CHEMICAL STUDIES IN HOST-VIRUS INTERACTIONS*

VIII. The Mutual Reactivation of T2r+ Virus Inactivated by Ultraviolet Light and the Synthesis of Desoxyribose Nucleic Acid

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It was shown by Hershey (1) and by Delbrück and Bailey (2) that in the infection of *Escherichia coli* by two different bacterial viruses of the T-even type, each of which contained two or more genetic differences, virus progeny were produced, the individuals of which contained any possible combination of the characters used. A related phenomenon, designated mutual reactivation, has been described by Luria and his colleagues (3, 4). Luria has shown that although infection of bacteria with a single ultraviolet-inactivated phage did not result in virus production, the multiple infection of *E. coli* with this ultraviolet-inactivated phage sometimes led to virus production under suitable conditions. These phenomena are of the greatest interest in the study of the mechanism of virus synthesis and genetic duplication, as well as of the interference phenomenon, in which it has been shown, among other things, that treatment of the host cell with an ultraviolet-irradiated virus will impair the ability of that cell to produce this virus or some others on subsequent treatment with active virus.

It has been proposed by Luria and Dulbecco (4) that a virus particle consists of a definite number of discrete material genetic units each of which can be lethally altered in a single random hit by ultraviolet irradiation, and that virus multiplication within the infected cell can occur if there is present at least one of each unit in non-lethal form. It was further proposed that the fulfillment of this requirement is adequate for virus production, regardless of the presence of inactive units. According to this hypothesis a particle hit in two sites will be effective to cause virus multiplication if the two active units are provided by another inactivated particle undamaged in these particular sites. The latter prediction has been tested and it was indeed found that the probability of production of active phage from inactive phage depends on the dose of radiation and on the multiplicity of infection in a manner similar to that predicted (4). Deviations occurred at high multiplicities, which were ascribed to a limitation in the efficiency of recombination when the active units were derived from more than two inactivated particles.

The most remarkable corollary of this theory is independently supported by other lines of evidence. This corollary states that an infecting virus particle is dissociated within the host to form a number of discrete genetic units; *i. e.*, that an infecting par-

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ticle loses its structural integrity as an organism before being used for reproduction. It may be noted that (*a*) infective virus has never been found after infection until late in the latent period, regardless of the method employed for the disruption of the infected host (5); (*b*) isotopically labelled virus used in infection has not been recovered in the viral progeny (6, 7) but most of the isotope has been recovered in nonsedimentable constituents (7); (*c*) the kinetics of synthesis of virus substances and virus was found to be linear throughout most of its course (5, 8, 9). Exponential increase would be anticipated if virus reproduction was comparable to most cellular reproduction in which growth occurs within an intact organism, followed by division. (*d*) No synthesizing or other enzymes essential to energy production and synthesis have been found in the phages, in agreement with (*c*) which suggests that virus synthesis depends on the host's enzymatic equipment. The specificity of virus synthesis within the host cell is presumably directed by the portions of an infecting virus. There can be no way of imagining this latter process, employing only the short-range forces known to chemistry, unless it is conceived that the numerous material virus fragments are separated from the large tadpole-shaped particle. In this virus, the nucleoproteins are in large measure organized within a distinct membrane.

In attempting to explain the selective bringing together of active genetic units, Luria and Dulbecco (4) proposed the hypothesis of "independent reproduction of individual units to form a 'gene pool', from which the new active particles could be derived." It has been shown by one of us (10) and Robinow[1] that an important viral constituent, desoxyribose nucleic acid (DNA), is not synthesized in bacteria infected with heavily irradiated T2. Luria and Dulbecco (4) correctly consider it important that this evidence be confirmed under the conditions of their experiment; *i.e.*, mutual reactivation. Such confirmation would then signify that there is no reproduction of active units when they are not all present or that at least part of the reproduction of the units may not involve an increase in desoxyribose nucleotides.

A test of the hypothesis of independent reproduction of gene pools was therefore made by examining the course of nucleic acid synthesis under conditions in which mutual reactivation could be essentially complete; *i.e.*, under conditions in which a complete number of active units were supplied to the host in several irradiation-inactivated particles. It was found that DNA synthesis was completely inhibited until an intact virus particle could be found within the cell; *i.e.*, until reactivation had occurred.

Materials and Methods

Phage System.—The system has been described in previous papers. *E. coli* strain B was freshly grown for each day's experiment in the glucose-NH_4^+ medium to 2×10^8 organisms per cc., as estimated turbidimetrically (11). The bacteria were infected at that concentration with the desired amount of purified active or irradiated $T2r^+$. Virus preparation, $T2r^+$–6 suspended in 0.85 per cent NaCl was used in most of the experiments. Its analytical characteristics have previously been described (12).

The irradiation of the virus was accomplished with the aid of the apparatus described and

[1] Robinow, cited by Luria and Dulbecco (4).

kindly loaned by Dr. W. Henle of this Hospital (13). The number of hits (n) or "lethal mutations" was determined from the relation, ratio of survivors to starting active population $s = e^{-n}$ (14). For the purposes of these experiments, n, the number of average hits per particle in any preparation was not permitted to exceed one-quarter of the total number of discrete units. This is given by Luria as 25 for T2r$^+$ (4). In most of the studies a preparation of T2r$^+$ was used which had been subjected to an average of 3.3 hits.

The numbers of completely active particles were determined by the plaque count method (15). Under the relatively high survival values used in these studies, this procedure measured virus at single infection.

Chemical Estimations.—The colorimetric estimation of DNA has been described (10). RNA was estimated by the method of Schneider (16).

Analysis of Ultraviolet Absorption.—We had observed several years earlier that a bacterial suspension at 2×10^8 per cc. yielded an ultraviolet absorption spectrum with a marked nucleoprotein peak and a maximum at 2600 Å. These and subsequent spectra were determined with the Beckman spectrophotometer. Racker and Adams have noted that the density of a suspension at 2600 Å of infected bacteria increased linearly whereas treatment of these bacteria with heavily irradiated phage inhibited the normal increase of density of a growing culture (17).

In pursuing these preliminary data it seemed desirable to know how the increase in density correlated with the DNA increment estimated chemically. In Fig. 1 are presented data on the ultraviolet absorption spectra of our host cells at 2×10^8 per cc., prepared as described above, a solution of sodium desoxyribonucleate of known P and DNA content, and of T2r$^+$ bacteriophage.

Suspensions of bacteria infected with active virus were removed from aerated cultures at 37° and examined within 1 minute at room temperature at 2600 Å in the Beckman spectrophotometer. The density increments were plotted directly against time, or these increments were converted to DNA from the data in Fig. 1; *i. e.*, a density increment of 0.01 was equivalent to 0.357 γ DNA per cc. and the DNA value thus obtained was plotted against time. In Fig. 2 is presented a comparison for infected cells of the increment in DNA estimated spectrophotometrically and the increment in DNA estimated chemically. The data in Fig. 2 were determined on a suspension of bacteria at a 2-fold multiplicity of infection. In numerous experiments, the slopes of the increment in DNA as determined by the two methods were essentially identical. However, as the multiplicity of infection was increased, the curves were less separated in time although indicating equal rates. This is discussed in a later section.

Turbidimetry of Infected Cells.—The effect of T2r$^+$ infection in this medium has been described (11). It can be noted that in the glucose medium a culture of cells infected with T2r$^+$ showed a marked initial drop in opacity (11) followed by a rapid recovery to the original level and a slow increase until lysis ensues. There was essentially no difference in these curves whether the multiplicity of infection was 2, 4, or 6. The small percentage of uninfected cells under conditions of 2-fold multiplicity (*ca.* 11 per cent) increased only slightly before they were infected at 30 minutes as a result of the first burst.

However, under identical conditions of 2-fold multiplicity with T2r$^+$ hit 3.3 times by irradiation, the culture showed a much smaller drop in opacity and increased its opacity more than in infection with active virus. This slight but significant difference was due to the multiplication of uninfected cells which went on unchecked for over an hour, since, as will be described below, the latent period and lysis under conditions of mutual reactivation were considerably delayed.

With 4- and 6-fold multiplicities, this factor was essentially eliminated, and the opacity of the culture increased at a constant rate. In addition at higher multiplicities, the differences between the two types of infected culture were more pronounced. Some of these data may be

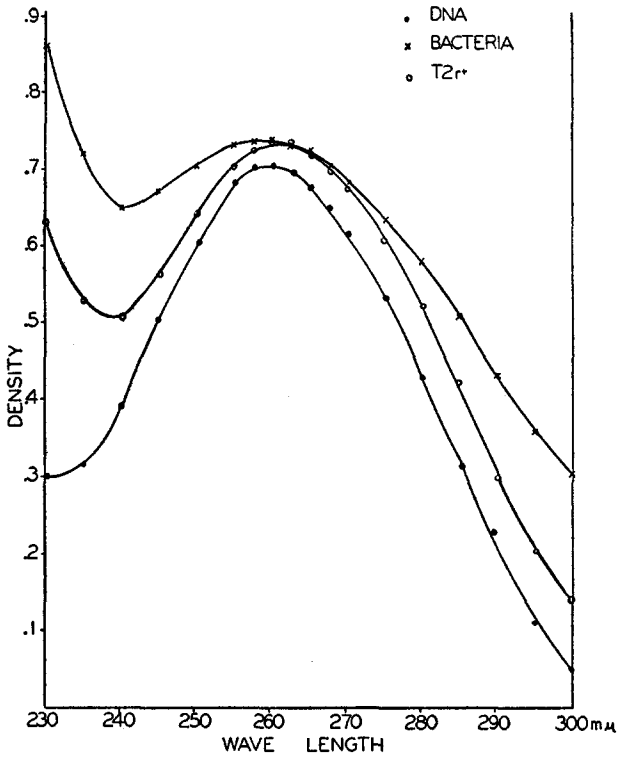

FIG. 1. A comparison of the ultraviolet absorption spectra of suspensions of *E. coli* strain B, T2r+ virus, and desoxyribose nucleic acid (DNA)

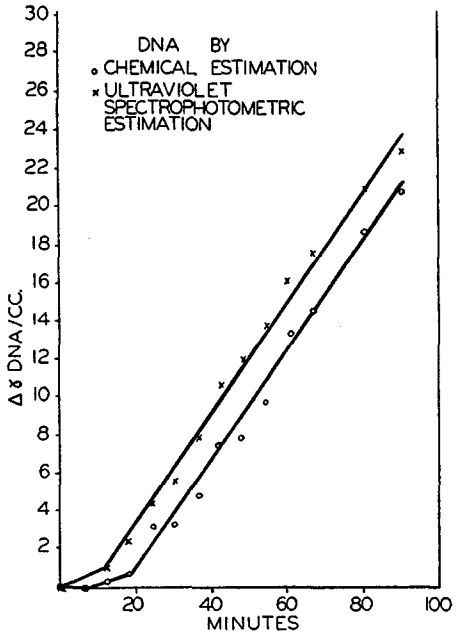

FIG. 2. A comparison of the synthesis of DNA in T2r+-infected cells, estimated chemically or from the ultraviolet absorption increment of the bacterial suspension.

seen in Fig. 3 in which is presented a turbidimetric study on infected and uninfected bacteria at a ratio of 6 of virus to bacteria.

The Effect of Multiplicity of Infection on DNA Synthesis.—Since the multiplicity of infection is important in establishing the reactivation, it was of interest to determine the effect of increasing multiplicity on DNA synthesis.

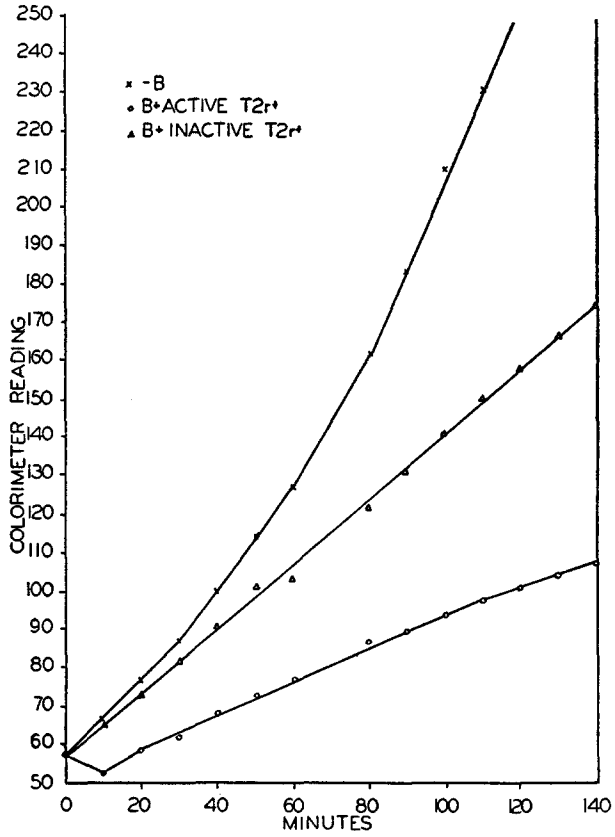

FIG. 3. A comparison of the increase of turbidity of cultures of bacteria without virus, or treated with active or irradiated T2r$^+$ (3.3 hits) at 6-fold multiplicity.

In Fig. 4A it can be seen that the time of inception of DNA synthesis, as determined chemically, was independent of the multiplicity. However, as the multiplicity increased the rate became maximal and constant earlier. Thus the curves for 4- and 6-fold multiplicity are similar and parallel to that for 2-fold multiplicity.

When a similar experiment was done, as in Fig. 4B, following nucleic acid synthesis spectrophotometrically, constant rates are seen to be established

in all cases up to 6-fold multiplicities at the same time, the curves being essentially identical. The slightly greater rate at the 2-fold multiplicity was due, as discussed earlier, to the slight increase in uninfected cells which became infected late. Thus in any one experiment in which the density of an infected culture at 2600 Å and its DNA content are being followed simultaneously, the separation of the curves describing DNA estimated from this density and the chemically estimated DNA will be a function of the multiplicity of infection.

DNA Synthesis under Conditions of Mutual Reactivation.—It may be estimated from the equations of Luria and Dulbecco on the probability of produc-

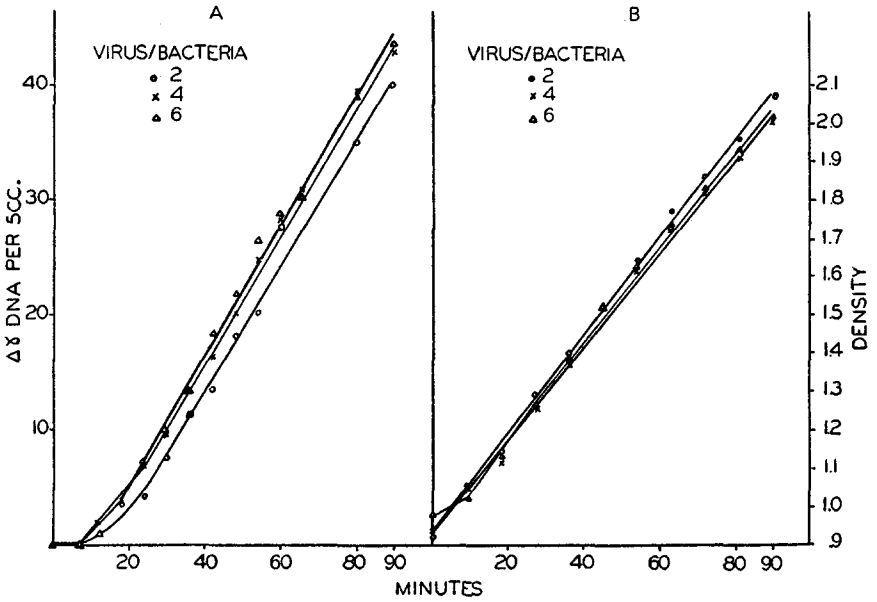

FIG. 4 A. A comparison of DNA synthesis in T2r$^+$-infected cells at different virus-cell ratios. B. A comparison of the increase in density at 2600 Å of the infected cells at these ratios.

ing an active infection under given conditions of multiplicity and of the number of hits that, with virus particles having 3.3 hits, mutual reactivation should be essentially complete at multiplicities of 3 and higher. The course of DNA synthesis was followed in cells at 2×10^8 per cc. to which were added irradiated T2r$^+$, averaging 3.3 hits per particle, at average multiplicities of 2, 4, and 6 virus particles per cc. Typical data are presented in Fig. 5A.

It can be seen that the start of DNA synthesis was delayed under these circumstances by at least 25 minutes. Of considerable interest however, is the fact that although the numbers of active units per cell were increased as the multiplicity increased, there was not a marked shift toward the normal state of affairs in the inception of DNA synthesis. On the contrary it was frequently

observed that as multiplicity increased, synthesis was even more delayed and occurred at a somewhat reduced rate. Since in the inactivated virus preparation about 5 per cent of the virus particles were unhit, at the 6-fold multiplicity about 25 per cent of the cells were infected with at least one completely active particle. Despite this, no early DNA synthesis at this multiplicity was detected although the analytical technique could have found it. Thus the presence of inactive virus of the homologous strain clearly interfered with DNA syn-

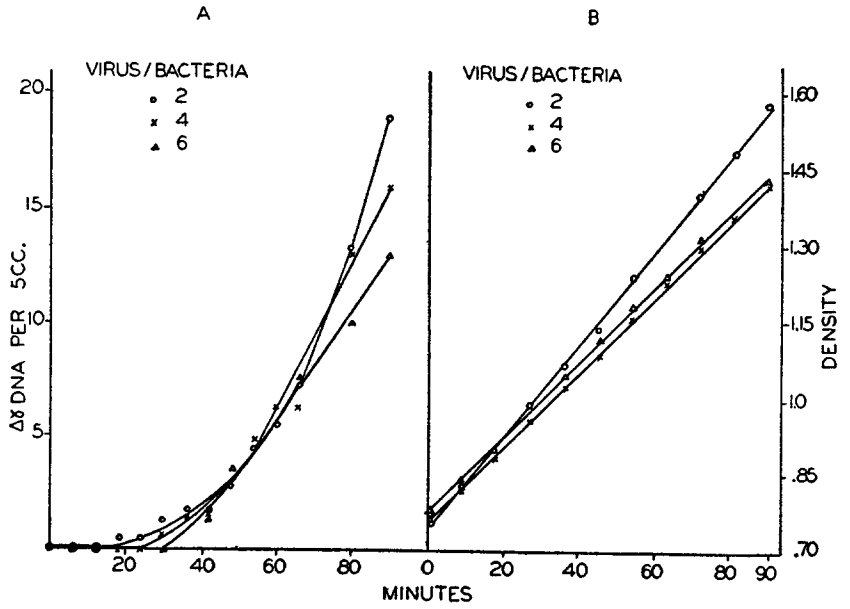

FIG. 5 A. A comparison of DNA synthesis in bacteria treated with irradiated T2r$^+$ at different virus-cell ratios. B. A comparison of the increase in density at 2600 Å of the cells treated with irradiated T2r$^+$ at these ratios.

thesis, and hence with virus synthesis, in cells simultaneously infected by completely active virus or containing a plethora of active units.

The question arose as to whether purine and pyrimidine synthesis as reflected in the ultraviolet absorption was comparably inhibited in these systems. As can be seen in Fig. 5B, no inhibition was noted in this synthesis.

The dissociation of the synthesis of ultraviolet-absorbing materials can be more clearly seen in Fig. 6, which presents DNA synthesis and the increment of ultraviolet-absorbing materials in cells treated at a 2-fold multiplicity with either active or irradiated T2r$^+$. It can be seen that for cells infected with active virus the two synthetic functions are only slightly separated in time, whereas in cells treated with inactive virus, the ultraviolet absorption increases

linearly from the beginning while the usual DNA synthesis curve begins after about 45 minutes.

It should be noted however, that in most mutual reactivation experiments, a slight DNA increase appeared at the time it occurred in normally infected cells and then rapidly levelled off at the new level until the major synthesis

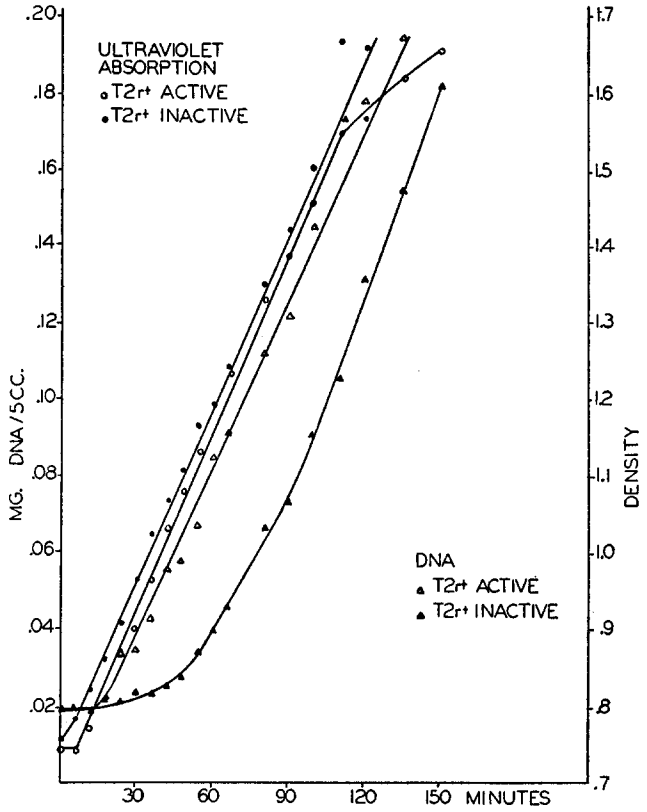

FIG. 6. A comparison of DNA synthesis and ultraviolet absorption increments at 2600 Å in cells treated with active or irradiated T2r$^+$ at a virus to cell ratio of 2.0.

began. Under conditions of 2-fold multiplicity, this increase was of the order of 10 to 20 per cent. Under conditions of 6-fold multiplicity however, it was considerably greater; *i.e.*, about 50 to 75 per cent.

An experiment with the latter multiplicity is presented in Fig. 7. It can be seen that the curves for DNA synthesis and ultraviolet increment were quite close in the infection with active virus. However, with inactive virus the increase in absorption at 2600 Å started immediately, and characteristically, at a greater rate than with active virus. DNA increased 50 per cent between 6 and

12 minutes, levelled off until 45 minutes and started again sharply at the same rate observed in cells infected with active virus.

The Ultraviolet-Absorbing Increment in Mutual Reactivation.—It was found that when an aerated culture of infected cells was removed from 37° to room temperature (about 25°) and the aeration stopped, there was no change in

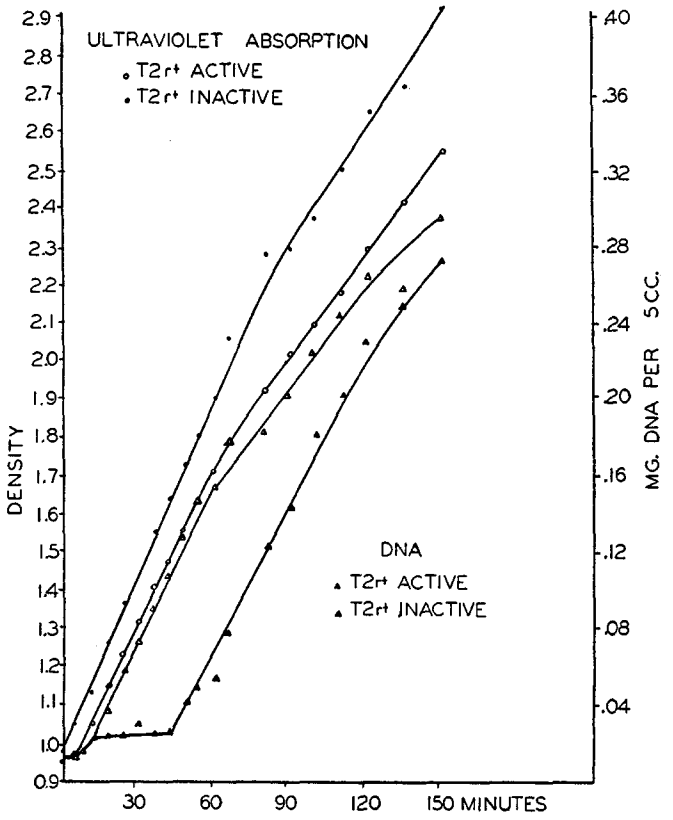

FIG. 7. A comparison of DNA synthesis and ultraviolet absorption increments at 2600 Å in cells treated with active or irradiated T2r$^+$ (virus:cell = 6.0).

ultraviolet absorption over a 10 minute period. Accordingly the absorption spectra of cells treated with inactive virus (3.3 hits) at 6-fold multiplicity were determined after 0 and 15 minute periods of infection. The curves are presented in Fig. 8. There is only a slight difference between the curve of increment and that of the bacteria. Thus the increment indicates a synthesis of material having an ultraviolet absorption spectrum quite similar to that of normal bacteria.

The Appearance of Intracellular Active Virus.—It had been assumed that

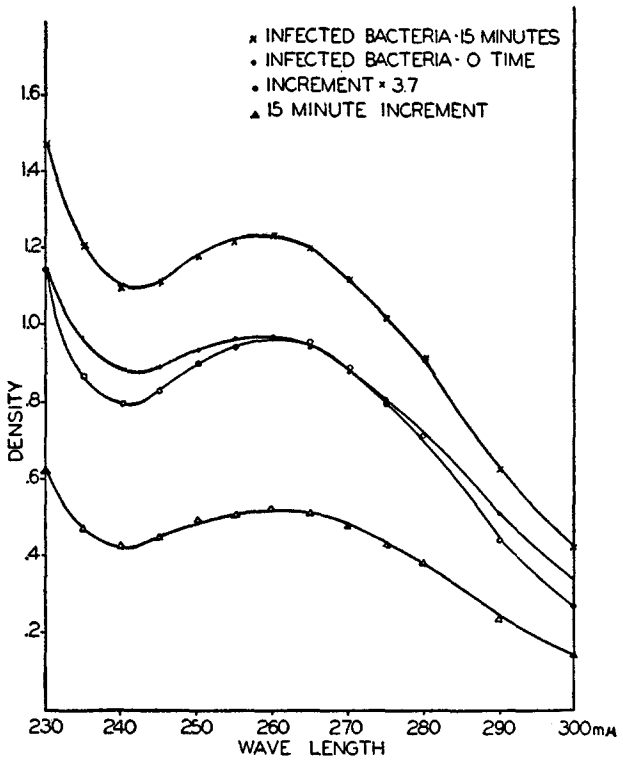

FIG. 8. A study of the ultraviolet absorption spectrum of the change after 15 minutes in cells treated with irradiated T2r$^+$ (virus:cell = 6.0).

TABLE I

The Estimation of Intracellular T2r$^+$ and DNA in Cells Treated with Irradiated T2r$^+$

Time	Operation
min.	
−200	Inoculated 90 cc. of medium with *E. coli*. Aerated at 37° until turbidity indicated 2.0 × 10^8 per cc.
0	Added 0.8 cc. of irradiated T2r$^+$ (3.3 hits) containing 1.25 × 10^{11} virus particles per cc. to bacterial culture A (virus:cell = 6.0).
15	Placed 0.1 cc. A in 2.4 cc. lysing medium (*Ly*)-*a*. *Ly* = 0.01 M NaCN in mineral medium (11). 0.1 cc. *a* + 1.9 cc. Ly = a_1. a_1 stored at 2° for 30 min. at 37° for 45 min. 0.1 cc. lysed a_1 was plated. Removed 5 cc. A to 0.5 cc. 50 per cent trichloroacetic acid as in DNA estimation (10).
	Repeat appropriate dilutions and estimations at 30, 45, 50, 55, 60, 70, 80, 90, 105, 120, 150, and 180 min.

mutual reactivation in this system could be observed under conditions comparable to those of Luria; *i.e.*, cells multiply infected with inactive T2r$^+$ would lyse and yield active virus, thereby providing an infectious center available for estimation in the usual plating technique. It was found that one-step growth curves could be readily obtained with the active T2r$^+$ but that plaques were unobtainable when bacteria treated with inactive T2r$^+$ under conditions suita-

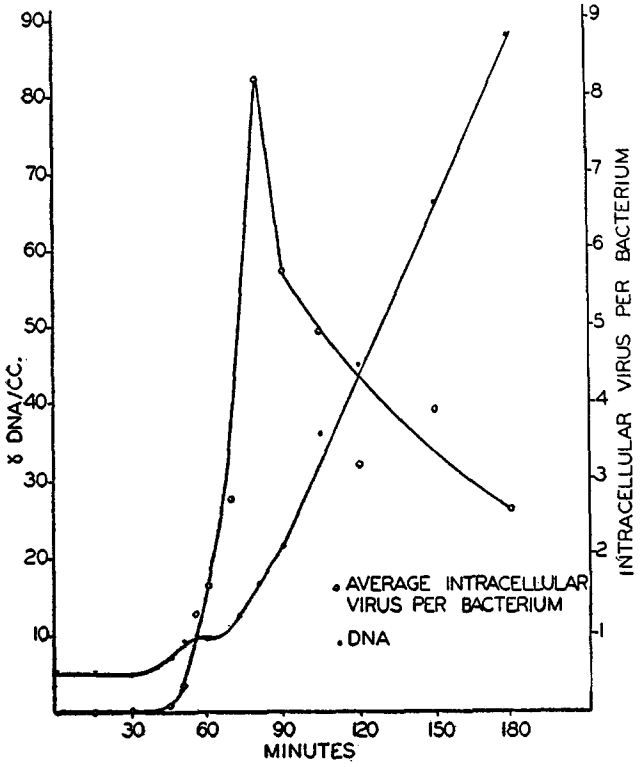

FIG. 9. A study of the formation of intracellular virus and DNA in cells treated with irradiated T2r$^+$ (virus:cell = 6.0).

ble for reactivation were plated in the usual way. It was soon found that despite the DNA synthesis in this system characteristic of virus synthesis, the cells did not lyse to liberate virus under the usual conditions.

Accordingly the cyanide technique of liberating intracellular virus (5) was used as described in Table I, while DNA estimations were run in the same culture. In Fig. 9 it can be seen that the reactivation process manifested by the time of appearance of about one particle per cell occurred in this experiment just a few minutes prior to the beginning of the major DNA synthesis.

As can be seen in Fig. 9, after the quantity of intracellular virus had reached

about 8 particles per cell, there was a sharp fall in the apparent intracellular virus content although DNA synthesis continued normally. Turbidimetric studies of the susceptibility of these cells to cyanide lysis revealed that, unlike cells infected with active virus, these cells became sharply more refractory to lysis with cyanide after 90 minutes or at about the time when apparent intracellular virus decreased. There is no indication at the present time whether this peculiar lysis inhibition is related to the particular r^+ strain used, or is a more general environmental effect.

DISCUSSION

It has been shown that in the period in which mutual reactivation occurred in this system (which can be taken as the time prior to the existence of one intact particle per cell) there was an inhibition of DNA synthesis. This inhibition appeared only after synthesis began, the synthesis apparently stopping less quickly as the multiplicity of inactivated particles increased from 3 to 6. It might be postulated that this slight initial synthesis corresponds to the time necessary for the damaged model (lethal unit) to interfere competitively at the enzyme site with the other active allelic models provided.

In all cases studied however, DNA synthesis was totally stopped sooner or later, without any synthesis referable to a definite proportion of undamaged genetic units. This may be taken to mean, as recognized by Luria and Dulbecco, either that the reproduction of genetic units requires the coordinated activity of all the undamaged genetic units, or that an increase in DNA has nothing to do with the reproduction of the active units. However, there are data which strongly suggest that the synthesis of DNA and the synthesis of genetic units are connected. These are summarized in another paper in this series (12). Nevertheless, it has not yet been shown whether virus protein was synthesized in this system during the inhibition of DNA synthesis.

This raises a closely related problem. It has been shown that under conditions of ultraviolet damage to a virus particle there is a block in the nucleic acid synthesis of the infected host. This block does not affect the synthesis of purine or pyrimidine, nor apparently that of protein, but definitely occurs at the level of DNA synthesis, suggesting that irradiation results in altered DNA which competes with unaltered DNA at the site of synthesis. This might be expected from the data of Zelle and Hollander (18) that the curve of the efficiency of ultraviolet irradiation in the inactivation of T2 plotted as a function of wave length appears to follow the nucleic acid absorption curve. The specificity of this block and of the site of irradiation damage would receive much support if it were shown that virus protein is synthesized in this system. However, this finding would leave the hypothesis of independent "gene pool" synthesis with the loophole that the active exchangeable and irradiation-damageable genetic units are uniquely protein. On the other hand if neither virus protein nor DNA were synthesized, the inhibition of DNA synthesis might be a secondary effect

produced by the block in protein synthesis. It should be noted in this connection that the metabolite antagonist, 5-methyltryptophane, which competes with tryptophane for protein synthesis, inhibits DNA synthesis as well as protein synthesis at concentrations capable of inhibiting T2 synthesis (10). The problems of the synthesis of intracellular virus protein and the relations to DNA synthesis are therefore clearly critical at this point in the solution of several fundamental genetic problems.

In an earlier paper (10) it was shown that most of the phosphorus assimilated under conditions of active virus production was found in the virus component, DNA. In the present paper, it would appear from the correlation of ultraviolet absorption and chemical data that most of the purines and pyrimidines synthesized under conditions of virus production may also be accounted for as newly synthesized DNA. Furthermore, the entire ultraviolet absorption curve of the bacterial suspension can suggest the type of synthesis proceeding under certain conditions. In another paper (19) the use of the ultraviolet absorption spectrum was found to be more sensitive and more accurate in certain microbiological assays.

SUMMARY

A method has been described for following purine and pyrimidine synthesis in intact cells by measuring the ultraviolet absorption of a bacterial suspension. It has been shown that in cells infected with active virus, the rate of increase of ultraviolet absorption at 2600 Å corresponded to that of DNA, although preceding DNA synthesis slightly. The amount of separation of the curves in time was determined by the multiplicity of infection.

Under conditions of mutual reactivation of ultraviolet-irradiated virus, DNA synthesis was inhibited but not the increase in ultraviolet absorption. Thus a continuing purine and pyrimidine synthesis was indicated by the increase in this function. When an intact virus particle could be found within these bacteria, however, DNA synthesis began at the rate characteristic of normal infections. Certain peculiarities of lysis in this system were noted.

The significance of these observations for various genetic theories has been discussed. It would appear that Luria's hypothesis of independent gene pool synthesis has probably been disproven. It has also been shown that the lesion produced by irradiation of a virus particle with ultraviolet light probably occurs in the DNA of the particle. The validity of these conclusions will depend on a knowledge of the relation of virus protein synthesis to DNA synthesis in this system.

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