Preliminary kinetic studies on virucidal activity of human serum suggest that more than one factor may limit the reaction. In view of the multicomponent nature of C′, experiments were performed to determine whether a C′ component as well as properdin is a limiting factor. Accordingly, a constant volume of Rp, which contains C′ components, was added to increasing quantities of serum. The virucidal activity of these mixtures was significantly less than that of serum alone, suggesting inhibition by high concentrations of serum constituents. Experiments are now being carried out using phage labeled with P^{32} or S^{35}, in order to elucidate the mechanism of phage neutralization by the properdin system.

Summary.—The phage-neutralizing action of fresh human serum is heat labile and is dependent on the four recognized components of complement, on properdin, and on a divalent cation(s). Phage neutralization by fresh serum may be of use in assaying properdin.

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*THE MECHANISM OF DNA REPLICATION AND GENETIC RECOMBINATION IN PHAGE**

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Communicated by A. Lwoff, April 12, 1956

INTRODUCTION

The hypothesis that desoxyribose nucleic acid (DNA) carries genetic information has been well established by experiments with the transforming factor of pneumococcus[1] and the functional separation of DNA and protein in bacterial viruses (phage).[2] This, together with the fact that the structure of the DNA molecule seems to be self-complementary,[3] has led to much speculation as to the mechanism by which it is duplicated. However, these speculations have not been very much

limited by experimental results. It has been shown[4] that there is little or no turnover of the atoms of DNA in growing *Escherichia coli*, but there are very few data on the question of how the atoms of this DNA are distributed among newly formed molecules or even newly formed organisms. Are these atoms found in one, in a few, or in many molecules after repeated replications?

Bacteriophage are particularly useful for investigations of this problem. Accepting the hypothesis that DNA is the genetic material of the phage, it seems likely that knowledge of the way in which the atoms of the parents are distributed among the progeny virus particles will help in answering this question. And since the DNA can be removed from these virus particles by artificial means, it is also possible to study the atomic distribution with the extracted material.

However, before considering the phage experiments, we will examine an idealized situation in which DNA molecules are labeled with P^{32} and allowed to reproduce in a nonlabeled cell growing in a nonlabeled medium. The various theories of replication can be classified according to the result each would predict for this experiment. Three possibilities are diagramed in Figure 1. In case *I* the parental

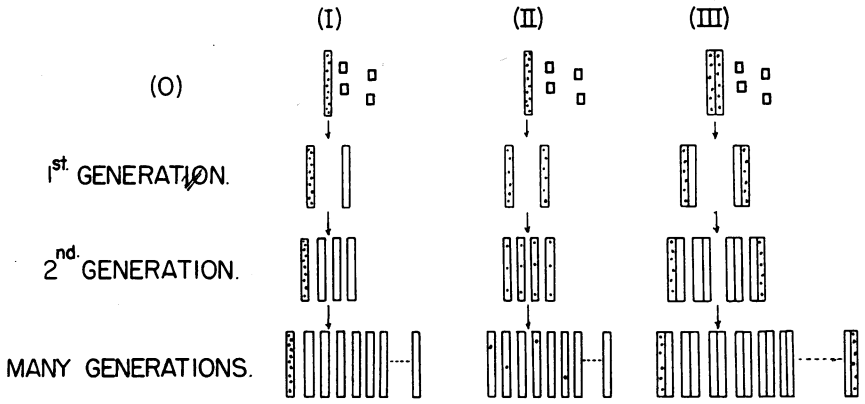

FIG. 1.—Three types of model for the replication process. The dots represent radioactive label, and the open squares represent the nonradioactive subunits used to build the new structure; (*o*) is the original labeled molecule. *I* is template-type replication which leaves the label in one molecule; *II* is a dispersive type of replication (see M. Delbrück, these PROCEEDINGS, **40**, 783, 1954); and *III* is a complementary type (see J. D. Watson and F. H. C. Crick, *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 123, 1953).

particle acts entirely as a template for the formation of its daughters. The P^{32} would continue to reside in one particle regardless of the number of replicas formed, and more and more completely nonradioactive particles would accumulate. In case *II* the atoms of the original structure are distributed equally between the two daughters at each doubling, and, when many particles had been formed, one with more than a single P^{32} atom would rarely be found. In case *III* we have indicated the results of the proposals of Watson and Crick[3] based on the complementary structure of DNA. The P^{32} is originally contained in the two complementary members of the double helix. When duplication occurs, these two members separate, and each makes its own complement from the nonradioactive subunits in the cell. Thus, after one generation, the radioactivity is distributed between the two daughters, as in case *II*. However, further growth would result only

in the formation of more new nonlabeled particles, since each member of the double helix is assumed to maintain its atoms intact. After many generations of growth there would still be two radioactive particles, each of which would have half the original number of P^{32} atoms.

In considering this labeling experiment with the phage T2, it must be noted that there is extensive genetic recombination during the vegetative phase of growth. Although there has not been any advance toward a solution of the problem of the molecular basis of genetic recombination, there are experimental data which can distinguish between certain general classes of schemes which might be involved. In the case of higher organisms, one has been led to the idea of breakage and reunion of already formed chromosomes as the basis of the recombination event. In the bacterial viruses, there is evidence[5-7] to suggest that the process occurs by some sort of partial replica formation which leaves the original structures intact. In this hypothesis the recombinants are thought of as formed *de novo*, partly under the control of one parental structure and partly under the control of another. These two classes of mechanisms lead to different predictions for the P^{32} tracer experiment if one assumes that the genetic structure is DNA. As shown in Figure 2, *A*, each mating event would distribute the P^{32} as it distributes the genes if crossing over involved breakage and reunion of preformed structures, while (Fig. 2, *B*) the partial-replica type of model will not disturb the label in the original structure.

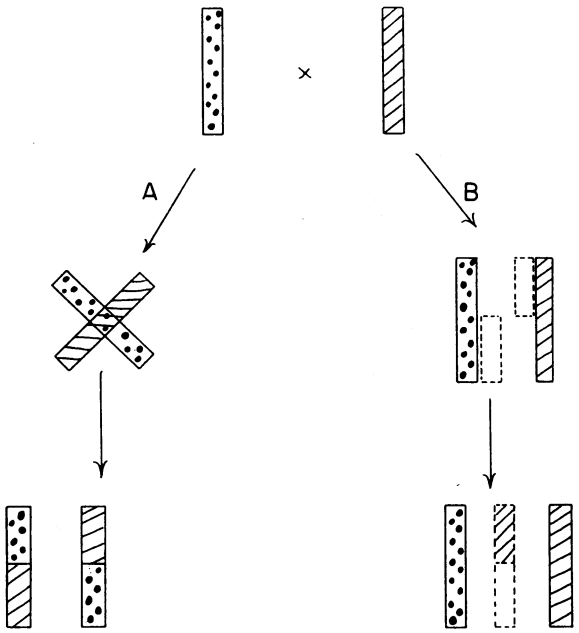

FIG. 2.—Two types of model for the production of genetic recombinants. The dots represent the radioactive label, and the cross hatching represents a second genetic composition. The structures outlined with dotted lines are newly synthesized. *A* represents the breakage-and-reunion type mechanism, and *B* represents partial replica formation.

The tracer experiment with phage is complicated by the fact that only about 40 per cent of the total phosphorus of the parental virus is transmitted to the progeny particles. However, since in one growth cycle there is extensive growth as well as several mating events per particle, we have the problem of distinguishing among the six possibilities obtainable by combining cases *I*, *II*, and *III* for the replication process with *A* and *B* for the recombination events.

In order to study the question directly, a method has been developed using an electron-sensitive photographic emulsion for the measurement of the radioactivity

of a single virus particle or a single DNA molecule, if it produces as few as fifteen disintegrations per month. This paper will give a brief description of the method and a preliminary report of the results obtained.

METHODS

The electron-sensitive photographic emulsion G-5, manufactured by Ilford, Ltd., was used in these experiments. The emulsion is supplied in the form of a gel which is liquefied by warming to 50° C. A layer of emulsion is poured onto a specially prepared microscope slide (also supplied by Ilford), chilled, and allowed to dry.

In our procedure, three spots of emulsion approximately 2 cm. in diameter are poured onto each microscope slide, each spot being contained by a glass ring. The glass rings are removed as soon as the emulsion has solidified, and the emulsion is freed from the ring by means of a sharp needle. When the first layer of emulsion has been dried, a second layer containing the P^{32}-labeled particle is poured on top, and, when this layer is dry, a third of pure emulsion is added. Thus a sandwich of about 200-μ thickness is formed, with the radioactive particle imbedded in the emulsion. The plates are stored under 10 cm. of lead at 5° C. and developed[8] after various exposure times.

When the developed plates are examined under a microscope, one observes stars consisting of several electron tracks emerging from a point in the emulsion. The number of tracks per star can be counted and should correspond to the number of P^{32} disintegrations which occurred in the particle during the exposure time. Since the particles used were the phage themselves and the DNA extracted from them, the tracks of a star all originate in volumes of less than a few cubic microns of the emulsion, and, therefore, background tracks which pass several microns away from the center cause no confusion. Tests with radioactive phage showed that 85–100 per cent of the total disintegration leads to countable tracks and that the method can be used for measuring radioactivity of particles with as few as ten to fifteen disintegrations per month. In general, no attempt was made to count stars with less than five tracks, since these could be simulated by a single electron undergoing a sharp scattering or by two tracks crossing each other.

In order to determine the number of radioactive particles in the solution tested, polonium 210 is added before the material is mixed with the emulsion. The alpha particles emitted by this isotope leave easily distinguishable tracks in the emulsion and can be used as indicators. The ratio of alpha tracks to stars is used to calculate the number of radioactive particles in the test solution.

Radioactive phage was obtained from bacteria grown in a tris-glucose medium[9] containing 0.1 mg. of neo-peptone (Difco) per milliliter and 1.5 μg. of added phosphorus per milliliter. Carrier-free P^{32} was added to give a specific activity of between 100 and 200 millicuries per milligram of phosphorus. A culture of *E. coli* B grown in this medium was infected with phage R2*r*1 at a multiplicity of approximately 0.25 phage per bacterium. After lysis of the culture, the lysate was treated with RNase and DNase, and the phage was purified by three or four cycles of high- and low-speed centrifugation.

Except where indicated, the methods used for the biological assays are those described by Adams.[10]

RESULTS

Tests of the Emulsion Method.—As part of the preliminary investigation into the technique using the photographic method, the purified radioactive virus was put into the emulsion. The P^{32} content of the virus particle was known from the activity of the medium combined with the total phosphorus content per phage, as measured by Hershey.[9] In addition, the rate of decay of the biological activity due to the incorporated P^{32} was measured. From this decay, the so-called "suicide rate," and the efficiency of the P^{32} disintegrations in inactivating the phage as previously measured by Hershey *et al.*[11] and by Stent,[12] the P^{32} per phage was also calculated. These values agreed within an experimental variation of about 15 per cent and also agreed with the P^{32} per phage calculated from the number of tracks per star and the exposure time. However, in some cases the exposure time for the original phage was so short that delays in the drying of the emulsion, especially in warm, humid weather, meant that agreement could be obtained only by allowing the P^{32} to decay before the virus particles were plated, so that longer exposure times were possible. When the standard deviation of the number of tracks per star in a given plate was determined, it was found to be equal to the square root of the mean number of tracks per star, as is to be expected for a process which is governed by the laws of random labeling and random decay.

Figure 3 gives the results of one calibration curve recently obtained in collaboration with Dr. C. A. Thomas, Jr. A labeled phage stock was adsorbed to sensitive cells at low multiplicity, washed by centrifugation, and plated in the emulsion. The data show the observed mean number of tracks per star as a function of the amount of the P^{32} which decayed before development. The slope gives a value for the total number of P^{32} atoms per phage which is in agreement with the value calculated from the "suicide" curve of the same stock.

Transfer Experiments.—The purified P^{32}-labeled phage were adsorbed to sensitive bacteria with less than one phage particle added per bacterium. This low multiplicity of infection was used so that any phage which had been inactivated during the purification would not be able to contribute its P^{32} to the progeny. The effectiveness of this procedure was demonstrated by an experiment in which the phage was stored for several days before the transfer experiment. Under those conditions most of the plaque-forming activity of the phage stock was lost, but the particles could still adsorb to bacteria. However, their P^{32} was not subsequently found in phage particles.

The adsorption was carried out in buffer at 37° C., and the cells were then sedimented at low speed (3,000 × *g*) for 5 minutes. They were resuspended in salt-free broth at 37° C. and centrifuged again to remove any phage which might be reversibly adsorbed. They were again resuspended in salt-free broth and aerated at 37°. In salt-free broth the newly formed phage were not readsorbed to cells, and lysis of the infected bacteria was completed after 35 minutes by the addition of 0.01 *M* KCN. The lysate was centrifuged at low speed to remove the uninfected cells and bacterial debris. The amount of P^{32} in this low-speed pellet was from 25 to 35 per cent of that in the lysate, depending on the amount of suicide which had occurred during the purification. The phage in the supernatent was purified either by high-speed centrifugation or by adsorption to sensitive cells and put into the nuclear emulsion.

Those first transfer phage were also used to infect new bacteria for a second transfer experiment. The second transfer was done in the same way as the first, except that the multiplicity of infection was about two phage per bacterium. The progeny of this second transfer was again plated in the nuclear emulsions. At every stage of the transfer experiment, biological assays were made of the uninfected bacteria, the infected bacteria, and the free phage, as well as Geiger-counter measurements of the total radioactivity of each fraction.

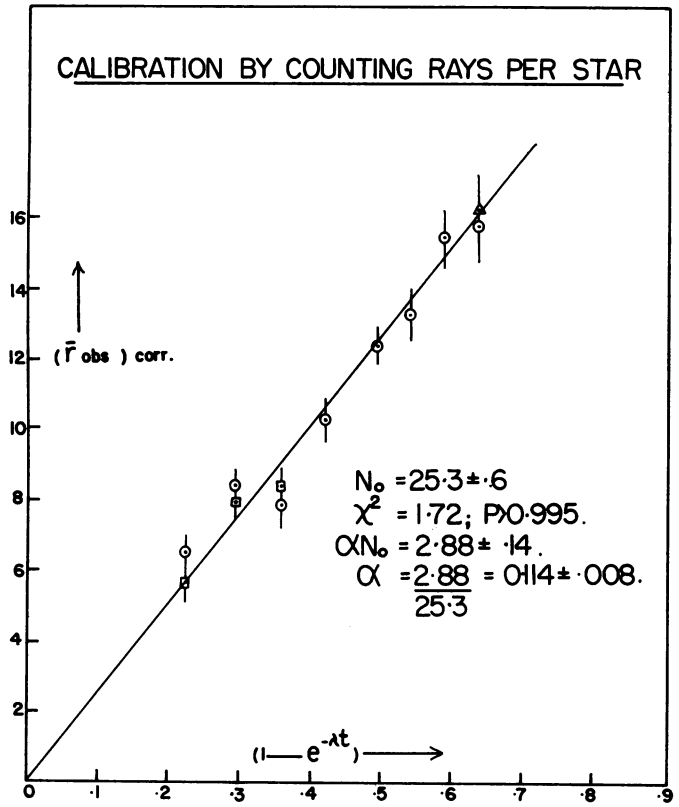

FIG. 3.—($\bar{r}$ *obs.*) *corr.* is the mean value of the star size corrected to take into account the fact that stars of less than 5 tracks are not counted. This correction is negligible for ($\bar{r}$ *obs.*) greater than 10. ($1 - e^{-\lambda t}$) is the fraction of the total P^{32} decaying during the exposure time. See Hershey *et al.*, *J. Gen. Physiol.*, **34**, 305, 1951, for the derivation of the suicide efficiency α. They find α to be about 0.10, to be compared to the value calculated from the star counts.

The results of these control measurements of biological activity and total radioactivity can be summarized as follows. Over 90 per cent of the biologically active phage was adsorbed to the cells, but only about 65–70 per cent of the radioactivity was adsorbed. The burst size in each transfer experiment was between 100 and 150 phages per infected bacterium, and 35–45 per cent of the radioactivity in viable parental phage was transferred to the progeny in each case. Those results are in agreement with similar experiments reported by others.[13]

The nuclear emulsion in which the first transfer progeny were plated showed stars which corresponded to 22 ± 3 per cent of the amount of P^{32} that was in the original parental phage. The number of these phage particles which produce stars was about one per infected bacterium. However, this latter number is subject to more experimental error than the size of the stars. The maximum error in the average star size is about ±15 per cent, while that in the count of the number of stars is about ±40 per cent. The phage of the progeny at the second transfer experiment also produced stars in the emulsion, and these stars corresponded to the same number of P^{32} atoms per phage as was found for the first-generation phage, that is, about 23 ± 3 per cent of the original phage P^{32}. The number of the stars per unit of total radioactivity, as measured in the Geiger counter, was the same in the second-generation progeny as in the first generation, with the limit of error again about 40 per cent.

In order to verify that the star-producing particles were actually phage, the lysates were allowed to adsorb to sensitive and resistant cells, and the infected cells were plated in the emulsion. The same size stars were observed in the plates on which phage adsorbed to sensitive cells were plated, but no stars were to be seen on plates in which the resistant cells were plated.

The distribution about the mean number of tracks per star was consistent with a Poisson distribution for both the first and second transfer phage progeny, as well as for the original labeled phage, as shown in Table 1.

TABLE 1

Material Put in Emulsion	$\bar{r}$ Mean No. Tracks per Star	σ^2 Variance of Distribution	n No. Counted	F Fraction P^{32} Decay during Exposure	N P^{32} in Star Former	N_0 P^{32} in Orig. Phage (from Suicide)†	N/N_0
Original phase	10.2	11	20	0.092	111	110	...
	17.1	20	20	.169	101	110	...
	10.0	9	20	.115	87	90	...
	17.5	22	20	.210	83.5	90	...
Orig. phage DNA*	13.2	13	20	.345	37.4	90	0.43
Orig. phage DNA	14.8	17	70	.150	99	245	.40
1st transfer phage	12.8	10	50	.230	56	245	.23
1st transfer DNA	13.8	11	29	.260	53	245	.22
2d transfer phage	16.5	12	18	.260	63	245	.26
Orig. phage DNA	12.75	12	40	.23	55	130	.42
1st transfer phage	19.3	17	50	.60	32	130	.25
1st transfer DNA	19.6	15	30	.60	33	130	.25
2d transfer phage	21.5	29	30	0.60	34	130	0.26

* "DNA" means the phage suspension after osmotic shock.
† The "efficiency of killing" was taken as 0.10.

Osmotic-Shock Experiments.—An osmotic-shock method[14] was used to disrupt the protein membranes of the phage and release the DNA into solution. The phages were put in a saturated NaCl solution and diluted rapidly with 20 volumes of added distilled water. This procedure reduced the viable titer of the phage to less than 3 per cent of its original value. If the phage stocks are sufficiently concentrated, one observes an increase in viscosity and a drop in light scattering after the shock, and the viscosity is reduced to that of the original suspension by the addition of DNAse. The osmotic shock was done on phage suspensions of various concentration and with various amounts of added nonradioactive phage as carrier. Also, the plating in the emulsion was done with one molar salt added. In all these cases the mean star size obtained was the same.

When the original uniformly labeled phage was subjected to osmotic shock, the number of stars produced by the solution was the same as that produced by the intact phage at the same dilution. However, the size of the stars dropped to about 40 per cent of those produced by the intact phage. When the progency of the first transfer experiment was similarly treated, the number of stars remained unchanged, but the star size also remained unchanged at its value corresponding to about 20 per cent of the original labeled phage. In both cases the variance of the observed star sizes was equal to the mean, thus indicating a Poisson distribution. In all cases where shocked material was plated after treatment with DNAse. no stars were observed.

These experiments indicate that the original phage contained one large piece of DNA with about 40 per cent of the P^{32} of the phage and a number of small pieces too small to be detected in these experiments. The molecular weight of the large piece would be about 45 million, and that of the small pieces would be less than 7–8 million. The fact that the star size does not change when the progeny phage is shocked seems to indicate that the P^{32} causing the stars is in a single DNA molecule with about 20 per cent of the P^{32} of the original phage.

The experiments on the DNA molecule will have to be repeated with other methods used for extraction. However, the difference in the results obtained for the parental and the progeny phage would appear to eliminate most of the obvious kinds of artifact, such as partial extraction, as being responsible for the existence of the stars. Also, the fact that sharp distributions (i.e., distributions which had a variance equal to the mean) were obtained for the star sizes indicates that the osmotic shock was not producing random degradation of a single DNA particle.

DISCUSSION

The fact that the molecular-weight distribution is so extremely nonuniform is surprising but not greatly at variance with other measurements on this material. The most reliable results for the molecular weight of DNA have been obtained by means of light-scattering studies[15] which determine a weight-average molecular weight. If we take 2.0×10^{-10} μg. as the DNA content of the phage,[9] then the molecular weight of the large piece would be 48 million and the weight average would be 19–25 million. However, regardless of whether the large piece is truly free DNA in solution, the discussion to follow requires only that it is a unique piece and not a random breakdown product. This conclusion is suggested by the difference observed between the effect of osmotic shock on the original phage and the effect on the first transfer progeny, as well as the narrow distribution found for the star size of the osmotically shocked material.

Making the assumption that the large piece of DNA is the genetic structure of the phage, we can, by combining our measurements of the size with Benzer's[16] finding that there are at least 20,000 possible gene mutations in this phage, conclude that there are, at most, four nucleotide pairs involved in the minimum distance of recombination.

The persistence of a piece of DNA with a high level of labeling seems to rule out any mechanism of duplication which implies repeated sharing of the atoms of the parental structure between the daughters. In our case each single-step growth cycle represents a multiplication of the DNA by a factor of 30–50, even before the

appearance of any mature phage. Therefore, two *growth cycles* correspond to multiplication by at least 1,000-fold.

The fact that there are single phage particles with about 20 per cent of the P^{32} in the original particles after both the first and the second growth cycle, with no drop in the activity of these labeled particles in the second cycle, does not, of course, imply that there is a 100 per cent transfer of efficiency for these particles, but only that the loss is due to an all-or-none transfer process. The additional finding that the fraction of the total radioactivity which is in stars remains unchanged during this second transfer is in agreement with the experiments of Maaløe and Watson,[17] showing equal transfer efficiency in the first and second vegetative cycles.

The simplest interpretation of all the data is in terms of the Crick-Watson type of complementary replication indicated in Figure 1, case *III*. The phage has a large piece of DNA of molecular weight about 40 million and a number of small pieces of molecular weight less than about 8 million. When growth occurs, this large piece splits and produces two particles, each of which has half the radioactivity of the original large one. The small pieces are distributed widely among the progeny phages and, therefore, no longer contribute to the formation of stars. The progeny phages which do produce stars are those which receive the half-labeled large piece. We assume (after Hershey)[18] that the 40 per cent transfer is the result of accumulated inefficiencies in the growth process which apply equally to the large and small pieces of DNA.

Stent and Jerne[19] reported some very ingenious experiments designed to determine the distribution of P^{32} among the phages of the first transfer progeny. Their method was based on observing the decrease in the transferability of the P^{32} in a second-generation transfer experiment. Their conclusion was that the P^{32} was distributed among 8–25 particles. Further studies[20] indicate that the decrease in transferability stops when it has dropped to about half its original value, and these results, if analyzed as a two-component system, are not in disagreement with those reported here.

The persistence of the highly labeled piece of DNA, particularly through the second-generation transfer, immediately suggests that genetic recombination does not occur by breaking and reunion of the DNA molecule. However, this conclusion is based on the assumption that the large piece of DNA is the chromosome of the phage. In order to test this assumption, experiments are being carried out in collaboration with Dr. C. A. Thomas, Jr., in which a P^{32}-labeled phage of one genotype is crossed with a nonlabeled phage of another genotype. Preliminary results indicate that there is a close and possibly complete association between the ability of the phage in the progeny to produce stars and the markers of the labeled parent.

Visconti and Delbrück[21] analyzed the kinetics of a phage cross under the assumption that the mating event is analogous to that in higher organisms and involves breakage and reunion of preformed chromosomes. With the additional assumption that the matings occur randomly in a pool of noninfective particles, presumably nucleic acid, they calculate that there would have to be, on the average, about 2.5 matings per particle in this pool before any appearance of infective phage. Since it seems reasonable that we are observing the structure which

carries the genetic information of the phage, one can conclude that this structure remains intact even during a phase in which genetic recombination is known to be taking place. If mating involved breakage and reunion, one can demonstrate that the star size would decrease to about 30 per cent during the second-generation transfer. If the mechanism for the production of recombinants involves partial replica formation the calculations of Visconti and Delbrück must be modified, but the problem can still be considered as one of population genetics, with matings taking place randomly in the pool. Under this assumption, matings would not imply any decrease in star size during the second transfer.

The detailed predictions inferred from the Crick-Watson model (Fig. 1, case *III*) cannot be checked by these experiments for the following reasons: First, it is not feasible to examine the DNA molecules of the phage after one doubling, but only after a complete growth cycle which yields a burst of approximately one hundred new phages. Second, the inefficiencies of the system which produce the transfer of only 40 per cent of the total parental phosphorus prevent one from observing whether there are really two labeled particles formed from each parental structure. For these reasons, experiments are being undertaken to study the distribution of the label of the DNA extracted from growing bacteria in which it is known that atoms once incorporated in DNA remain.

Throughout this discussion it has been assumed that DNA causes its own replication without the formation of any intermediates. Although experiments have been reported[12] suggesting that there is a transfer of genetic information to a non-DNA component, the evidence does not, at present, seem sufficiently compelling to require consideration of this extra complication.

The theoretical problem of how the DNA might replicate in spite of the fact that it must rotate along its length has been considered by Levinthal[22] and Crane and does not seem to be as serious as was believed.[23] However, no satisfactory detailed model has been suggested to explain how two such replicating structures could co-operate to make a recombinant entirely of new material. It is likely that the direct product of the mating is a heterozygous particle which is complementary in the Watson-Crick sense over most of its length but not complementary for a few mutated genes which lie on a short overlap region. This particle, which has the nucleotide order of one parent on one side of this overlap and that of the other parent on the opposite side, would duplicate to produce the observed progeny, but it is not clear what molecular interactions would be required for its formation.

SUMMARY

The experiments reported here suggest that the phage T2 contains in one large piece approximately 40 per cent of its DNA. This piece of DNA probably contains the genetic markers so far mapped, and it replicates to produce particles, each of which contains about half its atoms, but no further distributions of the original atoms take place with further replication. We have no information as to the size or function of the small pieces, except that each of them is less than about 8 per cent of the total in the phage. Thus we conclude that the large piece of DNA probably replicates in the complementary manner suggested by Crick and Watson and that genetic recombination takes place by the formation of partial replicas.

I should like to thank Drs. Lwoff and Monod, in whose laboratory much of this work was done, for their co-operation and encouragement. I also owe much to Drs. F. Jacob, D. Kaiser, and E. Wollman for their advice as well as for their help in carrying out the experiments. Great assistance in the storage and development of the emulsions was given by Mlle. Petit of the biophysics group of the Commissariat d'Énergie Atomique.

* This investigation was supported in part by research grants C-2159 (C2) and RG 4557 from the National Institutes of Health. A preliminary report of this work was published in the proceedings of the Basalli conference held at Pallanza, Italy, July, 1955 (*Rend. Ist. lombardo sci.* **89**, 192, 1955).

† Many of these experiments were performed while the author was a fellow of the National Foundation for Infantile Paralysis working at L'Institut Pasteur, Paris, France, Service de Physiologie Microbienne.

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THE PHYTOENE CONTENT OF TOMATOES

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Communicated by R. E. Clausen, May 14, 1956

With an appropriate genetic makeup, the fruit of the tomato, *L. esculentum*, is capable of synthesizing substantial amounts of carotenoid pigments. The common red-fruited varieties may contain 70–150 μg. of all-*trans* lycopene per gram of fresh fruit. Beta-orange types, first obtained by Lincoln and Porter,[1] contain comparable concentrations of β-carotene. A single gene difference between the two at the *B* locus determines orange versus red phenotypes. The present study is an outcome of the discovery by O. Brauer and C. M. Rick—made in-