Chloroplastal DNA has been extracted from aqueously and non-aqueously prepared chloroplast fractions from several higher plants. When examined in the analytical ultracentrifuge it was found to have a buoyant density of 1.697 g cm$^{-3}$ and in whole cell preparations to be within the gaussian distribution of the nuclear DNA (1.694 g cm$^{-3}$). This finding is a variance with other published results for chloroplastal DNA which associates this DNA with a heavy satellite at a buoyant density of 1.706 g cm$^{-3}$. This satellite was shown, from aqueous preparations to be not chloroplastal but mitochondrial in origin.

In an attempt to find out the genomic content of the chloroplastal DNA it was subjected to extensive renaturation. In contrast to the control DNAs it did not renature as a homogenous population but gave the appearance of a two population system. A simple analysis of this result was undertaken assuming that chloroplastal DNA is made up of a slow and a fast renaturing fraction. The true reaction rate, which is related to the genome content, was taken as that measured for each fraction divided by the square of its concentration ratio of the chloroplastal DNA.

This calculation put the genome content of the chloroplast DNA at 195 x 10$^6$ daltons which includes about eight copies each of 5.5 x 10$^6$ daltons and represents the fast fraction. This value is similar to that of a bacteriophage genome and so rules out bacterial contamination of the chloroplastal DNA. It was found however that chloroplasts have a similar DNA content to bacteria and so each chloroplast must carry several copies of the genome content. The genome repetition per chloroplast was estimated as a minimum of ten.

Renaturation of mitochondrial DNA was undertaken but was limited by the amount of DNA available. A tentative value for the genomic contents is
520 \times 10^6 \text{ daltons which includes 8 copies of } 10 \times 10^6 \text{ daltons.}
BIOPHYSICAL AND BIOCHEMICAL STUDIES ON

CHLOROPLAST DNA

Thesis

Submitted for

the degree of

DOCTOR OF PHILOSOPHY

by

RICHARD WELLS

University of Edinburgh,
August, 1968.
## CONTENTS

### CHAPTER 1  
**INTRODUCTION**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Genetics of Chloroplasts</td>
<td>1</td>
</tr>
<tr>
<td>B. Evidence for DNA in Chloroplasts</td>
<td>3</td>
</tr>
<tr>
<td>C. Evidence for RNA</td>
<td>6</td>
</tr>
<tr>
<td>D. Renaturation of DNA</td>
<td>7</td>
</tr>
</tbody>
</table>

### CHAPTER 2  
**METHODS AND MATERIALS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. List of Abbreviations</td>
<td>13</td>
</tr>
<tr>
<td>B. Chemicals</td>
<td>14</td>
</tr>
<tr>
<td>C. Light Microscopical Examination of Plant Fractions</td>
<td>14</td>
</tr>
<tr>
<td>D. Preparation of Non-aqueous Cell Fractions</td>
<td>15</td>
</tr>
<tr>
<td>E. The DNA, RNA and Protein Content of Non-aqueous Chloroplast Fractions</td>
<td>17</td>
</tr>
<tr>
<td>F. Extraction and Purification of DNA from non-aqueous Preparations</td>
<td>18</td>
</tr>
<tr>
<td>G. Aqueous Cell Fractions and Their Related DNA</td>
<td>23</td>
</tr>
<tr>
<td>H. Base analysis of DNA</td>
<td>36</td>
</tr>
<tr>
<td>I. Denaturation and Renaturation of DNA</td>
<td>37</td>
</tr>
<tr>
<td>J. Analytical Ultracentrifugation of DNA</td>
<td>42</td>
</tr>
</tbody>
</table>

### CHAPTER 3  
**RESULTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Purity of all fractions</td>
<td>46</td>
</tr>
<tr>
<td>B. Buoyant Density of DNA fractions</td>
<td>47</td>
</tr>
<tr>
<td>C. Base composition of DNA</td>
<td>48</td>
</tr>
<tr>
<td>D. Denaturation and Renaturation of Lettuce aqueous DNA Fractions</td>
<td>49</td>
</tr>
</tbody>
</table>
## CONTENTS (Contd.)

<table>
<thead>
<tr>
<th>CHAPTER 4</th>
<th>CONCLUSIONS AND DISCUSSION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>Identification of Chloroplastal DNA</td>
<td>52</td>
</tr>
<tr>
<td>B.</td>
<td>Renaturation of DNA</td>
<td>57</td>
</tr>
<tr>
<td>C.</td>
<td>Final Conclusions</td>
<td>64</td>
</tr>
</tbody>
</table>

Acknowledgement .......................... 66
Appendices .................................. 67
References .................................. 75
CHAPTER 1

INTRODUCTION

A) Genetics of Chloroplasts

Plastids are present in all green plants and may be concerned with storage, such as amyloplasts, with colouring such as chromoplasts or, most important of all, with the process of photosynthesis which is the responsibility of chloroplasts. These occur in some form in photosynthetic bacteria, protozoa and higher plants and vary widely in size, number and shape but are species specific for these characteristics.

Chloroplasts were one of the first subjects in which extrachromosomal inheritance was studied. In almost every plant species albino mutants or variegated individuals are known. In these cases the plants have either lost all recognizable plastids or have lost the ability to synthesize chlorophyll. Fully albino plants are often not viable but it is possible to observe inheritance characteristics from layers of albino cells in normal plants. In many instances the plastid mutants are inherited as a single chromosomal gene but the phenotypic effect of other chloroplast mutants can be explained only in terms of non-Mendelian genetics. Some of the earliest examples of this work was done on variegated plants of Mirabilis jalapa (Correns 1909, Bauer 1909). Other well known instances of non-Mendelian genetics are found in cases of paternal and maternal inheritance in plants, the classic example of the latter being the iojap mutant in Zea mays (Rhoades, 1946).
It is possible to correlate biochemical mutants of chloroplasts with their genetic segregation. One of the primary constituents of chloroplasts is chlorophyll for which many mutants are known and documented in the comprehensive review by Kirk 1967. Highkin (1950) showed a mutant which lacked the ability to synthesise chlorophyll B. A similar mutant has also been described by Hirono & Redei (1963) for Arabidopsis thaliana. Information known on the genetics of chlorophyll synthesis (Figure 1) shows it to be under nuclear control.

Another important constituent of chloroplasts is carotenoid and information on its biochemical pathway can be seen in Figure 2, and in common with chlorophyll, shows that all genes are under nuclear control. The localisation of genetic determinants responsible for many of the characteristics of chromoplasts, which are derived from chloroplasts, are particularly well documented (cf. Kirk, 1967) no less than nine separate genes being known, all of which segregate with mendelian genetics expected of nuclear genes.

There is also much evidence given by Kirk (1967) in his review to show that many of the steps governing the photosynthetic apparatus are also under nuclear control.

It must be pointed out however that the method of selection of chloroplast mutants favours those under nuclear control. This is so because a homozygous nuclear gene controlling a chloroplast character will affect all the chloroplasts in that cell and so will readily be identifiable. If however a chloroplast gene mutated it would only be likely to affect just its own chloroplast and so would
Figure 1. Genetic control of chlorophyll synthesis.
(reproduced from Kirk, 1967.)
Figure 2. Genetic control of carotenoid synthesis; a, In the leaf chloroplast, b, In the tomato fruit chromoplast. (reproduced from Kirk, 1967).
be extremely unlikely to be detected. Even if a system with only one chloroplast per cell is used there is a further complication in that, if there are chloroplast genes directing their own characteristics, the number of such gene copies per chloroplasts is not known. It may be, that to get such a gene in a homozygous situation would require the mutation of several identical gene copies, a process which could be time limited. The possibility of such reiterated genes being present in the chloroplast is strengthened by some of the work described in this thesis.

It is now accepted that the genetic material of all organisms so far studied, with the exception of RNA viruses, is DNA (see J.D. Watson, 1965). It would therefore be reasonable to suppose that if chloroplasts do have some autonomy of existence, and therefore carry their own genetic information, that they would have associated with them a DNA fraction. Such expectations appear to be justified and some of this evidence is presented below.

B) Evidence for DNA in chloroplasts

Early reports (Milovidov 1936, Yuasa 1938) indicated the presence of some cytoplasmic staining when examining the nucleus and about the same time Menke (1938, 1940) reported the presence of 0.7% phosphorus content of chloroplasts and a lamellar structure which had an absorption peak for ultraviolet light at 275 μm. Recent work has also shown DNA to be present in chloroplasts of *Chlamydomonas* (Ris & Plaut, 1962) and higher plants (Chiba 1951, Spieckerman 1957) by the technique of differential staining, whilst autoradiography has demonstrated the presence
of chloroplast DNA in marine algae (Stephenson & Sheridan, 1965), tobacco (McClendon, 1952) and Swiss chard (Kislev, Swift and Bogorad, 1965).

However, whilst these techniques confirm the presence of DNA in the chloroplasts, they give no indication of the origin or nature of this DNA for they cannot distinguish whether the DNA seen is truly chloroplastal or whether it is either nuclear or bacterial contamination. The use of biochemical identification of the DNA does not necessarily answer these questions but it does provide additional evidence of their origin.

DNA extracted by biochemical methods has been identified in Bryophyllum (Joussaume & Bourdu, 1964) and in Acetabularia where advantage can be taken of enucleation (Baltus & Brachet 1963, Gibor & Izawa 1963, Goffeau & Brachet 1965, Janouski 1965). Examinations with the analytical ultracentrifuge have revealed, in organisms containing chloroplasts, the presence of one or more satellite bands (Table 1) to the nuclear DNA band in cesium chloride gradients. These have been shown, usually by differential extraction, not to be nuclear contamination but to have an independent existence. The use of this technique and the identification of the chloroplast satellite has been elegantly shown in Euglena where colourless mutants, obtained by ultraviolet light or other techniques, which lacked any form of chloroplasts also lacked one of the two satellite bands of DNA. (Ray & Hanawalt 1965, Edelman, Schiff & Epstein, 1965). Later the second satellite band was identified as mitochondrial (Edelman, Epstein & Schiff, 1966).

In higher plants, DNA was extracted from the chloroplast
<table>
<thead>
<tr>
<th>Organism</th>
<th>Mainband Nuclear DNA</th>
<th>1st Satellite Chloroplastal DNA</th>
<th>2nd Satellite when identified as mitochondrial DNA</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydomonas</td>
<td>1.723</td>
<td>1.695</td>
<td>1.712</td>
<td>Chun et al., 1963.</td>
</tr>
<tr>
<td></td>
<td>1.721</td>
<td></td>
<td></td>
<td>Leff et al., 1963.</td>
</tr>
<tr>
<td>Chorella</td>
<td>1.716</td>
<td>1.695</td>
<td></td>
<td>Chun et al., 1965.</td>
</tr>
<tr>
<td>Beet</td>
<td>1.695</td>
<td>1.705</td>
<td>1.719</td>
<td>Chun et al., 1963.</td>
</tr>
<tr>
<td>Tobacco</td>
<td>1.690</td>
<td>1.703</td>
<td></td>
<td>Shipp et al., 1965</td>
</tr>
<tr>
<td>Ipomoea</td>
<td>1.692</td>
<td></td>
<td>1.706</td>
<td>&quot;</td>
</tr>
<tr>
<td>Allium</td>
<td>1.689</td>
<td></td>
<td>1.706</td>
<td>&quot;</td>
</tr>
<tr>
<td>Snapdragon</td>
<td>1.697</td>
<td>1.709</td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Swiss Chard</td>
<td>1.689</td>
<td>1.700</td>
<td></td>
<td>Kislev et al., 1965.</td>
</tr>
</tbody>
</table>
fraction of tobacco (Chiba, 1951, McClendon, 1952) and spinach (Baltus & Brachet, 1963, Chiba 1951, Pollard 1964). Information was also obtained on the nucleotide composition of Broad Bean chloroplast DNA (Kirk, 1963), whilst satellite bands (see Table 1) were observed in caesium chloride gradients for spinach, beet, tobacco and Swiss chard (Chun, Vaughan & Rich 1963, Kislev et al. 1965, Shipp, Kieras and Haselkorn, 1965).

Alternative evidence is provided by the differential incorporation of biochemical compounds, such as 5-bromouracil, into the two classes of DNA in Chlorella, one of which is associated with chloroplasts (Iwamura & Muto, 1964). This result could be explained by a difference in permeability of chloroplast and nuclear membranes. Other workers (Lyman, Epstein & Schiff, 1961) showed that the localisation of a micro-beam of ultraviolet light onto chloroplasts of Euglena produced white mutants, at wavelengths corresponding to nucleic acid action spectra, with no apparent damage to the rest of cell synthesis.

A differential effect of actinomycin D has also been shown in Euglena (McCalla & Allan 1964, Pogo & Pogo 1964), Broad Bean (Bogorad & Jacobson 1964) and Acetabularia (Schweiger & Berger, 1964) where it inhibited greening but did not affect the growth and so the rest of cell synthesis. Equivocal evidence was also obtained by DNA-DNA hybridisation which showed chloroplast DNA to be different from nuclear DNA (Shipp et al., 1965).

In only one of these cases is it possible to rule out nuclear contamination, that is for the enucleated Acetabularia. It was widely believed that in this case the DNA found was symptomatic
of bacteria adsorbed on to the outside of the Acetabularia but Gibor & Izawa (1963) showed that chloroplasts from sterile cultures contained $10^{-16}$ g of DNA per chloroplast. Other estimates for the amount of DNA per chloroplasts range from $10^{-15}$ g to $10^{-14}$ g for Euglena (Brawerman & Eisenstadt 1964, Edelman, Cowan, Epstein & Schiff 1964), Chlorella (Chun et al., 1963), Anthirrhinum (Ruppel & Wyk, 1965), tobacco and spinach (Chiba & Sugahara, 1957).

If Chloroplasts do have their own DNA, then, as DNA is known to code for RNA (see J.D. Watson, 1965), it is to be expected that chloroplasts also have their own RNA fraction distinct from the cytoplasmic (nuclear) RNA.

C) Evidence for RNA

RNA has been identified by various cytological techniques in the chloroplasts of green algae (Flaumenhaft, Conrad and Katz, 1960) and higher plants (Jacobson, Swift and Bogorad, 1963, Kislev et al. 1965, Littau 1958, Weier and Stocking, 1952).

Most reports on RNA in chloroplasts are based, however, on biochemical evidence. Extraction of nucleic acids from chloroplast fractions has shown RNA to be present in Allium and Antirrhinum (Ruppel, 1964), chinese cabbage (Clark, 1964), Corn (Orth & Cornwell, 1963), Sunflower (Sissakian & Odinstova, 1956), Spinach (Chiba 1951, Littleton & Tso, 1958) and Tobacco (App & Jagendorf, 1963, Dangeard 1945, Dubuy & Wood, 1943, Holden, 1952, McClendon, 1952, Sissakian & Odinstova, 1956, Stephens, Thimann & Zamenick, 1956). Biochemical techniques have also been used to show that in Euglena there is a change in the nucleotide composition of RNA on moving the organism from a dark to light environment (Brawerman, 1959)
and a similar difference was also observed between green and colourless cells (Pogo, Brawerman & Chagraff, 1962). Other workers have shown a difference in the nucleotide composition of the nuclear and chloroplast RNA fractions of tobacco (Parker, 1952), whilst evidence from experiments with Acetabularia suggest, from the maintenance of RNA turnover after enucleation, the possibility of extra nuclear RNA synthesis (Hammerling, Claus, Keck, Richter & Werz, 1958).

The presence of RNA in chloroplasts does not necessarily indicate that it is synthesised in the chloroplasts, for this RNA could be of nuclear origin. There is, however, one chloroplast RNA fraction which seems to differ from the bulk of the cytoplasmic RNA and that is the ribosomal RNA.

This difference can be seen in many ways. The incorporation of labelled amino acids in chloroplast preparations free of cytoplasmic ribosomes is taken as an indication of chloroplast ribosomes in tobacco (Alva & Jagendorf, 1963) and in Euglena (Eisenstadt & Brawerman, 1964), (Goffeau & Brachet, 1965). More definite proof is obtained from work on spinach (Lyttleton, 1962) and on Euglena (Brawerman, 1963) showing that the chloroplast ribosomes differ from the relative nuclear ribosomes in nucleotide composition (Table 2). Other differences observed are in sedimentation values between the two classes of ribosomes for Euglena (Brawerman, 1963, Eisenstadt & Brawerman, 1964), Chinese cabbage (Clark, 1964) and pea (Loening, 1967) (Table 3). Loening (1968) has also shown that there is a difference in the molecular weight of the ribosomal RNA from the chloroplast and cytoplasm (Table 4).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Nucleotide</th>
<th>Cytoplasmic (mole per cent)</th>
<th>Chloroplastal (mole per cent)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euglena</td>
<td>A</td>
<td>22.7</td>
<td>27.4</td>
<td>Brawerman (1963)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>27.1</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>29.5</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>19.6</td>
<td>24.6</td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>A</td>
<td>25.0</td>
<td>25.5</td>
<td>Littleton (1962)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>22.3</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>31.7</td>
<td>32.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>21.0</td>
<td>20.0</td>
<td></td>
</tr>
</tbody>
</table>
Table 3
Sedimentation Values for Ribosomal Sub-units

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cytoplasmic (Svedbergs $\times 10^{13}$)</th>
<th>Chloroplastal (Svedbergs $\times 10^{13}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euglena</td>
<td>49</td>
<td>43</td>
<td>Brawerman (1963)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>60</td>
<td>Eisenstadt &amp; Brawerman (1964)</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>83</td>
<td>68</td>
<td>Clark et al. (1964)</td>
</tr>
</tbody>
</table>

Table 4
Molecular Weight of Ribosomal RNA

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cytoplasmic (daltons $\times 10^{6}$)</th>
<th>Chloroplastal (daltons $\times 10^{6}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td>1.3</td>
<td>1.1</td>
<td>Loening (1968)</td>
</tr>
</tbody>
</table>
There appears to be a mass of evidence favouring the existence of autonomous DNA and RNA in chloroplasts. As they also are associated with a unique class of ribosomes it would seem logical to suppose that this RNA fraction is coded for by the chloroplastal DNA. It is obviously of interest to ask how much coding information is carried by the chloroplastal DNA and this can be approached by the use of renaturation kinetics on this DNA. (Bolton, Britten, Cowie, Roberts, Szafranski & Waring, 1965). It is no use, however, obtaining such a result if the DNA is of uncertain character and purity and so the first part of this thesis is concerned with the unambiguous identification of the chloroplastal DNA.

D) Renaturation of DNA

Complete denaturation of DNA, that is the separation of complementary strands of DNA, can be achieved by heat, high pH, low salt conditions or a combination of these factors (Marmur & Doty, 1962). Renaturation is the process by which the separated strands come back into register. Although the melting temperature for strand separation of DNA of similar base composition is independent of its source, the extent to which renaturation proceeds is greatly influenced by the type of DNA under study (Marmur & Doty, 1961). This becomes understandable when it is recognised that renaturation of any specific complementary strands during renaturation will depend on its concentration (Marmur & Lane, 1960). For a fixed quantity of DNA the concentration of such a specific nucleotide sequence will become smaller with increasing complexity of the species from which the DNA is isolated. This assumes that the
complexity of a species is reflected in its DNA and that the DNA under question contains no repeated sequences. Bacteria which are relatively simple will contain a more homogeneous distribution of DNA molecules than that of DNA isolated from higher plants or animals but less homogeneous than that extracted from bacteriophages. Thus, for a given concentration of DNA, renaturation would be expected to occur most readily with the bacteriophage DNA and to be least complete with DNA from a mammalian source such as calf thymus DNA. That this is so has been shown by Marmur & Doty (1961), who indeed found that bacteriophage DNA renatured faster than bacterial DNA, which in turn renatured much faster than mammalian DNA.

This raises the question of how representative the rate of renaturation is with relation to the complexity of the DNA under consideration. Wetmur & Davidson (1968) produced values for the kinetic complexity of DNA from various sources. The kinetic complexity is taken as the length of DNA represented by the renaturation data and was arrived at by theoretical considerations (Wetmur & Davidson, 1968). Such values, which assume the non repetition of the DNA sequences, are shown in Table 5. The average value for the kinetic complexity is compared with values for the analytical complexity in Table 6 (Wetmur & Davidson, 1968). The analytical complexity is equivalent to the true molecular weight of the DNA contained by the organism, or in the case of multicellular organisms, per cell and represents its "genomic content". It will be noted that over a range of $10^6$ in molecular weight no error larger than 2 is observed between the analytical and kinetic complexity values.
### Table 5

Kinetic Complexity, in Molecular Units for Various Organisms calculated from Renaturation Kinetics.

<table>
<thead>
<tr>
<th>DNA</th>
<th>An</th>
<th>K2 mole⁻¹ sec⁻¹</th>
<th>Kinetic Complexity daltons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat ascites</td>
<td>9.4</td>
<td>0.0026</td>
<td>2.3 × 10¹²</td>
</tr>
<tr>
<td>Tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>3.5</td>
<td>5.0</td>
<td>2.5 × 10⁹</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.75</td>
<td>4.7</td>
<td>2.7 × 10⁹</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.23</td>
<td>5.4</td>
<td>2.3 × 10⁹</td>
</tr>
<tr>
<td>T4</td>
<td>1.4</td>
<td>190</td>
<td>1.9 × 10⁸</td>
</tr>
<tr>
<td>T4</td>
<td>1.4</td>
<td>39</td>
<td>1.9 × 10⁸</td>
</tr>
<tr>
<td>T4</td>
<td>0.14</td>
<td>220</td>
<td>1.75 × 10⁸</td>
</tr>
<tr>
<td>T4</td>
<td>0.14</td>
<td>34</td>
<td>1.7 × 10⁸</td>
</tr>
<tr>
<td>N1</td>
<td>0.14</td>
<td>1490</td>
<td>2.6 × 10⁶</td>
</tr>
<tr>
<td>N1</td>
<td>0.14</td>
<td>330</td>
<td>2.2 × 10⁷</td>
</tr>
<tr>
<td>T7</td>
<td>1.4</td>
<td>1790</td>
<td>2.6 × 10⁷</td>
</tr>
<tr>
<td>T7</td>
<td>1.4</td>
<td>150</td>
<td>2.7 × 10⁷</td>
</tr>
<tr>
<td>T7</td>
<td>0.14</td>
<td>1620</td>
<td>2.4 × 10⁷</td>
</tr>
<tr>
<td>T7</td>
<td>0.14</td>
<td>320</td>
<td>2.1 × 10⁷</td>
</tr>
<tr>
<td>SV40</td>
<td>0.16</td>
<td>1080</td>
<td>5.4 × 10⁶</td>
</tr>
</tbody>
</table>

An = optical absorption and represents DNA concentration.

K2 = The second order renaturation constant.

This data is reproduced from the work of Wetmur & Davidson (1968).
<table>
<thead>
<tr>
<th>DNA</th>
<th>Analytical Complexity (daltons)</th>
<th>Kinetic Complexity (daltons)</th>
<th>Ratio kinetic/analytical</th>
<th>GC°/o (mole per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td>$1.3 \pm 0.1 \times 10^8$</td>
<td>$1.6 \times 10^8$</td>
<td>1.4</td>
<td>34</td>
</tr>
<tr>
<td>Rat ascites tumour</td>
<td>$2.5 \pm 0.5 \times 10^{12}$</td>
<td>$2.3 \times 10^{12}$</td>
<td>0.92</td>
<td>42</td>
</tr>
<tr>
<td>SV40</td>
<td>$3.3 \pm 0.1 \times 10^6$</td>
<td>$5.4 \times 10^6$</td>
<td>1.6</td>
<td>41</td>
</tr>
<tr>
<td>T7</td>
<td>$2.5 \pm 0.1 \times 10^7$</td>
<td>$2.5 \times 10^7$</td>
<td>1.0</td>
<td>49</td>
</tr>
<tr>
<td>E. coli</td>
<td>$2.5 \pm 0.5 \times 10^9$</td>
<td>$2.5 \times 10^9$</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td>N1</td>
<td>$3.3 \pm 0.15 \times 10^7$</td>
<td>$2.4 \times 10^7$</td>
<td>0.70</td>
<td>64</td>
</tr>
</tbody>
</table>

$GC^{°/o}$ = The guanine plus cytosine composition of the DNA.

This data is reproduced from the work of Wetmur and Davidson (1968).
The difference between the two values appears in some way to be connected with the guanine plus cytosine content of the DNA (Wetmur & Davidson, 1968) for the DNA fractions which are rich in guanine plus cytosine appear to renature faster than predicted.

Comparisons of kinetic complexity such as those made by Wetmur & Davidson (1968) can only be made under constant and appropriate conditions. Much information is in fact known on the effect of various parameters on the rate of renaturation and the following notes are in the main abstracted from their work.

i) Since the rate of renaturation is second order (Britten & Kohne, 1966), it is directly dependent on the concentration of the DNA in solution. This is a very critical test of the renaturation system under study.

ii) The maximum rate of renaturation occurs at a temperature 20°C to 30°C below the melting temperature. (Marmur & Doty, 1961, Wetmur & Davidson, 1968). The variation of renaturation rate with temperature produces a bell shaped curve with its peak at about 30°C below the melting temperature and a width at half maximum of 30°C. (Britten & Kohne, 1966).

iii) Whilst their theory (Wetmur & Davidson, 1968) predicted that for a second order reaction the rate of renaturation should be directly proportional to the length of the DNA, they found that the empirical second order rate constant for renaturation of a DNA sample obtained by shearing a high molecular weight DNA preparation is proportional to the square root of the single stranded molecular weight (or length). Wetmur & Davidson (1968) proposed that the proportion of the second order rate constant to the square root of the DNA length (single stranded) is due to an excluded
volume or steric hindrance effect; that is, the longer a DNA strand the more difficult it is for a second strand to interpenetrate and find complementary sites.

iv) The effect of ionic strength on the rate of renaturation of DNA has been studied by Marmur & Lane (1960) and Britten & Kohne (1966) who found that similar to the case for the melting temperature of DNA (Dove & Davidson, 1962) there is little effect at above 0.4 molar in a chloride system. There is, however, a large effect on the rate of renaturation at low salt concentrations. These effects are likely to be comparable in origin (cf. Wetmur & Davidson, 1968), reflecting changes in the electrostatic interactions between the two strands of the DNA polymer in solution.

v) There is a dependence on the pH of the solution at low values but in the range of pH 5 to 9 the rate of renaturation at 0.4 molar sodium ion is essentially independent of pH.

vi) Wetmur & Davidson (1968) found that there was an effect on rate of renaturation by viscosity. These results were observed with differing concentrations of sucrose and ethylene glycol and cause the renaturation to change from its previously observed second order effect.

Renaturation of the DNA consists mainly of two processes, that of nucleation followed by zippering of the DNA region in register. Nucleation is the bringing together of the two complementary strands to effect contact. It would be expected that the recognition of complementary strands requires a minimum number
of base pairs to effect nucleation and Walker (1968) gives the minimum number of such base pairs as six. Once the minimum nucleation region has been achieved the remaining bases of that complementary strand can be zippered up. If the rate of renaturation is dependent on nucleation then this process is expected to be second order (cf. Marmur, Rownd, & Schildkraut, 1963), whereas if the zippering of the DNA was rate limiting it would be expected to be first order. The expected second order kinetics are in fact obtained (Wetmur & Davidson, 1968) except in cases of high viscosity. Under these conditions it is believed by these authors that the rate of zippering is hydrodynamically limited due to the requirement that the remaining single stranded random coil regions, not yet base paired, must move through the solvent and rotate about the axis as the reaction takes place. Under normal conditions the reaction is initiated by a nucleation step, which is not hydrodynamically limited.

There is no doubt that under appropriate conditions the renaturation of DNA can give much information on the kinetic complexity and therefore on the analytical complexity of DNA. In transferring results from kinetic to analytical complexity it must be kept in mind that the guanine plus cytosine content of the DNA may affect its value significantly.
A) List of abbreviations

DNA. Deoxyribonucleic acid.
RNA. Ribonucleic acid.
DNase. Deoxyribonuclease.
RNase. Ribonuclease.
EDTA. Ethyldiaminetetraacetic acid.
SLS. Sodium lauryl sulphate.
SSC. Standard saline citrate.
Tris. Tris(hydroxymethyl)aminomethane.
4AS. 4-amino salicylate.
TIPNS. Tri-isopropyl napthalenesulphonate.

\( g = \) gram, \( mg = \) milligram, \( \mu g = \) microgram.
\( \mu m = \) millimicron, a unit of wavelength.
\( ml = \) millilitre.
\( \text{rev/min} = \) revolutions per minute.
\( (W/V) = \) weight for volume.
\( (V/V) = \) volume for volume.
\( \text{CsCl} = \) caesium chloride.
B) **Chemicals**

All chemicals with the exception of the following were obtained in analar form from British Drug Houses Ltd., Poole, Dorset, England.

These chemicals were obtained from Sigma Biochem. Inc., London, England.

DNase I DN-CRNase -A, albumin (bovine serum), Protease and tris.

C) **Light Microscopical Examination of Plant Fractions**

Examination was carried out either by phase or contrast optics on a Wild M20-42227 microscope (Heerbrugg, Switzerland). The maximum magnification available was ×1500 with the oil immersion lens. Contaminating nuclei were identified by the use of aceto-carmine stain (Darlington & La Cour, 1942). Estimates of chloroplast numbers in a sample were made by dilution and counting in a haemocytometer (improved Neubauer type, Hawksley Ltd., London, England.) with the aid of the microscope. An attempt was also made to count mitochondria by this method but this was unsatisfactory for two reasons. 1) The area under the cover slip is significantly greater than the size of a mitochondria and therefore when counting, it was necessary to go through a spectrum of focusing. 2) Any small piece of debris tends to be identified as mitochondrial. This method did however give an indication of the magnitude of the mitochondria to chloroplast ratio.
D) Preparation of Non-aqueous Cell Fractions

These methods were based upon non-aqueous cell fractionation by Biggin & Park (1961) and Stocking (1959). Leaves were washed, the midribs removed, excess water shaken off and then they were placed in liquid nitrogen held in a litre thermos flask. When all the leaves were in the liquid nitrogen they were transferred to a mortar (precooled with liquid nitrogen), and ground with a china pestle until the pieces were \( \frac{1}{4} \) to \( \frac{1}{2} \) cm. square. The leaves were maintained in their frozen state by the addition of more liquid nitrogen. These pieces were then lyophilised and when dry, they were placed in a china pulveriser drum and ground in a vibrational ball mill (Griffin & George Ltd., London, England), 1 litre drum size half filled with 2 cc. china cylindrical pellets, eccentric cam drive) until the pieces, when suspended in a non-aqueous liquid, passed through a 37\( \mu \) mesh and so were sub-cellular in size. The non-aqueous liquid used was a mixture of carbon tetrachloride and petroleum ether with volumes adjusted to specific gravity requirements. (The specific gravity was measured by a PSL 103 specific gravity hydrometer, Poulten, Selfe & Lee Ltd., Wickford, Essex, England).

Investigation was made on the distribution of cell organelles within the specific gravity fractions. A specific gravity fraction was obtained by centrifuging (2000 rev/min, 10 minutes, Sorvall RC-2, HB4 rotor) at the upper specific gravity limit (say 1.59) to remove all material of higher specific gravity and then lowering the specific gravity of the non-aqueous liquid to the lower limit (say 1.50) and centrifuging once more. The resulting pellet was
taken as representing material of the specific gravity range 1.50 - 1.59. A complete fractionation of a non-aqueous spinach preparation produced the following results, when examined in the light microscope. Nuclei were identified by staining.

Fraction 1. Specific gravity greater than 1.59.
Mainly fragments with a few nuclei.

Fraction 2. Specific gravity 1.50 - 1.59.
Big debris and clumps of cells with some trapped chloroplasts and visible nuclear contamination.

Fraction 3. Specific gravity 1.45 - 1.50.
Some cellular debris with significant quantities of "grey" material. A few chloroplasts seen were attached to debris. A low level of nuclear contamination.

Fraction 4. Specific gravity 1.40 - 1.45.
A low level of cellular debris. No free chloroplasts but a few were in clumps, probably associated with the debris. A significant proportion of nuclei.

Fraction 5. Specific gravity 1.35 - 1.40.
Very high number of nuclei. A small number of free chloroplasts observed.

Fraction 6. Specific gravity 1.30 - 1.35.
Mainly free chloroplasts with small debris. Very low number of nuclei.

Fraction 7. Specific gravity 1.00 - 1.30.
Very small debris, no nuclei and very few chloroplasts.

In the light of these results it was decided to take the fraction of specific gravity 1.30 - 1.35 as most representative for the chloroplasts, a similar range to Biggin & Park (1961). When the
fraction of specific gravity 1.00 - 1.30 was modified to 1.00 - 1.25, it was found that any remaining chloroplasts were removed and so investigation was also undertaken of this fraction. The required fractions were obtained as shown in Figure 3. All steps, with the exception of those in liquid nitrogen, were undertaken between 0°C and 4°C.

E) The DNA, RNA and Protein Content of Non-aqueous Chloroplast Fractions

Having separated what appeared to be a chloroplast pellet, it is obviously worth while to see if it contains any DNA. This was carried out in the following manner (Biggin & Park, 1961). 1g. of dried chloroplast powder was given two washes with methyl alcohol. The residue was treated with cold 5% (W/V) trichloroacetic acid for 30 minutes and then centrifuged (2000 rev/min in a Sorvall RC-2 centrifuge HB rotor) and the residue given two further washes of ethanol. The remaining ethanol was removed by a wash with an ethanol-ether mixture (1/1), followed by a final wash with ether. The residue, now a white pellet, was given an alkaline digest by incubation at 37°C with 0.3N KOH for 16 hours. The suspension was then acidified with the addition of perchloric and centrifuged as described above. The supernatant was taken as containing the RNA component and this was tested by measurement and spectral examination in a DB spectrophotometer (Beckman Ltd., Glenrothes, Scotland). The RNA was also measured by the Orcinol method (Drury, 1948). The precipitate, which at this stage was presumed to contain the DNA and protein, was given a 15 minute
Figure 3

Leaves (Fragments smaller than 37μ)

Pellet (discarded)
(Nuclear material, cell walls)

Petroleum ether-carbon tetrachloride (specific gravity 1.35)

Pellet (chloroplastal fraction)

Petroleum ether-carbon tetrachloride (sp. gr. 1.30)

Pellet (discarded)
(1.00 - 1.25 fraction)

Petroleum ether-carbon tetrachloride (sp. gr. 1.25)

Pellet (discarded)

Petroleum ether-carbon tetrachloride (sp. gr. 1.00)

Flow diagram of separation of chloroplasts and fraction of specific gravity 1.00 - 1.25.

All centrifuge steps were at 1,000 rev/min for 10 minutes in the Sorvall RC-2.
treatment with 5\(^{0}/\text{o}\) (W/V) perchloric acid at 90\(^{0}\)C. The solution was again centrifuged as above and washed with cold perchloric acid. This step was repeated and the two supernatants pooled together. The supernatant pool was tested for DNA both by ultraviolet absorption and by the diphenylamine reaction. (Burton, 1956, modification of the Dische 1930 reaction.) The residue was tested for proteins by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). The dry weight percentages obtained were 20.5\(^{0}/\text{o}\) for protein, 0.7\(^{0}/\text{o}\) for RNA and 0.07\(^{0}/\text{o}\) for the DNA. The RNA and DNA spectra have their ultraviolet absorption peaks (Figures 4 and 5) at 258 \(\text{m}\mu\) and 268 \(\text{m}\mu\) respectively, which is very near the expected value (Ogur & Rosen, 1949) and indicated only a low possible level of cross contamination.

F) Extraction and Purification of DNA from Non-aqueous Preparations

List of Reagents

**Saline - EDTA.** 0.15M NaCl, 0.1M EDTA, pH 8. Inhibits DNase activity (Marmur, 1961).

**Standard saline citrate.** 0.15M NaCl, 0.015M trisodium citrate, pH 7.0, helps to protect DNA structure (Marmur, 1961). Dissolve DNA in 1/10 SSC.

**Sodium perchlorate.** 5M. The high salt concentration helps to dissociate protein from nucleic acid (Lerman & Tolmach, 1957).

**Sodium lauryl sulphate.** 25\(^{0}/\text{o}\). Will lyse most non-metabolizing cells, inhibit enzyme action and denature some proteins. (Bolle & Kellenberger, 1958).

**Chloroform-isoamyl alcohol, 24:1, (V/V).** A deproteinising agent (Sevag, Lackman & Smollens, 1938). The chloroform causes surface
Figure 4. Ultraviolet spectrum of hydrolised RNA from non-aqueous chloroplast preparations.
Figure 5. Ultraviolet spectrum of hydrolised DNA from non-aqueous chloroplast preparations.
denaturation of the proteins. The isoamyl alcohol reduces foaming, aids the separation and maintains the stability of the layers upon centrifugation.

Ethyl alcohol, 95\%/o. Used to precipitate DNA following deproteinisation (Marmur, 1961).

Ribonuclease, 0.2\%/o, in 0.15M NaCl, pH 5.0. Preheated at 80\°C for 10 minutes to remove contaminating DNase. (Marmur, 1961).

Acetate-EDTA. 3.0M sodium acetate, 0.001M EDTA, pH 7.0. Provides the proper ionic environment for the isopropanol separation of DNA from RNA (cf. Marmur, 1961).

Isopropanol. Used to selectively precipitate DNA (Marmur, 1961).

Phenol. A deproteinizing agent. (Kirby, 1957). Distilled and saturated with SSC. Stored in a dark bottle at 0\°C.

Phenol-m-cresol. (1/1, V/V). A more powerful deproteinizing agent than phenol alone (Kirby, 1965).

4 Aminosalicylate, 6\%/o (W/V). Helps to release the DNA into the aqueous phase when used in conjunction with phenol. (Kirby, 1965).

Tri-isopropyl-napthalenesulphanate, 5\%/o (W/V). A suitable detergent for use with phenol, can be used at greater concentrations than SLS (Kirby, 1965).

Extraction and Purification of DNA.

i) Preliminary investigations.

The results of the hydrolisation experiment above suggested a significant amount of DNA in the chloroplastal fraction. It gave no indication however of the origin of this DNA. The following method was therefore used in an attempt to extract this DNA.

a) Preparations of dried chloroplast were suspended (10 ml/g) in a standard laboratory medium used for DNA extraction (1M NaCl,
0.01M EDTA, 2% (w/v) SLS, pH 7.5. The ratio of lysing media to dried chloroplast preparation is important and was the minimum volume that the pellet could be dispersed in. Marmur (1961) suggests that too great a dilution of the DNA may result in degradation of the DNA. From this stage a standard Marmur (1961) procedure was used as described below.

1) This describes lysing of the cells and was replaced by the step described above.

2) Add perchlorate to 1M.

3) Add an equal volume of chloroform-isopropyl alcohol and shake for 30 minutes.

4) Centrifuge for 5 minutes at 5,000 rev/min in the Sorvall.

5) Remove the top aqueous phase, add two volumes of ethyl alcohol, and spool the DNA from the interphase with a glass rod. The excess ethyl alcohol is removed from the spool by gently pressing it against the side of the beaker.

6) The DNA is redissolved in 10-15 mls. of 1/10 SSC and made up to SSC when fully dissolved. An equal volume of chloroform is added and given a 15 minute shake, followed by a 5 minute centrifugation at 2,000 rev/min in the Sorvall.

7) Repeat step 6 with the top aqueous layer until no interphase appears upon centrifugation.

8) Incubate with RNase (final concentration of 50 μg/ml) at 37°C for 30 minutes.

9) Repeat step 6 to remove any remaining proteins.

10) Repeat step 5 and redissolve DNA in 9 ml of 1/10 SSC.

11) Add 1 ml acetate EDTA.
12) Stir rapidly and add 0.54 volumes of Isopropanol, drop by drop into the vortex. The DNA should precipitate in fibrous form.

13) Remove acetate and salt by gently stirring the DNA spool in increasing (70%-95%) portions of ethyl alcohol.

14) Redissolve in solvent of choice.

Unfortunately this method could not be taken beyond step 5 as it was not possible to obtain a spool. The following modification was therefore made to the initial extraction step. 400 µg/ml of protease was added to the extraction medium in place of the SLS in the hope that the DNA might be freed from its protein sheath. This was incubated for 30 minutes at 0°C at which time the SLS was added and the extraction proceeded as above. This modification was however unsuccessful and no spool was obtained. One final modification was tried, that of substituting a phenol shake for the chloroform-isoamylalcohol (Kirby, 1957). This, however, made no difference and the expected spool did not materialise (Ruth Sager in discussion).

There could be several reasons why no DNA spool could be obtained.

1) Damage to the DNA while the leaf is being lyophilised.

2) The chloroplasts are not dispersing properly in the extraction medium and so the SLS cannot act to lyse them.

3) SLS is not an active agent in breaking open chloroplasts.

4) The DNA is being broken down by the action of an extremely fast DNase enzyme.

5) There is little if any DNA in chloroplasts.
There is ample evidence that this last statement is not true (cf. Kirk, 1967) and it is extremely unlikely that a DNase action would be that fast. Unless there is significant DNase action at 0°C, it is also extremely unlikely that the DNA is being broken down while the leaves are lyophilised. The favourite contender for the disappearance of the chloroplastal DNA was the nonsolubilisation of the chloroplasts and their consequent lack of lysis. There is, however, the further possibility that even though lysis occurs, there is not enough DNA to spool. An indication that this last statement might be true was provided by using the extraction techniques described above with the difference that the spooling stage was omitted and the DNA brought down by centrifugation (18 hours, 38,000 rev/min at 15°C in a Beckman 1-2 centrifuge (Marmur, 1961)). When this preparation was examined in the ultracentrifuge a DNA band was visible for the chloroplast preparation but it had all the appearances of low molecular weight nuclear DNA, for it banded at a very similar buoyant density, and the expected satellite band was not apparent. As the Marmur (1961) method yielded some DNA but only a small fraction of the expected amount, it was felt worthwhile to try a different method of extraction and that described below owes much to the methods of Kirby, most of which are listed in Hastings & Kirby (1966). All steps were at room temperature unless otherwise stipulated. Centrifugation was in a Sorvall RC-2, with an HB4 rotor. Volumes are given for an input of 1 g. of dried chloroplast material.

ii) 1) Add 10 ml of 0.2M NaCl, 1% (w/v) SLS, 6% (w/v) 4AS, 50 mM tris, pH 8.0.

2) Add an equal volume of phenol and homogenise for 15 seconds
in the omni mixer at full speed (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) at 0°C.

3) Add 10 ml of chloroform-isoamyl alcohol and shake for 15 minutes. Centrifuge at 2,000 rev/min for 15 minutes.

4) Precipitate the DNA from the top aqueous phase by the addition of ethyl alcohol to a final concentration of 70% (V/V). Leave overnight at 0°C. Remove the resulting precipitate by centrifugation (1,000 rev/min, 10 minutes).

5) Dissolve precipitate in 1 ml, or more if necessary, of 1/10 SSC. When fully dissolved, make up to 3M NaCl. Leave overnight at 0°C and remove resulting DNA precipitate (Hastings & Kirby, 1966) by centrifugation (1,000 rev/min, 10 minutes).

6) Redissolve in 1N 1/10 SSC (1 ml). Add an equal volume of phenol-m-cresol (1:1, V/V) to the supernatant and shake for 15 minutes. Centrifuge at 2,000 rev/min for 15 minutes. The interphase in which the DNA is expected to reside was resuspended in 1 ml 1/10 SSC, but no DNA was obtained. This indeed was a blow but the method was repeated with modifications to make it simpler.

iii) Modification of ii).

1) As step 1 in ii).

2) Add 1/2 volume phenol.

3) Make 3M with respect to NaCl. Leave overnight at 0°C. Centrifuge at 6,000 rev/min for 15 minutes.

4) The DNA was precipitated from the aqueous layer.

5) Redissolve the precipitate in 1 ml of 1/10 SSC and add an equal volume of phenol-m-cresol (1:1, V/V) and shake for 15 minutes. Centrifuge at 2,000 rev/min for 15 minutes.
6) All fractions of the gradient were tested for DNA content by ultraviolet absorption spectra and by analytical centrifugation. Some DNA was found in the aqueous phase, but of very low quantity (5%-10% of theoretical yield from hydrolysis experiment) and banded in CsCl gradients at a buoyant density value similar to that for the nuclear DNA. There still was no sign of the satellite band seen in whole cell preparations when examined in the analytical ultracentrifuge.

iv) This is a yet more modified procedure of ii), from which it was hoped that DNA losses would be minimised.

1) 10 ml of medium (step 1, ii)) was carefully mixed into the chloroplast powder very much like mixing milk into flour. It was gently stirred for 5 minutes.

2) Add an equal volume of phenol and stir by hand for 5 minutes. Centrifuge at 6,000 rev/min for 10 minutes.

3) The DNA was precipitated from the aqueous top layer as described in ii), step 4.

4) Redissolve in 1/10 SSC (1 ml). Add 1.25g of CsCl and when fully dissolved, let stand in an ice bath for 30 minutes. Centrifuge for 10 minutes at 8,000 rev/min. This gave an RNA pellet and protein surface skin.

5) The supernatant was carefully removed, avoiding both the pellet and surface skin. It was dripped into 20 ml of an isopropanol-water mixture (1:1, V/V) which was being rapidly stirred with a magnetic stirrer. A small fibrous spool was obtained from the vortex.
6) The DNA spool was given three ethyl alcohol washes and then redissolved in 1/10 SSC. When fully dissolved it was made up to SSC with the addition of 10 X SSC.

7) Dialyse for 12 hours against SSC with a dialysis bag which had been pretreated by a wash in ZnCl2 for 15 minutes followed by a boil for 5 minutes in EDTA (McBain & Steuer, 1936). This is believed to increase the holes in the dialysis bag, thus encouraging the removal of the small nucleotides. This step also helps to degrade any nucleases on the dialysis bag.

When this DNA was examined in the analytical ultracentrifuge it was found to have a similar but not identical buoyant density to the nuclear DNA. Examination of the DNA by means of a melting curve, that is the observation of ultraviolet absorbance at 260 μ showed that the hyperchromic rise in the DNA region (above 60°C in 1/10 SSC) indicated that there was only a DNA complement of 30% and that RNA accounted for nearly 50% of the total UV absorbance in the DNA preparation.

A repeat of this experiment with the final DNA solution being exposed to an RNase digest (50 μg/ml, 37°C, 30 minutes) (Marmur, 1961) followed by a protease digest (400 μg/ml, 37°C, 3 hours) improved the DNA to give a hyperchromicity rise of 30% with no detectable rise below 60°C. This shows a nearly clean preparation of DNA as the expected hyperchromicity rise was 38% (Marmur & Doty, 1962) but the satellite DNA expected was not observable. The amount of DNA recovered was about 25% of the figure obtained by hydrolysis, which seemed reasonable after all the purification steps, and the spectra for this DNA can be seen in Figure 6. From the analytical
ultracentrifuge results it appears that the DNA is still not of very high molecular weight. A further preparation was undertaken with one further modification, that of adding 5% of tri-isopropyl-napthalene sulphonate to the initial suspension medium. This chemical helps to release the DNA from any protein covering and is also a good nuclease inhibitor (Kirby & Cook, 1967). The buoyant density values for the chloroplast fraction were identical to those for the previous preparation but there was some improvement in the length of the DNA. It was felt that the really significant details of the extraction of DNA from chloroplast preparations was the initial mixing of the dried pellet and the suspension medium and the precipitation of the DNA rather than spooling. It was also noted that better results could be obtained if a chloroform-isoamyl alcohol step (24/1, v/v) was included before a phenol shake, as this seems to cause some clumping of the DNA suspension. To confirm these beliefs a final preparation of DNA was undertaken.

v) The final preparation

This method has taken something from all those already tried, and was kept as simple as possible. All these methods which seemed not to work with the chloroplastal DNA extraction were good when tried on whole cell DNA, and all showed the heavy satellite when examined in the analytical ultracentrifuge. It was thought that the DNA which had been extracted might indeed have been representative of the chloroplastal DNA and this was kept very much in mind for this preparation. The suspension medium was as for preparation
Figure 6. Ultraviolet spectrum of native DNA from non-aqueous chloroplast preparations.
i) and great care was taken to form a good mixture. The lysate was then allowed to stand at room temperature for 10 minutes (10°C). At this time one and a half volumes (with respect to lysate) of chloroform-isoamyl alcohol (24:1, V/V) were added and the mixture given a 10 minute shake. It was then centrifuged at 8,000 rev/min for 15 minutes (Sorvall RC-2, HB4 rotor). The top phase was mixed with an equal volume of phenol (saturated with SSC), given a 10 minute shake and then centrifuged again as above. The aqueous top layer was carefully removed and the DNA was precipitated by the addition of two volumes of ethanol, and overnight storage at -20°C. The DNA precipitate was recovered by centrifugation at 1,000 rev/min for 5 minutes in the Sorvall RC-2, HB4 rotor, given two ethanol washes and dissolved in 1/10 SSC (1/2 ml per gram of dried input material), which was made up to SSC when the DNA precipitate had fully dissolved. To lessen DNase activity the SSC suspension was given a 10 minute treatment at 60°C. It was rapidly cooled to 37°C and incubated for thirty minutes with 50 µg/ml RNase (made DNase free by a 5 minute treatment at 100°C with 2%, W/V, sodium Acetate, pH 5). At this time 400 µg/ml protease was added and the suspension given a further incubation for 3 hours at the same temperature. When the DNA was examined, it was found to be similar in all respects to that obtained from the methods described above. It was noticed, however, that the buoyant densities of the chloroplastal and whole cell DNA did differ to a small degree. It was decided to try and extract some chloroplast DNA by an aqueous means in order to assess this result.
The experimental material used for the initial preparations (i) and (ii) was spinach, but all further preparations were made from lettuce. This was because of its easier availability all round the year. The final experimental method was, however, repeated on spinach and extended to bean leaves and pea leaves.

G) Aqueous Cell Fractions and Their Related DNA

Preliminary experiments

1) Before attempting to extract chloroplastal DNA, a pilot experiment was run on the whole leaf tissue. The method was as follows. Leaves were washed and the stalks and midribs removed to make homogenisation easier. Leaves were then homogenised in a Waring blender type mixer (Atomix, Measuring and Scientific Equipment Ltd., London, England), in 5 litres of medium per kilogram of leaves (1M NaCl, 0.01M tris, 0.01M EDTA, 2% (w/v) SLS, 1% (w/v) mercapto ethanol, pH 7.5) for 15 seconds at full speed at 0°C. It was hoped by the use of the mercapto ethanol, a reducing agent, to prevent plant dies from damaging or adsorbing onto the DNA. The homogenate was incubated for 30 minutes at 70°C to liberate the DNA and prevent DNase action (Bolton E.T., personal communication). From this stage method v) for extraction of DNA from non-aqueous preparations was followed, with the exception of the lysis step. No spool was obtained for this type of preparation and it was thought likely that the DNA was being broken down under the action of the heat
treatment. The same experiment without the heat treatment was undertaken and a small spool was obtained. The yield, however, was too low for the whole cell preparation to justify its use on a chloroplast preparation.

ii) Honda medium as described by Boardman, Franki & Wildman (1966) was used as the homogenisation medium (2.5% (w/v), Ficoll, 5% (w/v), Dextran 40, 0.25M sucrose, 0.025M tris, pH 7.8 and the method described above (i) was used without the heat treatment. This method gave a better DNA yield but upon examination in the analytical centrifuge, it was found that the DNA was of low molecular weight. Another method, that of Chun, Vaughan & Rich (1963) was used and as it gave a promising result, it was repeated with a separation step included for the chloroplast fraction and is described below.

iii)  
1) 100 g of leaves were ground in a large mortar with sea sand and 140 ml of buffered sucrose solution (0.1M sucrose, 0.02M tris, 0.01M NaCl, 0.005M EDTA, pH 8.0).

2) Filter through four layers of cheesecloth and centrifuge for 10 minutes at 600 rev/min to obtain a white pellet (nuclei).

3) Centrifuge supernatant from 2) at 1,500 rev/min for 20 minutes and the pellet obtained was designated as chloroplastal.

4) The nuclear and chloroplastal pellets were separately resuspended in 0.15M NaCl, 0.1M EDTA, 1% (w/v) SLS, pH 7.4.
5) The method of Marmur (1961) (see section F, preparation 1)) was followed with the exception of the alcohol precipitation step. Instead the high perchlorate concentration was reduced by dialysis against 0.15M NaCl, 0.015M Na citrate buffer. The DNA from these two fractions appeared to have very similar buoyant densities. There was a suspicion, however, that the chloroplast fraction was contaminated with nuclei and this was confirmed when examined in the microscope. To clean up the chloroplastal pellet, an extra step was introduced of a sucrose pelleting. The chloroplast preparation was layered onto the top of a 1.7M sucrose in Beckman 25/1 tubes and centrifuged at 21,000 rev/min for 1 hour at 15°C (L-2, centrifuge, Beckman Instruments Ltd., Glenrothes, Scotland). The contaminating nuclei pelleted (Chauveau, Moule & Roullier, 1956) leaving the chloroplasts as a surface skin. DNA was extracted as described. The DNA from the two fractions was found to be similar but not identical in buoyant density. A small satellite was detectable in the chloroplast preparation under overload conditions for the analytical ultracentrifuge. It was difficult from these results to estimate how clean a preparation of chloroplastal DNA was being made if indeed it was chloroplastal DNA at all.

About this time the work of Suyama and Bonner (1966) was brought to notice. They had been successful in producing uncontaminated mitochondrial DNA from plants by use of a DNase step, and it was felt worthwhile to try a similar step for chloroplasts, for, if any DNA was obtained it should be free of contaminating nuclear DNA. DNA was indeed obtained and the method is described below.
iv) Aqueous fractionation of chloroplasts and mitochondria

Preparations of chloroplasts and mitochondria were based upon the protocol of Suyama & Bonner (1966). Leaves which had been washed had their midribs removed. All further steps were carried out between 0°C and 4°C. Leaves were then homogenised in a Waring Blender (Atomix, M.S.E., Ltd.) at the rate of 5 litres of homogenisation medium (0.3M mannitol, 0.05M tris-HCl buffer, 3mM EDTA, 0.05% (w/v) cysteine and 0.1% (w/v) bovine serum albumin pH adjusted to 7.2). The homogenate was filtered through four layers of cheesecloth and the chloroplastal and mitochondrial fractions prepared as shown in Figure 7.

Mitochondrial and chloroplastal fractions were resuspended in 3 volumes of homogenisation medium which was made up to 3 mM MgCl2. 10 μg/ml Dnase was added and the suspension kept in an ice bath for 30 minutes. The chloroplastal and mitochondrial fractions were twice pelleted in an excess volume (3:1) of saline EDTA (0.1M NaCl, 0.1M EDTA) at 400 rev/min and 2,000 rev/min, respectively (Griffin Christ Junior Centrifuge, Griffin & George Ltd.) for 15 minutes. These pellets, designated as clean chloroplastal and mitochondrial fractions, were resuspended in 3 volumes, relative to the pellet, of homogenisation medium for DNA extraction.

v) Aqueous preparation of nuclei

The initial steps were identical to those for the crude chloroplast pellet. Normally the nuclei are lysed by the action
Figure 7

Homogenate (after being passed through four layers of cheesecloth) 400 rev/min for 15 minutes

Pellet (chloroplasts with broken nuclei and chromatin contamination) 2,000 rev/min 15 minutes

Pellet

Pellet (discarded, Any remaining and broken chloroplasts.) 2000 rev/min 15 minutes

Pellet (mitochondrial fraction)

Supernatant (discarded)

Resuspended in 3 volumes of homogenisation medium.
600 rev/min, 10 minutes.

Flow diagram showing separation of chloroplastal and mitochondrial fractions from aqueous gradients. The first two centrifugations were undertaken in the Griffin-Christ centrifuge and all others in the Sorvall RC-2.
of the EDTA, and the nuclear DNA, then removed by a DNase digest. Leaves were homogenised as for chloroplasts in the isotonic homogenisation medium which was free of EDTA but contained 3mM CaCl2, which helps to preserve nuclei structure (Schneider & Peterman, 1950). The homogenate was centrifuged at 2,000 rev/min for 15 mins in the Griffin-Christ centrifuge and the resulting pellet gave a mixture of nuclei and chloroplasts. The nuclei were separated from the chloroplasts by differential centrifugation in a sucrose solution (Chauveau, Moule & Roullier, 1956). The nuclear and chloroplast pellets were resuspended in the modified homogenisation medium (10 ml. per 1000 g. fresh leaves). This suspension was equally divided and layered on to the top of three 25/1 Beckman L-2 centrifuge tubes, three quarters filled with a 2M sucrose, 3mM CaCl2 solution. The suspension was gently stirred into the top layer of the sucrose solution and then given a 60 minutes centrifugation at 25,000 rev/min, 15°C, in a Beckman L-2 centrifuge. This resulted in a pellet consisting predominantly of nuclei and this was resuspended in homogenisation medium (10 ml. per 1000 g. fresh leaves) ready for DNA extraction.

vi) **Final preparation of DNA**

Suspension of chloroplasts, mitochondria or nuclei were made up to SSC, 2% SLS, 0.2M tris, pH 8.5 and the DNA extracted by a method similar to that of Marmur (1961). The lysate was allowed to stand at room temperature (10°C) for 10 minutes, one and a half
volumes of chloroform-isoamyl alcohol (24:1, V/V) were added and the mixture shaken for 10 minutes. To this was added a further (lysate) volume of SSC saturated phenol and the whole mixture agitated for a further 10 minutes. The mixture was then centrifuged at 8,000 rev/min for 10 minutes in a Sorvall RC-2 centrifuge, HB4 rotor. The aqueous top layer was carefully removed and the DNA was precipitated by the addition of two volumes of ethyl alcohol. After storage at -20°C for 24 hours, the precipitate was recovered by centrifugation at 1,000 rev/min for 5 minutes in the Sorvall RC-2 HB4 rotor and dissolved in a small volume of 1/10 SSC (1 ml. per 1,000 g. original fresh weight), which was made up to SSC when the precipitate was fully dissolved. It was noted that at this point the solution was contaminated with a brown colouration.

The DNA was purified by the addition of 50 µg/ml RNase, made DNase free by a 5 minute treatment at 80°C in 2% (W/V) NaCl, pH 5 (Marmur, 1961) and incubated for 1 hour at 37°C. At this time 400 µg/ml of protease was added and the DNA solution given a further 3 hours incubation at the same temperature. These steps, which should remove any contaminating RNA or protein, seemed to have little effect on the contaminating colouration. The use of mercapto ethanol, TRNS or 4AS were tried out in the homogenisation medium but none of them had any significant effect on the contamination level. It was noted that if the DNA was banded in a preparative CsCl gradient, that some of the colouration pelleted whilst the rest spread through the gradient very much as if there was a gradient of molecule sizes, some of
which were so small that their back diffusion was as great, if not
greater, than their sedimentation value. It was decided to make
use of this and so, after the RNase and protease treatment, the
DNA was pelleted in a high salt solution (38,000 rev/min, 18 hours,
15°C, 5M NaCl in a Beckman L-2 centrifuge 40 head). It was hoped
that any colouration which pelleted with the DNA would subse-
quently pellet in the preparative CsCl gradient, thus separating
from the DNA. The pellet from the NaCl centrifugation was re-
suspended in 4 ml. of 1/10 SSC, to which was added 5.1 g. of
CsCl. The buoyant density was adjusted to 1.710 g.cm\(^{-3}\) by
examination of the CsCl solution in the Abbe refractometer
(Bellingham & Staley, Ltd., London, England). The DNA in CsCl
solution was then centrifuged at 42,000 rev/min for 60 hours at
25°C in an MSE superspeed 50 centrifuge, 2410 rotor (Measuring &
resulting gradient were collected by piercing the bottom of the
tube and collecting the resulting drops (slowly) in a series of
tubes. The position of the resulting DNA band was determined by
sampling the fractions for ultraviolet absorption. At this stage
the fractions containing the DNA appeared still to be contaminated
with about 20% background absorption, assumed to be the brown
colouration (Figure 8). The hyperchromicity of the DNA at this
stage was consistent with a 20% contamination level and was in
the region of 30%. It was hoped that the contaminating molecules
in the DNA fractions would be sufficiently small not to pellet
with the DNA when it was brought down by centrifugation. The DNA
containing fractions were pooled together and three volumes of
Figure 3. 0---O represents the DNA as measured by ultraviolet absorption. --- The estimated background contamination.
1/10 SSC added. The DNA was pelleted by centrifugation for 18 hours at 15°C in the L-2 centrifuge, 50 head and this resulted in a clear pellet at the end of the run. The hyperchromicity was consistent with the removal of the remaining contamination. DNA yields were determined at this point and found to be in the order of 150-250 μg and 30-50 μg per 1,000 g. leaves (fresh weight) for chloroplastal and mitochondrial fractions respectively. The nuclear DNA yield for the same input was in the order of 8 mg.

H) Base Analysis of DNA

The method of Wyatt (1955) was followed. 200 μg - 500 μg of DNA was hydrolysed by a 30 minute treatment at 175°C in 70% formic acid and the bases resuspended in 0.1 ml. of 1N HCl. This was then applied to a point at the top of the Whatman No. 1 paper in small amounts to minimise spread.

The paper was left in the chromatography tank with isopropanol-HCl in the bottom for one hour, to allow it to equilibrate and then the top section of the tank filled with isopropanol-HCl. The chromatogram was allowed to run for 24 hours and then removed and air dried. The position of the spots containing the bases was determined by shining an ultraviolet light at the paper. The positions of the spots were lightly ringed with a pencil and blanks were also ringed at the same distance from the front. The bases were eluted from the paper in the following manner. 0.1 ml. of 0.1N HCl was placed on the cut out piece of chromatography paper and it was wrapped in an open tube of silver foil, one end of which was pointed down a centrifuge tube and the other end bent over
the rim to prevent it slipping down the tube. The tube was then centrifuged for one minute at position one in a Gallenkamp Junior bench centrifuge (Gallenkamp Ltd., London, England). This step was repeated ten times and so resulted in each spot being eluted in 1 ml. of 0.1N HCl after ten washes. Identification of the bases was undertaken both from the Rf value (the position of the spot relative to the movement of the front) and from the ultraviolet spectrum. Comparison was made with standard values given by Wyatt (1955). Recovery of DNA in the form of bases was better than 95% of the original input. The mole percent was calculated from the extinction values at 260 μm or at the maximum wavelength from values given by Wyatt (1955) or from the Schwartz catalogue (1967). (Schwartz Bioresearch Inc., New York, U.S.A.)

I) Denaturation and Renaturation of DNA

There are a variety of systems which can be used to study renaturation of DNA, each of which provides information which is complementary and yet unique.

One such technique is the use of a hydroxyapatite column. Since renatured DNA binds to hydroxyapatite (Britten & Kohne, 1966) the kinetics of renaturation can be monitored by measurement of the amount of DNA which binds to the column as a function of time. Although the hydroxyapatite renaturation kinetics paralleled those by spectrophotometric means (see below) the hydroxyapatite reaction was faster. This can be explained in the following manner (Britten & Kohne, 1966). The optical method measures the fraction of the
length of the DNA that is actually paired. The hydroxyapatite method, however, measures the fraction of the total number of DNA molecules that have some portion of their length renatured. Due to random shearing of a large population of similar molecules, any one strand may react with another that has been sheared at a different point. Thus unpaired lengths will be present early on in the reaction, giving the impression of a larger amount of renaturation than is true. Hydroxyapatite is, however, particularly useful for fractionation of DNA on the basis of renaturation rates, especially where there are widely divergent renaturation rates present in the total DNA (Britten & Kohne, 1966). A technique not so commonly used but useful in cases of very fast renaturation is that of density gradients. Since renaturation of DNA results in a decrease of its buoyant density (cf. Marmur, Rownd & Schildkraut, 1963) to a value approaching its native buoyant density, the kinetics of renaturation can be followed by the return of the buoyant density of the renaturing DNA towards its native value. Use of this technique for the molecular weight of ribosomal cistrons has been made by Birnstiel, Jones & Loening (1968).

Probably the most widely used technique for DNA renaturation is spectrophotometric. Since the denaturation of DNA is accompanied by a hyperchromic shift because of the disruption of its ordered secondary structure (Marmur et al., 1963), renaturation or the restoration of the DNA to native properties can be conveniently followed by measuring the changes in hypochromicity (the loss of hyperchromicity) at an elevated temperature. This
was the system used for renaturation in this thesis. The instrument available for renaturation studies was a Beckman DB spectrophotometer with a heating jacket. The parameters for renaturation were in part determined by the system. The temperature characteristics of the instrument decreed that renaturation had to take place below 70°C. There also was no automatic readout on the instrument and therefore another serious limitation is the speed at which readings can be taken. The fastest renaturing fraction was found to be the chloroplastal DNA and therefore the conditions had to be suitable for this DNA. The size of the renaturing DNA used was that found to be most reproducible, for, under the sonication conditions used, there appeared to be a plateau of size against time of renaturation. It could be that this is caused by limiting hydrodynamic shear on the molecule. The maximum concentration of DNA is also limited by the instrument and the largest concentration that could conveniently be used was 60 μg/ml. Renaturation conditions used were therefore 1 x SSC, 60°C, 6 x 10^5 daltons and concentrations of DNA from 5 to 60 μg/ml.

Melting curves were carried out on an aliquot of all DNA fractions used for renaturation. These provide information on the purity of the DNA fraction, for any hyperchromicity observed below 60°C in 1/10 SSC indicated contaminating RNA. The presence of DNA of very low molecular weight could also be detected by an unusually shallow and early rise to the melting curve, but is quite distinct from the RNA effect. As a final check the hyperchromicity is an indication whether the DNA has present any ultraviolet absorbing impurities, and the expected hyperchromicity is in the order of
38% (Marmur & Doty, 1962). Melting of the DNA was carried out in 1/10 SSC by measuring the increase in ultraviolet absorbance at 260 nm with increasing temperature. The temperature was measured by direct immersion of a thermistor 1107 (Sasco Ltd., London, England), into the blank cell of the spectrophotometer. The thermistor represented one arm of a Wheatstone bridge circuit and was adjusted for a null point at 50°C. A calibration plot was made for the necessary range of temperature. Before use the reading was checked at two standard temperatures but it was found to be a very stable device with an accuracy of 1/5°C.

The melting temperature of a DNA fraction was taken as that which was equivalent to half the hyperchromicity. No DNA was used for renaturation studies that did not show at least 35% hyperchromicity. In order to make comparisons between renaturation of DNA fractions more meaningful, DNA was brought to a constant size by sonication. The DNA in solution, at the concentration required for that experiment, had nitrogen gas bubbled through it for 5 minutes to minimise free radical interactions upon sonication. The DNA solution was then given a 4 second pulse at full power (4 amp reading on the scale) with a Dawes Soniprobe 1130 A (Dawe Instruments Ltd., London, England). The sonication was carried out by the total immersion of the probe into the DNA solution, which was in the order of 1 ml, held in a Beckman L-2 centrifuge, 50 rotor tube. Temperature during sonication was maintained near 0°C by immersing the tube into a beaker holding an ice water mixture. Before use the probe was precooled with a similar mixture and given a short burst in distilled water near 0°C to clean it.
For renaturation studies the DNA was denatured by a 5 minute treatment in 1/10 SSC at 100°C. The spectrophotometer was preheated for a sufficient time to ensure equilibrium of temperature by passing hot water through a jacket around the cells. The temperature was monitored by the thermistor in the blank cell but it was found that under equilibrium condition the cell temperature was 5°C below that of the circulating water bath and this could be used as a reliable indicator. When the DNA was fully denatured, it was made up to SSC with the addition of 10 x SSC (preheated to 100°C). For denaturation the DNA solution had been placed in the spectrophotometer cell and so this was now transferred directly to the spectrophotometer. An objection arises here that the DNA solution is not at a constant temperature during the initial part of renaturation but experiments showed that within 1 minute the temperature in the cell was down to about 75°C. The temperature dependence of renaturation, however, is a broad plateau and by this time the renaturation reaction was proceeding at least at half speed. (Wetmur & Davidson, 1968). Effect was taken of a small drop in absorbance between 100°C and 60°C (Wetmur & Davidson, 1968). It was found that immersion of the spectrophotometer cell in water at 0°C for 5 seconds reduced the temperature to near 60°C but no significant difference could be observed between the renaturation curves with and without this treatment. As the value of the second order rate constant is calculated from the slope of a graph involving perhaps up to 20 readings, the first two points could safely be ignored. The value of the half-life
is dependent on the starting value of the ultraviolet absorbance which is not affected, though a small time error, estimated at maximum as 10%, may introduce an error of this magnitude. The half-life was taken as the time to return to 50% hyperchromicity (Bolton, Britten, Cowie, Roberts, Szafranski & Waring, 1965) and the second order renaturation constant can be calculated from the ultraviolet absorbance decay curve (Wetmur & Davidson, 1968) (see Appendix).

Whilst it is possible to compare renaturation results obtained by the method above with that of other workers, in order to calculate a value for the analytical complexity variables in parameters between the renaturation systems have to be taken into account. It was therefore felt more satisfactory to use as renaturation standards DNA's of known analytical complexity. The DNA standards chosen, T4 bacteriophage and E. coli DNA, were known to behave with the expected second order kinetics (Wetmur & Davidson, 1968) and to have no detectable repeated sequences (Britten & Kohne, 1966).

J) **Analytical Ultracentrifugation of DNA.**

1) **CsCl gradient density centrifugation.**

Analytical ultracentrifugation was carried out in a Beckman model E (Beckman Instruments Ltd., Glenrothes, Scotland) equipped with Schlieren and ultraviolet optics. An alternator attachment was provided, allowing automatic photography of two cells in each experiment. The method of Vinograd & Hearst (1962) was followed.
The cells used for ultracentrifugation were those of 12 mm light path, Kel-F centrepiece with a 4° sector. When used with two cells, special windows were used to deflect the light through the appropriate path in the alternator. Centrifugation was at 44,000 rev/min, 25° C for 20 hours. The speed was kept constant by an electronic speed control. Early experiments were undertaken with a mechanical speed control at 44,770 rev/min, but these values have been converted to the lower speed (see Appendix). The time of 20 hours was found necessary to allow the gradient to reach equilibrium. Examination of DNA samples were carried out on input levels of 1-3 μg/ml, except when DNA fractions were being sampled for minor satellites under overload conditions, when input levels as high as 20 μg/ml were used. Samples were prepared by adding 0.8 ml of the DNA solution to 1.02 g. of CsCl. The buoyant density was checked by means of the Abe refractometer and was adjusted to 1.715. This value is approximately the mean of the plant DNA buoyant density and that of the marker DNA.

Calibration of the analytical ultracentrifuge was undertaken by using two DNA fractions of known buoyant densities. When equilibrated, an ultraviolet photograph of the cell was taken (Film, Kodak CP8) and the negative scanned with a Joyce Loebl microdensitometer 111C (Joyce, Loeble Ltd., Gateshead-on-Tyne, England). This gives an expanded tracing of the centrifuge photograph and converts the DNA bands into peaks (as in Figures 8 and 9). The standard DNA's used were E. coli DNA and Micrococcus Lysodeikticus DNA. The buoyant density figures for these DNA's were taken from the work of Szybalski (1968) and
were 1.710 and 1.731 g. cm\(^{-3}\) respectively. If a linear density gradient is assumed, then a value for the buoyant density per cm. of the microdensitometer can be calculated. This figure came to 3.27 mg. cm\(^{-3}\) per cm. of microdensitometer tracing at a scale magnification of X 20. For experimental runs the *Micrococcus Lysodeikticus* DNA was used as a standard of buoyant density 1.731 g. cm\(^{-3}\) and all other DNA values referred to it by the figure above. These values assume that the two DNA bands were equally spaced about the mid position of the analytical cell, which should have been assured by the refractometer check. If, as occasionally happened, the bands were not equicentral then a correction factor was introduced into the calculation (see Appendix).

ii) **Analytical Zone Sedimentation**

The analytical zone centrifugation method (band sedimentation) followed was that of Vinograd, Bruner, Kent & Weigle (1963). The Kel-F centrepiece was modified as described by these authors. The single stranded DNA size was estimated from the sedimentation value (Studier, 1965) in alkali solutions (0.9M NaCl, 0.1M NaOH). A convenient speed for molecules of single stranded size 10^5-10^6 daltons was found to be 44,000 rev/min. Photographs were taken with the ultraviolet optics at intervals of two minutes and calculations of the sedimentation values (see Appendix) were made when the band was at least a third the way across the cell to allow equilibrium to be attained (Marmur et al., 1963). Photographs were traced out on the
Joyce Loebl microdensitometer and the mid-point of the peak was taken as representative of the DNA sample under investigation unless the tracing appeared to be very lopsided, suggesting a non-linear distribution of DNA sizes. DNA populations of this type are no use for renaturation studies as they provide an unnecessary complication and so were used for base analysis or another experiment not affected by the size variation of the molecule. The movement of the DNA band was taken as the mean distance travelled on the tracing for a four minute period. This was divided by the magnification factor which can be obtained from the microdensitometer tracing. The length of the ultracentrifuge cell limits the length of the photograph and consequently shows up as sharp transitions on the tracing. As the cell length is given by the manufacturers the magnification factor can be obtained directly. The DNA input for the sedimentation experiments was 0.05 ml of solution of DNA concentration 100 μg/ml.
CHAPTER 3

RESULTS

A) Purity of Cell Fractions

i) Non-aqueous fractions. The examination of these fractions in the light microscope is listed in materials and methods, B).

ii) Aqueous fractions. Light microscopical examination of chloroplastal or mitochondrial fractions did not show contamination by intact nuclei or nucleoli. A low number of nucleoli were identified when the EDTA level of the homogenisation medium (final preparation) was reduced to 1 mM.

When these fractions were examined in the electron microscope (by Dr. Shankarnarayan) the chloroplast fraction was seen to be free of nuclei and mitochondria and that over 50% of the chloroplasts contained an intact outer membrane. This finding is in good agreement with the examination in the light microscope where it was observed that, of the total chloroplast population, about 60% appeared to be well preserved and crescent shaped. The remainder were dull in colour and had a swollen appearance.

The mitochondrial fraction, when examined in the electron microscope was essentially free of contaminating material, except for a very low level of chloroplastal contamination (1 per 1,500 mitochondrial like particles in the haemocytometer).

iii) Number of chloroplasts. For lettuce preparations the mean number of chloroplasts was $1.2 \times 10^{11}$ per 1,000 g. of fresh leaves. The number of mitochondrial like particles for the same preparation was $5.2 \times 10^{11}$.
B) Buoyant Density of DNA Fractions

1) Non-aqueous preparations.

DNA from the total lyophilised spinach leaves banded in CsCl gradients at a buoyant density of $1.694 \text{ g cm}^{-3}$ (Figure 9a) with a minor satellite at a buoyant density of $1.706 \text{ g cm}^{-3}$. Early experiments with the chloroplastal DNA indicated that it had a similar buoyant density to the nuclear DNA. Closer examination of the DNA extracted by method v) showed the buoyant densities to be slightly different. This was confirmed when a sample of chloroplastal and nuclear DNA was run in each cell of the rotor in the analytical centrifuge. This ensures that the DNA has been examined under identical conditions, with the exception of the optical system. This was checked by reversing the cell contents but the difference of the order of $3 \text{ mg cm}^{-3}$ was still maintained between the buoyant density of chloroplastal and nuclear samples. As final proof, when the two cells were loaded with an identical DNA the same buoyant density result was returned. This gives the spinach chloroplast DNA a buoyant density value of $1.697 \text{ g cm}^{-3}$ (Figure 9b) which is distinct from both the nuclear value ($1.694 \text{ g cm}^{-3}$) and the heavy satellite ($1.706 \text{ g cm}^{-3}$). DNA prepared from the fraction of specific gravity $1.00 - 1.25$ (by method v)), which is low in chloroplast content, yielded a DNA band of low molecular weight in the buoyant density range $1.706 \text{ g cm}^{-3}$ (Figure 9c). Similar results were obtained with lettuce and with bean and sweet pea leaves (Table 7).
Figure 9. Microdensitometer tracings of DNA from non-aqueously prepared lettuce fractions in CsCl density gradients.

A) Whole Cell DNA.
B) DNA from the chloroplast fraction (specific gravity 1.30 - 1.35).
C) DNA from the fraction of specific gravity 1.00 - 1.25.
Table 7

Buoyant density of DNA from non-aqueously prepared plant fractions

<table>
<thead>
<tr>
<th></th>
<th>Nuclear (g. cm(^{-3}))</th>
<th>Chloroplastal (Specific gravity 1.30-1.35) (g. cm(^{-3}))</th>
<th>(Specific gravity 1.00-1.25) (g. cm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet pea</td>
<td>1.695</td>
<td>1.697</td>
<td>------</td>
</tr>
<tr>
<td>Bean (\textit{Vicia faba})</td>
<td>1.695</td>
<td>1.697</td>
<td>1.705</td>
</tr>
<tr>
<td>Spinach</td>
<td>1.694</td>
<td>1.697</td>
<td>1.706</td>
</tr>
<tr>
<td>Lettuce</td>
<td>1.694</td>
<td>1.697</td>
<td>1.706</td>
</tr>
</tbody>
</table>

Table 8

Buoyant density of DNA from aqueously prepared plant fractions

<table>
<thead>
<tr>
<th></th>
<th>Nuclear (g. cm(^{-3}))</th>
<th>Chloroplastal (g. cm(^{-3}))</th>
<th>Mitochondrial (g. cm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bean</td>
<td>1.695</td>
<td>1.697</td>
<td>1.705</td>
</tr>
<tr>
<td>Lettuce</td>
<td>1.694</td>
<td>1.697</td>
<td>1.706</td>
</tr>
</tbody>
</table>

All values to 0.001 g. cm\(^{-3}\)
ii) Aqueous Preparations

DNA prepared from lettuce chloroplastal fractions which had not been treated with DNase gave a broad band of buoyant density around 1.695 g.cm\(^{-3}\). When DNA of high enough molecular weight was used (\(20 \times 10^6\) daltons) this resolved into two bands, one at the buoyant density of nuclear DNA (1.694 g.cm\(^{-3}\)) and the other 2-4 mg/cm\(^3\) higher in buoyant density (Figure 10a). When the DNase step was included the chloroplast DNA formed a unimodal band at a buoyant density of 1.697 g.cm\(^{-3}\) (Figure 10d). Mitochondrial DNA fractions without the DNase step showed two bands of buoyant density 1.694 g.cm\(^{-3}\) and 1.706 g.cm\(^{-3}\) (Figure 10b). With the DNase step included, a unimodal band of buoyant density 1.706 g.cm\(^{-3}\) resulted (Figure 10e). The DNA removed by the DNase treatment was shown to be coincident in buoyant density (1.694 g.cm\(^{-3}\) (Figure 10c)) to that obtained from nuclei. The buoyant density value for the chloroplast DNA from lettuce and bean (Table 8) is identical to that obtained from the non-aqueous preparations and that of the mitochondrial DNA agrees well with the value obtained from the non-aqueous fraction of specific gravity 1.00 - 1.25 (Tables 7 and 8).

C) Base Composition of DNA

The base composition of DNA can be estimated both from the melting temperature (Marmur & Doty, 1962) and the buoyant density (Sueoka, Marmur & Doty, 1959). This, however, does not show up minor bases. It has been shown by other workers (Brawerman & Eisenstadt, 1964, Ray & Hanawalt, 1964, Ruppel & Wyk, 1965, Whitfield & Spencer, 1968) that Euglena and higher plant chloroplastal...
Figure 10. Microdensitometer tracings of DNA from aqueously prepared fractions in CsCl density gradients.

A) DNA from the chloroplast fraction without the DNase treatment.
B) DNA from the mitochondrial fraction without the DNase treatment.
C) DNA from nuclei.
D) DNA from the chloroplast fractions with the DNase treatment.
E) DNA from the mitochondrial fractions with the DNase treatment.
DNA can be differentiated from its relative nuclear DNA by the lack of a rare DNA base of 5-methyl cytosine which is present to several per cent in the nuclear DNA. This was confirmed for the lettuce DNA and the results are shown in Table 9.

D) Denaturation and Renaturation of Lettuce Aqueous DNA Fractions

Melting temperatures of 68°C, 69°C and 74°C in 1/10 SSC were obtained for nuclear chloroplastal and mitochondrial DNA respectively (Figure 11). In all cases the hyperchromicity was in excess of 35%. The progress of renaturation can be observed by the loss of hyperchromicity (hypochromicity) as the DNA returns to a double stranded structure and such decay curves for the lettuce DNA fractions are shown in Figure 12. The renaturation half-lives calculated from these curves are shown in Table 10, and is taken as the time to return 50% of the hyperchromicity. Also shown in Table 10 are renaturation half-lives for T4 phage and E. Coli DNA which were used as standards. These served both as a check that the renaturation system was working as expected and as a method of comparison. As expected, T4 and E. coli DNA regained 70-80% of the hyperchromicity (Bolton et al., 1965) and both the chloroplastal and mitochondrial DNA provide a similar figure. The nuclear DNA does renature to an extent of 20-30%. It is also possible to present the renaturation data in such a way (see Appendix) that the second order renaturation constant can be calculated (Wetmur & Davidson, 1968). Theoretically this gives a straight line plot and it can be seen in Figure 13 that the T4 and E. Coli DNA do give the predicted results.
Table 9

Base composition of DNA from aqueously prepared lettuce fractions.

<table>
<thead>
<tr>
<th></th>
<th>Nuclear (mole percent)</th>
<th>Chloroplastal (mole percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>28.5</td>
<td>31.1</td>
</tr>
<tr>
<td>Thymidine</td>
<td>28.3</td>
<td>25.8</td>
</tr>
<tr>
<td>Guanine</td>
<td>21.0</td>
<td>22.5</td>
</tr>
<tr>
<td>Cytosine</td>
<td>17.2</td>
<td>21.4</td>
</tr>
<tr>
<td>5-methyl cytosine</td>
<td>5.0</td>
<td>B.D.</td>
</tr>
</tbody>
</table>

B.D. = below detection, which is estimated as 0.3 mole percent.
Figure 11. Melting curves in 1/10 SSC of aqueously prepared lettuce DNA. Chloroplastal DNA (O---O---O). Nuclear DNA (●---●---●). Mitochondrial DNA (△ --- △ --- △).
### TABLE 10

Renaturation half-lives ($t_{1/2}$) of DNA fractions. (minutes)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 phage</td>
<td>18</td>
</tr>
<tr>
<td>E. coli</td>
<td>240</td>
</tr>
<tr>
<td>Lettuce chloroplasts (AQ)</td>
<td>15</td>
</tr>
<tr>
<td>Lettuce mitochondria (AQ)</td>
<td>42</td>
</tr>
</tbody>
</table>

The DNA was renatured at Tm = 25°C in 1 x SSC at concentrations of 5-50 µg/ml with a size of $6 \times 10^5$ daltons. Half-life figures above have been adjusted for a DNA concentration of 100 µg/ml assuming an inverse concentration ratio.
Figure 12. Renaturation decay curves of aqueously prepared lettuce DNA. Chloroplastal DNA (0---0---0). Nuclear DNA (Δ---Δ---Δ). Mitochondrial DNA (●---●---●).
Figure 13. A second order renaturation plot from the data of decay curves (Figure 12). DNA concentration adjusted to 100 µg/ml. T4 DNA (control (Δ--Δ--Δ). E.coli DNA (control) (x---x---x). Chloroplastal DNA (O---O---O). Mitochondrial DNA (Θ---Θ---Θ). Nuclear DNA (□---□---□).
Both the chloroplastal and mitochondrial fractions renature to the same extent as the T4 and E. coli but do not give the predicted straight line (Figure 12). For convenience these DNA fractions can be thought of as two DNA populations intermingled. This is clearly shown for the chloroplastal DNA in the alternative K2 plot (see Appendix). This kind of plot (Figure 14) gives a value for the second order renaturation constant (Table 11) which, under the same salt concentration and temperature, is independent of DNA concentration (Wetmur & Davidson, 1968) but dependent on DNA size. The ratio of the second order renaturation constants for the two populations should however, be independent of both DNA concentration and size. Table 12 shows a list of ratios for chloroplast DNA of varying sizes and gives a mean value of 2.5. Also shown are two experiments with mitochondrial DNA. An attempt was also made to calculate what percentage of the chloroplast DNA each population fraction represented in the following manner. Both fractions in Figure 14 were extrapolated to zero time. If the zero time value is assumed to represent the starting hyperchromicity of both fractions, then the difference between them should represent the first (fast) fraction (see Appendix). The hyperchromicity for this population fraction (fast) has been shown as a percentage of the total in Table 12 and has a mean value for the chloroplast DNA of 23%. The second order renaturation constant which can be directly read from the slope of Figure 14 assumes that all the DNA is identical. If, however, only half of the DNA represented by the hyperchromicity rise was to engage in renaturation, both axes of this graph would be affected, the one by the hyperchromicity
Figure 14. Alternative second order renaturation plot. Gives the value of $K_2$ directly and independently of DNA concentration. T4 DNA (control) (---). Chloroplastal DNA (O---O---O). Theoretical $K_2$ plot (X---X---X) from the $K_2$ values calculated in the results assuming the fast fraction is represented by 23% and the ratio of $K_2$ values between the two populations is 2.5.
Table 11

Second Order Renaturation Constant (K2) for DNA Fractions.

<table>
<thead>
<tr>
<th></th>
<th>K2 (mole(^{-1}) sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 phage</td>
<td>6.8</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.53</td>
</tr>
<tr>
<td>Lettuce chloroplasts slow fraction</td>
<td>4.6</td>
</tr>
<tr>
<td>Lettuce chloroplasts fast fraction</td>
<td>11.5</td>
</tr>
<tr>
<td>Lettuce mitochondria slow fraction</td>
<td>1.6</td>
</tr>
<tr>
<td>Lettuce mitochondria fast fraction</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Conditions of renaturation given in Table 10. The values for T4 and *E. coli* DNA were calculated from Figure 13 adjusted for concentration, or from the type of plot shown in Figure 14. Values for the chloroplasts and mitochondria were calculated from plots as shown in Figure 14.
Table 12

Renaturation data for chloroplastal and mitochondrial DNA when treated as a two population system.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>size of DNA (daltons)</th>
<th>K2 (slow fraction) (mole(^{-1}) sec(^{-1}))</th>
<th>(ratio K2 fast/K2 slow)</th>
<th>slow fraction (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplastal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 29</td>
<td>6 \times 10^5</td>
<td>4.6</td>
<td>2.4</td>
<td>24</td>
</tr>
<tr>
<td>30</td>
<td>6 \times 10^5</td>
<td>4.6</td>
<td>2.5</td>
<td>23</td>
</tr>
<tr>
<td>38</td>
<td>6 \times 10^5</td>
<td>4.4</td>
<td>2.9</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>1 \times 10^6</td>
<td>6.1</td>
<td>2.7</td>
<td>19</td>
</tr>
<tr>
<td>37</td>
<td>1 \times 10^6</td>
<td>6.2</td>
<td>2.4</td>
<td>29</td>
</tr>
<tr>
<td>36</td>
<td>1 \times 10^6</td>
<td>6.0</td>
<td>2.4</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>4 \times 10^6</td>
<td>11.4</td>
<td>2.8</td>
<td>15</td>
</tr>
<tr>
<td>17</td>
<td>18 \times 10^6*</td>
<td>25.2</td>
<td>3.2</td>
<td>17</td>
</tr>
<tr>
<td>35</td>
<td>38 \times 10^6*</td>
<td>39.4</td>
<td>2.2</td>
<td>28</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>6 \times 10^5</td>
<td>1.6</td>
<td>2.7</td>
<td>16</td>
</tr>
<tr>
<td>35</td>
<td>2 \times 10^6</td>
<td>2.5</td>
<td>2.8</td>
<td>18</td>
</tr>
</tbody>
</table>

\* DNA size estimated from band width in CsCl density gradients (Vinograd & Hearst, 1962).
change and the time axis by the apparent halving of the renaturation rate. The K2 value taken for each of the chloroplast DNA fractions was that calculated from the mean slopes for chloroplast fractions plotted as in Figure 13, divided by the squared fraction concentration (Table 12). This raises the K2 value for the slow fraction (0.77) from 4.6 to 7.7 mole\(^{-1}\) sec\(^{-1}\) and for the fast fraction from 11.5 to 217 mole\(^{-1}\) sec\(^{-1}\). There is a possibility that the non-linearity of the second order renaturation plot (Figure 12) is due to the fact that this DNA does not renature to second order. It is possible to examine this question by renaturation at various concentrations of DNA and in theory, when normalised, these should give identical results (Table 13).

Another question to be answered is whether the 70-80% hyperchromicity return of the chloroplast or mitochondrial DNA was partial renaturation of all the DNA fraction as found by other workers, (Britten & Kohne, 1966) or total renaturation of a fraction of the DNA. This question can be resolved, by examination of the DNA at a stage where no further renaturation occurred, in the analytical ultracentrifuge. In all cases, the results (Figure 15) were consistent with the partial renaturation of the whole DNA fraction, the renatured chloroplast fractions and mitochondrial resulting in unimodal band at buoyant density values of 1.699 g.cm\(^{-3}\) and 1.707 g.cm\(^{-3}\) respectively, which are very close to the native DNA values (1.697, 1.706 g.cm\(^{-3}\)).
Table 13

Second order renaturation constant (K2) for varying concentrations of lettuce chloroplast DNA (slow fraction).

<table>
<thead>
<tr>
<th>DNA concentration (μg/ml.)</th>
<th>K2 (mole⁻¹ sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>4.2</td>
</tr>
<tr>
<td>12</td>
<td>4.3</td>
</tr>
<tr>
<td>25</td>
<td>4.8</td>
</tr>
<tr>
<td>45</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Conditions and calculations as shown for Table 10.
Figure 15. Microdensitometer tracings of aqueously prepared lettuce DNA.

A) Top to bottom respectively show native, denatured and renatured nuclear DNA.

B) Top to bottom respectively show native, denatured and renatured chloroplastal DNA.

C) Top to bottom respectively shows native, denatured and renatured mitochondrial DNA.
CHAPTER 4

CONCLUSIONS AND DISCUSSION

This thesis falls naturally into two sections, the identification of the chloroplastal DNA and information from the renaturation kinetics on this DNA.

A. Identification of Chloroplastal DNA

At the start of this thesis all the published information (see Table 1) on chloroplastal DNA of higher plants suggested that it banded, in CsCl gradients, as a heavy satellite band to the nuclear DNA with a buoyant density in the range 1.700-1.706 g.cm\(^{-3}\), some 10 mg.cm\(^{-3}\) heavier than the related nuclear DNA. There were, however, some chinks of disagreement for Kirk (1963) showed that nuclear and chloroplastal DNA from Broad Bean had a very similar base composition, whilst Lyttleton & Pettersen (1964) concluded that if tobacco chloroplasts had any DNA it must be of identical base composition to that from the nucleus.

Early experiments with non-aqueous preparations of spinach chloroplast gave results which suggested that the DNA being extracted was nuclear contamination. As techniques were improved, it became clear that the DNA from chloroplast fractions with low nuclear contamination, banded in CsCl gradients at a buoyant density differing from both the nuclear value and the heavy satellite. This was a very surprising result at that time (early 1966) but even more surprising was the fact that DNA from the fraction of specific gravity 1.00 - 1.25 had a buoyant density
in the range of the heavy satellite even though, when examined in the light microscope, there were very few chloroplasts to be seen. There was the possibility that the DNA from this last fraction was truly chloroplastal, being extracted from a fraction comprising only of chloroplast fragments but with no nuclear contamination. It seemed strange, however, to have isolated a fraction of only chloroplast fragments when nuclear fragments must also have been plentiful. There is also the buoyant density difference to be explained between the chloroplast fraction and the nuclear DNA. It was established that this effect was not an optical artifact of the analytical ultracentrifuge and this finding was repeated for several higher plants.

The initial reason for using non-aqueous techniques were ease of separation of the chloroplasts and the hope that the extracted DNA would be of high molecular weight. This last hope was not to be fulfilled as can be seen from the microdensitometer tracings for these fractions. It was therefore decided as worthwhile to try aqueous extractions of chloroplastal DNA both in an attempt to repeat the non-aqueous results and once again to obtain DNA of higher molecular weight.

Initial results from aqueous preparations of chloroplasts confirmed the non-aqueous findings but still did not rule out the possibility of nuclear contamination. At this time a gleam of light appeared from the work of Suyama & Bonner (1966). They showed that DNA from mitochondrial fractions that had been treated with DNase gave unimodal bands in CsCl gradients at buoyant densities of 1.705 g. cm\(^{-3}\) for several higher plants, and furthermore,
that for *Brassica* the main band of the chloroplastal DNA differed from the nuclear by about $3 \text{ mg cm}^{-3}$. It was decided to extend this technique and use a DNase treatment on chloroplast fractions. This proved to be a great success and unimodal bands were apparent when this DNA was examined in the analytical ultracentrifuge. The buoyant density of this band was found to be identical to that of DNA from non-aqueously prepared chloroplasts. The possibility that this DNA was specifically resistant to DNase action was disproved by showing that if the chloroplast fraction is DNased after lysis of the chloroplasts, then no DNA is obtained. In addition to this it was confirmed that the buoyant density of the mitochondrial preparation was identical to that of the heavy satellite seen in whole cell preparations. This was the first time that unimodal bands of chloroplastal and mitochondrial DNA had been obtained for a higher plant. It was therefore concluded that, in addition to the nuclear DNA and the heavy satellite (mitochondrial), there existed another satellite associated with chloroplasts, whose buoyant density was such that it normally banded within the Gaussian distribution of the nuclear DNA (*Wells & Birnstiel, 1967*). A recent paper is in agreement with this finding (*Whitfield & Spencer, 1968*). The Gaussian distribution belief was particularly well demonstrated, for, in a chloroplast preparation yielding high molecular weight DNA, two bands could clearly be seen in the analytical ultracentrifuge, one corresponding in buoyant density to nuclear DNA and the other to chloroplastal DNA (Figure 10a). When the chloroplastal fraction was DNased, only the DNA associated with the chloroplasts remained. (Figure 10d). Yet a third satellite band was observed for lettuce DNA. This was of buoyant density $1.720 \text{ g cm}^{-3}$ and was only seen when the EDTA
content of the homogenisation medium for the aqueous preparations was lowered to 1 mM or less. Under these conditions nucleolus-like particles were identified in the light microscope and it is believed that this DNA could represent the ribosomal cistrons.

There is obviously a discrepancy between the results put forward in this thesis for the buoyant density of the chloroplast DNA and those of other workers. In all these cases the buoyant density value is more in keeping with a mitochondrial preparation (Suyama & Bonner, 1966, Wells & Birnstiel, 1967) and it is noted that they have as a common denominator the separation of chloroplasts by sucrose gradients. The whole cell preparations showed a heavy satellite DNA (1-2%) which was enriched to 5-15% for chloroplast fractions, the other 85-95% being taken as nuclear contamination. If, however, the chloroplastal DNA has a similar buoyant density to the nuclear DNA, then this finding could have been a major band of mainly chloroplastal DNA with the 5-15% satellite representing mitochondrial contamination. Such possible sticking of mitochondria to chloroplasts in sucrose gradients has been shown for Acetabularia by Green, Geilporn, Limbosch & Brachet (1967).

If published figures are taken for the amount of DNA in mitochondria as 40 x 10^6 daltons (Borst et al., 1967) and that in chloroplasts as 2,000 x 10^6 daltons (Chiba & Sugahara, 1957), it can be seen that a contamination level of only two mitochondria per chloroplasts would give a satellite band of 5%. If, however, the amount of DNA per mitochondria is in the order of 400 x 10^6 daltons (see below), then it would only need an extremely low level of contamination to achieve this result.
There is yet more support for associating the 1.697 g.cm\(^{-3}\) satellite as chloroplastal from the base composition of this DNA. Other workers have shown that chloroplastal DNA lacks the rare base 5-methyl cytosine which is found to several per cent in the related nuclear DNA (Brawerman & Eisenstadt, 1964, Ray & Hanawalt, 1964, Ruppel & Wik, 1965). The mitochondrial associated DNA (1.706 g.cm\(^{-3}\)) was, however, not tested in a similar manner owing to the scarcity of this material and therefore the lack of 5-methyl cytosine in the 1.697 g.cm\(^{-3}\) satellite only assures its non-nuclear origin without defining it.

The work of Green & Gordon (1966) is believed also to provide evidence, if unwitting, of a DNA satellite within the Gaussian distribution of the main (nuclear) band. They took leaves at a stage at which no further cell division would be expected and studied the turnover of DNA by means of radioactive label, in light and dark grown cells. This showed in dark grown cells a double peak of radioactivity one associated with the heavy satellite presumed to be of chloroplastal origin, and the other some 3 mg.cm\(^{-3}\) heavier than the nuclear DNA. When transferred to a light environment, both bands show an equal increase. The displacement of the major band is explained as contamination of the nuclear fraction by the 1.706 g.cm\(^{-3}\) (chloroplastal!) satellite. If the figure is analysed, assuming a regular Gaussian plot, it can readily be seen that this is not so. Further in the paper another "hot" fraction is attributed to contaminating bacteria with a DNA of buoyant density 1.701 g.cm\(^{-3}\), but careful examination puts the buoyant
density value as near to 1.698 g.cm\(^{-3}\). It is believed that their results can better be understood if the assumption is made of a mitochondrial DNA band at 1.706 g.cm\(^{-3}\) and a chloroplastal band at 1.697 g.cm\(^{-3}\) both being equally affected by the light environment.

With all this evidence in mind the 1.697 g.cm\(^{-3}\) DNA band has been identified as chloroplastal and the 1.706 g.cm\(^{-3}\) as mitochondrial. These results are shown in Table 14 with some of the satellites from other workers' results re-identified from the findings above. It will be noticed that, with the exception of Euglena there is excellent agreement for the buoyant density range of the chloroplastal DNA (1.695-1.697 g.cm\(^{-3}\)) and the mitochondrial DNA (1.705-1.709 g.cm\(^{-3}\)).

**B) Renaturation of DNA**

It is possible to estimate the "genomic content" from both the renaturation half-life (Bolton et al., 1965) and the second order rate constant (Wetmur & Davidson, 1968, analytical complexity). This calculation is normally a straightforward multiplication or division with reference to a known DNA standard. For the organelle DNA's described in this thesis, the situation is not so clear because the renaturation kinetics do not behave as expected.

The melting curves of these DNA fractions have shown them by the lack of hyperchromicity below 60\(^\circ\)C to be free of RNA (Hastings & Kirby, 1966) and by the hyperchromicity percentage
Table 14: Buoyant Density of DNA

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mainland Nuclear</th>
<th>1st Satellite Chloroplastal</th>
<th>2nd Satellite Mitochondrial</th>
<th>3rd Satellite Mitochondrial</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>1.695</td>
<td>1.694</td>
<td>1.694</td>
<td>1.705*</td>
<td>Chun et al., 1963</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whitfield &amp; Spencer, 1968</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.694</td>
<td></td>
<td></td>
<td>This thesis</td>
</tr>
<tr>
<td>Beet</td>
<td>1.695</td>
<td>1.692</td>
<td>1.705*</td>
<td>1.719</td>
<td>Chun et al., 1963</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Suyma &amp; Bonner, 1966</td>
</tr>
<tr>
<td>Tobacco</td>
<td>1.695</td>
<td>1.697</td>
<td>1.708*</td>
<td>1.719</td>
<td>Shipp et al., 1965 +</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tewari &amp; Wildman, 1966</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Green &amp; Gordon, 1967</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Whitfield &amp; Spencer, 1968</td>
</tr>
<tr>
<td>Sweet Pea</td>
<td>1.695</td>
<td>1.697</td>
<td></td>
<td></td>
<td>This thesis</td>
</tr>
<tr>
<td>Bean</td>
<td>1.695</td>
<td>1.697</td>
<td></td>
<td></td>
<td>This thesis</td>
</tr>
<tr>
<td>Phaseolus</td>
<td>1.691</td>
<td>1.692</td>
<td></td>
<td></td>
<td>Suyama &amp; Bonner, 1966</td>
</tr>
<tr>
<td>Ipomea</td>
<td>1.692</td>
<td>1.689</td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Allium</td>
<td>1.694</td>
<td>1.697</td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Swiss Chard</td>
<td>1.694</td>
<td>1.697</td>
<td></td>
<td></td>
<td>&quot;</td>
</tr>
<tr>
<td>Lettuce</td>
<td>1.695</td>
<td>1.697</td>
<td>1.706</td>
<td>1.720</td>
<td>Kislev et al., 1965 +</td>
</tr>
<tr>
<td>Carnation</td>
<td>1.695</td>
<td>1.697</td>
<td>1.706*</td>
<td></td>
<td>This thesis</td>
</tr>
<tr>
<td>Snapdragon</td>
<td>1.697</td>
<td></td>
<td></td>
<td></td>
<td>Green &amp; Gordon, 1967</td>
</tr>
<tr>
<td>Chlamydomonas</td>
<td>1.723</td>
<td>1.695</td>
<td>1.709*</td>
<td></td>
<td>Chun et al., 1963</td>
</tr>
<tr>
<td>Chorella</td>
<td>1.716</td>
<td>1.695</td>
<td>1.691</td>
<td></td>
<td>Edelman et al., 1966</td>
</tr>
<tr>
<td>Euglena</td>
<td>1.707</td>
<td>1.686</td>
<td>1.691</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* re-identified as mitochondrial DNA.
+ Buoyant density adjusted from recent value for marker DNA.
to be essentially free of other contaminating material. Renatur-
ation studies at differing concentrations of DNA have confirmed
that the kinetics are second order, and that the hypochromicity
of the organelle DNA's to be similar to the T4 and E. coli DNA
controls. In theory, (Wetmur & Davidson, 1968) the K2 plot
(Figures 13 and 14) should give a straight line and it can be
seen that this is so for the T4 and E. coli DNA controls. The
plot for the nuclear DNA is very much as expected with fast
initial renaturation of reiterated stretches (Bolton et al.,
1965). The K2 plot for the chloroplastic DNA is different from
both of these and suggests a two population system similar to
that found for rat ascites DNA (Wetmur & Davidson, 1968). The
final rate constant, the slow fraction, is assumed to represent
the non-repeated sequences, whilst the fast fraction is presumed
to represent reiterated sequences. The K2 values (Table 11)
for the slow and fast fractions are 4.6 and 11.5 mole$^{-1}$ sec$^{-1}$
respectively. To calculate the genomic content of the chioro-
plast DNA it is not sufficient just to find the genomic repre-
sentation of each of these fractions by direct calculation from
the slope (Figure 14) (Wetmur & Davidson, 1968) and add them, for
the ratio of the DNA represented by each fraction must be taken
into account.

The assumption was made (see results) that the contribution
of each fraction can be calculated from extrapolation of the slow
fraction to zero time and a mean value of 77% was obtained for
this fraction (Table 12). The effective K2 value for the slow
fraction was taken as the K2 value from Figure 14, (4.6 mole$^{-1}$
sec$^{-1}$) for this fraction divided by the square of the fraction
ratio. The square of the ratio \( (.77^2 \text{ for the slow fraction}) \) is taken, as both axes of the K2 plot are affected, the one by the amount of hyperchromicity and the other because the rate of renaturation is directly related to concentration (Bolton et al., 1965). This gives an adjusted K2 value of \( 7.7 \text{ mole}^{-1} \text{ sec}^{-1} \), which would be equivalent to an analytical complexity of \( 150 \times 10^6 \) daltons. As this represents 77% of the DNA fraction, this would indicate a total genome content of \( 195 \times 10^6 \) daltons. The adjusted K2 value for the fast fraction is \( 217 \text{ mole}^{-1} \text{ sec}^{-1} \), which would be equivalent to an analytical complexity of \( 5.3 \times 10^6 \) daltons. This fraction is however represented by a genome content of about \( 45 \times 10^6 \) daltons, (23% of the DNA content), and therefore is reiterated by about eight times relative to the main (slow) fraction.

It should be pointed out that the value for the reiterated stretches is very sensitive to the contribution this fraction makes to the total DNA. If, for instance, it is represented by only 20% instead of 23%, then the value of the analytical complexity for this fraction would be about \( 4 \times 10^6 \) daltons.

A tentative value for the genomic content of the mitochondrial DNA is \( 520 \times 10^6 \) daltons, which includes a fast fraction of 8 copies each of \( 10 \times 10^6 \) daltons.

The value for the genomic content obtained from the half-life value would be expected, from the shape of the K2 plot, to be less than that obtained from this plot. This is so because the half-life measurements are very much affected by the initial rate of hyperchromicity decay at which time the "fast" fraction is most active. This expectation is borne out for the "genomic
content" calculated from the half-life figures (Bolton et al., 1965) are $1.5 \times 10^6$ daltons for the chloroplasmal DNA and $4.2 \times 10^6$ daltons for the mitochondrial.

It is believed that the value from the K2 calculations is more representative and there is justification both for treating these organelle DNA's as a two population system and for the use of the concentration squared correction (see Appendix). Fig. 14 shows a theoretical plot for the chloroplasmal DNA compared to the results obtained. The theoretical plot was calculated assuming a two population system with adjusted K2 values as above. Theoretical decay curves were then calculated for each of the fractions and a composite decay curve made up from these, taking into account the percentage represented by each fraction. It can be seen that the K2 plot from this decay curve is in good agreement with that obtained for the chloroplasmal DNA.

If it is true that the chloroplasmal DNA is represented by two classes of DNA molecules it is pertinent to ask whether this is the true situation or an artifact. The various possibilities are enumerated below.

1) The results are an artifact of the renaturation system used. This is disproved by the renaturation of the T4 and E.coli DNA controls. The K2 value for T4 DNA from the work of Wetmur & Davidson (1968), when adjusted to the renaturation conditions used, is $6.4 \text{ mole}^{-1} \text{ sec.}^{-1}$ which compares well with the value obtained of $6.8 \text{ mole}^{-1} \text{ sec.}^{-1}$.
ii) The fast fraction is caused by RNA. This is disproved by the melting curve of the DNA.

iii) Either slow or fast fraction is bacterial in origin. This is not possible as the rate of renaturation of either fraction is much faster than that normally associated with bacteria (Bolton et al., 1965, Wetmur & Davidson, 1968).

iv) The slow fraction is contamination by nuclear DNA. This can be shown not to be true on two counts. The first is that examination of the high molecular weight chloroplastal DNA in the analytical centrifuge did not show any such DNA. Secondly, it has been shown that the amount of renaturation of the nuclear DNA is only about 20%, which is inconsistent with the 70% for chloroplastal DNA.

v) The fast fraction is mitochondrial DNA. As this fraction accounts for about 25% of the total chloroplastal DNA, it would clearly have been seen when examining the chloroplastal DNA in the analytical ultracentrifuge under overload conditions.

vi) A sonication effect.... the K2 plot suggesting two fractions for the chloroplastal DNA is observed on both sonicated and unsonicated DNA and is not observed on sonicated control DNA. If the two fractions represented the same DNA at two different lengths then, as the renaturation rate is affected by the square root of the size ratio (Wetmur & Davidson, 1968), the difference in molecular weights of these DNA's would have to be about 900 as the difference in renaturation rates is 30. This would mean a fast fraction of $5.4 \times 10^8$ daltons which was never seen in
sedimentation runs, or a slow fraction of $7 \times 10^2$ daltons which would not have pelleted when extracting the DNA. The fact that the ratio between the K2 values of the two fractions is maintained irrespective of the DNA size examined ($6 \times 10^5 - 38 \times 10^6$ daltons, Table 12) is a good indication that these two fractions are closely connected.

Similar arguments can be put forward for the mitochondrial DNA but as this experiment has only been carried out twice, not very much weight can be attached to this result. Renaturation kinetics apparently displaying the two population result may have been obtained by Adam, Blewett & Flamm (1968) from a DNA fraction of Amoeba which they believe to be of mitochondrial origin.

It is of interest to find out how many times a "genome content" of $195 \times 10^6$ daltons would be represented in each chloroplast. To do this we need to know the amount of DNA per chloroplasts, which can be calculated from the DNA yield divided by the number of chloroplasts. For an input of 1,000 g. fresh weight of leaves about 200 µg of DNA was obtained. If the number of chloroplasts is assumed to be those with intact double membranes, and therefore resistant to DNase action, then this figure is about $7 \times 10^{10}$ per 1,000 g. of leaves. The amount of DNA per chloroplasts is therefore $3 \times 10^{-15}$ g, which is equivalent to a molecular weight of $1.8 \times 10^9$ daltons. This would indicate that each "genome content" be represented 9-10 times in each chloroplasts, which would mean about 80 times for the fast fraction. This assumes 100% recovery of the chloroplast DNA, so this is a minimum figure.
It has been pointed out in the introduction that chloroplasts appear to have their own ribosomes and it is tempting to link the reiterated fast fraction with their production. The "genomic content" of $5.3 \times 10^6$ daltons is very close to the value of $4.6 \times 10^6$ daltons for the DNA representing the ribosomal precursor in plants (loening, 1968). From the base composition of the ribosomal RNA and in common with other ribosomal cistrons (Birnstiel et al., 1968) the DNA would be expected to have a buoyant density value around 1.720. No such DNA band was observed in analytical gradients of chloroplastal DNA fractions used for renaturation. As this DNA would account for some 25% of the chloroplastal DNA and is about $5 \times 10^6$ daltons in size, it was expected to show up in DNA preparations where the molecular size was around $2-3 \times 10^6$ daltons.

It must be pointed out that any value for "genomic content" could be either an over- or under-estimate. It is known that many enzyme molecules have common amino acid sequences, which would give common nucleotide sequences of DNA and indicate a lower apparent value for the genome content. On the other hand, Walker (1968) has suggested that there could be considerable "wobble" in reiterated stretches of DNA and this would give an apparent overstatement of the genomic content.

That there is also complete genome reiteration within a circular molecule has been shown for the Mycoplasma family. This shows a series of circular molecules varying from $444 \times 10^6$ daltons from \textit{M. arthriditis} to $1,200 \times 10^6$ daltons for \textit{M. gallipsepticum} (cf. Morowitz, Bode and Kirk, 1967) and seems
to show a periodicity of $400 \times 10^6$ daltons. A renaturation value for *M. gallisepticum* was found, however, to give a genome content of $400 \times 10^6$ daltons, suggesting its circular molecule is 3 genome copies and that other circles of this family could be multiples of two or three genome copies.

It should also be kept in mind that the "genome content" which is obtained from renaturation kinetics can be influenced by the guanine plus cytosine content of the DNA.

As a last piece of corroborating evidence (Woodcock & Fernandez-Moran, 1968) showed that when spinach chloroplasts were carefully handled and the DNA examined in the electron microscope, the preferred DNA size was 70 u which is equivalent to a molecular weight of $140 \times 10^6$ daltons, a very close value to the slow fraction of the chloroplastal DNA.

C) Final Conclusions

i) The heavy satellite DNA at 1.706-7 g.cm\(^{-3}\) seen in all higher plants examined is not chloroplastal but mitochondrial in origin.

ii) The chloroplastal DNA bands as a second heavy satellite of buoyant density 1.697 g. cm\(^{-3}\) but cannot normally be seen in whole cell preparations because it is within the Gaussian distribution of the nuclear DNA.

iii) The chloroplastal DNA represents a "genome content" of $195 \times 10^6$ daltons which includes eight reiterated stretches of $5.3 \times 10^6$ daltons.
iv) Each chloroplast has associated with it a minimum of ten "genome copies".

v) Tentatively, the mitochondrial DNA represents a "genome content" of $520 \times 10^6$ daltons which includes eight reiterated stretches of $10 \times 10^6$ daltons.

vi) The chloroplast DNA represents some 4% of the total cell DNA making the assumptions that chloroplastal DNA extracted represents 60% of the total (see above) and there are identical extraction losses for chloroplastal and nuclear DNA.
I wish to thank Dr. M.L. Birnstiel very much for his help, encouragement and advice throughout this project and especially on his insistence of the scientific method in research and the injection of realism into flights of imagination. I am also indebted to Dr. Shankar Narayan for very kindly examining samples in the electron microscope and to Dr. J.O. Bishop for the gifts of T4 and E. coli DNA.

I wish also to thank the University of Edinburgh for a studentship and help from the Macaulay Research Fellowship Fund to enable me to carry out this research.
Appendix 1

a) Analytical centrifugation.

The centrifugal force acting on the molecule is $\omega^2 r$ where
\[ \omega = \text{revolution in radians per second}. \]
\[ r = \text{radius to mid-point of cell}. \]

Under standard conditions of $44,000 \text{ rev/min.}$ and the DNA band in the centre of the cell buoyant density differences can be calculated from the figure of $3.27 \text{ mg. cm}^{-3}$ per cm. of the densitometer tracing.

If however the centrifugation has been carried out at $44,770 \text{ rev/min.}$ the buoyant density difference can be converted to the $44,000 \text{ rev/min.}$ series by multiplying by $\left( \frac{44,000}{44,770} \right)^2$.

If the mid-point of the marker DNA and the band under examination is not in the middle of the cell then the buoyant density difference can be converted by multiplying by $\frac{R}{R'}$ where $R$ is the true distance to the centre of the cell, and $R'$ the distance from the rotor centre to the mid-point of the two bands under examination.

To calculate the molecular weight from the band width (Vinograd & Hearst, 1962) the equation used was
\[ M = \frac{2,600}{\sigma^2} \]
where
\[ \sigma^2 = \text{the DNA band width } 2/3 \text{ from the base line when traced out on the microdensitometer (converted to actual width)}. \]
\[ 2,600 = \text{a constant for the centrifugation conditions used}. \]
B. Band Centrifugation

\[ \frac{\Delta R}{\Delta T} = \beta \omega^2 r. \]

\[ \frac{\Delta R}{\Delta T} \] = the rate of sedimentation cm. seconds\(^{-1}\).

\( W \) = speed of rotation in radians per second.

\( r \) = radius of rotation in cm.

\( \beta \) = the Svedberg constant for sedimentation. This can be converted into a molecular weight value for alkaline systems using the Studier (1965) equation.

\[ S_{20,w}^0 = 0.0528 M^{0.400} \]
Appendix 2 Renaturation Kinetics

Let $A_n$ be the absorbance of native DNA at the maximum of the ultraviolet spectrum.

Let $A_d$ be the absorbance of denatured DNA (above melting temperature).

Then within 5% (Wetmur & Davidson, 1968)

$$A_d - A_n = 0.36A_n.$$  \hspace{1cm} (1)

If $P_t$ is the total phosphate concentration then expressed in terms of absorbance (1 O.D. = 50 μg., 330 g = 1 mole 1⁻¹)

$$P_t = 1.47 \times 10^{-4} A_n \text{ mole 1}^{-1}.$$  \hspace{1cm} (2)

If $A_r$ is the absorbance of the denatured DNA in solution when $P$ is the concentration then

$$P = \frac{A_r - A_n P_t}{A_d - A_n}.$$  \hspace{1cm} (3)

If the renaturation follows second order kinetics then $K_2$ (the second order renaturation constant) can be defined as

$$-\frac{d(P)}{dt} = K_2 \left(\frac{P}{2}\right)^2$$  \hspace{1cm} (4)

as $\left(\frac{P}{2}\right)$ is equivalent to $\frac{1}{2}$ the DNA strands.

$$-\frac{d(P)}{P^2} = \frac{K_2 dt}{4}$$  \hspace{1cm} (5)

$$\therefore \quad \frac{1}{P} = \frac{K_2 t}{2} + C$$  \hspace{1cm} (6)
at \( T = 0 \quad C = \frac{1}{Pt} \quad (7) \)

\[ \therefore \quad \frac{Pt}{P} = \frac{K_2Pt.T}{2} + 1 \quad (8) \]

but

\[ \frac{Pt}{P} = \frac{AD - An}{AR - An} \quad (9) \]

\[ \therefore \quad \frac{AD - An}{AR - An} = \frac{K_2Pt.T}{2} + 1 \quad (10) \]

Substituting for \( Pt \) (2) and \( AD - An \) (1), we get

\[ \frac{1}{AR - An} = 2.04 \times 10^{-4} K_2 T \frac{1}{0.36 An} \quad (11) \]

It can readily be seen that if \( \frac{AD - An}{AR - An} \) is plotted against time (10) then a value of \( K_2 \) can be obtained, taking concentration into account. However, if equation (11) is used the value of \( K_2 \) can be obtained independently of DNA concentration.
Two Component System

$P_a = \text{Phosphate concentration of denatured bases of component (a)}.$

$P_b = \text{Phosphate concentration of denatured bases of component (b)}.$

$K_a = \text{Kinetic (second order) constant for component (a)}.$

$K_b = \text{Kinetic constant of component (b)}.$

\[ \therefore P_a + P_b = \frac{A_r - A_n}{A_b - A_n} \cdot P_t \quad (12) \]

from (6)

\[ \frac{1}{P_a} = \frac{1}{2} K_a T + C_a \quad (13) \]

\[ \frac{1}{P_b} = \frac{1}{2} K_b T + C_b \quad (14) \]

\[ \therefore P_a + P_b = \frac{1}{\frac{1}{2} K_a T + C_a} + \frac{1}{\frac{1}{2} K_b T + C_b} \quad (15) \]

\[ = \frac{\frac{1}{2} (K_a + K_b) T + C_a + C_b}{(\frac{1}{2} K_a T + C_a)(\frac{1}{2} K_b T + C_b)} \quad (16) \]

Substituting for $P_a + P_b$ from (12) and inverting

\[ \frac{A_d - A_n}{A_r - A_n} \frac{1}{P_t} = \frac{\frac{1}{2} K_a T + C_a)(\frac{1}{2} K_b T + C_b)}{(\frac{1}{2} K_a T + C_a)(\frac{1}{2} K_b T + C_b)} \quad (17) \]

\[ \therefore \frac{A_d - A_n}{A_r - A_n} \frac{1}{P_t} = \frac{\frac{1}{2} K_a K_b T^2 + \frac{1}{2} K_a C_b T + \frac{1}{2} K_b C_a T + C_a C_b}{C_a + C_b} \frac{1}{\frac{1}{2}(C_a + C_b) T + 1} \quad (18) \]

Expanding the denominator as $\frac{1}{1 + x} = 1 - x + \frac{1}{2} x^2$ and ignoring
all powers of $T$ greater than one

$$\frac{Ad - An}{Ar - An} \frac{1}{Pt} = \frac{1}{2} \left( \frac{Ca Kb + Cb Ka - (Ka + Kb)Ca Cb}{Ca + Cb} \right) T + \frac{Ca Cb}{Ca + Cb} \tag{19}$$

Taking this to represent the initial slope $Si$,

$$Si = \frac{1}{2} \frac{1}{Ca + Cb} \left( Ca Kb + Cb Ka - \frac{(Ka + Kb)Ca Cb}{Ca + Cb} \right) \tag{20}$$

If the initial rate $Ka$ is much greater than the final rate then (20) develops into

$$Si = \frac{1}{2} \frac{1}{Ca + Cb} \left( Cb Ka - \frac{Ka Ca Cb}{Ca + Cb} \right) \tag{21}$$

$$\therefore Si = \frac{1}{2} Ka \left( \frac{Cb}{Ca + Cb} \right)^2 \tag{22}$$

but from equations (13) and (14) at $t = 0$

$$Ca = \left( \frac{1}{Pa} \right) \bigg|_{t=0} \quad Cb = \left( \frac{1}{Pb} \right) \bigg|_{t=0} \tag{23}$$

$$\therefore \left( \frac{Gb}{Ca + Cb} \right)^2 = \left( \frac{1}{Pa + \frac{1}{Pb}} \right)^2 \bigg|_{t=0} = \left( \frac{1}{Pb + \frac{Pa}{Pb}} \right)^2 \tag{24}$$

$$\therefore \left( \frac{Gb}{Ca + Cb} \right)^2 = \left( \frac{Pa}{Pa + Pb} \right)^2 \bigg|_{t=0} \quad \text{But} \ (Pa + Pb)_{|t=0} = Pt . \tag{25}$$
\[
\left( \frac{C_b}{C_a + C_b} \right)^2 \quad \text{represents the concentration ratio of fraction (a) to the total.}
\]

\[
\frac{1}{\text{(concentration ratio)}^2} \quad \frac{A_d - A_n}{A_r - A_n} = \frac{K_a P_t}{T} + K
\]

\( K \) = constant.

\[
K_2 \text{ for fraction a is the slope of the graph divided by the concentration ratio squared.}
\]

Fraction b. When \( T \) is large, fraction a is assumed to be fully renatured. Therefore equation (17) becomes

\[
\frac{A_d - A_n}{A_r - A_n} \cdot \frac{1}{P_t} = \frac{1}{\frac{1}{2}K_b T + C_b} \frac{C_a}{C_a + C_b}
\]

\[
= \frac{1}{\frac{1}{2}K_b T + C_b + C_a} \frac{C_a^2}{C_a + C_b}
\]

\[
= C_a - \frac{C_a^2}{\frac{1}{2}K_b T + C_b + C_a} = C_a - \frac{C_a^2}{C_a + C_b} \cdot \frac{1}{\frac{1}{2}K_b T + C_a + C_b + 1}
\]

Expanding \( \frac{1}{\frac{1}{2}K_b T + C_a + C_b} + 1 \) as \( \frac{1}{1 + X} = 1 - X + \frac{1}{2}X^2 \)

\[
\frac{A_d - A_n}{A_r - A_n} \cdot \frac{1}{P_t} = C_a - \frac{C_a^2}{C_a + C_b} \left( 1 - \frac{\frac{1}{2}K_b T}{C_a + C_b} \right)
\]

\[
= C_a + \frac{1}{2} K_b T \left( \frac{C_a}{C_a + C_b} \right)^2
\]
\[
\frac{1}{2}kBT \left( \frac{Pb}{Pa + Pb} \right)^2 + Ca
\]  
(32)

Equation (32) is of the same form as (26),

\[
\frac{1}{(\text{concentration ratio})^2} \frac{Ad - An}{Ar - An} = \frac{KbT \cdot P}{2} \text{ constant.}
\]

It has therefore been shown that for limiting values the K2 value for fast and slow fractions can be obtained by taking the initial and final slopes and dividing by the relevant concentration ratio squared.
A Rapidly Renaturing Deoxyribonucleic Acid Component Associated with Chloroplast Preparations

By R. WELLS and M. L. BIRNSTIEL. (Epigenetics Research Laboratories, Institute of Animal Genetics, University of Edinburgh)

Chloroplasts were prepared non-aqueously (Biggin & Park, 1963; Stocking, 1959) or aqueously in isotonic solution according to the protocol of Suyama & Bonner (1966), which was modified to remove chromatin contamination, by the inclusion of a DNase treatment of the chloroplast suspension prior to DNA extraction. Light microscopic examination of the non-aqueous fractions showed the chloroplasts to be contaminated with cytoplasmic fragments but to be essentially free of nuclei. Aqueously prepared chloroplasts, when viewed in the electron microscope, were pure and did not reveal nuclear or mitochondrial material. About half the chloroplasts looked well preserved.

DNA from the chloroplastal fractions, prepared by either method, banded in CsCl at a density intermediate between nuclear DNA (buoyant density 1.691-1.692 g.cm.\(^{-3}\)) and the `heavy' satellite (1.703-1.705 g.cm.\(^{-3}\)) which was previously identified as chloroplastal DNA (Chun, Vaughan & Rich, 1963; Shipp, Kieras & Haselkorn, 1965). We find that in spinach, lettuce, broad bean and sweetpea our chloroplastal preparations yield a single DNA component with a buoyant density of 1.694-1.695 g.cm.\(^{-3}\), which is in good agreement with values reported for algal chloroplasts (Chun \textit{et al.} 1963; Ray & Hanawalt, 1965).

The mitochondrial pellet prepared from the chloroplastal supernatant contains the 1.705 satellite in agreement with other workers (Suyama & Bonner, 1966; Rabinowitz, Sinclair, DeSalle, Haselkorn & Swift, 1965).

The DNA associated with aqueously prepared chloroplasts renatures completely in 7min. ± 3min. (calculated for 100 µg/ml.) at 60° in 1 x SSC (Britten & Kohne, 1965). DNA from non-aqueously prepared chloroplasts renatured in the same time but was contaminated by a slowly renaturing fraction believed to be nuclear in origin. This renaturing time compares to 10min. for T4 DNA, 25min. for FPLO DNA and 450min. for \textit{E. coli} DNA (Britten & Kohne, 1965) and suggests a genomic content of approximately 80 \times 10^6 daltons, a value similar to that of some bacteriophages. This value is very different from nuclear (8 \times 10^{12} daltons), bacterial (6 \times 10^9 daltons) or mitochondrial DNA (9 \times 10^6 daltons) (Gibor & Grannick, 1964; Sinsheimer, 1957; Borst & Ruttenber, 1966) and argues in favour of the possibility that we are truly looking at chloroplastal DNA.

REFERENCES

REFERENCES (Contd.)

Edelman, M., Cowan, C.A., Epstein, H.T. & Schiff, J.H. (1964)
Green, B.R., Heilporn, V., Limbosch, S., Boloukhene, M. & Brachet, J.
Hamerling, J., Claus, H., Keck, K., Richter, G. & Werz, G. (1958)
Iwamura, T. (1960) in Progress in nucleic acid research, Academic
Kirby, K.S. (1965) Biochem. J. 96, 266.
REFERENCES (Contd.)


Lyttleton, J.W. & Ts'0, P.C.P. (1958) Arch. Biochem. Biophys. 73, 120.


REFERENCES (Contd.)


REFERENCES (Contd.)