; it is, furthermore, possible that certain intracellular "organisers" might have to be set up *de novo* subsequent to the introduction of the inducer before enzyme synthesis can get under way[24]. If now formation of either transport mechanism or intracellular "organiser", or of some other special feature connected with induced enzyme synthesis, were especially ^{32}P-decay-sensitive because it depended in some way directly on the integrity of the bacterial DNA, but if synthesis of the enzyme, once under way in the cell, could proceed independently of the DNA, then one could expect that the capacity of bacteria to continue the formation of *constitutive* enzymes, for which no external inducer needs to be added and whose synthesis is already under way in the cells prior to radioactive decay, be very much less sensitive to disintegration of incorporated ^{32}P atoms.

In order to examine this possibility, the capacity of ^{32}P-labeled *E. coli* to synthesize the constitutive enzymes β-galactosidase and L-threonine deaminase was followed at various stages of radioactive decay. β-galactosidase formation was studied in the constitutive mutant W1317 of strain K12 (λ) and L-threonine deaminase formation was studied in strain 15_T-[15]. For this purpose, uniformly ^{32}P-labeled cultures of these bacteria were grown in radioactive *H*-medium containing ^{32}P at 25 mc/mg and as before, aliquots frozen, stored and thawed from day to day for assay of enzyme-forming capacity. Although strain W1317 is lysogenic, carrying the λ prophage, and although phage development may be induced by radioactive decay, no difficulty should be encountered from this fact since, as has been shown previously[25], and as we could confirm in control experiments also under the conditions employed here, the early stages of lytic development of phage λ following its induction do not interfere with β-galactosidase synthesis. Accordingly, constitutive β-galactosidase synthesis measurements in the present experiments were carried out only during the first 60 minutes of growth following thawing on various days. No viability assays, however, were undertaken in the experiment with lysogenic bacteria since loss of colony forming ability by radioactive decay in this case reflects primarily the lysis of the cells following prophage induction rather than nuclear inactivation.

The survival of the capacity of radioactive W1317 bacteria to continue the synthesis of their constitutive β-galactosidase after having sustained various degrees of radioactive decay is presented in Fig. 5. It may be seen there that the synthesis of the constitutive β-galactosidase is suppressed by radioactive decay very much like the synthesis of the induced β-galactosidase shown in Fig. 2, in that the differential rate of enzyme synthesis manifested by the culture falls off progressively with the fraction of the incorporated ^{32}P atoms which have disintegrated by the time of assay. A precise quantitative comparison between the results of Figs. 2 and 5 concerning the *efficiency* with which decaying ^{32}P atoms suppress β-galactosidase forming

References p. 297.

capacity in the two cases cannot easily be made since no meaningful viability assays could be carried out for the lysogenic strain of Fig. 5. On the basis of the estimated specific activity of the growth media of both cultures, one may conclude, however, that the efficiency per ^{32}P disintegration of destroying the capacity for synthesis of either inducible or constitutive β-galactosidase synthesis is roughly the same. Also shown in Fig. 5 is the differential rate of constitutive β-galactosidase synthesis by two control cultures of W1317 grown in non-radioactive *H* medium to the same density as the radioactive cultures of the experiment itself. In one of these cultures, development of phage λ had been induced by exposure to ultraviolet light (UV). It is apparent from these controls that neither induction of λ development nor growth of the culture in the highly radioactive medium has any serious effect on the capacity of W1317 bacteria to synthesize their constitutive β-galactosidase and that the progressive decrease in enzyme formation observed in the radioactive bacteria is due to the lethal effects of ^{32}P decay during their storage.

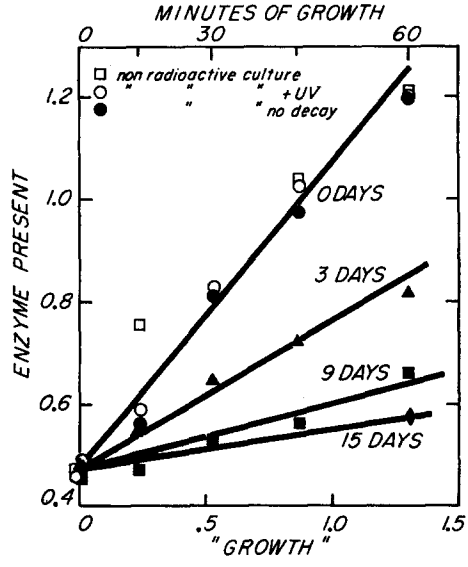

Fig. 5. Constitutive β-galactosidase synthesis by radioactive W1317 bacteria after ^{32}P decay and by non-radioactive cultures with and without UV-induced development of the λ prophage. The number of days for which ^{32}P decay had been allowed to occur is indicated on each curve. Ordinate and abscissa: as in Fig. 2.

The survival of the capacity of radioactive 15_T- bacteria to continue the synthesis of their constitutive L-threonine deaminase is presented in Fig. 3, where it may be seen that the differential rate of synthesis of this second constitutive enzyme is also suppressed about as rapidly by radioactive decay as the viability of the cells of this culture, similar to the observation on the induced enzymes presented in Fig. 3.

It may be concluded, therefore, that the high sensitivity to suppression by decay of incorporated ^{32}P atoms of the enzyme-forming capacity is not restricted to the synthesis of inducible enzymes but is obtained equally for the synthesis of constitutive enzymes for which no "permease" or "organiser" need be created *de novo*.

Effect of ^{32}P decay on the formation of ribonucleic acid and protein

In order to examine the extent to which the rapid loss in enzyme-forming capacity with radioactive decay observed in the preceding experiments reflects a generalized suppression of the macromolecular syntheses in cells which have sustained a lethal "hit" in their DNA, the effect of ^{32}P decay on the capacity of radioactive bacteria

References p. 297.

to carry out the synthesis of ribonucleic acid (RNA) and protein has been studied. For this purpose, a culture of 15_T- was grown in radioactive *H* medium containing ^{32}P at a level of 12 mc/mg, and aliquots frozen, stored, thawed from day to day and assayed for the rate at which the bacteria produce RNA and protein at various stages of radioactive decay. The rates of RNA and protein synthesis manifested by a control culture of 15_T- grown in non-radioactive *H* medium to an equivalent density was similarly assayed. Certain modifications of the previous procedures were employed for this experiment. Since the intensity of color developed by the orcinol[17] and Folin[16] tests employed here is less than that generated in the previous enzyme assays, an 8-fold greater concentration ($8 \cdot 10^7$/ml) of bacteria and a 10-times greater volume of suspension (1.0 ml) had to be stored in the frozen state. The medium into which the radioactive cultures were diluted tenfold prior to their being frozen was not minimal medium but *H* medium to which 3% glycerol had been added.

The results of this experiment are presented in Figs. 6 and 7. Fig. 6 shows the amount of RNA per ml contained, *i.e.* the intensity of color developed in the orcinol test, by cultures in which various amounts of ^{32}P decay had occurred, plotted against the time which elapsed, or the "growth" which occurred, since thawing. It may be seen that the radioactive culture which was thawed on the first day of the experiment before any appreciable ^{32}P decay had taken place and in which all the bacteria retain their viability synthesizes RNA at a normal rate, in that the amount of RNA doubles for every unit of "growth". This rate of RNA synthesis is the same as that manifested by a non-radioactive control in *H* medium which has never been frozen. The two cultures thawed on subsequent days, by which time extensive ^{32}P decay has taken place and in which only 19% and 3.9% of the individuals still retained their viability, are seen to have suffered a diminution of their capacity to synthesize RNA, although both cultures appear to have retained the ability to synthesize RNA for one or two division periods at a rate significantly greater than could be accounted for exclusively by the normal RNA synthesis of the surviving cells. (This statement must be qualified, inasmuch as the orcinol test as employed here detects, strictly speaking, only *ribose*

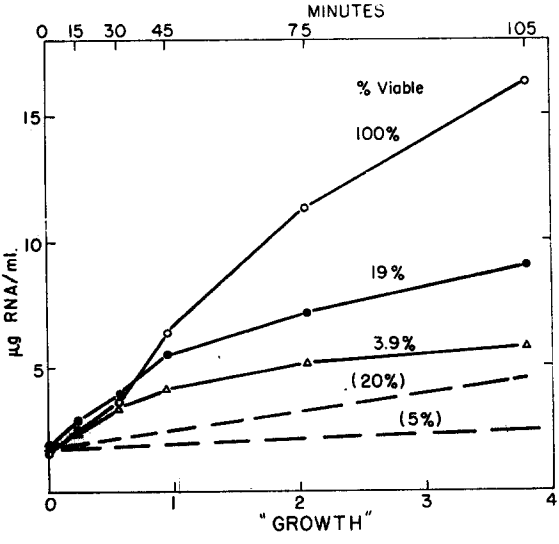

Fig. 6. Ribonucleic acid (RNA) synthesis by radioactive 15_T- bacteria after ^{32}P decay. The percentage of cells remaining viable is indicated on each curve. The broken lines show the expected RNA synthesis by 20% and 5%, respectively, of the initial cell population. Abscissa: as in Fig. 2.

References p. 297.

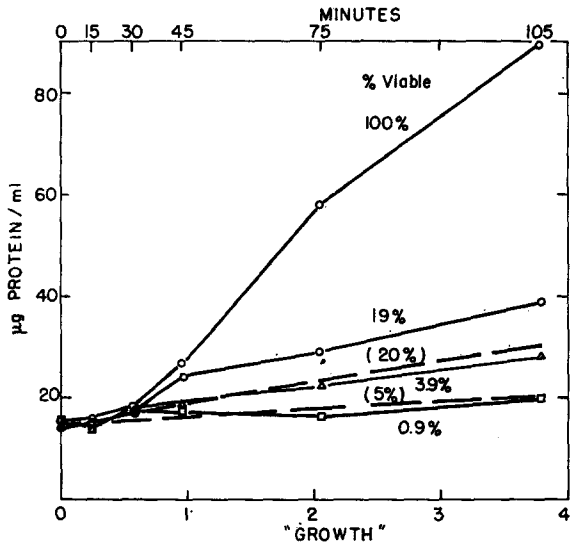

Fig. 7. Protein synthesis by radioactive 15T- bacteria after ^{32}P decay. The percentage of cells remaining viable is indicated on each curve. The broken lines show the expected protein synthesis by 20% and 5% respectively of the initial cell population. Abscissa: as in Fig. 2.

in molecules large enough to be precipitated by TCA, and the residual synthesis of orcinol-positive material in ^{32}P-inactivated bacteria may not be identical with the RNA normally produced.)

Fig. 7 shows the amount of protein per ml contained by the same three cultures, *i.e.* the intensity of color developed in the Folin test, after the same amounts of radioactive decay, as well as the protein content of a fourth culture in which even more extensive ^{32}P decay had been allowed to occur and in which only 0.9% of the cells still retained their viability. The results of this part of the experiment can be seen to be quite similar to those presented in Fig. 6, in that the 100% viable culture thawed on the first day of the experiment, before any appreciable decay, synthesizes protein at a normal rate, *i.e.* at a rate similar to that exhibited by a non-radioactive, non-frozen control culture. The cultures, on the other hand, in which only 19% and 3.9% respectively of the cells had retained their viability after ^{32}P decay are seen to synthesize TCA-insoluble, Folin-positive material at a very much reduced rate, albeit a rate significantly greater than that which could be accounted for exclusively by the synthetic activities of the cells having "survived" radioactive decay. Finally, an amount of ^{32}P decay so extensive that it suppresses the viability of all but 0.9% of the culture is seen to have reduced the capacity of the culture to synthesize protein to a negligible value.

One may infer from these observations, therefore, that the very few lethal disintegrations of ^{32}P atoms in the bacterial DNA destroy not only the ability of the cells to give rise to an indefinite succession of daughter cells or to form certain specific enzymes, but also have a widespread effect on the capacity of the bacteria to synthesize RNA and protein in general. It appears, however, that the temporary maintenance of RNA and protein synthesis in these bacteria is somewhat less sensitive to suppression by ^{32}P decay than survival or enzyme formation.

References p. 297.

DISCUSSION

The result of the experiments reported here, in which it was observed that decays of ^{32}P atoms incorporated into the DNA of *E. coli* cells eliminate the capacity for formation of specific enzymes, indicates that the integrity of the bacterial DNA is required for enzyme synthesis by the cell. The fact, furthermore, that the capacity to form a given enzyme, *i.e.* β-galactosidase, is suppressed by ^{32}P decay at the same rate as the viability, and that only a few hundred ^{32}P decays per bacterium in the DNA reduce the viability to 1%, suggests that the bacterial DNA, *i.e.* the bacterial nucleus, acts as a unit in enzyme synthesis: destruction of any part of the nucleus, and not only of the limited region of the genome which by means of genetic tests can be demonstrated to be concerned with the synthesis of β-galactosidase[26], suppresses synthesis of this enzyme. This conclusion is in harmony with previous observations concerning enzyme syntheses in bacteriophage-infected bacteria, in which it has been found that those bacteriophage strains, like the coliphage T2, which cause a rapid breakdown of the host nucleus in the course of their intracellular growth[27], arrest the formation of respiratory enzymes or β-galactosidase[28], whereas other phage types, like the coliphage λ, during whose growth the host nucleus preserves its integrity[29], still permit their host cells to continue synthesis of these same enzymes[25].

The present observation that ^{32}P decays likewise suppress the synthesis of polyribonucleotides and polypeptides suggests that the entire bacterial nucleus acts as a unit in controlling such polymerization reactions, since formation of these macromolecular species is seriously reduced after the nuclear DNA has been destroyed by radioactive decay. The fact that the capacity to form protein and RNA is somewhat more refractory to suppression by ^{32}P decay than the ability to synthesize certain enzymes might suggest that it is still possible for a ^{32}P-decay-damaged bacterial nucleus to elicit a limited synthesis of polypeptides which are now devoid of the functional specificity normally conferred upon them. In this respect, lightly ^{32}P-inactivated bacteria may resemble bacteriophage T2-infected cells, which, besides synthesizing bacteriophage-specific proteins, continue to form protein molecules with electrophoretic properties similar to those of normal bacterial proteins but presumably without normal enzymic activity[30, 31].

Finally, it must be noted that although ^{32}P-decay-inactivated bacteria appear no longer able to synthesize either certain enzymes or proteins or RNA, it must not be inferred that a few lethal ^{32}P disintegrations cause such extensive protoplasmic damage that the radioactive cells have been rendered entirely useless as biochemical systems. For, it has been found that in a ^{32}P-labeled culture of *E. coli* in which radioactive decay has abolished the colony-forming ability in 99.9% of the individuals almost every cell can still support the growth of infecting bacteriophage particles[32]. Such ^{32}P-inactivated bacteria are, therefore, still capable of synthesizing the bacteriophage nucleic acids and proteins, as if the healthy, undamaged DNA of the infecting bacteriophage particle supersedes the ^{32}P-damaged host cell nucleus in the control of phage-specific reactions.

SUMMARY

1. Aliquots of a ^{32}P-labeled culture of *Escherichia coli* which have been starved for phosphorus to various degrees and which contain various total and relative amounts of ribonucleic (RNA)

References p. 297.

and deoxyribonucleic (DNA) acid lose their viability with radioactive decay at the same final rate. This supports a previous conclusion that the disintegrations of ^{32}P atoms in the bacterial DNA are principally responsible for the decay-induced loss of viability of bacterial cells.

2. The capacity of ^{32}P-labeled cultures of *E. coli* to form two induced enzymes (β-galactosidase and D-serine deaminase) and two constitutive enzymes (β-galactosidase and L-threonine deaminase) is lost with radioactive decay at the same rate as viability. Radioactive decay, furthermore, destroys the capacity to form the two induced enzymes more rapidly in bacteria which are uniformly ^{32}P-labeled in all their phosphorylated constituents than in bacteria which are preferentially labeled in their RNA. ^{32}P-labeled bacterial populations in which the normal ratio of the amounts per cell of RNA to DNA has been reduced by phosphorus starvation, lose their enzyme-forming capacity at the same rate as non-starved, RNA-rich cells. These observations suggest that, as for loss of viability, decay of ^{32}P atoms in the bacterial DNA is principally responsible for loss of enzyme-forming capacity.

3. The capacity of ^{32}P-labeled *E. coli* cultures for synthesis of RNA and protein is also destroyed by radioactive decay, albeit at a rate which is somewhat less than the rate of loss of viability or capacity for enzyme formation of the same bacteria.

4. From these experiments it is concluded that the DNA of the bacterial cell functions *as an integral unit* in the synthesis of its enzymes.

REFERENCES

1 J. BRACHET, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 2, Academic Press. Inc., New York, 1955, p. 476.
2 R. D. HOTCHKISS, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 2, Academic Press, Inc., New York, 1955, p. 435.
3 A. B. PARDEE, *Proc. Natl. Acad. Sci. U.S.*, 40 (1954) 263.
4 S. SPIEGELMAN, H. O. HALVORSON AND R. BEN-ISHAI, in W. D. MCELROY AND B. GLASS, *Amino Acid Metabolism*, The Johns Hopkins Press, Baltimore, 1955, p. 124.
5 S. S. COHEN AND H. BARNER, *J. Bacteriol.*, 69 (1955) 59.
6 E. F. GALE AND J. P. FOLKES, *Biochem. J.*, 53 (1953) 483.
7 S. SPIEGELMAN, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, The Johns Hopkins Press, Baltimore, 1957, p. 232.
8 C. R. FUERST AND G. S. STENT, *J. Gen. Physiol.*, 40 (1956) 73.
9 E. M. WITKIN, *Cold Spring Harbor Symposia Quant. Biol.*, 16 (1951) 357.
10 J. LEDERBERG, in L. C. DUNN, *Genetics in the 20th Century*, Macmillan, New York, 1951, p. 263.
11 G. S. STENT AND C. R. FUERST, *J. Gen. Physiol.*, 38 (1955) 441.
12 D. FRASER AND E. A. JERREL, *J. Biol. Chem.*, 205 (1953) 291.
13 G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
14 J. LEDERBERG, *J. Bacteriol.*, 60 (1950) 381.
15 A. B. PARDEE AND L. S. PRESTIDGE, *J. Bacteriol.*, 70 (1955) 667.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
17 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
18 C. F. ROBINOW, in R. J. DUBOS, *The Bacterial Cell*, Harvard University Press, Cambridge, Mass., 1949, Appendix.
19 M. OGUR AND G. ROSEN, *Arch. Biochem.*, 25 (1950) 262.
20 M. COHN, J. MONOD, M. R. POLLOCK, S. SPIEGELMAN AND R. Y. STANIER, *Nature*, 172 (1953) 1096.
21 J. MONOD, A. M. PAPPENHEIMER JR. AND G. COHEN-BAZIRE, *Biochim. Biophys. Acta*, 9 (1952) 648.
22 H. V. RICKENBERG, G. N. COHEN, G. BUTTIN AND J. MONOD, *Ann. Inst. Pasteur*, 91 (1956) 829.
23 A. B. PARDEE, *J. Bacteriol.*, 73 (1957) 376.
24 M. R. POLLOCK, in E. F. GALE AND R. DAVIES, *Adaptation in Micro-organisms*, Cambridge University Press, Cambridge, England, 1953, p. 150.
25 L. SIMINOVITCH, *Ann. Inst. Pasteur*, 84 (1953) 265.
26 J. LEDERBERG, *Genetics*, 33 (1948) 617.
27 S. E. LURIA AND M. C. HUMAN, *J. Bacteriol.*, 59 (1950) 551.
28 S. BENZER, *Biochim. Biophys. Acta*, 11 (1953) 383.
29 E. KELLENBERGER, *Symposium on Bacterial Cytology, Intern. Congr. Microbiol.*, 6 (1953) 45.
30 M. ROSENBAUM, *Doctoral Dissertation*, University of Michigan, Ann Arbor, 1955.
31 A. B. PARDEE, unpublished.
32 G. S. STENT AND C. R. FUERST, *Virology*, 2 (1956) 737.

Received July 31st, 1957