STUDIES ON THE REPLICATION AND TRANSFER TO PROGENY OF THE DNA OF BACTERIOPHAGE T4

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ABSTRACT

The purpose of the present research has been to learn more about the mechanism of replication and ultimate fate of the DNA of the phage T4. The distribution of parental material in DNA molecules in the replicating pool and in the DNA obtained from progeny phage has been examined. The association of parental atoms with the r genotype has also been examined.

Phage in which the nitrogen and carbon were replaced by the stable heavy isotopes N^{15} and C^{13} were used to infect unlabeled bacteria. At various times after infection, aliquots of the culture were removed and lysed; finally, progeny phage were harvested and lysed. DNA molecules containing heavy atoms show a corresponding increase in their density. Separation is achieved by density-gradient centrifugation.

Most parental atoms transferred to progeny are found in DNA of light density. Following fragmentation, DNA containing parental atoms is found to have shifted to densities between those of light and half-heavy DNA. In the replicating pool, half-heavy DNA is also found. It is concluded that transferred DNA is incorporated into hybrid units which are attached to larger segments of completely new DNA. The parental atoms may comprise a part of one strand of a duplex DNA molecule.

An analysis was made of the density distribution of the viable progeny of a multiple infection with T4r and r+, one of which was labeled with N^{15} and C^{13}. At densities greater than that of the average progeny, relatively more progeny of the genotype of the labeled parent are found. Thus, parental atoms tend to remain associated with parental genotype.

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I. INTRODUCTION

When a bacteriophage particle infects a bacterium, its DNA enters the host cell. Most of the phage protein remains attached to the cell wall in such a manner that it can be stripped off without loss of ability of the infected cell to produce progeny (Hershey and Chase, 1952). Not more than 3% of the protein enters the cell with the DNA (Hershey, 1955). Thus, it is evidently the parental DNA which initiates the processes of synthesis of protein and DNA which lead ultimately to the release from the cell of mature progeny phage. By labeling the DNA of the infecting particles, it has been shown that a fraction of the label is transferred to progeny particles (Putnam and Kozloff, 1950).

The transfer of parental DNA is of interest because of its relationship to the mechanisms of DNA replication and genetic recombination. The double helical structure of DNA proposed by Watson and Crick (1953) suggested to the authors a semi-conservative mechanism of replication, whereby the two complementary strands of the parental DNA molecule are distributed intact, one to each of the two daughter molecules. Each daughter molecule is again composed of two complementary strands, one of parental origin and the other newly synthesized. Other mechanisms of replication have been proposed, in efforts toward a molecular understanding of genetic recombination. Genetic recombination could be the result of breakage of DNA molecules. In this case, it is not obligatory that it be associated with the process of replication, which could be conservative, semi-conservative, or dispersive. In any case, the replicated parental DNA would be fragmented and dispersed among the

daughter molecules. The copying-choice model for recombination postulates that daughter molecules may be partial replicas of two parent molecules. Parent molecules could conceivably be retained intact during such a process (conservative replication), or could be fragmented. These different hypotheses make more or less specific predictions regarding the fate of the parental DNA molecules, and the distribution of parental atoms among daughter molecules. This subject has been thoroughly discussed (Delbrück and Stent, 1957). The present introduction will be concerned with experiments designed to elucidate the transfer of the DNA of the two very similar phage, T2 and T4. There are several recent comprehensive reviews of this subject (Hershey and Burgi, 1956, Delbrück and Stent, 1957 and Stent 1959).

Only about 50% of the DNA of the infecting phage particle is transferred to progeny. These progeny, in turn, transfer about 50% of their inherited DNA to a second generation (Maaløe and Watson, 1951, and Hershey, Roesel, Chase, and Forman, 1951). This controverts the idea suggested by the incomplete transfer, namely that phage DNA is comprised of two fractions, only one of which is efficiently transferred to progeny. Were this the case, one would expect that the first generation progeny would receive only the transferrable fraction, which they would transfer to their progeny with an efficiency much greater than 50%. Most of the transferred DNA is incorporated into the first mature progeny. By the time that 100 progeny have been formed, the supply of parental precursors is nearly exhausted. Parental DNA evidently forms part of the early DNA pool, and, when phage particles are matured, it is withdrawn

by a process which does not detectably discriminate between parental and newly synthesized DNA. The incomplete transfer is, then, the result of random losses, which are due to technical difficulties inherent in the transfer measurements (Hershey and Burgi, 1956).

It is found that approximately 50% of the parental DNA is transferred to progeny, regardless of whether the parental phage are labeled with radioactive phosphorus, or with C^{14}-labeled adenine (Watson and Maaløe, 1953). If the infecting phage are uniformly labeled, then the relative C^{14} distribution among adenine, guanine, thymine, and hydroxymethylcytosine residues is the same in the progeny and parental phage. Moreover, the distribution is not affected by adding unlabeled purines or pyrimidines to the growth medium (Hershey, 1954 and Hershey and Burgi, 1956). This suggests that polymerized parental DNA, rather than single nucleotides, is transferred. The parental base ratios are therefore retained in the transferred DNA, even despite attempts to alter them.

In a mixed infection with unlabeled phage and P^{32}-labeled phage which have been subjected to ten phage-lethal hits by exposure to ultraviolet irradiation, about 50% of the transferred P^{32} is found in dead particles which are unable to transfer their P^{32} in single infection (Hershey and Burgi, 1956). At least a fraction of the transferred material must be of sufficient size to include UV damage. About 50% of the transferred P^{32} was found in live progeny, even if the parents had received up to 400 phage lethal hits. In this respect, the transferred DNA appears to contain one fraction which is immune to UV killing, perhaps

because it can function despite UV damage, or because it is transferred in pieces too small to contain, on the average, a UV damage.

In a mixed infection with phage, one of which is labeled with P^{32}, and one of which is a host-range mutant, it is found that the transferred isotope is associated preferentially with the genotype of the labeled parent, regardless of which parent is labeled and regardless of the ratio of parental genotypes (Hershey and Burgi, 1956). This suggests that a fraction of the transferred DNA is of sufficient size to contain genetic information. In both of these experiments, the fraction which remains associated with the marker (either UV damage or host-range genotype) comprises approximately 40% of the transferred material. Whether, in the two cases, it is the same 40% is not certain, although there is some evidence presented to indicate that it is the same. A second cycle of mixed infection decreases by half the fraction which is specifically associated with host range genotype. This may indicate that progressive transfers are associated with progressive fragmentation.

The genetically significant DNA of a single parental phage particle is not transferred in toto to a single phage progeny. If P^{32}-labeled bacteria in P^{32}-containing medium of high specific activity are infected with unlabeled phage, the progeny are subject to exponential killing as a result of the disintegration of the P^{32} which they have incorporated. This is known as the suicide effect. A P^{32}-containing bacterium infected with five unlabeled phage yields no progeny which are immune to the suicide effect. Thus, no progeny receive their entire complement of

DNA from the normal isotope parent (Hershey, Kamen, Kennedy and Gest, 1951).

If infecting phage contain sufficient P^{32}, their progeny may be expected to suffer inactivation as a result of the P^{32} which they inherit. By comparing the rate of inactivation of progeny to that of the parents, the amount of transferred P^{32} per progeny particle can be estimated. It has been found that the rate of inactivation of the progeny is not more than 0.2% of that of the parents (Stent and Jerne, 1955). Thus, the majority of progeny contain not more than this amount of transferred material. However, the rapid inactivation of only a small minority of progeny, each containing a large fraction of the transferred P^{32}, would not be detected by this method, because their death would not be detected. In order to study such a minority, advantage was taken of the finding that the suicide of P^{32}-containing phage is accompanied by a parallel loss of their ability to transfer their P^{32} to progeny in single infection; this is presumably because an infection in which the parental phage is dead is abortive and does not lead to the production of any progeny particles. The rate at which progeny lose the ability to transfer their inherited P^{32} to a second generation was measured. It was indeed found that a large fraction of the transferred P^{32} is contained in a few phage progeny, as evidenced by the rapid decrease in the fraction of inherited P^{32} transferred to a second generation. The rate of decrease has been used to estimate the amount of P^{32} contained in these progeny phage: it is assumed that 10% of the disintegrations suffered by these phage is lethal, as was true for their parents. The

results can be interpreted to mean that phage receiving transferred parental P^{32} fall into two categories with different rates of inactivation (Stent, Sato and Jerne, 1959). One category would contain approximately 60% of the transferred phosphorus in fragments comprising about 15% of the total DNA complement; the second category would contain the remainder of the transferred phosphorus, in fragments comprising perhaps 0.3% of the total DNA complement. The transfer of the inherited P^{32} in these first generation phage to a second generation has been analyzed by the same method. It has been found that the P^{32} may again be transferred into phage which contain 15% and 0.3% of the parental complement, in the same ratio as among the first generation progeny.

The most direct method which has been used to investigate the distribution of transferred parental DNA is radioautography (Levinthal, 1956). Phage which are very highly labeled with P^{32}, or the DNA from such phage, constitute point sources of radiation when imbedded in a radiosensitive photographic emulsion. They generate "stars", the rays of which are the tracks left by single beta emissions from disintegrating P^{32} atoms. The number of rays per star is statistically related to the P^{32} content of the source, which can be calculated from this number. With this technique it has been found that, following single infection with highly radioactive parental phage, some progeny produce large stars corresponding to $22 \pm 3\%$ of the parental phosphorus. The number of such particles is $1 \pm .4$ per parent. The remainder of the transferred P^{32} is evidently dispersed among progeny in fragments too small to be detected by this method. The first generation phage transfer a fraction

of the P^{32} which they inherited to a second generation, among which there are again particles containing 22±3% of the P^{32} of the grandparent. The fraction of transferred P^{32} which is transferred in this large subunit is the same for the first and second generation progeny. These results have been taken to indicate that phage DNA is comprised of one large subunit and at least 5 or 6 small subunits, all of which are transferred with equal efficiency; at least the large subunit is not fragmented when transferred.

If bacteria are multiply infected with phage differing in host range, one of which is highly labeled with P^{32}, and the progeny are put through a further cycle of single infection to eliminate phenotypic mixing, then 90% of the large subunits, identified as stars, are found to be in phage of the genotype of the radioactive parent (Levinthal and Thomas, 1957). The large subunit, here identified as carrier of the h genotype, is transferred without fragmentation and without discrimination. This is in contradiction to the finding of Hershey that the fraction of transferred parental DNA which is associated specifically with a host range marker is decreased to half following a second cycle of mixed infection (Hershey and Burgi, 1956).

The technique of autoradiography was used by Levinthal (1956) to study the mechanism of replication of the DNA of T4. However, technical difficulties associated with the isolation of the DNA invalidate the results of these experiments.

The experiments of Stent and of Levinthal described above are open to serious question because the techniques used require that the

parental phage contain enormous amounts of radioactive phosphorus (100 to 900 P^{32} atoms per phage). A significant fraction of the parental phage are dead, as shown by their inability to transfer P^{32} to progeny phage in single infection; these phage therefore do not contribute to the results of the transfer experiments. However, only 10% of the disintegrations are lethal; most of the parental phage have suffered some non-lethal disintegrations. Such disintegrations, or other effects associated with the high levels of P^{32}, may cause anomalies such as fragmentation during the replication process. Thus, the physical nature of the transferred subunits remains uncertain.

The technique of density-gradient centrifugation (Meselson, Stahl, and Vinograd, 1957) has been used to study the transfer of parental DNA among progeny molecules in the phages T7 (Meselson, 1959) and λ (Meselson and Weigle, 1961). Parental phage were heavily labeled with the stable heavy isotopes of nitrogen and carbon, N^{15} and C^{13}. Among the progeny, phage particles or DNA molecules containing transferred parental atoms can be distinguished by their increased density, which is proportional to the isotope content. In T7, a fraction of the DNA isolated from progeny phage is exactly half-heavy. Among the progeny phage, a discrete fraction are found which have a density indicating that half their DNA atoms are of parental origin. This suggests that the DNA of parental T7 contains two subunits, perhaps the two strands of a duplex DNA molecule, which are separated during replication and which may be incorporated intact, together with an equal amount of newly synthesized DNA, into a progeny particle.

Using the phage λ, Meselson and Weigle performed a cross between labeled and unlabeled phage differing in two genetic markers. Analysis of the density distributions of parental and recombinant genotypes among the progeny lead to the conclusions that the DNA complement of λ replicates semi-conservatively, and that genetic recombination occurs by breakage.

Recently a report has been published of the application of this technique to a study of the replication of the DNA of phage T4 (Kozinski, 1961). The results indicate that parental DNA is found in fragments with a density exactly intermediate between parental and progeny densities. These fragments are incorporated into molecules which are comprised mainly of newly synthesized DNA; the fragments are isolated by fragmenting the molecules by means of sonication. In order to distinguish parental from progeny DNA, the phage were grown in the presence of the thymine analog, 5-bromodeoxyuridine. The density of fully substituted phage DNA is increased 0.1 g./cm.^{3}. This analog is known to be mutagenic in phage (Litman and Pardee, 1956 and Freese, 1959), and may cause anomalous transfer of parental DNA. Also, it is incorporated into the phage in variable amounts, as indicated by the broad density distribution of the phage (Meselson, Stahl and Vinograd, 1957). Either of these factors could cause spurious results.

In the present experiments, the density-gradient technique has been used to investigate the fate of the DNA of the phage T4 during replication, and the structure and genetic significance of the subunits which are transferred to progeny. The density distribution of DNA in

the replicating pool has been examined. The DNA isolated from the progeny of a transfer experiment has been examined and subjected to various treatments in order to determine the state of the parental atoms. Finally, the specific association of r genotype with parental atoms has been examined by analyzing the density distribution of the progeny of a mixed infection with r and r+ phage, one of which was isotopically labeled. The density label has been N^{15}, or N^{15} and C^{13}; thus, the possible sources of error mentioned above are avoided.

II. MATERIALS AND METHODS

2.1 Bacteriophages

The bacteriophage $T4Bo_1^r$ is a shock resistant mutant of T4B, originally isolated by Dr. S. Brenner. The r mutant used was isolated from $T4Bo_1^r$ and was uncharacterized except that it was non-reverting. These phages were used in all experiments except the study of the intracellular DNA pool, in which $T4Br_{-240}$ was used. In experiments 1-19-1 and 1-19-2, the phage T6 was used as a density marker.

2.2 Bacteria

The host bacterium in all experiments was E. coli strain B. Plating bacteria were prepared by concentrating an exponentially growing culture to about 2×10^9 bacteria/ml. The indicators used in the pool DNA experiment were E. coli strains B and K12 for assays of total phage and wild type respectively. In other experiments, strain S/6 was used to assay T4r and +, and S/4 to assay T6. The agar layer plating technique was used throughout. Plates were incubated at 30°.

2.3 Media

Hershey broth contains, in grams per liter, nutrient broth 8, Bactopeptone 5, NaCl 5, glucose 1.

Fraser medium contains, in g/l, KH_2PO_4 4.5, Na_2HPO_4 10.5, NH_4Cl 3.0, glycerine 30, casamino acids 15, gelatin 0.03; after autoclaving, 3.0 ml. 0.5 M $CaCl_2$ and 3.0 ml. 1.0 M $MgSO_4$ are added.

SM, suspension medium, contains, in g/l, NaCl 25, Tris(hydroxymethylamino)methane 12.1, gelatin 0.05, and $MgSO_4$ 0.25, and is

adjusted to pH 7.4 with NaOH.

M9 medium contains, in g/l, KH_2PO_4 3, Na_2HPO_4 7, NH_4Cl 1; after autoclaving, 20 ml 10% glucose, 12 ml 25% NaCl, 0.2 ml 0.5 M $CaCl_2$, 0.3 ml 0.01 M $FeCl_3$, 1 ml 1 M $MgSO_4$ per liter are added.

Hershey tris medium is a synthetic medium buffered with tris-(hydroxymethylamino)methane, and used to grow phage labeled with radioactive phosphorus (Hershey, 1955).

Yeast synthetic medium contains, in g/l, sucrose 100, NH_4Cl 1.2, $MgSO_4 \cdot 7H_2O$ 0.4, KH_2PO_4 0.5, $CaCl_2 \cdot 2H_2O$ 0.13, and is adjusted to pH 6.5 by the addition of HCl.

Collection medium is tryptone broth, containing, in g/l, Bacto-tryptone 10, NaCl 5, and is adjusted to pH 7.4 with NaOH. Before use, 5 ml per liter of 1 M $MgSO_4$, and $CHCl_3$ to saturate, are added.

2.4 Heavy Media

99% N^{15} yeast extract was prepared by growing yeast in yeast synthetic medium containing $N^{15}H_4Cl$ of 99% isotopic purity. 700 ml medium was innoculated with 2×10^6 yeast cells, and grown at 37° with aeration to 8×10^7/ml. The generation time was 2 hours. After a titer of 10^7 cells/ml was reached, the pH of the culture was checked every half hour and adjusted by the addition of 1 M $NaHCO_3$. Centrifugation yielded 4.1 g wet packed cells. These were suspended in ethyl acetate, and allowed to autolyse at 37° 36 hours, with two subsequent additions of ethyl acetate. The suspension was centrifuged and the pellet washed twice. The combined supernatant and washings were filtered through a

0.45 micron Millipore filter, yielding 7 ml autolysate. The debris was hydrolyzed by resuspending in 2.5 M HCl and autoclaving 25 minutes. The hydrolysate was neutralized with $NaHCO_3$, centrifuged, and the pellet washed twice. The combined supernatant and washings were filtered through a Millipore filter and brought to a volume of 6 ml. The autolysate and hydrolysate contained 16 and 13 mg of nitrogen, respectively, 1/3 of the nitrogen in the medium. They were combined, and diluted 10 fold with M9 without NH_4Cl, to give a "yeast extract medium" in which bacteria grew with a generation time of 20 minutes at 37°.

99% N^{15}, 65% C^{13} algal hydrolysate was prepared from Ankistrodesmus grown in $N^{15}H_4Cl$ of 99% isotopic purity, and $C^{13}O_2$ of 65% isotopic purity, following the procedure of Davern (1959). This medium was dephosphorylated by the method of Astrachan and Volkin (1958). The resulting algal hydrolysate was diluted 50 fold in Hershey tris medium from which NH_4Cl and glucose had been omitted. From the specific activity of radioactive phage grown in this medium, a minimum phosphorus content of 1.5 μg/ml can be calculated, assuming all P^{32} which adsorbs to bacteria is in viable phage.

99% N^{15}, 93% C^{13} medium was prepared by Dr. M. Meselson, and kindly supplied by him in the form of an algal hydrolysate H2, and a yeast extract YE, prepared by growing yeast on algal hydrolysate (Meselson and Weigle, 1961). 0.5 ml H2, 1.0 ml YE and 2.8 mg. $N^{15}H_4Cl$ were added to 4.5 ml Hershey tris medium containing 10 μg/ml phosphorus as KH_2PO_4, and from which NH_4Cl and glucose had been omitted.

2.5 Phage Stocks

Phage stocks were prepared by infection of E. coli in Fraser medium. A fresh overnight culture was diluted 1000 fold into 1 liter medium, and grown to 2×10^8 cells/ml at 37°. Phage were added at a multiplicity of 0.1. After 2 hours, $CHCl_3$ was added, and the crude lysate was filtered through washed Celite. It was then centrifuged at 12,000 RPM for 90 minutes. The phage, resuspended overnight in 100 ml SM, were treated with 20 µg/ml DNase (Worthington) for 1/2 hour at room temperature, followed by 20 µg/ml RNase (Armour). They were centrifuged at 5,000 RPM 20 minutes, then 15,000 RPM 90 minutes. The purified phage stock, suspended in 30 ml SM, was filtered through a Millipore filter, and diluted to 150 ml to give a titer of about 4×10^{11}/ml. Stocks prepared in this way and stored at 4° keep a constant titer for months.

To prepare hot and heavy phage containing radioactive phosphorus, 99% N^{15} and 65% C^{13}, a modification of the method of Stahl (1956) was used. E. coli, which had been adapted to growth on algal hydrolysate, were grown overnight at 30° in Hershey tris medium containing 10 µg/ml of phosphorus as KH_2PO_4. The culture was diluted and grown 10 generations in Hershey tris medium to 10^8 cells/ml. The bacteria were centrifuged, and the pellet resuspended in Hershey tris medium from which glucose and NH_4Cl had been omitted. This was diluted 500 fold into 65% C^{13} medium containing 1-3 µc P^{32}/ml. At a titer of 2×10^8/ml, the bacteria were infected at a multiplicity of 0.1 with stock $T4Bo^r_1$, r or r+, which had been diluted 3,000 fold into Hershey tris medium con-

taining 5 μg/ml tryptophan, and without glucose or NH_4Cl. After 2 1/2 hours, the culture was saturated with $CHCl_3$. Phage were purified as described above, with the exceptions that filtration through Celite was omitted, and the phage were submitted to only one high speed centrifugation. Radioactive phage were used the day after they were prepared.

In experiment H and H 5, Oak Ridge P^{32}, carrier free, was dried, resuspended in a small volume of water, and purified with Dowex 50. In experiment H and H 6, P^{32} was supplied by Nuclear Consultants Corp., Glendale, Calif. It was carrier free, therapeutic grade, and was used without purification immediately upon receipt.

Heavy phage of 99% N^{15}, 93% C^{13} isotopic composition were prepared in the manner described above, with the exception that P^{32} was omitted. Phage titers of the crude lysates were about 8×10^9 for r phage and 3 times that for wild type. For this reason, heavy r+ were used in most experiments.

2.6 Preparation of Phage DNA

Phage lysates were purified as described above for heavy phage stocks. After centrifugation at 13,000 or 20,000 RPM at 0° for 90 minutes, the pellet was resuspended overnight in 0.1 ml SM. The phage were transferred to a vial with an additional 0.1 to 0.2 ml SM, and lysed by saturation with guanidine HCl. All preparations for analytical centrifugation, and some of those for preparative centrifugation, were dialyzed to remove the guanidine HCl. A piece of dialysis membrane was secured on the end of the vial, which was inverted in buffer (0.15 M

NaCl, 0.015 M Na_3Citrate, pH 7.8). In some cases, the volume was reduced by blowing a jet of air against the dialysis membrane. DNA solutions were clear and viscous. Except in the experiment on pool DNA, samples were transferred by pouring or with the large end of a 1 ml pipette, in which the diameter is about 2 mm.

To break the DNA molecules, samples were sucked rapidly in and out of a hypodermic syringe several times (number 23 or 26 needle). The hydrodynamic shear imposed by forcing through a hypodermic needle is sufficient to decrease the molecular weight of phage DNA (Davison, 1959).

DNA samples examined analytically were further degraded by sonication in a Raytheon Magnetostriction Oscillator. The centrifuge cell containing the sample was placed directly into the receptacle of the sonicator, with sufficient water to cover it. At regular intervals the cell was removed and the contents mixed by shaking.

The sample heated to 70° was placed, in the analytical cell, in a beaker of water at this temperature. Samples heated to 100° were removed from the cell and heated for 20 minutes in a vial. They were cooled rapidly by immersion in ice water. The density of such solutions was readjusted before centrifuging. The density of DNA heated to 100° increased about 0.014 g/cm^3. No DNA was observed at lighter densities.

2.7 Density Gradient Centrifugation

The technique of analytical density-gradient centrifugation described by Meselson, Stahl and Vinograd (1957) has been employed to

analyze the density distribution of some of the DNA samples. A Model E Spinco analytical ultracentrifuge with standard UV absorption optics was used at 44, 770 RPM and 25°, except as mentioned below. Samples were centrifuged at least 24 hours, with the exception of the samples of pool DNA, which were centrifuged 16 hours. Pictures were taken on Kodak Commercial film, and developed in Kodak developer D11. The results are presented in the form of densitometer tracings of the films, made with a Joyce Loebl Microdensitometer. The range of optical densities was always within the range of linear response of the film to log exposure, as ascertained by the use of the sector wedge described by Robkin, Meselson and Vinograd (1959). Minor irregularities in the tracings, caused by film grain, occasional dust particles, etc., have been smoothed over.

Except in the experiment on pool DNA, the centrifuge cell was loaded with the large end of a 1 ml pipette. The bottom window and centerpiece were held together in the housing by tightening the housing plug. The centerpiece was filled, and assembly of the cell was then completed.

For determination of the band shape of the DNA, the speed was decreased to 27, 690 RPM. Equilibrium was closely approached; tracings of films exposed at an interval of 24 hours were indistinguishable. The molecular weight calculation was made according to the method of Meselson, Stahl and Vinograd (1957). Sedimentation velocity was measured in a 12 mm cell with aluminum centerpiece, at 31, 410 RPM, in the analytical centrifuge.

Preparative density-gradient centrifugation, described by Weigle, Meselson and Paigen (1957), was employed to analyze samples of radioactive phage DNA, and of viable phage particles. Centrifugations were performed in the swinging bucket rotor SW 39 of a Spinco Model L preparative ultracentrifuge, equipped with a thermometer in the rotor chamber; all centrifugations were performed at 23-25°. Sample volume was 2 or 3 ml. The lusteroid tubes were filled with mineral oil to within 2-3 mm of the top to prevent their collapse. The initial density was so chosen that the bands formed in the bottom 1.5 ml of solution, except in the two centrifugations of experiment H and H 5, in which the band was higher. The bands were kept low in order to prevent their being preceded by a minor band or shoulder, comprising about 5% of the material. The above method for preventing this artifact was suggested by Dr. F. W. Stahl.

DNA samples were collected, 1 drop/tube, in 0.5 ml M9 from which salts and glucose had been omitted. The optical density at 260 mμ was measured with a Beckman Model DU spectrophotometer in a 1 ml quartz cuvette of 1 cm path length. The optical density scale is not included in the figures. The entire sample was dried on an aluminum planchet, and counted with a Nuclear Chicago scaler, gas flow counter, automatic sample changer and printer (Models 181A, D47, C110B and C111B respectively). For samples at or near possible densities of the DNA, the radioactivity has been calculated as counts per minute from the time required to count at least 800 disintegrations; for other samples this was decreased to a minimum of 400 disintegrations.

In the experiments on the distribution of viable phage particles, drops were collected individually in 1 ml collection medium. Assay of drops was discontinued at densities lighter than that of the band of light phage. It has always been found in the preliminary estimates of drop titers that the trail following the band continues to the top of the cell, with a slight increase in the last 1-3 drops. Recovery of viable phage particles was always essentially complete.

CsCl was obtained from American Potash and Chemical Corp., Los Angeles, Calif., trademark Trona, or from Maywood Chemical Works, Maywood, N. J., optical grade. The former CsCl was purified with acid washed activated charcoal before use; the Maywood CsCl was recrystallized. CsCl was dispensed in solutions of density 1.85-1.91 in 0.01 M tris. The pH was 8.4 or 7.5 for centrifugations of DNA or viable phage, respectively. DNA solutions were brought to the appropriate volume and density by the addition of Maywood CsCl solutions and water, or, more often, buffer containing 0.3 M NaCl and 0.01 M tris pH 8.4. Phage were suspended in Trona CsCl and Hershey broth containing 0.5, instead of 5 g/l of NaCl. Densities of solutions were calculated from the empirical relationship between density and refractive index, $\rho(25^{\circ}) = 10.8601\, n_D^{25} - 13.4974$ (Meselson, 1959). Measurements were made with a Zeiss refractometer at 25°.

In the following discussion, "heavy" phage or DNA refers to fully labeled material, "light" to material of normal isotopic composition, and "intermediate" to material which has a density exactly midway between heavy and light. Quotations will not be used with these words. The words band and peak are used interchangeably.

III. DISTRIBUTION OF PARENTAL ATOMS IN THE POOL OF REPLICATING PHAGE DNA

An investigation was undertaken of the distribution of atoms derived from parental phage in the DNA inside infected bacteria. Bacteria were grown at 37° from 5×10^4 to 2×10^8/ml in yeast extract medium of 99% N^{15} isotopic composition. KCN was added (2×10^{-3}M) before infection with 0.2 ml of a stock solution of T4B r_{-240}; the multiplicity was 9 phage/bacterium. Ten minutes later a 10 ml sample was removed and chilled for the zero time ultracentrifugation sample. The remainder of the culture (67 ml) was centrifuged at 2500 RPM for 5 minutes at 2°. The pellet was resuspended in 70 ml fresh medium at 37° at zero time. Thereafter, 10 ml samples were removed at 4 minute intervals, pipetting with a chilled pipette into chilled polyethylene centrifuge tubes (the time intervals and most especially the zero time may be inexact). The last sample was removed at 16 minutes after resuspension, at which time there were 20 T4 r/infected cell. At 30 minutes the remainder of the culture (30 ml) was saturated with $CHCl_3$ to yield progeny phage (burst size of 180).

In this experiment, bacteria were simultaneously infected at a multiplicity of 0.01 with wild type T4, in order to determine the fraction of parents contributing genetic markers to progeny phage. The r+ were assayed on the indicator strain K12, on which the phage of r genotype cannot grow. The results indicate that as many as 1/3 of the phage may be excluded from the genetic pool. It is also possible that some infected bacteria are unable to replicate the infecting phage DNA. These factors

could contribute to produce a class of "dead" parental phage DNA which is not replicating and appears unchanged through the course of the infection.

All samples except progeny phage were centrifuged 3 minutes at 3,000 RPM at 10^{o}. The pellets of infected bacteria were resuspended in 0.2 ml of buffer (0.1 M NaCl, 0.015 M Versene, pH 6.8), and lysed 15 minutes at room temperature following the addition of 0.1 ml 15% Duponol. Aliquots of 0.02 ml of the zero time and 4 minute samples, and 0.05 ml of the 8, 12 and 16 minute samples were added to a CsCl solution and centrifuged. The progeny were purified and lysed with guanidine HCl as described in sections 2.5 and 2.6. All samples were loaded into the centrifuge cell with a hypodermic syringe. This fact must be kept in mind in evaluating the data obtained.

Figure 1 is a densitometer tracing showing the density distribution of the DNA obtained from lysing the zero time sample. There are two peaks, separated by a density difference of 0.023 g/cm^3, exactly the difference between light phage DNA and N^{15}-bacterial DNA. (The reported buoyant densities in CsCl are: T4 DNA, 1.70, from Meselson, Stahl and Vinograd, 1957; N^{14} and N^{15} E. coli DNA, 1.710 and 1.724, respectively, from Meselson and Stahl, 1958.)

The distribution of the DNA 4 minutes after infection is shown in Fig. 2. There appears to be less material in the band of light phage DNA, and two new peaks have appeared. The denser of these differs in density by 0.013 g/cm^3; this is the expected density difference for light and heavy T4 DNA, calculated from the base analyses reported by

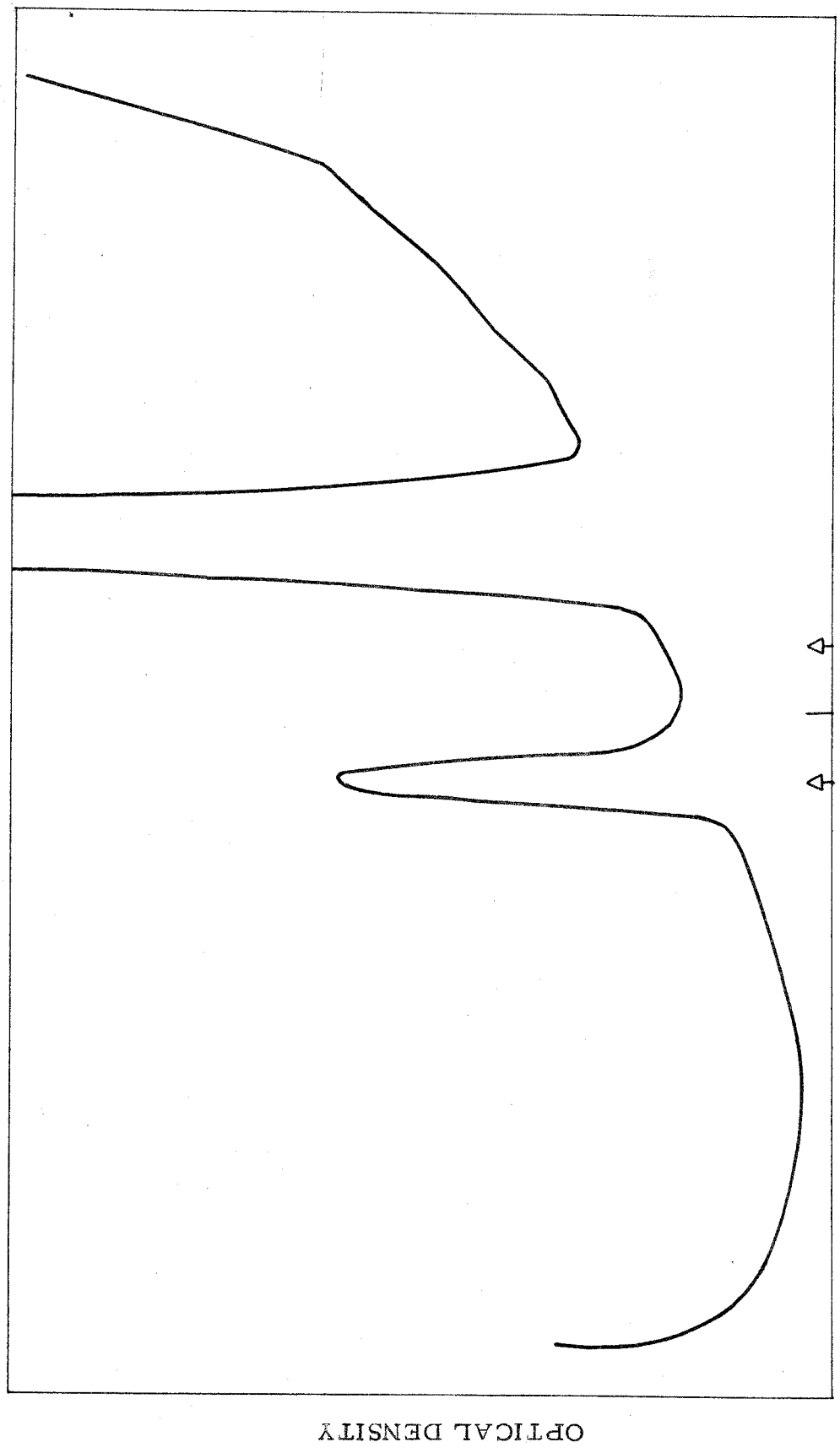

Fig. 1. Density distribution of DNA obtained from heavy bacteria, lysed zero minutes after infection with light phage.

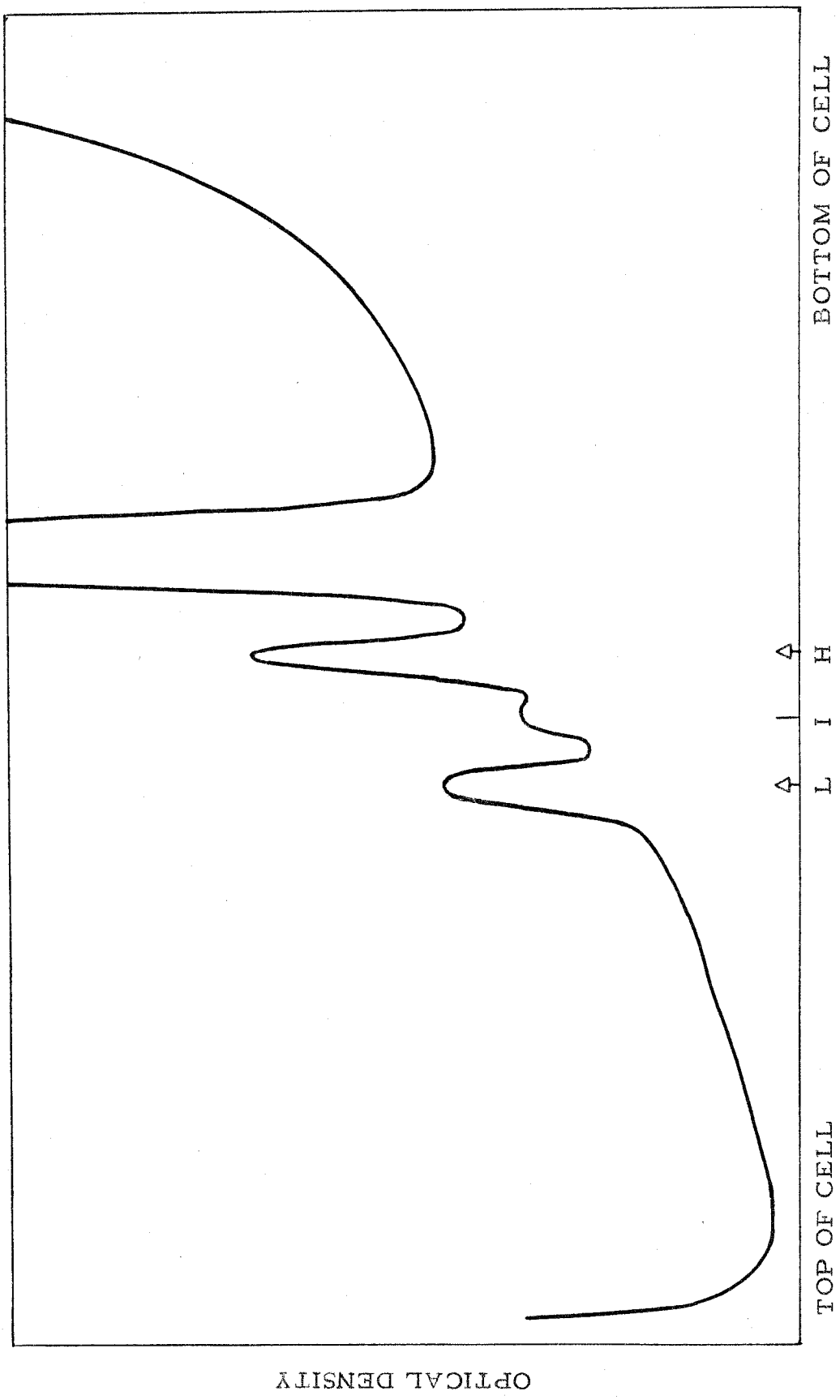

Fig. 2. Density distribution of DNA obtained from heavy bacteria, lysed four minutes after infection with light phage.

Wyatt and Cohen (1953) and the glucose content reported by Volkin (1954). There is, in addition, a small peak of intermediate density. In these and the following densitometer tracings, arrows are placed below the observed centers of the bands of light and heavy phage DNA, or at the calculated position if none is present; these arrows are marked by L and H. The position midway between these densities is called the intermediate density, and is indicated by a straight line marked by I.

Figures 3, 4 and 5 show the DNA distribution 8, 12 and 16 minutes after infection (2 1/2 times more material was centrifuged in these samples). During this time, the amount of material in the peak of heavy phage DNA increases, showing that the pool of progeny DNA is growing. Light parental DNA persists throughout this period; this may represent "dead" DNA which is not replicating, as was mentioned above. By 16 minutes, the presence of DNA of intermediate density is uncertain due to the large amount of heavy progeny DNA.

Figure 6 shows a mixture of DNA obtained from the progeny phage and from normal isotope phage. There appears to be a small amount of transferred material which is less dense than the bulk of the heavy DNA. However, the significance of such a shoulder is difficult to assess.

Preparative centrifugation of pool DNA in experiments incorporating both heavy isotopes and a radioactive marker showed that the recovery of material was very low, and variable. This was probably due to trapping of DNA in the curd produced by Duponol in CsCl. Further examination of the pool DNA was abandoned. Instead, the DNA obtained from the progeny phage produced in transfer experiments was examined.

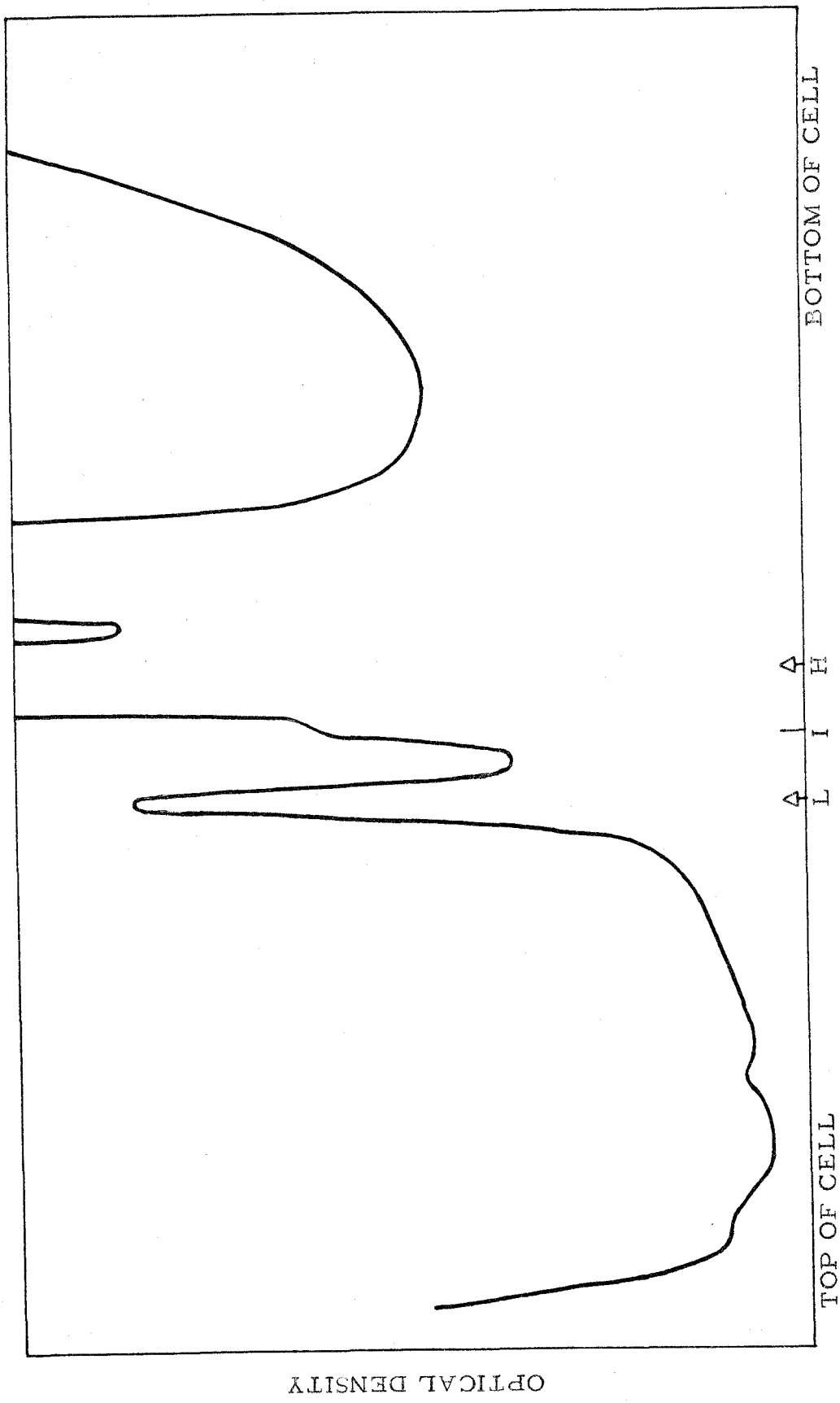

Fig. 3. Density distribution of DNA obtained from heavy bacteria, lysed eight minutes after infection with light phage.

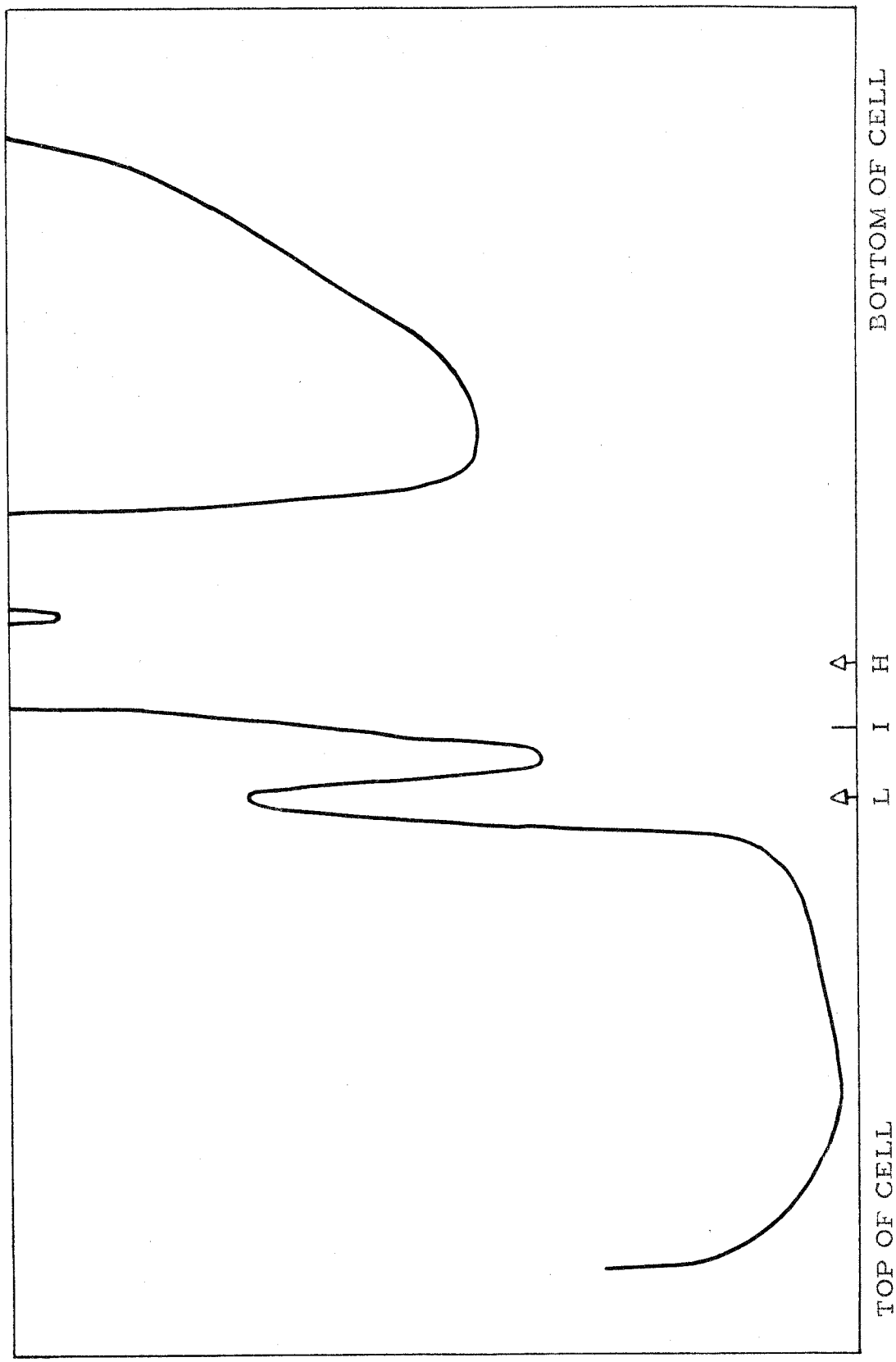

Fig. 4. Density distribution of DNA obtained from heavy bacteria, lysed twelve minutes after infection with light phage.

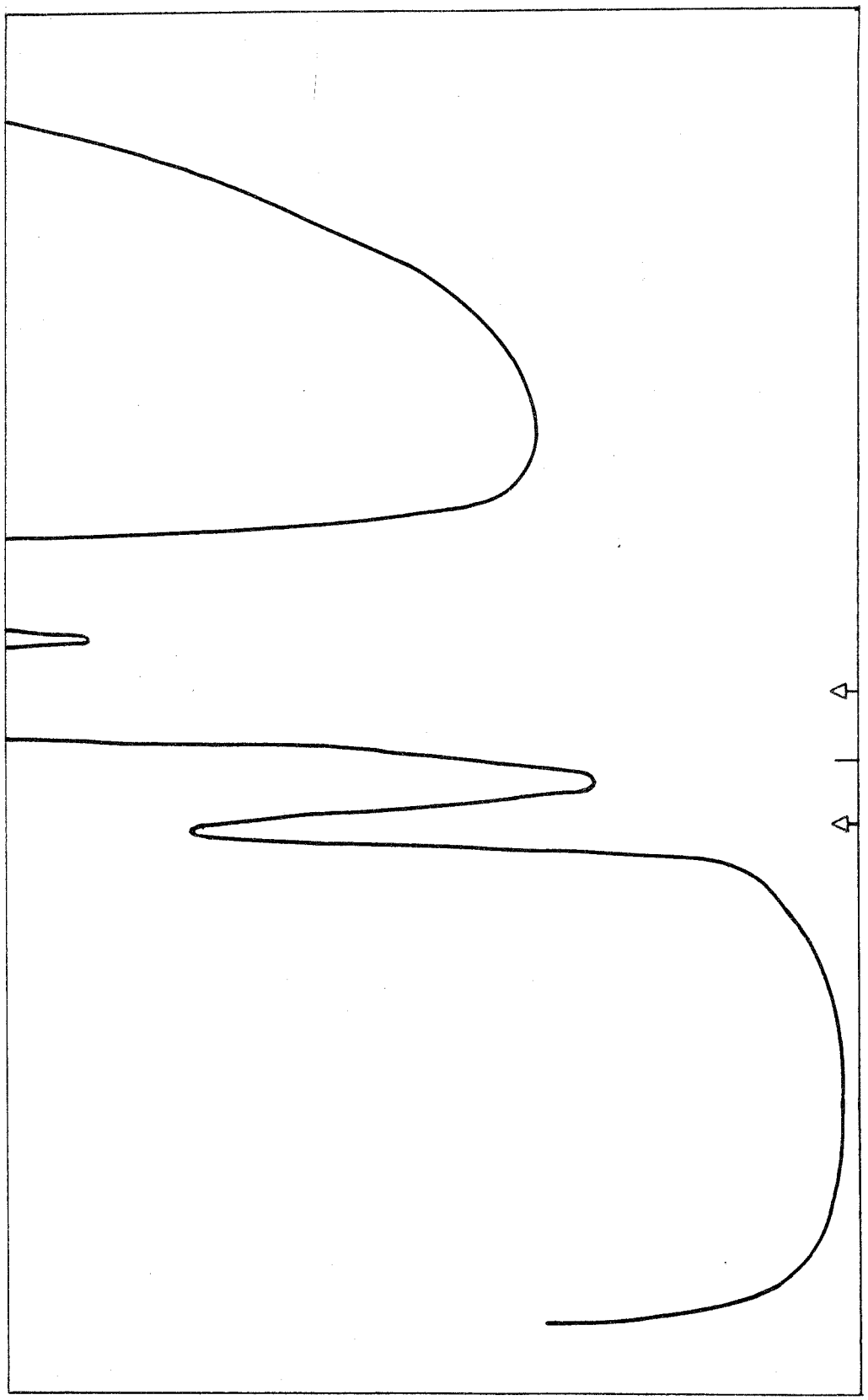

Fig. 5. Density distribution of DNA obtained from heavy bacteria, lysed sixteen minutes after infection with light phage.

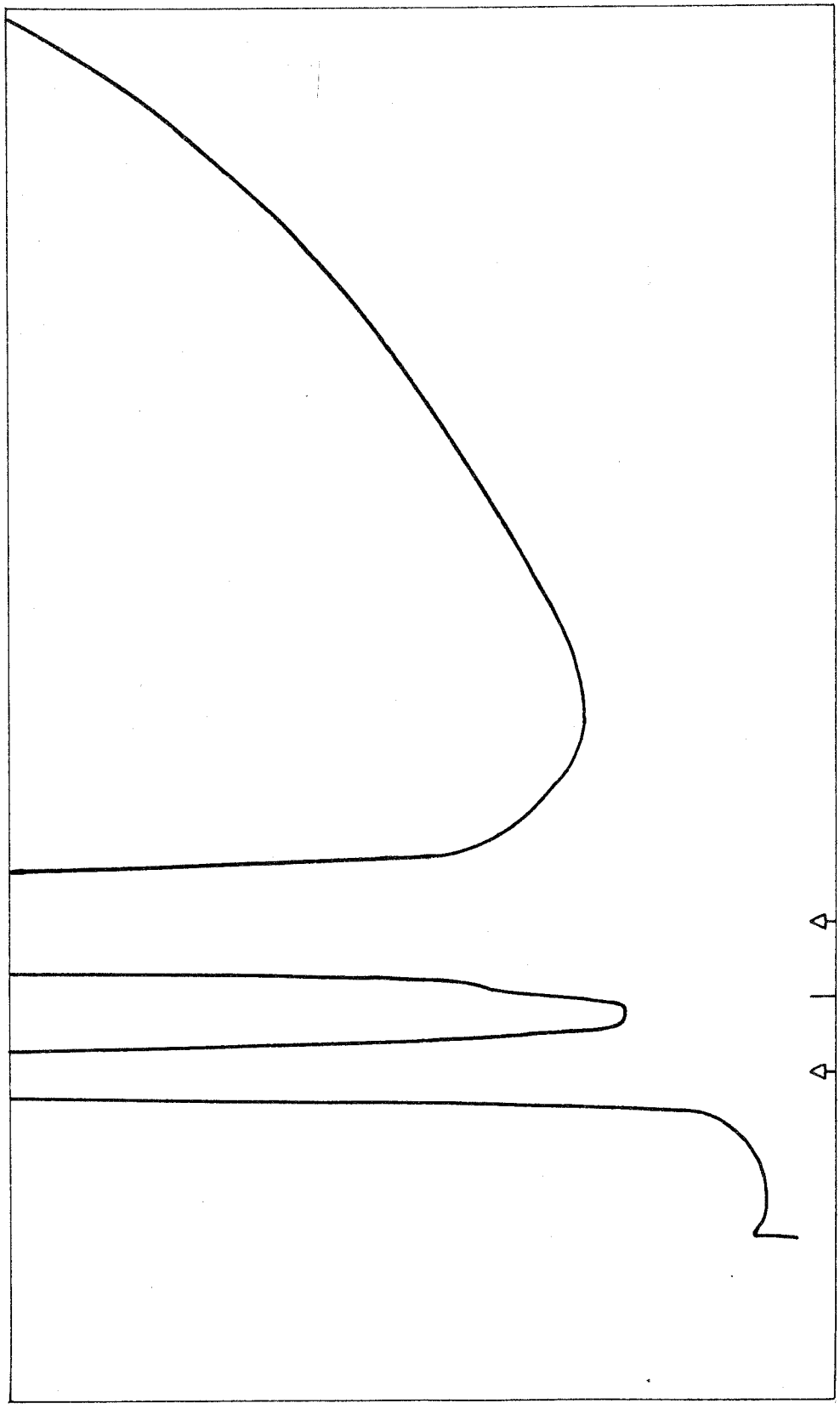

Fig. 6. Progeny phage DNA from Pool DNA Experiment, together with added normal isotope phage DNA.

Progeny phage DNA has the advantage that it can be purified in a state in which it is relatively well protected from mechanical damage, that is, while it is enclosed in the phage protein coat.

The following chapter describes the results of these experiments.

IV. DISTRIBUTION OF PARENTAL ATOMS IN PROGENY PHAGE DNA

The distribution of parental atoms in the DNA isolated from progeny phage has been examined by both analytical and preparative density-gradient centrifugation. In these experiments, the direction of transfer has been reversed; parental phage are isotopically labeled, whereas the host bacteria, the growth medium, and therefore the newly synthesized DNA are composed of normal isotopes. The analytical method shows the density distribution of the total progeny DNA, and changes in this distribution following various manipulations. DNA molecules containing parental atoms can be distinguished if they contain a fraction of heavy isotope sufficient to produce a detectable density alteration. Possible disturbances which may attend the preparative method, caused by deceleration or drop collection, are avoided. In the preparative method, parental phage contain radioactive phosphorus in addition to heavy isotopes. The distribution of P^{32} corresponds to the distribution of transferred parental atoms, unobscured by the newly synthesized DNA. The change in this distribution following various treatments can be determined.

4.1 Analytical Centrifugation of Progeny DNA

The transfer DNA analyzed in this series of experiments was obtained from progeny phage following infection of bacteria with heavy phage of 99% N^{15}, and 93% or 65% C^{13} isotopic composition, in experiments 8-3-4 and H and H 6, respectively. The heavy phage were prepared as described in section 2.5. Infected cells were suspended in low salt

Hershey broth containing 0.5, instead of 5 g NaCl/l. In this medium the phage are unable to adsorb to bacteria, although the time course of the infection is normal.

A fresh overnight culture of bacteria was diluted 500 fold into low salt Hershey broth, aerated at 30° 3 hours, and concentrated by centrifugation to approximately 10^{10}/ml in normal Hershey broth at 0°. The heavy phage, suspended in SM, were diluted with 3.5 volumes of salt free Hershey broth to adjust the salt concentration; 5 μg/ml tryptophan was added. To the phage at 30° was added 0.1 volume bacteria. After 1 minute about 90% of the phage had adsorbed to bacteria, and 10 volumes of salt free Hershey broth at 30° were added. The low salt concentration prevents further adsorption of parental phage, as well as loss of progeny by adsorption to bacterial debris. Infected bacteria were lysed with $CHCl_3$. Progeny phage were purified and their DNA isolated as described in sections 2.5 and 2.6. The details of these experiments are summarized in Table 1.

In the first series of centrifugations of DNA obtained in experiment 8-3-4, approximately 3×10^{10} phage units of progeny DNA were put into the centrifuge cell. This contained an estimated 10^9 units of transferred parental phage DNA (assuming 25% transfer at a burst size of 75). In the second series of centrifugations of this material, approximately 4 times more DNA was centrifuged.

A "dummy" experiment, 8-3-5, was performed in parallel with experiment 8-3-4. In the dummy experiment, the parental phage were of normal N^{14} C^{12} isotopic composition.

TABLE 1

Description of Experiments Included in Chapter IV

Experiment	Parental Phage*	P^{32} Content, Atoms/Phage	Multiplicity	Burst Size
8-3-4	Hr+	-	6.5	75
8-3-5	Lr+	-	4	64
H and H 5	Hr+	1	0.15	67
H and H 6	Hr+	3	8	170
H and H 6D	Lr+	1	14	180

*The parental phage are described by a capital L or H, indicating whether this parent was of light or heavy isotopic composition, respectively; the genotype is indicated to the right.

In the second experiment, H and H 6, the heavy parental phage contained radioactive phosphorus. The specific activity, estimated from P^{32} adsorbable to bacteria, was approximately 10^{-4} counts per minute per plaque forming unit, or a maximum of approximately 3 P^{32} atoms per phage, assuming all particles which adsorb to bacteria also make plaques. In the course of experiment H and H 6 (and H and H 5, to be discussed later) the fraction of P^{32} transferred to progeny phage, measured as P^{32} adsorbable to bacteria, was determined as a function of burst size. These data are presented in Fig. 7, and compared to the data of Watson and Maaløe, (1953).

DNA was isolated from progeny in 24 ml. of lysate containing a total of 4.5×10^{11} progeny phage and approximately 6×10^{9} transferred phage units of parental DNA. All of the material obtained was centrifuged.

A dummy experiment, H and H 6D, was performed in parallel with the above experiment. The amount of dummy transfer DNA centrifuged was equal to or somewhat greater than the amount of transfer DNA centrifuged.

The results of centrifugations of progeny DNA from experiment 8-3-4 are shown in the densitometer tracings of Figs. 8 to 13. Most of the untreated DNA (Fig. 8) is of approximately the density of light phage DNA. A small sharp peak is found with a density 0.049 g/cm^3 greater than that of light DNA. The density increase calculated for the Cs salt of T4 DNA of parental isotope composition is 0.050, assuming no selective solvation. It is assumed that this heavy DNA derives from unadsorbed

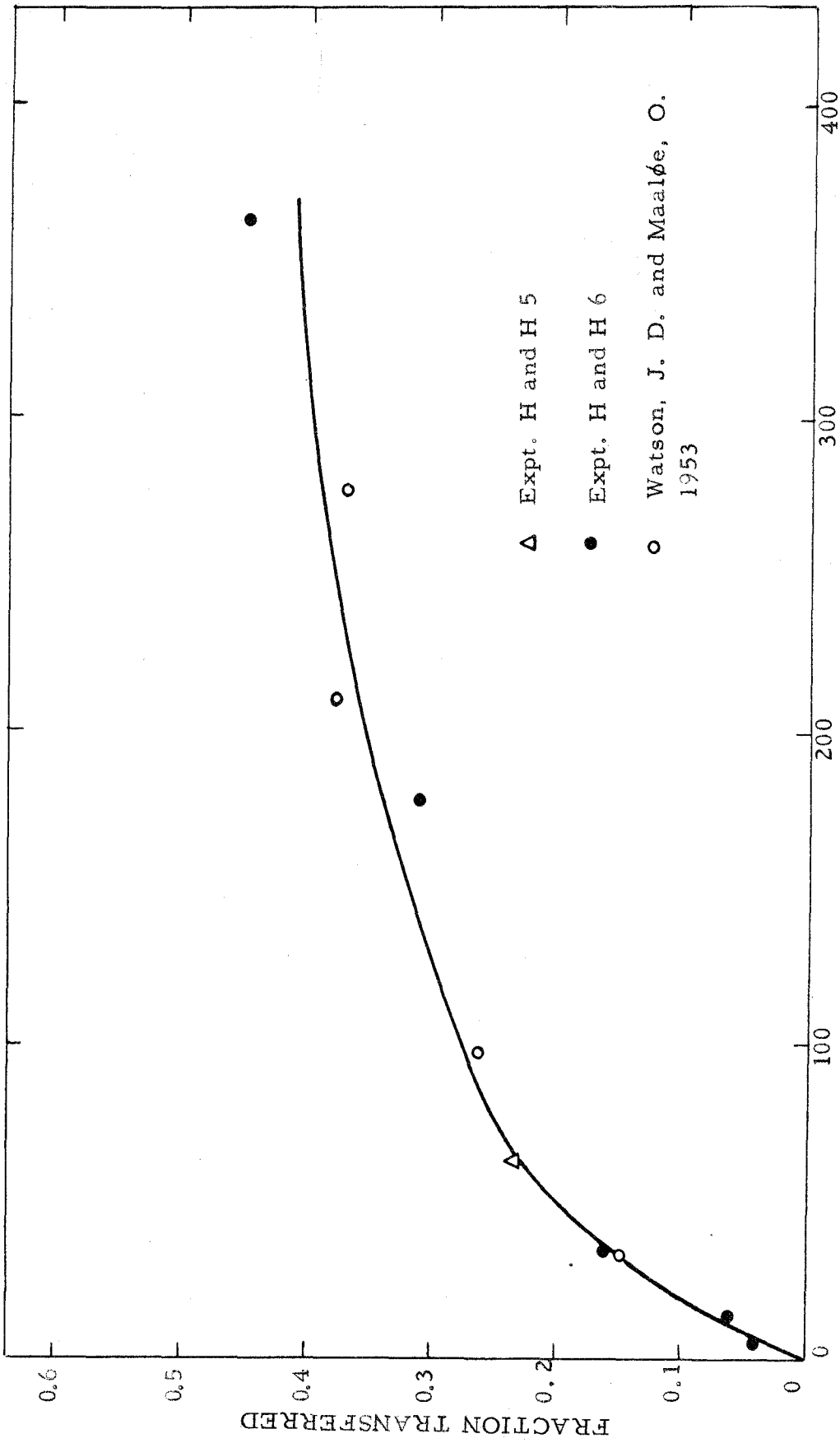

Fig. 7. Fraction of parental P^{32} transferred to progeny phage as a function of burst size.

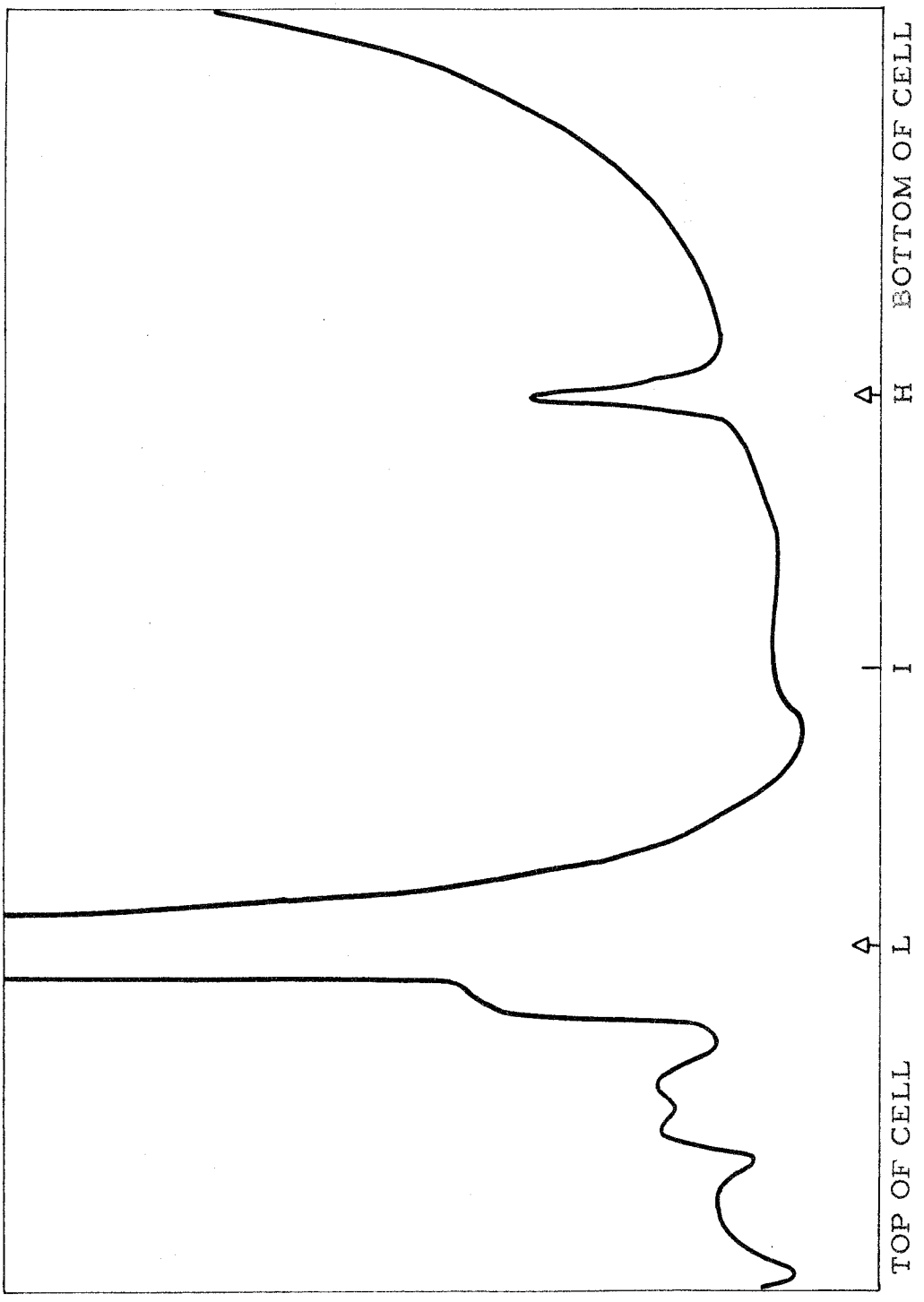

Fig. 8. Progeny DNA, untreated.
(Transfer experiment 8-3-4)

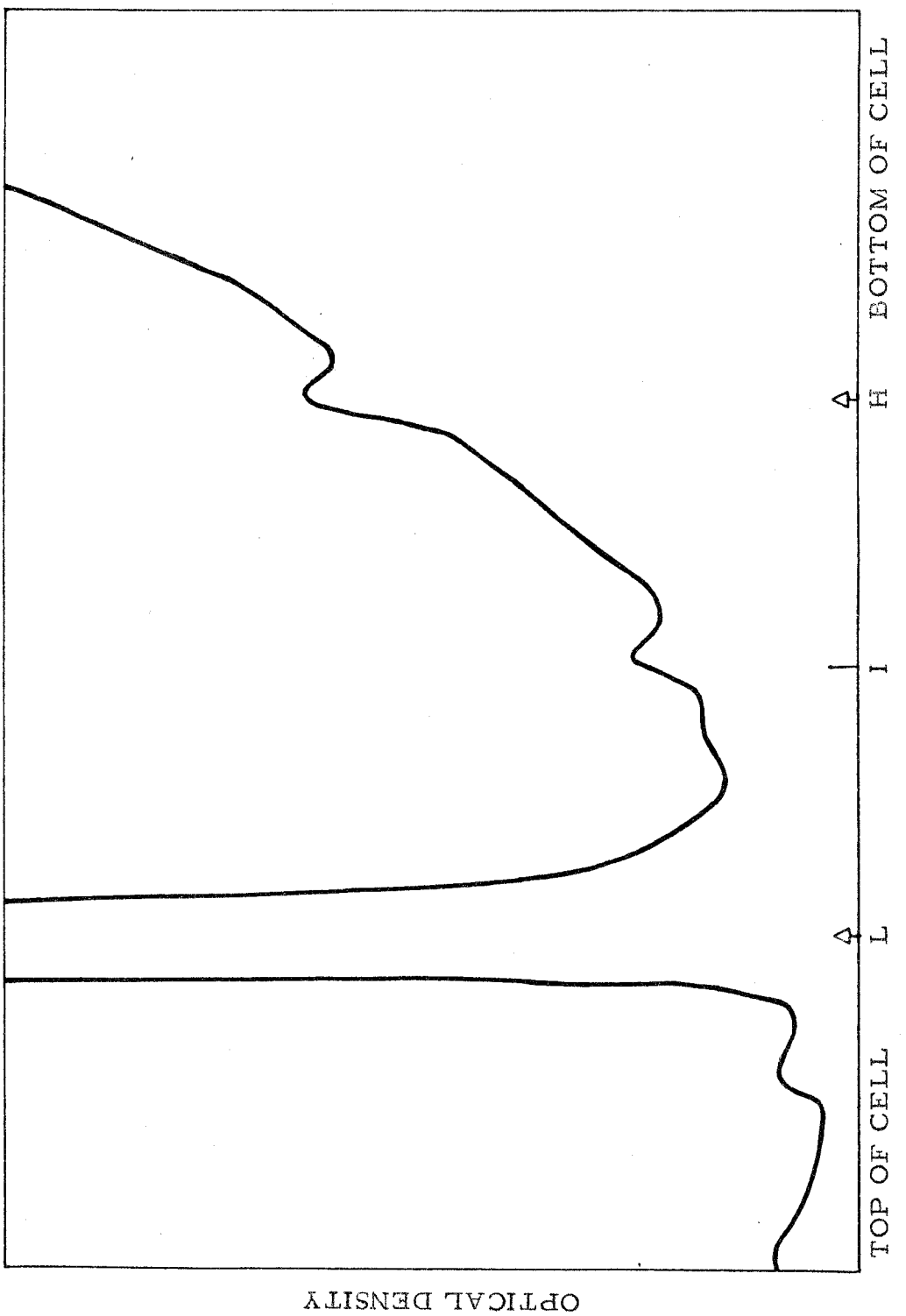

Fig. 9. Progeny DNA, passed through No. 23 needle.
(Transfer experiment 8-3-4)

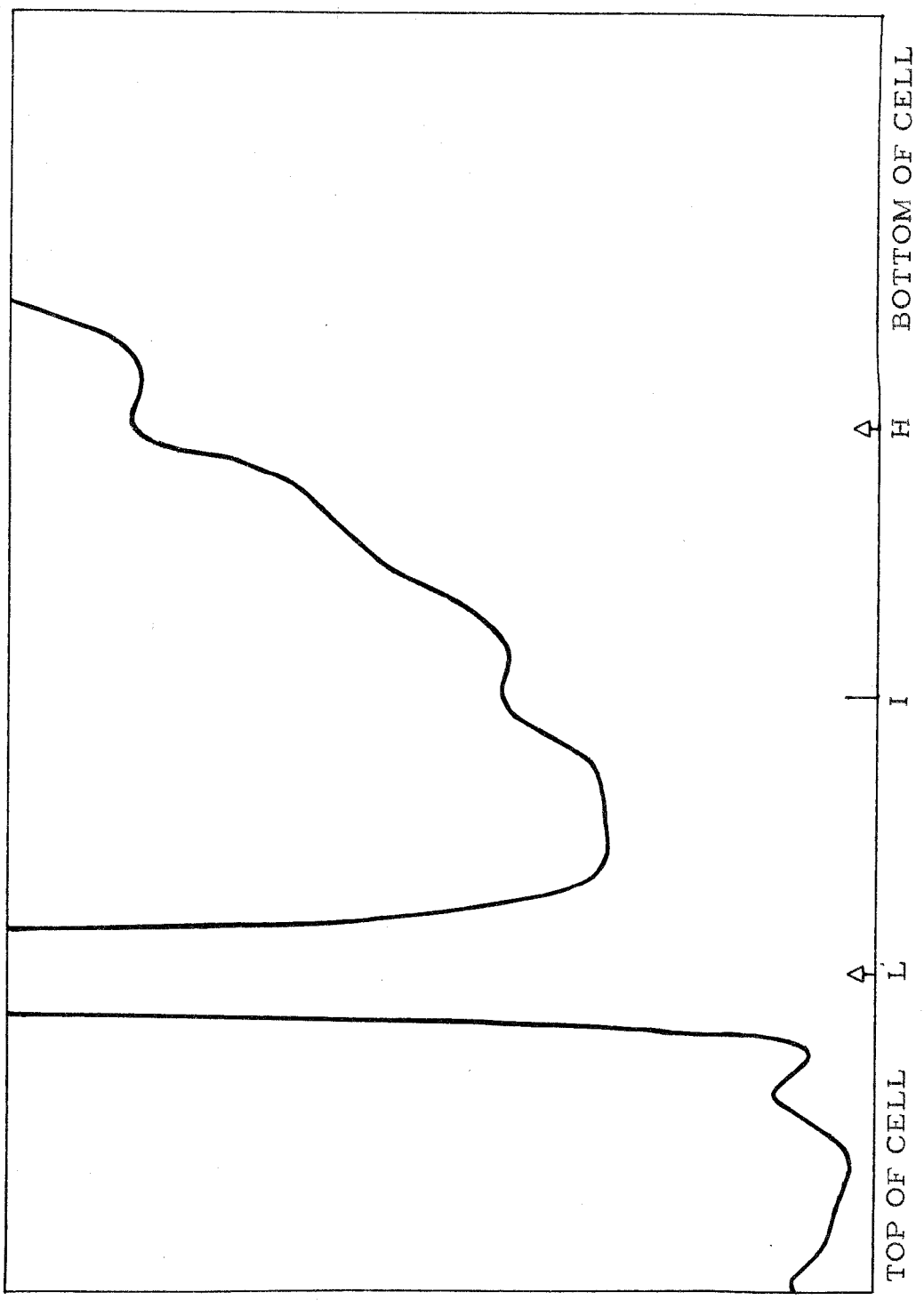

Fig. 10. Progeny DNA, passed through No. 23 and 26 needles.
(Transfer experiment 8-3-4)

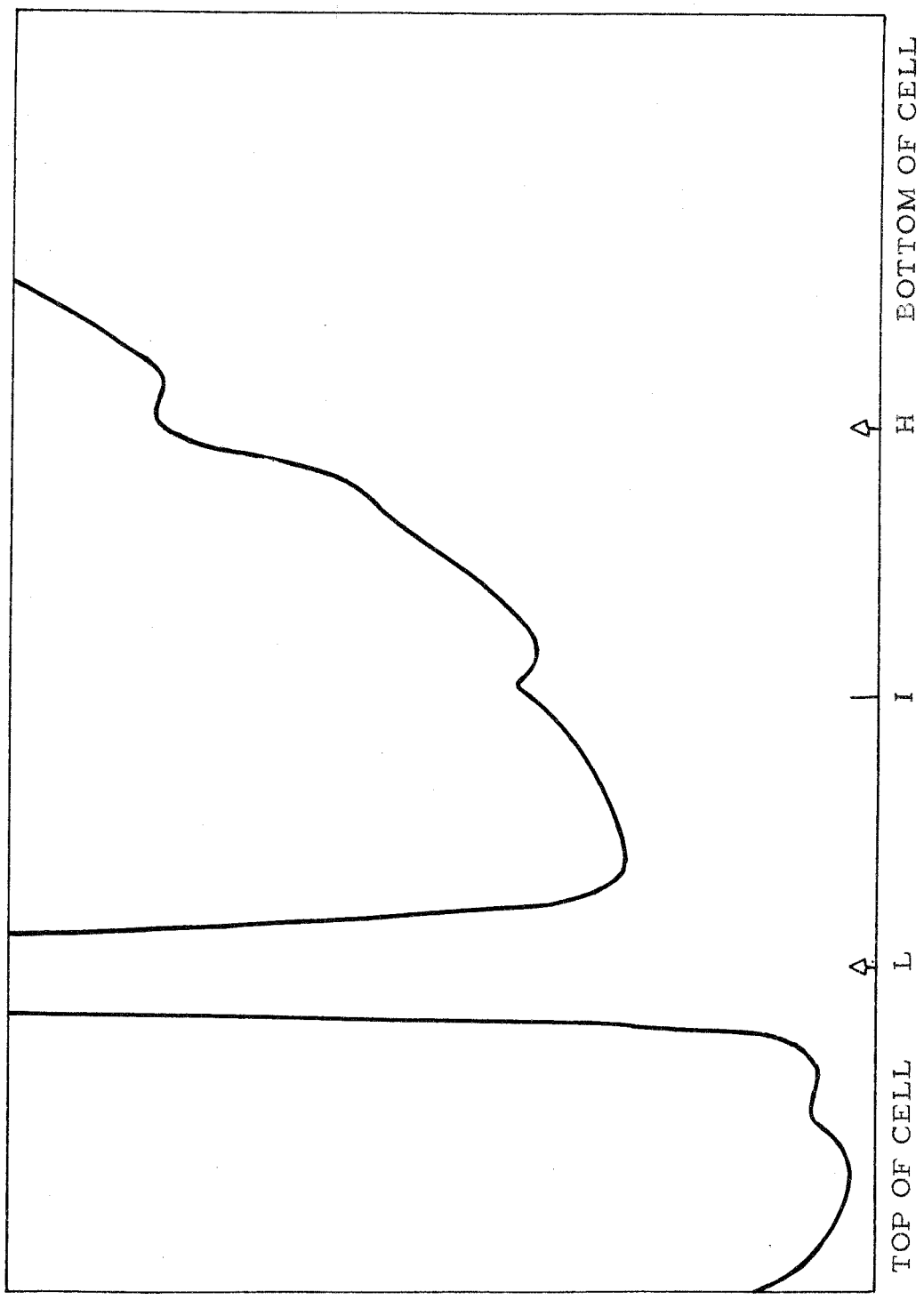

Fig. 11. Progeny DNA, passed through No. 23 and 26 needles, sonicated 15 seconds.
(Transfer experiment 8-3-4)

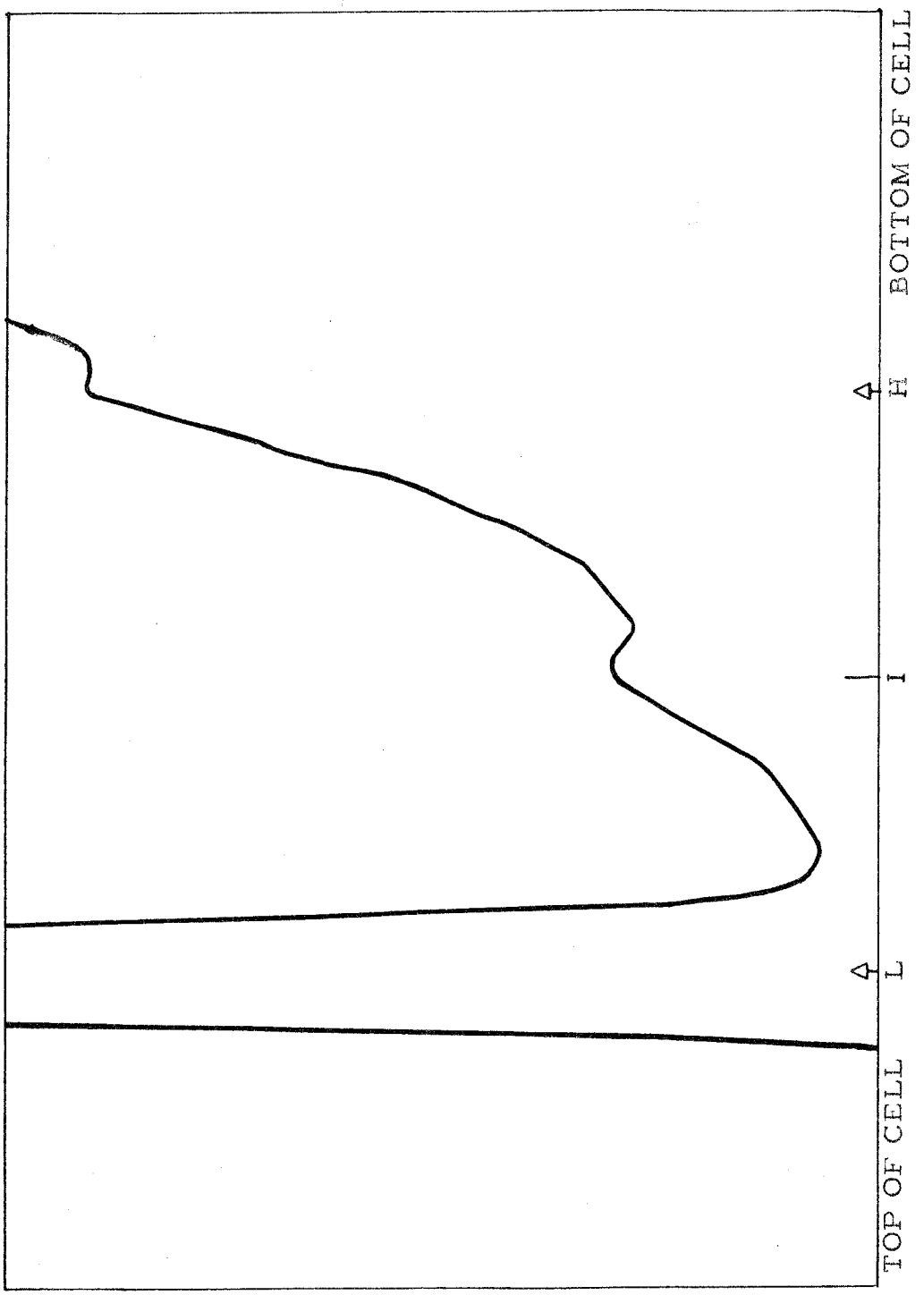

Fig. 12. Progeny DNA, passed through No. 23 and 26 needles, sonicated 30 minutes.
(Transfer experiment 8-3-4)

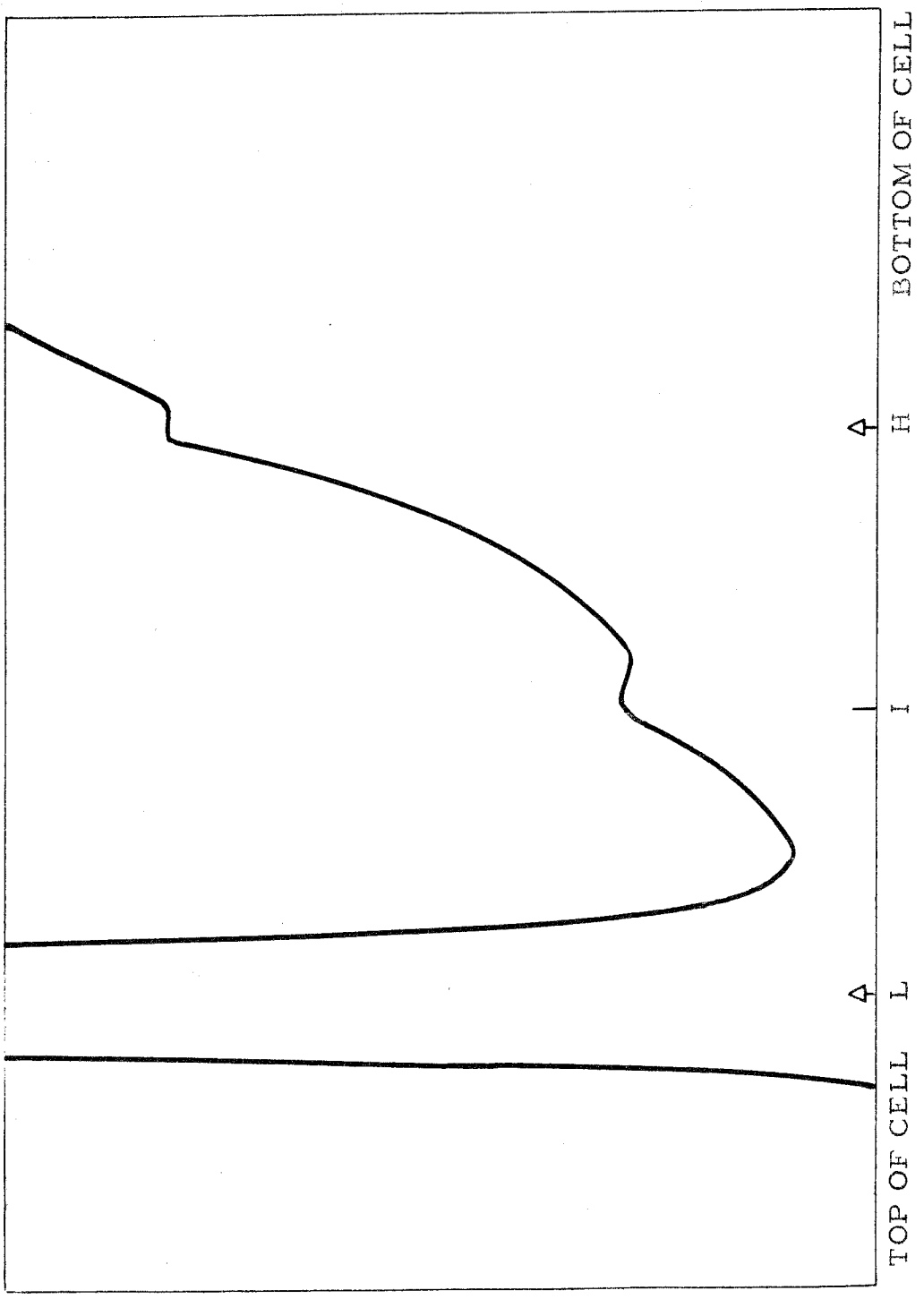

Fig. 13. Progeny DNA, passed through No. 23 and 26 needles, sonicated 2 hours.
(Transfer experiment 8-3-4)

parental phage isolated with the progeny. There is also a small amount of DNA of approximately intermediate density.

Little change is observed in the distribution of the DNA following passage through a hypodermic needle or sonication for 15 seconds (Figs. 9 to 11). Sonication for 30 minutes increases the fraction of material in the band of intermediate density (Fig. 11). This can be seen if the intermediate density peak is compared to the heavy peak in each figure. It contains less material than the heavy peak until the 30 minute period of sonication, after which it contains more. Sonication for an additional 2 hours produces no further effect (Fig. 12).

Another sample containing 4 times the previous amount of progeny DNA from experiment 8-3-4 was centrifuged. The photometer tracings (Figs. 14 to 18) are more convincing than those of Figs. 8 to 13, and show the same effects. The untreated DNA is mainly of light density, but the band is evidently skewed toward higher density (Fig. 14). There is a band of parental density. There is also material at densities between light and heavy.

No apparent change is produced by passing the sample twice in and out of a hypodermic with a number 26 hypodermic needle at moderate speed, apart from broadening the parental band (Fig. 15); nor is a change produced by heating to 70° (Fig. 16). Sonicating for 1/2 hour approximately doubles the amount of material in the intermediate density peak (Fig. 17). This is accompanied by a loss of much of the material in the heavy shoulder of the band of light DNA. The band of heavy DNA is broader, but has suffered no detectable change in the

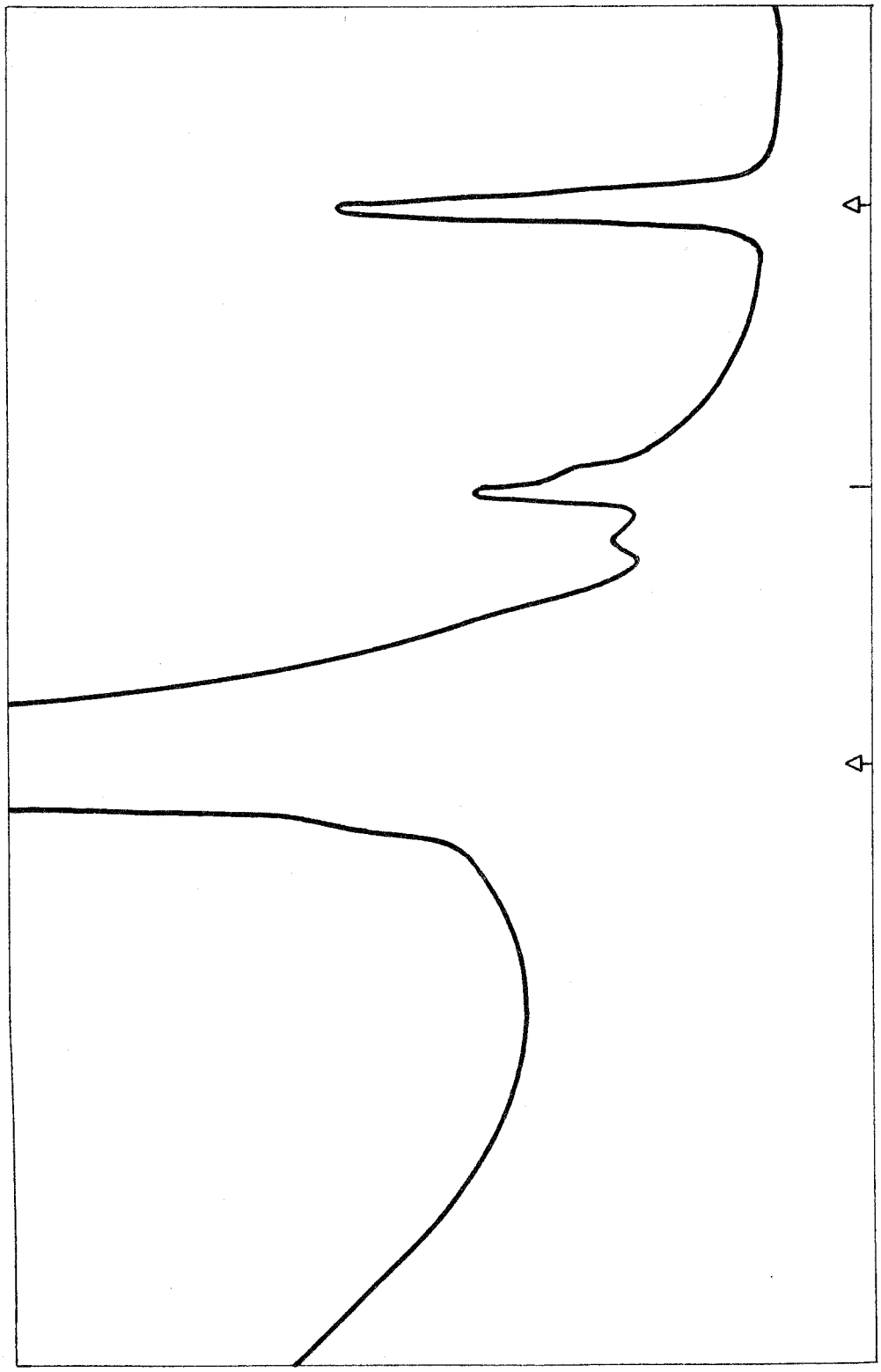

Fig. 14. Progeny DNA, untreated.
(Transfer experiment 8-3-4)

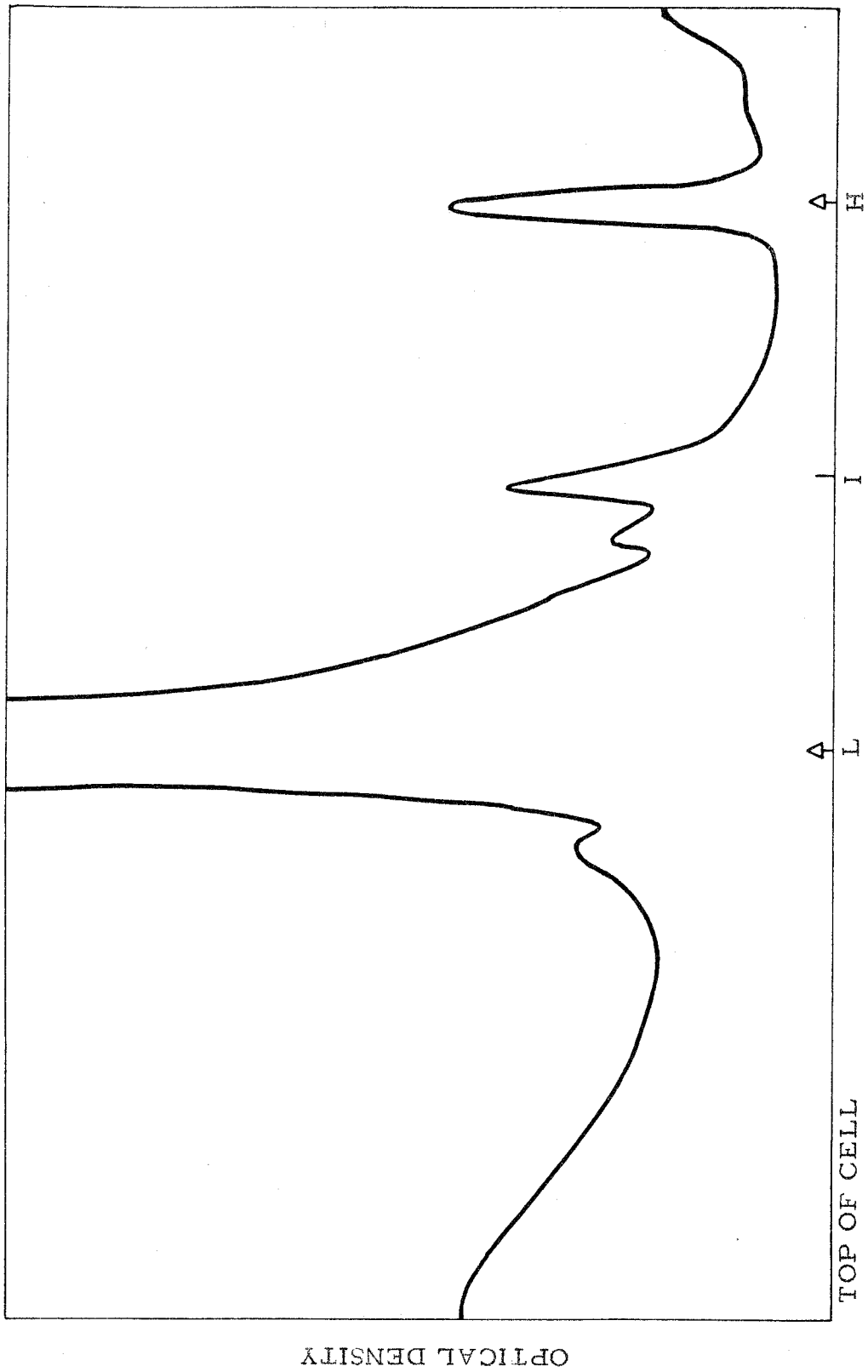

Fig. 15. Progeny DNA, passed through No. 26 needle.
(Transfer experiment 8-3-4)

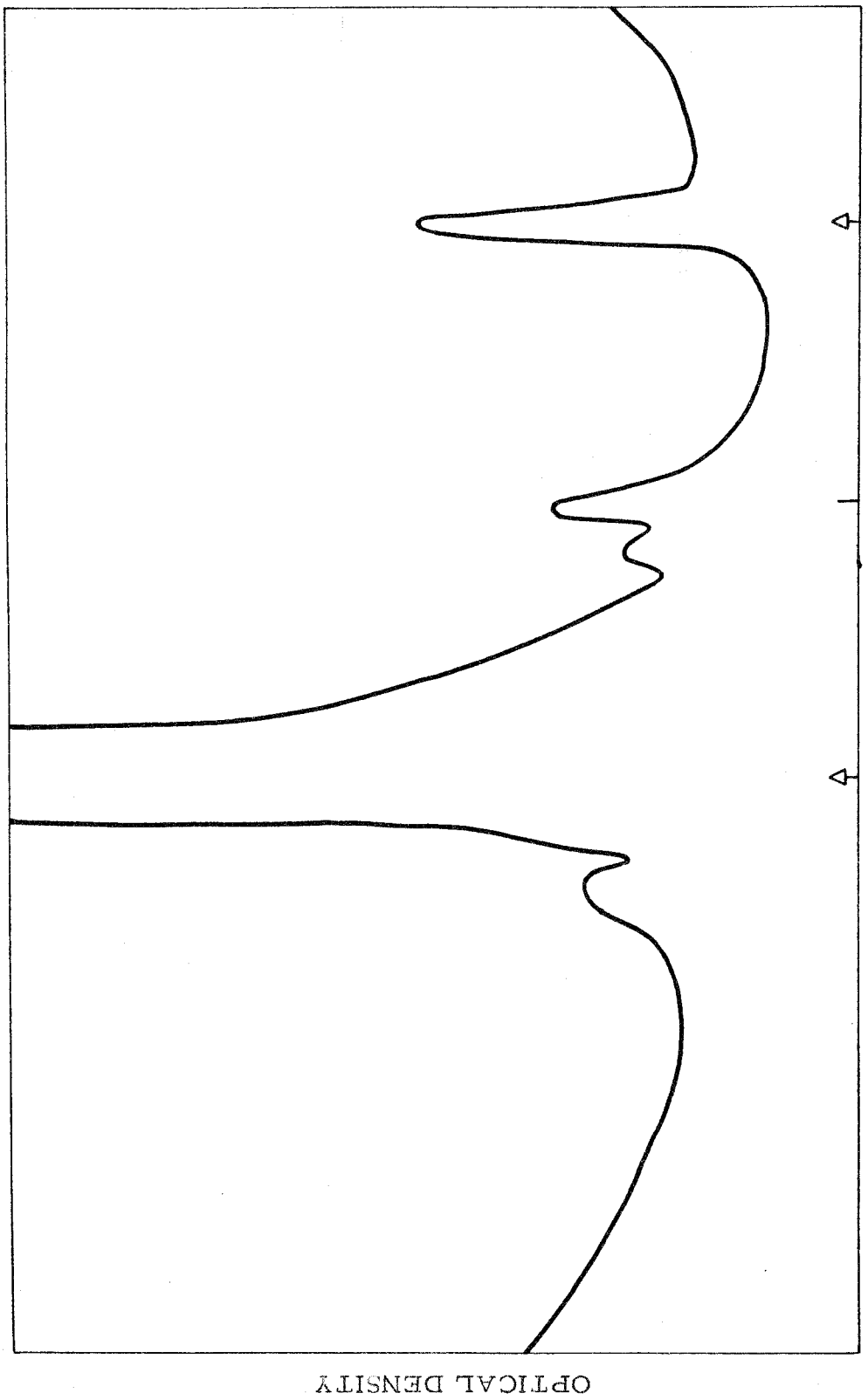

Fig. 16. Progeny DNA, passed through No. 26 needle, heated 70°.
(Transfer experiment 8-3-4)

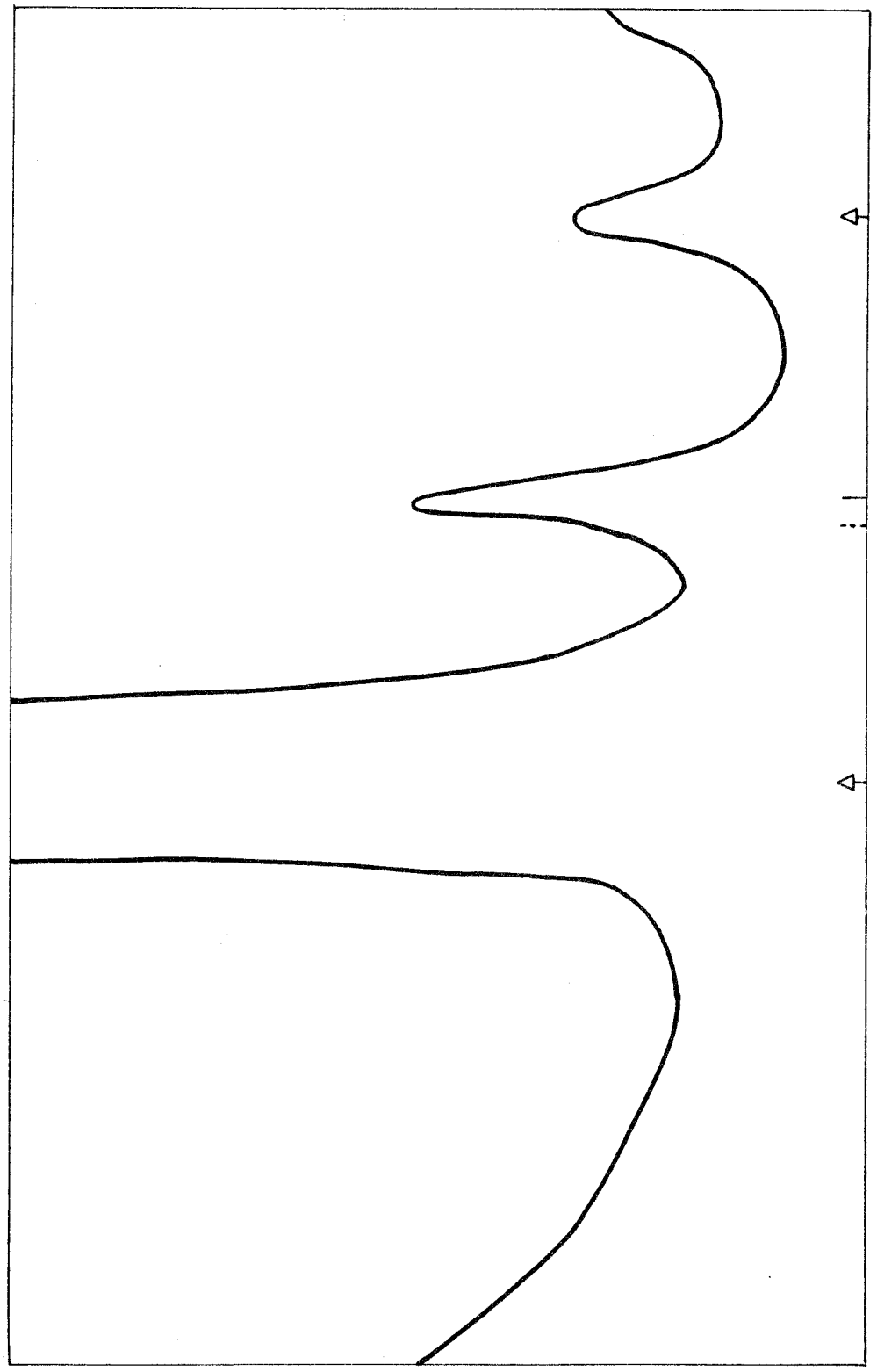

Fig. 17. Progeny DNA, passed through No. 26 needle, heated 70°, sonicated 1/2 hour.
(Transfer experiment 8-3-4)

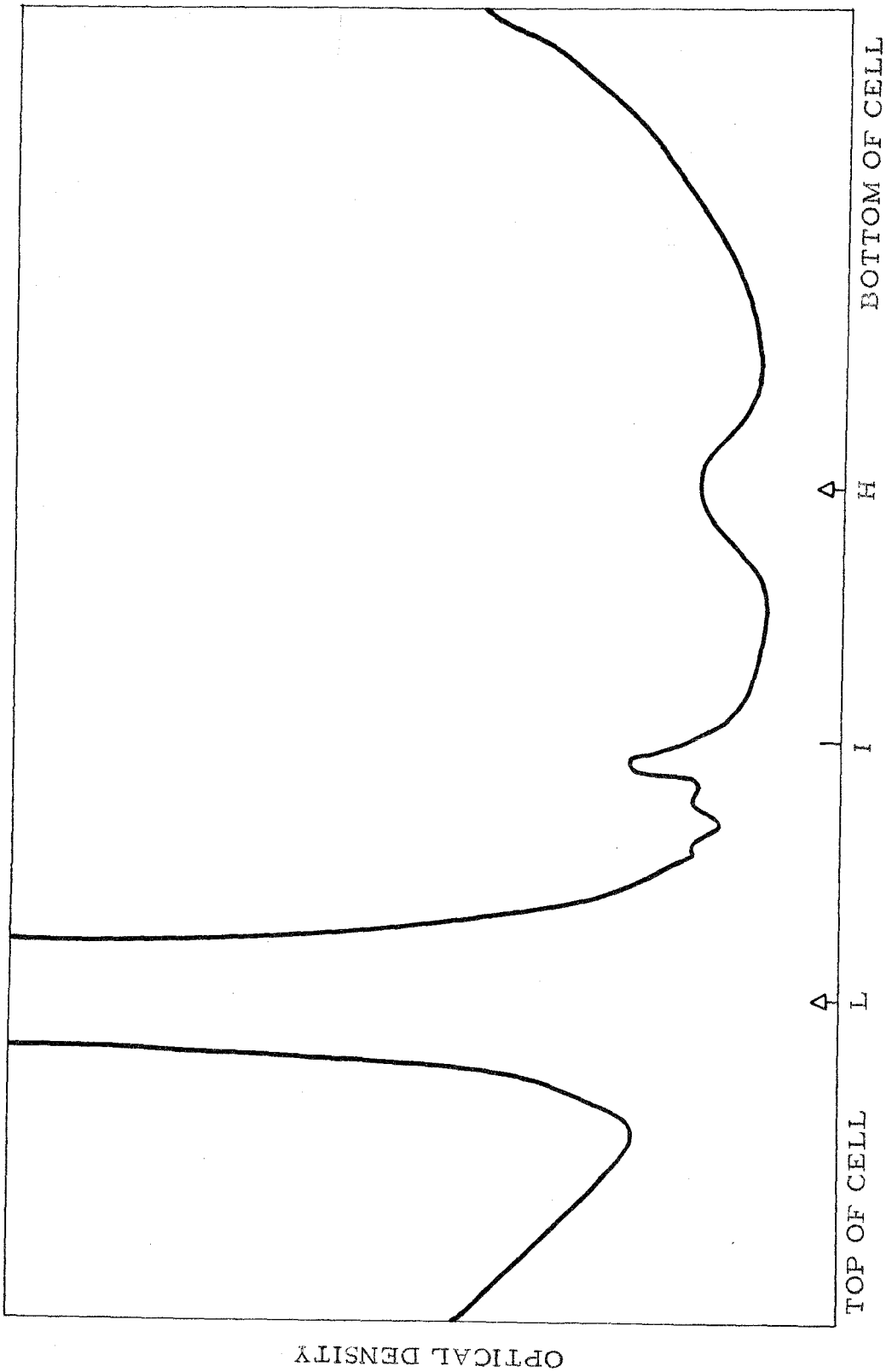

Fig. 18. Progeny DNA, passed through No. 26 needle, heated 70°, sonicated 1/2 hour, heated 100° 20 minutes.
(Transfer experiment 8-3-4)

amount of material it contains. After heating the sample to 100°, the DNA appears to be specifically lost from the band of intermediate density (Fig. 18). The apparent decrease in size of the peak is exaggerated by an overall loss of material incurred in the manipulation.

Because of the observation that much of the transferred parental DNA in the untreated sample is found at approximately the density of light phage DNA, the band shape of this light DNA was determined. It was hoped that the skewing on the heavy side of the band could be used to determine the distribution of parental atoms among these approximately light molecules. Therefore, a small aliquot of the transfer DNA was centrifuged at 27,690 RPM until equilibrium was closely approached. The photometer tracing is shown in Fig. 19. It can be seen that the band is indeed skewed on the heavy side.

Fig. 20 is a tracing of a similar sample of progeny DNA obtained from the dummy experiment 8-3-5, in which no heavy isotope was introduced. It can be seen that this band is also skewed on the heavy side. The skewing is less in this case. However, estimates of the distribution of parental atoms in the progeny DNA by the method of subtraction are clearly hazardous and have not been attempted. A similar observation of skewing of the band of light phage DNA on the heavy side has been reported (Hearst, 1961).

From these tracings a minimum molecular weight has been calculated. For the Cs salt of the DNA this is 64×10^6, or 44×10^6 for the Na salt. This was calculated from a measurement of the band width of the completely light, dummy DNA. (Sedimentation of a sample of the dummy DNA having an optical density at 260 mu of 1 indicated a

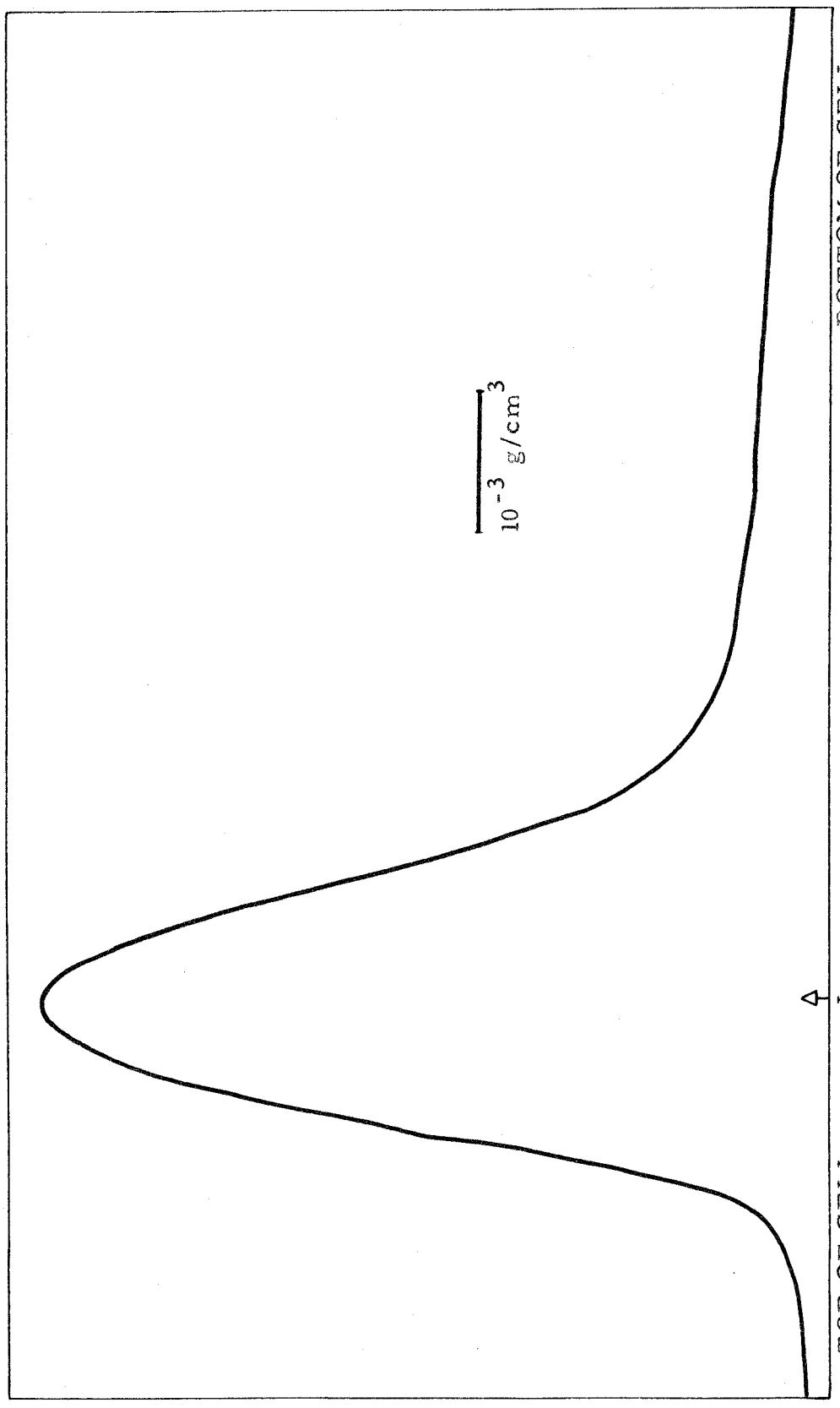

Fig. 19. Progeny DNA, untreated
(Transfer experiment 8-3-4)

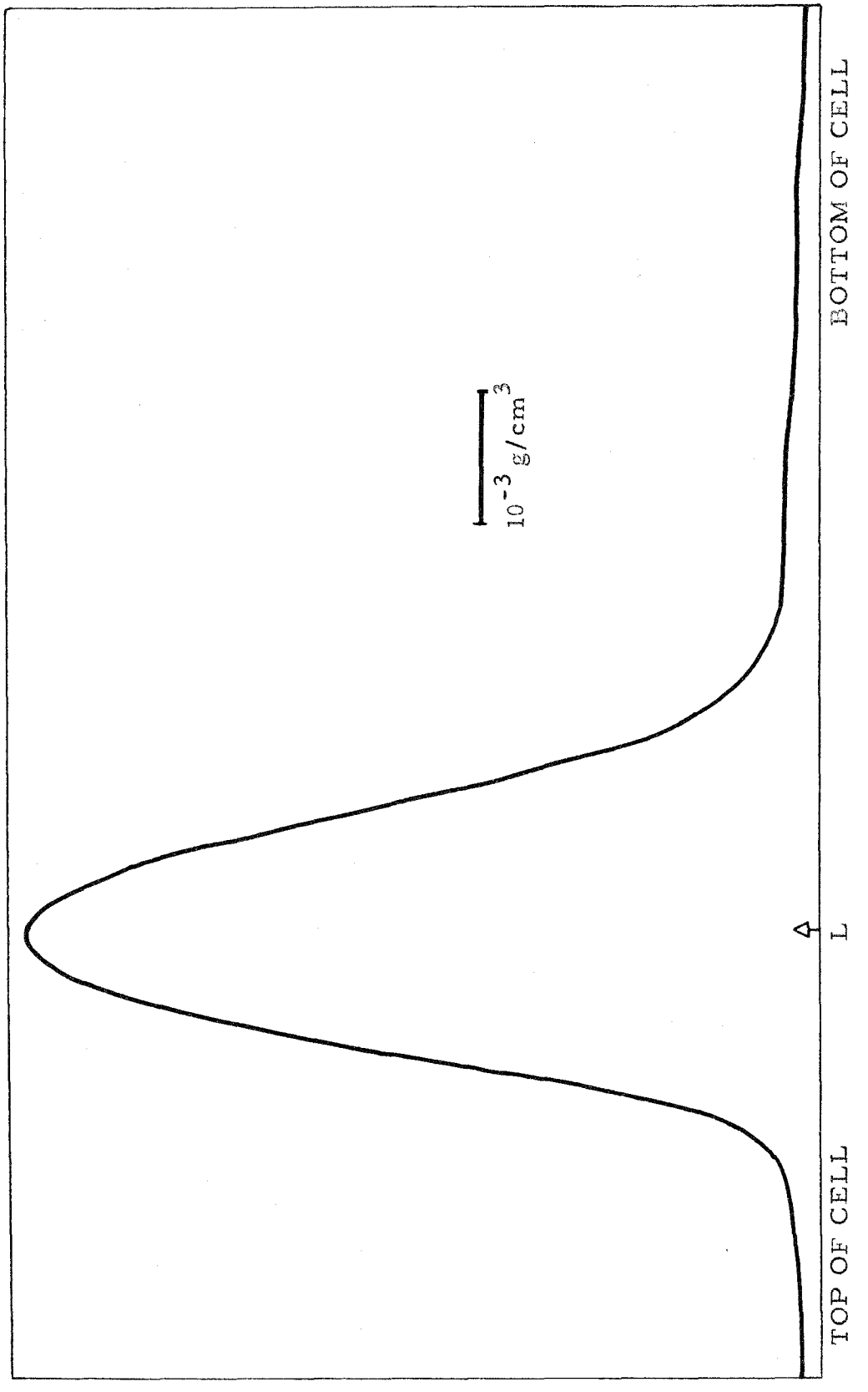

Fig. 20. Dummy progeny DNA, untreated
(Dummy experiment 8-3-5)

sedimentation coefficient of 54 S.) The samples of dummy and transfer DNA are apparently of about the same molecular weight, as indicated by the similar band widths.

The density distribution of progeny DNA from experiment H and H 6 is shown in Fig. 21. Most of the DNA is found at the density of light phage DNA. There is a sharp band of DNA with a density 0.036 g/cm^3 greater than that of light DNA. The density increase calculated for the Cs salt of phage DNA which is isotopically 99% N^{15} and 65% C^{13} is 0.039, assuming no selective solvation. It is assumed that this DNA is derived from unadsorbed parental phage. There also appear to be small amounts of DNA of densities between light and heavy.

Figs. 22 to 24 show the effects of several treatments on this DNA. After passing the sample rapidly through a number 26 needle 4 times, some of the DNA which previously appeared at light density is distributed between light and intermediate densities, with a slight maximum at intermediate density or a little lighter (Fig. 22). Treatment with a number 26 needle in this experiment, as well as in other experiments to be described, has released DNA from the band of light DNA. It is not clear why this effect is not observed in the experiment described above.

Sonication separates little if any DNA from the main band of light DNA (Fig. 23). However, the distribution of the liberated DNA appears to shift so that a larger fraction is found near intermediate density.

Subsequent heating to 100^o causes the disappearance of most of

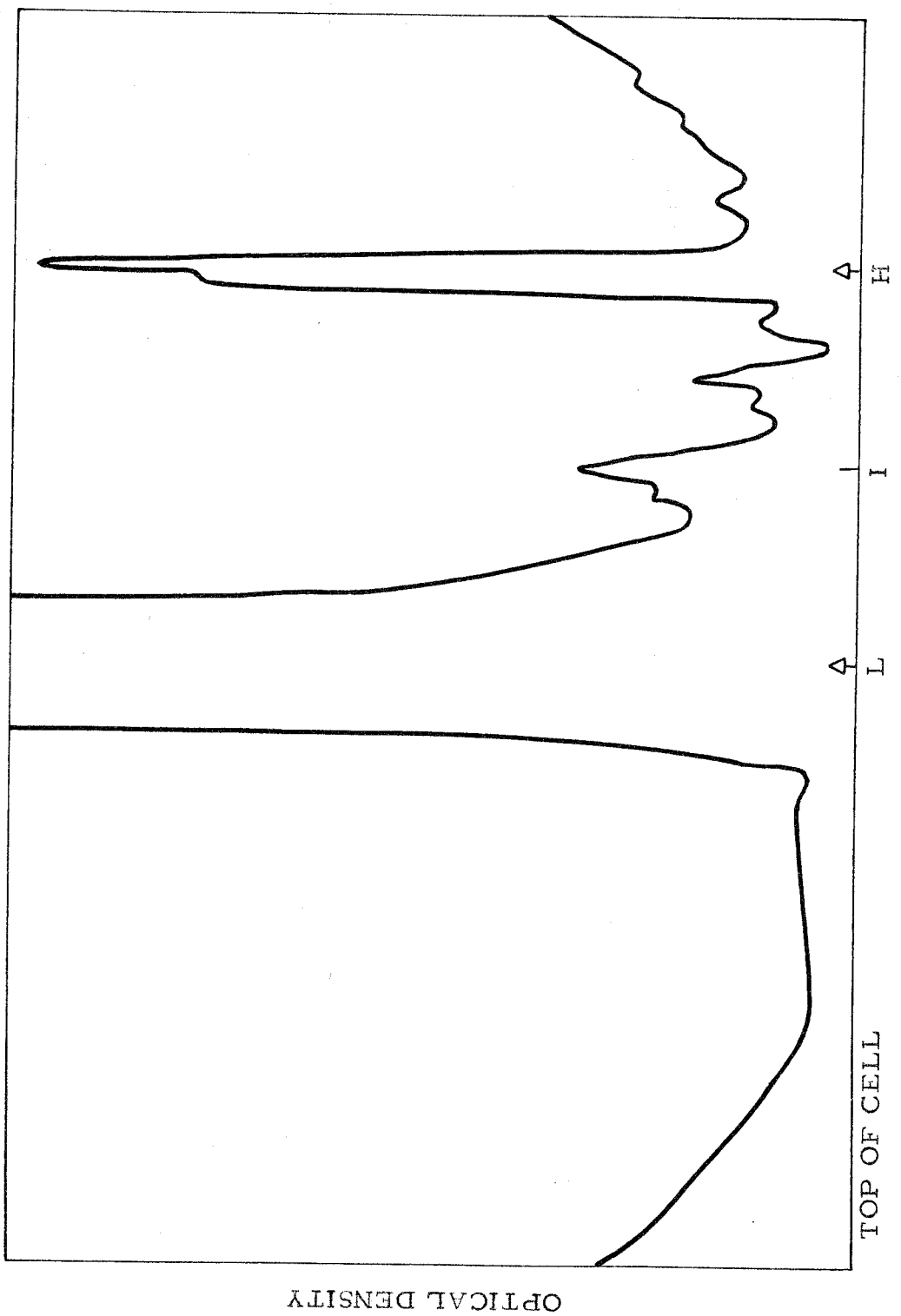

Fig. 21. Progeny DNA, untreated
(Transfer experiment H and H 6)

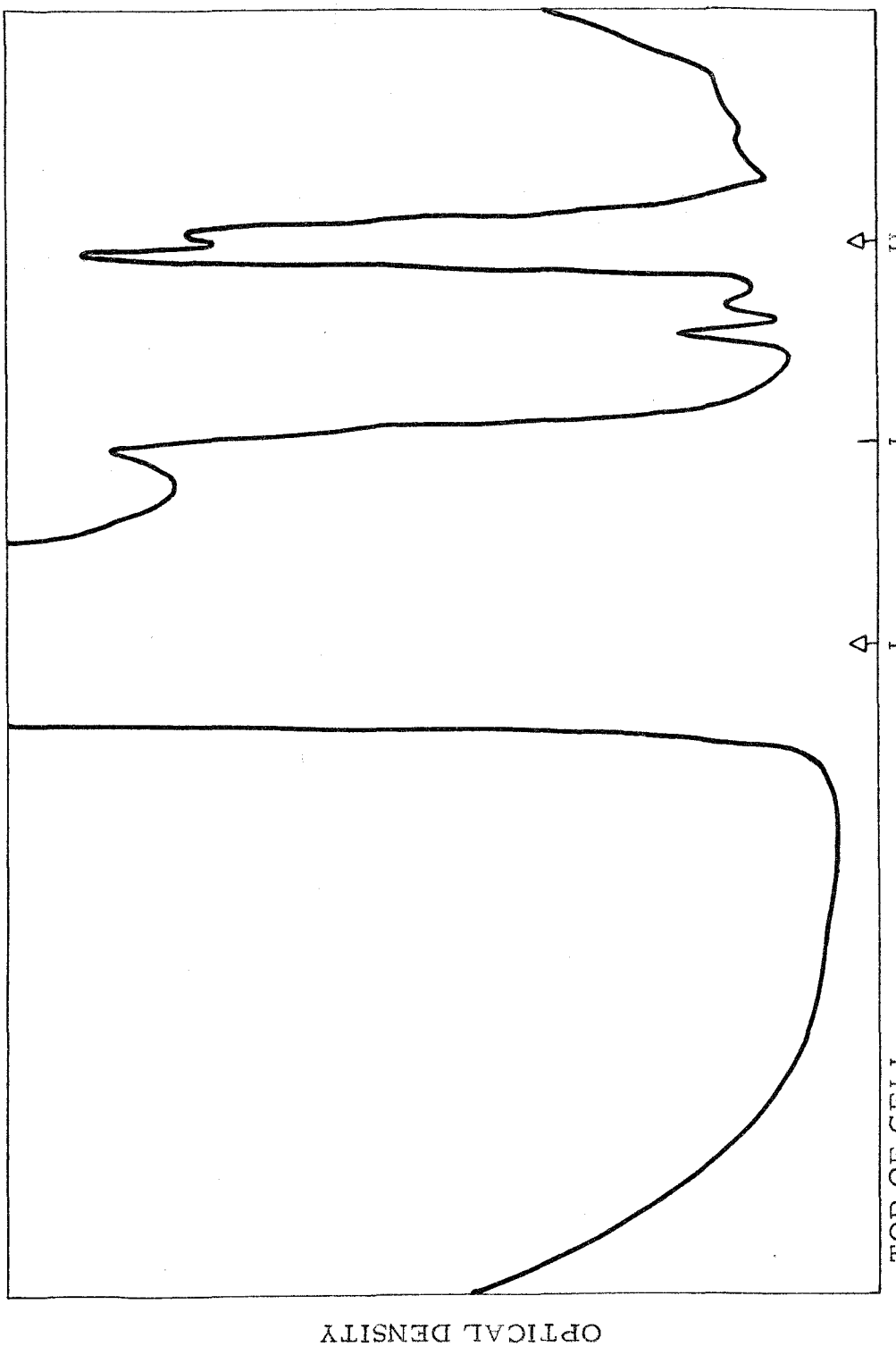

Fig. 22. Progeny DNA, passed through No. 26 needle.
(Transfer experiment H and H 6)

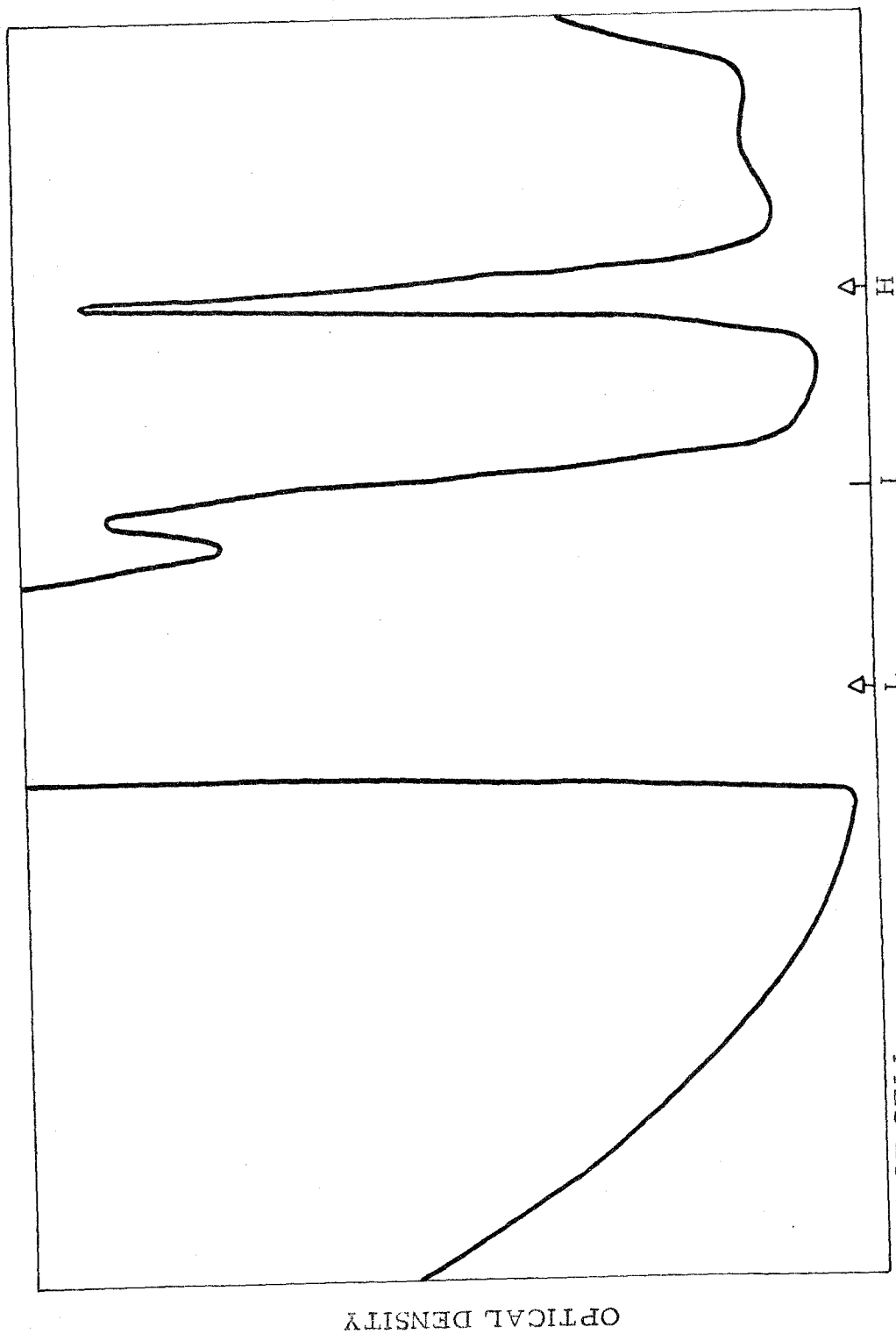

Fig. 23. Progeny DNA, passed through No. 26 needle, sonicated 15 minutes.
(Transfer experiment H and H 6)

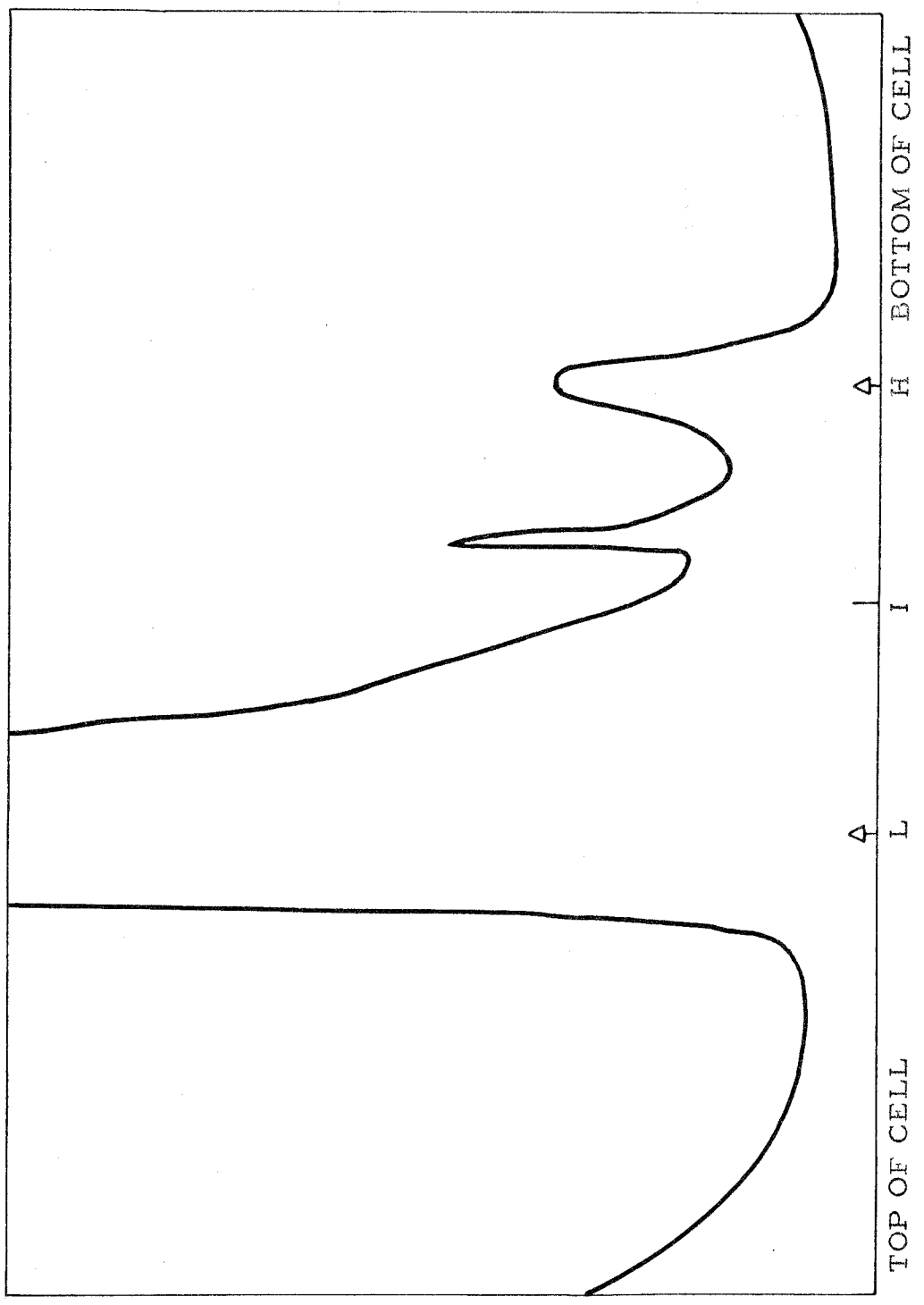

Fig. 24. Progeny DNA, passed through No. 26 needle, sonicated 15 minutes, and heated 100° 20 minutes. (Transfer experiment H and H 6)

the DNA which had been found between light and intermediate densities. The light band now has a long tail on the heavy side, which extends to the density of completely heavy DNA. The band at heavy density is very broad, and has suffered a considerable loss of material, which may be attributable to physical losses incurred during the manipulation. The source of the peak between heavy and light DNA is not clear. It is very sharp, but were it an aggregate of heavy and light DNA, the density should be closer to that of light DNA. Moreover, the material recovered from this centrifugation has been analyzed by means of preparative centrifugation (Fig. 32). It would be expected that such a narrow band of relatively high specific activity would be observed; there is no indication of its presence.

The results of centrifugations of the DNA obtained from the progeny phage of the dummy experiment H and H 6D, in which there was no heavy isotope introduced, are seen in Figs. 25 to 27. There are no small bands of DNA between the densities of heavy and light phage DNA, either in the case of the untreated progeny DNA, or of this DNA after treatment with a number 26 needle followed by 30 minutes sonication, or after heating to 100^{o} for 20 minutes. The bands seen in the previous samples cannot therefore reflect a property of normal isotope phage DNA which is observed when very large amounts of it are analysed.

4.2 Preparative Centrifugation of Progeny DNA

In the first experiment to be described, H and H 5, bacteria were grown to a titer of 2×10^{8}/ml at 30^{o} in M9 containing 0.084% Difco Casamino acids. They were centrifuged and resuspended at a concentration

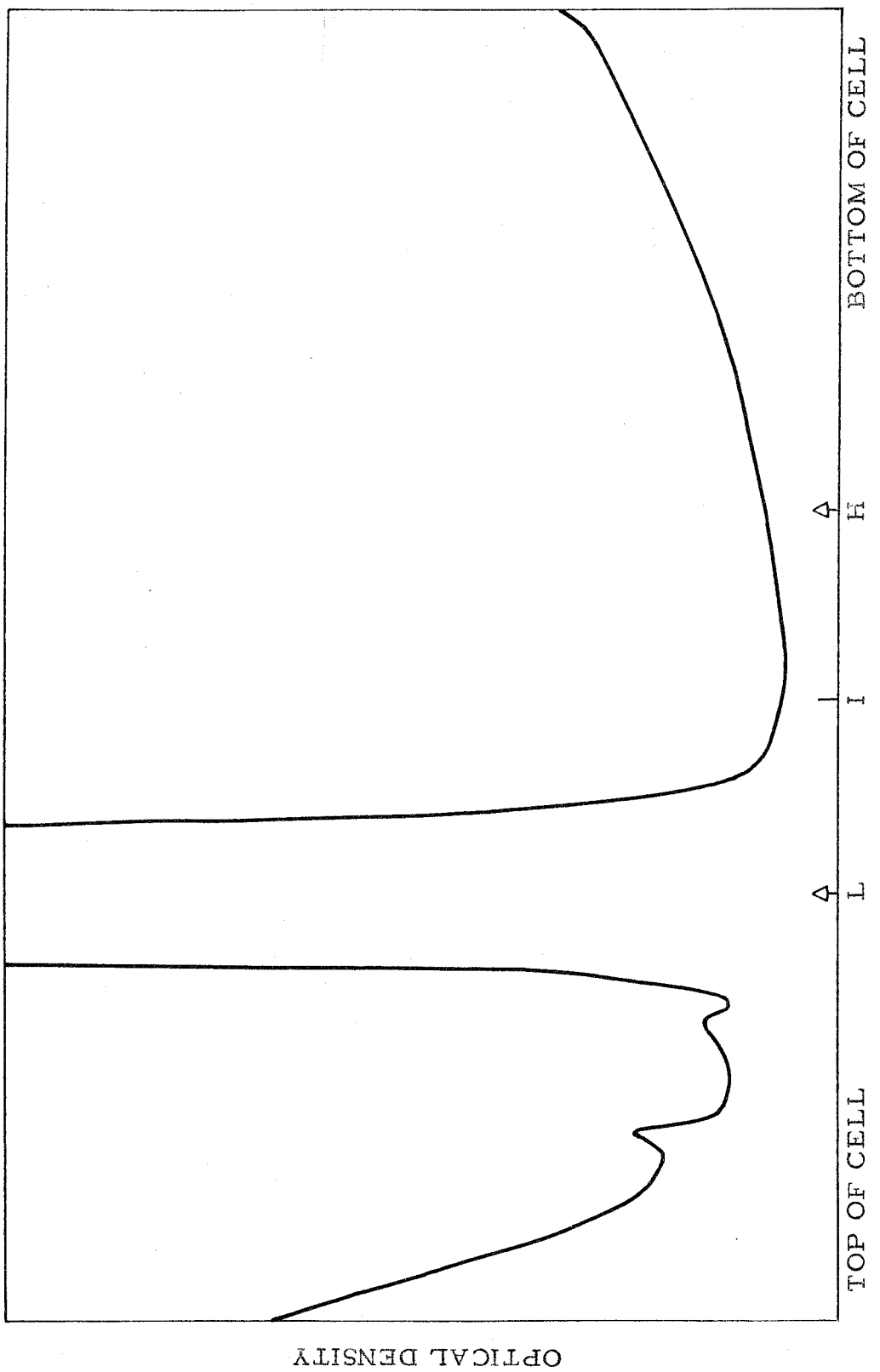

Fig. 25. Dummy progeny DNA, untreated.
(Dummy experiment HandH 6D)

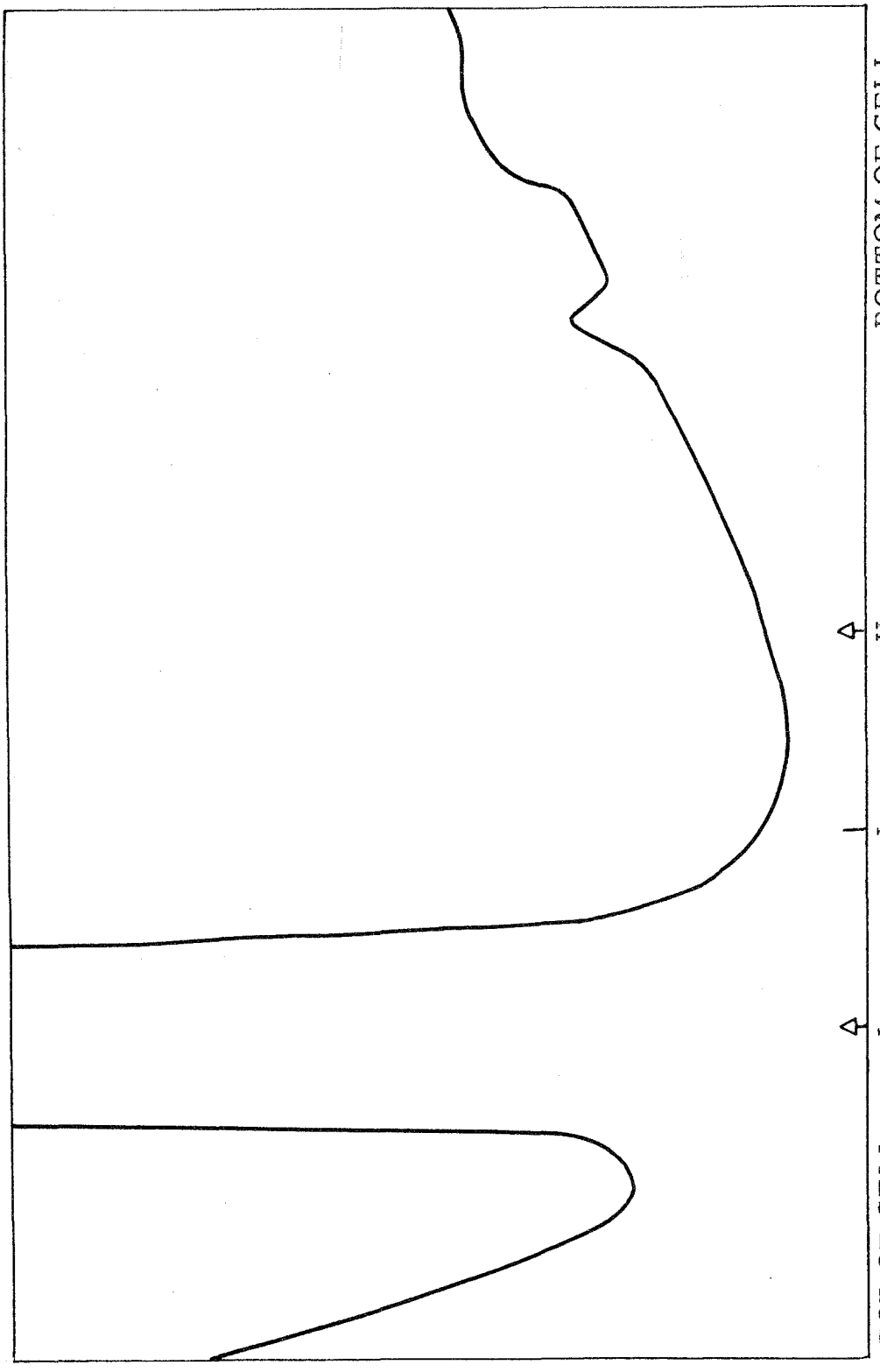

Fig. 26. Dummy progeny DNA, passed through No. 26 needle, sonicated 15 minutes.
(Dummy experiment H and H 6D)

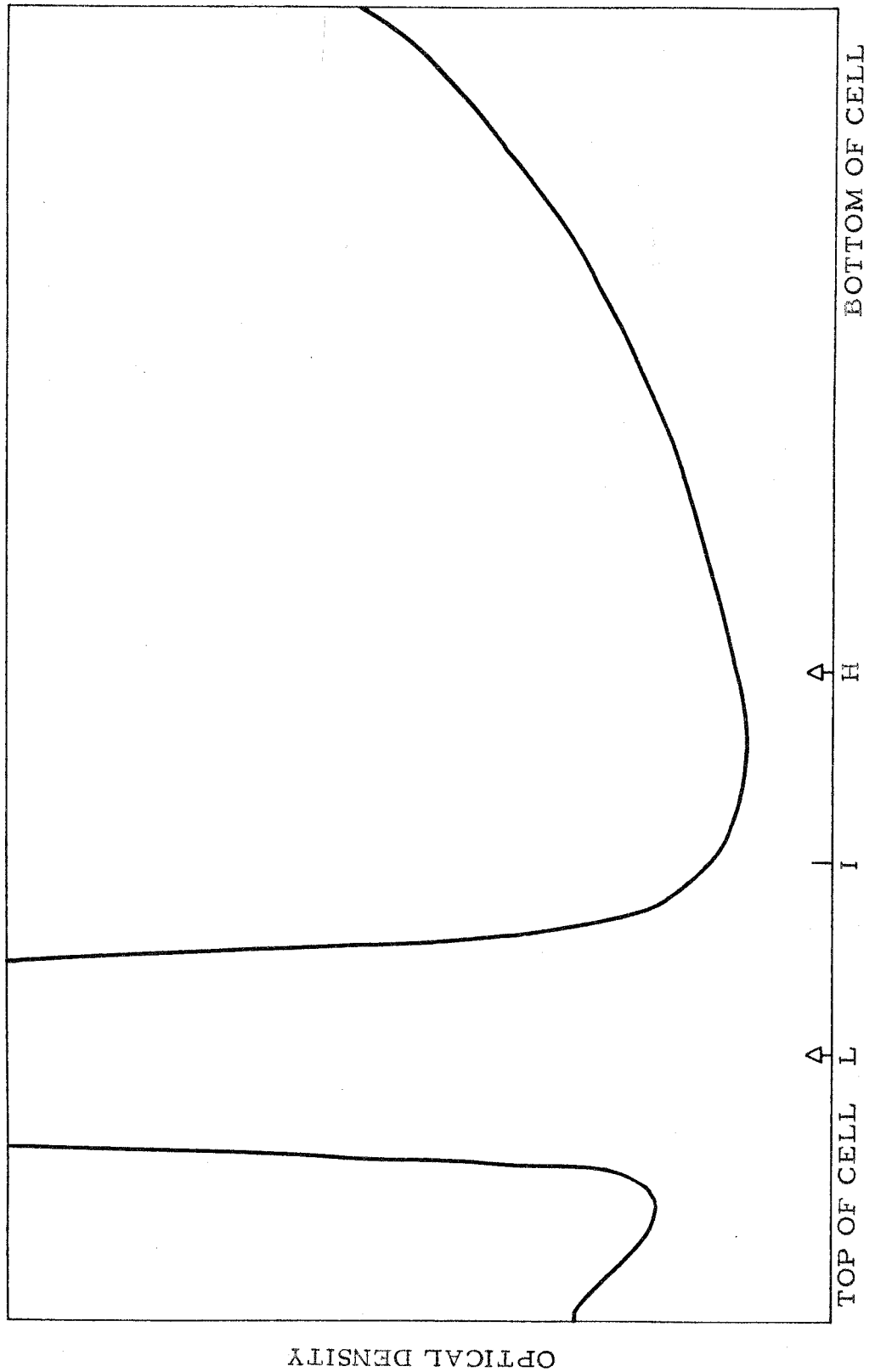

Fig. 27. Dummy progeny DNA, passed through No. 26 needle, sonicated 15 minutes, and heated 100° 20 minutes. (Dummy experiment H and H 6D)

of 4×10^8/ml in fresh medium to which was added tryptophan (5 μg/ml) and KCN (2×10^{-3} M). Preparation of the phage, $T4Bo^r_1$ of 99% N^{15}, 65% C^{13} isotopic composition, is detailed in section 2.5. They contained radioactive phosphorus, specific activity approximately 3×10^{-5} disintegrations per minute per plaque forming unit, assayed as P^{32} adsorbable to bacteria. This corresponds to a maximum of approximately 1 P^{32} atom per phage, assuming all P^{32} is in viable phage particles.

The bacteria were infected with phage at a multiplicity of 0.15. After 10 minutes, the adsorption tube was chilled in ice, centrifuged at 4^o 10 minutes at 3200 RPM, resuspended in cold buffer, and recentrifuged. The pellet was resuspended in a small volume of cold buffer. 27 volumes of fresh medium at 30^o were added at zero time. At 37 minutes the remaining 140 ml of culture was lysed by the addition of $CHCl_3$ with a burst size of 67. DNase (35 μg/ml) was added to the lysate; after 6 minutes RNase (35 μg/ml) was added; after 1 minute 14 g $(NH_4)_2SO_4$ was added to prevent adsorption of progeny phage to bacterial debris. The solution was immediately filtered through a Millipore filter. Two 25 ml aliquots of the lysate were centrifuged at 15,000 RPM 2 hours; the phage pellets were resuspended overnight. To each was added 0.01 ml of a stock solution of $T4Bo^r_1$, approximately doubling the optical density at 260 mμ. The phage were lysed by saturating with guanidine HCl, and brought to density 1.72 as described in section 2.7. One sample was passed several times through a number 24 needle. They were centrifuged at 35,000 RPM at 25^o. During the

collection of drops, the density was determined several times by measuring the refractive index of small aliquots withdrawn for this purpose.

The second experiment, H and H 6, was described in section 4.1 above. 5 ml of the infected culture was reserved for these centrifugations. Growth was allowed to proceed for three hours after infection, at which time $CHCl_3$ was added to complete lysis. The burst size was 360, twice that of the analytical centrifuge samples. 30 ml of the dummy culture in which parental phage were cold and light was lysed after 3 hours with a burst size of 400. All the samples of progeny phage were purified and lysed in parallel, as described in sections 2.5 and 2.6. The small volume of parental phage remaining after the completion of the experiment (about 0.05 ml) had already been purified in preparation and was lysed directly.

The progeny DNA and an aliquot of dummy progeny DNA were combined. Appropriate volumes of CsCl and buffer were added to give 6 ml of a solution of density 1.715, pH 8.4, containing 3×10^4 counts/minute and having an optical density at 260 mμ of 0.4. The optical density, 75% of which was contributed by the dummy transfer DNA, was used as a density marker, indicating the position of completely light phage DNA. 2 ml of the untreated solution was centrifuged. The remaining 4 ml was passed rapidly 4 times in and out of a 5 ml syringe with a 26 needle. 2 ml of this solution was centrifuged.

Aliquots of the samples recovered from the transfer and dummy analytical centrifugations were mixed. These samples had both been passed through a number 26 needle, sonicated, and heated to 100° for

20 minutes. Appropriate volumes of CsCl and buffer were added to obtain a density of 1.735 g/cm^3 and a volume of 2 ml. This sample contained 3.4x10^3 counts/minute and had an optical density at 260 mμ of 0.4.

The analytical centrifugations of light dummy DNA showed that such DNA does not contain components which band at densities between light and heavy. In the preparative centrifugations, the parental phage DNA was centrifuged to ascertain that it was not the source of bands of densities between light and heavy.

To an aliquot of the DNA obtained from parental phage particles was added light dummy progeny DNA to provide a density marker. The sample was brought to density 1.715, pH 8.4 and 6 ml volume. It contained 4x10^4 counts/minute and had an optical density of 0.15, contributed entirely by dummy DNA. 2 ml was centrifuged untreated. The remaining 4 ml was passed through a 26 needle. 2 ml of this solution was centrifuged. The remaining 2 ml was heated to 100° 20 minutes, and adjusted to density 1.731 before centrifuging.

All samples were centrifuged at 35,000 RPM, 25°, 45 hours. The recovery of P^{32} and optical density in these samples is presented in Table 2. P^{32} losses are generally less than 10%, except in the sample of parental DNA heated to 100°. In this case, the losses incurred in the additional manipulations have not been taken into consideration, although they might be expected to be of the order of 10 to 20%.

In Fig. 28 is shown the density distribution of P^{32} in DNA isolated from progeny phage following infection of cold, light bacteria with hot

TABLE 2

Recovery of Samples Following Preparative Centrifugation

Experiment	Sample	Fraction Recovered P^{32}	Fraction Recovered O.D.
H and H 5	Progeny DNA, untreated	0.90	0.64[1]
H and H 5	Progeny DNA, passed through No. 26 needle	1.02	0.72[1]
H and H 6	Progeny DNA, untreated	0.90	0.88
H and H 6	Progeny DNA, passed through No. 26 needle	0.92	0.86
H and H 6	Progeny DNA, recovered from analytical centrifugations, passed through No. 26 needle, sonicated, heated 100° 20 min.	0.96	0.67[2]
H and H 6	Parental DNA, untreated	1.00	1.02
H and H 6	Parental DNA, passed through No. 26 needle	0.99	0.86
H and H 6	Parental DNA, passed through No. 26 needle, heated 100° 20 min.	0.67[3]	0.53[3]

[1]Estimation of recovery is inaccurate because total optical density in sample was small.

[2]The DNA added for the optical density marker was dummy DNA recovered from analytical centrifugations. Some of this optical density is an accumulation from the many manipulations and adds to the background. Recovery is estimated from total O.D. added and total recovered in the peak only.

[3]No estimate of mechanical losses accompanying the manipulations is included. The loss may be 10-20%.

and heavy phage in experiment H and H 5. The optical density distribution is provided by the addition of DNA obtained from normal light isotope phage. (The optical density scale is not drawn on the figures). It can be seen that the density distributions of P^{32} and of the optical density marker are approximately the same; that is, parental atoms are found in DNA molecules of approximately light density.

In Fig. 29 is shown the density distribution of the mixture of transfer and normal isotope DNA following passage through a number 24 needle. The density of the light DNA, assayed as optical density, is not affected by this treatment. However, the P^{32} is now found in DNA of density greater than that of the light DNA.

The density distributions of P^{32} and optical density following centrifugation of untreated progeny DNA from experiment H and H 6 are shown in Fig. 30. The P^{32} is found at two densities. The smaller peak corresponds in position to parental DNA, and presumably derives from unadsorbed parental phage isolated with progeny phage. In this experiment, unlike H and H 5, the infected cells were not freed of unadsorbed phage by centrifuging and washing. The completely heavy DNA, comprising about 1/4 of the total P^{32}, provides an additional density marker. The peak of the P^{32} band at light density corresponds very closely to that of the optical density band. Thus, transferred parental P^{32} atoms are, in large part, found in DNA molecules with the density of light, unlabeled phage DNA. Since there are 12 drops between the light and heavy peak, it is reasonable that the major portion of transferred DNA must be incorporated into molecules with less than

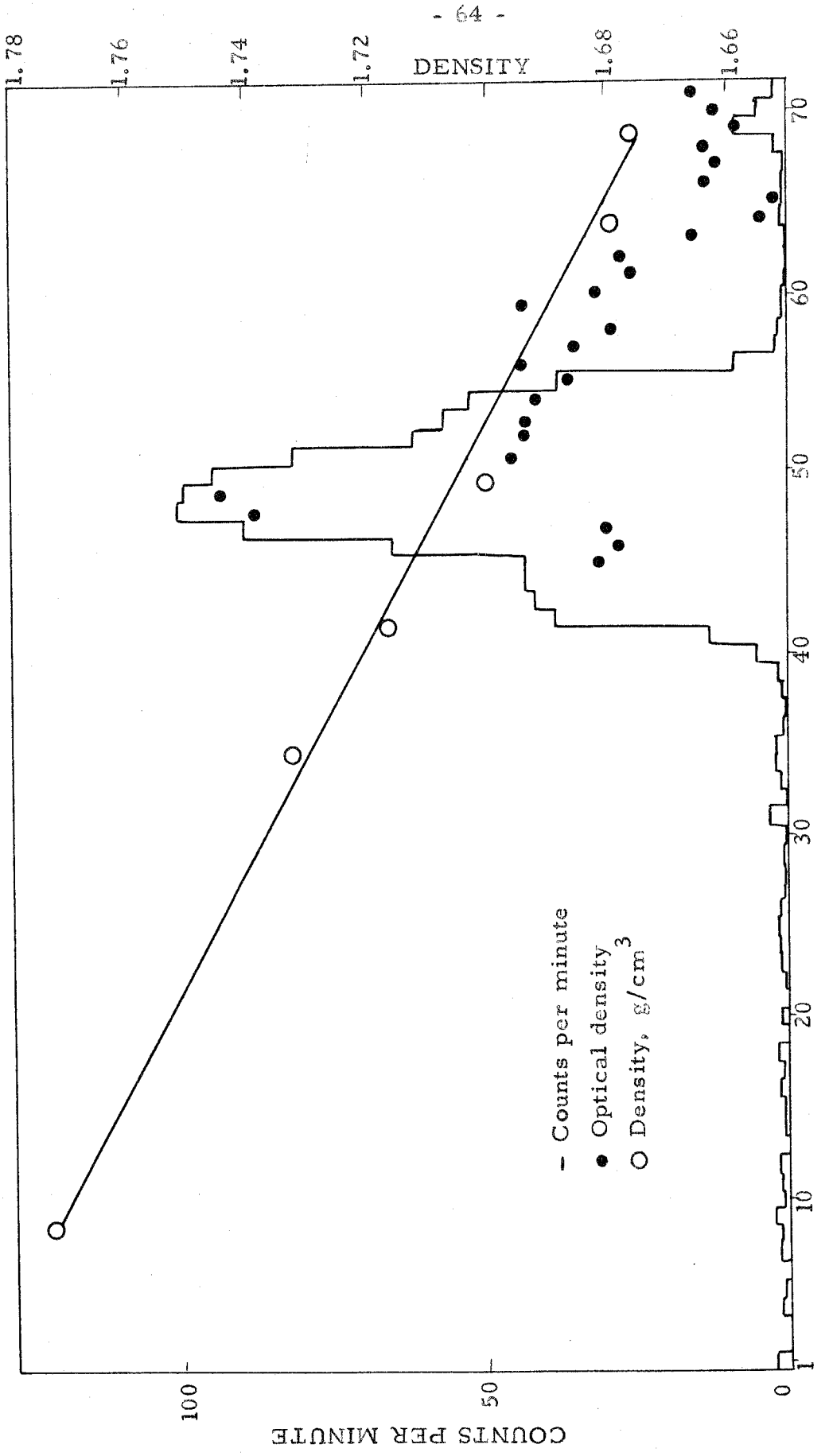

Fig. 28. Progeny DNA, untreated.
(Transfer experiment H and H 5)

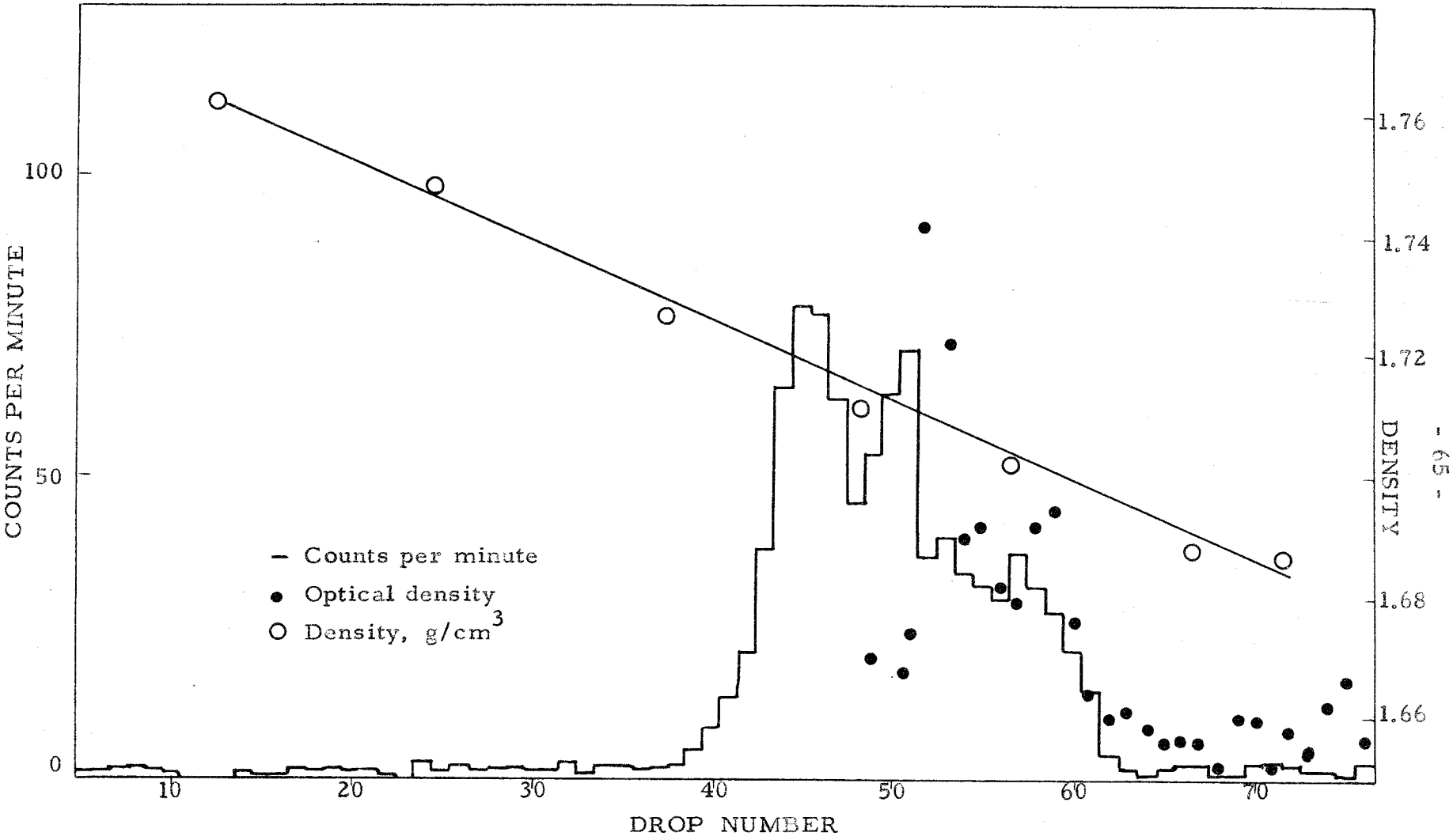

Fig. 29. Progeny DNA, passed through No. 24 needle
(Transfer experiment H and H 5)

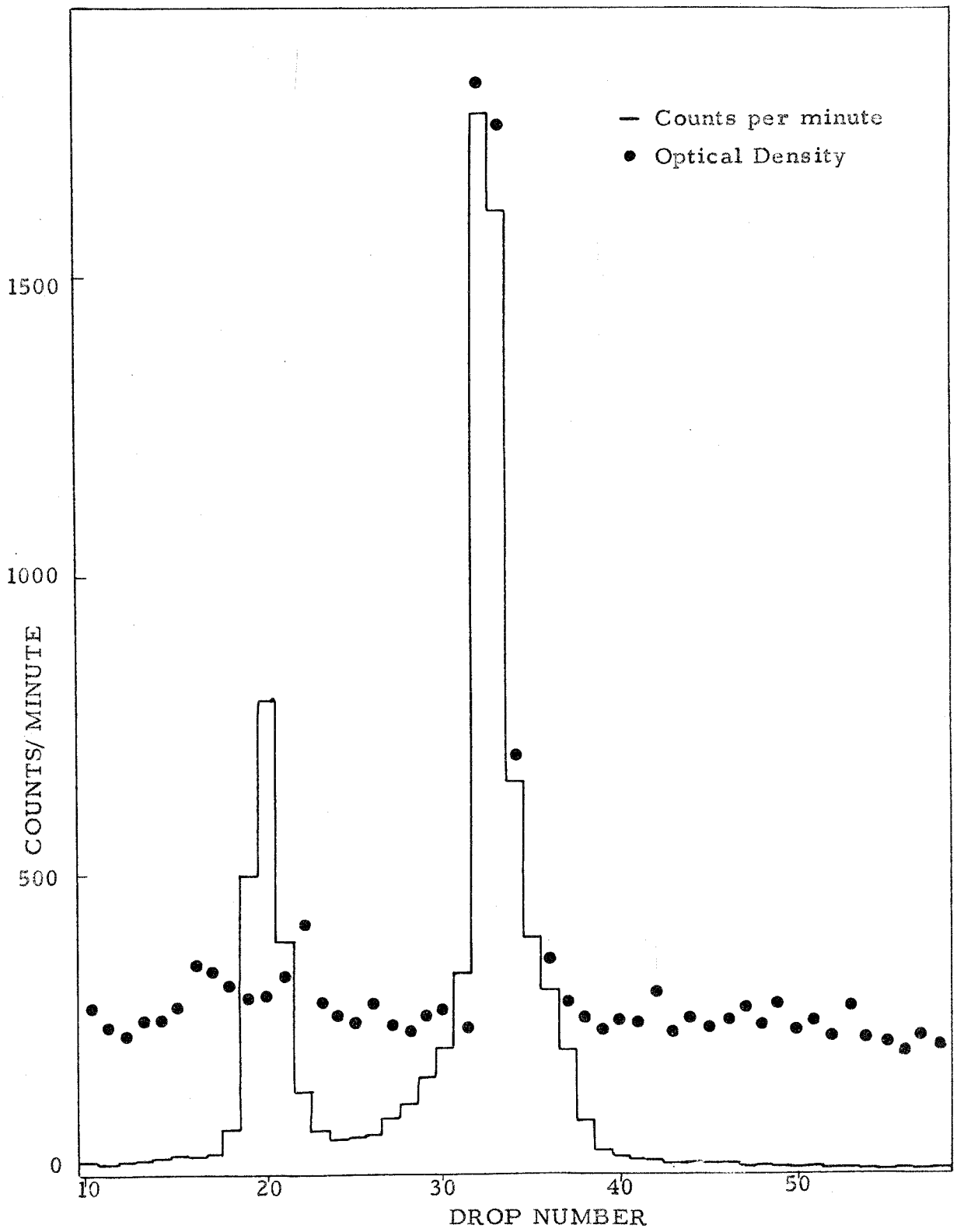

Fig. 30. Progeny DNA, untreated
(Transfer experiment H and H 6)

1/12, or 8%, heavy parental atoms. If as much as 1 out of 12 of the DNA atoms were heavy, the P^{32} peak would be shifted 1 drop toward the peak of completely heavy DNA, and away from the peak of light DNA.

The leading edge of the light P^{32} peak is much less sharp than the leading edge of either the heavy P^{32} peak or the optical density peak. The material in this leading edge must represent transferred parental phosphorus which is in DNA molecules of densities between light and heavy, containing 8% or more of parental atoms. For the most part, this heavier DNA is found between light and intermediate densities. It is difficult to estimate the fraction of the P^{32} in the leading edge, but probably not more than 20% of the transferred P^{32} in the lighter peak has a density detectably greater than that of DNA composed only of normal isotopes.

In the photometer tracing of untreated progeny DNA from this experiment, there are small peaks between the peaks of light and heavy DNA (Fig. 21). In the distribution of Fig. 30 these are not present. This may be due in part to the poorer resolution of preparative centrifugation. Small peaks in the photometer tracing will be distributed among 2 or 3 drops, causing blurring. Another contributing factor is the change in specific activity with density. That is, the optical density associated with a given amount of P^{32} found at intermediate density will be twice that associated with the same amount of P^{32} at completely heavy density, because the P^{32}-containing DNA at intermediate density must be associated with an equal amount of unlabeled DNA. The intermediate

density peak in Fig. 21, the largest of the minor peaks, contains approximately 1/4 the optical density of the heavy peak. This is 1/8 the P^{32}. Moreover, this peak is broader than the heavy peak. These factors could conspire to place this and the other small peaks just below the limits of visibility in the preparative centrifugation.

In Fig. 31 is seen the change in density distribution brought about by rapidly passing a sample identical to that of Fig. 30 in and out of a number 26 needle 4 times. The heavy P^{32} peak and the (light) optical density peak are both broader, presumably indicating a decrease in molecular weight. The distance between them remains unchanged, as expected. The P^{32} in the lighter peak now has a very broad density distribution, with the maximum shifted to approximately intermediate density. It may be noted that, although most of the P^{32} has shifted to densities greater than that of light phage DNA, much of the P^{32}-containing DNA is at or near light density, because the small fraction of P^{32} at light density must be in molecules in which most of the atoms are the normal isotopes. These results are in agreement with the results of the corresponding analytical centrifugation (Fig. 22).

An aliquot of progeny DNA, recovered from analytical centrifugation, was subjected to preparative centrifugation. (The photometer tracing is seen in Fig. 24.) This sample had been treated with a number 26 needle, sonicated 15 minutes, and heated to 100° for 20 minutes. In Fig. 32, it can be seen that these treatments have caused there to be a broad density distribution of P^{32}, extending from light to heavy densities. The largest fraction of the P^{32}-containing DNA is at or near

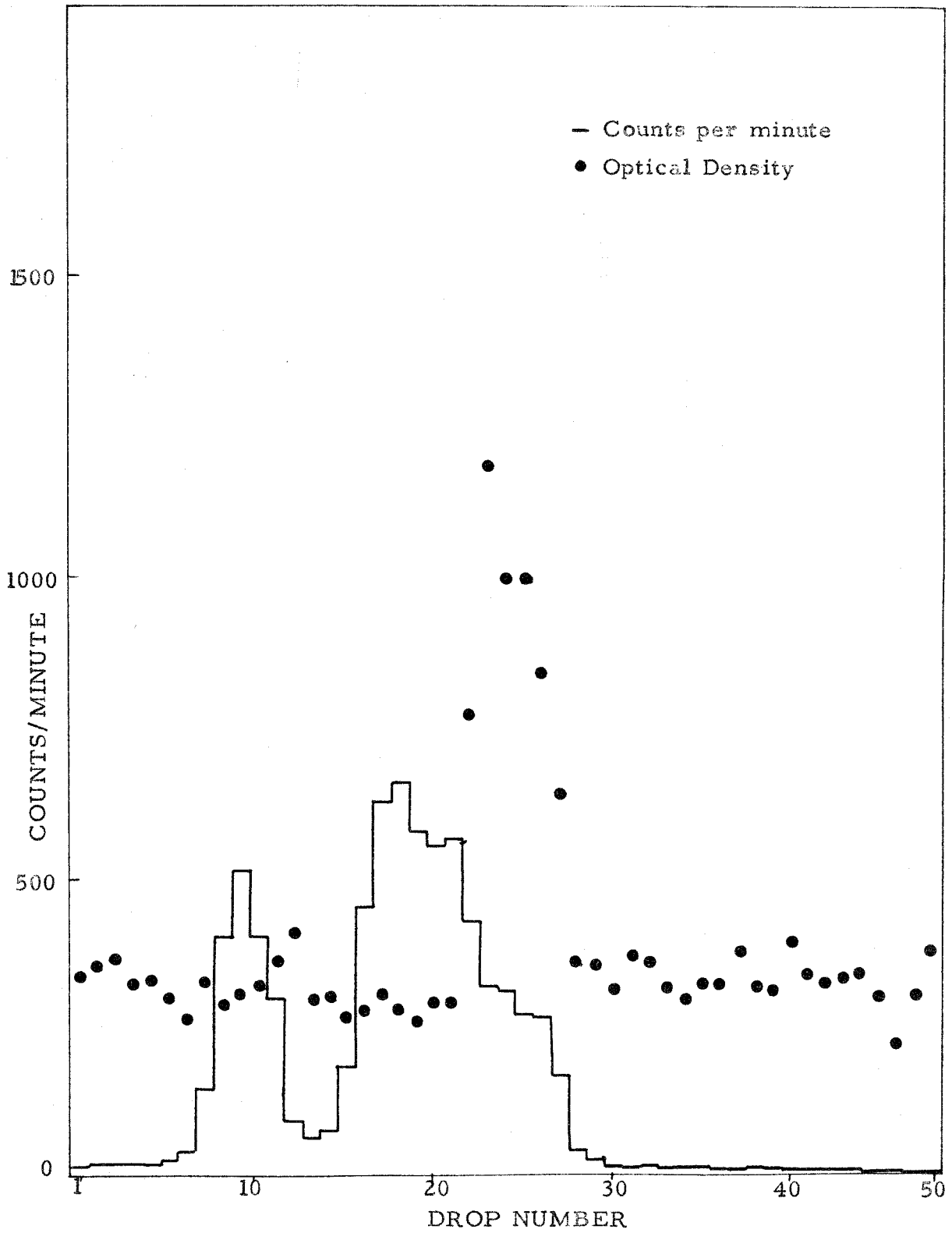

Fig. 31. Progeny DNA, passed through No. 26 needle.
(Transfer experiment H and H 6)

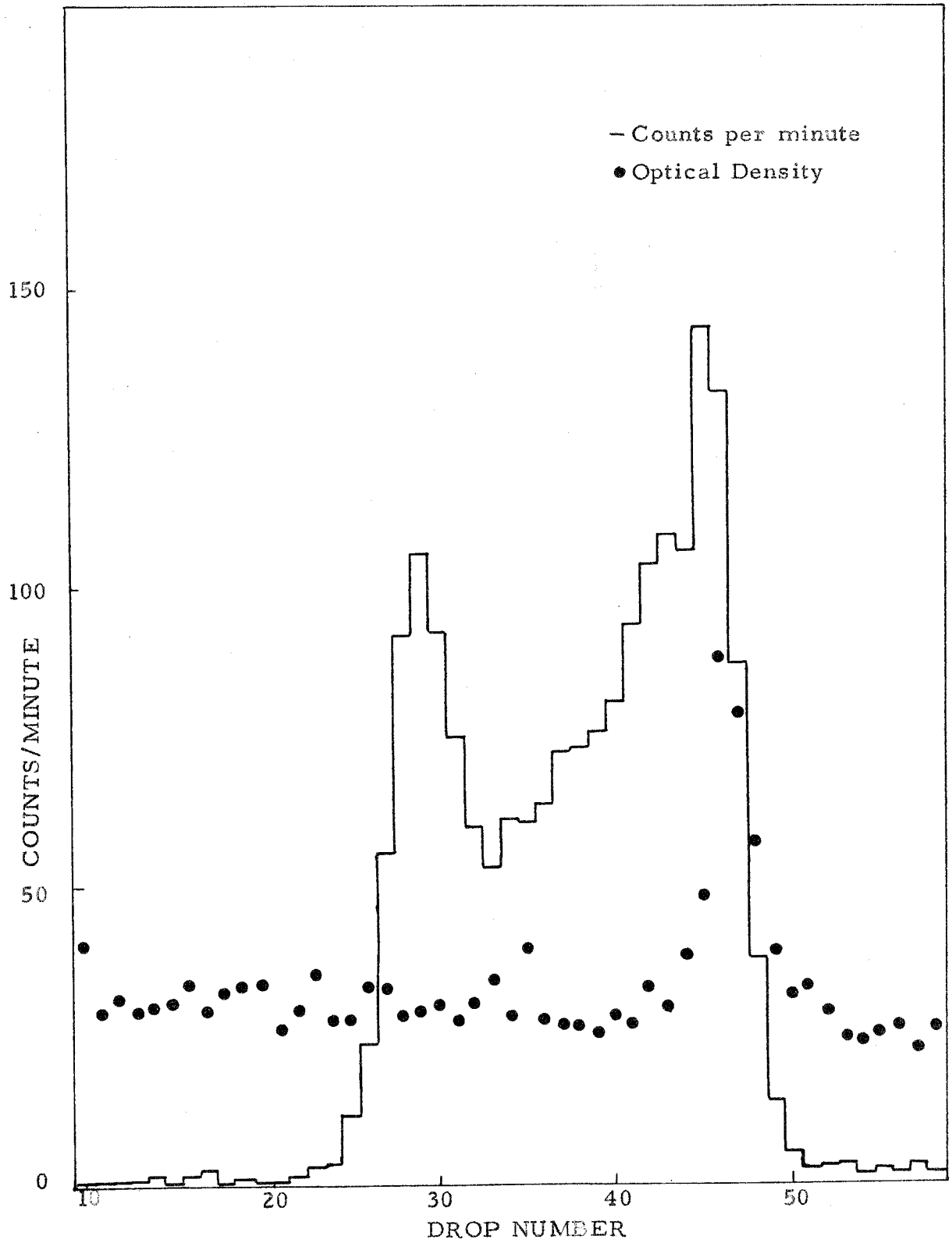

Fig. 32. Progeny DNA, reoovered after analytical centrifugations. Passed through No. 26 needle, sonicated, heated 100°. (Transfer experiment H and H 6)

light density. This is in accord with the distribution shown in the densitometer tracing.

In Figs. 33 to 35 are shown the P^{32} and optical density distributions of mixtures of DNA isolated from the parental phage and cold, light phage. These controls are included to ascertain whether the hot and heavy DNA from unadsorbed parental phage contains hot DNA of lighter densities. Such lighter DNA might be a component of the parental phage DNA, or might arise from aggregation. Since most of the DNA present is of light density, aggregates of hot and heavy DNA would contain light DNA, and therefore suffer a density shift.

The mixture of parental and light DNA was centrifuged untreated, after passage through a number 26 hypodermic needle, and after passage through a number 26 needle followed by heating to 100^{o}, as described above. Untreated parental DNA gives a single sharp band with little spread (Fig. 33). This has been the observation with the DNA obtained from other preparations of hot and heavy phage, including that of experiment H and H 5; these figures are not included. Parental DNA which has been passed through a number 26 needle, whether or not it has been heated to 100^{o}, forms a wider (single) band, as expected on the basis of the reduced molecular weight. There is no indication of extensive aggregation following these treatments (Figs. 34 and 35). The tail after the band of heated DNA could be an artifact introduced in collecting drops, or it could be caused by aggregation of a small fraction of the DNA. The poor optical density distribution of Fig. 35 is presumably due to UV absorbing material accumulated during the manipulations, rendering the band (at about drop 45) almost indistinguishable.

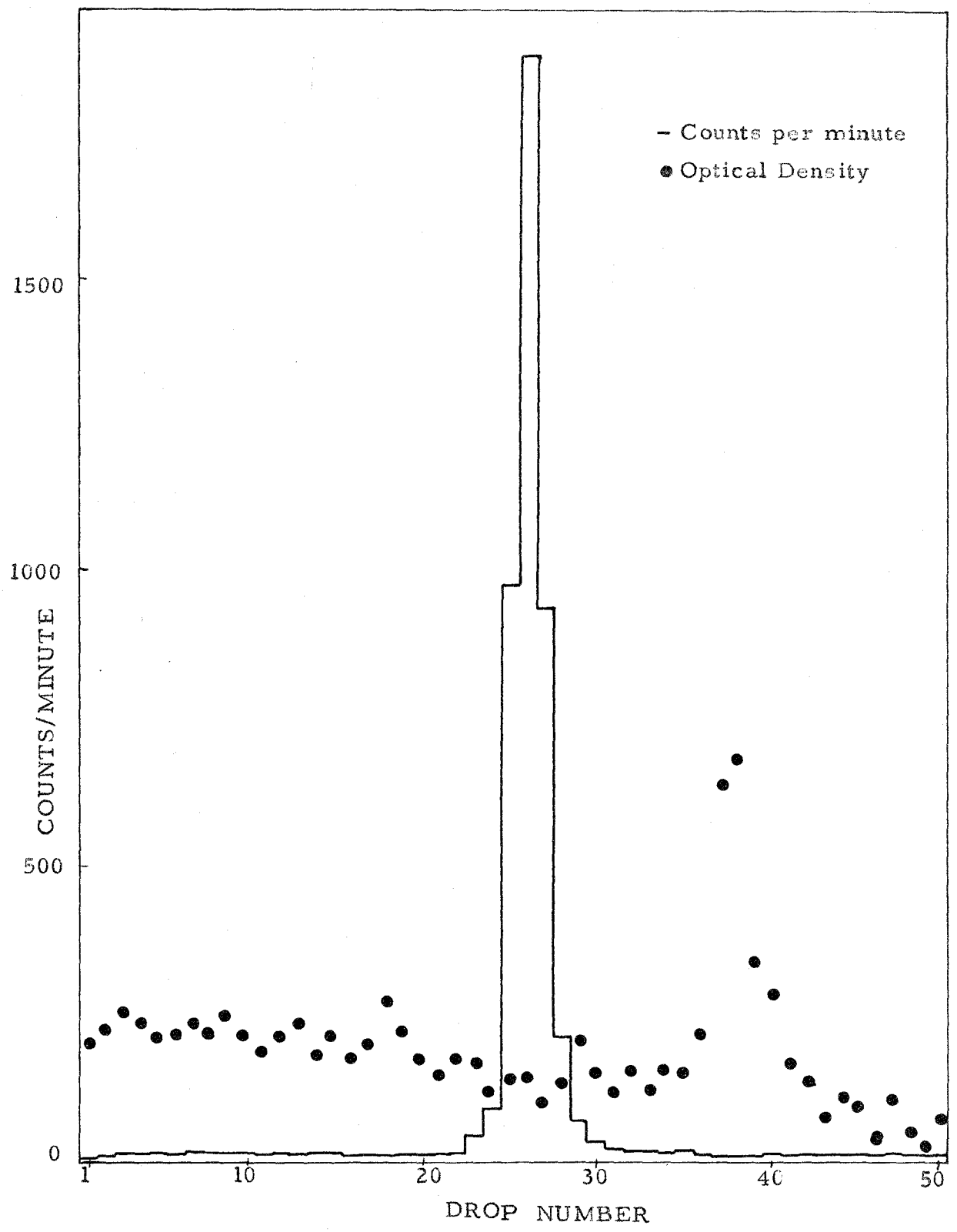

Fig. 33. Parental DNA, untreated
(Transfer experiment H and H 6)

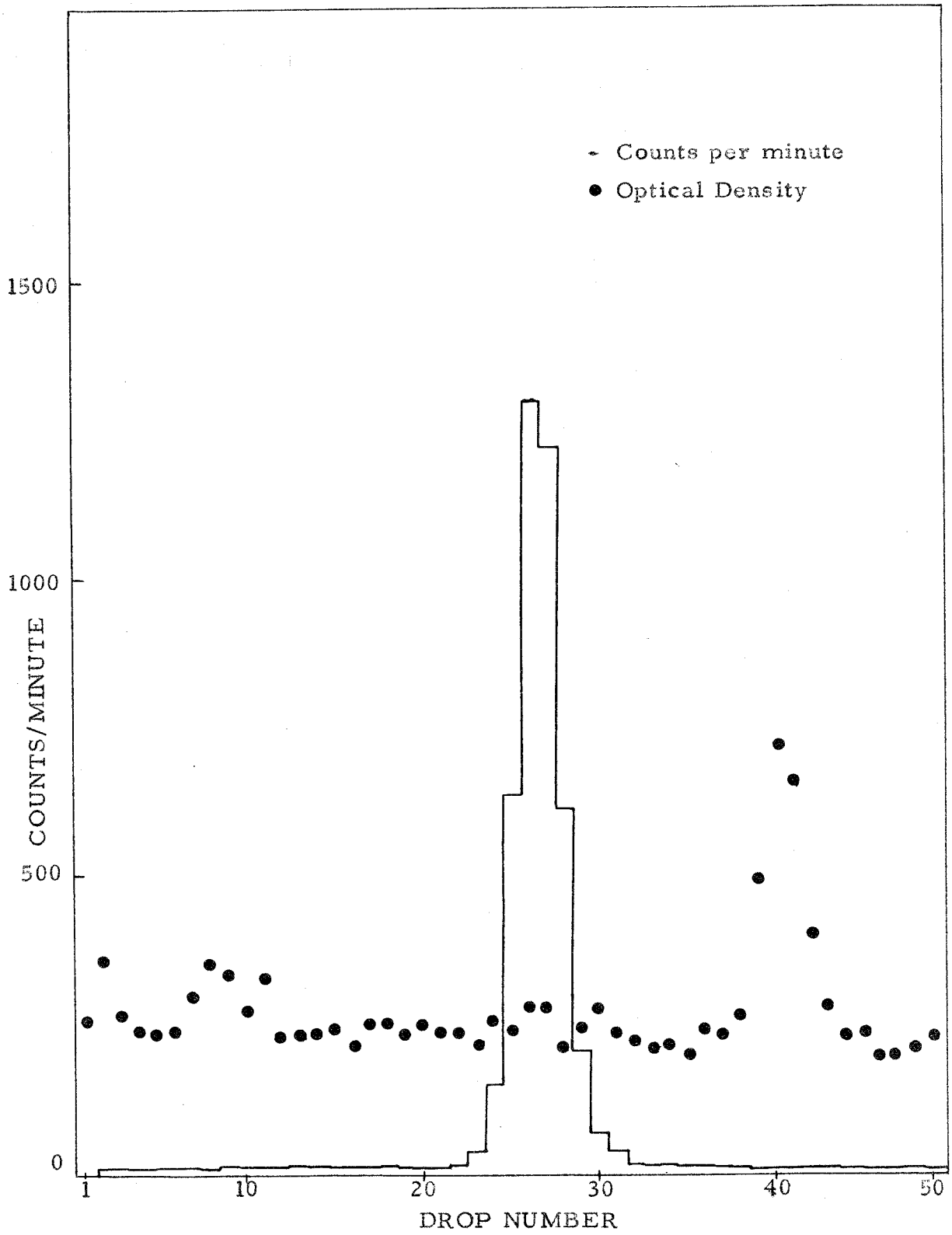

Fig. 34. Parental DNA, passed through No. 26 needle.
(Transfer experiment H and H 6)

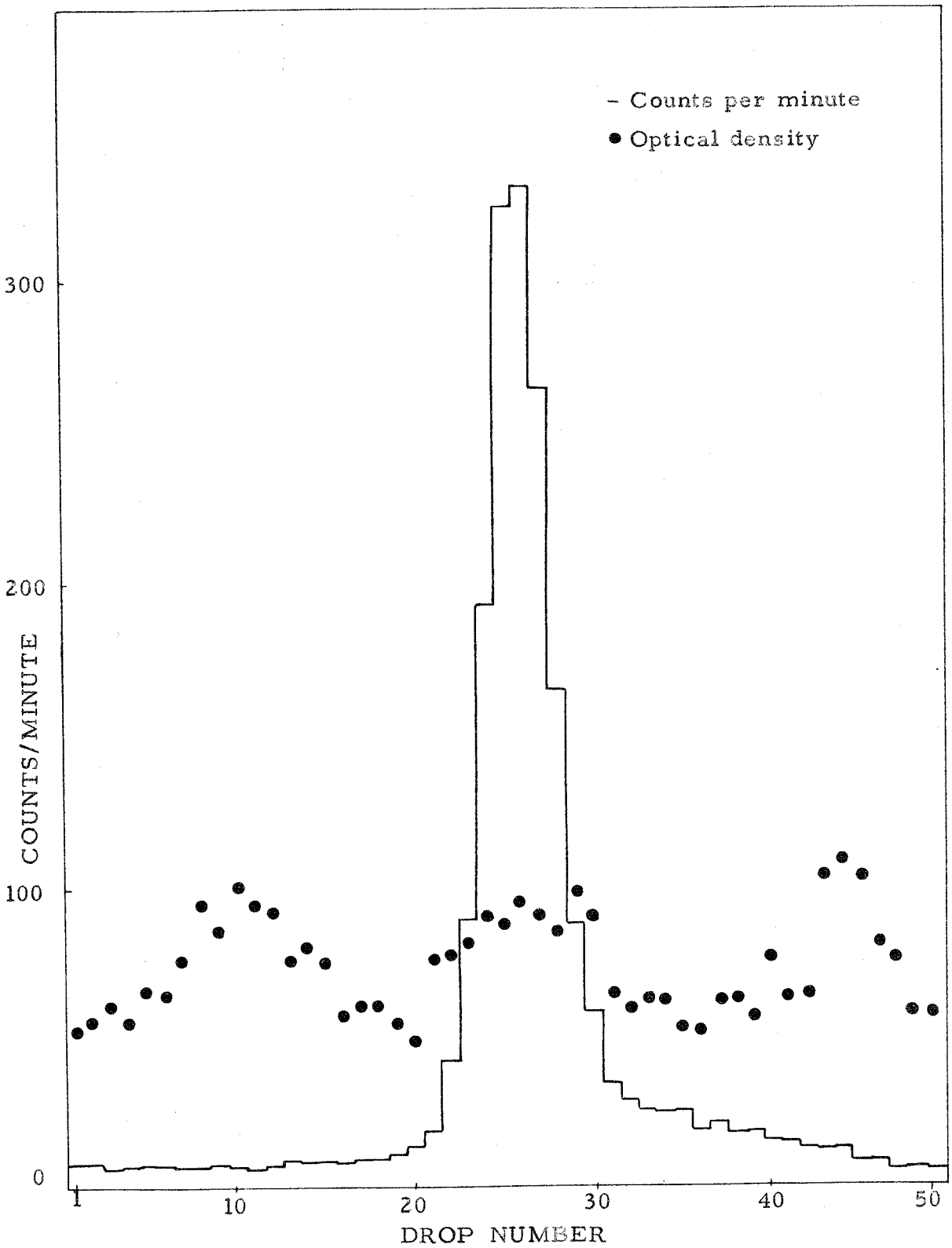

Fig. 35. Parental DNA, passed through No. 26 needle, heated 100° 20 minutes.
(Transer experiment H and H 6)

V. DISTRIBUTION OF PARENTAL ATOMS IN VIABLE PROGENY

An investigation was undertaken of the density distribution of viable progeny phage obtained after infection of light bacteria with heavy phage particles. The density distribution of such progeny phage cannot be determined with high resolution. This is in part because the phage T4, despite their greater particle weight, form a band in CsCl which is at least as wide as the DNA band. Furthermore, the light protein coat common to all progeny phage effectively dilutes heavy isotope in the DNA. The buoyant density of T4 in CsCl (1.49 g/cm^3) is approximately the arithmetic average of the densities of the protein and DNA. The density of phage containing light protein and DNA with 99% N^{15}, 93% C^{13} isotopic label, is calculated as 1.515 g/cm^3. The increase of 0.025 g/cm^3 is half the difference between light and heavy phage DNA.

In the experiments to be described, the bacteria were prematurely lysed, giving bursts of between 3 and 25. The purpose was to obtain phage with the largest possible fraction of parental atoms. As can be seen in Fig. 7, the fraction of atoms transferred to progeny is approximately a linear function of burst size, if this is not greater than 20 to 40; that is, dilution of transferred atoms is approximately constant for small burst sizes.

It was necessary to compare the density distribution of the progeny phage to a reference distribution, provided by another phage. This phage should be identifiable by its genotype, but otherwise should differ only in that it contains no heavy isotope. It was found that differences in

density distribution can be detected between phage of the genotypes r+ and r if they are the progeny of two separate infections performed in parallel. The density distributions are identical if the phage are obtained from the simultaneous infection of bacteria with parental phage of both genotypes. Bacteria were therefore infected with a heavy and a light parent, the two differing in genotype. In this experiment, differences in density distribution between the progeny of the two genotypes are observed only if heavy parental atoms are transferred preferentially to progeny of one genotype.

The progeny used in these analyses were obtained from infections carried out in the same manner as described for experiment 8-3-4 in section 4.1. Parental phage, multiplicity of infection and burst size are detailed in Table 3. Between 23 and 26 minutes after bacteria and phage were mixed, growth was stopped by the addition of cold medium. The infected bacteria were collected by centrifugation. In experiments 11-15-1 and -2, and 1-19-1 and -2, the infected cells were washed once with cold medium. In all experiments, the infected cells were then suspended in 1 ml medium and lysed by the addition of $CHCl_3$. The crude lysate was brought to a volume of 3 ml and density 1.458 by the addition of CsCl solution and 0.3 ml 1 M $MgSO_4$, and additional medium. The solutions were kept at 45° for 20 to 30 minutes before centrifuging. Heating in 0.1 M $MgSO_4$ reduces the band width of the phage; it does not detectably reduce the phage titer.

Samples were centrifuged at least 20 hours at 30,000 RPM, which is long enough to insure a very close approach to equilibrium.

TABLE 3

Description of Experiments Included in Chapter V

Experiment	Parental Phage*	Parental Ratio, r+/r	Total Multiplicity	Total Burst Size
8-3-1	Lr+, Hr	0.6	6.3	24
8-3-2	Hr+, Lr	0.7	4.8	10
8-3-3	Lr+, Lr	0.9	5.2	20
11-15-1	Hr+, Lr	1.0	10	3.4
11-15-2	Lr+, Lr	1.2	19	15
1-19-1	Hr+, Lr	1.0	20	3.4
1-19-2	Lr+, Lr	1.0	20	9.6

*The parental phage are described by a capital L or H, indicating whether this parent was of light or heavy isotopic composition, respectively; the genotype is indicated to the right.

The drops were collected and analyzed for the ratio of genotypes. The dilutions for each genotype are identical. Dilution errors cancel, and the root mean square error can be calculated from the number of plaques counted. For the ratio of r+ / r, this error is $\sqrt{N(N-r)/r}\,/\,r$, where N is the total number of plaques counted, r is the number of r plaques counted, and $(N-r)/r$ is the ratio r+ / r.

In Figs. 36, 37 and 38 are shown the density distributions of progeny phage obtained from infections with heavy r+ and light r, heavy r and light r+, and the dummy infection with light r+ and r, respectively. Figs. 37 and 38 show the only poor curves obtained in this series of experiments. All other distributions resemble that of Fig. 36; they are not included. The distorted distributions shown are presumably the result of mechanical agitation of the samples preceding or during the collection of drops.

In transfer experiments, the density distribution of phage of the genotype of the heavy parent is bimodal. The peak at greater density arises from unadsorbed parental phage; the density is about that calculated for a phage with both heavy DNA and heavy protein. The light peak corresponds in density to that of light phage; its maximum is the same as the maximum for the progeny of the other genotype. However, in comparison to the distribution of these phage, the distribution of progeny of the genotype of the heavy parent is skewed on the heavy side.

Fig. 39 shows the density distribution of the heavy parental phage of r+ genotype, centrifuged in a solution with the same final density as that in other centrifugations. The dashed arrow indicates the approx-

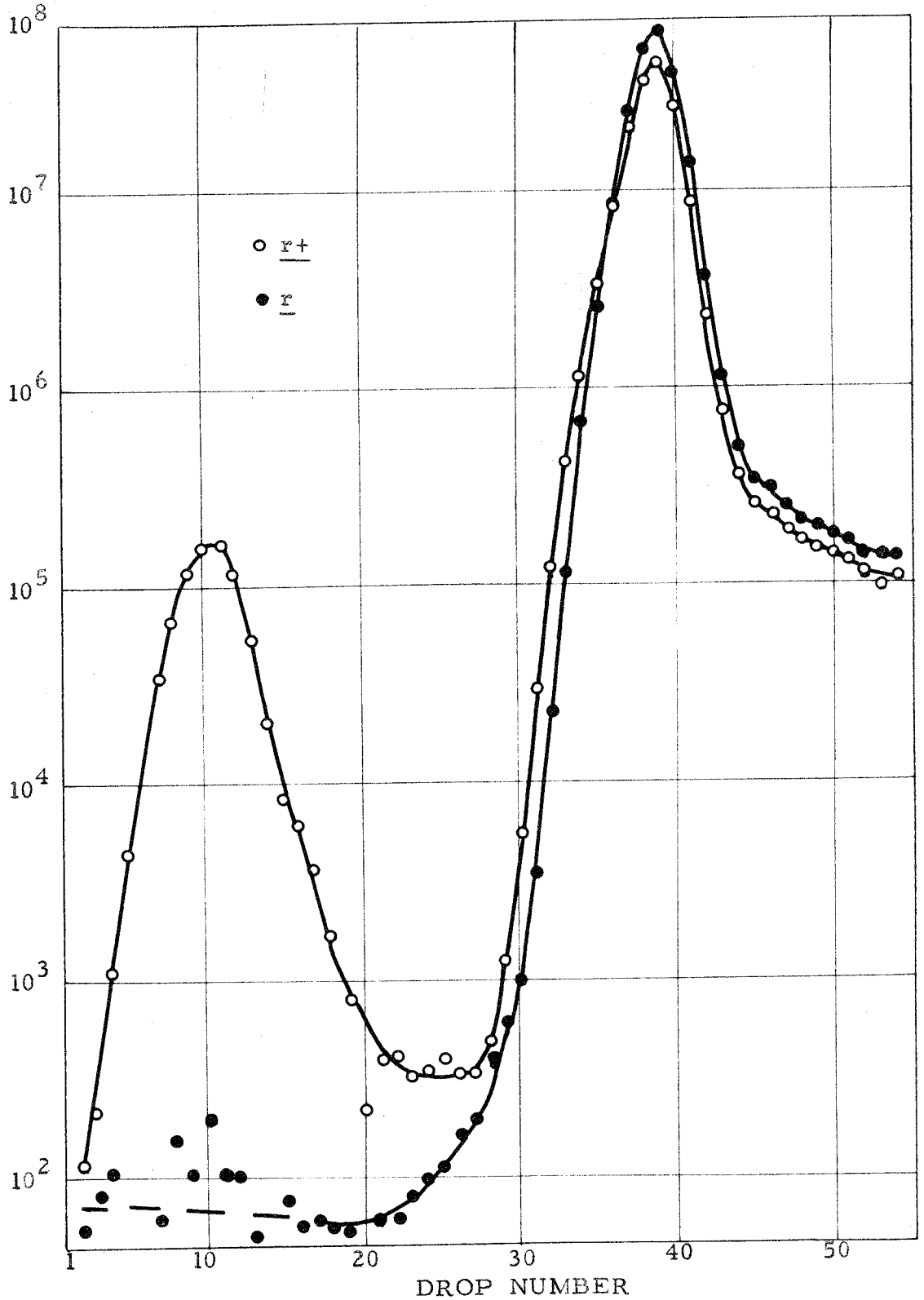

Fig. 36. Density distribution of progeny of infection with heavy r+, light r parental phage. (Transfer experiment 8-3-2)

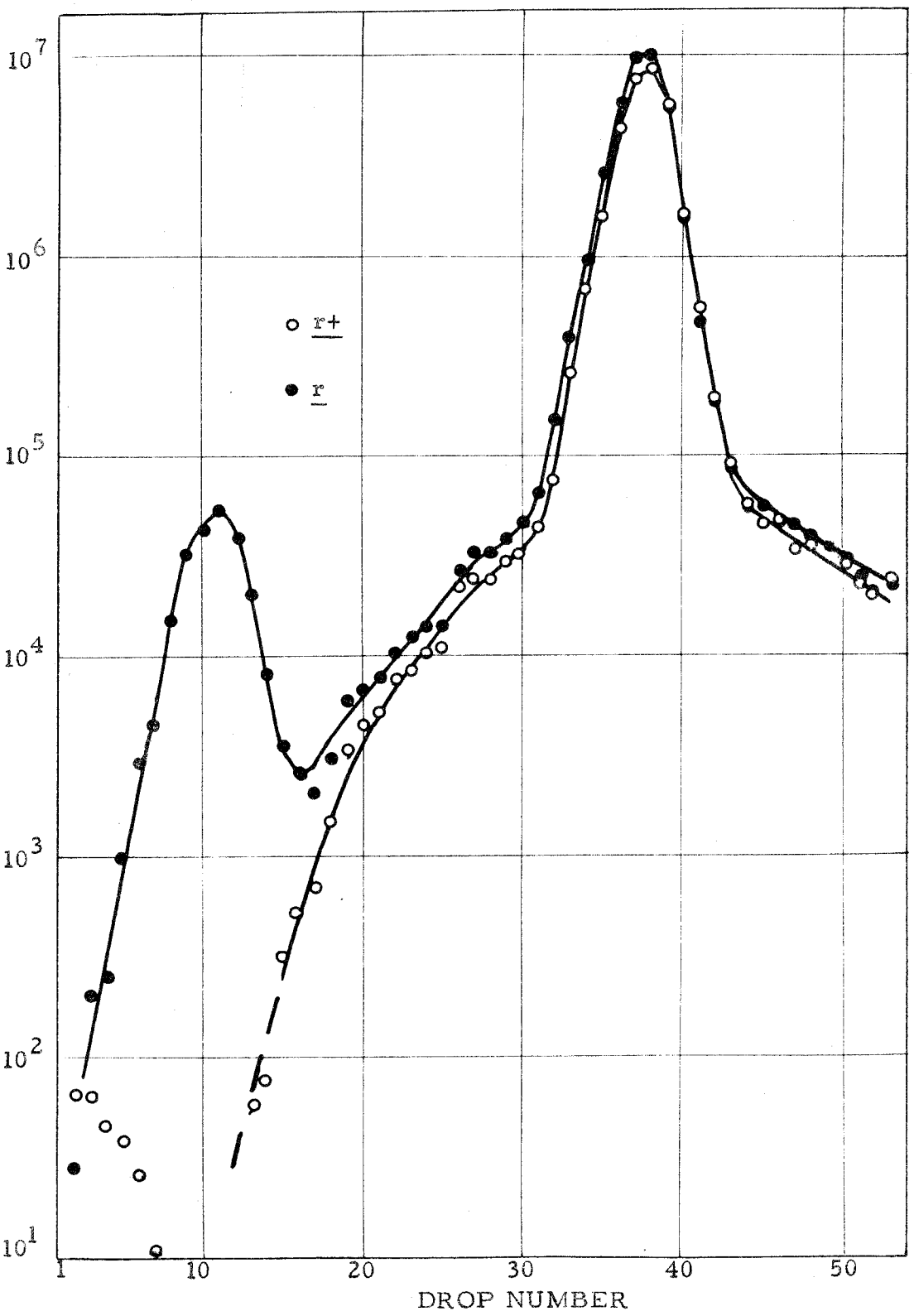

Fig. 37. Density distribution of progeny of infection with heavy r, light r+ parental phage. (Transfer experiment 8-3-1)

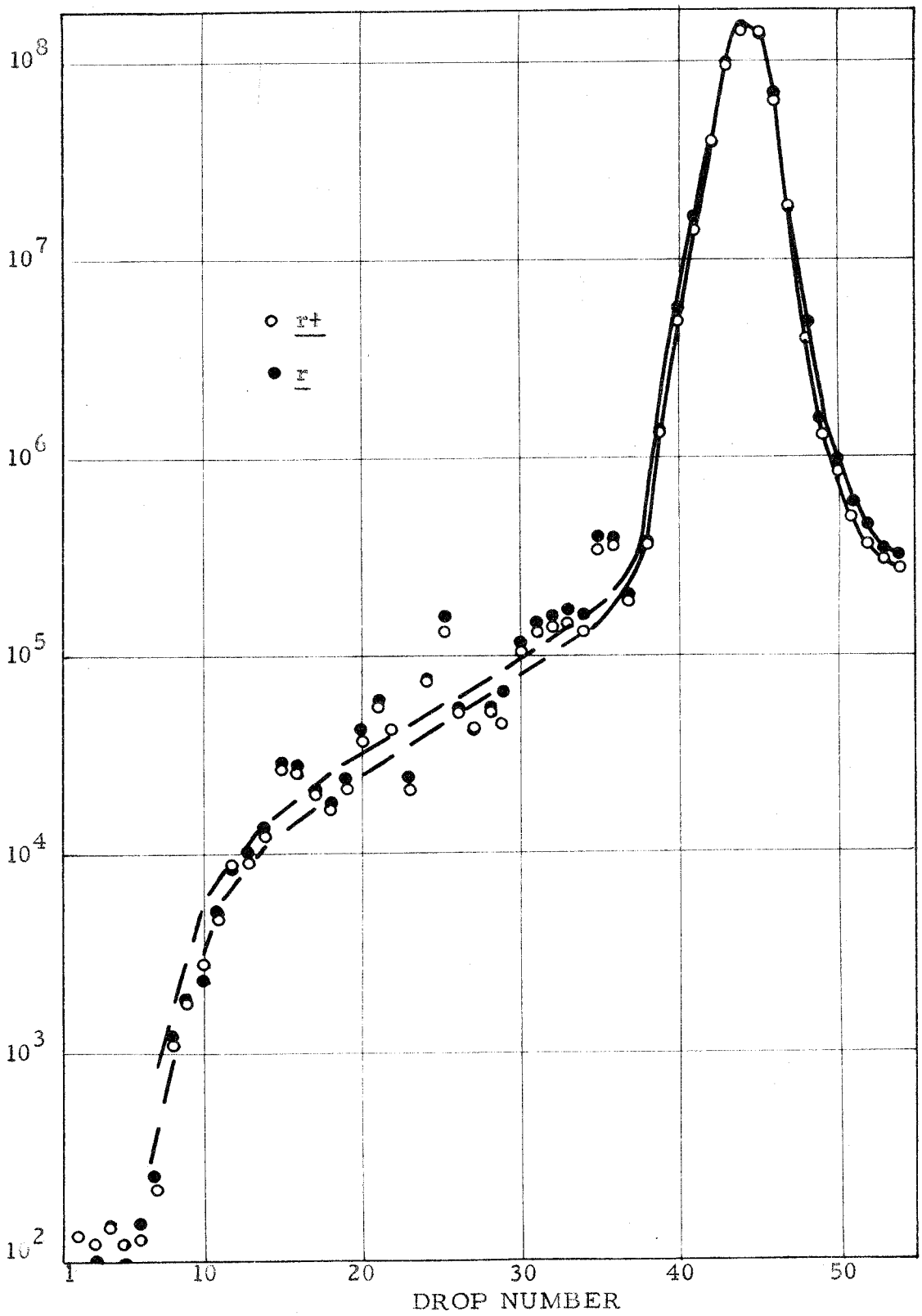

Fig. 38. Density distribution of progeny of infection with light r+, light r parental phage. (Dummy experiment 8-3-3)

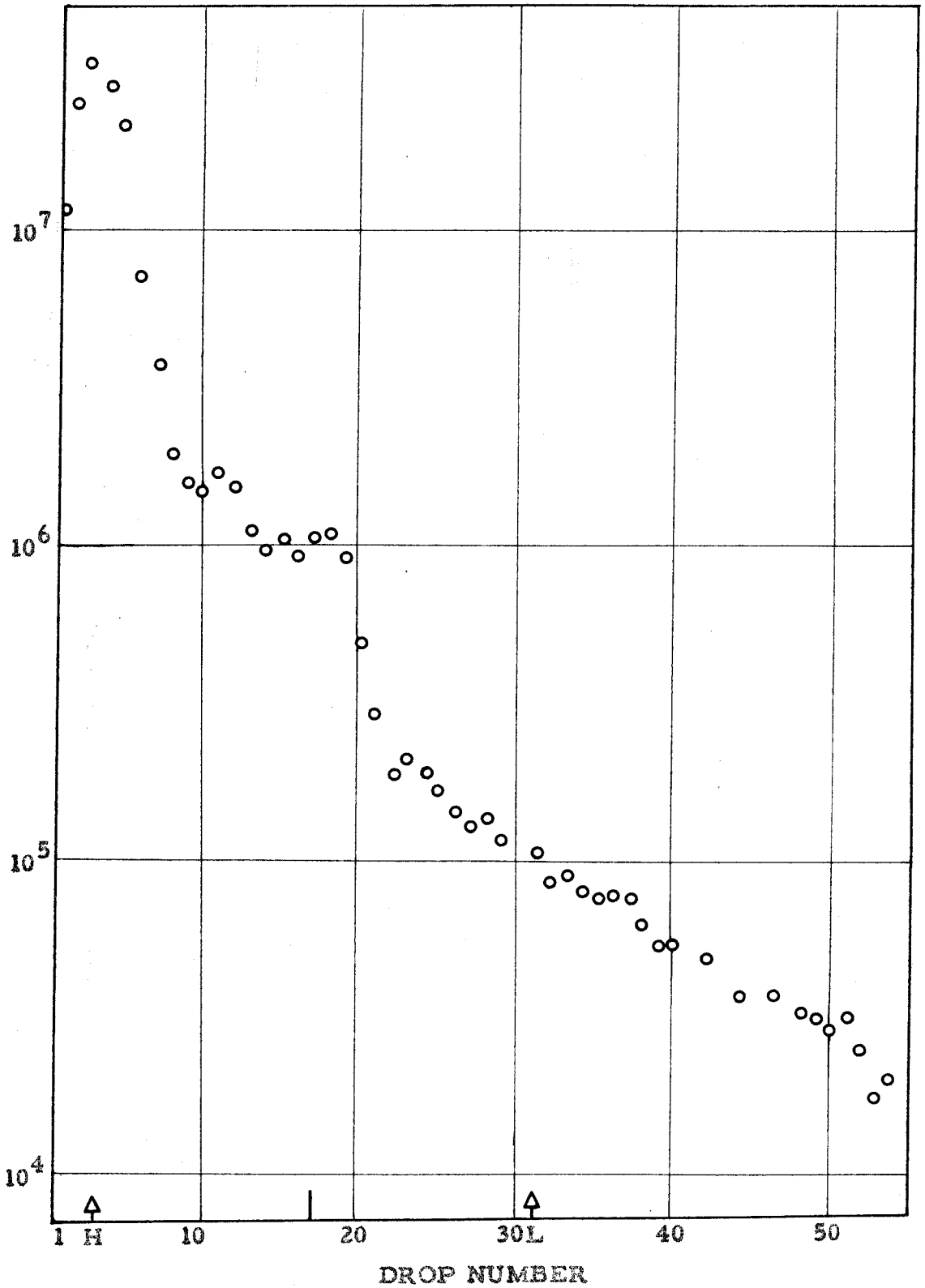

Fig. 39. Density distribution of heavy r+ phage.

imate position where light phage would band. Midway between this position and the maximum of the band of parental phage is the density of a phage half of whose atoms are heavy and half light. This position is the heaviest density permissible for progeny phage, and is marked by a line. The distribution indicates that parental phage probably do not cause anomalies in the distribution of progeny phage in the range of densities which will be considered in the present discussion.

Fig. 40 presents the log of the ratio of r+ / r as a function of density, among progeny of an infection by heavy r+ and light r parents. The density distribution of the progeny was seen in Fig. 36; the two curves are derived from the same data. Figure 40 is merely a magnification of the linear vertical distances between the r+ and r points of Fig. 36. The abscissa are the same.

In Figs. 41 to 43 are presented other density distributions of the log of the ratio of genotypes, obtained from other experiments. The data of Fig. 41 are the same as those for which the phage distribution was presented in Fig. 37. This is the only experiment in which the heavy parent was of r genotype. In Fig. 41, the ratio r / r+ is presented, while in all other cases the ratio is r+ / r.

The log ratio curves are presented, rather than the distributions themselves, because they are a more sensitive test of the reproducibility of the results; this is difficult to assess from graphs on 7-cycle log paper. Furthermore, the root mean square error of the ratio is shown. It is therefore possible to judge the significance of differences between the distributions of the two genotypes of progeny. The arrow on the left

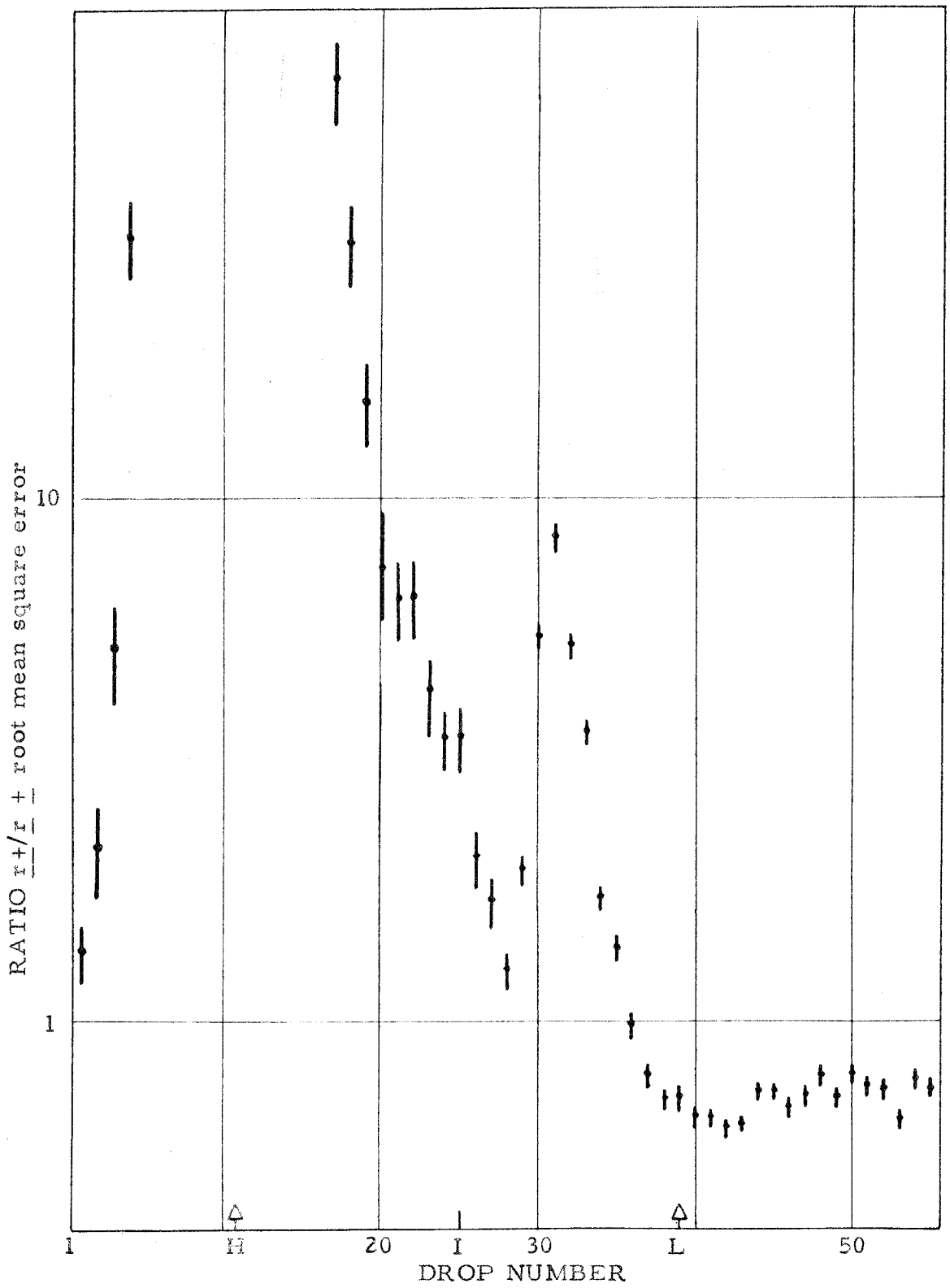

Fig. 40. Density distribution of log r+/r ratio among progeny of infection with heavy r+, light r parental phage. (Transfer experiment 8-3-2)

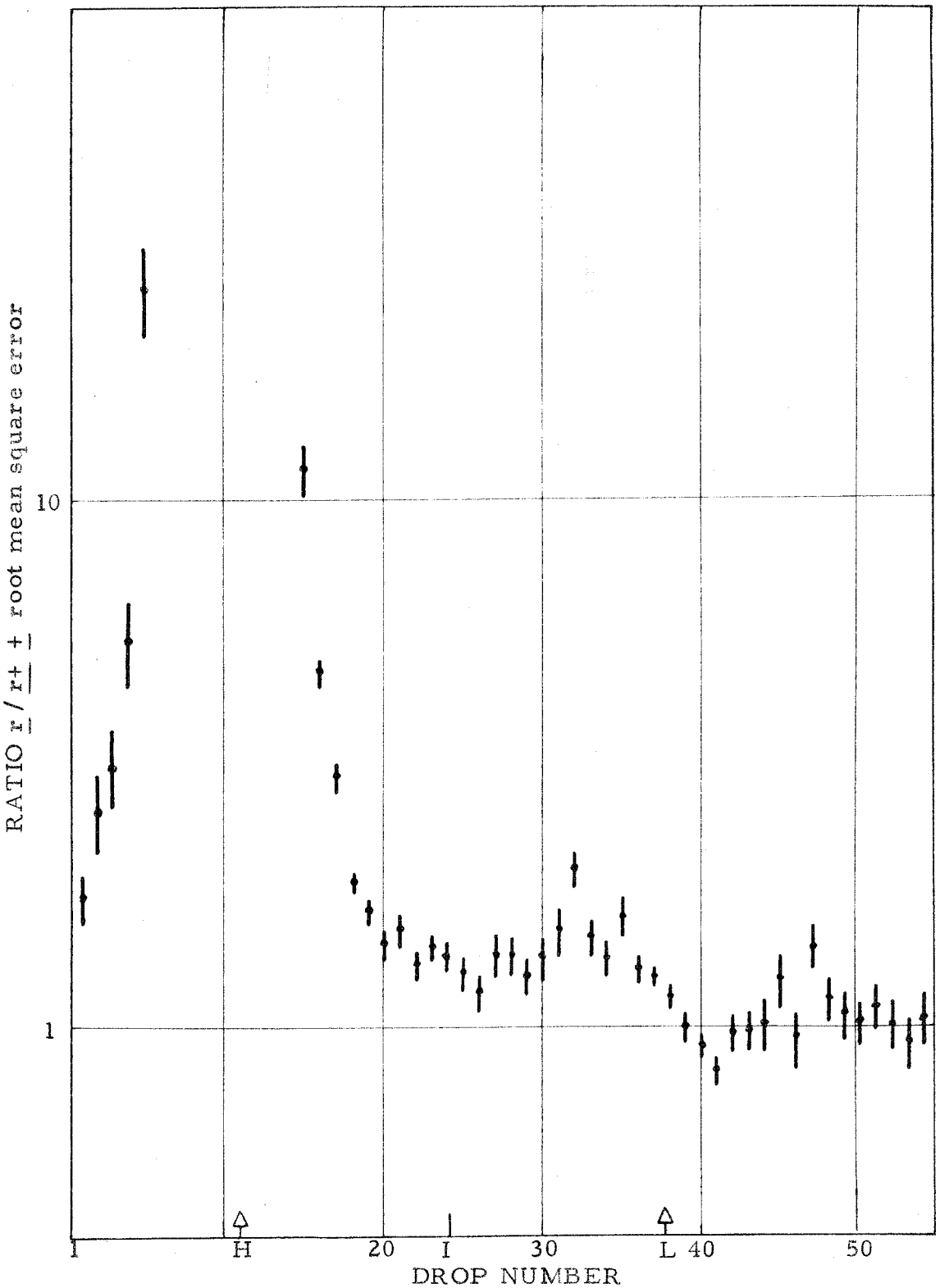

Fig. 41. Density distribution of log r/r+ ratio among progeny of infection with heavy r and light r+ parental phage. (Transfer experiment 8-3-1)

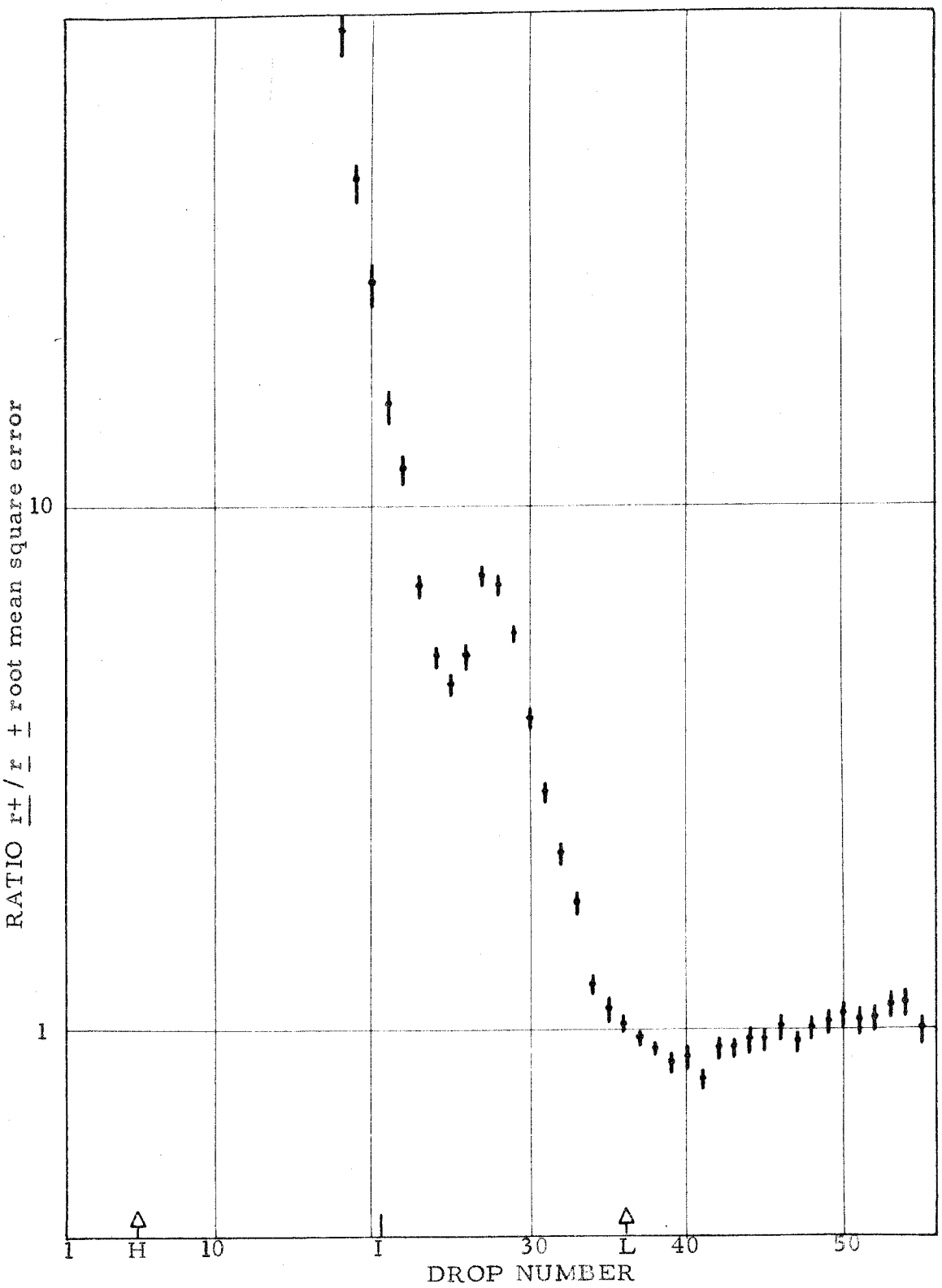

Fig. 42. Density distribution of log r+/r ratio among progeny of infection with heavy r+ and light r parental phage. (Transfer experiment 11-15-1)

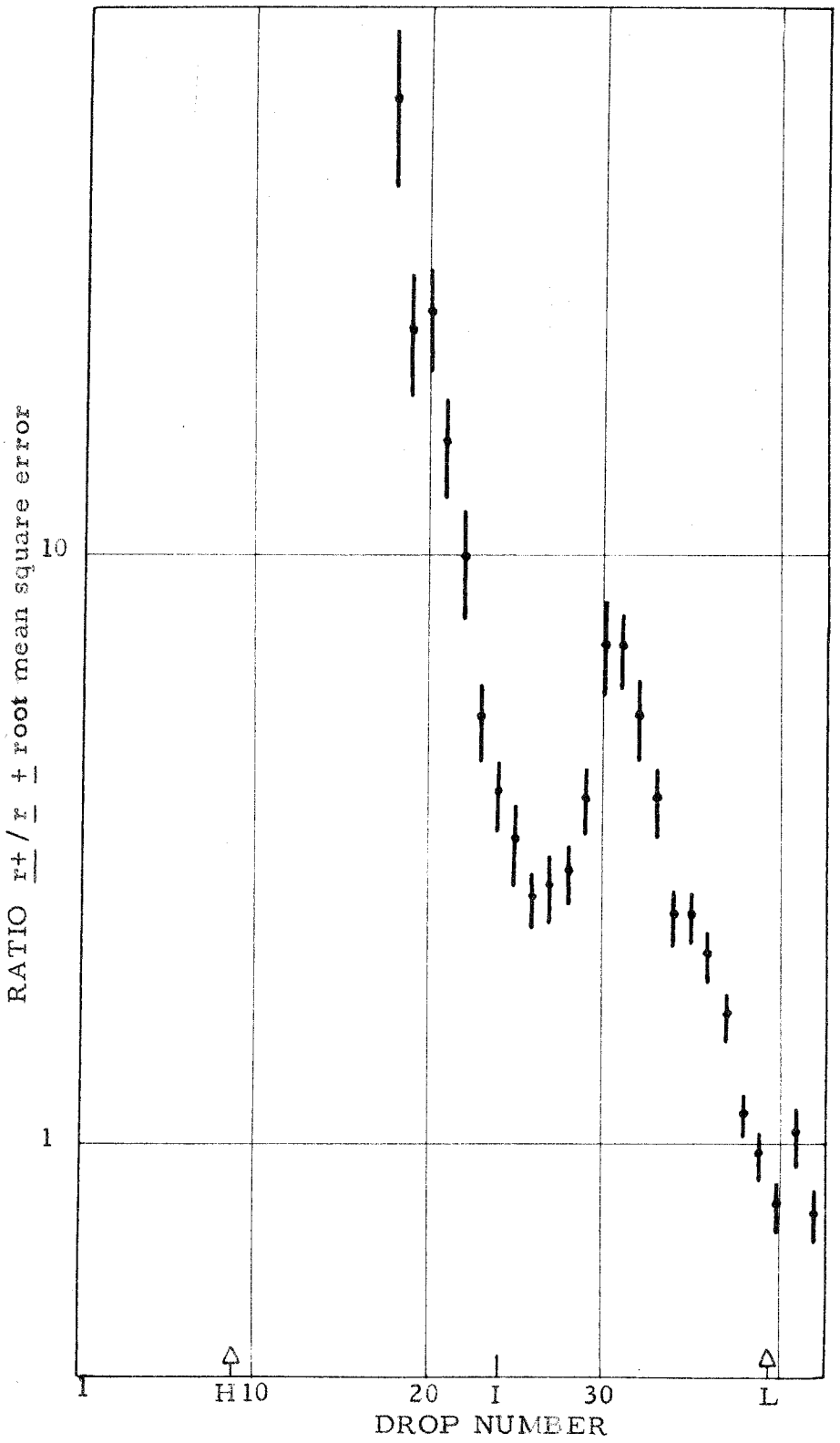

Fig. 43. Density distribution of log r+/r ratio among progeny of infection with heavy r+ and light r parental phage. (Transfer experiment 1-19-1)

of the figures marks the position of the maximum of the band of heavy parental phage. The arrow on the right marks the position of the maximum of the band of light progeny phage. Midway between is the heaviest density permissible for progeny phage, that of a particle with a light protein coat and DNA of heavy parental density; this position is marked by a line. These markers are designated by the capital letters H, L and I, respectively.

In all curves, the ratio of genotypes is approximately constant at densities lighter than that of light phage. At densities greater than this, the ratios increase sharply, and then again decrease. The rise in the curve following this drop is attributed to unadsorbed heavy parental phage being compared to a small lead of progeny phage of the other genotype.

Figs. 44 to 46 present the ratio curves obtained from analyzing the density distribution of progeny phage obtained in three independent crosses of r+ and r, both of normal light isotopic composition. The scale is the same as that of Figs. 40 to 43. In Figs. 44 to 46, it can be seen that the ratio of genotypes is essentially independent of density; in other words, in this case the density distributions of the progeny of r+ and r genotypes are identical.

Following the analysis of the density distributions of progeny in experiments 11-15-1 and -2, and 1-19-1 and -2, some of the drops at densities greater than that of light phage were recentrifuged. In the transfer experiments 11-15-1 and 1-19-1, the maxima of heavy parental and light progeny phage are separated by about 28 drops. 7 drops from the

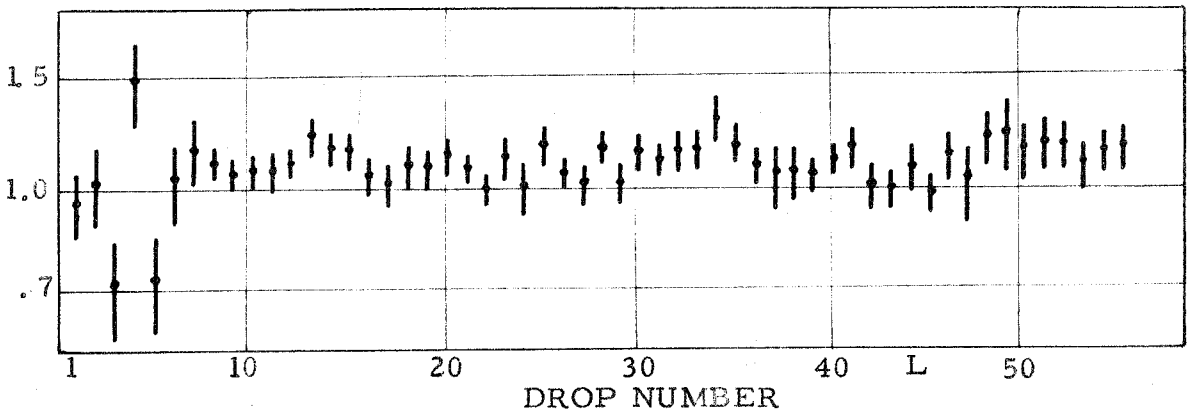

Fig. 44. Density distribution of log r+/r ratio among progeny of infection with light r+ and light r parental phage. (Dummy experiment 8-3-3)

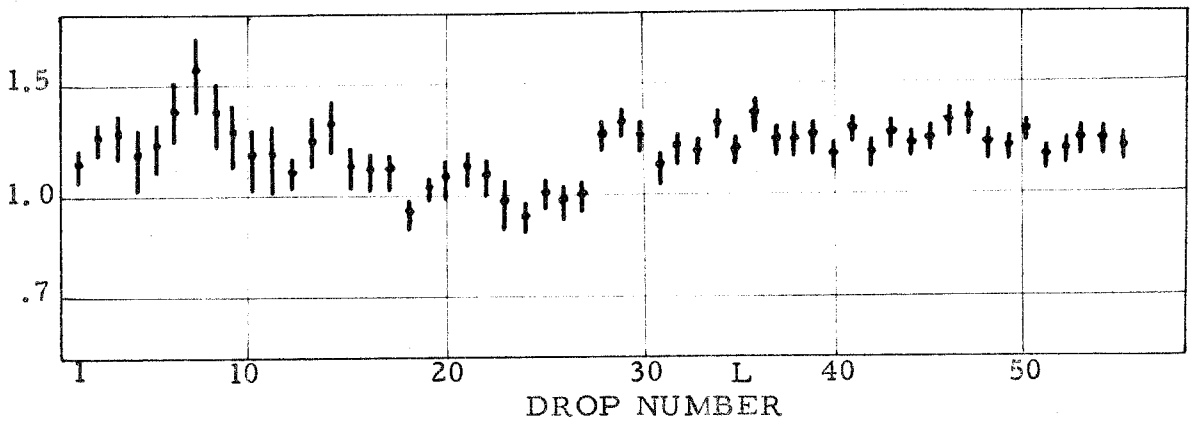

Fig. 45. Density distribution of log r+/r ratio among progeny of infection with light r+ and light r parental phage. (Dummy experiment 11-15-2)

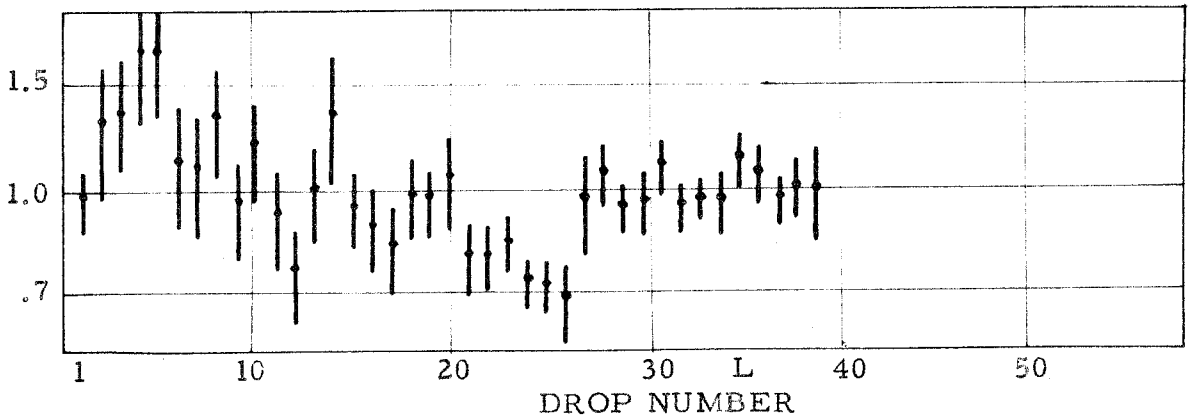

Fig. 46. Density distribution of log r+/r ratio among progeny of infection with light r+ and light r parental phage. (Dummy experiment 1-19-2)

light maximum is 1/4 the total density increment, and corresponds to a progeny particle with 1/2 its DNA derived from the heavy parent. At densities greater than this, the trail of heavy parental phage could constitute a significant fraction of the r+. Moreover, there are too few r phage to analyze the redistribution adequately. Therefore, the drops displaced by 7 and 3 from the maxima were rebanded. These are called the "1/2 heavy" and "1/4 heavy" densities, respectively. In the dummy transfer experiments, 11-15-2 and 1-19-2, the drops at a density corresponding to the "1/2 heavy" density were rebanded. In this case, the presence of phage at this density is due to the width of the band of T4. In experiment 1-19, the original samples which were centrifuged had added to them a small aliquot of T6 as a density marker. This phage does not make plaques on the indicator S/6 used in analyzing T4, but can be assayed on S/4, on which T4 does not make plaques. It has, by chance, the further advantage that the peak is at exactly the "1/2 heavy" density. T6 was also included in the rebanded samples from 1-19; its density is indicated by a labeled arrow.

The distribution obtained by rebanding phage from the dummy experiments in which both parents contained only light isotopes are presented first, because it is impossible otherwise to understand the results of rebanding drops from the transfer experiments. Figs. 47 and 48 show the density distribution of the phage found at "1/2 heavy" density after centrifugation of the progeny of the dummy transfer experiments, 11-15-2 and 1-19-2. In Fig. 47, a large fraction of the phage reband at about the same position in the cell as that from which they

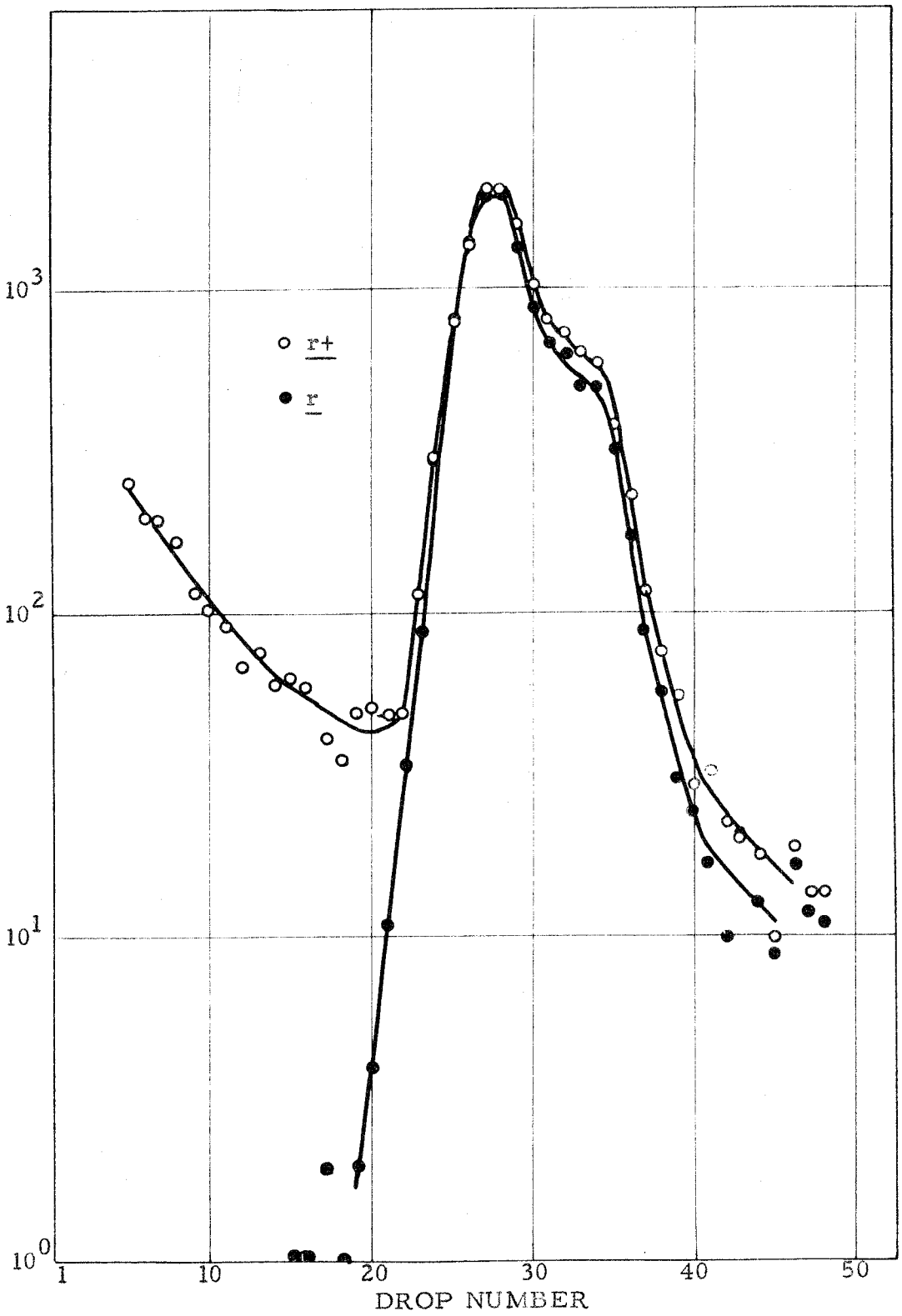

Fig. 47. Density distribution of rebanded progeny of "1/2 heavy" density. Light r+, light r parental phage. (Dummy experiment 11-15-2)

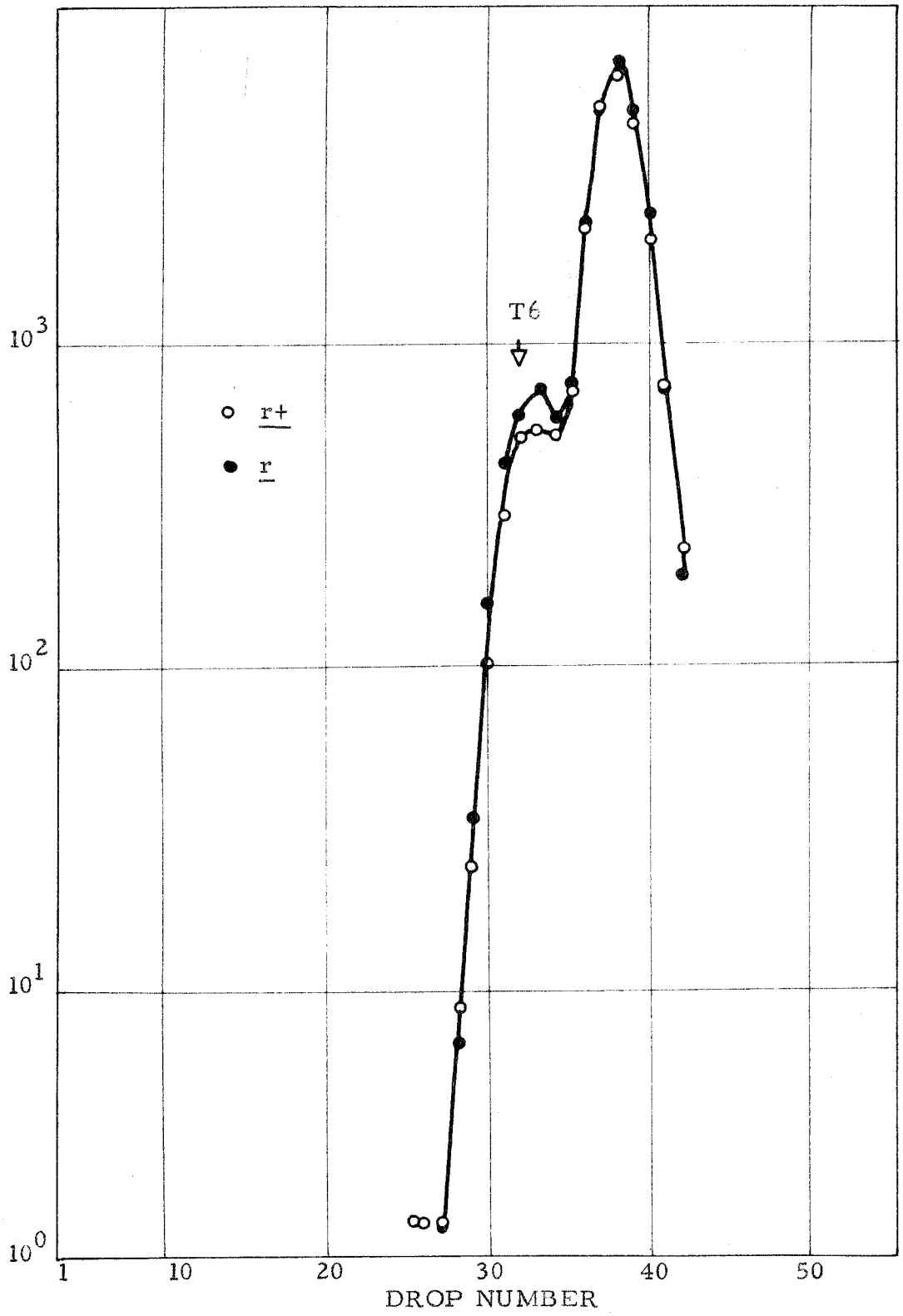

Fig. 48. Density distribution of rebanded progeny of "1/2 heavy" density. Light r+, light r parental phage.
(Dummy experiment 1-19-2)

came; the remainder are found at lighter densities. (The lead of heavy r+ phage is due to the presence of heavy parental phage added as an unsuccessful density marker.) Because of the lack of a density marker, it is not possible to determine the actual densities of the phage. However, in the second rebanding, the addition of the T6 makes the assignment of densities unambiguous. A small fraction of the phage appear at a somewhat lighter density than that from which the drop was taken; most of the phage appear at approximately the density of light phage (Fig. 48). In order to be certain that this distribution was not the result of disturbances occurring before or during the collection of drops, another aliquot of the dummy phage of "1/2 heavy" density was rebanded; the distribution was the same.

The band of light phage is very broad. Evidently the phage which appear at a density far from the mean density are comprised of a variable ratio of two kinds, those which reband at approximately the same density as before, and those which reband at lighter densities, close to or at the mean density of light phage. In the original dummy distributions, the phage of r+ and r genotypes have approximately identical distributions, as shown by the lack of dependence of the ratio of genotypes on density. In the rebandings as well, r+ and r phage have approximately identical distributions.

The density distributions obtained from rebanding the "1/2 heavy" and "1/4 heavy" density drops from the transfer experiments are shown in Figs. 49 to 52. Most of the phage reband at approximately the same density as that at which they originally appeared. The remainder appear

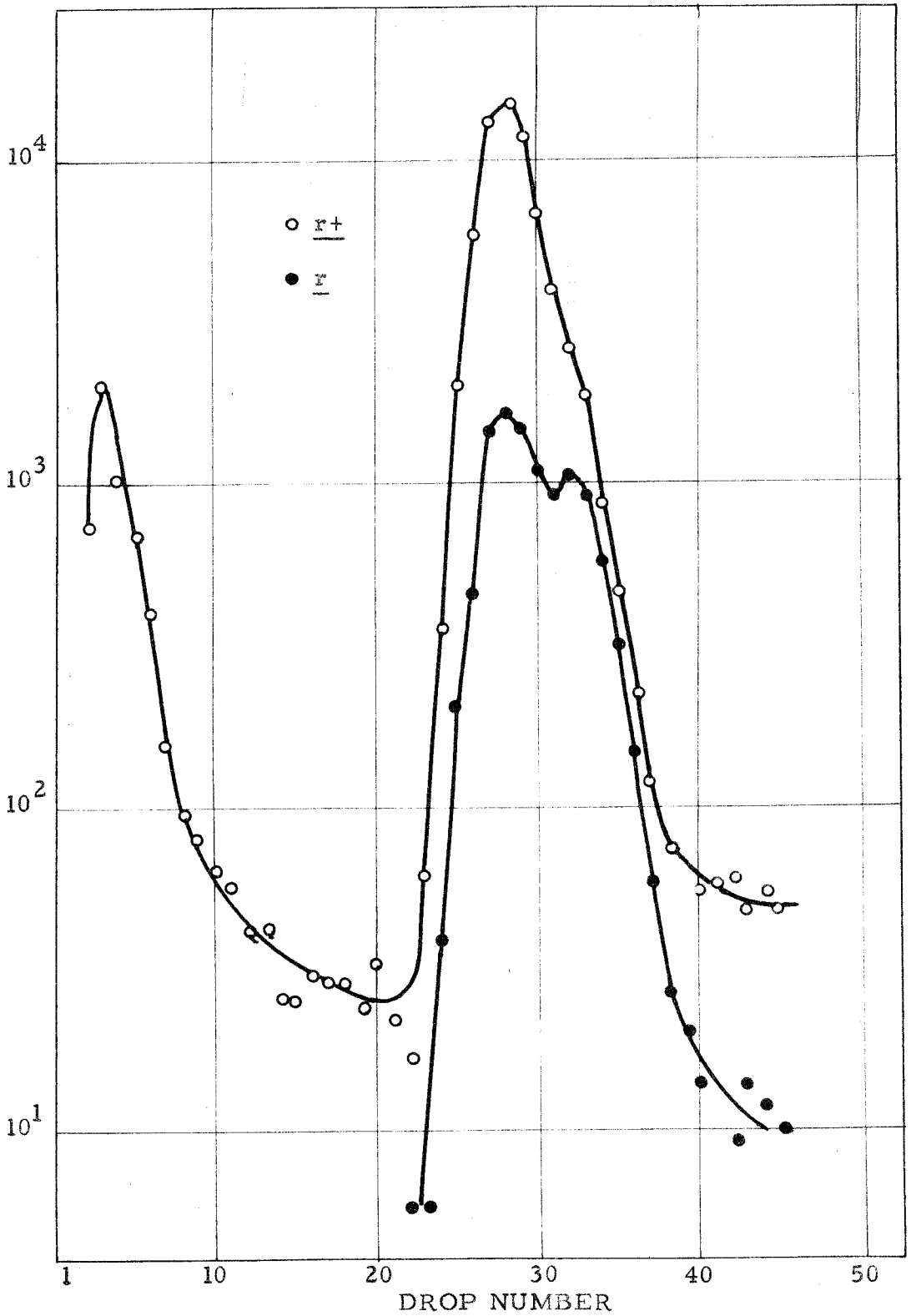

Fig. 49. Density distribution of rebanded progeny of "1/2 heavy" density. Heavy r+, light r parental phage. (Transfer experiment 11-15-1)

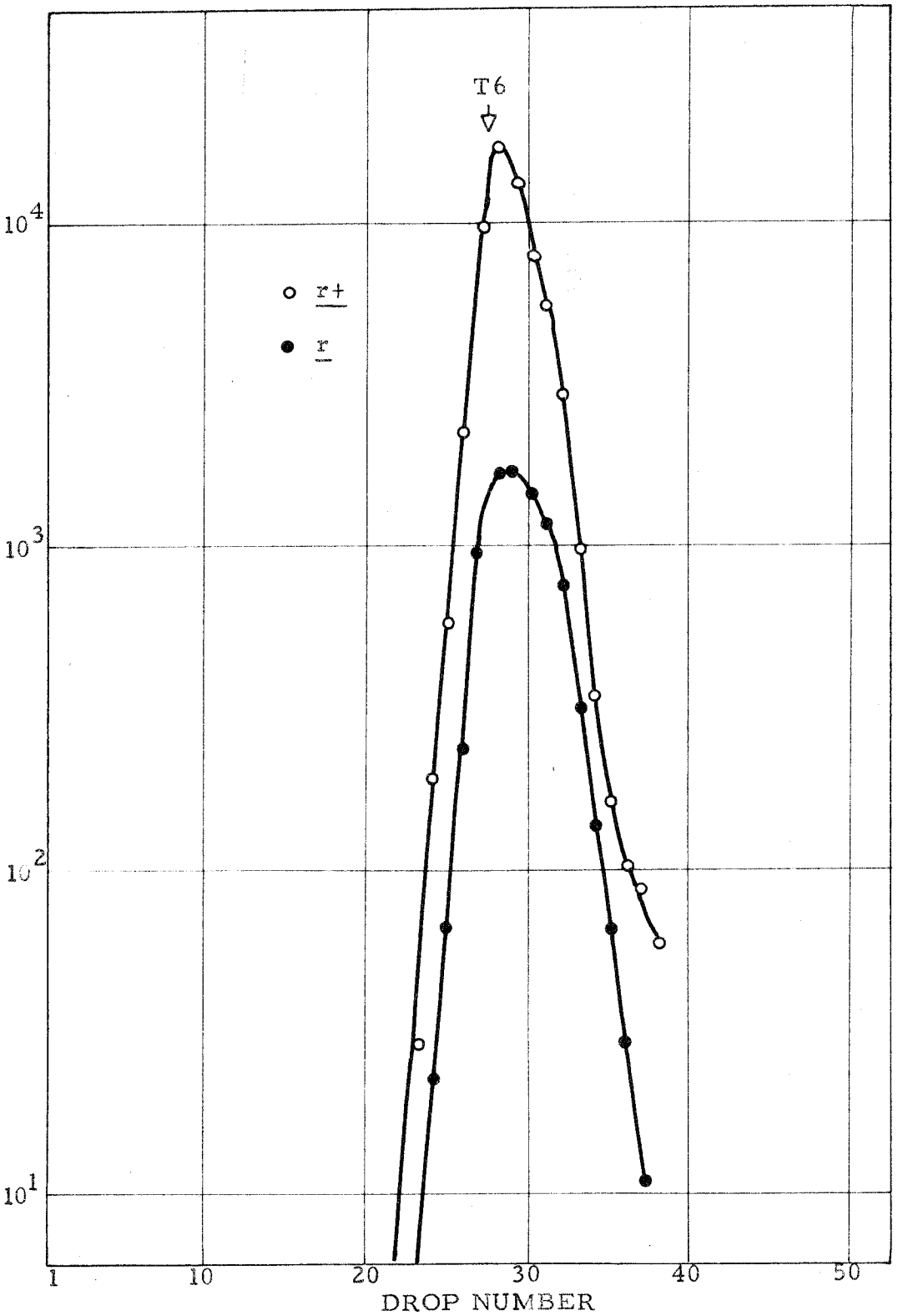

Fig. 50. Density distribution of rebanded progeny of "1/2 heavy" density. Heavy r+, light r parental phage. (Transfer experiment 1-19-1)

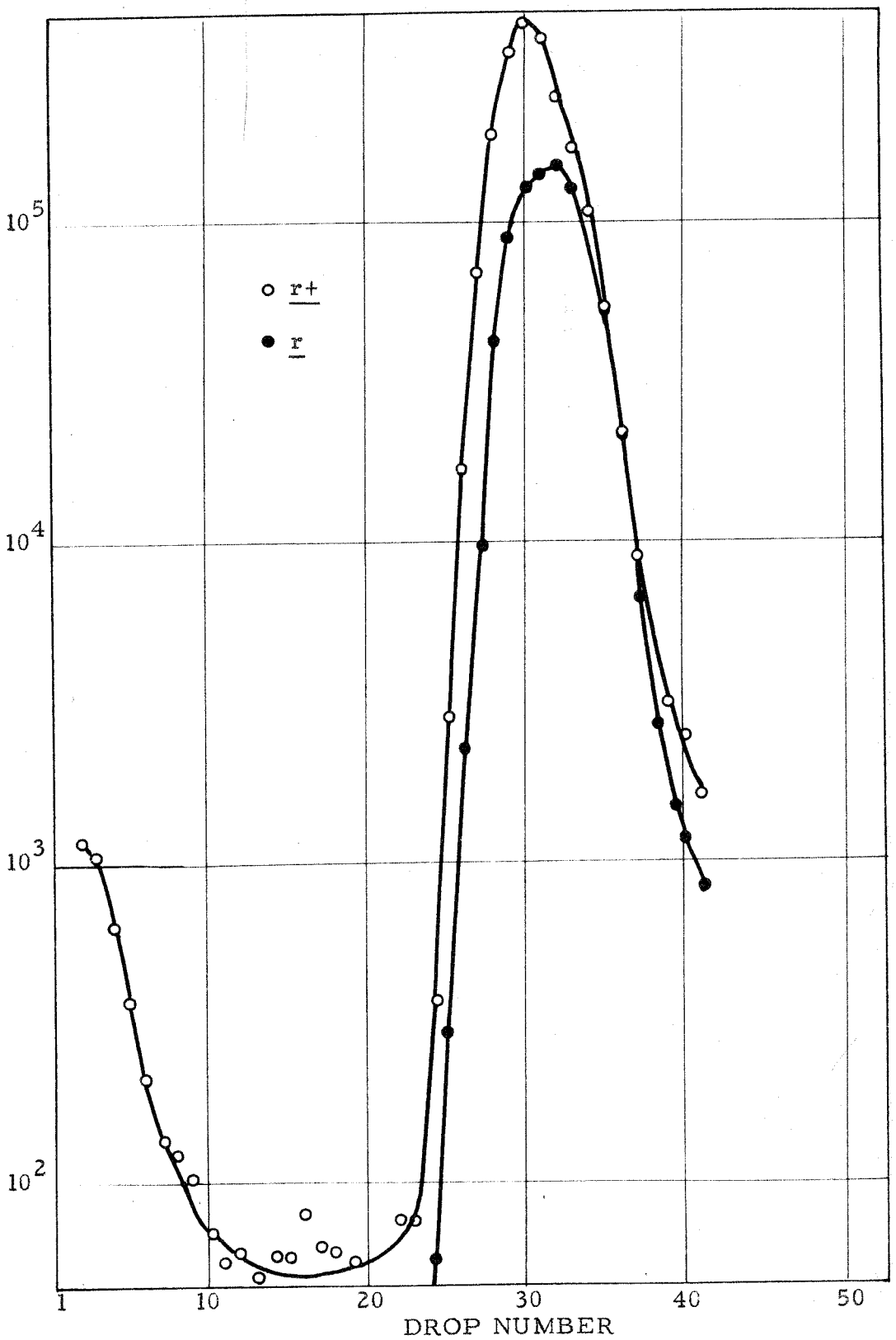

Fig. 51. Density distribution of rebanded progeny of "1/4 heavy" density. Heavy r+, light r parental phage.
(Transfer experiment 11-15-1)

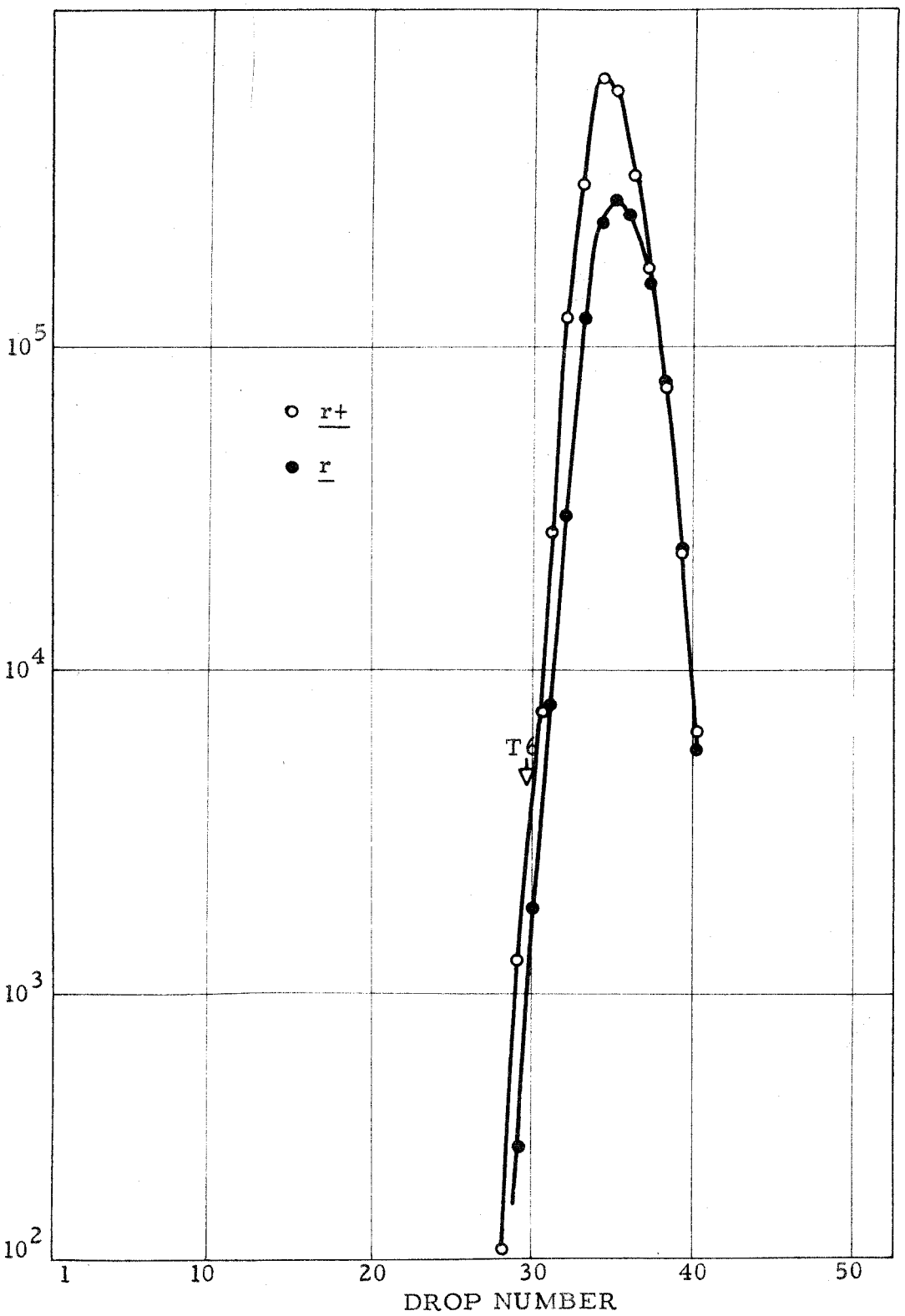

Fig. 52. Density distribution of rebanded progeny of "1/4 heavy" density. Heavy r+, light r parental phage. (Transfer experiment 1-19-1)

at lighter densities. However, unlike the distributions from the rebanded dummy drops, in the transfer distributions, r+ and r are not the same. In general, r+, the genotype associated with the heavy parent, is of greater density.

VI. CONCLUSIONS AND DISCUSSION

Most heavy parental DNA transferred to progeny is found in molecules with the density of light DNA. This must be due to association of heavy atoms with material of lighter density. It is presumed that this light material is newly synthesized DNA, and not, for example, a protein which is associated specifically with transferred DNA. The analyses of progeny DNA do not include any evidence on this point. However, in the examination of DNA in the replicating pool, the direction of transfer was reversed. Parental phage were of normal isotopic composition, while newly synthesized DNA contained N^{15}, and therefore had a greater density. At no time after infection is there an indication of a band at a density lighter than that of the parental DNA, as would be expected if parental DNA became associated with protein. Moreover, Kozinski (1961) has found that transferred parental material, which is of normal composition, has the same density as the mass of DNA isolated from progeny phage; progeny DNA is heavy due to the incorporation of 5-bromodeoxyuridine. Thus, transferred parental atoms comprise a part of molecules with the density of newly synthesized DNA, regardless of the direction of transfer. This must mean that they are associated with segments of newly synthesized DNA. The parental atoms are usually sufficiently diluted to cause no detectable change in the density of the progeny DNA.

However, the parental atoms are evidently not distributed in the newly synthesized DNA in the form of isolated nucleotides or small polynucleotides; but rather, there must be regions in which a large

fraction of the atoms are derived from parental DNA. These regions are at least partially separated from the segments of light DNA to which they were previously attached when the molecules are broken by passage through a hypodermic needle or by sonicating. Some of the DNA fragments then have a sufficient fraction of heavy parental atoms to produce a noticeable increase in density.

The maximum density which fragments attain is just intermediate between the densities of light and heavy DNA. In experiment H and H 6, the parental DNA contained P^{32} in order to distinguish it from newly synthesized DNA. It can be seen that the degree of fragmentation achieved with a number 26 hypodermic needle is sufficient to produce a noticeable increase in the density of most of the transferred parental DNA (Fig. 31). This is in accord with the density distribution found in the analytical centrifugation of this material (Fig. 22). This sample was further fragmented by sonication (Fig. 23). Sonication causes only a small amount of material which had remained at the density of light DNA to increase in density to an observable extent. However, DNA whose density had been increased by the first treatment seems to suffer a slight further increase; it still does not acquire a density greater than intermediate density. Presumably further breaks cannot increase the density of molecules containing transferred atoms beyond this point. It is concluded that parental DNA may be associated with less dense material from which it cannot be dissociated by fragmentation.

Most of the transferred parental DNA is found, in untreated progeny DNA, associated with large segments of light DNA, and not

some other material. However, the much smaller fragments of intermediate density could be composed of parental DNA associated with a less dense material such as protein, without producing a detectible density alteration in the intact material. In the transfer experiments, the parental phage were of 99% N^{15}, and either 93% or 65% C^{13} isotopic composition. In both cases, at least part, and perhaps all of the transferred parental DNA is in segments of intermediate density. The amount of protein necessary to bring the 93% C^{13} DNA to intermediate density would, if associated with 65% C^{13} DNA, lower its density below the intermediate density; conversely, the amount of protein necessary to bring the 65% C^{13} DNA to intermediate density would leave the 93% C^{13} DNA at a density greater than intermediate. The difference is calculated as 0.005 g/cm^3. In the analytical centrifugations, the DNA is not as far as 1/2 of 0.005 g/cm^3 from intermediate density. Kozinski found that the density of transferred parental atoms could be <u>decreased</u> by sonication, because intermediate density is, in the reverse direction transfer, lighter than the density of newly synthesized progeny DNA. It can therefore be concluded that the transferred DNA is associated with a minimum of an equal volume of newly synthesized DNA, in such a manner that it cannot be dissociated by fragmentation. It is apparent that this suggests that the transferred parental DNA is found in "hybrid" segments, in which single parental strands are paired with newly synthesized single strands, and attached in some manner, presumably lengthwise, to very long segments of completely new DNA. The entire molecule, which has a minimum molecular weight of approximately

45 million as calculated from the band width, most frequently contains less than 8% parental atoms; or, in other words, not more than 16% of a single strand is usually of parental origin. (The fraction of material that is of parental origin was estimated from the position of transferred parental P^{32} in the preparative centrifugations of progeny DNA, as was described in section 4.2.)

Cocito and Hershey (1960) studied the transfer of DNA glucose from parental to offspring phage T2. Using C^{14} labeled parental phage, they concluded that labeled glucose was transferred with the same efficiency as labeled adenine. In the present experiments in which the parental phage contain 93% C^{13}, hybrid DNA in which all, instead of only half of the glucose was light would have a density detectably lower than intermediate density. Fig. 17 shows the distribution of sonicated transfer DNA in which the parental phage contained 93% C^{13}. As in other figures, intermediate density is indicated by a straight line between the arrows marking the centers of the bands of light and heavy DNA. The dashed line indicates the density calculated for a molecule hybrid except for containing only light glucose. The observed density of the hybrid DNA indicates that half the glucose is parental. The processes leading to the association of the parental strand with the light progeny strand evidently occur without removal of glucose in T4. This substantiates the conclusion of Cocito and Hershey.

An attempt was made to separate parental from light atoms by heating fragmented DNA to 100^{o}. It has been shown by Meselson and Stahl (1958) that the heavy and light subunits of hybrid DNA, obtained

in a transfer experiment with E. coli, can be separated by heating to 100° in CsCl. In the present experiments, fragmentation has not produced a distinct class of DNA of intermediate density. Parental atoms are found in molecules with a distribution of densities between light and intermediate. Nevertheless, it was hoped that the distribution of parental atoms following heating could be used to define more closely the way in which the parental and light DNA are associated. The distribution of P^{32} in the heated fragmented material (Fig. 32) indicates that the density of some of the DNA molecules containing parental atoms is now greater than intermediate density. This conclusion comes from the observation that, in this distribution, a larger fraction of the total P^{32} is between intermediate and heavy densities than was the case before heating. However, the increase in the amount of P^{32} at light densities indicates aggregation of a part of the material; this is the only way in which the density of the P^{32} can be decreased. Very little aggregation was observed in the control centrifugation of a mixture of parental and dummy DNA which had been passed through a hypodermic needle and then heated to 100° (Fig. 35). This may be because the concentration of DNA in the transfer sample which was subjected to analytical centrifugation had a DNA concentration 5 to 10 times that of the control. The amount of aggregation would be expected to be concentration dependent (Marmur and Lane, 1960). Because of the aggregation, it is impossible to analyze the results in any detail. It can only be said that heating to 100° has probably caused some of the transferred DNA to appear at densities greater than intermediate density. It seems likely

that parental DNA is transferred as single strands comprising a fraction of the length of duplex molecules, and that heating the fragmented molecules in CsCl causes separation of at least some of the strands, as it is presumed to do in buffer (Marmur and Doty, 1959).

The above discussion has been concerned with the transferred DNA found in molecules the density of which is very close to that of newly synthesized DNA; in all experiments, this seems to comprise most of the transferred DNA. However, in all experiments, some progeny DNA is also found at densities greater than light density. In the analytical centrifugations of progeny DNA from experiments 8-3-4 and H and H 6 (Figs. 8, 14 and 21) and in the preparative centrifugation of the progeny DNA from H and H 6 (Fig. 30), some material is found at the density of parental DNA. In these experiments, no attempt was made to remove unadsorbed parental phage. The DNA obtained from these heavy phage, which were isolated along with progeny phage, serves as a density marker; because of its presence, it is impossible to determine whether any parental DNA was transferred to progeny intact, and therefore without suffering a density change. Fig. 31 presents the density distribution of progeny DNA after passage through a number 26 needle. Within the limits of resolution, no parental P^{32}, (that is, less than 10%), which in the untreated sample had been found at light density, has acquired a density close to that of parental DNA. Therefore, among the fragments containing parental atoms, none can be detected in which both strands are of parental origin. In the preparative centrifugation of progeny DNA from H and H 5, no P^{32} is found at or near the expected density of

parental DNA, either before or after passage through a number 24 needle (Figs. 28 and 29). A peak containing 5% of the transferred P^{32} would almost certainly be detected. In this case, the infected cells were centrifuged to remove unadsorbed phage. It should be noted that only in this experiment were the bacteria singly infected; in all other experiments of this group, the multiplicity was between 4 and 14 (see Table 1). It can be concluded that, in single infection, little if any DNA is transferred in large segments which contain only parental atoms.

In all centrifugations of untreated progeny DNA, material is found at densities between those of parental and newly synthesized DNA. In the analytical centrifugations, there are small peaks between light and heavy densities. In the preparative centrifugations there is a lead which extends from light to intermediate density. As was explained in section 4.2, these peaks could be just below the limits of resolution of the preparative method. These peaks could be the result of aggregation. In particular, material found between light and intermediate densities could indicate that a fraction of the untreated DNA has suffered sufficient fragmentation to release segments with an increased density. As these possibilities cannot be ruled out, it seems unprofitable to discuss the possible biological significance of the DNA found at densities between heavy and light in the untreated samples.

The finding that parental DNA is transferred to progeny in molecules containing hybrid segments should be reflected in the disappearance of parental DNA from the pool of replicating phage DNA within infected bacteria. In fact, light parental DNA seems to decrease in amount while

the amount of DNA of progeny density increases during the period of the infection in which the pool was examined. Parental DNA does not completely disappear, which may indicate that at least a fraction of the infecting phage is dead, in the sense that its DNA does not replicate. (This was discussed in more detail in Chapter III.) Only a part of the loss of material from the band of parental DNA can be accounted for by the appearance of the band of DNA at intermediate density. Therefore either a fraction of the parental DNA must be sufficiently degraded so that it does not band, or else some parental DNA must form a part of molecules with the density of newly synthesized DNA. The latter explanation seems more likely because preparative centrifugations of fragmented progeny DNA indicate that at least the majority of transferred parental atoms are incorporated into hybrid fragments which are large enough to band. The parental DNA, then, must be fragmented and rapidly incorporated into molecules composed, for the most part, of newly synthesized DNA. The pool evidently does not contain DNA fragments which are joined together only prior to maturation of progeny phage.

The distribution of transferred parental atoms among progeny phage has been examined by studying the density distribution of the progeny. In the dummy control experiments, in which bacteria were simultaneously infected with T4$\underline{r+}$ and $\underline{r}$, both of normal isotopic composition, the density distributions of the progeny of $\underline{r+}$ and $\underline{r}$ genotypes are identical. This is clear from the observation that the ratio of the genotypes is a constant, independent of density. If, however, one of the parental phage contains heavy isotopes, the ratio of genotypes among

the progeny is found to depend strongly on density. The maxima in the distributions of the progeny of each genotype coincide, and the ratio of genotypes is approximately constant at this and at lighter densities. However, at greater densities there is a sharp increase in the ratio of phage of the genotype of the heavy parent to phage of the genotype of the light parent. In comparison to the latter distribution, the distribution of phage of the genotype of the heavy parent is skewed on the heavy side of the maximum. Thus, this distribution includes a component which is evidently comprised of progeny containing heavy parental DNA which emerges associated specifically with parental genotype. This result does not seem to depend on the parental genotype with which the heavy isotope is introduced, nor on the ratio of parental phage, which, in these experiments, has been kept close to unity.

It is noted parenthetically that the sharp decrease in the ratios which is always seen at the density of a progeny phage containing only half-heavy DNA is evidently due to the fact that there is, at this point, a decrease in the slope of the distribution of progeny of the genotype of the light parent. The subsequent increase in ratios at still higher densities is due to unadsorbed heavy parental phage.

The conclusion that there is a phage component containing heavy parental DNA which emerges associated specifically with parental genotype is supported by the rebanding experiments. Rebanding of phage from the dummy control shows that the small fraction of phage found at a density considerably heavier than the mean density are comprised of two kinds, those which reband at approximately the same heavy density,

and those which reband close to the mean density. The ratio of the two species is very different in the two rebandings done; it is not known whether this is due to biological or technical variation, or both. In both rebandings, the distributions of r+ and r progeny are essentially identical, indicating that whatever factors contribute to the distributions do not discriminate with respect to genotype.

From this result it could be inferred that when drops from the distribution of progeny phage from the transfer experiments were rebanded, it would again be found that a fraction of the rebanded phage return to approximately their original density and the remainder reband closer to the mean density of light phage. This was indeed found. However, when drops from the transfer experiments were rebanded, phage of the genotype of the heavy parent have a relatively large component which rebands at the heavier density. This is consistent with the conclusion that progeny of this genotype include a component which has a mean density greater than the mean density of the majority of progeny.

By subtracting the distribution of progeny of the genotype of the light parent from that of the heavy parent, the density distribution of this component can be found. Two such difference distributions are presented in Figs. 53 and 54, together with the corresponding distributions of the progeny of the genotype of the light (r) parent, provided as a reference. Compared to other figures, the abscissa have been multiplied times 4. The difference distributions cannot be meaningfully examined at densities greater than those shown because of interference by unadsorbed heavy parental phage. (It was necessary to correct for

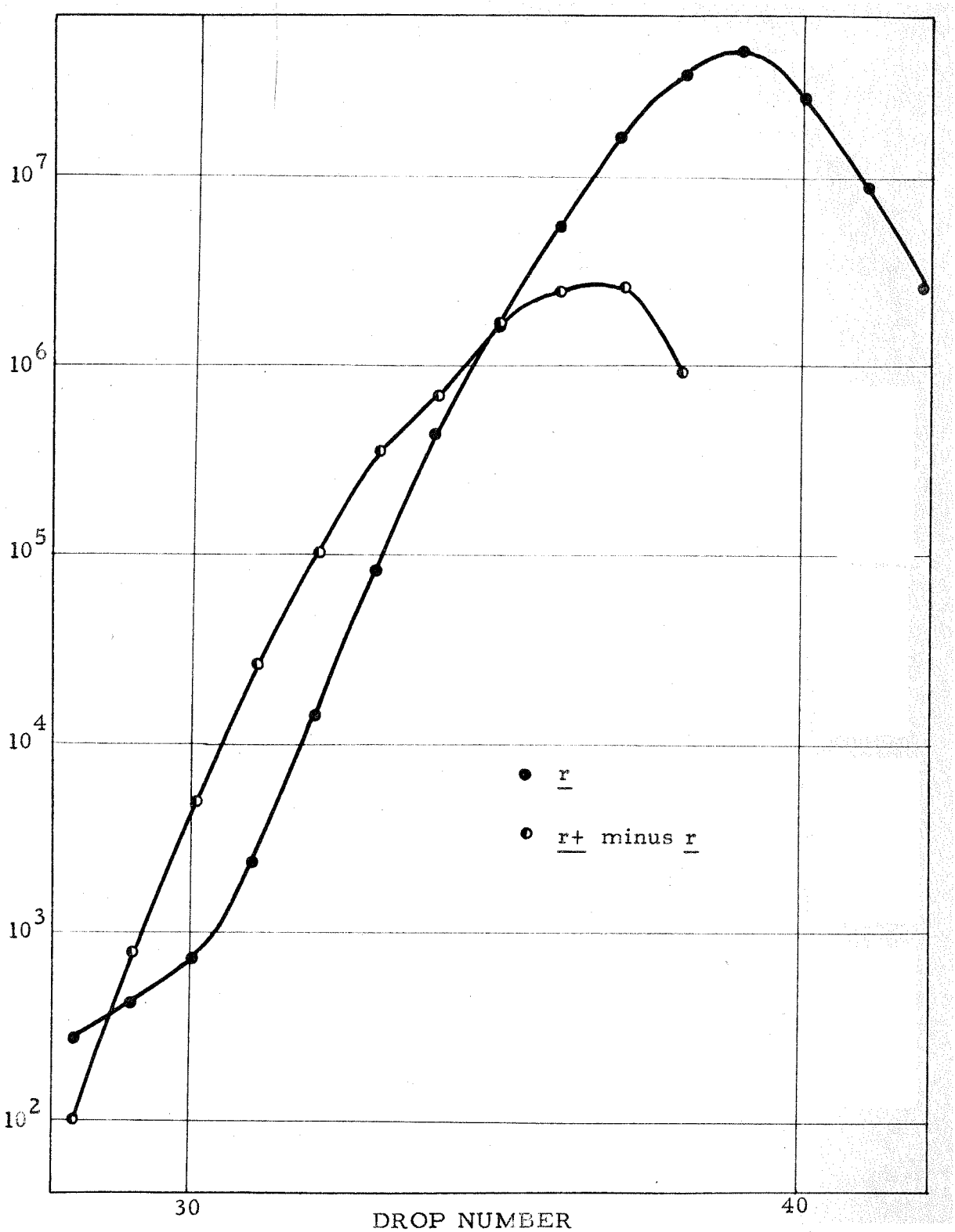

Fig. 53. Distribution of r progeny, and difference between r+ and r distributions.
(Transfer experiment 8-3-2)

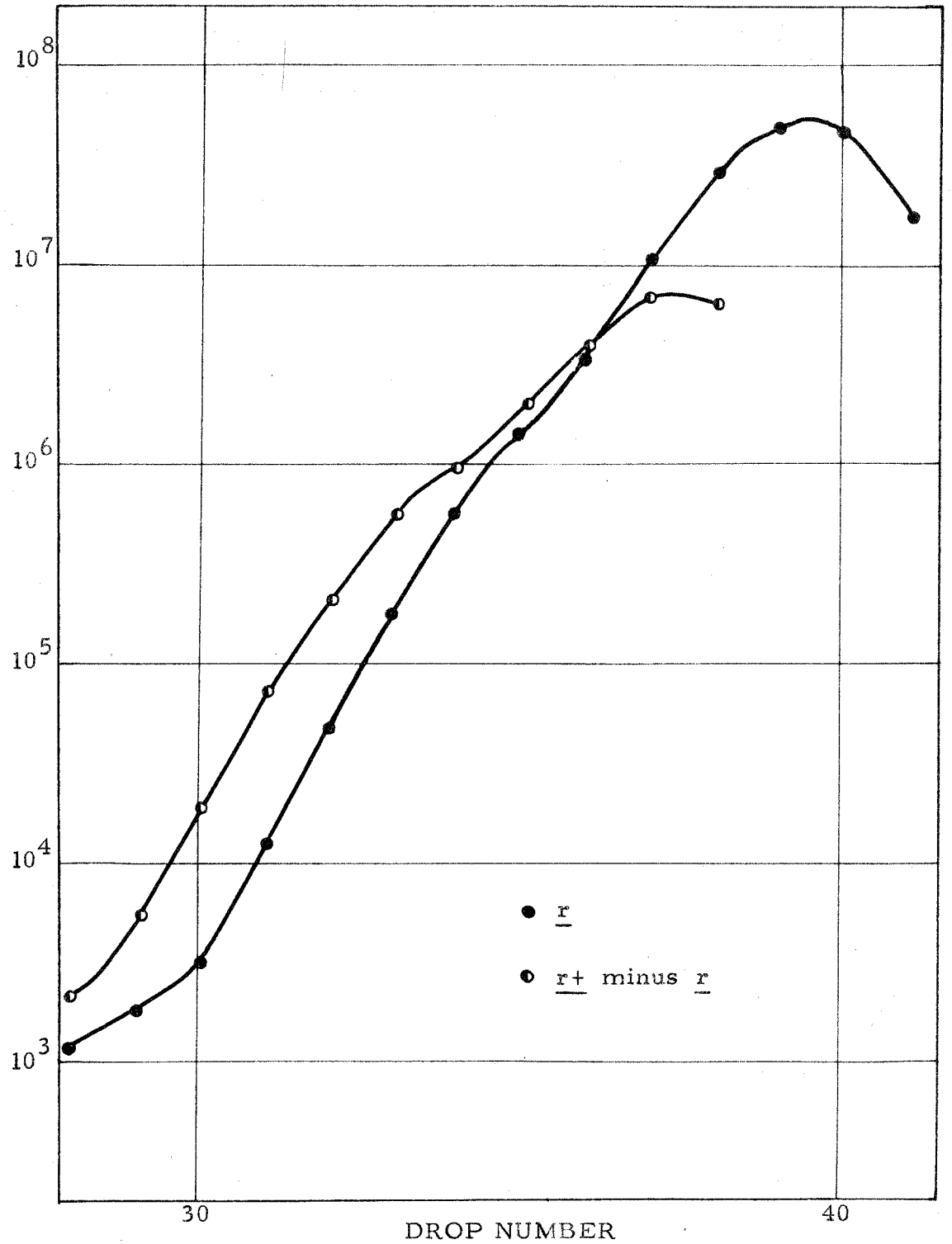

Fig. 54. Distribution of r progeny, and difference between r+ and r distributions.
(Transfer experiment 1-19-1)

the unequal numbers of progeny of the two genotypes in experiment 8-3-2, obtained because the ratio of parental phage differed from unity; the distribution of progeny of the light genotype has been multiplied by the ratio of heavy to light parents. This renders the distributions of the progeny identical at the density of their maxima and at lighter densities.)

The maxima of the difference distributions are displaced by 2 drops from the maxima of the reference distributions. In these experiments, there are about 14 1/2 drops between the density of light phage, and the calculated density of progeny phage containing heavy DNA. The 2 drop displacement suggests that, among those progeny containing parental DNA which emerges associated specifically with parental genotype, the majority receive about 14% of the parental complement of DNA, and few if any receive more than about twice this amount.

It is not possible to determine the fraction of progeny of the genotype of the heavy parent which receive half or more than half of their DNA complement from the parental phage. This is in part because of the poor resolution, and in part because it is not possible to determine the relative contributions of progeny phage and of unadsorbed parental phage in the trough between the peaks of parental and progeny phage. However, an upper limit can be set based on the total number of phage found at these densities. Of the progeny phage of the genotype of the heavy parent, approximately 10% contain isotope which has emerged associated specifically with genotype. Of these 10%, about 2% are found at the density of phage containing only hybrid DNA, and about 0.01% are found at the density of phage containing only heavy parental DNA. It is

likely that most of the 2% of phage of hybrid DNA density contain less than 50% parental DNA; they are probably at this density because of the width of bands of phage with 15-30% of their DNA derived from the heavy parent. The tail of the peak of heavy parental phage probably contributes substantially (or even totally) to the group of phage at the density which could indicate that they are progeny containing only parental DNA.

The conclusion was reached from the preparative centrifugation of progeny DNA that at least 80% of the transferred parental DNA is in fragments comprising not more than about 8% of the parental complement. The two groups of experiments differ in that the progeny of the infections with r+ and r parental phage were obtained by very early lysis of the infected cells; it may be that the size distribution of transferred DNA is a function of the length of time before it is withdrawn from the pool. However, within the resolution of these experiments, the two sets of data seem to be compatible simply on the assumption that larger fragments of transferred DNA have a proportionately greater probability of emerging specifically associated with parental genotype. The extensive fragmentation indicated by all of the experiments presented does not seem to be compatible with the data which have suggested that large segments of the DNA complement of parental phage particles (e.g. 25%) are transferred intact to progeny (Levinthal 1956 and Stent, Sato and Jerne 1959).

In conclusion, the data suggest that the DNA of the infecting T4 particle is replicated semiconservatively during its passage through the bacterial cell. In this respect it resembles the replication of the DNA

of the phages T7 (Meselson, 1959) and λ (Meselson and Weigle, 1961). T4 further resembles λ in that the transferred DNA is fragmented. It has been shown that the fragmentation of λ DNA is associated with the process of genetic recombination. The data of Kellenberger, Zichichi and Weigle (1961) indicate that, during a single cycle of infection, the DNA of the parental λ particle suffers, on the average, about one break. This is consistent with the low frequency of genetic recombination in λ. The present data suggest that single strands of parental T4 DNA suffer an average of at least 5 breaks. The frequency of recombination in this phage is much higher than in λ, and it may be that in T4 also, DNA breakage is associated with recombination. In the pool of replicating DNA, parental fragments do not persist, but are rapidly incorporated into molecules containing a large fraction of newly synthesized material. This may mean that fragments do not enter a pool in which they are mixed before being joined together. If this is so, then a breakage model could be consistent with recombination between pairs, or small groups, of genomes. The present data cannot decide whether recombination is associated with breakage of DNA molecules in the phage T4, as it is in λ. However, they require that any model for recombination take into account the extensive fragmentation of T4 DNA which occurs in the course of its replication.

REFERENCES

Astrachan, L. and Volkin, E. (1958) Biochim. Biophys. Acta 29, 536-544.

Cocito, C. and Hershey, A. D. (1960) Biochim. Biophys. Acta 37, 543-544.

Davern, C. (1959) Doctoral Dissertation, California Institute of Technology.

Davison, P. F. (1959) Proc. Nat. Acad. Sci. 45, 1560-1568.

Delbrück, M. and Stent, G. S. (1957) in The Chemical Basis of Heredity, 699-737, ed. by McElroy, W. D. and Glass, B., Johns Hopkins Press.

Freese, E. (1959) Brookhaven Symp. in Biol. 12, 63-69.

Hearst, J. (1961) Personal communication.

Hershey, A. D. (1954) J. Gen. Physiol. 38, 145-148.

Hershey, A. D. (1955) Vir. 1, 108-127.

Hershey, A. D. and Burgi, E. (1956) Cold Spring Harbor Symp. Quant. Biol. XXI, 91-101.

Hershey, A. D. and Chase, M. (1952) J. Gen. Physiol. 36, 777-789.

Hershey, A. S., Kamen, M. D., Kennedy, J. W. and Gest, H. (1951) J. Gen. Physiol. 34, 305-319.

Hershey, A. D., Roesel, C., Chase, M., Forman, S. (1951) Carnegie Inst. Wash. Yearbook 50, 195-200.

Kellenberger, G., Zichichi, M. L., and Weigle, J. J. (1961) Proc. Nat. Acad. Sci., in press.

Kozinski, A. W. (1961) Vir. 13, 124-134.

Levinthal, C. (1956) Proc. Nat. Acad. Sci. 42, 394-404.

Levinthal, C. and Thomas, C. A. Jr. (1957) in The Chemical Basis of Heredity, 737-742, ed. by McElroy, W. D. and Glass, B., Johns Hopkins Press.

Litman, R. M. and Pardee, A. B. (1956) Nature 178, 529-531.

Maaløe, O. and Watson, J. D. (1951) Proc. Nat. Acad. Sci. 37, 507-513.

Marmur, J. and Doty, P. (1959) Nature 183, 1427-1429.

Marmur, J. and Lane, D. (1960) Proc. Nat. Acad. Sci. 46, 453-461.

Meselson, M. (1959) in The Cell Nucleus (the Faraday Soc.), 240-245, Butterworth and Co., Ltd.

Meselson, M. and Stahl, F. W. (1958) Proc. Nat. Acad. Sci. 44, 671-682.

Meselson, M., Stahl, F. W. and Vinograd, J. (1957) Proc. Nat. Acad. Sci. 43, 581-588.

Meselson, M. and Weigle, J. (1961) Proc. Nat. Acad. Sci., in press.

Putnam, F. W. and Kozloff, L. M. (1950) J. Biol. Chem. 182, 243-250.

Robkin, E., Meselson, M. and Vinograd, J. (1959) J. Am. Chem. Soc. 81, 1305-1307.

Stahl, F. W. (1956) Vir. 2, 206-234.

Stent, G. S. (1959) in The Viruses, Vol. 2, 237-280, ed. by Burnet, F. M. and Stanley, W. M., Academic Press.

Stent, G. S. and Jerne, N.K. (1955) Proc. Nat. Acad. Sci. 41, 704-709.

Stent, G. S., Sato, G. H. and Jerne, N. K. (1959) J. Mol. Biol. 1, 134-146.

Volkin, E. (1954) J. Am. Chem. Soc. 76, 5892-5893.

Watson, J. D. and Crick, F. H. C. (1953) Cold Spring Harbor Symp. Quant. Biol. XVIII, 123-132.

Watson, J. D. and Maaløe, O. (1953) Biochim. Biophys. Acta 10, 432-442.

Weigle, J., Meselson, M. and Paigen, K. (1957), J. Mol. Biol. 1, 379-386.

Wyatt, G. R. and Cohen, S. S. (1953) Biochem. J. 55, 774-782.