

THE MOLECULAR WEIGHT OF PBS 1 DNA

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OF PBS 1 DNA

By

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

May 1967

MASTER OF SCIENCE (1967)
(Biology)

McMASTER UNIVERSITY
Hamilton, Ontario.

TITLE: The Molecular Weight of PBS 1 DNA

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NUMBER OF PAGES: vii, 83

SCOPE AND CONTENTS:

That bacteriophage PBS 1 contains DNA of a considerable size is suggested by the size of its head, and also by the fairly high frequencies with which it performs joint transduction of genetic markers. Density differences between the active, plaque-forming particles and the transducing particles of PBS 1 facilitated a separation of the two which served as a preliminary to investigating the DNA content of each. The molecular weight of DNA obtained from active particles is estimated to be 1.9×10^8 . Tentative evidence is offered for the presence of a large fragment of host DNA within the head of transducing PBS 1.

PREFACE

This thesis describes studies undertaken in the Department of Biology, McMaster University, from September, 1965, to April, 1967. No similar thesis has been submitted to any other university.

I would like to express my thanks to my supervisor, Dr. I. Takahashi, for his invaluable and unfailing interest and advice; to Dr. H. Yamagishi, who has been of practical assistance on many occasions, and to the Department of Biology for financial support during the period of study. I would also like to thank my typists, Mrs. Jean I. Wheatley and Miss Joyce Barrett for their helpfulness and efficiency.

A handwritten signature in cursive script that reads "Barbara Hockett". The signature is written in dark ink and is positioned above the typed name and affiliation.

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April, 1967

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CHAPTER ONE

INTRODUCTION

Prior to the advent of the electron microscope, nothing was known of bacteriophage morphology, and estimates of particle size put forward from studies of such physical properties as filterability (Elford, 1938) and rates of diffusion and sedimentation (Putnam, 1953) were only tentative. It was with the development of fine technique in electron microscopy, and its use by such workers as E. Kellenberger, T.F. Anderson, and R.C. Williams, that it was first possible to describe the spherical or polyhedral head and cylindrical tail structures common to most phages, and to measure their dimensions accurately. Anderson (1949) went on to use electron microscopy in the study of the particles of T-even phages which had been disrupted by osmotic shock, and revealed that the phage head consists of an outer membrane, the content of which disperses readily once the membrane is split.

It had been known for some time that protein and nucleic acid, usually deoxyribonucleic acid (DNA), are components of phage particles (Schlesinger, 1934), but it was not until the analytical work of Taylor (1946) and Cohen (1947) that it was known that they are almost exclusively responsible for phage structure. By osmotically shocking T₂ phages, as described by Anderson, purifying the empty membranes ('ghosts'), applying to them proteases and deoxyribonuclease (DNAase) respectively, and observing the results, Herriot (1951) was able to suggest that the

'ghost' of a phage particle is the protein constituent, and that the content of the head is the DNA constituent. The appearance of a network of fibres, 20⁰Å in diameter, round the 'ghosts' in electron micrographs of disrupted T₆ phage prepared by Williams (1953), further indicated that the content of the head comprises DNA. That this is indeed so was conclusively demonstrated by Garen (in Hershey, 1955), who showed that although DNAase has no effect on intact T₂ phage particles, it can and does act on extracted DNA and on particles whose heads are broken open.

A primary genetic role has been assigned to DNA ever since the classic discovery of Avery, MacLeod and McCarty (1944) that DNA extracted from an encapsulated strain of pneumococcus would permanently transform unencapsulated cells into encapsulated cells. It is no surprise, therefore, that the DNA known to be present in phage particles became singled out as the important, active component, and, further, that the findings establishing the localized, protected position of the DNA suggested an organization within the phage head that might be analogous to the nuclear arrangements of other organisms.

The first real evidence to prove that phage DNA is the genetic material - provided by an elegant isotopic study carried out with T₂ phage (Hershey and Chase, 1952) - also emphasized the ultimate importance of DNA. Hershey and Chase prepared T₂ particles in which the protein coat was labelled with ³⁵S, and the DNA was labelled with ³²P. Host cells were infected with the T₂, and the fate of the protein and DNA components of the phage particles followed by measuring the subsequent distribution of their characteristic radioactivities. It was found that the DNA entered the host cell and that its entry was followed by pro-

liferation of phage particles. The protein coats did not gain entry, but remained attached to the outer wall of the host cell. When the protein coats were detached from the cell wall by agitating cultures of newly infected bacteria, phage multiplication continued unaffected. Thus it was shown that the protein coat cannot be assigned any genetic function; the DNA alone is responsible for the reproduction of the phage.

Knowledge of the organization of DNA within the phage head was clarified, to a certain degree, with the initiation of research into the nature of intact DNA molecules, which were first successfully extracted from phages by Mandell and Hershey (1960), using T_2 and T_4 . For example, by using an autoradiographic method, Rubenstein, Thomas and Hershey (1961) were able to measure the number of ^{32}P atoms in a labelled T_2 DNA molecule and, from the specific activity, calculate the total phosphorus content per molecule - a value that can be correlated with the total phosphorus content of a single whole T_2 particle in order to obtain an estimate of the number of molecules per particle. It was shown that one T_2 DNA molecule accounts for virtually all of the phosphorus of the T_2 particle, which signifies the presence of but one molecule of DNA per particle. Such a finding is consistent with the idea of a 'phage chromosome', and relates to the fact that all known genetic markers of the T-even phages fall into one linkage group (Streisinger and Bruce, 1960). Since phages other than T_2 have also, more recently, been found to contain single DNA molecules, it seems likely that all phages conform to the same pattern.

The discovery that phage DNA is monomolecular had, and still has, an obvious corollary: the determination of the actual size of the DNA molecules. So far this has been attempted in three major ways, each of which is outlined below.

1. Electron microscopy.

This is the only method in which measurements are made directly, but is a method beset with difficulties since it demands the production of films bearing unbroken, single DNA molecules. A technique designed to overcome the difficulties has been investigated with some success by Beer (1961). It involves streaking DNA solutions of controlled concentration, ionic strength and pH along weakly basic ion exchange membranes made of a copolymer of styrene and vinylpyridine.

Another technique, devised by Kleinschmidt, Lang, Jacherts and Zahn (1962), consists of subjecting well separated phage particles to osmotic shock while they are floating on water covered by a molecular film of protein. The phage DNA molecules slowly diffuse out from the phage 'ghosts' across the protein layer in undisturbed array.

2. Autoradiography.

This is a method developed by Cairns (1961) in which the size of the DNA molecule is measured indirectly from an autoradiographic image. The method depends upon the fact that tritium gives rise to electrons whose mean range in autoradiographic emulsion is short - less than 1μ . A ^3H -labelled DNA molecule thus produces a distinct and measurable line of grains in adjacent autoradiographic film over a period of several weeks. Since the DNA molecules require to be arranged in an

orderly fashion on a solid surface prior to the application of autoradiographic film, this method presents the same problems as does electron microscopy of DNA molecules. Cairns reports surmounting the difficulties by using the copolymer membrane and streaking technique of Beer (1961).

3. Determination of molecular weight.

The usual configuration of the DNA molecule is regular, as illustrated by the commonly accepted model of Watson and Crick (1953), in which two polynucleotide chains are precisely twisted about each other in a double helix. Since this is so, the molecular weight of a particular DNA is a measure of the relative size (in units of length) of the individual molecules of which it is composed.

Determination of the molecular weight of phage DNA has been attempted using a variety of the techniques designed for the investigation of macromolecules in general. However, not all of the techniques have been found applicable to DNA, especially phage DNA, which is of such large molecular size that the interaction of molecules leads to unreliable results. It has been necessary to devise new methods. The methods that have achieved widest application in work with phage DNA are discussed in this section.

One of the conventional ways of determining the molecular weight of a DNA is from the viscosity of DNA solutions. The viscosity of a solution is proportional to (a) the concentration of molecules present, and a factor (b) which depends upon the shape and dimensions of the molecule. Since DNA molecules have a simple geometrical shape, by calculating their effective behaviour from the laws of hydrodynamics,

factor (b) can be modified so that it depends upon molecular dimensions alone. Usually, intrinsic viscosity is determined. This is the limiting value of viscosity when the concentration of DNA is zero, and by its use the interactions of molecules do not have to be accounted for and the molecular weight of DNA is directly related to viscosity. Two of the many workers who have employed the intrinsic viscosity method are Thomas and Matheson (1960) who used the classical capillary-type viscometer to make measurements of T_4 DNA, at low concentrations, for subsequent extrapolation to zero.

Another standard way of estimating the molecular weight of a DNA is by observing its sedimentation behaviour under conditions in which the gravitational potential energy is raised by centrifugation. The DNA molecules may be observed once their average motion has stopped, as in the sedimentation-equilibrium technique; on the other hand, the average drift velocity of the molecules away from the rotational axis may be measured, as in the sedimentation-velocity technique.

A particular modification of the sedimentation-equilibrium technique developed by Meselson, Stahl and Vinograd (1957) has been used quite extensively, as, for example, by Thomas and Pinkerton (1962), who worked with the DNAs of phages T_2 , T_4 , T_5 , T_7 , and P_{22} . The technique itself is one in which the DNA is spun in a concentrated solution of a heavy salt, such as caesium chloride, whose density is approximately the same as the density of the DNA. A density gradient is set up in the medium during the course of the centrifugation, and the DNA forms a band at the level at which the sum of the forces acting on a given molecule is zero; in other words, where the density of the medium precisely

corresponds to the density of the DNA. According to Meselson, Stahl and Vinograd (1957), the concentration distribution of a single species of DNA within the band at equilibrium is Gaussian, and the standard deviation of the Gaussian band is inversely proportional to the square root of the molecular weight. By photographing the band in the ultraviolet range and obtaining optical density tracings from the film on a densitometer, the distribution of the DNA can be estimated quantitatively and the molecular weight value readily derived.

In addition to this 'band width' method, the sedimentation-velocity technique has been applied to some advantage, for instance by Burgi and Hershey (1961) using T_4 DNA, and by Thomas and Berns (1961) using T_2 and T_4 DNAs. In this technique the boundary of the sedimenting DNA is detected by means of an ultraviolet optical method during centrifugation in order to determine the velocity of the DNA molecules. Linear acceleration can also be determined since it relates to the angular speed and the distance from the axis, both of which are known values. The ratio of sedimentation velocity to acceleration is defined as the sedimentation coefficient. This is known to vary according to the molecular weight of the sedimenting material, and the relationship has been expressed in a number of different ways. The following equation, relating sedimentation coefficient to molecular weight in a simple manner, was derived from conventional sedimentation measurements of samples of thymus DNA made by Doty, McGill and Rice (1958):

$$S = aM^k$$

where S = sedimentation coefficient

M = molecular weight

a = constant

k = constant

The samples of thymus DNA ranged in molecular weight from 0.3×10^6 to 7.0×10^6 , and gave the following values for the constants:

$$a = 0.063$$

$$k = 0.37$$

A method for determining the molecular weight of a DNA, which differs considerably from the classical methods, was introduced by Levinthal and Thomas (1957) and put to valuable use by Rubenstein, Thomas and Hershey (1961). By this method the total phosphorus content of an individual DNA molecule is determined by a process of molecular autoradiography, mentioned earlier; then, on the basis that the DNA contains a certain proportion of phosphorus, measurable by simple analysis, the molecular weight of the DNA is calculated. Rubenstein, Thomas and Hershey applied the method to the DNA of T_2 phage. They state that the autoradiographic measurements are reproducible and subject to a purely statistical error of only a few percent. That the molecular weight value they obtained for T_2 DNA is reliable is indicated by the fact that estimates they made based on direct measurements of the amount of DNA per plaque-forming particle supported their results. Rubenstein, Thomas and Hershey also made determinations of the molecular weight of T_2 DNA using the standard techniques. By comparison with the result of the newer method, results obtained from the intrinsic

viscosity, 'band width' and sedimentation velocity methods were all too low. To amply illustrate the discrepancy, measurements made on T_2 DNA which had been degraded to varying degrees to give a molecular weight range of 0.3×10^8 to 1.3×10^8 gave the following values for the constants in the equation of Doty, McGill and Rice:

$$a = 0.0024$$

$$k = 0.543$$

Thus it was indicated that though of some value, especially in combination with each other, the conventional methods fall far short of an ideal when high molecular weight compounds such as DNA are the materials under investigation.

It was the shortcoming of the sedimentation method in particular, and the limited applicability of most other available methods, which led Burgi and Hershey (1963) to propose a new and better sedimentation technique for estimating the molecular weights of DNAs. This is now the more commonly used of all the useful techniques for estimating the molecular size of a DNA. It consists of the sedimentation of two DNAs, differentially labelled with radioisotopes, in a previously prepared sucrose density gradient (in which convective disturbances are reduced). One of the DNAs is of known molecular weight and acts as reference to the other DNA, the unknown. The relative positions of the DNAs, after appropriate centrifugation time, can be determined by fractionating the gradient and assaying each fraction for radioactivity. Measurements made by Burgi and Hershey using the DNAs of T_2 , T_5 and λ phages have shown the following relationship to be of general application:

$$D_2/D_1 = (M_2/M_1)^{0.35}$$

where D = distance sedimented

M = molecular weight

Determinations of molecular size have now been made for a number of different phages. One of the smallest phages, ϕ X-174, having a head 30m μ in diameter, contains a DNA molecule of molecular weight 1.7×10^6 (Sinsheimer, 1959). At the other extreme, phage T₂, whose 65 x 95m μ head dimensions are the same as the head dimensions of T₄ (Williams and Fraser, 1953), one of the largest phages known (Luria, 1962), contains a much larger DNA molecule. The molecular weight of T₂ DNA is 1.3×10^8 (Rubenstein, Thomas and Hershey, 1961) and, prior to the findings presented in this thesis, was the highest molecular weight known for DNA from any source.

The work presented in this thesis concerns the molecular size of the DNA of bacteriophage PBS 1, which was isolated and reported by Takahashi (1961). Its important characteristics are summarized below.

1. The host range of PBS 1 is limited to strains of Bacillus subtilis. Phage PBS 1 is able to infect all derivatives of the Marburg strain of B. subtilis (Takahashi, 1961, 1963), which has been used most extensively in genetic studies.
2. Phage PBS 1 is a temperate bacteriophage. Plaques formed in a layer of sensitive bacteria have a central ring of bacterial growth surrounded by a turbid halo (Takahashi, 1961).

3. Phage PBS 1 is a transducing bacteriophage, performing generalized transduction of such characteristics as streptomycin resistance, prototrophy and sporogenesis (Takahashi, 1961) between the Marburg strains of B. subtilis only (Takahashi, 1963). It is capable of transducing, jointly, two or more genetic markers at a fairly high frequency (Takahashi, 1966).
4. Structurally, PBS 1 has a head and a tail which are similar to those of T-even phages; however, PBS 1 is of extremely large dimensions. Takahashi (1963) reported that the head is approximately 100m μ in diameter. Recent observations made by Eiserling (1967) have established the precise dimensions of PBS 1, and the head is now known to be 120m μ in diameter.
5. The DNA of phage PBS 1 is unusual in that uracil replaces thymine entirely (Takahashi and Marmur, 1963a).

It was thought that an investigation of the DNA of PBS 1 would prove interesting, since the large size of the head of the PBS 1 particle suggested that PBS 1 DNA might be larger in molecular size than T₂ DNA. In event of PBS 1 DNA being found larger than T₂ DNA, there would not only be an extension to the known upper limits of size for DNA molecules, but, in addition, it would be proved conclusively that temperate phages do not always contain DNA of smaller size than the DNA of T-even phages - as was the generalization of Jacob and Wollman (1959) from evidence provided by Smith and Siminovitch (in Lwoff, 1953) from analyses of a number of temperate phages.

This thesis, then, describes an attempt to characterize PBS 1 according to the physical nature of the DNA 'chromosome' it contains. Active, plaque-forming particles and transducing particles were studied separately in order that a comparison of their DNAs might be made, and in the hope that knowledge of the structure of the DNA of the transducing particle would throw some light on the mechanism of transduction and its genetic significance.

CHAPTER TWO
MATERIALS AND METHODS

I. MATERIALS

1. Bacteriophages

<u>bacteriophage</u>	<u>host</u>	<u>origin</u>
PBS 1	<u>Bacillus subtilis</u>	Takahashi (1961)
T ₂	<u>Escherichia coli</u>	Demerec and Fano (1945)

2. Bacteria

Bacillus subtilis

<u>strain</u>	<u>origin</u>	<u>genotype</u>
SB19E	SB19 (E.W.Nester)	prototrophic
A26	W	pyrimidine ⁻
168T	168 (J.L.Farmer)	indole ⁻ thymine ⁻
A7	W	methionine ⁻

Escherichia coli

<u>strain</u>	<u>origin</u>	<u>genotype</u>
B	C.Fuerst	prototrophic

3. Media and special solutions(a) For the propagation of bacterial stocks(i) Tryptose blood agar base (Difco)

Difco preparation	33g
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Distilled water	1000ml
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The suspension was heated to boiling before autoclaving.

(b) For the propagation of bacteriophages(ii) Nutrient broth (Difco) for the preparation of PBS 1 lysate using

B. subtilis 19E (prototrophic) as host.

Difco preparation	8g
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Distilled water	1000ml
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The broth was autoclaved without prior heating.

(iii) Penassay broth (Difco), also for the preparation of PBS 1 lysate

using B. subtilis 19E as host.

Difco preparation	17.5g
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Distilled water	1000ml
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The broth was autoclaved without prior heating.

- (iv) Tris medium, also for the preparation of PBS 1 lysate using B. subtilis 19E as host.

Ammonium sulphate	2g
Magnesium sulphate	0.2g
Sodium citrate	1g
Casamino acids (Difco)	0.5g
Yeast extract (Difco)	0.5g
Tris buffer, 1M, pH 7.5	50ml
Potassium phosphate buffer, 0.5M, pH 7.4	2ml
Distilled water	to 975ml

After autoclaving, the following sterile solutions were added:

Ferric chloride solution, 0.5% (final concentration 0.00025%)	0.5ml
Glucose solution, 10% (final concentration 0.25%)	25ml

- (v) Nutrient broth (Difco) supplemented with uracil for the preparation of PBS 1 lysate using B. subtilis A26 (uracil⁻) as host.

Difco preparation	8g
Distilled water	950ml

After autoclaving, the following sterile solution was added:

Uracil solution, 100 μ g per ml (final concentration 5 μ g per ml)	50ml
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- (vi) 1/2 Minimal medium supplemented with Casamino acids, tryptophan, and thymine for the preparation of PBS 1 lysate using B. subtilis 168T (indole⁻, thymine⁻) as host.

½ Minimal medium:

Dipotassium phosphate	7g
Monopotassium phosphate	3g
Ammonium sulphate	1g
Sodium citrate	0.5g
Magnesium sulphate, hydrated	0.1g
Distilled water	to 950ml

After autoclaving, the following sterile solutions were added:

Casamino acids (Difco), in solution, 5% (final concentration 0.1%)	20ml
Tryptophan solution, 0.1% (final concentration 25µg per ml)	25ml
Thymine solution, 1.12% (final concentration 56µg per ml)	5ml

(vii) Tris medium supplemented with thymine, also for the preparation of PBS 1 lysate using B. subtilis 168T as host.

Tris medium as described in (iv), but made up to 990ml instead of 1000ml.

After autoclaving, the following sterile solution was added:

Thymine solution, 1% (final concentration 100µg per ml)	10ml
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(viii) Tris-glucose-Casamino acids medium (Hershey, 1955) low in phosphorus content for the preparation of T₂ lysate using E. coli B as host.

Stock salt solution:

Sodium chloride	54g
Potassium chloride	30g

Ammonium chloride	11g
Calcium chloride solution, 0.1M	10ml
Magnesium chloride solution, 2.5M	4ml
Ferric chloride solution, 0.1M	1ml
Distilled water	to 1000ml

Tris-glucose-Casamino acids medium:

Stock salt solution	100ml
Tris buffer, 1M, pH 7.4	100ml
Sodium sulphate solution, 0.1M	1.6ml
Distilled water	to 900ml

After autoclaving, the following sterile solutions were added:

Glucose solution, 4% (final concentration 0.2%)	50ml
Casamino acids (Difco), in solution, 1% (final concentration 0.05%)	50ml

Note: According to H. Yamagishi, Casamino acids (Difco) contains 14 μ g total phosphorus per mg. Thus tris-glucose-Casamino acids medium contains only 7 μ g total phosphorus per ml.

- (c) For the detection of contaminants in millipore-filtered lysates
- (ix) Tryptose blood agar base (Difco)
See (i)
- (d) For the assay of lysates

- (x) Penassay broth (Difco) for the culture of indicator bacteria for both PBS 1 and T₂ assays.

See (iii)

- (xi) Adsorption medium for the preparation of dilution series in the PBS 1 assay procedure.

Sodium chloride	4g
Potassium sulphate	5g
Disodium phosphate	3g
Monopotassium phosphate	1.5g
Magnesium sulphate, hydrated	0.12g
Yeast extract (Difco)	1g
Distilled water	to 1000ml

After autoclaving, the following sterile solutions were added:

Calcium chloride solution, 1% (final concentration 0.001%)	1ml
Ferric chloride solution, 0.5% (final concentration 0.001%)	2ml

- (xii) T₂ medium for the preparation of dilution series in the T₂ assay procedure.

Nutrient broth (Difco)	8g
Sodium chloride	5g
Dextrose	1g
Distilled water	to 1000ml

The medium was autoclaved without prior heating.

(xiii) Tryptose blood agar base (Difco) for the base agar in the PBS 1 assay plate.

See (i)

(xiv) 1% Tryptose blood agar base (Takahashi, 1963) for the soft agar layer in the PBS 1 assay plate.

Sodium chloride	5g
Tryptose (Difco)	10g
Beef extract (Difco)	3g
Agar (Difco)	10g
Distilled water	to 1000ml

The suspension was heated to boiling before autoclaving.

(xv) T₂ base agar

Sodium chloride	8g
Sodium citrate	2g
Glucose	1.3g
Tryptone (Difco)	13g
Agar (Difco)	10g
Distilled water	to 1000ml

The suspension was heated to boiling before autoclaving.

(xvi) T₂ soft agar

Sodium chloride	8g
Sodium citrate	2g

Glucose	3g
Tryptone (Difco)	13g
Agar (Difco)	6.5g
Distilled water	to 1000ml

The suspension was heated to boiling before autoclaving.

(e) For transduction experiments

(xvii) Penassay broth (Difco) for culturing recipient bacteria.

See (iii)

(xviii) Minimal agar

Dipotassium phosphate	14g
Monopotassium phosphate	6g
Ammonium sulphate	2g
Sodium citrate	1g
Magnesium sulphate, hydrated	0.2g
Agar (Difco)	15g
Distilled water	to 950ml

The suspension was heated to boiling before autoclaving. After autoclaving, the following sterile solution was added:

Glucose solution, 10% (final concentration 0.5%)	50ml
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(f) For the suspension of phage particles

(xix) Saline sodium citrate (SSC)

Sodium chloride	8.5g
Sodium citrate	4.4g
Distilled water	to 1000ml

When the solutes were dissolved, the pH of the solution was adjusted to 7.0 with dilute hydrochloric acid. The solution was autoclaved.

(xx) Suspending medium

Sodium chloride	4g
Magnesium sulphate, hydrated	0.12g
Gelatin	0.05g
Tris buffer, 1M, pH 7.5	6ml
Distilled water	to 1000ml

The medium was autoclaved without prior heating.

(g) For caesium chloride density gradients(xxi) Stock caesium chloride solution

Caesium chloride (optical grade, Hershaw Chemicals Co.)	130g
Tris buffer, 0.02M, pH 8.0	70ml

(h) For sucrose density gradients

(xxii) Sucrose, in solution in SSC, 5% (w/v)

(xxiii) Sucrose, in solution in SSC, 20% (w/v)

II. METHODS

1. Maintenance of bacterial stocks

Bacterial stocks were kept as streak cultures on tryptose blood agar base. Stocks were stored at room temperature, and propagated every 3 or 4 days by transferring to fresh medium and incubating at 37°C for 16-18 hours.

When required, freshly streaked and incubated cultures were used such that inocula consisted essentially of vegetative cells.

2. Preparation and maintenance of stock bacteriophage lysates

(a) PBS 1

PBS 1 segregates at a high frequency clear-plaque type PBS 2, which differs from PBS 1 not only in plaque morphology but also in that it is less efficient in transduction (Takahashi, 1963). For this reason, stock lysates were always prepared from isolated plaques of PBS 1, and sufficient quantities were made for use in a complete series of experiments.

Procedure:

B. subtilis 19E was grown in nutrient broth at 37°C, with aeration, for 5-6 hours, diluted 20 times in nutrient broth, and distributed into test tubes in 3ml aliquots. The diluted

culture was infected with PBS 1 by adding to each test tube one plaque, picked off a fresh assay plate with a pasteur pipette. Only those plaques exhibiting a ring of bacterial growth surrounded by a turbid halo were isolated. The infected cultures were incubated at 37°C, without aeration, for 16-18 hours.

The resultant crude lysates were used for the preparation of large-volume lysates in the following manner (Takahashi, 1963). A fresh culture of B. subtilis 19E grown in nutrient broth at 37°C, with aeration, for 5-6 hours, was diluted 20 times in nutrient broth, and distributed into 250ml flasks in 20ml aliquots. Each aliquot of diluted culture was infected with 5ml of crude PBS 1 lysate, incubated at 37°C, with aeration, for 1 hour, then further incubated at 37°C, without aeration, for 16-18 hours.

Lysates were pooled and rendered cell-free by preliminary low-speed centrifugation at 6,000 r.p.m. for 10 minutes followed by passage of the supernatant through a millipore filter membrane (pore size 0.45 μ). After filtration, a sample of the lysate, 0.5-1.0ml in volume, was plated on to tryptose blood agar base and incubated at 37°C for 48 hours to confirm the absence of bacteria. Cell-free lysates were stored in the frozen state at -35°C after addition of the protective agent dimethyl sulphoxide (final concentration 10%).

The procedure for the preparation of stock PBS 1 lysate in tris medium is the same as described above.

Where stock PBS 1 lysate is mentioned in the text, nutrient broth medium is implied unless otherwise stated.

(b) T₂

In comparison to PBS 1, T₂ shows considerable genetic stability. Fresh stock lysates were therefore prepared directly from pre-existing stocks, in contrast to the plaque isolation method used in the preparation of stock PBS 1 lysates.

Procedure:

E. coli B was grown in tris-glucose-Casamino acids medium at 37°C, without aeration, for 10-18 hours, in order to obtain a stationary-phase culture. The culture was diluted 25 times in tris-glucose-Casamino acids medium, distributed into 250ml flasks in 25ml aliquots, and further incubated at 37°C, but with aeration, for 2 hours. The resultant cultures were in the early logarithmic phase of growth and contained approximately 1×10^8 cells per ml. At this point they were infected with stock T₂ lysate at a multiplicity of infection of 1.0, and incubated at 37°C, with aeration, until lysis occurred. Lysis usually occurred after 3-4 hours' incubation; however, if no lysis became apparent after 5 hours, incubation was stopped. After removal from the incubator, 0.3ml of chloroform was added to all flasks to complete the process of lysis.

Lysates were pooled and rendered cell-free by preliminary low-speed centrifugation at 6,000 r.p.m. for 10 minutes followed by passage of the supernatant through a millipore filter membrane (pore size 0.45μ). After filtration, a sample of the lysate, 0.5-1.0ml in volume, was plated on to tryptose blood agar base and incubated at 37°C for 48 hours to confirm the absence of bacteria. Cell-

free lysates were stored at 4°C.

3. Assay of lysates

(a) PBS 1

The assay technique used for PBS 1 was a modification of the pre-adsorption method of Potter and Nelson (1952) as described by Takahashi (1963). Medium Y was replaced by adsorption medium.

Procedure:

B. subtilis 19E was grown in Penassay broth at 37°C, with aeration, for 5-6 hours. Samples of the culture, 0.1ml in volume, were diluted 15 times in adsorption medium in 15 x 150 mm test tubes, to give 1.5ml diluted culture containing approximately 5×10^7 cells per tube. A 3ml sample of lysate, diluted to an appropriate degree in adsorption medium, was added to each tube, and the mixture allowed to stand at room temperature for 5 minutes. A 4.5ml aliquot of 1% tryptose blood agar base (previously melted and cooled to 45-50°C) was then added to each tube and mixed in thoroughly using a 10ml wide-tip pipette. Fresh tryptose blood agar base plates were overlaid with 3ml of this phage + indicator bacteria + soft agar mixture. All assay plates were made in duplicate, and incubated at 30°C for 16-18 hours. The final concentration of agar in the soft agar layer was 0.5%. By this method, the number of plaques that developed on each plate represented the number of plaque-forming

particles per ml of diluted lysate tested.

(b) T₂

The assay technique used for T₂ was the soft agar layer method of Adams (1959). The suspension of indicator bacteria described by Adams was replaced by a liquid culture of the bacteria, the 1.5% base agar was replaced by 1.0% base agar, and the 0.7% soft agar was replaced by 0.65% soft agar.

Procedure:

T₂ soft agar, which had been previously melted and cooled to 45-50°C, was pipetted in 2.5ml aliquots into 11 x 100 mm test tubes kept in a 45° water bath. E. coli B was grown in Penassay broth at 37°C, with aeration, for 2-2½ hours, and an 0.05ml sample of the culture (approximately 2 x 10⁷ cells) added to each of the tubes of agar. An 0.1ml sample of lysate, diluted to an appropriate degree in T₂ medium, was also added to each tube. Immediately following the addition of lysate to a tube, the contents of the tube were mixed, by rolling the tube between the hands, then poured over fresh thin plates of T₂ base agar. All assay plates were made in duplicate, and incubated at 37°C for 16-18 hours. By this method, the number of plaques that developed on each plate represented the number of plaque-forming particles per 0.1ml of diluted lysate tested.

4. Preparation of unlabelled phage lysates

(a) PBS 1

Procedure:

A fresh culture of B. subtilis 19E grown in Penassay broth at 37°C, with aeration, for 5-6 hours, was diluted 20 times in Penassay broth, and distributed into 2500ml flasks in 200ml aliquots. The diluted culture contained approximately 2.5×10^7 cells per ml. Each aliquot of diluted culture was infected with stock PBS 1 lysate at a multiplicity of infection of 0.1, incubated at 37°C, with aeration, for 1 hour, then left at room temperature for 16-18 hours. The crude lysate was stored at 4°C.

(b) T₂

The procedure described for the preparation of stock T₂ lysate was followed, with the modification that after addition of chloroform, the lysate was not centrifuged and millipore filtered, but stored in its crude form at 4°C.

5. Preparation of labelled phage lysates

(a) Preparation of ³H-labelled PBS 1

Since PBS 1 DNA contains uracil in place of thymine, the DNA was labelled with ^3H -uridine.

The host strain used in the preparation was a pyrimidine⁻ B. subtilis mutant, A26, which was grown on a minimal uracil supplement until the point of infection, when ^3H -uridine was added and readily incorporated into the multiplying phages.

Procedure:

B. subtilis A26 was grown in nutrient broth supplemented with uracil (5 $\mu\text{g}/\text{ml}$) at 37°C, with aeration, for 5-6 hours. A 10ml sample of the culture, containing approximately 5×10^8 cells per ml, was infected with stock PBS 1 lysate at a multiplicity of infection of 0.1, and the volume made up to 20ml with nutrient broth. At this stage 500 μc of ^3H -uridine was added. Incubation at 37°C was continued; for 1 hour, with aeration, and for a subsequent 16-18 hours without aeration. The crude lysate was stored at 4°C.

(b) Preparation of doubly labelled PBS 1

The presence of thymine in B. subtilis DNA and its absence in PBS 1 DNA made it possible to differentiate PBS 1 transducing particles from active, non-transducing PBS 1 phage using ^3H -thymidine as label. The active PBS 1 particles were labelled with ^{32}P -inorganic phosphate.

The host strain used in the lysate preparation was a thymine⁻ B. subtilis mutant, 168T, which was grown on a minimal

thymine supplement, and readily incorporated ^3H -thymidine added during growth. Removal of the ^3H -labelled host cells to a non-radioactive medium, and subsequent infection with PBS 1, ensured that resultant ^3H -labelled PBS 1 was transducing in nature. Label added in the form of ^{32}P -inorganic phosphate ($^{32}\text{PO}_4^-$) at the point of infection of PBS 1 was incorporated by active particles only.

Procedure:

B. subtilis 168T was grown in $\frac{1}{2}$ minimal medium supplemented with Casamino acids, tryptophan, and thymine ($56\mu\text{g}/\text{ml}$) at 37°C , with aeration, for 2 hours. The culture was diluted 5 times in the same medium. To 20ml of diluted culture was added $220\mu\text{c}$ of ^3H -thymidine. Incubation was continued at 37°C , with aeration, for 4-5 hours. The culture was centrifuged at 6,000 r.p.m. for 10 minutes, and the resultant pellet of cells resuspended in 5ml of tris medium supplemented with thymine ($100\mu\text{g}/\text{ml}$) to give approximately 2×10^9 cells per ml. The cells were infected with PBS 1 stock lysate (prepared in tris medium) at a multiplicity of infection of 2.0, and the volume of the infected culture was made up to 20ml with tris medium. At this stage $25\mu\text{c}$ of $^{32}\text{PO}_4^-$ was added. After 2 hours' incubation at 37°C , with aeration, 0.3ml of chloroform was added to complete the process of lysis. The crude lysate was stored at 4°C .

(c) Preparation of ^{32}P -labelled T_2

The method used for the labelling of bacteriophages $\phi 80$ and $\phi 80\text{pt}_1$ (Yamagishi, Yoshizako and Sato, 1966) was used in this work for labelling T_2 . Tris-glucose-Casamino acids medium in which T_2 was propagated is low in phosphorus content. Label added in the form of ^{32}P -inorganic phosphate at the point of infection of T_2 was readily incorporated into the DNA of the proliferating phages.

Procedure:

The procedure described for the preparation of stock T_2 lysate was followed, with the following modifications:

- (i) When T_2 phage was added to the early logarithmic phase culture of *E. coli* B, 60-100 μc of $^{32}\text{PO}_4^-$ was also added to each 25ml sample of culture.
- (ii) After addition of chloroform, the labelled lysate was not centrifuged and millipore filtered, but stored in its crude form at 4°C .

6. Purification of phages

(a) Preliminary purification by differential centrifugation

Phage particles were subjected to preliminary purification using a modification of the differential centrifugation method used by Takahashi and Marmur (1963b) for PBS 2.

Procedure:

All centrifugation was done on a Sorvall RC-2 centrifuge at 2°C. Lysates were first centrifuged at 6,000 r.p.m. for 10 minutes, to sediment bacteria and bacterial debris. The supernatant liquid was then centrifuged at 17,000 r.p.m. for 1 hour to collect the phage particles. The resultant pellets of phage particles were resuspended in 1ml of either SSC (used at the beginning of the work) or suspending medium (used later on in the work in an attempt to afford the phages more protection). Resuspension was brought about as rapidly as possible without employing violent motion. With constant, gentle shaking, the process took 1-2 hours.

(b) Purification by caesium chloride density gradient centrifugation

The caesium chloride density gradient technique was proposed for the separation of macromolecules by Meselson, Stahl and Vinograd (1957). Later, the technique was extended to use with phage particles (Weigle, Meselson and Paigen, 1959).

The method involves the production of a stable concentration gradient of caesium chloride in solution by centrifuging an original solution at high speed until the opposing tendencies of sedimentation and diffusion have reached equilibrium. In the direction of the centrifugal force there then exists a gradual density gradient, the result of both the concentration gradient and compression of

the liquid. Small particles, introduced into a density gradient whose range of density includes the density of the particulate material, are driven to the region where the sum of the forces acting on a given particle is zero. The effective density of the particulate material is defined by Meselson, Stahl and Vinograd (1957) as the density of the solution in that region. The particles form a band at right angles to the line of the gradient. In the case of phage particles of a particular density species, this is a very narrow band since the particle weight is relatively high and the only force opposing the banding of particles at equilibrium is that of Brownian motion. Thus any lack of homogeneity amongst phages in a caesium chloride density gradient is readily detected, and a particular band of relatively 'pure' phage can be isolated by this technique.

In order to purify suspensions of PBS 1 and T₂, the final concentration of caesium chloride solutions used for the gradients had to be such that the densities of the solutions were equivalent to the densities of the respective phages. The necessary measurement of density was greatly facilitated by use of the equation given by Weigle, Meselson and Paigen (1959), which expresses a linear relationship between density and refractive index, the latter of which can easily be determined with accuracy using a refractometer.

$$\rho^{20^{\circ}\text{C}} = 10.860 \eta_{\text{D}}^{25^{\circ}\text{C}} - 13.500$$

where ρ = density

η_{D} = refractive index

Procedure:

The refractive index of stock caesium chloride solution was measured and found to be 1.419, corresponding to a density of 1.903g cm^{-3} . A mixture of 0.9ml stock caesium chloride solution and 1.1ml SSC, having a refractive index of 1.374, and a corresponding density of 1.422g cm^{-3} , was found suitable for the centrifugation of PBS 1. Similarly, a mixture of 1.1ml stock caesium chloride solution and 0.9ml SSC, having a refractive index of 1.383, and a corresponding density of 1.500g cm^{-3} , was found suitable for the centrifugation of T_2 . The proportion of the respective phage suspensions in the SSC was varied from experiment to experiment, according to the concentration of the phage suspension being used, or, in the case of radioactive suspensions, according to the specific activity.

Mixtures were made up in 13 x 50 mm nitrocellulose ultracentrifuge tubes. The components of each tube were blended thoroughly and covered with mineral oil to the point where the tube was almost full. Tubes to be centrifuged in the same rotor were carefully balanced by the addition or subtraction of mineral oil. Where there were only one or two experimental tubes, additional 'dummy' tubes were prepared and similarly balanced so that all three buckets of the SW50 rotor would be occupied. Centrifugation was at 30,000 r.p.m. for 20 hours in a Beckman L-2 65 ultracentrifuge kept at 20°C.

Following centrifugation the nitrocellulose tubes were punctured at the base using a fine pin, and the contents were fractionated (original technique of Weigle, Meselson and Paigen, 1959).

Using the apparatus shown in Figure I, 3-drop or 4-drop fractions were collected in 10 x 75 mm test tubes containing 1ml of SSC.

The unit for regulating the drop sizes to a uniform volume was used only if quantitative results were required. Without the use of the drop size-regulation unit, each 2ml caesium chloride sample gave rise to approximately 75 drops, whereas with its use, each 2ml caesium chloride sample gave rise to approximately 110 drops.

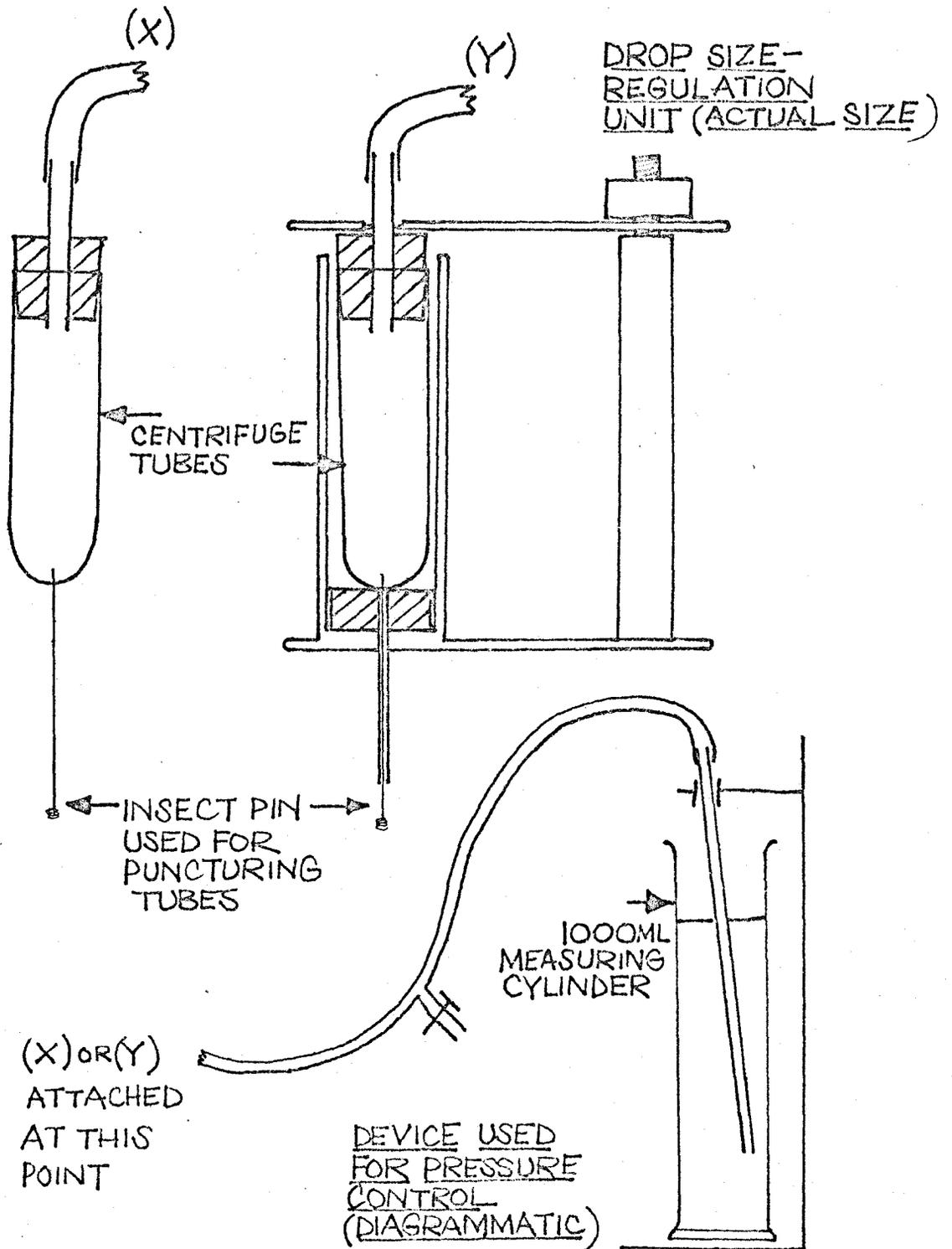
7. Measurement of optical density

The optical density (O.D.) values of caesium chloride density gradient fractions were measured in order to determine, quantitatively, the distribution of the suspended phage particles they contained. Phages consist of a DNA 'chromosome' and a surrounding protein coat. Deoxyribonucleic acid absorption is maximum at 260m μ ; protein absorption (which depends mainly on the content of aromatic amino acid residues) is maximum at 280m μ . On a weight basis, however, the peak absorption of protein is only $\frac{1}{10}$ or less that of DNA, so that for a combination of the two, as found in phage particles, the absorption contribution of DNA dominates the collective absorption curve. Thus O.D. values of fractions were measured at a wavelength of 260m μ , on a Beckman DU spectrophotometer. All measurements were made against an SSC blank.

8. Measurement of transducing activity

The basic method followed for measuring transducing activity

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was that proposed by Takahashi (1963) for estimating numbers of prototrophic transductants using the PBS 1 - B. subtilis system. A methionine⁻ B. subtilis mutant, A7, was used as recipient.

Procedure:

Recipient cells of B. subtilis A7 were grown in Penassay broth at 37°C, with aeration, for 4 hours. Transduction was carried out by mixing 0.9ml of recipient bacterial culture (approximately 5×10^8 cells) with 0.1ml of respective phage samples in 50ml flasks, and incubating the mixtures with aeration, at 37°C. After 30 minutes, 0.1ml samples of the mixtures were spread on to minimal agar plates, and the plates incubated at 37°C. As control, 0.1ml of untreated culture was spread on to a minimal agar plate and similarly incubated. Duplicate plates were made for each phage sample used, and also for the control. Incubation was continued for two days. Control plates were checked for the absence of bacterial colonies. Colonies (derived from prototrophic transductants) that were present on the experimental plates were carefully counted. Transducing activity was expressed as the number of transductants per ml of sample tested.

9. Assay of radioactivity

In instances where particular fractions of phage particles, from caesium chloride density gradients, were required for DNA

extraction and molecular weight determinations, the phages put in the gradients were specially labelled, and the desired fractions identified by their characteristic radioactivity.

Procedure:

A pasteur pipette, of which the ultimate 1cm was marked off, was used for sampling a standard volume of each fraction in a series. Samples were transferred to Schleicher and Schuell number 589 (20mm) filter paper discs, held in planchets in trays of 20. The filter discs had previously been marked with identifying symbols (in pencil) and the corresponding planchets marked similarly (in wax crayon). Filter discs were dried under an infra-red lamp, then washed: twice in 5% trichloroacetic acid, for 15 minutes each washing, and lastly in 95% ethanol, for 15 minutes. Washings were carried out in 250ml beakers kept in an ice bath (Tomizawa and Anraku, 1964). Filter discs were replaced in their respective planchets and again dried under the infra-red lamp. They were then placed in glass counting vials containing the fluors 1, 4,-bis-2(5-phenyloxazolyl)-benzene and 2,5-diphenyloxazole in toluene. The radioactivity of each sample, in c.p.m., was measured in a Nuclear-Chicago Series 725 scintillation counter.

10. Extraction of DNA molecules

The agent used to release DNA from phage particles was phenol. Phenol brings about the denaturation of protein and

dissolves away the coat of a phage particle very effectively, leaving the central DNA core free. Phenol was used by Gierer and Schramm (1956) for tobacco mosaic virus DNA extraction, and later by Mandell and Hershey (1960) who reported its use in the extraction of T_2 and T_4 DNAs, and enlarged on the extraction procedure. The procedure followed in the present work differed from that described by Mandell and Hershey in several ways. In contrast to Mandell's and Hershey's suspension of phage particles in saline phosphate buffer, caesium chloride density gradient fractions were used for the DNA extraction, and thus the phage was effectively suspended in a caesium chloride solution - SSC - (suspending medium) mixture. In the present work, a detergent and a chelating agent, both for the suppression of DNAase activity, were added to the phage suspension prior to the addition of phenol. The phenol used was SSC-saturated, not water-saturated. However, the major difference was that the mechanical shaking of the extraction mixtures described by Mandell and Hershey was discarded in favour of the more gentle roller tube method of Frankel (1963), in which the extraction mixtures, in tubes, are attached to a small wheel that rotates them about their long axis.

Note: Tests were made to determine the effect of caesium chloride on DNA extraction. It was found that there is a relationship of inverse proportionality between the amount of caesium chloride present during extraction, and the amount of DNA extracted. However, dialysis of caesium chloride density gradient fractions

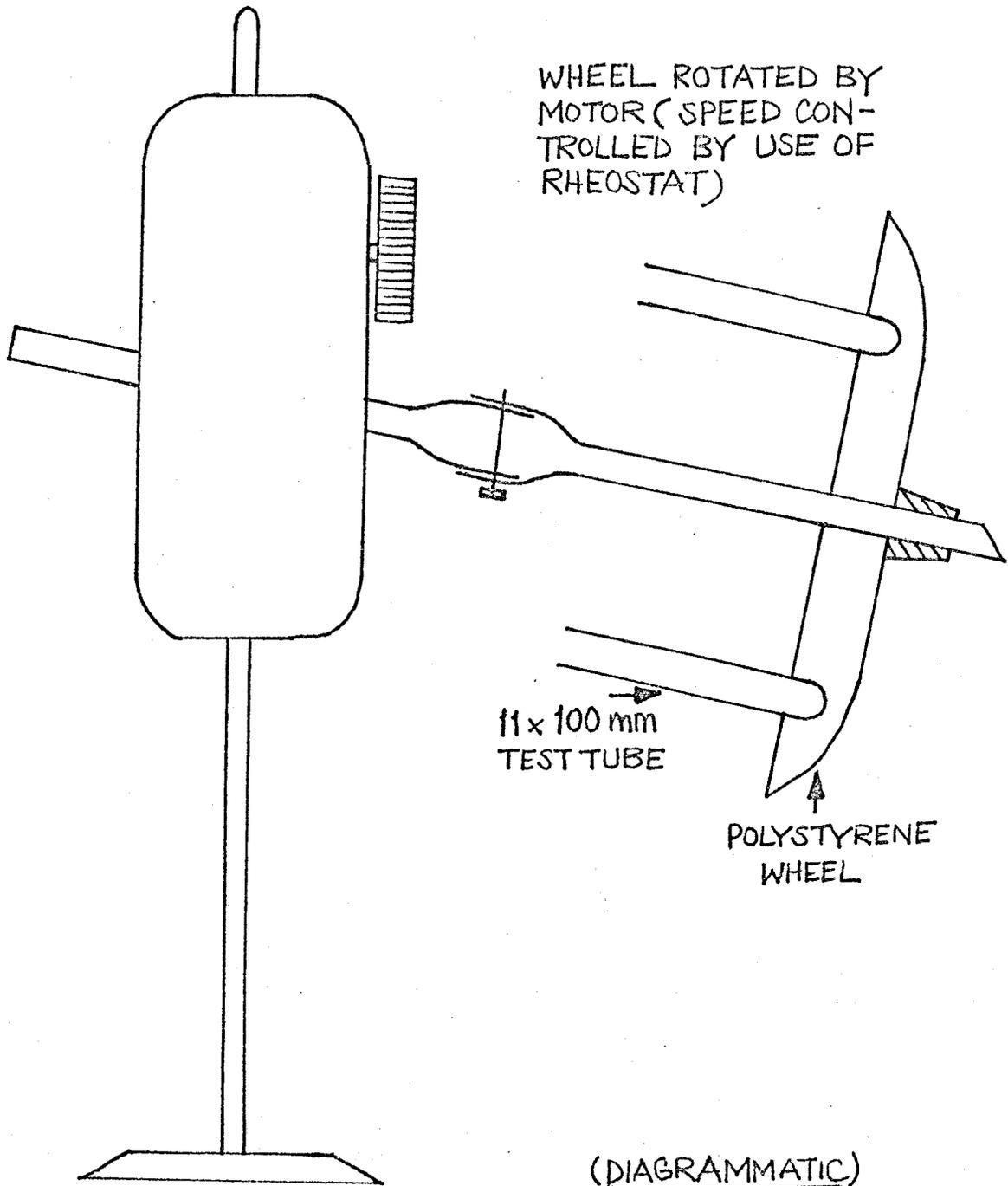
against SSC to remove caesium chloride, appeared to cause a drastic reduction in the amount of intact DNA molecules subsequently extracted, even though the overall yield of DNA was high. Possibly dialysis weakened the phage particles to a certain extent, rendering them highly vulnerable and open to mechanical damage. The dialysis process was discarded, finally, reduced efficiency of DNA extraction being regarded as more desirable than increased DNA damage.

Procedure:

To 1ml of caesium chloride density gradient fraction in an 11 x 100 mm test tube, were added 0.04ml of 5% sodium lauryl sulphate, to give a final concentration of 0.2%, and 0.02ml of 0.1M ethylenediamine tetracetate (EDTA), to give a final concentration of 2×10^{-3} M. One ml of phenol, freshly saturated with SSC, was also added. Tubes were carefully inserted into holes cut in the polystyrene wheel of the roller tube apparatus (Figure II) which was adjusted such that the tubes were at an angle of approximately 10° from the horizontal. The wheel was rotated at a speed of 50-60 r.p.m.. After 45 minutes the tubes were removed and centrifuged briefly at 2000 r.p.m. to separate out the phenol and water layers.

Note: The next step, the isolation of the upper aqueous layer containing the DNA, and its dialysis against SSC for the necessary removal of dissolved phenol, was found to be highly critical. From preliminary work it was found that at this stage DNA molecules were subject to shearing forces that caused them to break. Sources of

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such shearing forces were looked for, with a view to remedying them as far as possible. Two processes were found to be causative of DNA shear, and each was modified until successful results were achieved. The finer points of technique finally employed for this stage of the work were thus considerably different from those employed at the start.

The first process that was questioned was that of the physical removal of the DNA in solution from extraction tube to dialysis tubing, and its subsequent removal from dialysis tubing to a fresh vessel. These transfers were at that time being carried out by pipetting, using disposable 1ml pipettes having a 3mm bore, narrowing at the tip to 2mm. Pipettes were operated by screw delivery. It was realised that such pipetting was likely to impose considerable hydrodynamic shear upon the DNA (Davison, 1959).

The second process that was put to question was that of the dialysis itself. At that time, dialysing solutions were secured in the 5mm dialysis tubing very compactly, and were allowed to move freely in 250ml beakers containing SSC which was circulated by means of a magnetic stirring apparatus. Dialysis was continued for 18-24 hours, and the SSC was changed at least 4 times during this period. Since previous work had shown that phage particles, more resistant by far than DNA molecules, could be adversely affected by dialysis, it seemed highly likely that the dialysis process was a potential source of shear degradation. In addition to focussing attention on the technical details of pipetting and dialysis, it was also noted that there is a critical concentration of DNA below which

hydrodynamic shear is very great, and above which molecules exert a protective influence upon each other (Hershey and Burgi, 1960). Solutions of DNA used in the present work were often, at that time, of concentrations well below the critical value quoted by Hershey and Burgi for T₂ DNA (4μg/ml). This was a result of low concentrations of phage particles in the caesium chloride density gradient fractions used for extraction, and occurred especially with ³H-labelled PBS 1 DNA since the yield of ³H-labelled phages was always poor.

The modified procedure to which the foregoing considerations gave rise is given below.

The upper aqueous layer containing the DNA was removed by pipetting, using the same disposable 1ml pipette with a 3mm bore as was used previously, but this time with the narrowed tip cut off cleanly (Rubenstein, Thomas and Hershey, 1961). The pipette was operated by screw delivery, making sure that movement of liquid up the pipette was smooth and very slow (approximate rate of 0.5ml per minute). The DNA solution was pipetted out slowly into prepared 5mm dialysis tubing with similar smoothness. The open end of the dialysis tubing containing the DNA solution was tied some distance above the solution's upper meniscus, in order that the solution might be under no unnecessary pressure. This method of securing the dialysis tubing served two further purposes. It enabled the tubing to be fixed in one position during dialysis, thus preventing buffetting due to the circulation of the SSC solution induced by

use of a magnetic stirrer; it also facilitated transfer of the contents at the end of the dialysis period. Dialysis was carried out in a 500ml flask; the dialysis tubing was kept in a fixed position by tying it to a glass rod laid over the narrow neck of the flask. Suspended in such a way, the dialysis tubing tended to lie against the wall of the flask and was undisturbed by currents in the SSC. The SSC was changed at least 4 times over a period of 18-24 hours. After dialysis, the DNA solution was not transferred by pipetting. Rather, the end of the dialysis tubing not occupied by DNA solution was cut and the solution slowly run into a 10 x 75 mm test tube using a small glass funnel to prevent dripping. Phenol-free DNA solutions were kept at 4°C.

To avoid the detrimental effect of processing low concentrations of DNA, caesium chloride density gradient fractions with a low particle content were supplemented with 'carrier' phages prior to DNA extraction. 'Carrier' phages used were non-radioactive - their sole function was to raise the DNA concentration. 'Carrier' bacteriophages are analogous to the 'carrier' DNA of Rubenstein, Thomas and Hershey (1961) and Davison, Freifelder, Hede and Levinthal (1961).

11. Measurement of the molecular weight of DNA by zone centrifugation in sucrose density gradient

The molecular weight of DNA was determined from its sedimentation rate, according to the method of Burgi and Hershey (1963). The method involves zone centrifugation of a reference DNA of known molecular weight, together with the DNA whose molecular weight is to be determined, each at low concentration, in a linear sucrose density gradient. The unknown molecular weight can be measured in terms of the known molecular weight from the ratio of distances sedimented, as expressed by the equation:

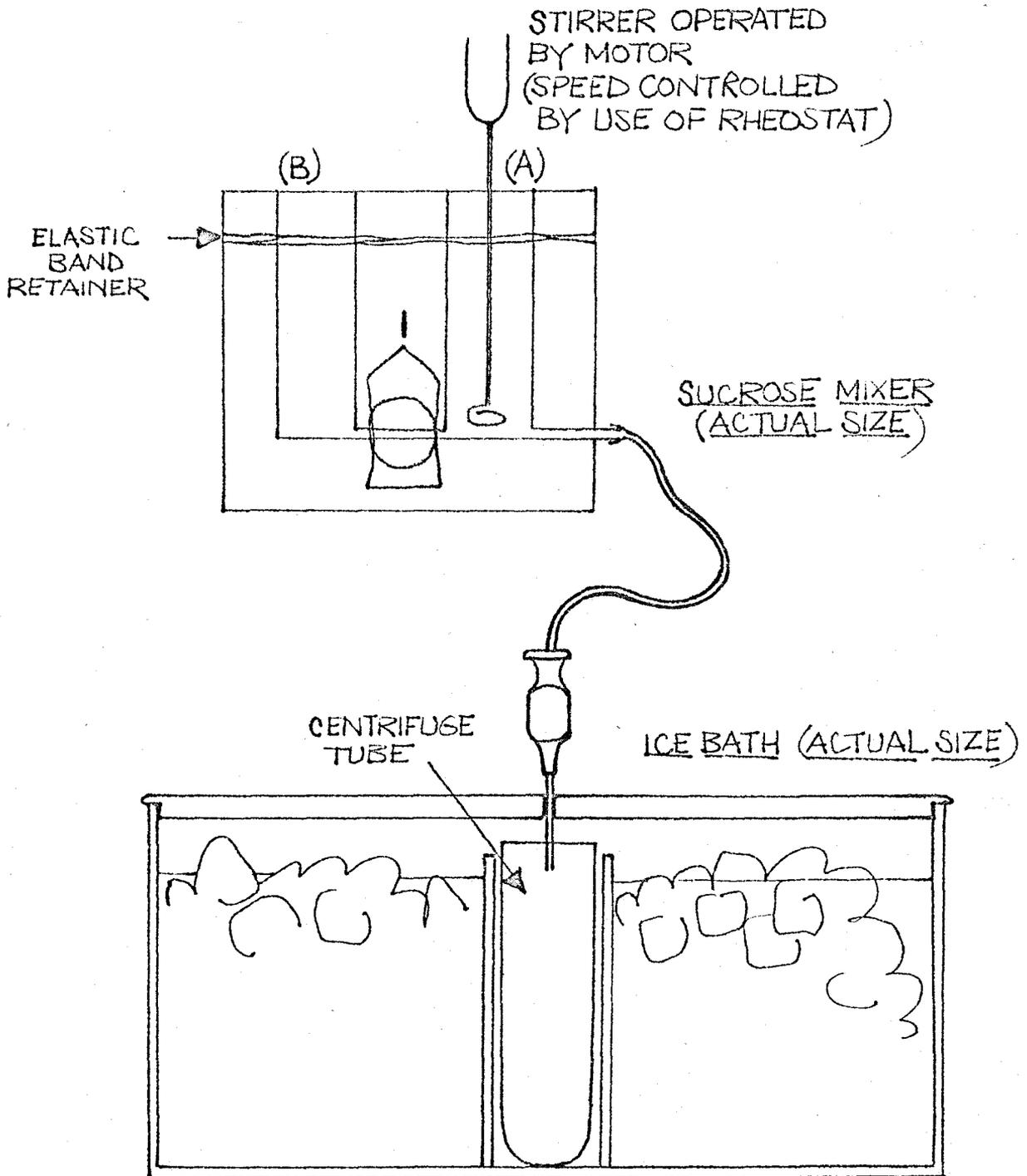
$$D_2 / D_1 = (M_2 / M_1)^{0.35}$$

where D = distance sedimented

M = molecular weight

Linear sucrose density gradients of 5-20% sucrose were used, after the method developed by Britten and Roberts (1960) for sedimenting and separating ribosomes of E. coli. The mixing device used for the preparation of the gradients was similar to the one described by Britten and Roberts - a modified version of the mixing system of Bock and Ling (1954). The mixer and its necessary accompaniments, a stirring apparatus, and an ice bath for the reception of the sucrose by ultracentrifuge tubes, are represented in Figure III. The mixer contained two chambers of equal volume, connected by a narrow channel. One chamber (B) contained the 5% sucrose; the other (A) contained the 20% sucrose. It was from the latter chamber that sucrose was drawn out via exit tubing. During the manufacture of a gradient, the exit

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tubing was turned down and fitted into one of the slots in the lid of the ice bath, below which the ultracentrifuge tubes were positioned. The ice bath was precisely constructed such that when the exit tubing was so fixed, its tip rested along the inner surface of the ultracentrifuge tube below. Sucrose thus ran down the exit tubing and into the ultracentrifuge tube without any appreciable disturbance.

A concentration gradient (and therefore a density gradient) was formed in the ultracentrifuge tubes as they filled up, due to the constant equilibration of the two sucrose columns and the rapid mixing of the contents of chamber A as sucrose was removed. Such a gradient is stable for many hours (Britten and Roberts, 1960). Stability was ensured by use of an ice bath, not only for preparing gradients, but also for storing them when they were not used immediately.

The molecular weight of PBS 1 DNA was estimated using T_2 DNA as reference. The molecular weight of T_2 DNA is reported to be 1.3×10^8 (Rubenstein, Thomas and Hershey, 1961).

Procedure:

With the stop-cock of the mixer at the closed position, chamber A was filled with 2.4ml 20% (w/v) sucrose in SSC, and chamber B with 2.5ml 5% (w/v) sucrose in SSC. After starting the mixing motor and regulating the stirring speed to approximately 150 r.p.m., the stop-cock was moved to the open position, and light pressure

applied to chamber B using the tip of the finger. In this way, the air in the connecting chamber was expelled, leaving the columns of sucrose free to achieve equilibrium without resistance. The exit tubing was then moved from its position in the elastic band retainer at the top of the mixer to the operating position in the ice bath lid. Gradients were collected in 13 x 50mm nitrocellulose ultracentrifuge tubes. Samples of both the reference DNA solution (T_2) and the experimental DNA solution (PBS 1), were layered on to the top of a sucrose gradient immediately prior to centrifuging. Solutions of DNA were transferred using disposable 1ml pipettes with cut-off tips, operated by slow screw delivery. The combined volume of the DNA solutions added was never allowed to exceed 0.2ml (Burgi and Hershey, 1963) and their combined DNA content never allowed to exceed 2 μ g in weight (Tomizawa and Anraku, 1964). In addition, since the T_2 DNA solution (32 P-labelled) tended to have a higher specific activity as well as a higher concentration than the PBS 1 DNA solution (3 H-labelled), it was diluted so that the volume added would represent approximately the same radioactivity in c.p.m. as would be represented by the PBS 1 DNA solution added. Thus the determination of both the concentration and the specific activity of DNA in a particular solution were necessary preliminaries to DNA addition. Concentrations were determined by measuring O.D. values of $\frac{1}{10}$ dilutions of DNA solutions against an SSC blank on a Beckman DU spectrophotometer at wavelength 260m μ , then applying the following relationship: O.D. of 0.2 (260m μ) for a $\frac{1}{10}$ dilution represents 100 μ g of DNA per ml of original solution. It was for this procedure that it was necessary to dialyse

away phenol from DNA extracts - phenol's absorption maximum is close to that of DNA. Radioactivities were determined by subjecting 0.02ml samples of DNA solutions to the procedure given earlier.

Once DNA solutions had been added to the sucrose gradients the tubes were centrifuged at 30,000 r.p.m. for 3 hours in a Beckman L-2 65 ultracentrifuge kept at 10°C. Tubes centrifuged in the same rotor could not be balanced individually. However, where there were only 1 or 2 experimental tubes, additional 'dummy' tubes were prepared so that all three buckets of the SW50 rotor were occupied. Following centrifugation, the nitrocellulose tubes were punctured and fractionated, using the apparatus shown in Figure I. The drop size-regulation unit was always included. Eight-drop fractions were collected directly on to the filter discs used in scintillation counting. Each 5ml sucrose sample gave rise to approximately 280 drops; thus fractions usually numbered 35. The radioactivities of the fractions were determined as described earlier. Counting was repeated 3 times and counts for each sample averaged.

The radioactivity of fractions was not, however, expressed as simple c.p.m., but as the recovery of total input of radioactivity. In order to calculate this value the sum c.p.m. for a range of fractions was totalled, then the c.p.m. value for each fraction within the range expressed as a fraction of the total. The range of fractions was so chosen that all relevant and important fractions were included, while fractions from the top and bottom of the tube excluded. The latter always contained a high proportion of radioactivity as a result of physical phenomena other than simple sedimentation. The range extended from approximately fraction number 6 to approximately fraction

number 30.

The positions in the sucrose gradient reached by the DNAs were well defined. "Owing to the compact bands of sedimenting DNA.....the distances sedimented can be measured rather precisely. In a satisfactory run, the bulk of a native bacteriophage DNA is recovered from a zone 2 or 3mm wide" (Burgi and Hershey, 1963). Peaks could be placed to an accuracy of one whole fraction. In order to place the peaks even more precisely, probability curves were plotted using the recovery of total input data. Peaks could then be placed to an accuracy of 0.1 fraction.

The distance travelled by a DNA in the sucrose gradient was expressed in fraction units to the nearest 0.1 fraction. The starting position was taken as the top of the sucrose gradient (not the top of the DNA solution added) and the finishing position as the point of peak radioactivity. Thus the following equation was applied:

$$D = \left(a \times \frac{4.9}{4.9 + b} \right) - c$$

where D = distance sedimented in fractions.

a = total number of fractions in the sucrose density gradient
i.e. distance (in fractions) from top to bottom of the
gradient.

b = volume of DNA solution applied in ml.

c = position (in fractions) of peak radioactivity i.e.

distance from the peak to the bottom of the gradient.

12. Aseptic technique

Standard aseptic technique was employed during the preparation of all stock bacterial cultures and bacteriophage lysates. Stocks were stored under aseptic conditions. Aseptic technique was also employed during the preparation of lysates to be used directly in experimental work. In the subsequent treatment of bacteriophage particles, however, the preservation of absolutely aseptic conditions was impossible. For example, the nitrocellulose tubes used in caesium chloride density gradient centrifugation could not be conveniently sterilised, and were used in the unsterile condition. Thus when caesium chloride density gradient fractions were subjected to transducing activity measurements, particular attention was paid to the control plates on which colonies would be found if contaminants had been introduced. On the other hand, when caesium chloride density gradient fractions were required for their content of labelled bacteriophage particles, contaminants (non-radioactive) obviously made no difference to the final results. In such cases there was no need to employ an aseptic technique at all; nevertheless, where possible, materials and glassware were sterilised by autoclaving, as a safeguard against DNAase activity.

13. The time factor

In the course of the work it was found that extracted DNA molecules, especially those of PBS 1, were so fragile that they were not stable when stored in solution at 4°C. This phenomenon was

obviously related to the difficulties encountered during the extraction procedure. All procedures subsequent to the preparation of labelled lysates were, therefore, performed as quickly and continuously as possible. With practice, the labelling of lysates, purification of particles, DNA extraction, and the measurement of molecular weight by sucrose density gradient centrifugation, could all be completed within 4 days.

CHAPTER THREE

PRELIMINARY SEPARATION OF ACTIVE AND TRANSDUCING PARTICLES OF PBS 1

A previously published report (Takahashi, 1963) indicates that the PBS 1 lysate is a heterogeneous mixture of phage particles which, because of their differing densities, separate out readily in caesium chloride density gradients. One density species was reported to be solely transducing, having no plaque-forming activity. In reviewing the above results, it was thought likely that by using the caesium chloride density gradient technique, active, plaque-forming PBS 1 and transducing PBS 1 could be obtained relatively uncontaminated with each other. If such separation were possible, it would not only be the preliminary to determining the molecular size of DNA from active PBS 1 particles unambiguously, but would also be the first step towards a comparison of such DNA with the DNA of transducing particles.

The following experiment was conducted in order to verify the results obtained by Takahashi, and also to characterize the separated components of PBS 1 lysate according to plaque-forming and transducing activities.

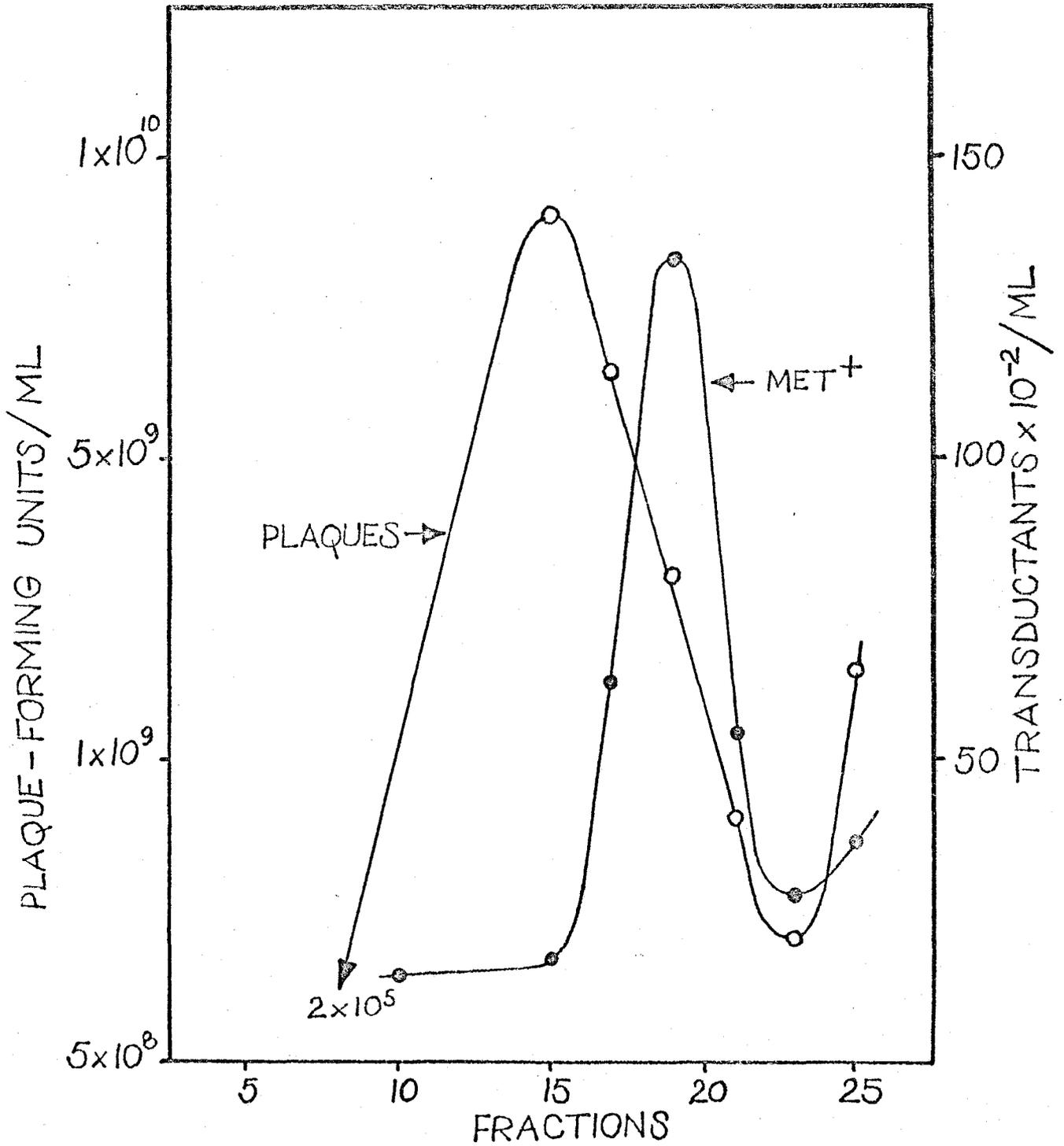
Stock PBS 1 was propagated on strain SB19E in 500ml of Penassay broth. The resultant lysate was subjected to differential centrifugation in order to remove bacterial cells and cell debris, and to collect the phage particles. The phage particles were resuspended in suspending

medium. A sample of the phage suspension was centrifuged in a caesium chloride density gradient. After centrifugation was completed, the centrifuge tube was punctured and 4-drop fractions were collected in 1ml aliquots of suspending medium. Optical density measurements and assays of both plaque-forming particles and transducing activities were made on the fractions. Strain A7 (methionine⁻) was used as recipient in the transduction experiments. Results are given in Figure IV.

From the results it is clear that PBS 1 lysate is, indeed, a heterogeneous mixture of particles. The peak of plaque-forming activity is separated by three fractions from the peak of transducing activity, the latter occurring in a region of lower density at a higher level in the centrifuge tube. The O.D. peak (not shown in Fig. IV) coincides with the plaque-forming activity peak, indicating that the plaque-forming particles constitute the majority of the phage particles.

The separation that is obtained is amply adequate for the purpose of isolating the respective plaque-forming and transducing peak fractions. This fact is put to use in the following experiments, in which purified active PBS 1 and purified transducing PBS 1 are respectively selected for investigations of their DNA.

PLAQUE-FORMING AND TRANSDUCING ACTIVITIES OF PBS 1 PARTICLES AFTER CENTRIFUGATION IN A CAESIUM CHLORIDE DENSITY GRADIENT



CHAPTER FOUR

THE DNA OF ACTIVE PBS 1 PARTICLES

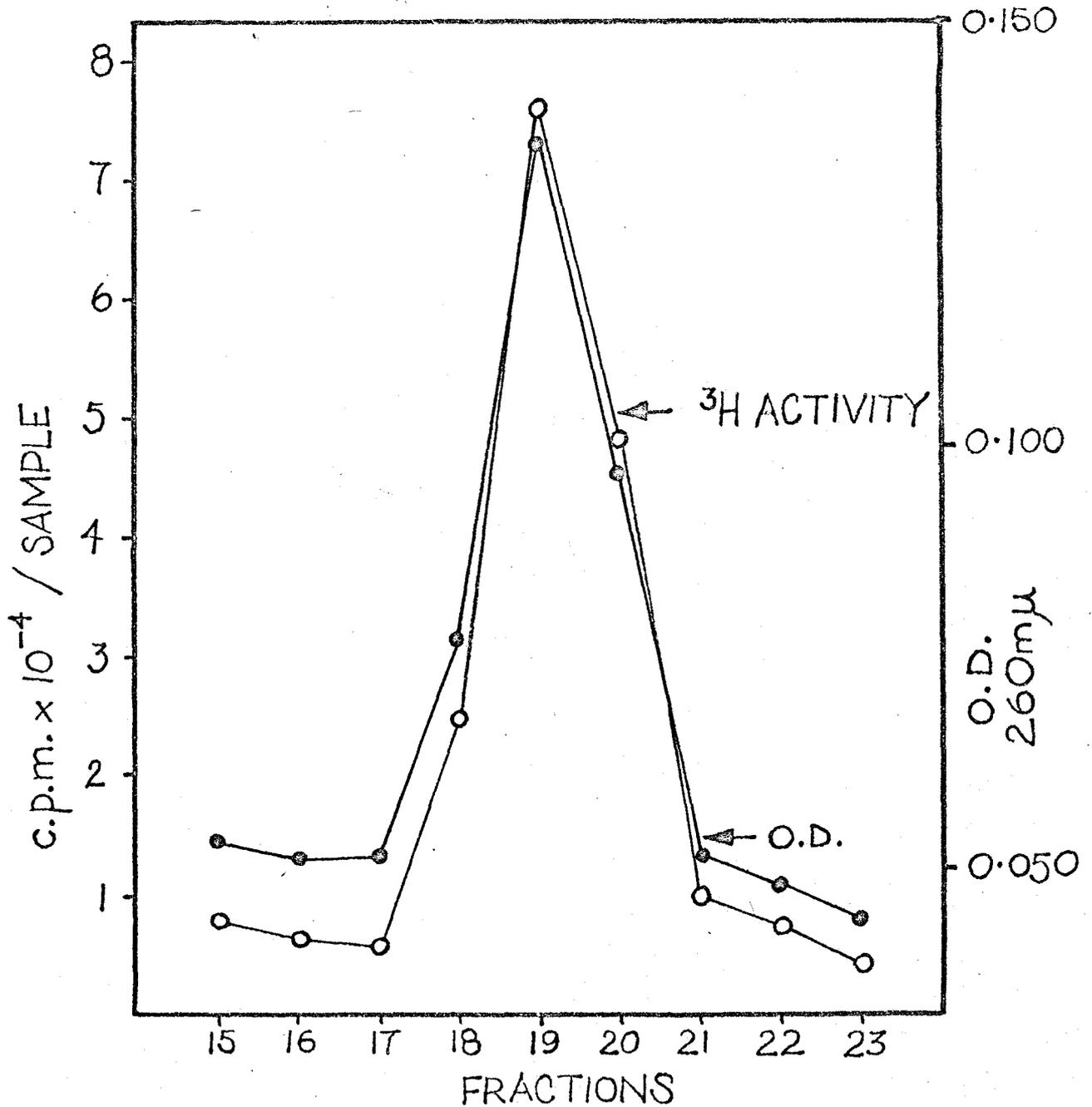
The major intention of the work reported in this thesis - the determination of the molecular size of the DNA of active PBS 1 - was fulfilled by making measurements of molecular weight according to the method of Burgi and Hershey (1963), using DNA extracted from purified active PBS 1 particles, together with T_2 DNA as reference. The basic experimental procedure was repeated a number of times for confirmation of results.

For each experiment a 3H -labelled PBS 1 lysate and a ^{32}P -labelled T_2 lysate were prepared such that the final volumes were 20ml and 25ml respectively. Both lysates were subjected to differential centrifugation for the collection of the phage particles, then the resultant pellets were resuspended in SSC or suspending medium. Samples of the phage suspensions were separately centrifuged in caesium chloride density gradients. After centrifugation was completed, the centrifuge tubes were punctured and 3-drop fractions were collected in 1ml aliquots of SSC. At this stage a number of points were established which gave rise to a simplified procedure.

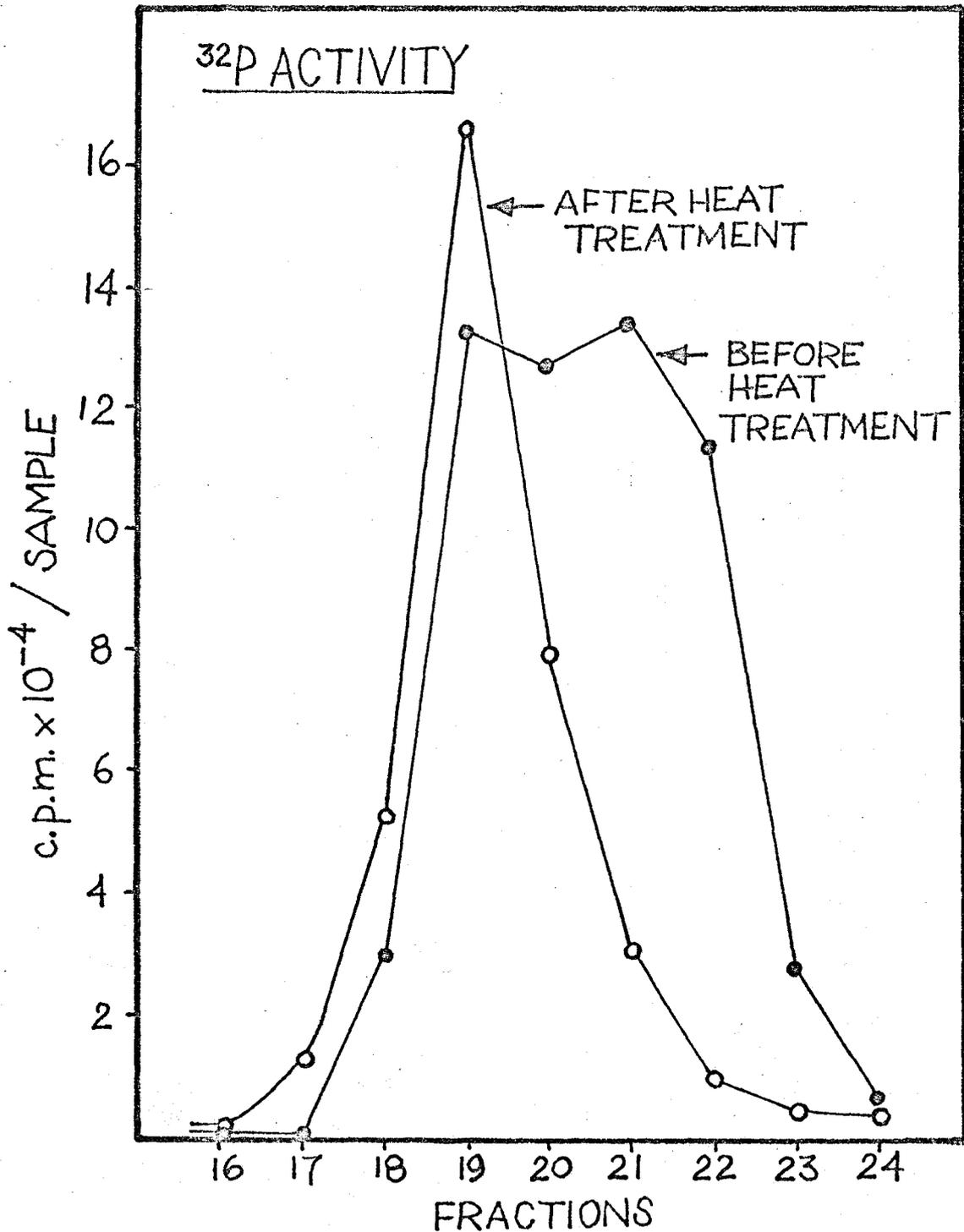
1. Assays of radioactivity and measurements of O.D. made on fractions of the caesium chloride density gradient containing PBS 1, established the fact that the peak of 3H activity and the O.D. peak coincide

- (Fig. V). Thus the fraction at the peak of ^3H activity was known to be the fraction of highest plaque-forming activity, containing purified active PBS 1 particles; as such, it was taken for DNA extraction.
2. Assays of radioactivity made on fractions of the caesium chloride density gradient containing T_2 , revealed that there was a double peak of ^{32}P activity. On investigation it was found that if the phage-caesium chloride suspension was heat treated at 45°C for 30 minutes prior to centrifugation (Tomizawa and Anraku, 1964), a single ^{32}P activity peak was subsequently formed (Fig. VI). This indicated that the T_2 lysate contained a proportion of particles that were resistant to the penetration of caesium chloride at room temperature. These particles were able to absorb caesium chloride fully when kept at 45°C for 30 minutes. Since, after heat treatment, T_2 appeared as a homogeneous density species, it was established that purification of T_2 by caesium chloride density gradient centrifugation was not necessary. However, the procedure was continued, heat treatment included, as a check on the homogeneity of the T_2 particles being used. The fraction taken for DNA extraction was the ^{32}P activity peak.
 3. The radioactivity peaks obtained for both PBS 1 and T_2 were always sharply defined. Therefore it was not essential for the gradients to be fractionated in a quantitative manner, as they were initially, and use of the drop size-regulation unit for this procedure was discontinued.

DISTRIBUTION OF RADIOACTIVITY AND O.D.
AFTER CENTRIFUGATION OF ³H-LABELLED
PBS 1 PARTICLES IN A CAESIUM CHLORIDE
DENSITY GRADIENT



EFFECT OF HEAT TREATMENT ON T₂ PARTICLES
IN A CAESIUM CHLORIDE DENSITY GRADIENT



4. Both PBS 1 and T_2 invariably banded in the centre of their respective gradients. Thus it was necessary to assay for radioactivity only those fractions from the middle portion of the tube. Routinely, 15 fractions from the middle of the total range of 25 fractions were assayed for radioactivity.

The selected fractions of PBS 1 and T_2 particles were subjected to DNA extraction. The resultant DNA solutions were dialysed against SSC. Appropriate samples of both PBS 1 and T_2 DNA solutions were layered on to the top of a sucrose density gradient. After centrifugation was completed, the centrifuge tube was punctured and fractions were collected. The radioactivity of fractions was measured directly.

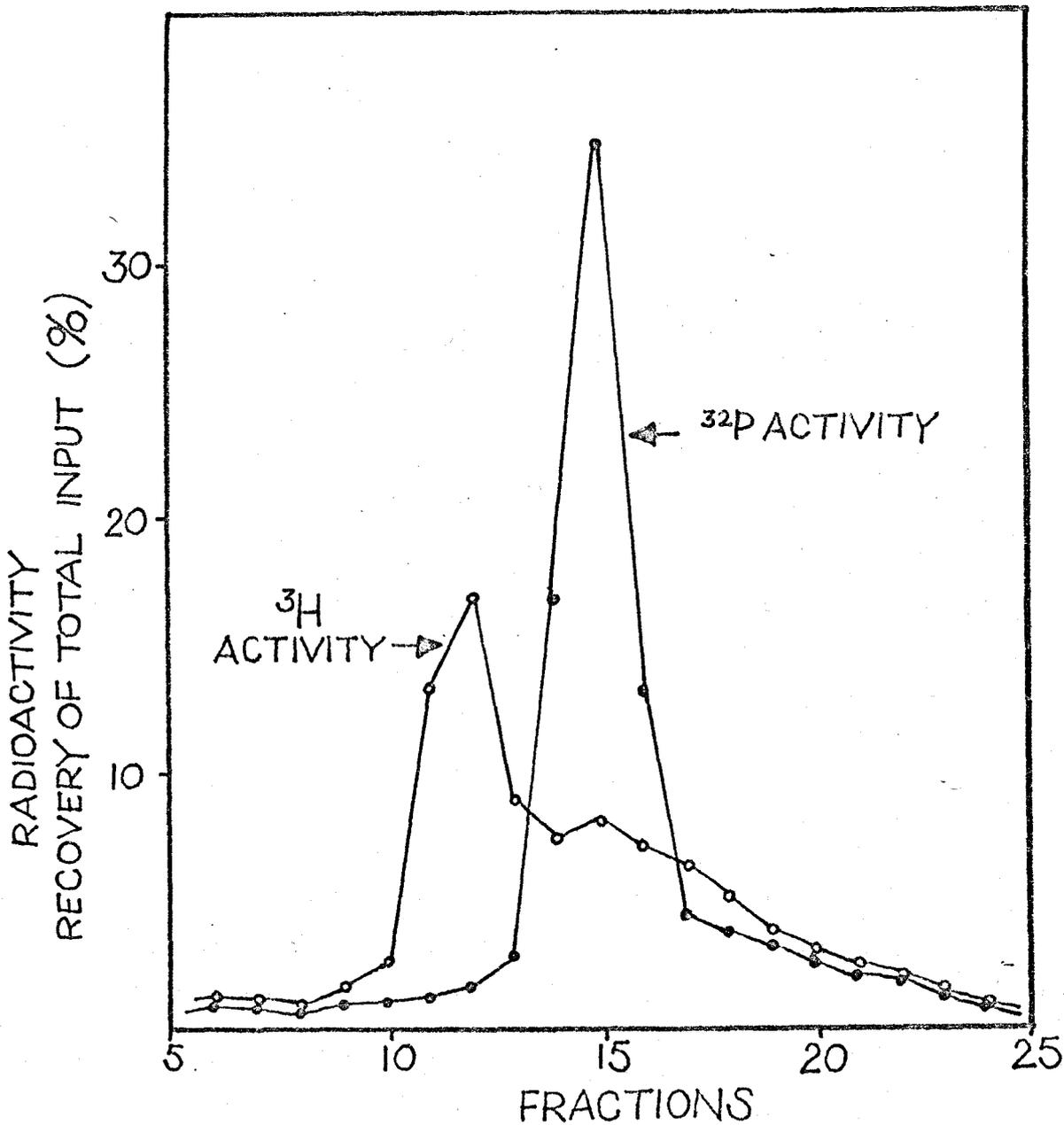
The radioactivity of 3H and ^{32}P in each fraction, expressed as the recovery of total input of radioactivity, was plotted directly against fraction number. From such plots the distributions and peak positions of 3H and ^{32}P activities were readily observable. The sedimentation profile of ^{32}P activity - corresponding to the distribution of T_2 DNA, the reference DNA - was always sharp and symmetrical, and comprised virtually all of the ^{32}P activity. Thus it was known that the T_2 DNA was in the form of unfragmented molecules. On the other hand, in earlier experiments, the sedimentation profile of 3H activity corresponded to a distribution of PBS 1 DNA that was far from normal: a considerable amount of 3H activity was always found in fractions which followed the peak fraction. This indicated that some of the PBS 1 DNA was fragmented during the extraction procedure. Figure VII illustrates a typical result obtained in these earlier experiments. Unfragmented

PBS 1 DNA, as shown by a sharp and symmetrical ^3H activity distribution comprising virtually all of the ^3H activity, was obtained only after the DNA extraction procedure had been modified in a number of ways. Mention has already been made of these modifications. Figure VIII shows the sedimentation profile of ^3H -PBS 1 DNA extracted by the improved technique.

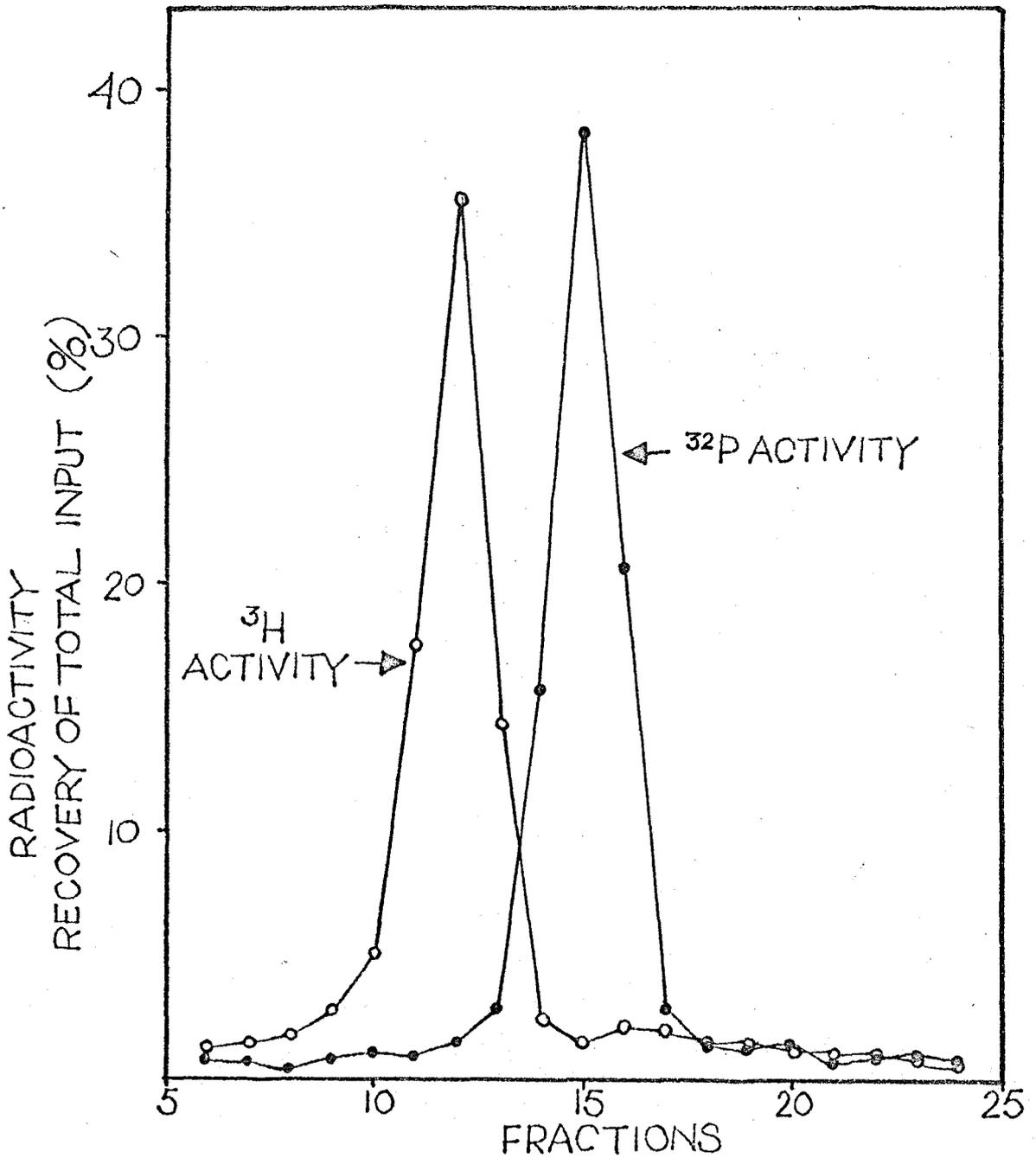
In order to determine the peak positions of both ^3H and ^{32}P activities to an accuracy of 0.1 fraction, the recovery of total input of radioactivity was plotted on probability paper (Fig. IX). The distances travelled by the DNAs in the sucrose gradient were calculated from the respective peak positions, and expressed in fraction units to the nearest 0.1 fraction. Since the starting position of the DNAs was taken as the surface of the sucrose gradient, these calculations involved taking into consideration the volume of DNA solutions added. Together with the known molecular weight value for T_2 DNA, the values for distances sedimented were substituted into the equation of Burgi and Hershey (1963), which was solved for the molecular weight of the unknown - the DNA of active PBS 1. Results are given in Table I.

From the results it is clear that the DNA of active PBS 1 particles has a molecular weight of 1.9×10^8 as determined by the zone sedimentation technique. Results are based upon the assumption that the molecules of PBS 1 DNA are linear.

ZONE SEDIMENTATION PROFILE OF ^3H -DNA FROM ACTIVE PBS 1 PARTICLES AND ^{32}P -T₂ DNA AS OBTAINED PRIOR TO MODIFICATION OF THE DNA EXTRACTION TECHNIQUE



ZONE SEDIMENTATION PROFILE OF ^3H -DNA FROM ACTIVE PBS 1 PARTICLES AND ^{32}P -T₂ DNA AS OBTAINED AFTER MODIFICATION OF THE DNA EXTRACTION TECHNIQUE



PROBABILITY PLOT OF THE RECOVERY OF TOTAL INPUT OF RADIOACTIVITY PRESENTED IN FIGURE VIII

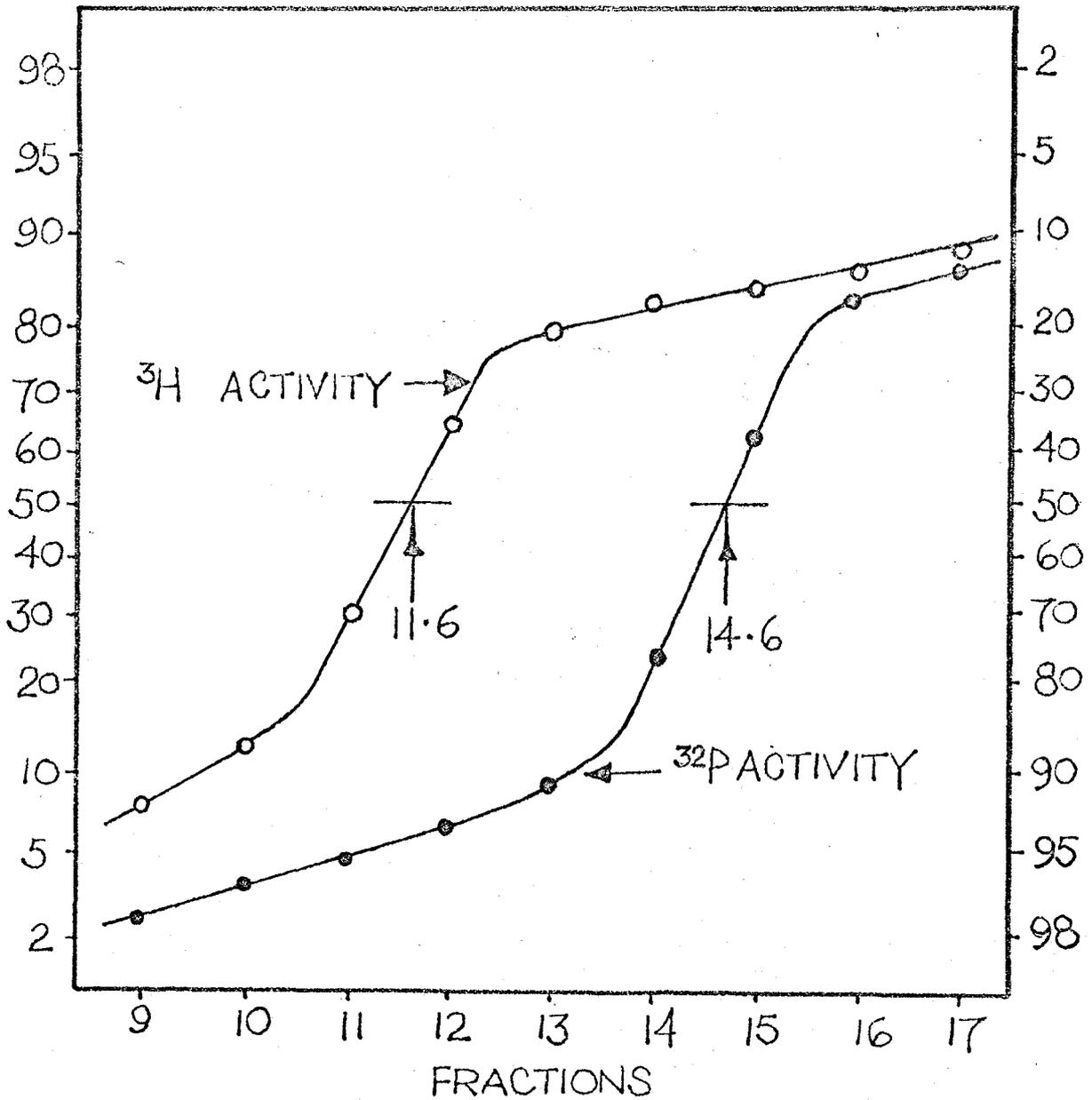


TABLE I

The molecular weight of DNA obtained from active PBS 1 particles

experiment #	D ₁ - distance sedimented by T ₂ DNA (fractions)	D ₂ - distance sedimented by PBS 1 DNA (fractions)	M ₂ - molecular weight of PBS 1 DNA (= 0.35 $\sqrt{D_2/D_1}$ x M ₁ [*])
1.	19.6	22.9	2.0 x 10 ⁸
2.	19.0	21.6	1.9 x 10 ⁸
3.	19.6	22.6	2.0 x 10 ⁸
4.	19.9	22.5	1.8 x 10 ⁸
		Mean molecular weight	<hr/> 1.9 x 10 ⁸ <hr/>

* M₁ = the molecular weight of T₂ DNA. The reported molecular weight of T₂ DNA is 1.3 x 10⁸ (Rubenstein, Thomas and Hershey, 1961)

CHAPTER FIVE

THE DNA OF TRANSDUCING PBS 1 PARTICLES

A further intention of the work presented in this thesis - an investigation of the size, or sizes, of DNA molecules obtained from transducing PBS 1 - was carried out by repeating the procedure of Burgi and Hershey (1963) for measuring the molecular weights of DNAs, this time using DNA extracted from purified transducing PBS 1 particles, together with T₂ DNA as reference. Since this part of the work was not repeated, its results are not substantiated by confirmatory evidence; they are put forward only tentatively as a basis for discussion.

A doubly labelled PBS 1 lysate - in which the active particles were labelled with ³²P and the transducing particles were selectively labelled with ³H - was prepared such that the final volume was 20ml. The lysate was subjected to differential centrifugation for the collection of the phage particles, then the resultant pellets were resuspended in SSC. In order to separate the transducing particles from the active particles, a sample of the phage suspension was centrifuged in a caesium chloride density gradient. After centrifugation was completed, the centrifuge tube was punctured and 3-drop fractions were collected in 1ml aliquots of SSC. Assays of radioactivity and measurements of O.D. were made on all the resultant fractions. Contrary to expectations, the ³²P activity of the gradient was not concentrated at the position of the O.D.

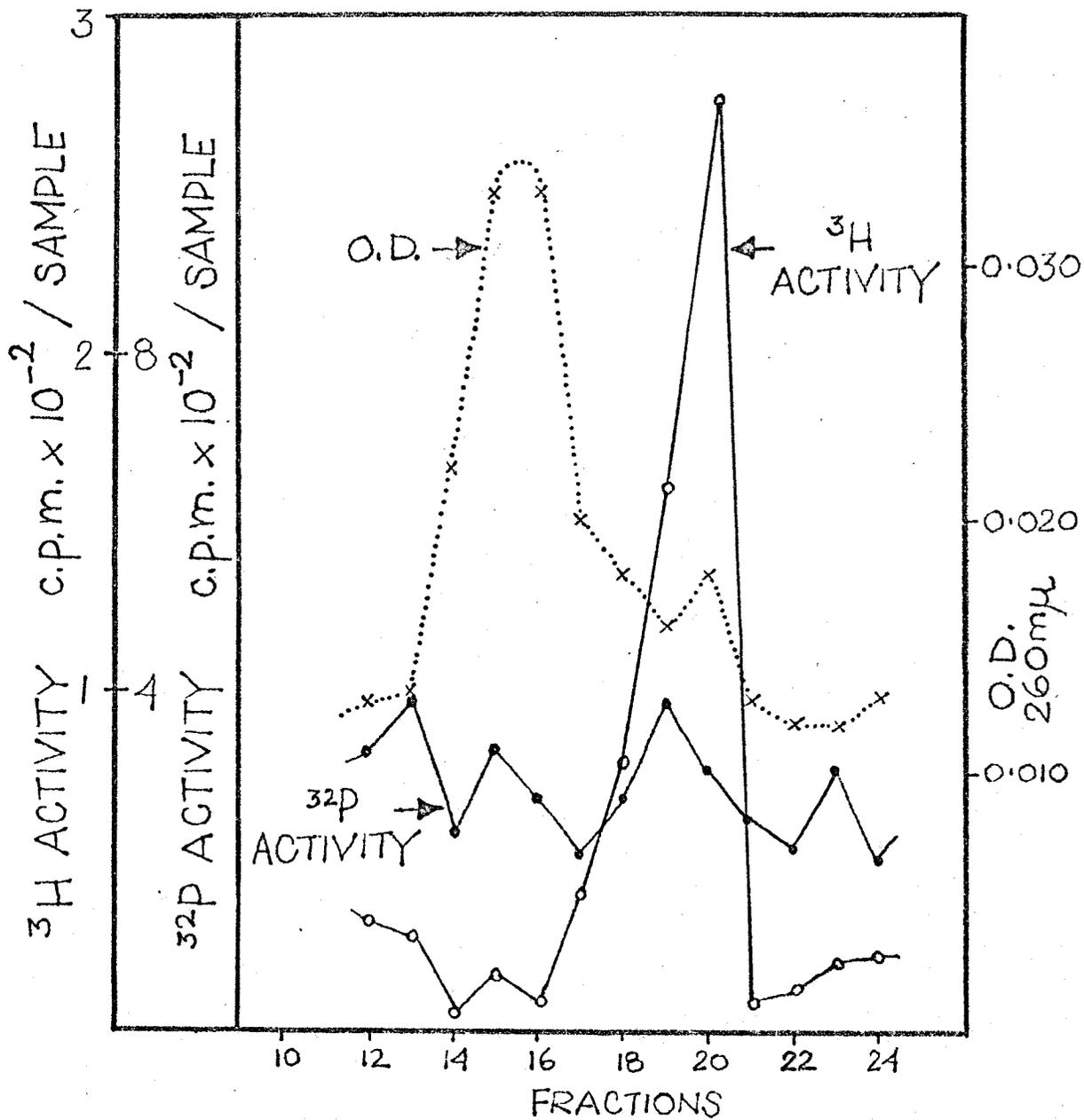
peak where the majority of particles (active particles) would be situated; rather, there was no well defined peak at all. This result cannot be explained. In spite of the lack of a ^{32}P activity peak to mark the contrasting density level of active particles, the fraction of highest transducing activity was known to be at the ^3H activity peak, since the latter was clear and sharp, and situated in the expected position - 3-4 fractions distant from the O.D. peak in a region of lower density (Fig. X). The absence of any detectable ^3H activity at the O.D. peak demonstrated that the transducing particles were selectively labelled with ^3H . The fraction taken for the purpose of DNA extraction was the ^3H activity peak fraction.

Particles of T_2 phage were labelled with ^{32}P and purified as described in the previous chapter.

After the selected fractions of ^3H -labelled PBS 1 transducing particles and ^{32}P -labelled T_2 particles had been subjected to DNA extraction, the resultant DNA solutions were dialysed against SSC. Appropriate samples of both PBS 1 and T_2 DNA solutions were layered on to the top of a sucrose density gradient. After centrifugation was completed the centrifuge tube was punctured and fractions were collected. The radioactivity of fractions was measured directly.

The radioactivity of ^3H and ^{32}P in each fraction, expressed as the recovery of total input of radioactivity, was plotted directly against fraction number. Figure XI shows the result of this plot. The sedimentation profile of ^{32}P activity - corresponding to the distribution of T_2 DNA, the reference DNA - is sharp and symmetrical, and comprises virtually all of the ^{32}P activity, as expected. The sedimentation

DISTRIBUTION OF RADIOACTIVITY AND O.D.
AFTER CENTRIFUGATION OF DOUBLY LAB-
ELLED PBS 1 PARTICLES IN A CAESIUM
CHLORIDE DENSITY GRADIENT



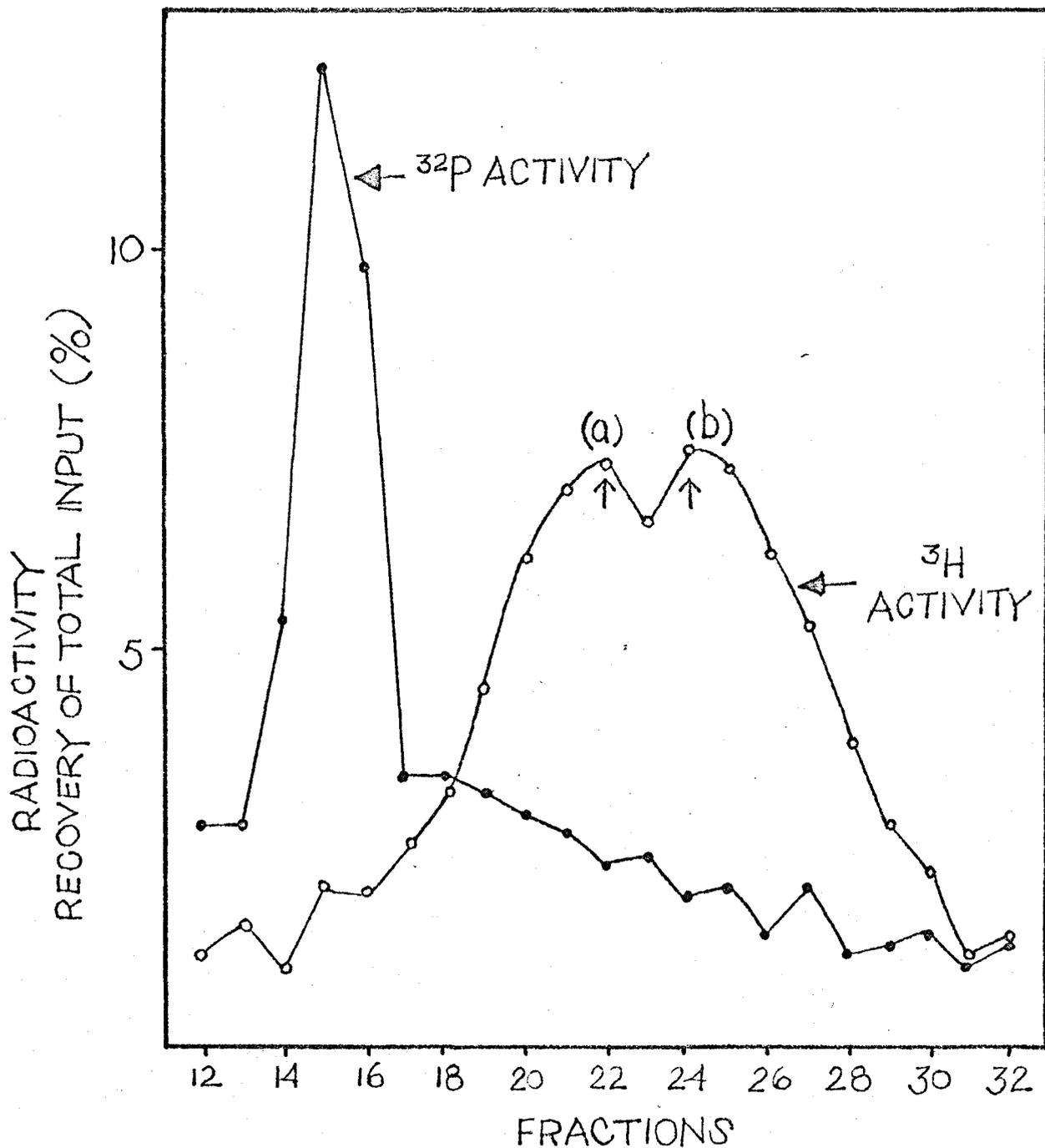
profile of ^3H activity, in contrast, corresponds to a broad distribution of transducing PBS 1 DNA which gives the appearance of rising in two peaks, (a) and (b), very close together.

In order to determine the peak position of ^{32}P activity to an accuracy of 0.1 fraction, the recovery of total input of radioactivity was plotted on probability paper (Fig. XII). The distance travelled by the T_2 DNA in the sucrose gradient was calculated from the ^{32}P peak position and expressed in fraction units to the nearest 0.1 fraction. The distances travelled by the transducing PBS 1 DNA were calculated for positions (a) and (b). Since the starting position of the DNAs was taken as the surface of the sucrose gradient, these calculations involved taking into consideration the volume of DNA solutions added. Together with the known molecular weight value for T_2 DNA, the values for distances sedimented were appropriately substituted into the equation of Burgi and Hershey (1963), to solve for the molecular weights of transducing PBS 1 DNA at positions (a) and (b). Results are given in Table II.

From the results the molecular weights attributable to the DNA of transducing PBS 1 at the positions (a) and (b) are as follows:

- position (a) - molecular weight 4.5×10^7
- position (b) - molecular weight 2.9×10^7 .

ZONE SEDIMENTATION PROFILE OF ^3H -DNA FROM TRANSDUCING PBS 1 PARTICLES AND ^{32}P - T_2 DNA



PROBABILITY PLOT OF THE RECOVERY
OF TOTAL INPUT OF ^{32}P ACTIVITY
(T_2 DNA) PRESENTED IN FIGURE XI

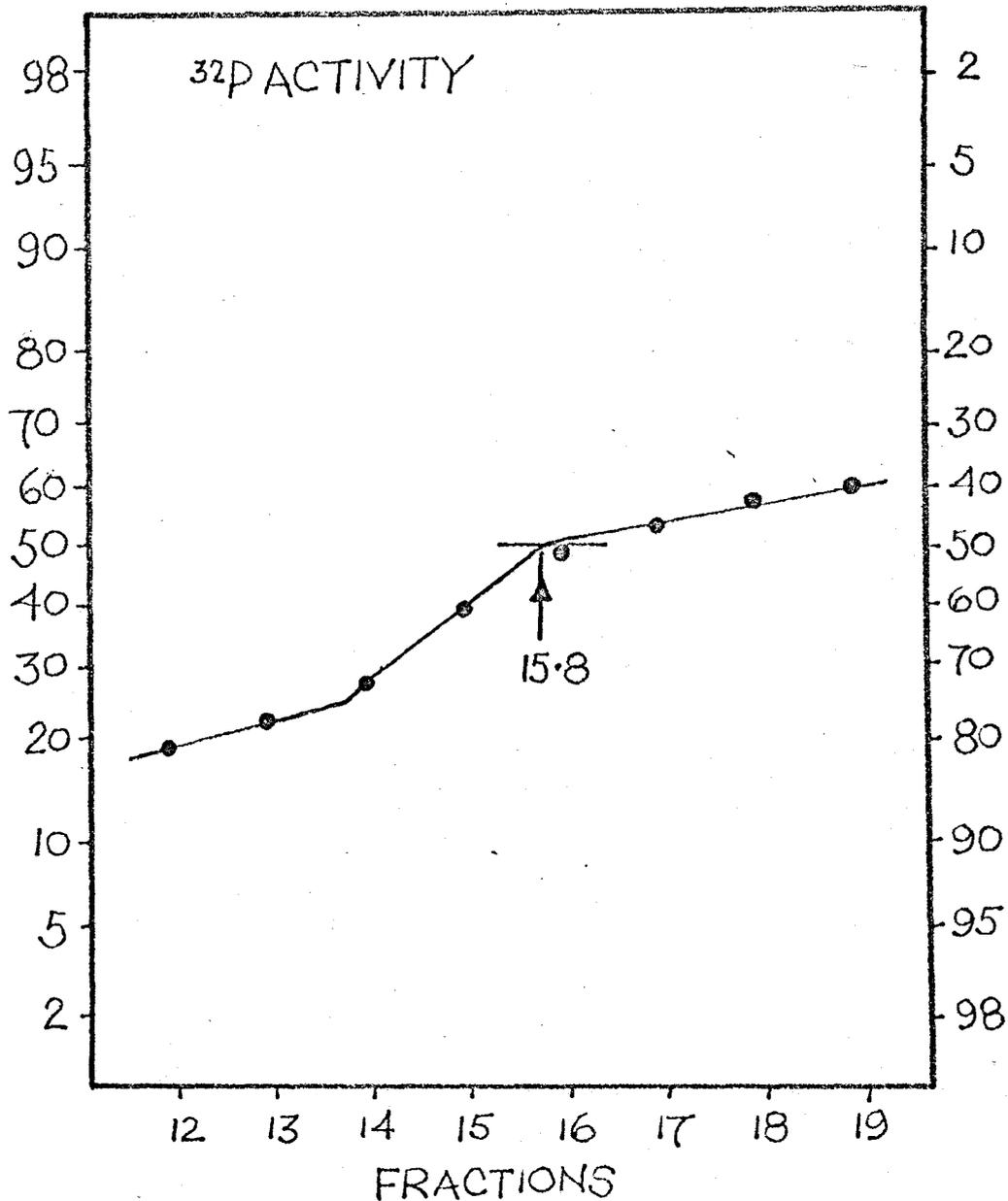


TABLE II

Molecular weights of DNA obtained from transducing PBS 1 particles

position of PBS 1 DNA	D_1 - distance sedimented by T_2 DNA (fractions)	D_2 - distance sedimented by PBS 1 DNA (fractions)	M_2 - molecular weight of PBS 1 DNA (= $0.35 \sqrt{D_2/D_1} \times M_1^*$)
peak (a)	19.8	13.6	4.5×10^7
peak (b)	19.8	11.6	2.9×10^7

* M_1 = the molecular weight of T_2 DNA. The reported molecular weight of T_2 DNA is 1.3×10^8 (Rubenstein, Thomas and Hershey, 1961)

CHAPTER SIX

SUMMARY AND DISCUSSION

The zone sedimentation technique of Burgi and Hershey (1963) for determining the molecular weight of a DNA is a particularly advantageous technique. As shown in the previous two chapters, its application provides an accurate description of the DNA distribution after sedimentation, and from the shape of the distribution curve it can be deduced whether or not the DNA is homogeneous with respect to molecular size. Molecular weight can be calculated for DNA at any position in the distribution. Thus, a value can be obtained for the molecular weight of a homogeneous DNA or, alternatively, values can be obtained to express the approximate molecular weight of a heterogeneous DNA.

Applying the zone sedimentation technique to DNA obtained from active PBS 1 particles according to the improved extraction method, the sedimentation profile of the DNA shows a sharply defined, normal distribution which accounts for virtually all of the DNA of its kind present in the sucrose gradient (Fig. VIII). This indicates that the DNA of active PBS 1 is unfragmented, and homogeneous with respect to molecular size. The latter fact was predicted on the basis that the DNA of an active phage particle is a 'chromosome' which reproduces itself during the replication procedure; also by analogy with those other phages whose DNA contents have been well studied, for example T_2 phage.

On account of its homogeneity, the DNA of active PBS 1 can be attributed a single molecular weight. Four independent determinations give closely corresponding results, of which the mean value is 1.9×10^8 (Table I). The recent finding of McHattie (personal communication) affords evidence to support this value. McHattie observed DNA from active PBS 1 under the electron microscope, and found it to consist of linear molecules, 82μ in length. On the assumption that PBS 1 DNA is uniformly doubly helical, and that a fragment 1μ in length represents a molecular weight of about 2.5×10^6 *, a molecular length of 82μ corresponds to an approximately molecular weight of 2×10^8 .

At 1.9×10^8 , the molecular weight of the DNA of active PBS 1 is 1.5 times the molecular weight of T_2 DNA. This is in keeping with the statement of Eiserling (1967), that the internal volume of the PBS 1 head could be as large as $5 \times 10^8 \text{ \AA}^3$, and that, by comparison, the internal volume of the head of phage T_4D (similar in dimensions to T_2) is approximately $2 \times 10^8 \text{ \AA}^3$.

It is clear that PBS 1 contains the largest DNA molecule known at the present time. More importantly, PBS 1 possesses the largest known 'phage chromosome', and unless a considerable proportion of the 'chromosome' is genetically inactive, it is likely that PBS 1 possesses a correspondingly greater number of genes than the other phages with which we are now acquainted. According to Eiserling (1967), PBS 1 has one of

* Based upon the following accepted values:

Length of T_2 DNA - 52μ (Cairns, 1961)

Molecular weight of T_2 DNA - 1.3×10^8 (Rubenstein, Thomas and Hershey, 1961).

the most complex and specialised of all known phage structures. Consequently, PBS 1 lends itself to further and fuller study.

Applying the zone sedimentation technique to DNA extracted from transducing PBS 1 particles, the distribution pattern of the DNA is found to be well defined but broad. It culminates in two apparent peaks, very close together, situated considerably higher in the sucrose gradient than the T_2 DNA peak (Fig. XII, (a) and (b)). The significance of the two peaks is considered questionable. In view of the fact that the results under discussion are the results of a solitary experiment, detailed interpretation of the peaks - separated by only one fraction - is not merited. It can, however, be deduced that the DNA from transducing PBS 1 represented in the distribution is heterogeneous with respect to molecular size. It is quite likely that this DNA is the result of originally larger molecules being fragmented during the extraction procedure.

It must be borne in mind that the distribution of DNA from transducing PBS 1, shown in Figure XI and discussed above, is measured by ^3H activity only. It is helpful at this point to reconsider the technique employed for radioactively labelling the original transducing particles, in order to be assured that it is only bacterial DNA that is ^3H labelled and therefore detected in the sucrose gradient fractions.

In preparing the labelled lysate of PBS 1, host cells of strain 168T that had been labelled with ^3H -methyl-thymidine were removed to fresh, non-radioactive medium before infection with PBS 1. In this way, the transducing particles of PBS 1, which contain a fragment of host DNA in place of phage DNA, must have been selectively labelled with ^3H .

That this was actually so is borne out by the fact that the active PBS 1 particles (at the position of the O.D. peak after caesium chloride density gradient centrifugation) contained no detectable ^3H label (Fig. X). The labelling technique also involved adding ^{32}P -inorganic phosphate at the point of infection, as it was intended to label the active phage particles with ^{32}P . However, the ^{32}P -labelling was unsuccessful in the present study. Since the radioactive thymidine added was labelled at the methyl group, and since PBS 1 DNA contains uracil in place of thymine, mixed labelling with ^{32}P and ^3H is improbable. Furthermore, recent data of Yamagishi and Takahashi (unpublished results) show that the distribution of ^3H is identical to that of transducing activity when doubly labelled PBS 1 particles are centrifuged in a caesium chloride density gradient.

Knowing, then, that the data given in Figure XI and Table II represent the behaviour of host DNA fragments only, interpretation of the data is facilitated. The fragments of host DNA extracted vary in size within fairly wide limits, but the greater proportion of fragments have molecular weight values between 2.9×10^7 and 4.5×10^7 . Taking the molecular weight of an average gene as 1.0×10^6 (Watson, 1965), the majority of fragments thus represent 29-45 genes each.

It is not clear from the present data whether the transducing particles contain several molecules of host DNA of the above size, or whether they contain a single large segment of the host chromosome whose molecular size is equivalent to that of active PBS 1 DNA. The recent work of Dubnau, Goldthwaite, Smith and Marmur (1967) suggests that the latter is the case. In an extensive investigation, in which trans-

duction and transformation techniques were used to map the B. subtilis chromosome, they found that the distance between the most distal genetic markers that are cotransduced by PBS 1 corresponds approximately to a molecular weight value of 2×10^8 .

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