

THE BASIS OF CELL-TO-CELL TRANSFORMATION IN *PARAMECIUM* *BURSARIA*

II. INVESTIGATION INTO THE MOLECULAR NATURE OF THE TRANSFORMING AGENT

C. A. CULLIS*

School of Biological Sciences, University of East Anglia, Norwich, England

SUMMARY

A mating type transformation occurring in syngen 4 of *Paramecium bursaria* has been further investigated. The transformation event was not reversed or prevented by acridine or ionizing radiations. The transformation has not been accomplished with cell-free extracts including the microinjection of cytoplasmic extracts.

The nucleic acids of normal and transformed cells have been compared. No differences were found. Both types had 2 major ribosomal RNAs of molecular weights 1.29×10^6 and 0.70×10^6 Daltons. In both types a particular RNA species, with molecular weight 1.0×10^6 Daltons, was found only in sexually competent cells. The DNA had a buoyant density in caesium chloride of 1.689 g cm^{-3} and a T_m in $0.1 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M trisodium citrate) of 65.2°C . The renaturation kinetics showed 2 types of DNA - a fast-renaturing component, comprising 13% of the total DNA, with a complexity of 1.84×10^9 Daltons, and a slow-renaturing component with a complexity of 1.6×10^{11} Daltons, in both normal and transformed cells. Possible models for the action of the transforming agent are discussed.

INTRODUCTION

A mating type transformation has been described in syngen 4 of *Paramecium bursaria* (Bomford, 1967) and is described in the accompanying paper. It has been shown in the latter paper that cytoplasmic exchange is necessary during abortive conjugation if the transformation is to occur (Cullis, 1972). The transformation is also accompanied by the acquisition of the ability to produce a mating type substance not previously formed (Bomford, 1967; Cullis, 1972). Experiments were designed to try to isolate the active agent and to determine the nature of the transforming agent, and how the mating type transformation was brought about.

MATERIALS AND METHODS

The stocks of *P. bursaria* and culture methods have been previously described (Cullis, 1971). Matings carried out in the presence of nucleases or acridine, were performed in Dryl's salts solution (Dryl, 1959) with the enzyme or dye also dissolved in Dryl's solution.

* Present address: John Innes Institute, Colney Lane, Norwich, England.

Microinjection technique

Injection needles were pulled from 1-mm Pyrex glass such that the orifice diameter was between 1 and 5 μm . The needle was attached to a de Fonbrune micromanipulator and filled with liquid paraffin. The material to be injected was drawn into the tip of the needle and the receiving animal had the material plus a small drop of liquid paraffin injected. The latter can be seen as a droplet in the cytoplasm and was taken as an indication of successful injection. The following fractions of transformed cells were used to inject into normal cells: (i) direct cytoplasmic transfer from transformed to normal cells; (ii) a homogenate of transformed cells; and (iii) a homogenate of transformed cells, spun at 500 g, to remove *Chlorellae* and large pieces of pellicle. For description of injection technique see Beale, Knowles & Tait (1972).

RNA extraction

The cells were collected by centrifugation in a rotor similar to that described by Martz (1962). They were washed twice in sterile Dryl's solution and concentrated to approximately 2×10^6 cells per ml. RNA preparations were made from about 5×10^6 cells. The cells were lysed by the addition of *p*-aminosalicylate (6% w/v), sodium lauryl sulphate (1% w/v), and magnesium chloride (5 mM) was added. The mixture was spun at 100 g for 5 min to remove the *Chlorellae*. The supernatant was deproteinized 3 times with a phenol/cresol mixture (phenol 500 g, *m*-cresol 70 ml, water 55 ml, 8-hydroxyquinoline 0.5 g; Kirby, 1965).

After the first deproteinization the aqueous phase was made 3% with respect to sodium chloride (w/v). The nucleic acids were precipitated with 2 vol. cold ethanol, washed with 0.1 M potassium acetate/5 mM magnesium chloride/70% ethanol and stored under this at -20°C until used.

Polyacrylamide gel electrophoresis

The method followed was that described by Loening (1968a). The buffer used for preparing the gels was: 0.08 M Tris, 0.066 M sodium acetate, 0.004 M EDTA (disodium salt) adjusted to pH 7.8 with glacial acetic acid. The electrophoresis buffer was a 1:1 dilution of the gel buffer plus sodium lauryl sulphate (0.2% w/v) and magnesium chloride (5 mM); 9-cm gels were used and run for 3 h. The nucleic acid precipitate was dissolved in the electrophoresis buffer, made 20% (w/v) with sucrose and 50 μl layered on to the gel. After electrophoresis the gel was scanned in a Joyce-Loebel chromoscan at 254 nm.

Preparation of DNA

(i) The procedure to the precipitation of the nucleic acids was the same as for the extraction of RNA; 5×10^6 to 1×10^7 cells were used in each preparation. The precipitate was dissolved in 0.1 \times SSC (1 \times SSC = 0.15 M sodium chloride, 0.015 M trisodium citrate), RNase was added to a final concentration of 50 $\mu\text{g}/\text{ml}$ and the mixture incubated at 37°C for 30 min. Predigested pronase (1 mg/ml) was added and the mixture again incubated for 30 min at 37°C , after which it was deproteinized with 0.5 vol. of phenol/cresol and the DNA precipitated from the aqueous layer with 2 vol. of cold ethanol. The DNA was redissolved in 0.1 \times SSC, dialysed against a 1000-fold excess of this buffer at 4°C and stored over a drop of chloroform at 4°C . Assuming the macronucleus to contain 1.5×10^{-10} g of DNA (Cullis, 1971) the above method gave a yield of 30% of the total DNA estimated to be present initially.

(ii) DNA was extracted over hydroxylapatite as described by Britten, Pavich & Smith (1969). This method was used as the phenol method had been shown to selectively remove high A-T satellites in certain salt environments (Smith, Martinez & Ratliff, 1970).

(iii) For some analytical gradients a sarcosyl lysate was used. 10^4 paramecia were suspended in 0.2 ml of 0.1 M Tris, 0.1 M EDTA (disodium salt) and 0.5% Sarcosyl NL97 at 27°C for 2 h. This solution was added to 1.304 g of saturated caesium chloride at 20°C and the total weight of solution made up to 1.526 g by addition of further buffer; 0.5 ml of the final mixture was used for analytical centrifugation.

Analytical caesium chloride centrifugation

The method of Vinograd & Hearst (1962) was used. A Spinco model E analytical centrifuge was used at 44 770 rev/min. Each cell was equipped with a 1° negative upper window. *Micrococcus lysodeikticus* (density 1.731 g cm⁻³), *Escherichia coli* (density 1.710 g cm⁻³) and *Aerobacter aerogenes* (density 1.718 g cm⁻³) were used as density standards in neutral gradients. Alkaline caesium chloride gradients were made by adding 0.5 ml DNA solution, plus 0.1 ml N NaOH to 0.840 g caesium chloride. The density of this solution was 1.750 g cm⁻³. Centrifugation was for 18–22 h at 25 °C. Photographs were taken with ultraviolet light (265 nm) and the negatives scanned with a Joyce-Loebel double beam microdensitometer. Alkaline buoyant densities were calculated from the initial densities of the solution, other buoyant densities were calculated from the formula of Sueoka (1961). No corrections for pressure effects were made.

Thermal denaturation of DNA

Seventy-five microgrammes of DNA in 2.5 ml of 0.1 × SSC were placed in stoppered quartz cuvettes and then heated in a Unicam SP 800 spectrophotometer fitted with a Honeywell continuous recorder. The temperature was controlled using a Haake circulating temperature bath. The temperature was usually raised in 0.2 °C steps. The guanine and cytosine (G + C) content of the DNA was calculated from the relationship $G + C = 2.44 (T_m - 81.5 - 16.6 \log_{10} [Na^+])$ (Schildkraut & Lifson, 1965), where T_m = the temperature at which the hyperchromicity has reached 50% of its final value and $[Na^+]$ = sodium ion concentration.

Renaturation of DNA

The renaturation reaction was followed spectrophotometrically, as described by Wetmur & Davison (1968), after alkaline denaturation. The DNA was sheared to a mol. wt. of 0.7×10^6 Daltons. The complexity of the DNA was calculated from the relationship

$$N_D = \frac{5.5 \times 10^8}{k_2} \times (S_{20,w}^{pH13})^{1.85} \text{ Daltons} \quad (\text{Wetmur \& Davison, 1968}).$$

N_D = Kinetic complexity, $S_{20,w}^{pH13}$ = sedimentation coefficient, k_2 = second-order rate constant obtained from the slope of the line $1/A - A_\infty$ v.t., where A = absorbance at time t , and A_∞ is the absorbance at infinite time. The molecular weight was obtained from the band width in equilibrium caesium chloride centrifugation (Daniel, 1969).

RESULTS

Cell-free transformation

Three methods were tried to obtain transformed cells without abortive conjugation. These were: (i) Growing normal cells in the presence of a brei of transformed cells (Bomford, 1967). (ii) The mating of normal cells in a brei of transformed cells. No transformed clones were found in the clones formed from the exconjugants of 87 pairs. (iii) The injection of cytoplasm of transformed cells into normal cells. No transformed cells were found in clones obtained from the progeny of 81 cells successfully injected. Thus, so far, no transformation has been brought about except by the natural cytoplasmic exchange during abortive conjugation.

A number of treatments were applied to cells undergoing abortive conjugation in an attempt to prevent transformation: (1) Matings were carried out in the presence of DNase (500 µg/ml), RNase (500 µg/ml), or acridine orange (5 µg/ml). (2) Pairs were irradiated for 0.5 h at 7 cm from a Hanovia portable ultraviolet lamp. (3) Pairs

were irradiated with 3.86 C kg^{-1} ($15\ 000 \text{ R}$) from a ^{60}Co source. (4) Pairs were placed at $32\ ^\circ\text{C}$ immediately after pair formation. In each of the treatments, clones from 20 pairs of exconjugants were tested, but none altered the proportion of animals which became transformed in a particular cross. This does not rule out the possibility that the agent is affected by the above treatments but the dose required to inactivate the transforming agent may be greater than the lethal dose for the paramecium.

Characterization of RNA from normal and transformed cells

RNA extracted from normal and transformed cells was run on 2.2, 2.4 and 5% polyacrylamide gels. No differences were obtained in the comparison between RNA from normal cells and that from transformed cells. However a difference was found between the RNA extracted from sexually competent* cells and that extracted from incompetent cells. This difference was most clearly illustrated on 2.4% gels.

RNA from sexually incompetent cells

RNA from normal and transformed cells which were incompetent at the time of extraction was run for 3 h on 2.4% gels (Fig. 1A, B); 3 RNA bands (2, 3, 5) and one, DNA band (1) were observed in each case. The RNA bands were identified as ribosomal RNA (band 2, 1.29×10^6 Daltons; band 3, 0.70×10^6 Daltons) and unresolved 5-s ribosomal RNA and 4-s RNA. These latter 2 components were resolved on 5% gels.

RNA from sexually competent cells

When RNA, extracted from sexually competent cells, was run on 2.4% gels, 4 RNA bands were observed (Fig. 1C); this gel was run for 2.5 h. Three bands were the same as those observed in the RNA from incompetent cells (2, 3, 5); the fourth (4), which had a molecular weight calculated as 1.0×10^5 Daltons, was found only in competent cells and disappeared when competent cells became incompetent. It is suggested here that this species of RNA is, in some way, connected with the appearance of sexual competence in the strains of *P. bursaria* investigated. In a given culture, sampled at different times, this RNA species was either present (when cells were competent) or absent (when the cells became incompetent).

Characterization of DNA from normal and transformed cells

A comparison of the banding pattern and the buoyant density of DNA, from normal and transformed cells, obtained in caesium chloride was made. In each case a single band of average density 1.689 g cm^{-3} was obtained. In gradients of sarcosyl lysates of whole cells a second band with a density of 1.669 g cm^{-3} was observed which was assumed to be glycogen (Fig. 2). A similar band has been observed in whole cell DNA preparations from several strains of *Tetrahymena pyriformis* (Flavell & Jones, 1970). DNA having a buoyant density of 1.689 has an estimated G + C content of 29%. In alkaline gradients the DNA from both cell types produced a single band with a

* Competent cells are those able to undergo the mating reaction. In mass culture cells became competent about 7 days after they were last fed provided the appropriate light conditions were present.

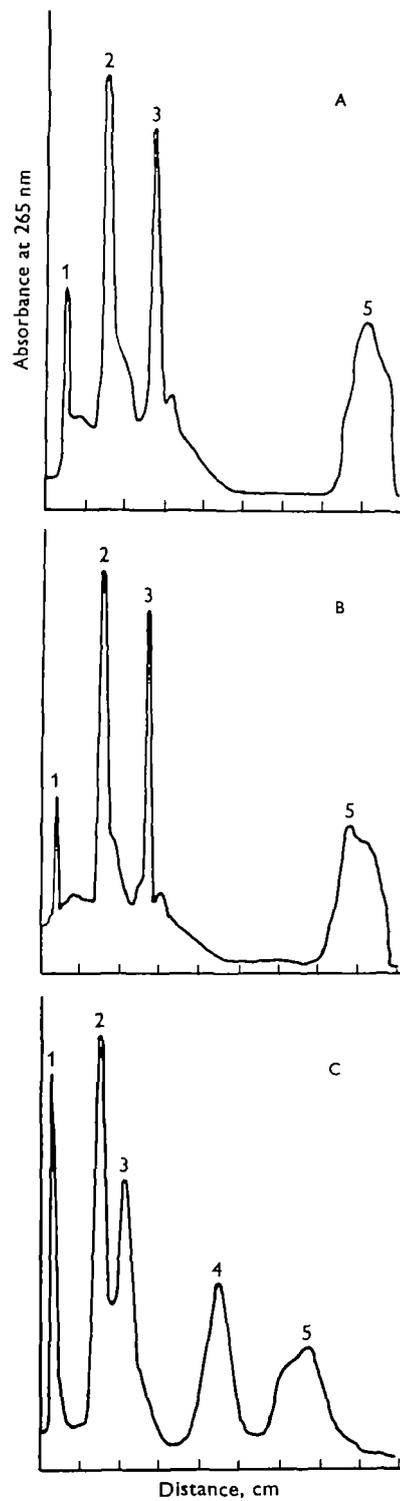


Fig. 1. RNA from mating types III, IIs and sexually competent IIs (curves A, B and C respectively) run on 2.4% polyacrylamide gels for 3, 3, and 2.5 h respectively.

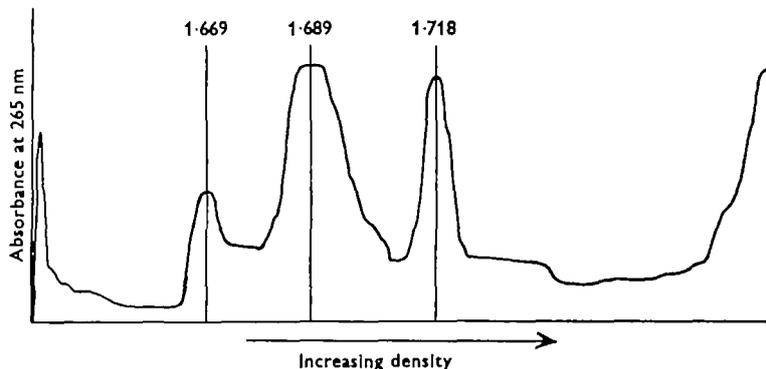


Fig. 2. CsCl gradient spun at 44770 rev/min for 20 h containing sarcosyl lysate of *P. bursaria* with *A. aerogenes* DNA added as a marker ($\delta = 1.718$).

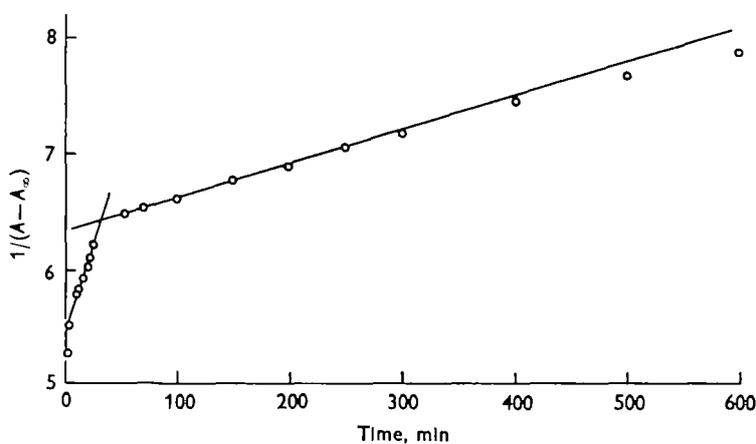


Fig. 3. Renaturation of *P. bursaria* DNA at 116 $\mu\text{g/ml}$.

density of 1.750 g cm^{-3} . No DNA bands from the *Chlorellae* were observed as the latter were removed before extraction. DNA extracted from the *Chlorellae* gave a band with a density of 1.727 g cm^{-3} .

Thermal denaturation of DNA

DNA prepared was by either method (i) or (ii) described in Materials and Methods. It was dissolved in $0.1 \times \text{SSC}$ at approximately $30 \mu\text{g/ml}$. The DNAs from several normal mating types (I, II, III, IV, VII) as well as from transformed cells were denatured. All gave a single step transition with a T_m of $65.2 \pm 0.1 \text{ }^\circ\text{C}$ in $0.1 \times \text{SSC}$. This would be equivalent with a DNA containing 29% (G+C) which agrees with the estimate obtained from the buoyant density.

Table 1. Kinetic complexity of *P. bursaria* DNA. k_2 values are corrected for proportions of DNA present in *Paramecium* DNA, - 13% for fast reaction, 87% for slow reaction; but not to standard sedimentation coefficient

DNA source	k_2 , l. mol ⁻¹ s ⁻¹	Complexity, Daltons
<i>Aerobacter</i>	9.6	2.8×10^9
<i>Paramecium</i> , fast reaction	32.7	0.84×10^9
<i>Paramecium</i> , slow reaction	0.172	1.6×10^{11}

Renaturation of DNA

DNA from normal cells of mating types II and III and from transformed cells, was allowed to renature under the conditions described in Materials and Methods; the reactions were allowed to take place at 30 $\mu\text{g/ml}$ and approximately 120 $\mu\text{g/ml}$. The different concentrations were used to separate a fast reaction, due to highly repeated sequences, and a slow reaction due to unique sequences, if these types of DNA were present (Britten & Kohne, 1968; Searcy & MacInnis, 1970). At the lower concentration the second-order reciprocal plot gave a single straight line, while at the higher concentration a biphasic curve was obtained (Fig. 3). The fast reaction at the higher concentration was taken to be due to the reassociation of the repeated sequences, and the slow reaction to the reassociation of the unique sequences. The second-order rate constants, 32.7 l. mol⁻¹ s⁻¹ and 0.172 l. mol⁻¹ s⁻¹, for the fast and slow components respectively give complexities calculated at 0.84×10^9 and 1.6×10^{11} Daltons for the repeated and unique sequences respectively. With the repeated sequences comprising some 13% of the total DNA, these values are in close agreement with those obtained for different stocks of *P. aurelia* (Allen & Gibson, 1972). *Aerobacter aerogenes* DNA was renatured under the same conditions as a control and a value for the complexity of this DNA was calculated as 2.8×10^9 Daltons (Table 1). However, no differences were observed in the renaturation of the DNA from normal and transformed cells. The same values were obtained for the complexities of the repeated and unique sequences and for the proportion of repeated sequences present, in both types of DNA.

DISCUSSION

It has been shown that cytoplasmic transfer during abortive conjugation was necessary for the mating type transformation to occur (Cullis, 1972). The transformation caused new information in the transformed cell, that is the ability to make a new mating type substance, to be expressed (Cullis, 1972; Bomford, 1967). This information could not be shown to be present before transformation (Bomford, 1967). The nucleic acids of normal and transformed cells were characterized to determine if there were any differences between the 2 cell types. No differences were observed. If the transforming agent caused differences of less than 1% in the total DNA of the cell these would not have been found by the methods used. However, an RNA species,

confined to sexually competent cells of both normal and transformed types, was found. The role of this RNA in the development of sexual competence is not known. The appearance of this RNA in transformed cells suggested that this part of the mating cycle was not altered by the transformation.

The following information about the nucleic acids of *P. bursaria* has been obtained. The major ribosomal RNA components have molecular weights of 1.29×10^6 and 0.69×10^6 Daltons, which are the same as those previously presented for *P. aurelia* (Loening, 1968*b*). The value of the guanine + cytosine content, 29%, is low, as is that for other ciliates; 26% G + C in *P. aurelia* (Gibson, Chance & Williams, 1971). The value for the genome size, 1.6×10^{11} Daltons compares with values of $1.8-2.5 \times 10^{11}$ Daltons for various strains of *P. aurelia* (Allen & Gibson, 1972) and $0.4-1.2 \times 10^{11}$ Daltons for various strains of *T. pyriformis* (Allen & Gibson, 1972; Flavell & Jones, 1970). The failure to obtain transformation without abortive conjugation, and inability to detect differences between normal and transformed cells limit speculation on the nature of the transforming agent. A comparison of the mating type transformation with other systems, such as episomal transfer or transformation is not possible until the nature of the transforming agent has been determined. This would require the preparation of an infective, cell free fraction, from transformed cells.

The author gratefully acknowledges the support of the Commonwealth Scholarship Commission, for the award of a Commonwealth Scholarship, and the University of East Anglia, for a Postgraduate studentship. The help and advice from Dr I. Gibson during this work is also acknowledged. I also express thanks to Professor G. H. Beale and J. Knowles for instructions in microinjection of paramecia.

REFERENCES

- ALLEN, S. L. & GIBSON, I. (1972). Genome amplification and gene expression in the ciliate macronucleus. *Biochem. Genet.* (in the Press).
- BEALE, G. H., KNOWLES, J. K. C. & TAIT, A. (1972). Mitochondrial genetics in *Paramecium*. *Nature, Lond.* **235**, 396-397.
- BOMFORD, R. G. (1967). Stable changes of mating type after abortive conjugation in *Paramecium bursaria*. *Expl Cell Res.* **47**, 30-41.
- BRITTEN, R. J. & KOHNE, D. E. (1968). Repeated sequences in DNA. *Science, N.Y.* **161**, 529-540.
- BRITTEN, R. J., PAVICH, M. & SMITH, J. (1969). A new method for DNA purification. *Carnegie Instn Yearbook* **68**, 400-403.
- CULLIS, C. A. (1971). *Transformation in Paramecium bursaria*. Ph.D. Thesis, University of East Anglia.
- CULLIS, C. A. (1972). The basis of cell-to-cell transformation in *Paramecium bursaria*. I. Transfer of cytoplasmic material. *J. Cell Sci.* **11**, 601-609.
- DANIEL, E. (1969). Molecular weight of DNA from sedimentation equilibrium in CsCl density gradient. *Biopolymers* **8**, 553-554.
- DRYL, S. (1959). Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogeny and conjugation. *J. Protozool.*, Suppl. **6**, 25.
- FLAVELL, R. A. & JONES, I. G. (1970). Mitochondrial deoxyribonucleic acid from *Tetrahymena pyriformis* and its kinetic complexity. *Biochem. J.* **116**, 811-817.
- GIBSON, I., CHANCE, M. L. & WILLIAMS, J. M. (1971). Extranuclear DNA and the endosymbionts of *P. aurelia*. *Nature, New Biol.* **234**, 75-77.
- KIRBY, K. S. (1965). Isolation and characterisation of ribosomal ribonucleic acid. *Biochem. J.* **96**, 266-269.

- LOENING, U. E. (1968*a*). Fractionation of high molecular weight RNA. In *Chromatographic and Electrophoretic Techniques*, vol. 2 (ed. I. Smith), pp. 437-442. London: Heinemann.
- LOENING, U. E. (1968*b*). Molecular weights of ribosomal RNA in relation to evolution. *J. molec. Biol.* **38**, 355-365.
- MARTZ, E. (1962). A centrifuge for rapid concentration of large fragile cells without extensive lysis. *J. Protozool.* **13**, 380-382.
- SCHILDKRAUT, C. L. & LIFSON, F. (1965). Dependence of the melting temperature of DNA on salt concentration. *Biopolymers* **3**, 195-208.
- SEARCY, D. G. & MACINNIS, A. J. (1970). Hybridization and renaturation of non repetitive DNA of higher organisms. *Biochim. biophys. Acta* **209**, 574-577.
- SMITH, D. A., MARTINEZ, A. A. & RATCLIFF, R. L. (1970). Deproteinisation with phenol of alternating polydeoxyadenylate-deoxythymidylate and other DNA-like polymers. *Analyt. Biochem.* **38**, 85-89.
- SUEOKA, N. (1961). Variation and heterogeneity of base composition of deoxyribonucleic acids: A compilation of old and new data. *J. molec. Biol.* **3**, 31-40.
- VINOGRAD, J. & HEARST, J. E. (1962). Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Fortschr. Chem. org. NatStoffe* **20**, 372-422.
- WETMUR, J. G. & DAVISON, N. (1968). Kinetics of renaturation of DNA. *J. molec. Biol.* **31**, 349-370.

(Received 23 December 1971)