THE ISOLATION AND BIOPHYSICAL CHARACTERISATION OF DNA FROM PROTEUS MIRABILIS (13/S suc^+)

by

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LIST OF SYMBOLS AND ABBREVIATIONS

S_c	:	uncorrected sedimentation coefficient, measured at concentration C
$S_{20,w}$	:	sedimentation coefficient, converted to a standard basis
$S^o_{20,w}$	:	limit sedimentation coefficient
η_t	:	viscosity of solvent at t^oC
η^o_{20}	:	viscosity of water at 20^oC
η^o_t	:	viscosity of water at t^oC
ρ_t	:	density of solvent at t^oC
ρ^o_{20}	:	density of water at 20^oC
D_c	:	uncorrected diffusion coefficient measured at concentration C
$D_{20,w}$	:	diffusion coefficient converted to a standard basis
DNA		Deoxyribonucleic acid
EDTA		ethylenediamine tetra-acetate
RNA		Ribonucleic acid
SLS		sodium lauryl sulphate
Tris		Tris - (hydroxymethyl - aminomethane)

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CHAPTER I

INTRODUCTION

The general acceptance of DNA as the universal carrier of genetic information has led to an intensive study of DNA composition and structure. While the empirical chemical composition of DNA has long been known, the macromolecular nature has only recently been established. Hence it became necessary to examine the configuration and dimensions of these particles. As the essential biochemical mechanisms operate within this region, the importance of such a study becomes apparent.

Special techniques are necessary for the characterisation of such large molecules. For example, spectral methods, such as infra-red spectroscopy used extensively for the study of small molecules, lose much of their efficiency in the macromolecular domain. They indicate the location of atoms or atom groups but give only vague indications of the whole assembly which they constitute.

Many specialised techniques used for the characterisation of macromolecules have been developed for sphere-like particles. Attempts to apply these methods to the nucleic acid field have frequently led to erroneous results. For example, Loring (1), assuming a spherical molecule, reported a molecular weight of 17,000 for yeast nucleic acid. Myrbäck and Jorpes (2) obtained a molecular weight of 1,360 and made the wrong deduction that this nucleic acid occurred in the cell as a tetranucleotide. It was not until Desreux (3) showed that the axial ratio of DNA may be as high as 9:1 that methods of investigation were reconsidered. Furthermore, modifications in instrumentation became necessary.

Although nucleic acids, especially DNA, have been isolated and characterised from a multitude of different organisms, a comparison of the published data is difficult. This is due to workers, even at present, employing isolation methods which are known to degrade DNA (4). Furthermore, data on molecular weights for example, are of no practical value, unless it is specifically stated how they were derived, because considerable fluctuations are possible, depending on the method employed. However, a comparison of data of highly polymerised DNA from P. mirabilis, maize and bacteriophage (5, 6), show that the DNAs are identical regarding molecular weight and other physical characteristics. Such a comparison is justified only when similar methods of

investigation are employed. Differences among these DNAs can apparently be detected only by means of base sequence determinations or base ratio analysis.

Compositional studies of macromolecules have aided the understanding of many concepts on the molecular level. For example, the equimolarity of adenine and thymine and of guanine and cytosine in DNA, which had been deduced from base analysis (7), provided essential information for the formulation of the Watson-Crick structure of DNA (8). Furthermore, the first physical evidence of the existence of mRNA was the similarity of its base composition to that of DNA (9).

Microorganisms possess DNAs that vary widely in base composition (10, 11). Extensive analysis of the base composition of different species have revealed that organisms which are very closely related, according to the criteria of numerical taxonomy, have DNA base compositions which are similar (12). The existence of these qualitative differences among the DNAs of different species revealed the possibility of their biological specificity. Today, therefore, microorganisms attract ever increasing attention as the organism in which the role of nucleic acid activity is most clearly revealed. It was, in fact, in microorganisms that the role of nucleic acids in the processes of protein synthesis, intermediary metabolism and heredity was recognised and studied (13, 14, 15).

The DNA base composition of higher plants, vertebrates and invertebrates show little variation (16). This, however, does not imply that these DNA molecules lack specificity, as true specificity expresses itself structurally through the nucleotide sequence. Therefore, in order to elucidate the biochemical mechanisms of heredity and protein synthesis, the nucleotide sequence must first be established.. To date only limited success has been achieved (17, 18).

An elegant approach to the problem of detecting and estimating the similarities of different DNAs of different origin has been provided by the demonstration that mRNA can form specific complexes with homologous DNA. This was originally shown for the T2-RNA formed in phage infected bacteria and T2-DNA (19, 20, 21). It was later observed also for RNA of normal bacteria and homologous DNA (22, 23). This finding was used as an approach to the measurement of genetic relatedness among organisms by McCarthy and Bolton (24).

At present it seems as though the maximum information which may be obtained from the base ratios of DNA has been fully utilised. However, many of

the aspects of DNA specificity still remain obscure and the urgent need for base sequence determination methods become increasingly apparent.

Most, if not all studies of cellular systems involving nucleic acids have previously been restricted to bacteria, since they grow rapidly and large numbers are readily available. These studies began in 1928 when Griffith (25) succeeded in transforming specific types of Pneumococcus. It was, however, not until 1944 when Avery, McLeod and McCarty (13) showed that the transforming principle was DNA that nucleic acids were intensively studied. The extremely important phenomenon of transformation was later shown to operate also in other bacteria (26, 27). The possibility cannot be rejected that transformations are of more general biological importance, and not limited to bacteria, but also apply to higher plants and animals. The reason for suggesting such a possibility is that many cellular mechanisms initially elucidated in bacteria, have been found to operate in higher organisms (28). It thus becomes apparent that as much information as possible must be obtained from microorganisms before moving to higher forms of life.

The object of this thesis was to substantiate available data on DNA from microorganisms. Proteus mirabilis (13/S suc^+) provided a suitable source of DNA as protoplasts of this organism are readily available and can be lysed under very mild conditions.

With this purpose in mind, the isolation and characterisation of DNA from P. mirabilis (13/S suc^+) was undertaken. Certain physico-chemical properties were determined and these results are reported.

CHAPTER II

EXPERIMENTAL PROCEDURES AND RESULTS

2.1 MATERIALS

2.1.1 Proteus mirabilis (13/S suc^+) protoplasts

The organisms were kindly prepared by Professor J.N. Coetzee of The Department of Microbiology, Institute of Pathology, University of Pretoria.

2.1.2 Buffers and Solvents

For the sake of convenience, the buffers will be designated as A, B and C.

Buffer A: 0.15 M NaCl - 1% SLS - 10 mM EDTA - 0.1 M Tris (pH9).

Buffer B: 1 M NaCl - 1% SLS - 10 mM EDTA - 0.1 M Tris (pH9).

Buffer C: 5 mM NaCl - 1% SLS - 10 mM EDTA - 0.1 M Tris (pH9).

The solvents used were the following:

Saline-citrate : 0.15 M NaCl - 0.015 M citrate (pH7) and dilute saline-citrate : 15 mM NaCl - 1.5 mM citrate (pH7).

2.1.3 Phenol

A water saturated phenol solution was used for deproteinisation purposes (27, 29, 30). Prior to use the phenol was distilled over zinc dust in vacuum.

2.1.4 Ethanol

Absolute ethanol was used for precipitation of the DNA (31).

2.1.5 Ribonuclease

A 0.2 per cent solution of pancreatic RNase in 0.15 M NaCl was used to hydrolyse the RNA present in crude DNA solutions (27). The RNase (four times recrystallised) was obtained from Seravac Laboratories (Pty.) Ltd., Cape Town.

2.1.6 Disodium phenolphthalein diphosphate

This reagent was obtained from L. Light and Co. Ltd., Colnbrook, England and stored below $4^{o}C$.

2.1.7 Activated Charcoal

Charcoal (Norit) was obtained from The Coleman and Bell Co., Norwood, O., U.S.A., and treated as follows prior to use: 100g of charcoal was mechanically stirred with 400 ml of 2 M NaCl for 30 min. After centrifugation at 2,500 xg for 10 min, the charcoal was stirred with 0.15 M NaCl for 30 min and centrifuged as before. The equilibration with 0.15 M NaCl was repeated and the charcoal air-dried. The material was stored in the dry form.

2.2 METHODS

2.2.1 ISOLATION

Several procedures for the isolation of the DNA were tested (see Flowsheet 1 page 6). The aim was to find a procedure by which a good yield of highly polymerised homogeneous DNA could be obtained. The procedure described below was found to be the most suitable and for the sake of clarity this will be described in more detail. However, brief reference will be made to procedures which were less efficient.

2.2.1.1 Extraction (Step 1)

Owing to the extreme lability of protoplasts with respect to changes in ionic strength (32, 33, 34), no difficulty was encountered in disintegrating the cells. Lysis was effected with ease by suspending the cells in any of the buffers described in 2.1.2. However, the yield and limit sedimentation coefficient of the DNA obtained by extraction with these buffers differed markedly (see Table 1).

TABLE 1 YIELD AND LIMIT SEDIMENTATION COEFFICIENT OF THE DNA EXTRACTED WITH BUFFERS DESCRIBED IN 2.1.2

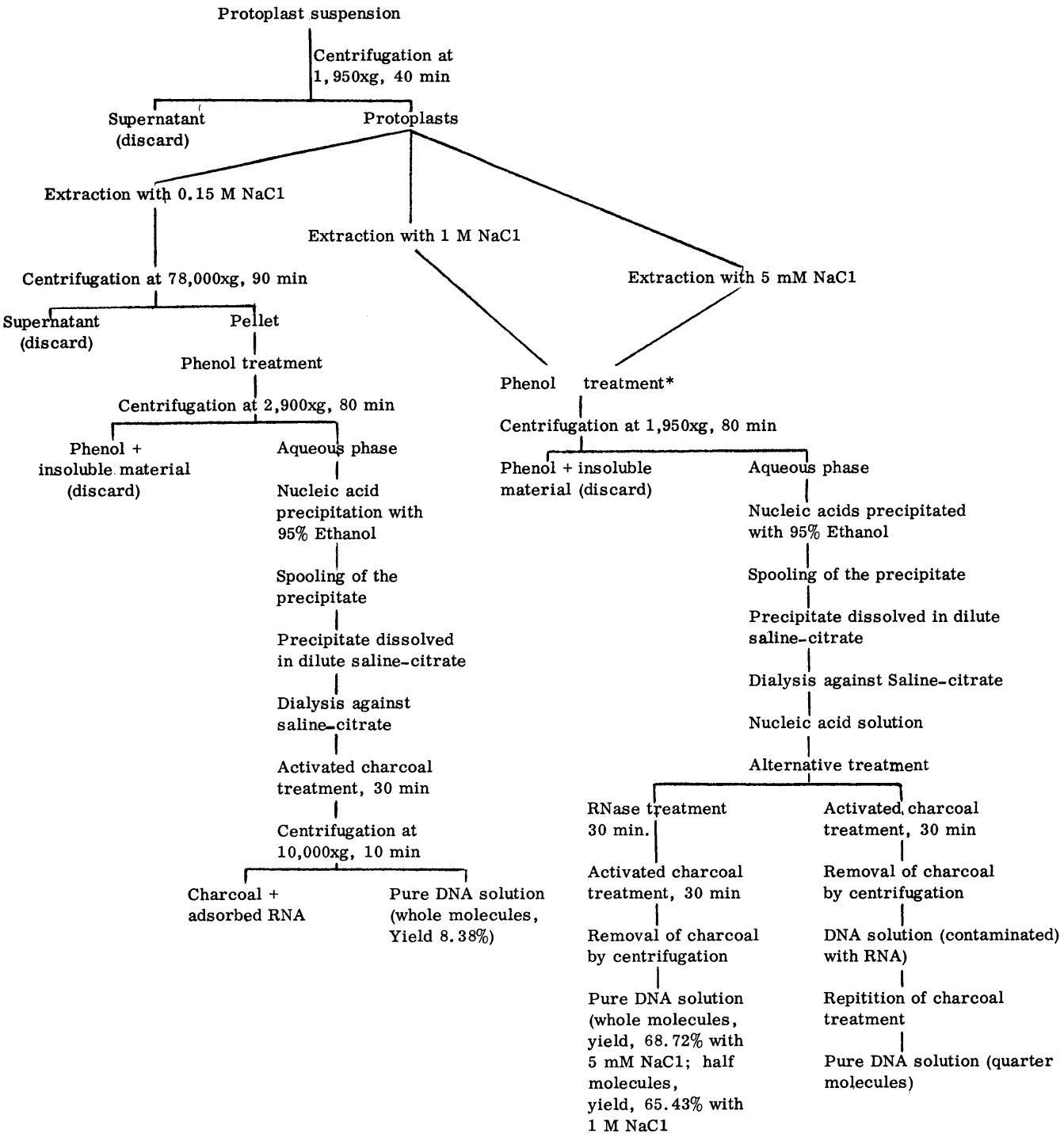
Buffer	DNA yield (percentage of total)	$S^{o}_{20,w} \times 10^{13}$ sec
A	8.38	62.5
B	65.43	42.0
C	68.72	62.5

Although a very low yield was obtained when buffer A was employed, this procedure offered the advantage of partial separation of the deoxypentose from the pentose nucleoprotein during the early stages of the isolation. By extracting with buffer A, which contains 0.15M NaCl, the deoxypentose nucleoprotein was obtained in an insoluble form (35). Centrifugation of the extract at 78,000xg for 90 min. left most of the pentose nucleoprotein in the supernatant, while approximately 98% of the total DNA was found in the gelatinous precipitate. Most of the DNA, however, was lost during the phenol deproteinisation step (see Table 2).

Extraction with buffer B gave a good yield of homogeneous DNA but it had a lower molecular weight than the DNA obtained with buffer C, containing 5mM NaCl. The extraction with 5 mM NaCl was carried out in the following

FLOWSHEET 1

FLOWSHEET OF THE ISOLATION PROCEDURES OF DNA FROM P. MIRABILIS (13/S suc$^+$)



* Each extractant was separately treated as indicated.

manner. Protoplasts were harvested from approximately 8l of cell suspension by centrifugation in a Christ centrifuge at 1,950xg for 40 min. The packed cells (30g) were suspended in 150 ml of buffer C and stored at 12°C overnight. Microscopic examination of the highly viscous cell suspension revealed that virtually all of the protoplasts were lysed.

2.2.1.2 Deproteinisation (Step 2)

To deproteinise the lysate, 150 ml of a 90% phenol solution was added. The mixture was cautiously shaken by hand in the cold room at 4°C for 30 min. After centrifugation at 2,900xg for 80 min in a Christ centrifuge, the mixture separated into three layers. The lower light brown fraction contained the phenol, while the upper, very viscous, aqueous layer contained the nucleic acids. Between these layers a white gel-like material, consisting mainly of denatured protein was present. The upper aqueous phase was carefully removed by means of a pipette with a large orifice in order to minimise shearing (36). The total volume of this fraction was 150 ml. The absorption spectrum revealed the presence of phenol.

2.2.1.3 Precipitation of the DNA (Step 3)

A volume of 300 ml cold ethanol was cautiously layered on to the viscous aqueous phase, containing the extracted nucleic acids. A white, thread-like precipitate formed at the interphase. The ethanol and aqueous layers were mixed cautiously by shaking the mixture by hand in the cold room. After complete mixing of the two layers, a vast quantity of thread-like precipitate formed. This precipitate could be removed easily by winding on to a glass rod. The thread coated rod was placed in 15 ml of dilute saline-citrate solution and stored under refrigeration. After about 2 hours the glass rod could be removed, leaving the precipitate behind. Complete solvation of the precipitate was achieved only after about 2 weeks. During this time the solution was mixed cautiously, a few times a day. The resulting solution was very viscous. The phenol and other interfering substances of low molecular weight were removed by dialysis. For this purpose cellulose dialysis tubing (No. 4465A2, size 27/32 inches), suspended in 1l of saline-citrate at 4°C was used. After 4 buffer changes, made at 2 hour intervals, no phenol could be detected in the dialysate.

2.2.1.4 Removal of the RNA (Step 4)

The separation of DNA from RNA according to the method of Kirby (37), which makes use of disodium phenolphthalein diphosphate, proved to be unsuccessful. The most effective separation was achieved with activated

charcoal (38). Purification by treatment with crystalline RNase, according to the method of Saito and Miura (27), left some enzyme-resistant portions which could not be removed by dialysis and subsequent charcoal treatment was necessary to obtain a pure preparation. Purification on activated charcoal was performed in the following manner. To every 10 ml of the crude DNA preparation, 0.41g of activated charcoal, suspended in 0.3 ml of saline-citrate, was added. The mixture was cautiously agitated by hand in the cold room for 30 min, taking precaution against shearing (36). Owing to the extreme viscosity of the crude DNA preparations, occasional dilution was necessary to promote mixing with the charcoal. The charcoal was easily removed by centrifugation for 10 min at 10,000xg. Purification of the DNA, extracted with 5 mM NaCl or 1 M NaCl necessitated three such treatments but, unfortunately, this also led to degradation of high polymer DNA. By combining the RNase and activated charcoal treatments, a pure, high molecular weight DNA was obtained.

2.2.1.5 Comparison of the isolation methods

For the sake of convenience, the comparison will be presented in tabular form (see Table 2).

TABLE 2 COMPARISON OF THE DNA ISOLATION PROCEDURES SHOWING THE AMOUNT OF DNA, EXPRESSED AS PERCENTAGE DNA OF THE TOTAL*, PRESENT IN THE VARIOUS STEPS OF ISOLATION. ISOLATIONS A, B AND C REPRESENT THE PROCEDURES EMPLOYING BUFFERS A, B AND C, RESPECTIVELY

Step of isolation	DNA content (percentage of total)		
	Isolation A	Isolation B	Isolation C
Cytoplasmic supernatant**	1.6	-	-
Deproteinisation			
(a) Phenol phase	0.035	0	0
(b) Protein interphase	88.62	30.94	25.10
(c) Aqueous phase	9.15	67.81	73.07
Precipitation			
(a) Ethanol supernatant	0.46	0.60	0.31
(b) Nucleic acid precipitate	9.08	66.47	70.68
Pure DNA preparation	8.38	65.43	68.72

* The total DNA content was 6.1 mg/g dry cells (Average of 4 determinations).

** Supernatant after removal of the deoxypentose nucleoprotein by centrifugation at 78,000xg for 90 min (see 2.2.1.1).

2.2.1.6 Concentration and purity determinations (Step 5).

(a) Concentration determination

The concentration of DNA in crude preparations was determined by the Burton modification (39) of the Dische diphenylamine reaction. The total DNA content of the protoplasts was determined according to Kupila, Bryan and Stern (40). The DNA concentration of pure preparations was determined gravimetrically or calculated from the phosphorus content. Phosphorus was determined according to Dryer, Tammes and Routh (41). This method is suitable for the determination of phosphorus down to 0.2 µg and employs N-phenyl-p-phynelenediamine as reducing agent. The concentration of DNA in preparations that were investigated in the analytical ultracentrifuge, was determined from a plot of absorbancy, measured at 254 mµ, versus concentration (see Fig. 1).

(b) Purity determination

The presence of RNA and protein was established by the orcinol (42) and Folin-Ciocalteu (43) reactions. Considerable information concerning the protein and RNA content of DNA preparations, was obtained from ultraviolet spectra (see 2.2.2.5). Indication of the degree of purity was obtained also by comparing determined atomic N/P ratios with those calculated from the distribution of the purines and pyrimidines actually found in the DNA (see 2.2.2.6). Nitrogen was determined according to Filipowicz and Gross (44). The experimentally determined as well as the calculated nitrogen and phosphorus values together with the atomic N/P ratios for pure and impure DNA preparations are summarised in Table 3.

TABLE 3 ELEMENTARY COMPOSITION OF PURE AND IMPURE DNA PREPARATIONS (PREPARATIONS 1 AND 2, RESPECTIVELY)

Preparation	Nitrogen %		Phosphorus %		Atomic $\frac{N}{P}$ ratio	
	Found	Calculated	Found	Calculated	Found	Calculated
1	15.43	15.13	9.31	9.17	3.67	3.63
2	18.99	15.13	9.71	9.17	4.24	3.63

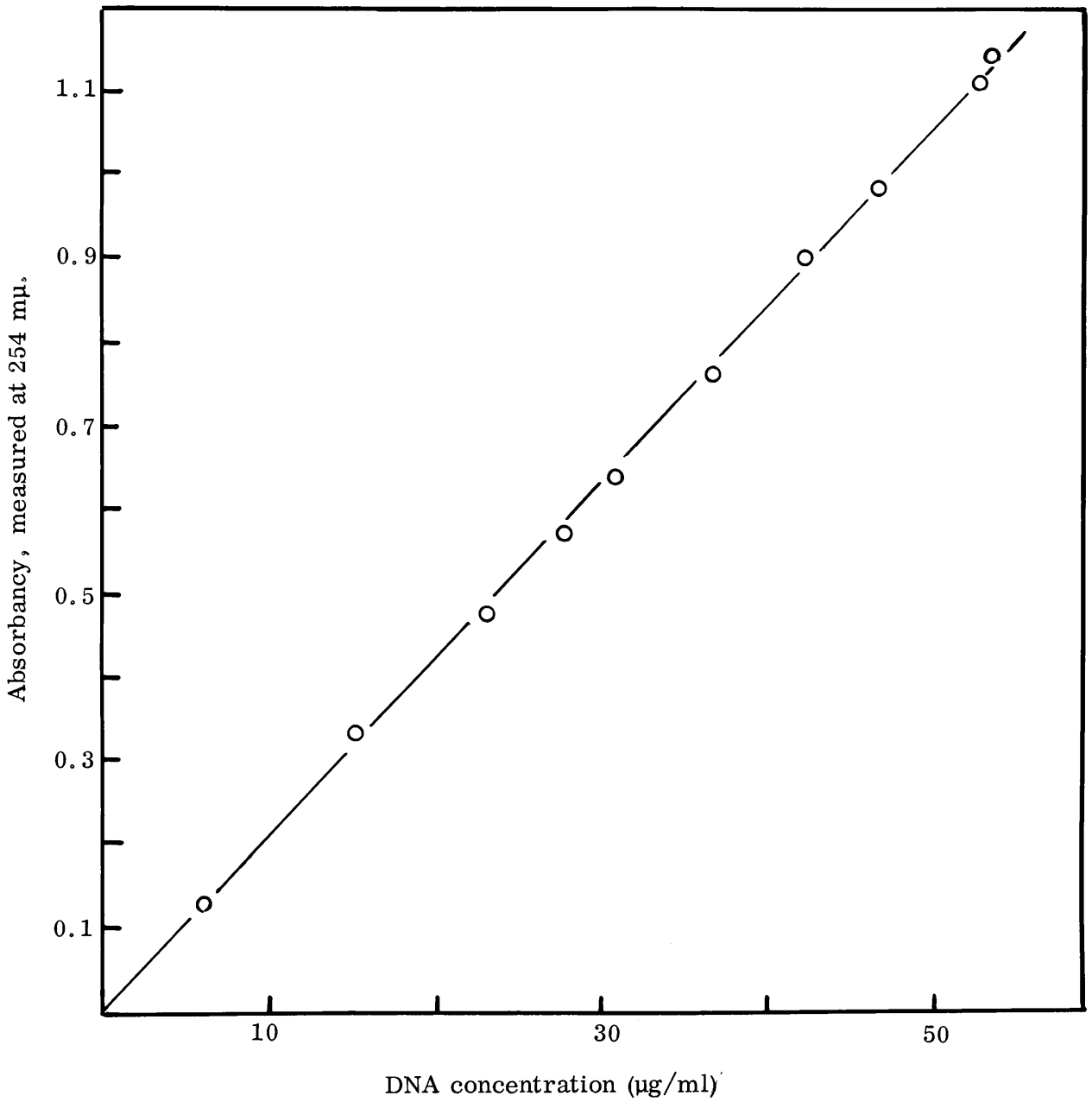


Fig. 1. Absorbancy, measured at 254 mμ versus DNA concentration. The concentrations were determined gravimetrically. The solvent was saline-citrate.

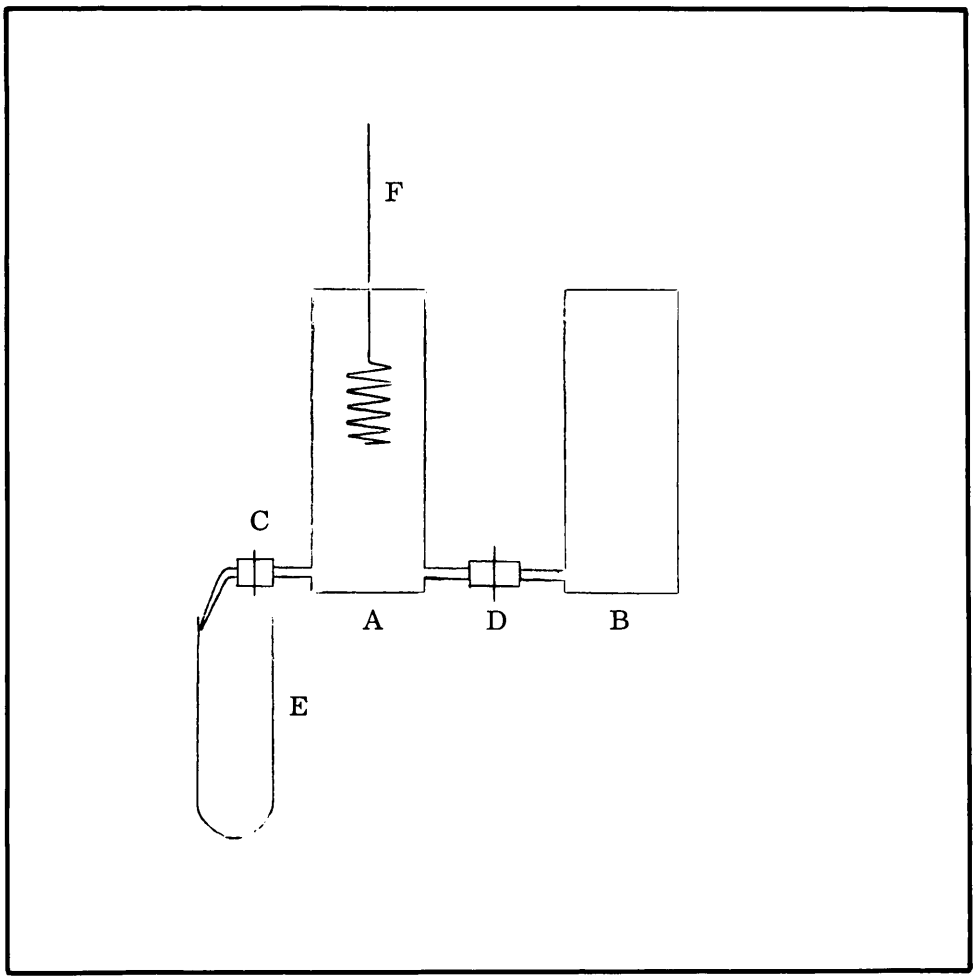


Fig. 2. Line diagram of gradient making device.

A : mixing chamber.
B : second chamber.
C and D : stopcocks.
E : tube for type SW39L swinging bucket rotor.
F : platinum wire used for mixing.

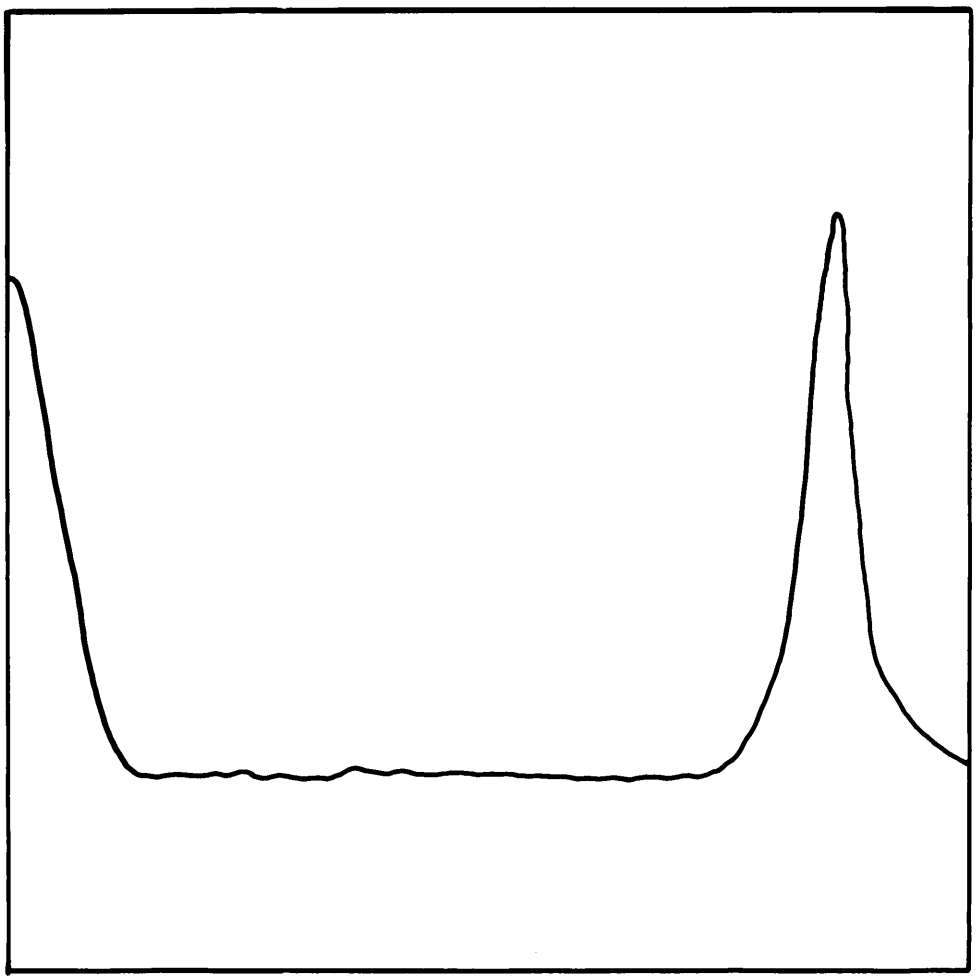


Fig. 3. Diagrammatic recording of a homogeneous DNA preparation after sucrose density gradient centrifugation.

2.2.1.7 Homogeneity determination (Step 6)

Sedimentation velocity centrifugation in the analytical centrifuge (see 2.2.2.1) and sucrose density gradient centrifugation (45) were used to determine the homogeneity of DNA preparations. For density gradient centrigufation, a gradient was used, which consisted of 5% to 20% sucrose. Gradients were formed in a device constructed from perspex and consisting of two cylinders, connected as shown in Fig. 2. The diameter of the outflow was ca. 0.5 mm. With the stopcocks C and D closed, 2.4 ml of 5% sucrose was placed in chamber B and 2.4 ml of 20% sucrose in A. Both stopcocks were then opened while the solution in chamber A was mixed with a spiral platinum wire that was moved up and down by means of an electrical motor. The gradient was allowed to flow directly into a centrifuge tube, E. The freshly prepared gradient was kept in the cold room for 2 hours. Then 0.15 ml of a DNA solution having an absorbancy of 1.0 to 1.4 at 254 mμ, was carefully layered on the gradient. The centrifugation was performed in a Spinco Model L ultracentrifuge at 30,000 r.p.m. for 2.5 hours. At the end of this period the distribution of the material within the tube was determined by a method similar to that described by Brakke (46) (see Fig. 3).

2.2.2 CHARACTERISATION

2.2.2.1 Determination of the sedimentation coefficient

Concentrated DNA solutions show hydrodynamical interactions (47). Extrapolation to zero concentration thus becomes necessary if correct values of the sedimentation coefficients are to be determined. To make the extrapolation practicable, very dilute solutions have to be used. At such high dilutions however, refractive optical methods are not able to detect changes of concentration. This necessitates the use of a more sensitive method based, for instance, on the high absorptivity of DNA in the ultra-violet region.

In this work, sedimentation coefficients were determined in a Spinco Model E ultracentrifuge equipped with a RTIC temperature control system. Absorption optics were employed, using ultra-violet transmitting filters. In later determinations a monochromator was used. Due to resonance absorption, by the mercury vapour and absorption by the glass envelope the 253.7 mμ line from the H85A3 light source is rather weak (48). Very long exposure times (70-90 sec) were thus necessary when the monochromator was used. By making use of the 265.2 mμ line, satisfactory exposure times (7-8 sec) comparable to those obtained with the filters, were obtained. All determinations were done with a 12 mm path length cell with aluminium centre piece

in an An-D rotor. The filling hole of the centre piece was drilled out to accommodate a 17 gauge hypodermic needle. The sedimentation pattern, however, did not differ from that obtained when a 22 gauge needle was used. To minimise shear forces (36) the DNA solution was allowed to flow into the cell, over a period of one minute or more.

The speed of rotation during the experiments was obtained from the odometer readings, The speed setting for all the determinations was 29,000 r.p.m., and the rotor temperature was adjusted as close as possible to 20°C. For each experiment, nine photographs were taken at 4 min intervals, using Kodak commercial films, (see Plate 1). The photographic records of the sedimenting boundaries were transcribed into density versus distance plots with a Beckman Analytrol type R6 microdensitometer.

The enlargement factor was determined with a calibration cell and was found to be 8.503. Fig. 4 shows a typical tracing obtained from a velocity sedimentation experiment, using a concentration of 40 µg/ml. Such a solution has an absorbancy of 0.85 at 254 mµ. This produced a 4 cm deflection on the tracing. Since a deflection of only 1 cm can be used for accurate measurements, satisfactory results could be obtained even at concentrations of about 10 µg/ml only. DNA samples were studied at concentrations between 11.8 and 45 µg/ml in saline-citrate. Sedimentation coefficients were calculated from the position of the boundary corresponding to the midpoint of the concentration gradient (49).

To facilitate comparison of experimental values of the sedimentation coefficients, measured with different solvents or at various temperatures, the results were converted to a standard basis corresponding to a reference solvent, having the viscosity and density of water at 20°C. The effect of pressure, on the density and viscosity was not considered as aqueous solvents were used. Corrections for radial dilution were found to be insignificant. An example of such a calculation is given in Appendix 1. Sedimentation coefficients of whole, half and quarter molecules at various concentrations are summarised in Table 4. The half molecules were obtained when 1 M NaCl was used for extraction while quarter molecules were produced as a result of prolonged shaking during removal of RNA with charcoal.

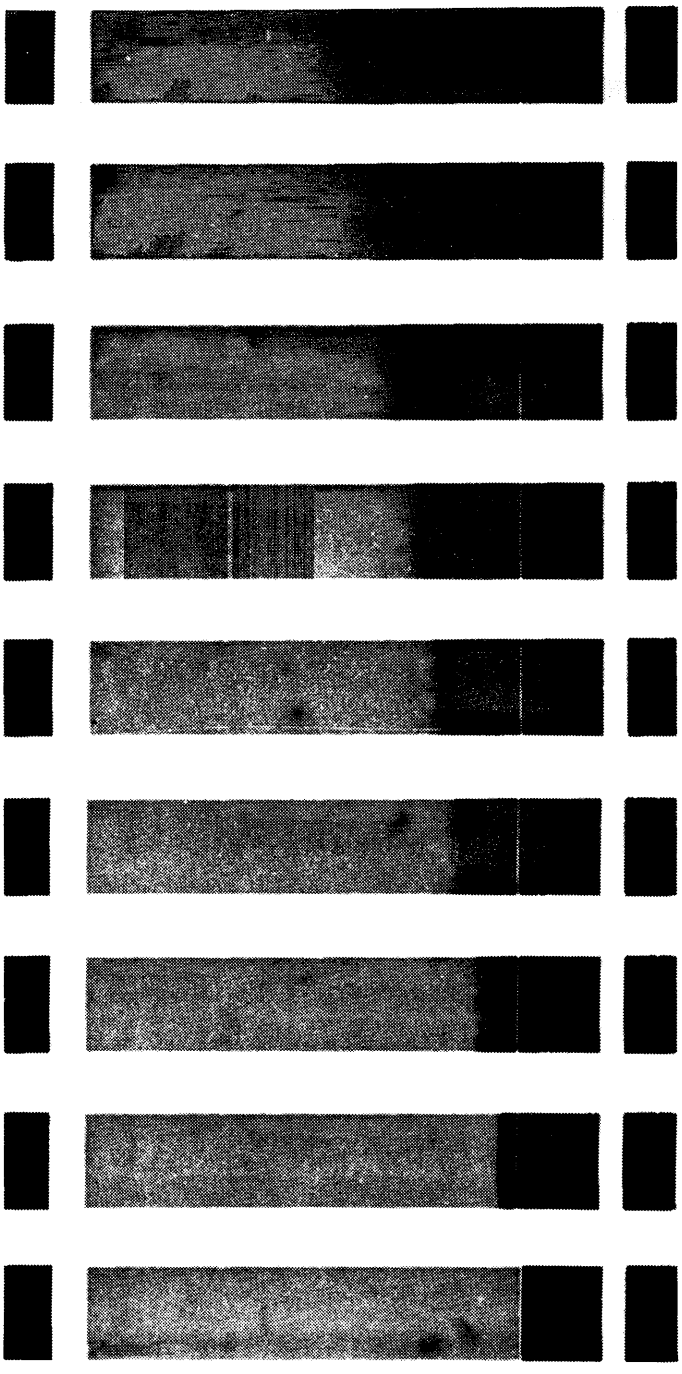


Plate 1. Photographic exposures taken during a sedimentation determination of DNA. The concentration was 40 µg/ml, the rotorspeed 29,000 r.p.m. and the exposure time, 8 sec.

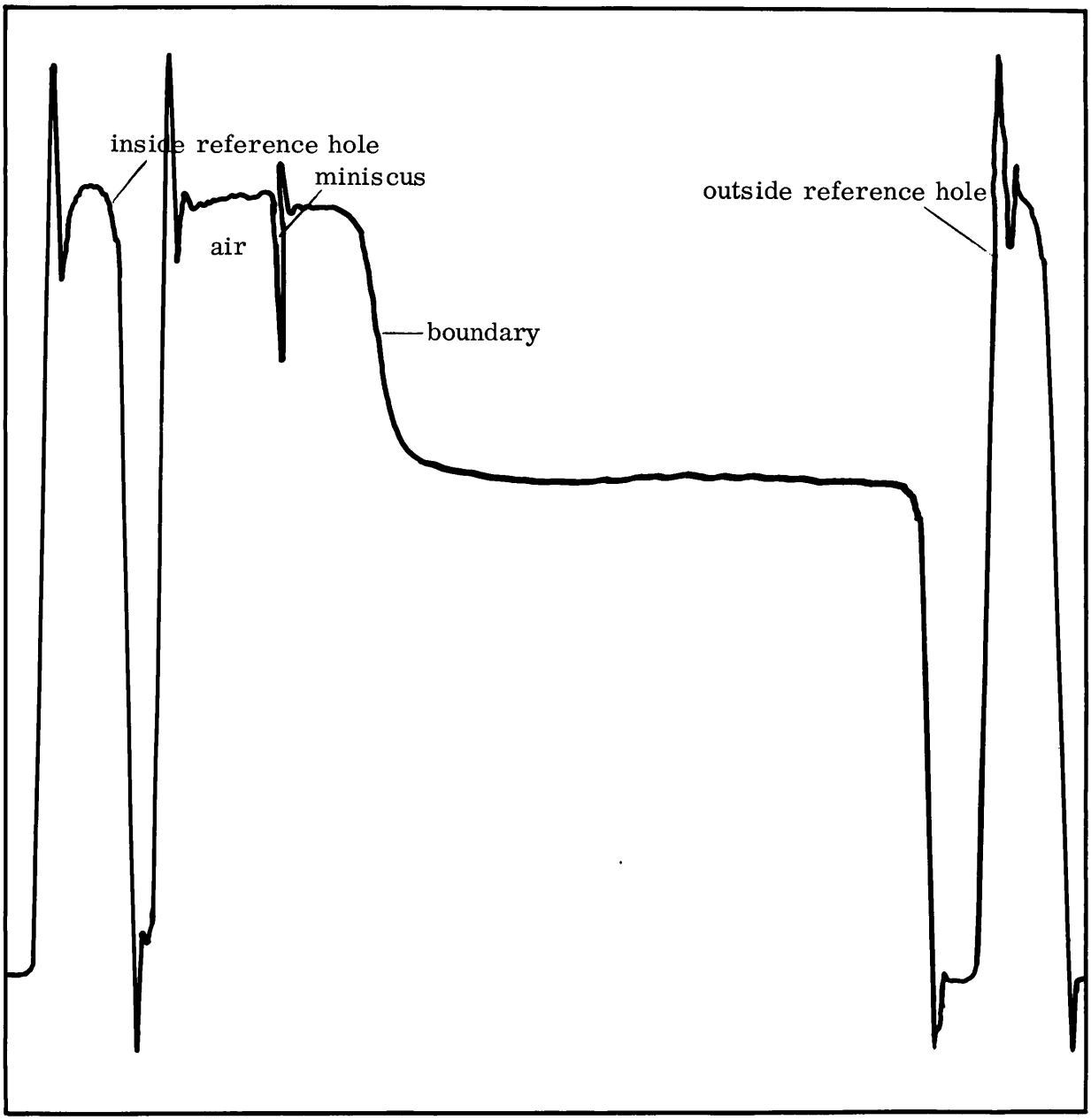


Fig. 4. Typical densitometer tracing of a photograph taken after 8 min during a sedimentation determination of DNA. The concentration was 40 µg/ml, rotorspeed 29,000 r.p.m. and the exposure time 8 sec.

TABLE 4 SEDIMENTATION COEFFICIENTS AT VARIOUS CONCENTRATIONS OF WHOLE, HALF AND QUARTER MOLECULES FROM P. MIRABILIS (13/S suc$^+$)

Whole molecules		Half molecules		Quarter molecules	
Concentration µg/ml	$S_{20,w} \times 10^{13}$ sec	Concentration µg/ml	$S_{20,w} \times 10^{13}$ sec	Concentration µg/ml	$S_{20,w} \times 10^{13}$ sec
15.1	43.2	20.2	26.3	11.8	23.4
21.0	38.1	25.8	23.5	25.1	20.5
23.2	37.2	31.0	21.5	34.9	18.8
28.5	34.5	36.0	20.1	40.0	17.5
37.5	32.5	45.2	18.7	45.3	17.2
40.0	31.3				
42.5	30.0				

The data in Table 4 are plotted in Fig. 5 which shows the marked dependence of the sedimentation coefficient on concentration. A plot which may also be used to determine $S^o_{20,w}$ is that of $^1/S_{20,w}$ versus C (see Fig. 6). The values of $S^o_{20,w}$, obtained from Fig. 5, for whole, half and quarter molecules are summarised in Table 5.

TABLE 5 $S^o_{20,w}$ FOR WHOLE, HALF AND QUARTER DNA MOLECULES

Type of DNA	$S^o_{20,w}$
Whole molecules	62.5
Half molecules	42.0
Quarter molecules	29.0

A comparison of $S^o_{20,w}$ of DNA from various sources is presented in Table 6.

TABLE 6 EXAMPLES OF $S^o_{20,w}$ FOR DNA FROM VARIOUS SOURCES

Source	$S^{\hat{o}}_{20,w}$	Reference
Proteus mirabilis (13/S suc$^+$)	62.5	This investigation
Coli phage T2	29.5	50
Coli phage T2	63.0	51
Calf Thymus	14.8	52
Calf Thymus	29.0	53
Escherichia coli	33.3	54
Diplococcus pneumoniae	25.8	55

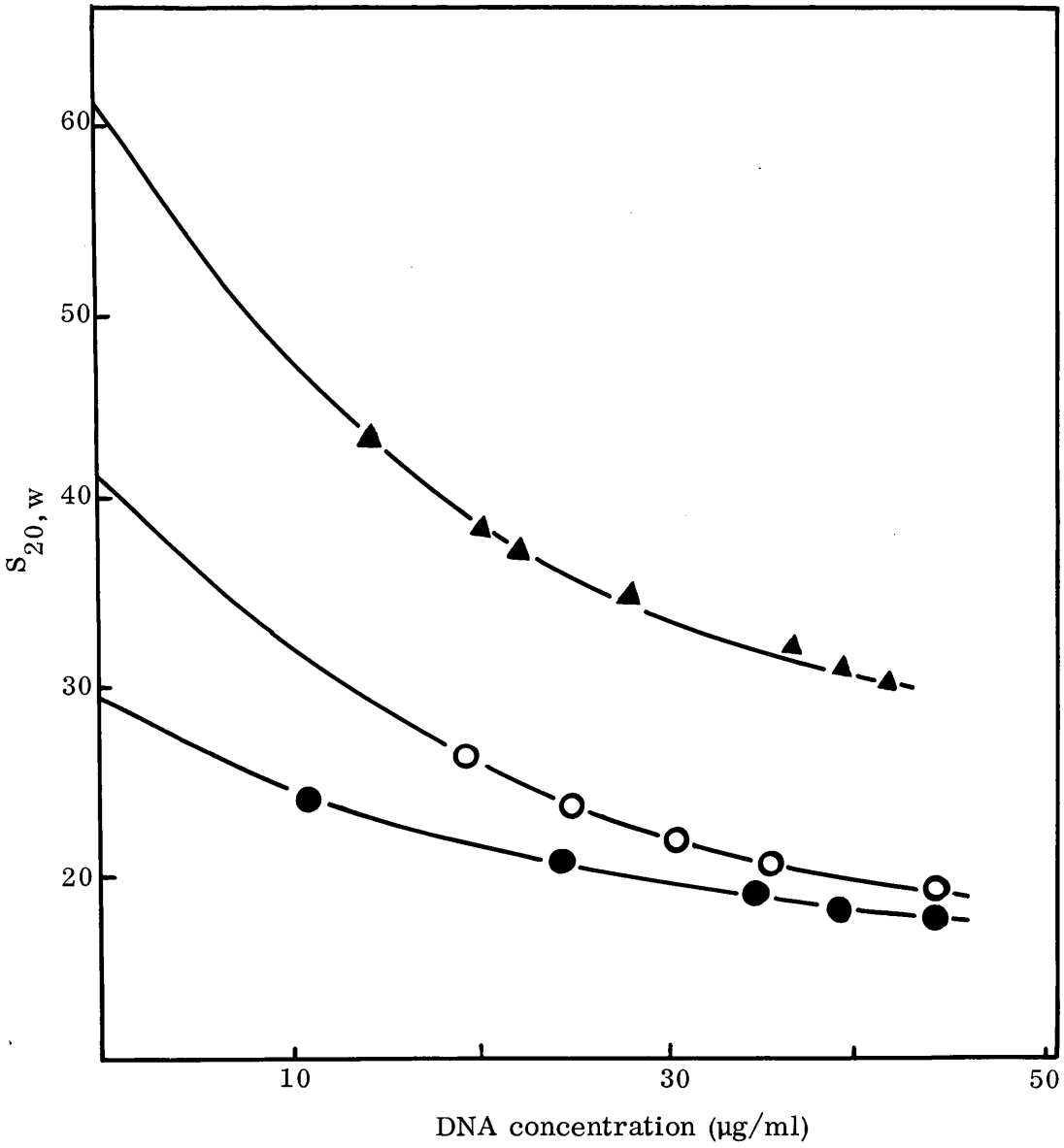


Fig. 5. Determination of the limit sedimentation coefficient ($S^{o}_{20,w}$) of whole, half and quarter DNA molecules from P.mirabilis (13/S suc^{+}). The whole, half and quarter molecules are represented by ▲ , O and ● , respectively.

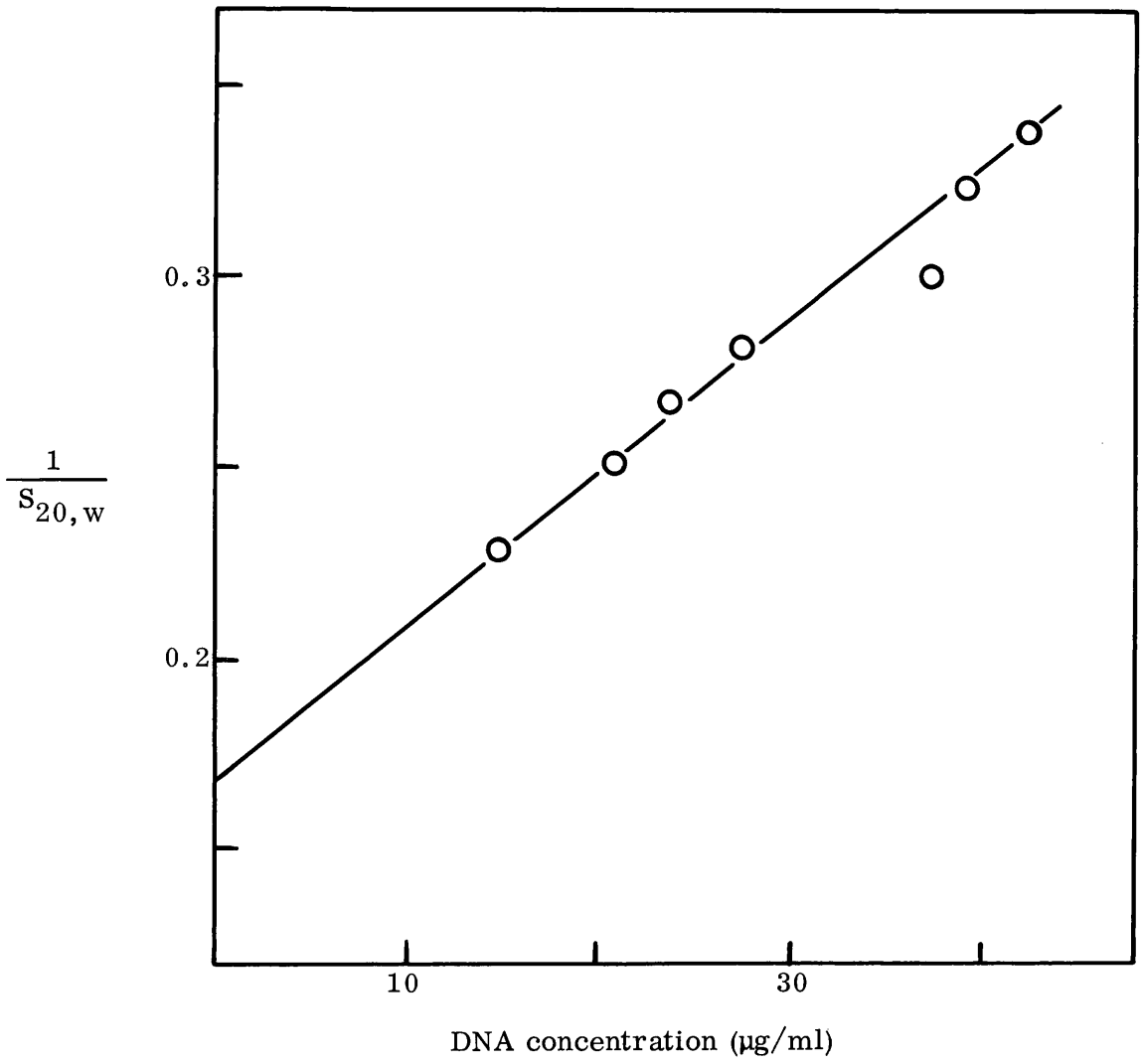


Fig. 6. Example of a plot, $1/S_{20,w}$ versus concentration. The $S_{20,w}$ values are those for whole molecules, measured at the indicated concentration.

2.2.2.2 Determination of the diffusion coefficient

Many workers have experienced difficulty in the determination of the diffusion coefficient of DNA. Extensive investigations were made by Cecil and Ogston (56) and Kahler (57), using the Gouy and Schlieren methods. Both these methods require relatively concentrated solutions and are suitable for use with proteins. However, in the case of DNA, where low concentrations are essential for the elimination of hydrodynamical and coulombian interactions neither the Gouy nor Schlieren methods are satisfactory.

Diffusion coefficient determinations based on the boundary spreading in sedimentation velocity experiments (58), are unsatisfactory. This is due to the marked dependence of the sedimentation on concentration, which influences the shape of the boundary. The equation, derived by Fujita (59) which accounts for this concentration dependence, also proved to be inadequate, since equation 1 was found to be non-linear (see Fig. 7).

$$S_{20,w} = S^{o}_{20,w} (1 - kc) \qquad (1)$$

Analysis of the boundary spreading observed during a prolonged low-speed centrifugation was eventually used for the determination of the diffusion coefficient (60). Ultra-violet optics were employed, which enables much lower concentrations to be investigated. Of consequence too, is the fact that the boundary shape is less influenced by sedimentation at low speeds.

All determinations were performed in the Model E analytical ultracentrifuge. Since low-speed centrifugation is necessary, the heavier An-J rotor was employed to eliminate precession. A standard 12 mm path length cell with aluminium centre piece was used in all the determinations. Convective disturbances, due to temperature fluctuations were eliminated by performing the experiments at 5^{o}C. Whole molecules of DNA were studied at three different concentrations (22.3, 33.0 and 47.5 µg/ml) in saline-citrate.

A permanent plateau region between the miniscus and the boundary was first established by a short period of high-speed centrifugation (17,980 r.p.m.). To ascertain the position of the boundary, photographs were taken when the rotor had attained the high speed setting. After the boundary had moved about 0.5 mm from the miniscus, the rotor was decelerated to 4,059 r.p.m. To prevent deterioration of the boundary the "slow brake" setting was used.

The first photograph used for the diffusion coefficient determination was taken about 40 min after the low speed setting had been reached.

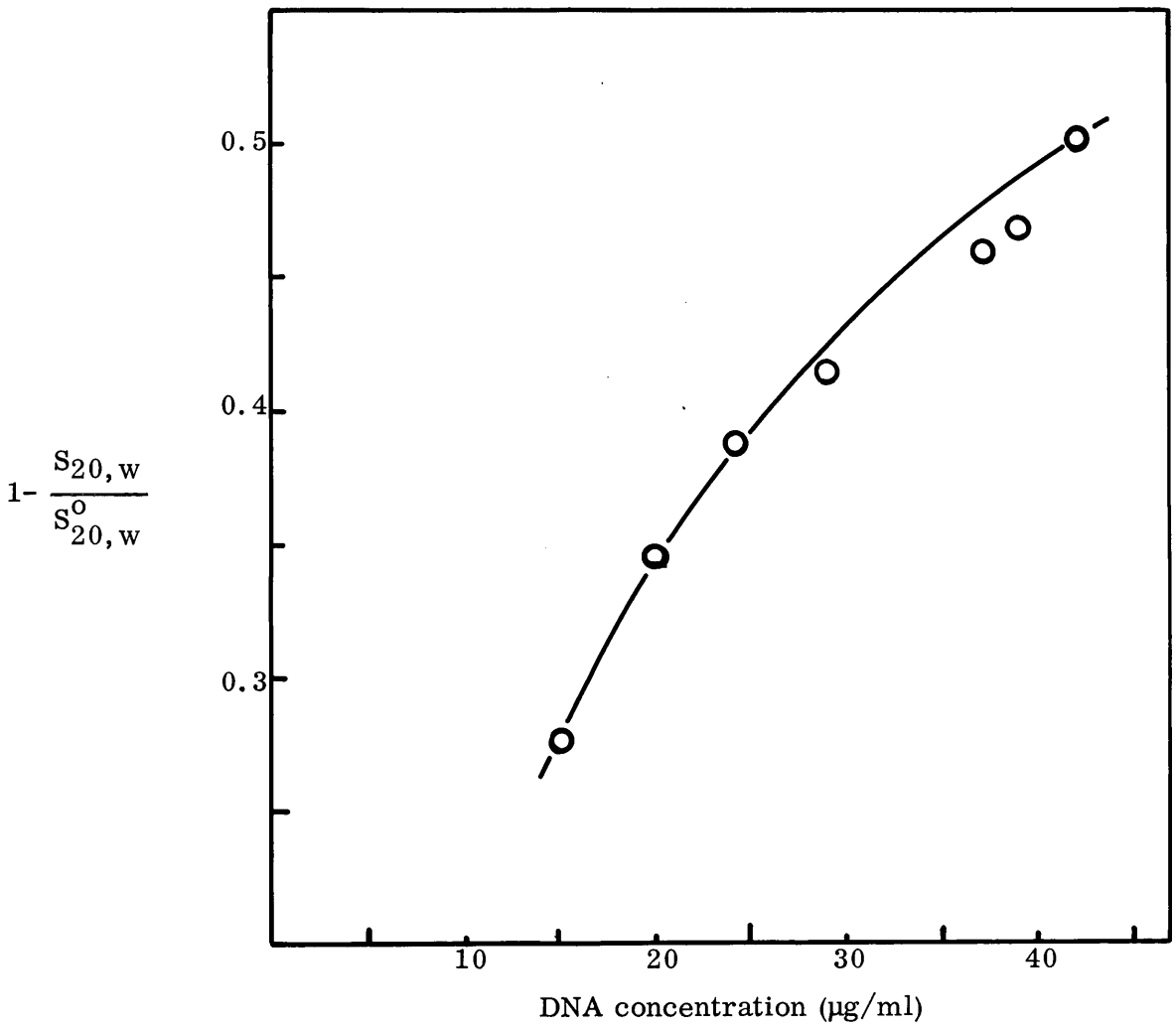


Fig. 7. Plot of $(1-S_{20,w}/S^{o}_{20,w})$ versus concentration to illustrate the invalidity of Fujita's equation.

Thereafter photographic exposures were made at 64 min intervals during the course of the low-speed centrifugation. Photographic records were converted into density versus distance plots with a recording densitometer (see Fig. 8).

Diffusion coefficients were calculated from the spreading of the boundaries and the equations for a homogeneous boundary diffusing in a centrifugal field (61).

$$D = \frac{\bar{u}^2 \, (1 - S^o_{20,w}\omega^2 t)}{4y^2 t} \qquad (2)$$

$$\frac{C}{C_o} = \tfrac{1}{2}\left[1 - \frac{2}{\sqrt{\pi}} \int_o^y e^{-y^2} \, dy \right] \qquad (3)$$

In equation 2, $\bar{u}$ is the mean distance in cm, at a time t, from a level in the boundary where the concentration ratio, C/C_o, is 0.5 to the equidistant levels with concentration ratios, C/C_o, of 0.2 and 0.8, respectively. The factor y may be obtained from tables giving the numerical values for the function

$$\phi(y) = \frac{2}{\sqrt{\pi}} \int_o^y e^{-y^2} dy = 1 - \frac{2C}{C_o}$$

for definite values of C/C_o (61). The influence of a centrifugal field on the diffusion process is given by $(1 - S^o_{20,w}\,\omega^2 t)$. In this factor $S^o_{20,w}$ is the sedimentation coefficient measured at low speed. The sedimentation coefficients were found to be much lower than those measured at 29,000 r.p.m., where the diffusion is negligible; ω is the angular velocity and t the time from the start of the experiment. Zero time was taken at the precise moment the rotor was taken to high speed. Values of $\bar{u}$ were determined from the photodensitometer tracings. An example of a diffusion coefficient calculation is given in Appendix 2. All the diffusion coefficients were corrected to $D_{20,w}$. Values of $D_{20,w}$ at different concentrations are given in Table 7.

TABLE 7 VALUES OF $D_{20,w}$ AT DIFFERENT CONCENTRATIONS

Concentration µg/ml	$D_{20,w} \times 10^7$ cm^2/sec
22.3	0.242
33.0	0.263
47.5	0.254

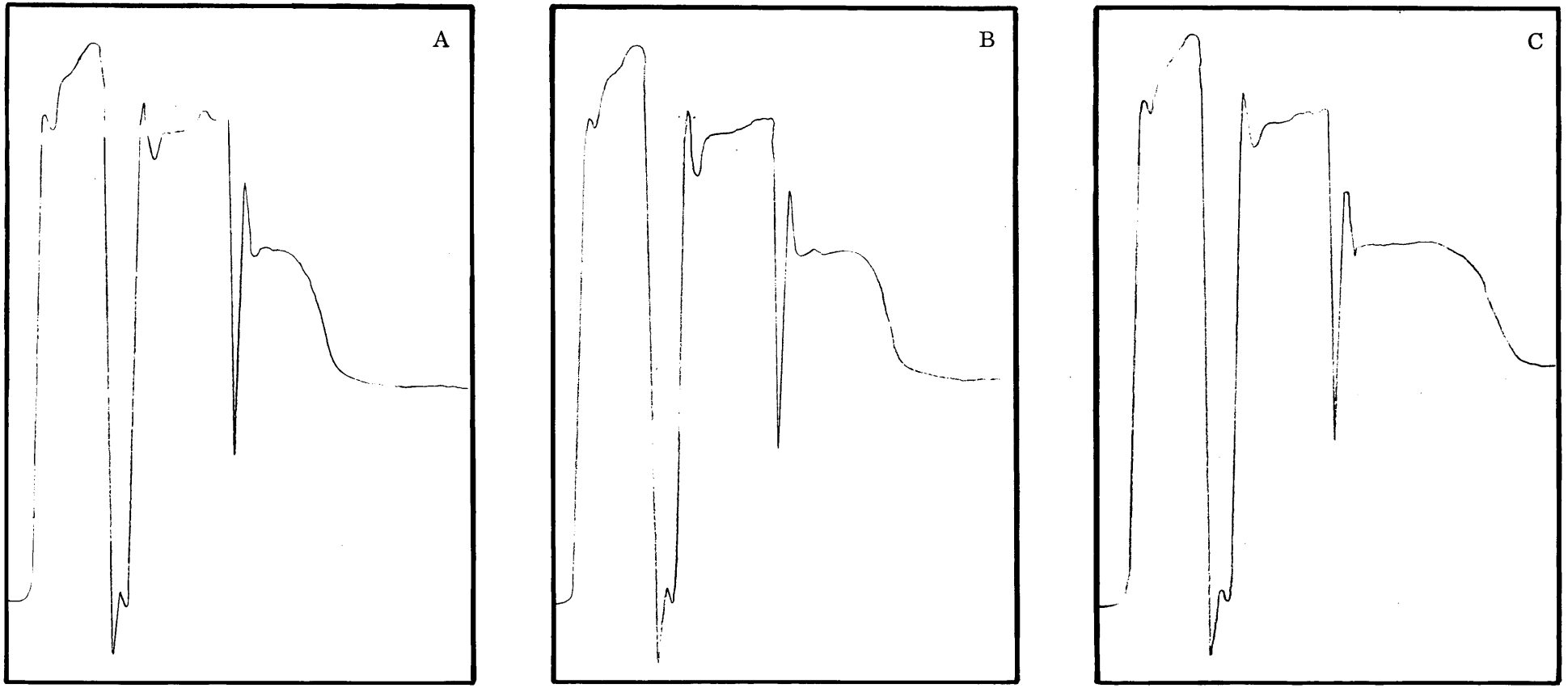


Fig. 8. Ultra-violet absorption patterns of DNA during a diffusion experiment at 4,079 r.p.m. The tracings show boundary spreading at different time intervals; A after 174 min, B after 495 min and C after 1198 min.

$D_{20,w}$ appears to be independent of concentration. The average value was thus calculated as $D_{20,w} = 0.253 \times 10^{-7}$ cm^2/sec.

2.2.2.3 Determination of the intrinsic viscosity

Viscosity determinations are important in the study of high polymers. Using simple viscosity measurements, certain qualitative conclusions may be made as to the general form of the macromolecules in solution.

Viscosity data alone are difficult to interpret since the effective hydrodynamic volume (62) depends on the configuration of the macromolecule in solution which, in turn, is influenced by polymer-solvent interactions. A solution in which the polymer molecule is loosely extended has a higher intrinsic viscosity than a solution in which the polymer molecules attract each other more strongly than they attract solvent molecules.

Einstein (63) showed that the viscosity η of a dilute suspension of small rigid spheres is given by equation 4.

$$\eta = \eta_o \left(1 + \frac{5}{2}\phi\right) \text{ or } \left(\frac{\eta/\eta_o - 1}{\phi}\right) = \frac{\eta_{sp}}{\phi} = 5/2 \qquad (4)$$

where η_o = viscosity of the solvent, ϕ = total volume of spheres / ml of suspension and η_{sp} = specific viscosity.

Thus the specific viscosity of a suspension of spherical particles is equal to 2.5 times the true volume fraction of the particles. If these particles were composed of 80% by volume of water the volume fraction of the solid matter would be only $\frac{1}{5}$ of the true volume fraction. Therefore, the intrinsic viscosity would be equal to 12.5, when concentration is expressed as volume fraction of solid matter. It is not common to find biological materials composed of very much more than 80% by volume of water. Hence one should not expect intrinsic viscosities of spherical biological materials to have values much greater than 12.5. Deoxyribonucleic acids, however, have intrinsic viscosities which are more than ten times this value. Today it is generally believed that the high intrinsic viscosities are caused by extreme departure of these molecules from the spherical shape (64).

It is not always possible to calculate the volume occupied by a polymer in an unambiguous manner. Therefore, concentrations are generally expressed in terms of weight of polymer per unit volume (usually 100 ml). When the η_{sp}/c ratio varies with the concentration, it is necessary to extra-

polate η sp/c to zero concentration, to obtain the intrinsic viscosity, $[\eta]$.

$$[\eta] = \lim_{C \to o} \frac{\eta_{sp}}{C} = \lim_{C \to o} \frac{1}{C} \ln \left(\frac{\eta}{\eta_o}\right) \qquad (5)$$

where C is the solute concentration in g/dl. By expanding the logarithmic function ln (η/η_o) as an infinite series, it can be shown that η sp/C and 1/C ln (η/η_o) extrapolate to the same limit at zero concentration.

$$\ln \eta/\eta_o = \ln (1 + \eta_{sp}) = \eta_{sp} - \frac{\eta^2_{sp}}{2} + \ldots . \qquad (6)$$

In this series the second and higher order terms in η sp become negligible compared to the first as the concentration approaches zero and the functions in equation 5 thus extrapolate to the same limit.

In this investigation, viscosities were measured at 20°C in a Zimm-Crothers model A low shear rotating cylinder-type viscometer (65, 66). Measurements were made at a shear stress of 1.8×10^{-3} dyne/cm^2 on 4 different DNA solutions, the concentrations of which ranged from 1.5 to 2.8×10^{-3} g/dl. The solvent was saline-citrate. The intrinsic viscosity of the DNA was obtained by plotting η sp/C and (1/C) ln (η/η_o) versus C (see Table 8 and Fig. 9). The advantage of the double extrapolation is that the intercept may be determined more precisely than by using only one straight line.

TABLE 8 RESULTS OF THE VISCOSITY MEASUREMENTS ON DNA (THE DATA ARE PLOTTED IN FIG. 9)

$C \times 10^3$ g/dl	η/η_o	$\eta/\eta_o - 1$	$\frac{1}{C} \ln \eta/\eta_o$	$\frac{\eta_{sp}}{c}$ dl/g
1.50	1.398	0.398	223.5	265.4
2.35	1.617	0.617	204.6	262.5
2.55	1.670	0.670	201.2	262.8
2.80	1.734	0.734	196.5	262.1

The viscosity observations on DNA can be represented by equation 7 (67).

$$\eta_{sp/c} = [\eta] + k'[\eta]^2 C \qquad (7)$$

where C is the DNA concentration in g/dl and $[\eta]$ is the intrinsic viscosity in dl/g and k' is the Huggins constant. The ratio η sp/C was found to be independent of concentration in the range used in this work and consequently

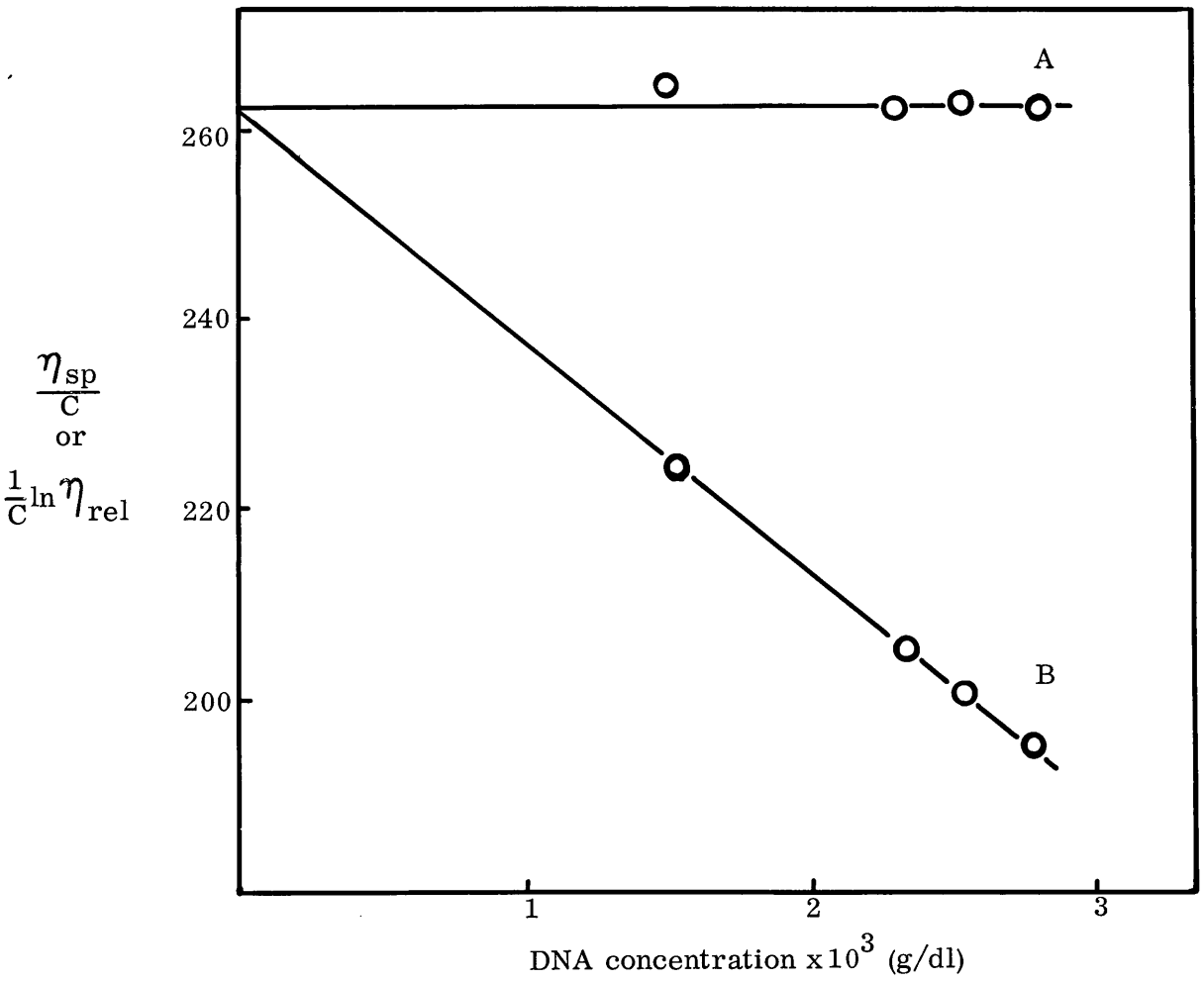


Fig. 9. Ratio of specific viscosity, η_{sp}, or logarithm of relative viscosity, η_{rel}, to concentration, plotted against concentration, C (see Table 8). The plots are indicated by A and B, respectively.

the Huggins constant equals zero and therefore $\eta_{sp/C} = [\eta]$.

2.2.2.4. Determination of the partial specific volume.

The partial specific volume of a solute may be defined as the volume increase suffered by a very large volume of solution upon the addition of 1g of solute. A useful way of expressing the partial specific volume is

$$\text{Partial specific volume} = \frac{dV}{dw}$$

where dV is the infinitesimal increase in the volume of a solution due to the addition of an infinitesimal weight of the solute. When the partial specific volume is independent of the concentration, the apparent partial specific volume may be used instead of the true partial specific volume (49). The apparent partial specific volume of the solute may be determined directly from density measurements, both on solution, of known concentration, and on the solvent. A variety of procedures exist for determining solution densities (49, 68).

In this work, densities were obtained through the use of a density gradient column consisting of bromobenzene and white kerosene (69). This method requires only very small volumes of solution and by selecting a suitable gradient, relatively low concentrations are required. Both the bromobenzene (refractive index : 1.559-1.561 and weight/ml at 20°C : 1.49 - 1.50g) and the kerosene were saturated with water by shaking 5 times with 2 volumes of distilled water in a separating funnel, followed by storage overnight over anhydrous calcium chloride. This saturation of the liquids is necessary to prevent evaporation of water from the solutions, the densities of which are to be determined. After filtering the liquids through filter paper, the lower and upper phases of the gradient were prepared. The lighter solution was carefully layered over 190 ml of the heavier one in a cylinderical column (see Fig. 10) up to a suitable height above the upper bulb. After three hours, the two solutions were partially mixed by gentle stirring with a paddle glass rod, so as to produce a linear density gradient. The column was then allowed to stand for 48 hours in order to smooth out any abrupt density gradients by diffusion. Since the gradients can be disrupted by temperature fluctuations, the columns were kept in a water bath, the temperature of which was held constant at 20°C $\pm$ 0.001 °C (see Fig. 10). The linearity of the column was tested with suitable potassium chloride (KCl) solutions. Analytical grade KCl solutions with S.G. of 1.0046, 1.0110 and 1.0239, respectively, were used. Drops (0.2 ml) of these solutions were delivered by means

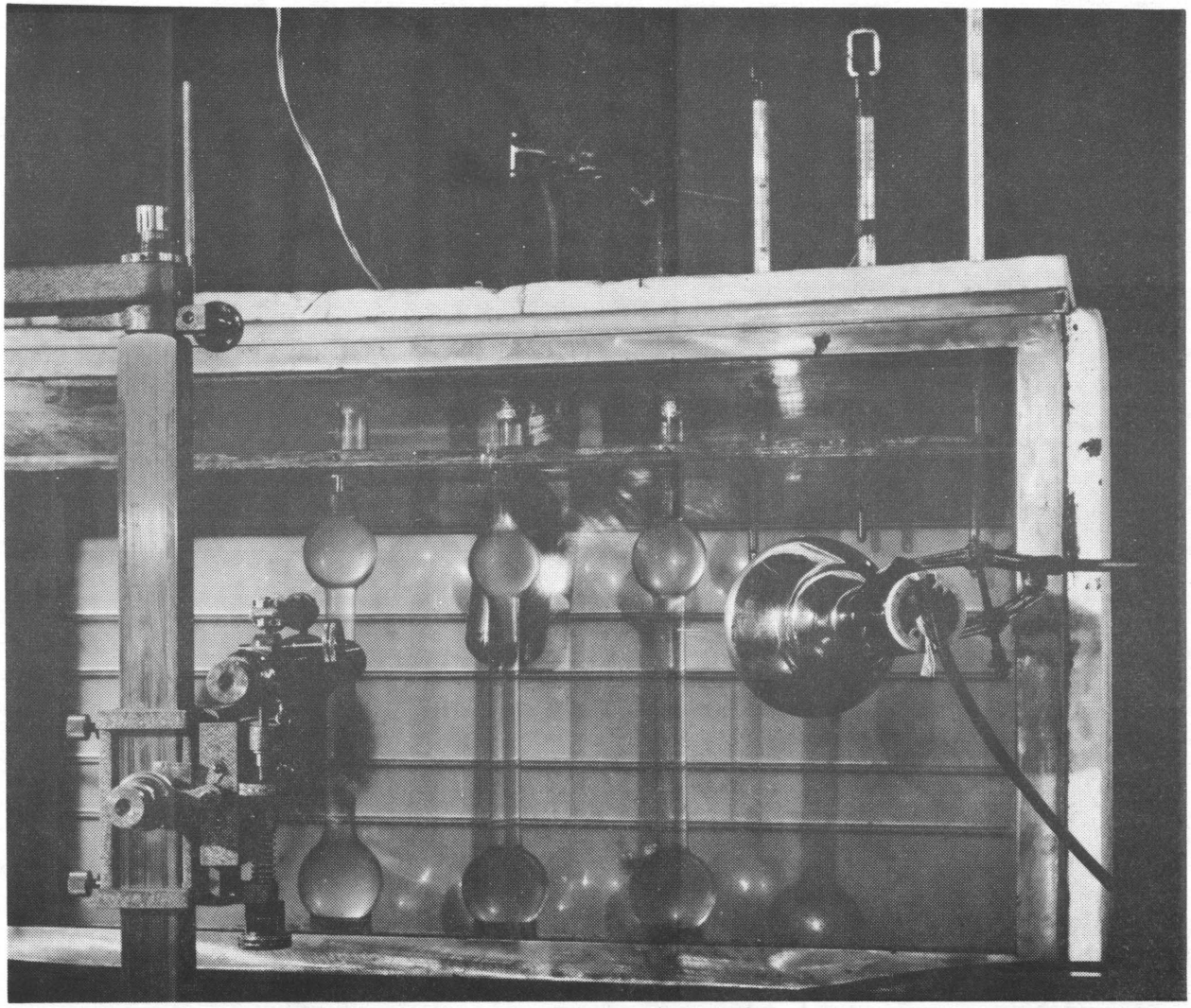


Fig. 10. Apparatus used for the determination of the partial specific volume of DNA.

of a hypodermic syringe, first 3 drops of the heavier standard, followed by 3 drops each of the less denser solutions. The position of the drops, after reaching equilibrium, which requires about 20 min, were determined with a cathetometer. The measurements were made from a reference mark made immediately below the upper bulb of the column.

Several gradients were tested (See Table 9). Plots of density versus height, which is the distance between the reference mark and the position of the drops, were made to check the gradients (see Fig. 11).

TABLE 9 COMPOSITION OF 3 DIFFERENT GRADIENTS A, B AND C

	Bromobenzene			Kerosene		
	A	B	C	A	B	C
Lighter phase	70	75	70	180	175	180
Heavy phase	100	95	90	150	155	160

The gradient with the smallest slope, viz. B, was selected for use, because larger height differences between drops could be obtained. This type of gradient is especially suitable for very low solute concentrations. The drops were removed by means of a thin glass rod with a small piece of damp filter paper attached to the tip.

The density of a given unknown solution d, was calculated by use of equation 8 (70).

$$d = d_1 + \frac{h - h_1}{h_2 - h_1} (d_2 - d_1) \qquad (8)$$

where h, h_1, h_2 are, respectively, the positions of the unknown drop, the lower density standard, d_1 and the higher density standard, d_2. Both the density of the DNA solution and solvent were determined in this manner. Potassium chloride solutions of densities 1.0046 (d_1) and 1.0110 (d_2) were used as the lower and higher density standards, respectively. The solvent was 0.15 M NaCl.

The apparent specific volume was determined by substituting in equation 9.

$$Vapp = \frac{1}{do} - \frac{1}{x}\left(\frac{d - do}{do}\right) \qquad (9)$$

where d and do are the densities of solution and solvent respectively and x is the concentration of DNA in g/ml. The apparent specific volume of 3 DNA solutions of different concentrations was determined. Values obtained showed an independent relationship with respect to concentration (see Table 10).

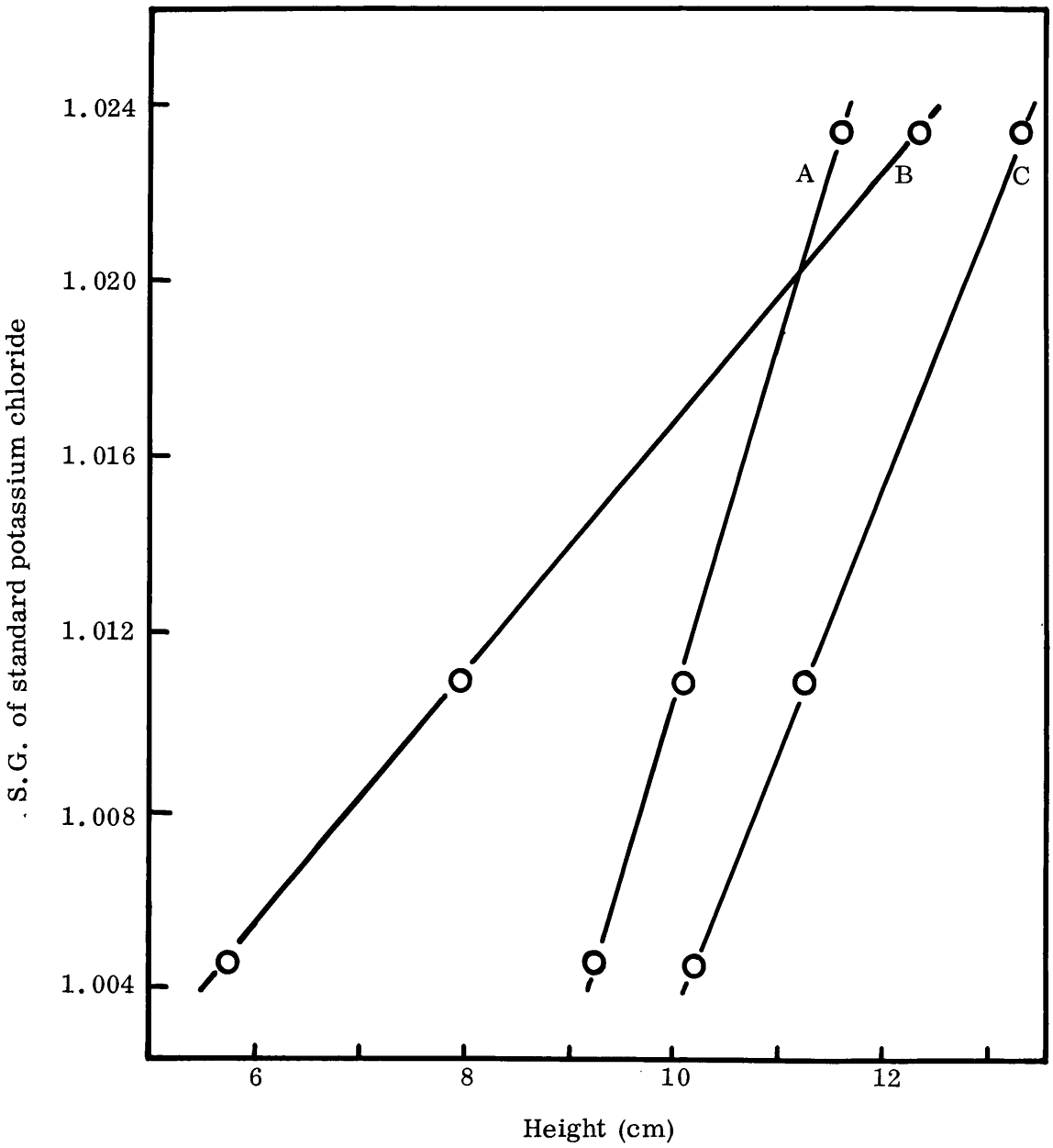


Fig. 11. Plot of S.G. of standard potassium chloride solutions versus their positions in the column in order to test the linearity and steepness of the gradients. The composition of the gradients shown are given in Table 9.

TABLE 10 VALUES OF Vapp FOR DIFFERENT DNA CONCENTRATIONS

DNA Concentration g/ml	Vapp
0.00198	0.5638
0.00132	0.5626
0.00099	0.5559

The average value for Vapp is 0.5608

2.2.2.5 Ultra-violet absorption

Ultra-violet light is strongly absorbed by nucleic acids (71). This property may be utilised in sedimentation, diffusion, concentration and nitrogen base determinations.

The absorbancy is markedly influenced by the concentration of the nucleic acid, its degree of nativeness, temperature, salt content and pH of the solution (72, 73). Furthermore, the spectra of high polymers of DNA are not simply the sum of the respective monomeric units (74). This observation is somewhat unexpected, for when chromophores are joined by saturated links, these links act as insulators of the π electrons and the two chromophores act independently. Thus, the maximum absorption of diphenylethylene is closely represented by twice that of methylbenzene at the same molar concentration (74).

Compounds are known however, of the structure described above, which show distinct spectroscopic evidence of interaction between the chromophores. In these compounds, the resonators are close enough to permit the π electron clouds to interact and thus alter the spectra of both chromophores. In the DNA molecule, the bases are stacked parallel at short distances (75), which makes overlapping of π orbitals possible. During denaturation however, the chromophores become too far apart and electron overlapping is prevented. Another factor to be considered is a change in the total effective area of the chromophoric groups, presented to the light beam. In the DNA molecule, the nitrogen bases are shielded by the sugar and phosphorus groups (76). The effective chromophoric area would thus be expected to be less than for a random array of monomeric units.

Because of the variable water content of nucleic acids, Chargaff and Zamenhoff (77) suggested an atomic absorptivity with respect to phorphorus at 259 mμ, E(P), rather than a molar absorptivity. The E(P) is defined as

$$E(P) = A / (c \times d)$$

where A is the absorbancy of the solution, C is its concentration in g atoms of phosphorus per litre and d is the optical path length. Native DNA has a E(P) value of about 6.5×10^3 whereas DNA, denatured by heat, acid or other means, exhibit values which are considerably higher (78).

Much information may be obtained from ultra-violet spectra of DNA with respect to purity and state of denaturation. Spectral data may be presented in various ways (74). A plot of absorbancy versus wavelength may be obtained with commercial instruments such as the Beckman DK2A ratio recording instrument, which was used in this work. A plot of great usefulness in the qualitative identification and comparison of curves is the log absorbancy versus wavelength plot. Homogeneous, highly polymerised DNA preparations studied in this work, showed identical shaped spectra regardless of concentration when plotted in this way (see Fig. 12), whereas the spectra of DNA preparations contaminated with RNA differed in shape (see Fig. 13).

The absorbancy versus wavelength plot for pure, highly polymerised DNA had the following characteristics: $\lambda_{max}^{\text{saline-citrate}}$ 258 mμ, $\lambda_{min}^{\text{saline-citrate}}$ 235 mμ and $A\frac{max.}{trough}$ 1.6. These characteristics remained constant for different isolations. Depending on the degree of RNA contamination, the $A\frac{max.}{trough}$ increased, λ min shifted to a shorter wavelength (a hipsochromic shift) and the λ max to a longer wavelength (a batochromic shift). The $A\frac{max.}{trough}$ represents the ratio of the absorbancies at λ max. (the peak) and at λ min. (the trough). The usefullness of these characteristics thus immediately becomes apparent.

2.2.2.6 Nitrogen base determinations.

Qualitative determination of the nitrogen bases was achieved by thin-layer chromatographic separation of the bases after acid hydrolysis of pure DNA (79, 80).

To hydrolyse the DNA (79), 7.8 mg of dry DNA which was previously dialysed against distilled water, was weighed in a pyrex tube (9 cm x 1 cm). Then 0.5 ml of 98% formic acid was added and the tube sealed off under vacuum. The tube was then heated in an oven at 175°C for 30 min. After cooling, the tube was cautiously opened and the hydrolysate evaporated to dryness in the same tube. Aqueous N hydrochloric acid (0.2 ml) was then added and the hydrolysate dissolved. The chromatographic plates were prepared in the following way: 10g of commercial grade Avicel (microcrystalline) was suspended in 20 ml of distilled water and shaken for a few minutes until a homogeneous slurry was obtained. This was poured onto clean glass plates (8.8 cm x 13.3 cm) and dried overnight at room temperature. With a micro-pipette, 10 μl of the hydrolysate

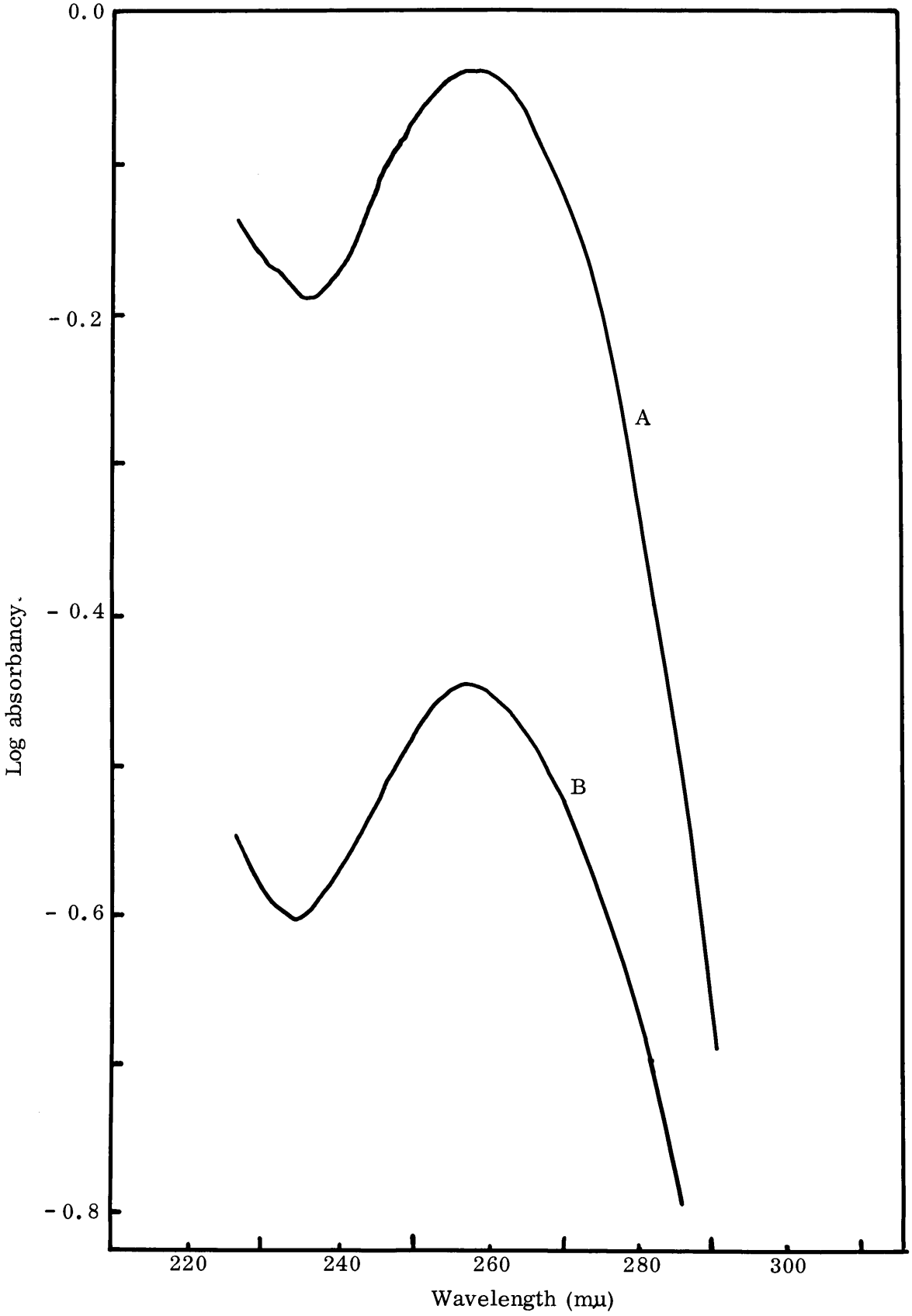


Fig. 12. Plot of the log absorbancy versus wavelength for pure, highly polymerised DNA at different concentrations; A is 42 μg/ml and B, 16.5 μg/ml.

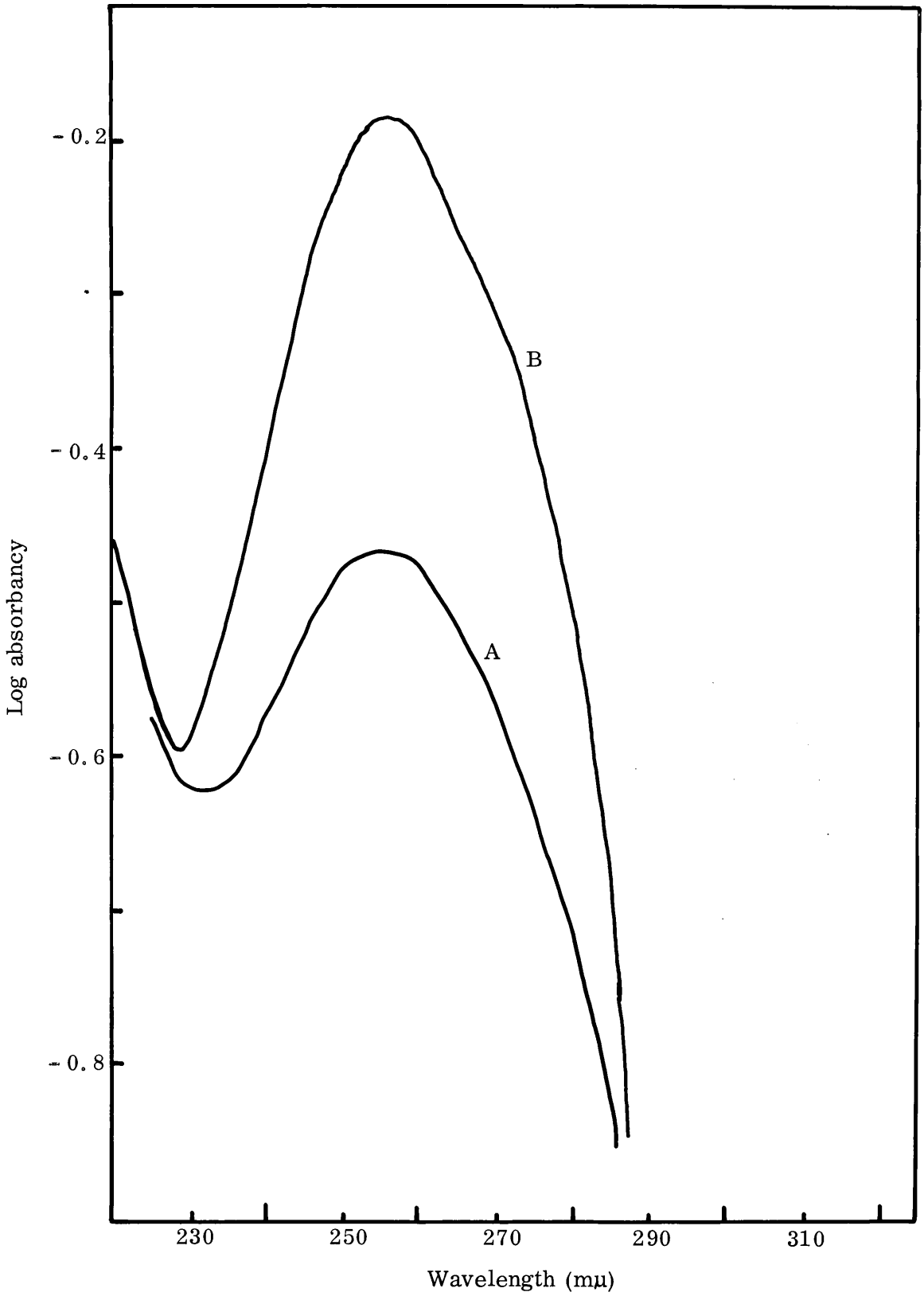


Fig. 13. Log absorbancy versus wavelength plot for pure, highly polymerised DNA (plot A) and for DNA contaminated with RNA (plot B).

was applied 2.5 cm from one end of the plate. A mixture, 10 µ moles each of adenine, guanine, cytosine and thymine, was used as standard. The chromatography was performed at room temperature in a chromatographic tank (10 cm x 10 cm x 15 cm), containing 25 ml of an aqueous solution consisting of 65% (V/V) of iso-propanol and hydrochloric acid (2.0 N in the whole volume) as the developing solvent. After the solvent had moved about 10 cm, the glass plate was removed, air-dried and the spots detected with the aid of a Fluotest unit (Quarzlampen Gesellschaft M.B.H., Hanau). Ultra-violet radiation with wavelength of 254 mµ was employed. Comparison of the hydrolysate spots with those of the standards, showed that only adenine, thymine, cytosine and guanine were present.

The base composition of the DNA, expressed in terms of percentage of guanine plus cytosine (G-C) bases, was determined from the thermal denaturation, Tm (81). The Tm value may be obtained easily by following the absorbancy at 260 mµ as a function of temperature of the DNA solution. In the determination of the DNA base composition, approximately 40 µg/ml of pure DNA in saline-citrate was placed in glass-stoppered quartz cuvettes, with 1 cm light path. The cuvettes were placed in a constant temperature cell holder of the Beckman DK2A spectrophotometer and the temperature raised by circulating water through the coil surrounding the cuvettes. Recording of absorbancy at 260 mµ was started at 25°C. The temperature was then raised in 1 to 2°C intervals, allowing about 10 min for equilibration at each temperature. No further increase in the absorbancy was recorded at temperatures above 88°C. A plot of relative absorbancy, which is the absorbancy at each temperature divided by the absorbancy at 25°C, versus temperature is shown in Fig. 14. The midpoint of the hyperchromic rise corresponds to the Tm value. The value obtained was 84.5°C, which could be reproduced within 0.5°C. The mole per cent (G-C) may be evaluated readily from the relation between the Tm and the (G-C) content, determined by other means, of DNA samples from various sources (81). The relation, when saline-citrate is the solvent, may be represented by equation 10.

$$Tm = 69.3 + 0.41\ (G-C) \qquad (10)$$

Substitution of the Tm value (84.5°C) in this equation yields a (G-C) content of 37.1 mole per cent.

The DNA base composition of Proteus organisms and other members of the Enterobacteriaceae is presented in Table 11.

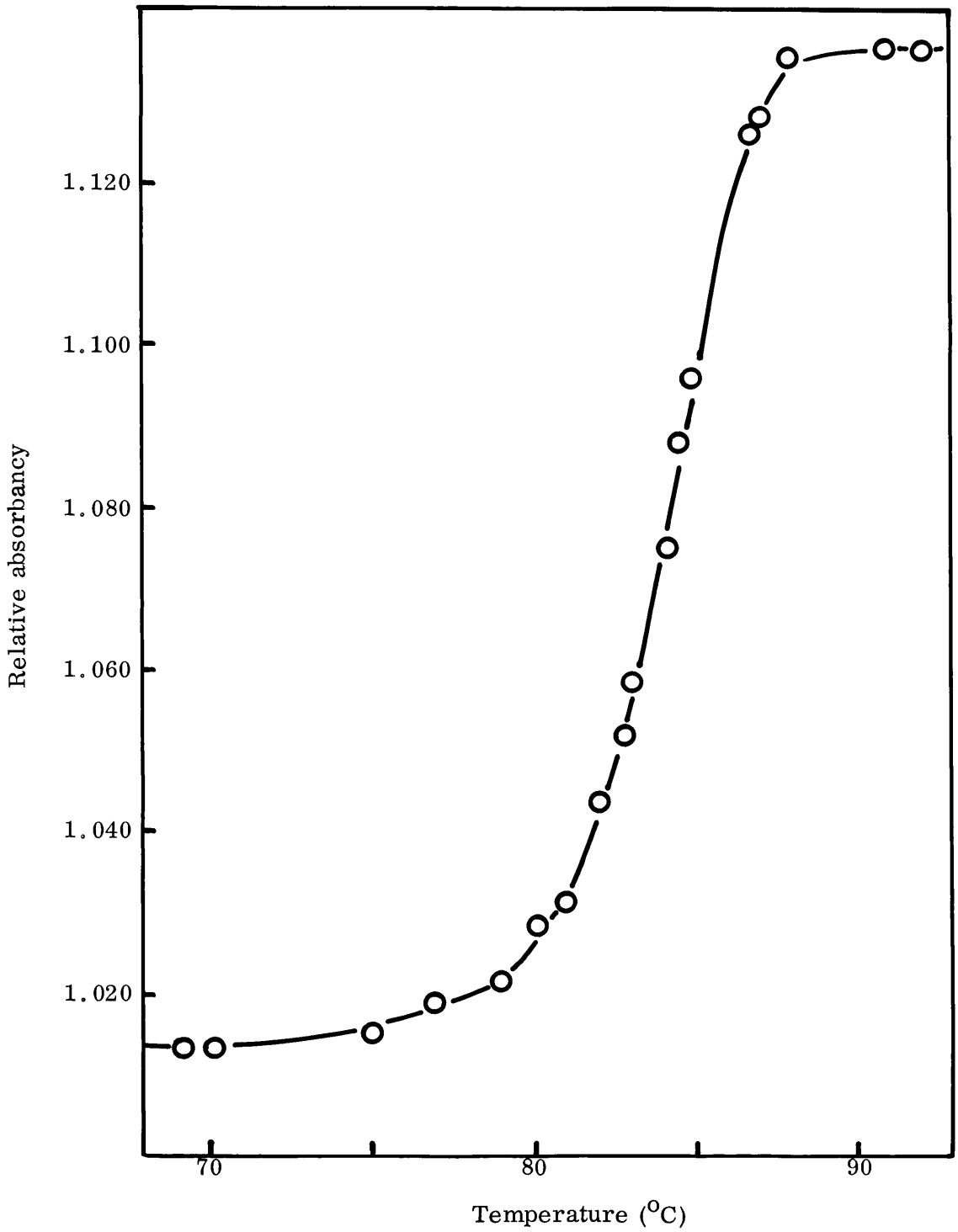


Fig. 14. Relative absorbancy versus temperature for DNA from P. mirabilis (13/S suc$^+$) for DNA concentration : 40 µg/ml; solvent : saline-citrate.

TABLE 11 DEOXYRIBONUCLEIC ACID BASE COMPOSITION OF PROTEUS, ESCHERICHIA, SALMONELLA AND SHIGELLA ORGANISMS

Organism	% (G-C)	Reference
Proteus mirabilis (13/S suc^+)	37.1	this investigation
P. vulgaris	36.5	82
P. rettgeri	39.0	83
P. morganii	54	11
Escherichia coli	52	11
Salmonella typhimurium	54	11
Shigella dysenteriae	54	11

2.2.2.7 Determination of the molecular weight

The molecular weight was calculated by substitution of the various experimentally determined, physical constants in four different equations. The molecular weights of whole, half and quarter molecules obtained from these equations are presented in Table 12. Since $D_{20,w}$, $[\eta]$ and $\overline{V}$ of the half and quarter molecules were not determined only equations 12 and 13 were used to calculate the molecular weight of these molecules.

The equations employed were the following:

(i) The Svedberg equation (61)

$$M = \frac{RTS}{D(1-\overline{V}\rho)} \qquad (11)$$

Where M is the molecular weight, R is the gas constant, T is the absolute temperature, S is the sedimentation coefficient, D is the diffusion coefficient, $\overline{V}$ is the partial specific volume and ρ is the density of the solvent.

(ii) The equation of Rubenstein, Thomas and Hershey (84)

These workers found a straight line relation between autoradiographically determined molecular weights and sedimentation for bacteriophage T2 DNA and its breakage products. This relation is given by the following equation:

$$S^{o}_{20,w} = 0.00244\ M^{0.543} \qquad (12)$$

where $S^{o}_{20,w}$ and M have the usual meaning.

(iii) The equation of Eigner and Doty (85)

An emperical relationship between the sedimentation constant and molecular weight was observed by Eigner and Doty who determined molecular

weights by means of light scattering.

$$S^{o}_{20,w} = 0.034M^{0.405} \quad .(13)$$

where $S^{o}_{20,w}$ and M have the usual meaning.

(iv) The equation of Crothers and Zimm (66)

The following equation was derived by these workers from a combination of sedimentation and viscosity data:

$$M^{2/3} = \frac{(S^{o}_{20,w} - 2.7)([\eta] + 5)^{1/3}\eta_o N}{2.27 \times 10^{19}(1 - \bar{V}\rho)} \quad (14)$$

where η_o is the solvent viscosity with density ρ, $\bar{V}$ is the partial specific volume of the DNA and N is Avogadro's number. The symbols M and $S^{o}_{20,w}$ have the usual meaning.

TABLE 12 MOLECULAR WEIGHTS OF WHOLE, HALF AND QUARTER MOLECULES OF DNA FROM P. MIRABILIS (13/S suc^{+}) CALCULATED FROM VARIOUS EQUATIONS

Type of DNA	Molecular weight x 10^{-6}			
	Eq. 11	Eq. 12	Eq. 13	Eq. 14
Whole molecules	13.66	132.0	114.3	112.0
Half molecules	-	63.0	43.15	-
Quarter molecules	-	31.92	17.22	-

CHAPTER III

DISCUSSION

The disruption of bacterial cells, in order to liberate the cell constituents, is a problem which presents many practical difficulties (86). Many techniques for disrupting bacterial cells are available but it must be stressed that successful disruption does not necessarily imply that the liberated cell constituents have biochemical properties similar to those exhibited in the intact cell. This is the result of mechanical damage caused by these techniques. A procedure which seems to be eminently suited for the preparation of DNA from bacteria is one which involves preparation of protoplasts. Once the protoplasts have been prepared, the DNA may be extracted quite easily because the protoplasts are extremely sensitive to changes in ionic strength of the surrounding medium.

Such extractions, however, may cause damage to DNA as shown by the fact that extraction with 1M NaCl resulted in degradation of the DNA. This degradation may be caused by the shrinkage of the protoplasm when the cells are placed in hypertonic medium. Apparently no degradation of the DNA occurred when 5 mM NaCl and 0.15 M NaCl were employed. The extremely low yield obtained with 0.15 M NaCl may be due to the fact that the dissociation and, thus, solubility of the deoxypentose nucleoprotein is depressed in physiological saline solutions. Evidently only slight dissociation of the nucleoprotein and subsequent release of the DNA into the aqueous phase occurred during the deproteinisation step. Consequently most DNA was lost in the protein interphase after the phenol treatment.

Virtually no DNA was lost during the ethanol precipitation step. This step however, is not without criticism since partial denaturation of the DNA occurs in ethanol solution. However, the studies of Herskovits, Singer and Geiduschek (87), revealed that this denaturation is virtually completely reversible. The exceptionally long time required to dissolve ethanol-denatured DNA in dilute saline-citrate solution is probably due to aggregation of the DNA molecules.

The removal of RNA was one of the main obstacles encountered during the purification of the extracted DNA. Partial separation of the RNA and DNA was achieved during the early stages of the isolation procedure, employing 0.15 M NaCl, since the solubilities of deoxypentose and pentose

nucleoproteins vary markedly in physiological saline solution. Consequently, the crude DNA preparations contained less RNA, which could be removed by only one charcoal treatment. Crude DNA preparations obtained by extraction with 1 M NaCl or 5 mM NaCl, contained considerably more RNA since the deoxypentose and pentose nucleoproteins have similar solubilities in these extractants. Consequently, complete removal of the RNA was troublesome, necessitating three charcoal treatments. The DNA preparation so obtained however, was degraded, presumably caused by the excessive shaking involved.

The failure of phenolphthalein diphosphate to separate the RNA from the DNA is difficult to explain. A possible explanation may be that interactions between the DNA and RNA occur which inhibit complete precipitation by this reagent. Such interactions may be expected, especially when the preparations contain large amounts of mRNA.

The yield of pure DNA, expressed as percentage of the total DNA present in the cells, was practically similar for the two procedures employing 5 mM or 1 M NaCl (see Table 2). Although the yield was fairly high, a considerable loss occurred during the deproteinisation step. This is probably due to incomplete dissociation of the nucleoprotein and as a result of the DNA being trapped in the mass of denatured protein after the phenol treatment. Further extraction with phenol may be considered but it should be kept in mind that this will subject the DNA to further shearing forces. A comparison of the DNA yield, expressed as mg DNA/g dry cells, with that of other bacteria is difficult because the DNA content of microbial cells vary considerably with the age, rate of growth of the cells and conditions of cultivation.

At present, no method exists by which the nativeness of a DNA preparation may be established. Transformation ability of a DNA preparation is not a reliable indication, since DNAs with molecular weight as low as 500,000 are capable of inducing specific transformation (13). On the other hand, a high molecular weight DNA preparation may also not be a criterion of the nativeness of the isolated DNA, since the highly polymerised state may be an artifact of the isolation procedure.

The determined sedimentation coefficient and intrinsic viscosity of P. mirabilis (13/S suc$^+$) fit well into the relationship which exists between $S^o_{20,w}$ and $[\eta]$ of DNA from various sources (85). A comparison of the value of the diffusion coefficient, determined in this work, with data in the literature is impossible. Most values reported, of which there are only very few, have been determined by methods used extensively for proteins and viruses.

The values reported, vary from 0.2 to 0.8 x 10^{-7} cm^2/sec. The value obtained in this work was 0.253 x 10^{-7} cm^2/sec. Much lower values are to be expected if one takes into account the extreme asymmetry of highly polymerised DNA. The molecular weights calculated from the Rubenstein, Thomas and Hershey, Eigner and Doty, Crothers and Zimm and Svedberg equations show interesting discrepancies. The value for whole molecules according to the Svedberg equation, is about ten times lower than the average value obtained from the other equations. This difference in molecular weight may be either due to an error in the value of the diffusion coefficient or to the unjustified use of the Svedberg equation, because this equation was derived for sphere-like molecules. It is furthermore interesting to note that the equation of Rubenstein, Thomas and Hershey indicates that the molecules with sedimentation coefficients of 42S and 29S are half and quarter molecules, respectively. The equation of Eigner and Doty does not. This may be due to the fact that the equation of Rubenstein, Thomas and Hershey is applicable over a wide range of molecular weights, whereas the Eigner and Doty equation is only applicable to the high molecular weight ranges.

The base composition found for P. mirabilis (13/S suc^+) is in close agreement with that of other species of Proteus, excepting P. morganii. This supports the findings of Sueoka (88) which show that organisms which are phenotypically similar, resemble one another in DNA base composition. A comparison of the base composition of representative members of the Enterobacteriaceae with that of P. morganii shows that the latter organism has a base composition similar to those of the Escherichia, Shigella and Salmonella groups. This similarity of base composition could indicate previously unrecognised homologies. A systematic analysis of the base ratios of DNA from bacteria could eventually provide the ultimate basis for their classification. It should be realised however that no final conclusions can be drawn without base sequence analysis.

CHAPTER IV

CONCLUSIONS

Protoplasts of Proteus mirabilis (13/S suc$^+$) offered an ideal source of DNA, since they could be lysed under mild conditions. The extraction of the DNA was easily effected by buffers containing 5 mM, 0.15 M or 1 M sodium chloride. However, only one of these extractants gave satisfactory DNA preparations.

During the purification of the DNA, two main problems were encountered. (1) A considerable loss of DNA occurred during the deproteinisation, especially when 0.15 M NaCl was employed. This renders 0.15 M NaCl unsuitable as extractant. (2) The RNA was difficult to remove. The crude DNA preparations, obtained by employing 5 mM or 1 M NaCl contained a vast amount of RNA which could not be removed by activated charcoal alone. However, RNase digestion, followed by adsorption of the digestion products on activated charcoal, proved to be satisfactory.

A very poor yield (viz. 8.38%) of DNA was obtained when 0.15 M NaCl was employed. The yield obtained when 5 mM NaCl or 1 M NaCl was employed, was 68.72% and 65.43%, respectively. In all cases pure, homogeneous preparations were obtained.

The sedimentation coefficient (62.5 S) and intrinsic viscosity (262.5 dl/g) of the whole molecules are comparable with values of DNA from T2 and T4 Coli phage and maize. The diffusion coefficient was found to be 0.253×10^{-7} cm^2/sec. The average molecular weight, which is the average of the values obtained from the equations of Rubenstein, Thomas and Hershey, Eigner and Doty and Crothers and Zimm was 119.4. . The Svedberg equation is apparently unsuitable for the calculation of the molecular weight of asymmetric molecules. Degradation of the DNA resulted when 1 M NaCl was used as extractant. The molecular weight of these degraded molecules was 63 million. Quarter molecules were obtained as a result of excessive shaking of the DNA solutions during the charcoal treatments. A comparison of biophysical constants obtained in this work with those in the literature reveal that many of the reported values are probably those for half and quarter molecules.

The base composition of P. mirabilis (13/S suc$^+$), viz. 37.1 mole per cent of guanine plus cytosine, is in good agreement with those of other Proteus species.

CHAPTER V

SUMMARY

Deoxyribonucleic acid was extracted from Proteus mirabilis ($13/S\ suc^{+}$) protoplasts with buffers containing sodium chloride, ethylene-diaminetetra acetate, sodium lauryl sulphate and Tris (hydroxymethyl) aminomethane. Different results were obtained when the sodium chloride concentration in these buffers was varied, while the concentrations of the other buffer components were kept constant.

Deproteinisation was achieved with phenol, while the RNA was removed by RNase treatment, followed by adsorption of the digestion products on activated charcoal. Phenolphthalein diphosphate proved to be unsuccessful in removing the RNA. Extraction with buffers containing 1 M NaCl and 5 mM NaCl gave fairly good DNA yields, viz. 65.43% and 68.72%, respectively. Extraction with 0.15 M NaCl gave a very poor yield (8.38%), the greatest loss occurring during the deproteinisation.

The purity of the DNA preparations was determined according to chemical assay methods, while the homogeneity was determined by sucrose density gradient and sedimentation velocity centrifugation. All these results indicated pure, homogeneous preparations.

The biophysical constants of the DNA were determined. The sedimentation and diffusion coefficients of the whole molecules were 62.5S and $0.253 \times 10^{-7} cm^2/sec$, respectively. The sedimentation coefficients were determined at 29,500 r.p.m. The diffusion coefficients were calculated from the boundary spreading at low centrifugal forces. In both cases ultra-violet optics were employed. The intrinsic viscosity (262.5 dl/g) was determined at a shear stress of 1.8×10^{-3} dynes/cm^2 in a rotating cylinder viscometer. The partial specific volume (0.5608) was calculated from the solvent and solution densities, which were determined in density gradient columns.

The molecular weight was calculated from the equations of Svedberg, Eigner and Doty, Rubenstein, Thomas and Hershey and according to the Crothers and Zimm equation. The values obtained from the above-mentioned equations for whole molecules were 13×10^6, 114.3×10^6, 132×10^6 and 112×10^6, respectively. Half molecules were obtained when 1 M NaCl was the extractant while quarter molecules were produced when inadequate precautions were taken to avoid shear forces during the isolation procedures.

The base ratio, determined from the melting temperature of the DNA was 37.1 mole per cent of guanine plus cytosine.

APPENDIX 1

DETERMINATION OF THE SEDIMENTATION COEFFICIENT OF DNA

Sample : DNA from P. mirabilis (13/S suc$^+$) Date of isolation : 3 Jan. 1965 Solvent : Saline-citrate Concentration : 23 µg/ml

Run No. : 219

Date : 1 Feb. 1965

Rotor type : An-D

Cell type : 12 mm - Aluminium centre piece

Temperature : 20.1°C

Speed : 29,590 r.p.m.

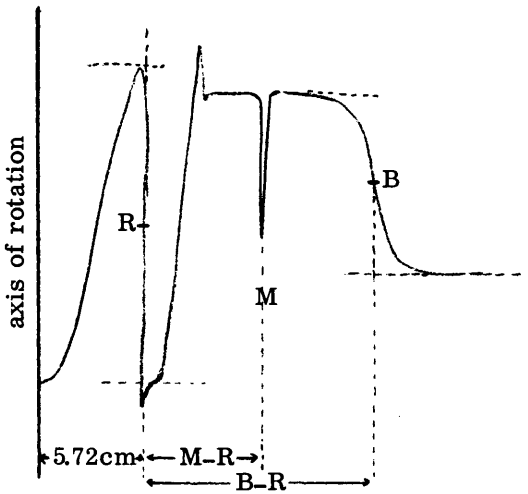
ω^2 : 9.548×10^6 rad^2/sec^2

Optics: U.V. with chlorine and bromine filters

Exposure time: 8 sec

Photo intervals: 4 min

Enlargement factor : 8.03



$$X = 5.72 + \frac{M-R}{8.03} + \frac{B-M}{8.03}$$

Photo No.	t min	B-R cm	M-R cm	B-M cm	$\frac{B-M}{8.03}$ cm	$\frac{M-R}{8.03}$ cm	X cm	$\frac{Xn+2}{Xn}$
1	0	2.30	1.85	0.45	0.0561	0.2304	6.0065	1.0166
2	4	2.7	1.80	0.90	0.1121	0.2242	6.0563	1.0165
3	8	3.1	1.80	1.30	0.1619	0.2242	6.1061	1.0163
4	12	3.5	1.80	1.70	0.2117	0.2242	6.1559	1.0162
5	16	3.9	1.80	2.10	0.2615	0.2242	6.2057	1.0171
6	20	4.3	1.80	2.50	0.3113	0.2242	6.2559	1.0169
7	24	4.75	1.80	2.95	0.3675	0.2242	6.3116	1.0158
8	28	5.15	1.80	3.35	0.4172	0.2242	6.3614	av.= 1.0165
9	32	5.55	1.80	3.75	0.4670	0.2242	6.4112	

$$S_c = \frac{2.303 \log (X_{n+2}/X_n)}{\omega^2 (t_3 - t_1)}$$

$$= 36.18 \times 10^{-13} \text{ sec}$$

$$S_{20,w} = S_c \cdot \frac{\eta^\circ_t}{\eta^\circ_{20}} \cdot \frac{\eta_t}{\eta^\circ_t} \cdot \frac{(1-V_{20}\rho^\circ_{20})}{(1-V_t \rho_t)}$$

$$= (36.18)(0.998)(1.023)(1.007)$$

$$= 37.2 \times 10^{-13} \text{ sec}$$

Corrections for dilution and pressure were insignificant, that is (Sc) app = Sc

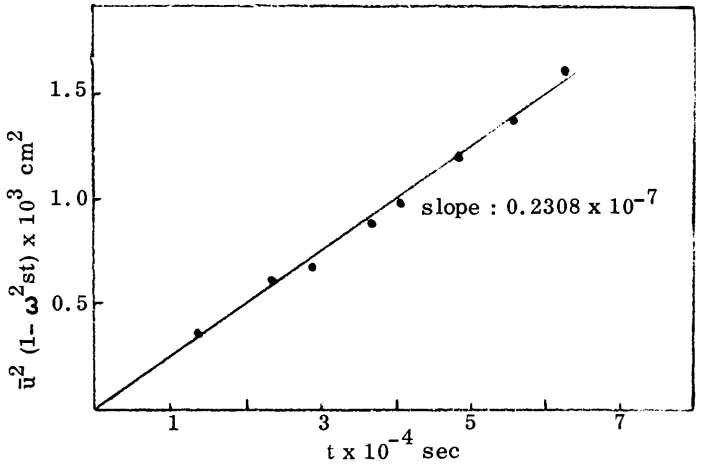
The symbols are explained on page (ii).

APPENDIX 2

DETERMINATION OF THE DIFFUSION COEFFICIENT OF DNA

Sample: DNA from P. mirabilis (13/S suc$^+$) Date of isolation: 4 July 1964 Solvent: Saline-Citrate Concentration: 33 µg/ml

Run No.:	56
Date:	25 Aug. 1964
Rotor type:	An-J
Cell type:	12 mm-aluminium centre piece
Temperature:	5°C
Speed:	4,057 r.p.m.
ω^2 :	1.805×10^5 rad^2/sec^2
s :	(measured at 4057 r.p.m.): 21.54×10^{-13} sec
$\omega^2 s$:	3.881×10^{-7} rad^2/sec
Optics:	U.V. - chlorine and bromine filters
Exposure:	8 sec
Photo intervals:	64 min
Enlargement:	8.03



t x 10^{-4} sec	$\bar{u}$* cm	$\bar{u}^2 \times 10^3$ cm^2	$\omega^2 ts \times 10^3$	$(1 - \omega^2 st)$*	$\bar{u}^2 (1-\omega^2 st) \times 10^3$ cm^2
1.4029	0.02179	0.3490	5.444	0.9946	0.347
2.1713	0.02420	0.6200	8.426	0.9917	0.613
2.9397	0.02491	0.6205	11.410	0.9886	0.616
3.7081	0.02988	0.8927	14.390	0.9856	0.878
4.0923	0.03113	0.9691	15.880	0.9841	0.955
4.8607	0.03400	1.1560	18.870	0.9811	1.156
5.6291	0.03736	1.3960	21.840	0.9782	1.366
6.3975	0.03985	1.5880	24.820	0.9752	1.549

* See 2.2.2.2. The distances from the boundary to the axis of rotation were measured from the densitometer tracings as shown in Appendix 1. A plot of $\bar{u}^2 (1 - \omega^2 st)$ versus time is shown in the accompanying figure.

$$D_c = \frac{\bar{u}^2 (1 - \omega^2 t)}{4 y^2 t}$$

$$= \frac{0.2308 \times 10^{-7}}{1.4166}$$

$$= 0.1629 \times 10^{-7} \text{ cm}^2/\text{sec}$$

$$D_{20,w} = D_c \cdot \frac{\eta_t^\circ}{\eta_{20}^\circ} \cdot \frac{\eta_t}{\eta_t^\circ} \cdot \frac{(273 + 20)}{(273 + 5)}$$

$$= (0.1629 \times 10^{-7})\ (1.505)\ (1.018)\ (1.054)$$

$$= 0.263 \times 10^{-7} \text{ cm}^2/\text{sec}$$

The symbols are explained on page (ii) .

REFERENCES

1 LORING, H.S., J. Biol. Chem., 128 (1939), Sci. Proc., p. 61.

2 MYRBÄCK, K., and JORPES, E., Hoppe-Seyl. 2., 237, 159 (1935).

3 DESREUX, V., Trans. Faraday Soc., 46, 790 (1950).

4 SAVITSKY, J.P., and STAND, F., Biochim. Biophys. Acta, 114, 419 (1966).

5 PITOUT, M.J. Doctoral thesis, University of Pretoria, 1966.

6 ATEN, J.B.T., and COHEN, J.A., J. Mol. Biol., 12, 537 (1965).

7 CHARGAFF, E., Federation Proc., 10, 654 (1951).

8 WATSON, J.D., and CRICK, F.H.C., Cold Spring Harbor Symposia Quant. Biol. 18, 123 (1953).

9 VOLKIN, E., and ASTRACHAN, L., Virology, 2, 149 (1956).

10 SUEOKA, N., Proc. Natl. Acad. Sci. U.S., 48, 582 (1962).

11 BELOZERSKY, A.N., and SPIRIN, A.S., Nature, 182, 111 (1958).

12 MARMUR, J., FALKOW, S., and MANDEL, M., Ann. Rev. Microbiol., 17, 329 (1963).

13 AVERY, O.T., MACLEOD, C.M., and McCARTY, M., J.Exptl. Med., 79, 137 (1944).

14 JACOB, F., and MONOD, J., J.Mol. Biol., 3, 318 (1961).

15 MONOD, J., and JACOB, F., Cold Spring Harbor Sumposia Quant. Biol., 26, 389 (1961).

16 CHARGAFF, E., in E. CHARGAFF, E., and J.N. DAVIDSON (Editors), The Nucleic Acids, Academic Press, New York, Vol. 1., 1955, p. 307.

17 SHAPIRO, H.S., and CHARGAFF, E., Biochim et Biophys. Acta, 23, 451 (1957).

18 JOSSE, J., KAISER, A. D., and KORNBERG, A., J. Biol. Chem., 236, 864 (1961).

19 SCHILDKRAUT, C.L., WIERZCHOWSKI, K.L., MARMUR, J., GREE GREEN, D.M., and DOTY, P., Virology, 18, 43 (1962).

19

20 HALL, B.D., and SPIEGELMAN, S., Proc. Natl. Acad. Sci. U.S., 47, 137 (1961).

21 SPIEGELMAN, S., HALL, B.D., and STORCK, R., Proc. Natl. Acad. Sci. U.S., 47, 1135 (1961).

22 SCHULMAN, H.M., and BONNER, D.M., Proc. Natl. Acad. Sci. U.S., 48, 53 (1962).

23 HAYASHI, M., and SPIEGELMAN, S., Proc. Natl. Acad. Sci. U.S., 47, 1564 (1961).

24 McCARTHY, B.J., and BOLTON, E.T., Proc. Natl. Acad. Sci. U.S., 50, 156 (1963).

25 GRIFFITH, F., J. Hyg., Cambridge, Eng., 27, 113 (1928).

26 KAISER, A.D., and HOGNESS, S., J. Mol. Biol., 2, 392 (1960).

27 SAITO, H., and MIURA, K., Biochim. Biophys. Acta, 72, 619 (1963).

28 FELIX, K., in A Symposium on Molecular Biology Ed. by ZIRKLE, R.E., University of Chicago Press, 1959.

29 KIRBY, K.S., Bioch. J. 64, 405 (1956).

30 COLTER, J.S., BROWN, R.A., and ELLEM, K.A.O., Biochim. Biophys. Acta, 55, 31 (1962).

31 LEWIN, S., Lab. Prac., 13, 315 (1964).

32 LEDERBERG, J., Proc. Natl. Acad. Sci. U.S., 42, 574 (1956).

33 FRASER, P., MAHLER, H.R., SCHUG, A.L., and THOMAS, C.A., Proc. Natl. Acad. Sci. U.S., 43, 939 (1957).

34 SPIZIZEN, J., Proc. Natl. Acad. Sci. U.S., 43, 694 (1957).

35 FRICK, G., Biochim. Biophys. Acta, 3, 103 (1949).

36 DAVIDSON, P.F., Proc. Natl. Acad. Sci. U.S., 45, 1560 (1959).

37 KIRBY, K.S., Biochim. Biophys. Acta, 47, 18 (1961).

38 ZAMENHOF, S., and CHARGAFF, E., Nature, 168, 604 (1951).

39 BURTON, K., Bioch. J., 62, 315 (1956).

40 KUPILA, S., BRYAN, A.M. and STERN, H., Plant Phys., 36, 212 (1961).

41 DRYER, R.L., TAMMES, A.R., ROUTH, J.I., J. Biol. Chem. 225, 177 (1957).

42 SMILLIE, R.M., and KROTKOV, G., Can. J. Botany, 38, 31 (1960.

43 MILLER, G.L., Anal. Chem., 31, 964 (1959).

44 FILIPOWICZ, B., GROSS, M., and SKOCZYLAS, B., Anal. Bioch., 5, 187 (1963).

45 DE DUVE, C., BERTHET, J., and BEAUFAY, H., Prog. Biophys. and Biophys. Chem., 9, 326 (1959).

46 BRAKKE, M.K., Arch. Biochem. Biophys., 107, 388 (1964).

47 SADRON, C.L., in E. CHARGAFF, E., and J.N. DAVIDSON (Editors) The Nucleic acids, Academic Press, New York, Vol 3, 1960, p.1.

48 SHACHMAN, H.K., GROPPER, L., HANLON, S., PUTNEY, F.' Arch. Biochem. Biophys, 99, 175 (1962).

49 SCHACHMAN, H.K., in S.P. Colowick and N.O. Kaplan (Editors). Methods in Enzymology, Academic Press, New York, Vol. 4, 1957, p.32.

50 FLEISCHMAN, J.B., J. Mol. Biol., 2, 226 (1960).

51 BURGI, E., and HERSHEY, A.D., J. Mol. Biol. 3, 458 (1961).

52 KOENIG, V. L., and PERRINGS, J.D., J. Colloid Sci., 8, 452 (1953).

53 SHOOTER, K.V., and BUTLER, J.A.V., Trans. Faraday Soc., 52, 734 (1956).

54 EIGNER, J. SCHILDKRAUT, C., and DOTY, P., Biochim. Biophys. Acta, 55, (1962).

55 CAVALEIRI, L.F., and ROSENBERG, B.H., J. Amer. Chem. Soc., 81, 5136 (1959).

56 CECIL, R., and OGSTON, A.G., J. Chem. Soc. 1382 (1948).

57 KAHLER, H.J., J. Phys. Chem., 52, 676 (1948).

58 BALDWIN, R. L., Bioch. J., 65, 503 (1957).

59 FUJITA, H., J Chem. Phys., 57, 194 (1956).

60 MÖLLER, W.J., Proc. Natl. Acad. Sci. U.S., 51, 501 (1964).

61 SVEDBERG, T., and PEDERSON, K.O., The Ultracentrifuge, Oxford University Press, 1940.

62 ALFREY, T, BARTOVICS, A. and MARK, H, J Amer. Chem. Soc., 64, 1557 (1942).

63. EINSTEIN, A., Ann. Physik. 34, 591 (1911).

64 FRISCH, H. L., and SIMHA, R., in R. EIRICH (Editor), Rheology Theory and Applications, Academic Press, New York, Vol. 1, 1956, 0. 525.

65 ZIMM, B.H., and CROTHERS, D.M., Proc. Nat. Acad. Sci. U.S., 48, 905 (1962).

66 CROTHERS, D.M. and ZIMM, B.H., J. Mol. Biol., 12, 525 (1965).

67 SCHRUGGS, R.L., and ROSS, P.D., Biopolymers, 2, 593 (1964).

68 MacINNES, D.A., DAYHOFF, M.O., and RAY, B.R., Rev. Sci. Instr., 22, 642 (1951).

69 LINDERSTRØM-LANG, K., and LANZ, H., Compt. rend. trav. lab. Carlsberg Sér. chim., 21, 315 (1938).

70 MARKHAM, R., Adv. in Virus Res 9, 241 (1962).

71 SHUGAR, D., in E. CHARGAFF, and J.N. DAVIDSON (Editors), The Nucleic acids Academic Press, New York, Vol. 3, 1960, p 39.

72 LAWLEY, P.D., Biochim. Biophys. Acta, 21, 481 (1956).

73 LALAND, S.G., LEE, W.A., OXEREND, W.G., and PEACOCKE, A.R., Biochim. Biophys. Acta, 14, 356 (1954).

74 SCOTT, J.F., in G. OSTER and A.W. POLLISTER (Editors) Physical Techniques in Biological Research, Academic Press, New York, Vol. 1, 1955, p. 131.

75 FURBERG, S., Acta Chem. Scand., 6, 637 (1952).

76 WATSON, J.D., and CRICK, F.H.G., Nature, 171, 737 (1953).

77 CHARGAFF, E., and ZAMENHOFF, S., J. Biol. Chem., 173, 327 (1948).

78 LEWIN, S., Lab. Prac., 13, 196 (1964).

79 WYATT, G.R., Bioch. J., 48, 584 (1951).

80 GRIPPO, P., IACCARINO, M., ROSSI, M., and SCARANO, E., Biochim. Biophys. Acta, 95, 1 (1965).

81 MARMUR, J., and DOTY, P., J. Mol. Biol. 5, 109 (1962).

82 BELOZERSKY, A.N., and SPIRIN., A. S., in E. CHARGAFF and J.N. DAVIDSON, (Editors), The Nucleic acids, Academic Press, New York, Vol. 3, 1960, p 147.

83 FALKOW, S., RYMAN, I.R., and WASHINGTON, O., J. Bacteriol., 83, 1318 (1962).

84 RUBENSTEIN, I., THOMAS, C.A., and HERSHEY, A.D., Proc. Natl. Acad. Sci. U.S., 47, 1113 (1961).

85 EIGNER, J., and DOTY, P., J. Mol. Biol. 12, 549 (1965).

86 HUGO, W.B., Bact. Rev., 18, 87 (1954).

87 HERSKOVITS, T.T., and SINGER, S.J., GEIDUSCHEK, E.P., Arch. Biochem. Biophys., 94, 99 (1961).

88 SUEOKA, N, J. Mol. Biol., 3, 31 (1961).

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