DNA was extracted from Drosophila melanogaster according to the published procedures of other workers; however the product was usually degraded and heavily contaminated. A new method was therefore developed for the extraction of DNA from Drosophila. Adult flies were homogenised in a buffer solution at neutral pH and containing EDTA. The DNA-containing fractions were sedimented at 12,000 x g for 10 minutes. The DNA was then deproteinised and purified by treatment with ribonuclease and further deproteinisation. The DNA extracted was of high purity and of very high molecular weight (up to 110 x 10^6 daltons). This method has also been applied successfully to other insects.

The Drosophila DNA was used as a primer for the synthesis of C labelled complementary RNA (cRNA) by Micrococcus lysodeikticus RNA polymerase in vitro. When the cRNA was annealed with denatured Drosophila DNA under conditions of high ionic strength and high temperature a molecular cRNA-DNA hybrid was formed between them. Such hybrids are formed only between DNA and its homologous complementary RNA. Different methods of assaying the cRNA-DNA hybrids were tested. The most successful procedure was to anneal the cRNA with denatured DNA in free solution and to trap the cRNA-DNA hybrid by filtration onto nitrocellulose membranes or millipore filters.

The optimum conditions for the formation of the cRNA-DNA hybrid were determined. It was found that the percentage of the cRNA which was bound by the DNA increased with increasing DNA:RNA ratios and that a maximum of 28% of the cRNA could be bound by the DNA at DNA:RNA ratios of 300:1 or more. It seems probable that the cRNA bound to the DNA consists mainly of reiterated nucleotide sequences.

Denatured Drosophila DNA was annealed with an excess of cRNA to determine what fraction of the DNA could be covered by cRNA in a cRNA-DNA hybrid, indicating the percentage of DNA which had been transcribed into cRNA. At an RNA:DNA ratio of 20:1 7% of the DNA was covered with cRNA. However this was not a saturation value and it is almost certain that at higher RNA:DNA ratios a larger fraction of the DNA would be covered with cRNA. Thus 7% is a minimum value for percentage transcription of the DNA.

When DNA from other organisms, a bacteriophage (T1), an amphibian (Xenopus), a mammal (Rat) and other insects (Schistocerca and Aedes) was annealed with Drosophila cRNA no cRNA-DNA hybrids were formed. Thus virtually no homologies can be detected between Drosophila cRNA and DNA from other genera. Since the DNA-cRNA hybridisation reaction appears to be so highly specific the prospects are encouraging that it may be extended to investigate genomal differences between and perhaps within species of Drosophila. In addition it may be used to study the transcription of DNA by RNA polymerase.
STUDIES ON THE NUCLEIC ACIDS OF
DROSOPHILA

by
Margaret Birmstiel

A Thesis Presented for the Degree of Doctor of Philosophy
of the University of Edinburgh.

Institute of Animal Genetics
Edinburgh, Scotland.

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## PART I

**EXTRACTION OF DNA**

### INTRODUCTION

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HYBRIDISATION STUDIES

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**Abbreviations**

A, C, G, T, U.  
the bases adenine, cytosine, guanine, thymine, uracil or their corresponding nucleotides.

ATP, CTP, GTP, UTP.  
adenosine triphosphate, cytidine triphosphate, guanosine triphosphate, uridine triphosphate.

poly-A, poly-U.  
polyadenylic acid, polyuridylic acid.

deaDNA  
deoxynucleic acid.

dNase  
deoxynuclease.

DNA  
deoxyribonucleoprotein.

DNA  
disodium ethylene-diamine-tetra-acetic acid.

MAK  
methylated-albumen-kieselguhr.

NDS  
naphthalene 1:5 disulphonate.

PCA  
perchloric acid.

RNA  
ribonucleic acid.

cRNA  
complementary ribonucleic acid.

mRNA  
messenger ribonucleic acid.

sRNA  
soluble ribonucleic acid.

RNase  
ribonuclease.

RNP  
ribonucleoprotein.

S  
Svedberg unit (10^{-13} secs). Unit of sedimentation coefficient.

S_{20,w}^{0}  
sedimentation coefficient at zero concentration, calculated for 20°C. in water.

SSC  
standard saline citrate. 0.15 M NaCl + 0.015 M sodium citrate pH 7.0.

0.01 SSC, 0.1 SSC  
1/100th or 1/10th dilutions of SSC.

2 x SSC, 6 x SSC, 12 x SSC, 18 x SSC  
corresponding concentrations of SSC.
**Abbreviations (Contd.)**

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<tr>
<td>SLS</td>
<td>sodium lauryl sulphate</td>
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<tr>
<td>$T_m$</td>
<td>melting temperature. (Temperature at which the double-stranded polynucleotide chain is 50% dissociated.)</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid.</td>
</tr>
<tr>
<td>TWEST</td>
<td>0.1% Tween 80 + 0.05 M EDTA + 0.15 M NaCl + tris-HCl pH 6.8.</td>
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The experiments to be described here represent the beginning of a research programme to develop quantitative methods for estimating genetic differences within and between populations of *Drosophila*.

Variation between the genomes of a population is believed to arise principally from mutation, deletion and duplication of the base-paired nucleotide sequences of the DNA. Ideally we should like to express genetic differences quantitatively in terms of the number of altered base-pairs in the DNA.

Although classical genetic techniques have been used to study divergence within populations they do not give a quantititative estimate of changes in the genetic material itself, namely the DNA of the chromosomes. Thus, we may envisage that a single point mutation in an important enzyme might produce profound morphological changes. Alternatively, mutational events might produce fundamental changes in the internal metabolism which could not be detected by classical genetic methods. Furthermore, many base-pair substitutions do not produce a detectable genetic mutation because they do not affect a critical part of the protein coded on the DNA.

Within the last few years techniques have become available for studying the nucleotide sequences of DNA. In 1953 Watson and Crick (1953a & b) proposed that the DNA molecule consisted of a double-stranded helix with the strands held together by hydrogen bonds between the bases on the different strands, adenine
pairing with thymine and guanine with cytosine. Because of this highly ordered molecular structure the nucleotide sequence in one strand determines the nucleotide sequence on the other strand and the two strands are complementary.

When DNA in aqueous solution is heated to temperatures above its melting temperature it denatures, that is, the hydrogen bonds between the base pairs are broken and the two strands separate to form random coils (Marmur and Doty, 1959). Theoretically we might expect that if the denatured DNA solution is then cooled very slowly the complementary nucleotide sequences will come together through hydrogen bonding between the base pairs and the DNA will renature. Since the base pairing of the nucleotide sequences is very precise this would then provide us with a test system for DNA homology: complementary strands from homologous DNA should renature completely whereas heterologous DNA should not renature at all.

Marmur and Doty (1961) showed that denaturation of homologous denatured DNA did, in fact, occur. Denatured DNA from bacteriophage and very small bacteria renatured almost completely; however, DNA from larger bacteria renatured to a lesser extent and DNA from mammalian tissues hardly at all. They pointed out that this was due to the increased complexity of DNA from more advanced forms since renaturation depends on the concentration of the individual specific complementary strands during renaturation (Marmur and Lane, 1960; Marmur and Doty, 1961).

**DNA–DNA hybridisation studies on bacteria**

Schildkraut et al. (1961) attempted to use the renaturation of homologous DNA as a tool to study homologies amongst different
species of bacteria. Denatured DNAs from two different sources were annealed together in solution so that renaturation could occur. One of the DNAs was labelled with $^{15}$N and deuterium. This high density DNA could be separated from the non-labelled DNA in a cesium chloride gradient. When labelled and non-labelled DNAs from *B. subtilis* were annealed together three species of DNA were recovered, together with some unrenatured DNA, namely renatured low density DNA, renatured high density DNA and a hybrid formed by hydrogen bonding of a low density DNA strand with a high density DNA strand. This hybrid separated at an intermediate density on the cesium chloride gradient. However, in some cases where DNA from two different species were annealed they found that 'aggregates' rather than true hybrids were formed. These aggregates probably consisted of DNA strands only partially bound together since unlike the true hybrids they could be digested by *E. coli* phosphodiesterase which is specific for single-stranded DNA (Schildkraut *et al.*, 1961). When DNAs from closely related bacterial species such as *B. subtilis* and *B. natto* or *E. coli* and *Shigella dysenteriae* were annealed true hybrids formed between them. However in the case of *E. coli* and *Salmonella typhimurium*, two members of the *Enterobacteriaceae* which might be expected to show some homology, no true hybrid could be detected although aggregates were formed which were digested by phosphodiesterase.

When *B. subtilis* DNA was annealed with thymus DNA no hybrids or aggregates were formed, the DNA from *B. subtilis* renaturing while calf thymus DNA remained denatured.

These experiments demonstrated that DNA homologies could be identified by the technique of DNA–DNA hybridisation. However the
method is not suited for routine assay since it is both tedious and expensive, and cannot easily be put on a quantitative basis because of renaturation in DNA from a single source.

Following the discovery by Bautz and Hall (1962) that phage DNA attached to nitrocellulose columns could be used for hybridization studies, Bolton and McCarthy (1962) developed a method in which high molecular weight denatured DNA was mechanically trapped in a solidified agar gel and annealed with labelled RNA or DNA at 60°C. For DNA-DNA hybridization the DNA in free solution was first sheared to a molecular weight of around 500,000 to allow it to penetrate the agar gel. This is equivalent to roughly 1,000 nucleotide pairs and will contain sufficient information to code for three medium sized proteins (McLaren and Walker, 1965). It is therefore roughly the same size as polycistronic messenger RNA.

Using this technique Bolton and McCarthy (1962) explored the homologies between the DNA of E. coli and other organisms. Using an excess of E. coli DNA in the column they found that 40% of the sheared E. coli DNA could be bound to the DNA-agar column. McClaren and Walker (1965) obtained a similar value of 50%. The inability of DNA-agar to bind 100% of homologous DNA fragments may be due to some leaching out of the high molecular weight DNA from the agar which then hybridizes with the fragmented DNA in solution and also to the renaturation of the DNA fragments in solution (Cowie and McCarthy, 1963). It has been shown that preincubation of sheared DNA prior to annealing with DNA-agar reduces its binding capacity (McCarthy and Bolton, 1964; Martin and Hoyer, 1966).
However Walker and McLaren (1965a) have questioned these conclusions on the basis of their results with mouse and rat DNA. They consider that renaturation of the sheared DNA is not significant at levels used for hybridization (Walker and McLaren, 1965a).

However, when E. coli DNA-agar is annealed with an excess of sheared E. coli DNA 80% of the DNA-agar sites can be covered (Cowie and McCarthy, 1963; McCarthy and Bolton, 1964) indicating complete homology. Failure to bind 100% may be attributed to non-availability of some of the DNA embedded in agar (McCarthy and Bolton, 1964). As might be expected from the findings of Schildkraut et al. (1961) no hybridization occurred between E. coli DNA and phage, thymus or mouse liver DNA (McCarthy and Bolton, 1963). In another paper McCarthy and Bolton (1964) showed that E. coli DNA-agar did not bind E. coli DNA unless both DNAs had been denatured and the DNA in free solution was sheared. These findings taken together suggest that the hybridization of E. coli DNA to itself is highly specific.

When an excess of E. coli DNA in agar is annealed with sheared DNA from other bacteria some cross hybridization occurs. Since E. coli DNA-agar binds only 40% of E. coli DNA results have to be "normalized", that is, expressed as a fraction of the reaction with E. coli DNA to obtain a quantitative estimate of homology. Thus 26% of the DNA from Shigella dysentericae may be bound to E. coli DNA (McCarthy and Bolton, 1963) and consequently a 70% homology with E. coli can be claimed when the results are "normalized". This agrees with the findings of Schildkraut et al. (1961) who also observed extensive cross reaction.
between the DNA of *E. coli* and *Shigella dysenteriae*. A very interesting result is the finding of a 70% homology between *E. coli* and *S. typhimurium* by McCarthy and Bolton (1963) since Schildkraut et al. (1961) could detect no hybridization between these two species. Schildkraut et al. (1961) observed extensive aggregation between these two but the complex was completely digested by phosphodiesterase and was not considered to be a true hybrid. In addition, the genetic evidence (Demerec, 1965) suggests that there is little homology between the nucleotide sequences of *E. coli* and *S. typhimurium*. This raises the question of whether the binding of DNA by DNA-agar columns is really due to formation of specific hybrids. McCarthy, Bolton and co-workers consider the binding is specific since no cross-reaction is observed between bacteria and higher organisms, but this may not be a valid test. Schildkraut et al. (1961) observed that neither hybridization nor aggregation occurred with DNAs from such disparate species. The McCarthy and Bolton group also claim that incubation at 60°C, as carried out in their experiments, minimizes non-specific DNA-DNA associations (Bolton and McCarthy, 1964; McCarthy and Bolton, 1964; Hoyer, McCarthy and Bolton, 1964). Thus the melting curve for *E. coli* DNA-DNA duplexes formed at 60°C closely resembles that for native DNA, the duplexes having a $T_m$ of 75°C in 0.1 SSC compared with 71°C for native DNA in 0.1 SSC (Bolton and McCarthy, 1964). However McCarthy and Bolton (1963) also reported that when *E. coli* DNA-agar was annealed with a mixture of homologous and related (*Aerobacter aerogenes*) sheared DNAs the bound heterologous DNA was eluted first from the column at 75°C. They suggested that this showed the heterologous DNA was bound less well than was homologous DNA, i.e.
the specificity of hybridisation was imperfect. Studies on DNA from higher organisms (Walker and McLaren, 1965a; Martin and Hoyer, 1966) indicate that the specificity of hybridisation is dependent on the annealing temperature and that hybrids formed at 60°C have a T_m some 14°C lower than that of native DNA. Walker and McLaren (1965) therefore considered that some of the DNA bound to DNA-agar might be present as "loose" ends or "loops" of unpaired DNA. The marked lowering of the T_m in the mammalian system is in contrast to the result of Bolton and McCarthy (1964). It may be that the simpler bacterial DNAs more readily form stable helical regions than does heterologous mammalian DNA.

Thus there is some doubt as to whether the DNA-DNA interaction is due to specific hybrid formation. For this reason estimates of genetic relatedness obtained by this means should be treated with caution. These estimates are also rendered dubious by the fact that quantitative duplex formation does not occur within homologous DNA and this raises the question that the hybridisation reaction may be selective for certain classes of DNA molecules.

**DNA-DNA hybridisation studies on higher organisms.**

The above criticisms apply to studies on hybridisation of DNA from higher organisms. Hoyer et al. (1964) used the DNA-agar technique to investigate genetic relationships between various vertebrates. Using mouse DNA they found that at a concentration of long DNA (in agar) to DNA fragments of 100:1 or more a maximum of 25% of the sheared DNA could be bound. They attributed the failure to bind more to leaching out of high molecular weight DNA from the agar column and DNA-DNA interactions amongst the frag-
ments. McLaren and Walker (1965) obtained a similar value of about 30% binding of mouse DNA fragments. The Carnegie Institute group (Bolton et al., 1965) who incubated high molecular weight mouse DNA with sheared mouse DNA in a ratio of 500:1 in free solution, trapped the produce by filtration on cellulose acetate filters, found that some 60% of the DNA fragments were retained on the filter after incubation for 18 hrs. at 60°C. Since no precautions were taken against physical trapping of the DNA fragments this value may well be too high. Hoyer et al. (1964) also claimed that when mouse DNA-agar was incubated with an excess of sheared mouse DNA 70% of the long DNA could be covered indicating that most of it was available for hybridisation. However, Walker and McLaren (1965a) also working with mouse found that only 50% of the long DNA was available for hybridisation. They concluded that the figures obtained by Hoyer et al. (1964) were too high due to the presence of unpaired ends and loops of DNA.

To demonstrate the specificity of the DNA complexes formed at 60°C in their experiments Hoyer et al. (1964) incubated mouse DNA-agar with a mixture of sheared mouse and bacterial DNA. The bound DNA was then eluted from the complex by a stepwise increase in temperature. The bound homologous mouse DNA was eluted over a temperature range of 50-70°C whereas the bacterial DNA was nearly all removed by washing at 36°C and the remainder eluted at 50°C. Walker and McLaren (1965a) also found that DNA-DNA interactions to 60°C showed greater specificity than those carried out at lower temperatures. This may arise from the fact that longer complementary sequences are needed to give stability at higher
temperatures (Walker and McLaren, 1965a; Martin and Hoyer, 1966). Martin and Hoyer (1966) suggested from their results that comparison of the 40°/60° binding ratio for two heterologous DNAs might be used as an indication of species homology.

Walker and McLaren (1965a) reported that the Tm for mouse-duplexes formed at 60°C was some 14°C lower than the Tm of native mouse DNA. Martin and Hoyer (1966) confirmed these results by incubating sheared DNA by itself at 60°C. Successive fractions were melted out with a stepwise temperature gradient and their percentage binding to long DNA in agar measured. The Tm of renatured fragments determined in this way was found to be 13°C lower than that for native DNA. They concluded from this that the renaturation of the DNA was imperfect.

The Carnegie Institute group (Bolton et al., 1965) who also found a reduction of Tm of some 10°C for renatured mouse DNA compared to native, attempted to estimate the extent of mismatching in mouse-mouse duplexes formed at 60°C. To calibrate their system they first prepared irradiated poly-U, determined the percentage of damaged bases and annealed the irradiated poly-U with poly-A. By measuring the decrease in Tm of the irradiated poly-U:poly-A duplexes compared to that of the normal poly-U:poly-A hybrid they were able to relate the reduction in Tm to the number of mismatched bases. From their results they calculated that the reduction of the Tm in the mouse-mouse system indicated that 1 in 7 of the nucleotides in the associated regions were mismatched. Precisely matched regions appeared to be of the order of 20-50 nucleotides in length.

The low hypochromicity of mouse DNA renatured at 60°C suggests
that only $1/3$ of the nucleotides were paired (Bolton et al., 1965). The broad melting curve of renatured mouse DNA also indicates that there must be considerable variation in the degree of pairing of different strands, or different regions of individual strands. Since Bolton et al. (1965) claim that some 60% of sheared mouse DNA may be bound by high molecular weight mouse DNA they conclude that individual strands are paired for only part of their length (Bolton et al., 1965). Analysis of renatured sheared mouse DNA on a CsCl gradient yields a product with a density intermediate between that of native and denatured mouse DNA, indicating that the renatured DNA contains 50% precisely matched sequences and 50% unmatched sequences. Thus the picture that emerges from this work is that mouse-mouse duplexes contain a considerable number of mismatched sequences interspersed amongst the paired regions. While the quantitative determinations of Bolton et al. (1965) may be criticised it is clear from their evidence, taken with that of Walker and McLaren (1965a) and Martin and Hoyer (1966) that non-specific binding of mammalian DNA occurs even at 60°C, consequently the validity of results obtained by DNA-DNA hybridisations at this temperature is in question.

Hoyer et al. (1964) tried to measure the homology between DNA from mouse and man and DNAs from certain other vertebrates using the DNA-agar technique. As expected DNAs from closely related species showed the greatest degree of hybridisation. It was also found (Hoyer et al., 1964) that the addition of sheared heterologous DNA inhibited the binding of sheared mouse DNA to mouse DNA-agar, the most nearly related species competing most effectively for DNA sites. Similar results have been obtained in competition
experiments between DNAs of different plants (Bolton et al., 1965). Near relatives such as guinea pig and hamster did not compete additively with mouse DNA for sites on the mouse DNA-agar, presumably because the sequences for which they were competing were held in common between them. On the other hand, human and rodent DNA showed an additive competition effect with mouse DNA because they bound to different sites on the mouse DNA-agar.

The presence of homologous DNA sequences among different vertebrates was also shown by pre-selection of the sheared DNA on a heterologous DNA-agar. Thus, sheared mouse DNA was annealed with bovine DNA embedded in agar. The bound DNA was then recovered and challenged with DNA-agar prepared from guinea pig, mouse, salmon or calf. In all cases there was an increased binding of bovine-selected mouse DNA compared with unselected mouse DNA indicating that the nucleotide sequences hybridising with bovine DNA were those held in common with other vertebrates. The increased binding of mouse DNA-agar with bovine-selected mouse compared to non-selected mouse DNA is attributed to an unequal selection of base sequences in the first incubation.

Although DNA homology may be demonstrated in this way it is extremely difficult to put the results on a quantitative basis. Hoyer et al. (1964) found that human DNA-agar incubated with a mixture of sheared labelled human and mouse DNA bound 18% of human fragments and 5% of the mouse fragments. Similarly, mouse DNA-agar bound 6% human DNA and 22% mouse DNA. From "normalisation" i.e., expressing the reaction between heterologous DNAs as a percentage of the homologous DNA interaction they concluded that man and mouse shared 25% of their nucleotide sequences in common.
Normalisation is only valid if one assumes the DNA bound is representative of the total DNA and that the remainder fails to bind through DNA-DNA interaction and to loss of high molecular weight DNA from the agar column. A hint that the binding process might be selective came from the experiment cited above in which bovine selected mouse DNA annealed better with mouse DNA agar than did non-selected mouse DNA (Hoyer et al., 1964).

McLaren and Walker (1965) investigated the nature of the bound and unbound DNA in some detail. (McLaren and Walker, 1965; Walker and McLaren, 1965a; McLaren and Walker, 1966). They found that mouse DNA-agar bound only 30% of mouse DNA fragments (a value close to that of Royer et al. (1964)). If the bound fraction was released and reincubated with the second batch of mouse DNA-agar 40-50% of this could be rebound. However, only 10% of the original unbound fraction could be bound upon a second incubation. It was concluded that the initial DNA preparation contained a large fraction of "unbindeble" DNA (McLaren and Walker, 1965). If the mouse DNA bound through two successive hybridisation cycles was then released and annealed again with a third batch of DNA-agar the percentage binding was increased to 50-60%. This suggests that the mouse DNA-agar selects for a fraction of high binding capacity.

The failure of a large fraction of DNA to bind does not appear to be due to the presence of RNA or protein, to depurination or the size of the sheared DNA (Walker and McLaren, 1965a). Walker and McLaren (1965a) also consider that contrary to the suggestions of Cowie and McCarthy (1963) failure to bind cannot be attributed to interactions between different strands of the sheared DNA since
pre-incubation of sheared DNA at the concentrations used for hybridisation reduces the binding capacity only slightly.

However, Martin and Hoyer (1966) point out that since up to 15% of long DNA may leach out of the agar during incubation this long DNA in free solution may interact with the sheared DNA and prevent its binding to the DNA-agar.

If sheared denatured mouse DNA is fractionated on hydroxyapatite (Walker and McLaren, 1965b), two fractions are recovered: a "stable" fraction eluting at the same point as native DNA, and a "labile" fraction. The "labile" fraction has a melting curve characteristic of a single stranded molecule while the stable fraction appears to be partly duplex in nature since the melting curve resembles that of native DNA although it melts over a wider range and the $T_m$ is 5.5°C lower. The "stable" mouse DNA fraction showed a high binding capacity, 40% of it hybridising to mouse DNA-agar (that is that it binds better than unfractionated mouse DNA) (McLaren and Walker, 1966). It therefore seems that in the case of mouse failure to bind all the sheared DNA is due to the fact that the DNA-agar technique selects for a group of rapidly renaturing molecules. This was confirmed by Martin and Hoyer (1966). When the bound fraction of mouse DNA was recovered from the DNA-agar and self-annealed at 60°C the melting curve of the product resembled that of self-annealed unselected mouse DNA fragments but the $T_m$ was some 5°C lower (Martin and Hoyer, 1966). Thus the bound DNA fraction differs from the total DNA indicating selection of a special class of fragments by the DNA-agar. The experiments of Walker and McLaren with rat and mouse (McLaren and Walker, 1966) suggest this high binding fraction may show species specificity.
It has been suggested (McLaren and Walker, 1966; Martin and Hoyer, 1966) that this rapidly renaturing fraction of high binding capacity may represent the satellite DNA which represents some 10-20% of the DNA in mouse (Bolton et al., 1965). This satellite is thought to consist of some 2 million reiterated sequences each sequence 250 base pairs in length, its simple structure accounting for its high binding and renaturing capacity.

The Carnegie Institute group have pointed out that renaturation of mammalian DNA occurs more rapidly than would be expected for such a heterologous DNA (Bolton et al., 1965). Since the rate of renaturation is dependent on the concentration of the individual nucleotide sequences (Nygaard and Hall, 1964) and mammalian DNA is roughly 1000 times more complex than bacterial DNA we would expect mammalian DNA to renature 1000 times more slowly than bacterial DNA (Bolton et al., 1965). Instead renaturation proceeds at about the same rate in both cases. Bolton et al. (1965) consider that this is due to the presence of 'families' of reiterated sequences within the DNA. Each family would contain some 1000 repeating units and several 1000 such families would occur within the genome of the mouse. Because the individual sequences in these families are in high concentration, by virtue of their reiteration, rapid renaturation can take place between homologous families. These families renature at different rates, the fastest at a rate 100,000 times faster and the slowest 1000 times faster than would be predicted for completely heterologous DNA. Bolton et al. (1965) attribute the hybridisation within mammalian DNA to the renaturation of these families of reiterated sequences. The non-renaturing regions scattered between the families of reiterations
are presumably highly heterogeneous and thus are in such low concentration in the genome as a whole that the chances of two complementary sequences colliding are very poor. Consequently these regions do not hybridise under the reaction conditions employed. It seems probable that many of the families of reiterations have sequences in common so that different non-renaturing regions adjoin identical reiterations. Otherwise if the families of reiterations were all unique then renaturation of these sequences would align the complementary heterogeneous regions. This could bring about "zippering-up" of the complementary DNA chains and would result in nearly complete hybridisation.

When mammalian DNA is annealed in solution the rapidly renaturing regions on the different strands bind together forming networks (Bolton et al., 1965). There appears to be a lower limit to the size of DNA fragments capable of forming networks. The majority of DNA fragments of 1,300 nucleotides long contain at least one such region capable of renaturation and since more than 2/3 of the fragments enter into network formation it is probable that most fragments of this size have more than one such region. Since fragments 600 nucleotides long do not form networks the Carnegie Institute group (Bolton et al., 1965) suggest that the rapidly renaturing regions occur at spacings 300-1000 nucleotides apart. (It is interesting that 300 nucleotides would code for a protein of 100 amino-acids i.e. such a stretch is roughly the size of a gene.) These rapidly renaturing regions, of which the mouse satellite DNA is a prominent example may be equated with the DNA of high binding capacity reported by Walker and McLaren (1965a) and the heterologous DNA stretches between these regions with their
'unbindable' fraction (McLaren and Walker, 1965; Walker and McLaren 1965a). Thus the DNA-DNA hybridisation reaction occurs amongst a selected class of DNA molecules.

These findings mean that the results of Hoyer et al. (1964) need reassessment. Thus increased binding of DNA fragments by heterologous DNAs after pre-selection on DNA-agar does not give a quantitative measure of the overall homology between them. In particular quantitative estimates obtained by "normalisation" are not valid since only a selected class of molecules are hybridised. At present therefore results obtained from DNA-DNA hybridisation are not truly quantitative both because of selective hybridisation and also because of the specificity of the hybrid formed is in some doubt.

**DNA-RNA hybridisation studies**

Another method which has been used for determination of homologies is the hybridisation of DNA with RNA. Cellular RNA is synthesised on a DNA template and is complementary to it. DNA and RNA may therefore hybridise through the formation of hydrogen bonds in a manner analogous to DNA-DNA hybridisation.

Several techniques have been developed for the estimation of RNA-DNA hybrids; nitrocellulose-columns (Bautz and Hall, 1962) DNA-agar columns (Bolton and McCarthy, 1962) CsCl gradient separation (Hall and Spiegelman, 1961) trapping of the hybrid on nitrocellulose membranes (Nygaard and Hall, 1963) isolation on MAK columns (Hayashi et al., 1965) and hybridisation with DNA immobilised on membranes (Gillespie and Spiegelman, 1965). With these techniques RNA-DNA hybridisations have been exploited to
establish numerous points which have included (1) the identity if an RNA species (Attardi et al., 1963; Hall and Spiegelman, 1961; Bautz and Hall, 1962; Imamoto et al., 1965) (2) the nature of the DNA template coding for RNA (Green, 1964; Tocchini-Valentini et al., 1963), (3) the site of ribosomal RNA synthesis in the cell (Ritossa and Spiegelman, 1965; Wallace and Birnstiel, 1966) (4) that different DNA cistrons code for the two species of ribosomal RNA (Yankofsky and Spiegelman, 1963; Chipchase and Birnstiel, 1963).

DNA-RNA hybridisations have also been used to demonstrate genetic homologies in bacteria (Bolton and McCarthy, 1962; McCarthy and Bolton, 1963). When pulse-labelled RNA was extracted from Proteus vulgaris and incubated with an excess of P. vulgaris DNA embedded in agar, 33% of the label could be bound (Bolton and McCarthy, 1962). This hybridisation was specific since P. vulgaris RNA did not hybridise with DNA from T2 phage, thymus or Pseudomonas aeruginosa. Failure to hybridise all the RNA with P. vulgaris DNA was attributed to the presence of ribosomal RNA precursor in the pulse-labelled RNA. Only a very small fraction (ca. 0.3%) of DNA codes for ribosomal RNA (Yankofsky and Spiegelman, 1962a and b) consequently these sequences are quickly saturated and no sites are available to bind the remaining ribosomal RNA. The unbound RNA had a base composition resembling that of ribosomal RNA (Bolton and McCarthy, 1962). In addition if the bacterial cellular RNA (80% of which is ribosomal RNA) was uniformly labelled with $P^{32}$ very little $P^{32}$ RNA bound compared to pulse-labelled RNA. When pulse-labelled E. coli RNA was incubated with E. coli DNA 27% of the label could be bound (McCarthy and Bolton,
If the bound RNA was recovered and reincubated with a second batch of *E. coli* DNA 76% of this RNA could be bound. On the other hand only 6% of the unbound RNA could be hybridised in a second incubation. McCarthy and Bolton (1963) concluded from this that about 30% of the pulse-labelled RNA is present as messenger RNA and that the hybridisation procedure is about 80% efficient. *E. coli* pulse-labelled RNA also binds to DNA from other species of bacteria. If the hybridisation values are "normalised" DNA from *Shigella dysenterica* and *Salmonella typhimurium* binds 88% and 87% as much pulse-labelled *E. coli* RNA as does *E. coli* DNA. These values are similar to the 70% homology between *E. coli* and *Sh. dysenterica* or *S. typhimurium* estimated from DNA-DNA interactions (McCarthy and Bolton, 1963). However, they conflict with the evidence of Schildkraut et al. (1961) who found that no true hybrids were formed between DNA from *E. coli* and *S. typhimurium*.

It has already been pointed out that the DNA-agar technique for measuring genetic homology has serious defects, chiefly non-specific binding of heterologous material and selectivity in hybridisation. Similar criticisms may also be levelled at the DNA-RNA hybridisation method. Thus Yankofsky and Spiegelman (1962a, b) have shown that RNA-DNA duplexes formed after incubation in solution involve spurious hybrids consisting of RNA strands held to the DNA by small scattered complementary segments as well as the true hybrids formed between long complementary stretches of RNA and DNA. The RNA of the spurious hybrids may be digested by ribonuclease while true RNA-DNA hybrids are RNase resistant (Yankofsky and Spiegelman 1962a,b). This provides a simple method for testing the specificity of the RNA-DNA complex. In the
experiments to be described in the later sections it was found that 10–20% of the RNA bound to DNA after incubation in free solution could be digested with RNase and an even larger portion of the RNA bound to DNA immobilised on membranes (Gillespie and Spiegelman, 1965) was RNase sensitive. McCarthy and Bolton (1963) did not employ an RNase digestion step and did not provide evidence for the specificity of their RNA–DNA hybrids other than the failure of the bacterial RNA to bind to vertebrate DNA (McCarthy and Bolton, 1963). Although the melting curve of the E. coli RNA–DNA hybrids in agar is clearly that of a duplex structure (Bolton and McCarthy, 1964) the $T_m$ is $4^\circ{}C$ lower than that of the corresponding DNA–DNA hybrid, suggesting that the RNA–DNA hybrid is less stable (Bolton and McCarthy, 1964). It is therefore possible that the hybridisation values obtained by McCarthy and Bolton (1963) are too high due to some non-specific pairing.

The major objection to the use of RNA which has been pulse-labelled in vivo for the study of genetic relatedness by RNA–DNA hybridisation is that quantitative comparisons can only be made if (1) the pulse labelled RNA is a perfect copy of the DNA (i.e. all sequences present in the DNA are represented in the RNA) and (2) if the different RNA species are labelled to the same extent. These two requirements may be more or less fulfilled by pulse-labelled RNA obtained from a rapidly growing population of bacteria of mixed age. However, in the case of higher organisms it is exceedingly unlikely that a complete transcription of all DNA sequences into RNA occurs at any point in the life cycle; consequently a complete spectrum of pulse-labelled RNA species cannot be obtained. In addition the rate of transcription and the pool sizes of different RNAs may differ, yielding pulse-
labelled RNA species of varying specific activity. For these reasons *in vivo* pulse-labelled RNA cannot be used for the study of genetic relatedness in higher organisms.

An alternative method of obtaining an RNA copy of the genome of a higher organism for use in hybridisation studies is to synthesize RNA *in vitro* using the DNA of the organism as a template together with the 4 ribo-nucleotide triphosphates and an RNA polymerase. This approach has been investigated in the present work using *Drosophila* DNA as the template and RNA polymerase prepared from *Micrococcus lysodeikticus*. For convenience the RNA product of the *in vitro* system will be designated cRNA (complementary RNA) as distinct from mRNA (strictly messenger RNA) which we will equate with the *in vivo* pulse-labelled RNA.

The use of *in vitro* synthesised cRNA for DNA-RNA hybridisation has the advantage that all cRNA species may be expected to have a uniform specific activity (varying only with the base ratio of the RNA if the label is present in the nucleotide residues) and may therefore be used directly for quantitative comparisons. Whether all the nucleotide sequences present in the DNA are transcribed into cRNA is less certain. The relationship of cRNA to its DNA template is discussed in detail in the Introduction to Part II.

A DNA-cRNA hybridisation system similar to the one to be investigated here has been exploited most successfully by Paul and Gilmour (1966a and b) although they were not studying genetic relatedness. They prepared cRNA using *M. lysodeikticus* RNA polymerase and calf thymus DNA as template. It was shown by extrapolation that when the DNA was annealed with an excess of cRNA 50% of the DNA could be covered with RNA. Since
M. lysodeikticus polymerase transcribes both strands of the DNA (Geiduschek et al., 1961; Geiduschek et al., 1962) we would theoretically expect 100% coverage of the DNA. This discrepancy is discussed later. For the time being it may be taken that Paul and Gilmours results show that a large fraction of the DNA is transcribed into cRNA by M. lysodeikticus polymerase.

Paul and Gilmour (1966a) extended their findings to show that if cRNA was prepared on a calf thymus chromatin template the chromatin cRNA could cover only 5% of the DNA. They concluded from this that the template available for transcription into RNA in the chromatin preparation was only 5-10% of the total DNA. In subsequent experiments (Paul and Gilmour, 1966b) they showed that RNA extracted from calf thymus nuclei competed with chromatin-primed cRNA for the same DNA sites indicating that the in vitro synthesised cRNA contains the same RNA species as the mRNA present in vivo. In further competition experiments using cRNA and mRNA prepared from rabbit thymus and bone marrow they showed that the DNA templates available for RNA synthesis in the living cell are organ specific.

Thus the work of Paul and Gilmour indicates that a large fraction of the DNA may be transcribed by M. lysodeikticus polymerase and that the cRNA product resembles the mRNA present in the living cells. In addition the success of these experiments shows that DNA-cRNA hybridisation is a technically feasible method for studying DNA from different sources.

DNA-cRNA hybridisation therefore appears a promising alternative to DNA-DNA hybridisation for the measurement of
genetic relatedness. Two immediate advantages of the cRNA-DNA system are apparent, (1) a stringent test is available for the specificity of hybrid formation, namely the RNase resistance of the hybrid. Such a test is not readily available for the DNA-DNA system. (2) Once the system is established it is not necessary to prepare labelled DNA to measure the reaction. This means that this technique may be extended to organisms whose DNA cannot be readily labelled.

The ultimate goal of the present research is to discover whether the DNA-cRNA hybridisation technique may be used to study genetic differences and to see how far the genetic homologies revealed by this technique are in accord with homologies estimated by genetic and taxonomic criteria. Eventually it is hoped to extend the DNA-cRNA hybridisation technique to study genetic differences amongst species, sub-species and selected classes within a species of Drosophila. The first steps in this project were to extract pure DNA from Drosophila, to use this DNA as a template for the synthesis of Drosophila cRNA by M.lysodeikticus polymerase, to study the optimum conditions for hybridisation of Drosophila cRNA with Drosophila DNA and to show how far this hybridisation was specific for Drosophila.
PART I.

EXTRACTION OF DNA
PART I
INTRODUCTION

The extraction of high molecular weight DNA has become a standard procedure in many laboratories within the last 6-7 years since the publication of a general method by Marmur (1961). However it is only since the work of Mead (1964) and principally since that of Ritossa and Spiegelman (1965) that Drosophila DNA has been extracted in quantities sufficient for biochemical studies. The fact that such a genetically interesting material should have been neglected for so long speaks for the difficulty of preparing DNA from Drosophila.

The newly emerged adult male Drosophila has a weight of approximately 0.9 mg and contains 7 µg of DNA (Church, 1965; Church and Robertson, 1966). The newly emerged adult female has a weight of about 1.2 mg and contains roughly 1 µg of DNA (Church and Robertson, 1966). In the female both weight and DNA content rise after emergence due to feeding and egg development. As a rough approximation therefore 10 g. of Drosophila contains 10,000 flies and a total of 10 mg. DNA. The highest yield claimed for the extraction of DNA from Drosophila is 2.83 mg. from 10 g. flies (Kirby, 1961) subsequently lowered to 1.5-2 mg. per 10 g. flies (Hastings and Kirby, 1966). Travaglini and Meloni (1962) reported a yield of only 1 mg. per 10 g. flies, all other workers maintaining a discreet silence as to the yield of DNA from Drosophila. While a recovery of only 15-20% of the DNA as obtained by Hastings and Kirby (1966) might be tolerable in a material available in large quantities it is something of a nuisance in Drosophila since it is moderately difficult to obtain more than
30 g. of Drosophila at a time. Flies cannot be accumulated in the deep freeze for subsequent extraction since these yield a degraded product (Church, 1965).

The extraction of DNA from Drosophila is bedevilled by four major problems. (1) The release of cellular material from the chitinous exoskeleton: since Drosophila is very small and is surrounded by impermeable chitin large quantities of DNA-containing cellular material may be trapped within the exoskeleton, particularly if homogenisation is carried out in the presence of a protein precipitant such as phenol. (2) The potent DNase present in Drosophila: it is essential to inactivate or remove the DNase present in Drosophila right from the beginning of the extraction. The enzyme appears to differ in its properties from the DNase of bovine pancreas whose characteristics have been extensively studied (McCarty, 1946; Kunitz, 1950). So far it has not been possible to inactivate completely the Drosophila enzyme by chemical inhibitors. (3) The high RNA∶DNA ratio of Drosophila: Church and Robertson (1966) reported an RNA∶DNA ratio of about 7∶1 for newly emerged adults of D. melanogaster Pacific strain. With a mixed population of varying age taken from the population cages values nearer 10∶1 have been obtained. If we compare this with other tissues commonly used for DNA preparation we find they have significantly lower RNA∶DNA ratios: thus, rat liver has an RNA∶DNA ratio of about 4∶1 (Davidson, 1953), calf thymus 0.4∶1 (Davidson, 1953) and E. coli 2∶1 (Vendrely, 1946). This large excess of RNA in Drosophila makes purification of the DNA difficult and experience has shown that unless steps are taken to remove the bulk of the RNA in the initial stages of extraction it is almost
impossible to obtain RNA-free DNA. (4) The difficulty of separating DNA from protein in *Drosophila*. *Drosophila* has a relatively low DNA content. From figures published by Church (1965) it may be calculated that in newly emerged adult females the DNA forms only about 3 per cent of the dry weight. Much of this dry weight material is contributed by protein and this high protein/DNA ratio is unfavourable for extraction. When DNA is de-proteinized by precipitation of the associated proteins a considerable amount of DNA becomes occluded and precipitates with the denatured protein. Clearly the greater the excess of protein the more DNA will be physically trapped. Even with bacteria such as *E. coli* which has a much higher DNA content (some 1% of the dry weight (Vendrely 1946)) only a 50% recovery of the DNA may be expected if the proteins are removed by denaturation and precipitation (Marmur 1961). In my opinion the release of DNA from the protein forms the major obstacle to improving the recovery of DNA from *Drosophila*. The other three difficulties may be circumvented by various means but so far no completely satisfactory method has been found for the deproteination of the DNA.

In addition to the general hazards outlined above the present work is handicapped by the fact that DNA of very high molecular weight was required. Relatively little is known about the effect of the molecular size of the DNA on transcription and RNA–DNA hybridisation in a heterogeneous system such as *Drosophila*. Cohen et al. (1966) using *E. coli* RNA polymerase with phage DNA as a template found that sonication of the DNA led to a decrease in RNA synthesis and that the average length of the RNA was also
reduced as a result of a decreased synthetic rate. Karkas and Chargaff (1966) using a thymus DNA template and *E. coli* RNA polymerase found that sonication reduced the template activity to around 40% of that obtained with high molecular weight DNA. Bremer et al. (1966) reported that reduction in the size of T₄ DNA template from $120 \times 10^6$ to $12 \times 10^6$ by sonication or shearing did not effect the rate of RNA synthesis by *E. coli* polymerase. However $12 \times 10^6$ is still comparatively large DNA and may be above the critical length for maximum RNA synthesis. In preliminary experiments it was found that *Drosophila* DNA of low molecular weight (less than 12 S) did not prime as well in the polymerase reaction as the preparations of high molecular weight which were obtained in later experiments. Admittedly these early preparations of DNA may have been contaminated by RNA which could have inhibited the priming activity. However, *Drosophila* DNA of high molecular weight (larger than 25 S) primed as well as did DNA from other higher organisms such as rat and mouse. Since it seemed probable that degraded DNA had less template activity than high molecular weight DNA it was considered advisable to prepare DNA of maximum possible size. Furthermore if the polymerase can only attach to certain specific sites on the DNA (Bremer et al., 1966; Richardson, 1966) disruption of the DNA chain by degradation may interfere with the stoichiometry of transcription. Thus Green (1964) showed that, *in vitro*, *E. coli* polymerase transcribed only the sense strand of T₄ DNA but sonication of the DNA to around 800,000 molecular weight (9.7S) caused unequal transcription of both strands.

In order to obtain DNA of high molecular weight from *Drosophila*
all violent techniques of grinding and shaking were avoided. Unfortunately these precautions increase the difficulty of liberating DNA from the exoskeleton and from the proteins.

The methods available for the extraction of the DNA from *Drosophila* may be classified in three main groups. (1) Extraction in cesium chloride, (2) methods based on the phenol extraction technique of Kirby and co-workers, (3) methods requiring a preliminary preparation of a "nuclear" fraction.

In the following section various attempts to repeat and improve on these different methods will be described and their advantages and shortcomings discussed. In each case it is of particular interest to see how the different approaches attempt to combat the major problems of extraction outlined above.
METHODS I

Chemicals and solutions

Analar CsCl was repurified by dissolving 100 g. CsCl in 100 ml. distilled water and filtering through filter paper to remove gross impurities. 500 ml. of absolute alcohol were added and the solution chilled for several hours. The precipitated CsCl was collected by filtration and the crystals dried in the oven at 200-300°C. Water-saturated phenol was prepared by re-distilling B.D.H. crystalline phenol; the distillate was shaken with distilled water and kept in the refrigerator. Ether was redistilled over ferrous sulphate. 10 ml. ethanol was added per 500 ml. of redistilled ether and the mixture stored over water in the refrigerator.

The solution TWEEN used for the preparation of 'nuclear' fractions contains 0.1% Tween 80 (culture grade), 0.05 M EDTA, 0.15 M NaCl and 0.05 M tris-HCl buffer pH 6.8.

Pancreatic ribonuclease (pRNase) was obtained from Sigma Chemical Corp. Before use this was dissolved in a small volume of SSC pH 5.0 and boiled for 10 mins. to destroy any DNase present. Pronase, purchased from Kodak Ltd. was dissolved in water and pre-incubated by itself for 1 hr. at 37°C before use to digest any nucleases present.

T4 DNA and yeast sRNA were the gift of Dr. John Bishop.

All solutions of Drosophila DNA were handled with extreme care. Violent shaking was avoided and all pipetting was carried out with wide-bore pipettes to reduce shearing.
Culture of Drosophila

D. melanogaster (Pacific strain) were grown as cage populations at 25°C on the usual corn-meal-molasses-yeast medium. Some 6-10 of these cages were maintained yielding 20-30 g. Drosophila per week. With the large populations used there was an inevitable accumulation of old pupae cases. To eliminate this contamination and also to reduce contamination by yeast the flies were anaesthetised with CO₂ gas, transferred to a clean cage and spread out in numerous small beakers; this enabled the adult Drosophila to fly off leaving the pupae cases behind in the beakers which were then withdrawn. The adults in the cage were kept overnight with sucrose solution as the only food source. Under these conditions one would expect most of the yeast present in the gut of the flies to have been digested by the time of DNA extraction.

For collection the flies were anaesthetised with CO₂ gas and kept under anaesthesia until homogenisation either by covering the collecting vessel with aluminium foil and gently blowing in CO₂ gas from time to time or by placing a small lump of dry ice in the vessel. It was found that if the flies were kept in the deep-freeze (-20°C) for a day or so before DNA extraction there was a noticeable lowering of the yield. Church (1965) reported that DNA extracted from deep-frozen flies was degraded compared to DNA from fresh flies. Accordingly the flies were always collected immediately before use. The brief exposure to solid CO₂ during collection did not appear to have any deleterious effect: the flies recovered rapidly after removal of the CO₂ and DNA extraction was not impaired.
Estimation of DNA and RNA content

a) Schmidt-Thannhauser method. DNA and RNA were determined by a modification (Fleck and Munro, 1962) of the method of Schmidt and Thannhauser (1945). The cellular material was precipitated in 0.3 N PCA at 0°C for 30 mins., sedimented at 2000 x g. and the pellet resuspended and centrifuged twice more in cold 0.3 N PCA to remove oligonucleotides. The precipitate was then exposed to 1 N NaOH for 1 hr. at room temperature to hydrolyse the RNA (Scott and Froccostoro, 1952; Fleck and Munro, 1962). The solution was neutralised by the addition of PCA and brought to 0.3 N in PCA. This precipitates the DNA and protein leaving the ribonucleotides in solution. The RNA content was then determined from the U.V. absorption of this solution at 260 μμ. The pellet was washed once in 0.3 N PCA and kept for 15 mins. in 0.5 N PCA at 70°C to digest the DNA. The acid-resistant material was sedimented and the DNA present in the supernatant determined from its absorption at 260 μμ.

b) Diphenylamine method. This method was used to determine DNA in solution and also to determine the DNA content of acid-precipitated cellular material where an estimate of the RNA content was not required. Cellular material was precipitated and washed twice with PCA as described above and then hydrolysed in 0.5 N PCA for 15 mins. at 70°C. The unhydrolysed material was sedimented and the supernatant used for the determination of DNA. Neutral solutions of DNA were also brought to 0.5 N PCA and hydrolysed under the above conditions before estimation. The DNA content of the hydrolysate was determined by the diphenylamine method of Burton (1956). Diphenylamine, purchased
from BDH was twice recrystallised from hexane before use. A.R. glacial acetic acid was used and the aqueous acetaldehyde was made up freshly before use from freshly distilled or recently purchased acetaldehyde. The absorption of the reaction mixture at 600 \( \text{m} \mu \) and 660 \( \text{m} \mu \) was measured and the difference between them used to calculate the DNA content. The reaction was calibrated each time with salmon sperm DNA as a standard.

**Determination of \( ^{14}C \) DNA or \( ^{14}C \) RNA counts**

\( ^{14}C \) DNA or \( ^{14}C \) RNA, together with 500 \( \mu \)g. carrier RNA was precipitated in 5\% TCA and left to stand 20 mins. in ice. The precipitate was then collected on oxoid filters (Oxo Ltd.) under gentle suction. The tube which originally held the precipitated material was rinsed twice with 5\% TCA and the washings transferred to the filters. The precipitated material on the filters was then washed 5 times with 2 ml. aliquots of 5\% TCA under suction. The filters were then washed twice with ether:ethanol (2:1) mixture and stuck onto planchets with upholstery adhesive. They were then dried for 20 mins. at 70\(^\circ\)C and counted on a Nuclear-Chicago gas-flow counter.

**Estimation of the size of DNA**

a) **Elution from MAK**

A comparison of the size of the DNA obtained by different treatments was made by running the DNA together with yeast sRNA as marker on an MAK column (Mandell and Hershey, 1960) and determining the point at which the DNA eluted from the column. Methylated albumen and the MAK columns were prepared according to Mandell and Hershey (1960). Elution was carried out with a linear salt gradient of 0.1-2.0 M NaCl in 0.05 M potassium phosphate buffer
pH 6.7. The flow rate was 1 ml./min. and 6 ml. fractions were collected. The O.D. of the fractions was read at 260 mμ to determine the position of the sRNA and DNA fractions. The presence of DNA was confirmed by the diphenylamine reaction where necessary.

b) Determination of S value.

This was carried out according to the method of Bruner and Vinograd (1965). This method is the only one available which gives reliable measurements for DNA of very high molecular weight. In addition it gives a direct measurement of the S value at zero concentration ($S^0$). 50 μl of a Drosophila DNA solution (100 μg./ml.) in 0.1 SSC was inserted into the hole at the side of a Spinco Model E cell sector. The cell was then assembled and the cell sector completely filled with 2 M CaCl in 0.1 SSC. The cell was centrifuged at 20,410 rpm at 20°C and time lapse photographs (U.V. optics) taken at intervals of 16 mins. A tracing was made of the photographic record on a Joyce-Loebl densitometer and the $S^0_{20,W}$ value calculated according to Hearst and Vinograd (1962).
RESULTS

1. Cesium Chloride Extraction

This method is based on the procedure of Travaglini and Meloni (1962). In this procedure the tissue is ground in saturated CsCl. The high ionic strength of the CsCl brings about the dissociation of the DNA from its accompanying protein and also inhibits the DNase present in the tissue. After grinding the CsCl homogenate is adjusted to a density of 1.4 g.cm\(^{-3}\). Upon centrifugation this yields a gradient of 1.3 to 1.5 g.cm\(^{-3}\). Under these conditions DNA (density 1.5 g.cm\(^{-3}\)) and RNA (2.0 g.cm\(^{-3}\)) are pelleted, while protein and lipoproteins rise to the top of the gradient.

Method 1.

4 g. Drosophila were ground with 4 ml. saturated cesium chloride in 0.05 M potassium phosphate buffer pH 6.4 + 0.01 M sodium citrate on an MSE homogenizer for 25 seconds at maximum speed. The homogenate was filtered through gauze to remove debris and the density adjusted to 1.4 g.cm\(^{-3}\) in a final volume of 10 ml. The homogenate was centrifuged for 24 hours at 86,000 x g. at +1°C in the Spinco No.40 rotor. A reddish pellet formed at the bottom of the tube. The supernatant was poured off and the pellet resuspended in 1 M NaCl + 0.05 potassium phosphate buffer pH 6.8 and transferred to an MAK column (Fig. 1). The DNA containing fractions were identified by the diphenylamine reaction.

This procedure gave a yield of approximately 1.2 mg. per 10 g. Drosophila (Table I). Since the DNA content of Drosophila is approximately 10 mg. per 10 g. this rather poor recovery indicates that only a small fraction of the DNA is released from the protein by the cesium chloride. The DNA eluted from the MAK column was found to have a size of 18 S determined by analytical centrifugation.

This method has a number of disadvantages. The weight of flies which can be handled in this manner is relatively small and...
Fig. 1.

MAK elution profile of *Drosophila* DNA prepared by Cs Cl fractionation (Travaglini and Meloni, 1962):

MAK fractionation as described in Methods I. The fractions containing DNA were identified by the diphenylamine reaction.

- o U.V. absorption at 260 μ
- x Diphenylamine reaction. O.D. 600 - O.D. 660

(No corrections have been made for O.D.'s larger than 2.0).
Fig. 1.
the yield per gram is no better than that obtained by other methods (Table I). Furthermore the DNA is pelleted together with nucleoproteins and RNA and is apparently contaminated by DNase since if the pellets are left overnight in the refrigerator the DNA is degraded. For large scale production of the DNA the pelleted material would therefore require considerable further purification and this would inevitably reduce the yield of DNA.

The method was therefore modified in an attempt to overcome the above disadvantages, first by preparing a "nuclear" fraction from Drosophila so that larger quantities of material could be handled and second by centrifuging in a CsCl solution of density of 1.7 g cm\(^{-3}\). Under these conditions the RNA pellets while the DNA forms a band in the centre of the tube.

**Method 2**

7 gms. of Drosophila were ground in 40 ml. of TWST and a "nuclear" fraction prepared as described on page 59. The "nuclear" fraction was dispersed in 7 ml. SSC and brought to a final density of 1.7 g cm\(^{-3}\) with CsCl. Upon addition of the CsCl the suspension became extremely viscous and sticky. The suspension was centrifuged for 46 hours at 33,000 rpm (86,000 x g) at +5°C in the Spinco No. 39 rotor. After centrifugation a distinct RNA pellet could be seen and a thick band of protein was present at the top of the tube. The DNA formed a viscous band in the centre of the tube. The tubes were punctured and eight-drop fractions collected. The DNA-containing fractions could be identified by their extreme viscosity. The DNA fractions were dialysed against SSC and the presence of DNA confirmed by U.V. determinations. The fractions were bulked and any residual protein removed by shaking with 1% SLS and an equal volume of water-saturated phenol. The emulsion was centrifuged, the supernatant withdrawn and the phenol extracted with ether as described on page 59.

The yield of DNA was 7 mg. per 10 gm. of flies (Table I). The fact that this is lower than the yield of DNA from the preceding method is probably due in part to some loss of DNA-containing material during preparation of the "nuclear" fraction and
### Table I

Extraction of Drosophila DNA by different procedures

<table>
<thead>
<tr>
<th>Method</th>
<th>Yield in mg/10g. flies</th>
<th>Size</th>
<th>% RNA contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsCl Method 1</td>
<td>1.2</td>
<td>18 S</td>
<td>Not measured</td>
</tr>
<tr>
<td>Method 2</td>
<td>0.7</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>Phenol Method 1</td>
<td>1.5</td>
<td>33 S</td>
<td>20% - 0</td>
</tr>
<tr>
<td>Method 2a</td>
<td>0.7</td>
<td>forms small fibrous ppt.</td>
<td>50%</td>
</tr>
<tr>
<td>Method 2b</td>
<td>1.06</td>
<td>forms small fibrous ppt</td>
<td>70%</td>
</tr>
</tbody>
</table>

**Extraction from "nuclear" fraction**

- **Method of Mead (1964)**: 1.30 forms flocculent ppt. 50%
- **Method of Ritossa and Spiegelman (1966)**: 0.7 forms flocculent Not measured ppt.
- **60°C heat treatment + pronase deproteinisation**: 0.55 - > 30 S 0.7
- **60°C heat treatment + chloroform/iso-amyl alcohol deproteinisation at neutral pH**: 0.7 > 30 S "

**Standard extraction procedure:**

- **60°C heat treatment + chloroform/iso-amyl alcohol deproteinisation at pH 8.5**: 1.2 - 30 - 56 S 1.5
- **Preliminary chloroform/iso-amyl alcohol deproteinisation at pH 8.5 followed by 60°C heat treatment**: 1.2 - 0
also to a loss of DNA during the deproteinisation step. The cesium chloride method is therefore not suitable for extraction of DNA on a large scale. It can however be of use where only very small quantities (less than 5 gms.) of material are available.

2. Phenol Methods

The use of phenol as a deproteinising agent, together with extraction of the DNA by an organic salt solution, has been developed by Kirby and his co-workers. The tissue is ground in the presence of phenol and an organic salt solution. The phenol acts as a deproteinising agent and inhibits the DNase. Kirby found that the choice of organic salt greatly affected the release of the DNA from the nucleoprotein. At 0.3 M concentration hydrophilic salts, such as naphthalene 1:5 disulphorate (NDS) and phenolphthalein diphosphate release very little DNA into solution (Kirby 1956, 1961, 1964). It was suggested that the NDS could not adsorb to the nucleoprotein because of the highly charged hydrophilic groups at opposite ends of the molecule (Kirby, 1956) and thus could not disrupt the chromosome structure (1966). Phenolphthalein diphosphate may precipitate DNA by cross-linking the strands through the phosphate groups (Kirby, 1961).

Lipophilic salts however produce a good release of DNA from the protein. This is maximised if the salt has some chelating properties as well (Kirby, 1964). As a lipophilic salt 4-aminosalicylate has been most widely used but dithiocarbamate or a mixture of napthalene-2-sulphonate and EDTA are also effective. It is possible that the chromosome is covered by lipoprotein with some metal linkages (Kirby, 1956, 1964) which may be disrupted by lipophilic substances or chelating agents. The addition of 8-hydroxyquinoline, which has chelating properties, to the phenol
also improves the release of DNA from the protein (Kirby, 1964).

The different properties of lipophilic and hydrophilic salts have been exploited in a method to purify DNA from RNA. When the tissue is ground in a mixture of phenol and a hydrophilic salt such as NDS, the RNA is released into solution (Kirby 1961, 1964, 1965). The DNA, however, forms an insoluble precipitate which may be collected by centrifugation. Since the DNA does not enter into solution it is protected from shearing during the grinding process (Kirby, 1964). The DNA may then be released from the precipitated DNA by extraction with a lipophilic salt solution.

**Method I.**

The method used here was a modification of the procedure of Church (1965) which is based on the method of Hastings and Kirby (1966).

10 gm. or more of adult *Drosophila* were ground with 5 volumes of a 1:1 mixture of 5% NDS and 1% 8-hydroxyquinoline in 90% phenol for 30 seconds in a Waring blender at slow speed. The mixture was then stirred briskly for 10 minutes at a temperature of about 15°C to release the DNA and RNA from the chitinous exoskeleton. The mixture was centrifuged for 20 minutes at full speed on an MSE tabletop centrifuge in the cold room. Upon centrifugation the mixture separated into three phases: an upper aqueous phase containing the RNA, a creamy pink interface containing the DNA and a brown phenolic phase containing the chitinous exoskeletons. The aqueous phase was withdrawn with a pipette and the interface scooped up with a spatula or wide-bore pipette and transferred to a round bottom flask with 1 ml. of 90% phenol + 1% 8-hydroxyquinoline and 5-10 ml. of cold (5-10°C) 6% 4-amino-salicylate solution. The phenolic phase was stirred for a further 30 minutes at 15-20°C with an equal volume of 0.5% NDS to release any DNA trapped in the exoskeletons. After centrifugation the interface was collected as before and added to the suspension in 4-amino-salicylate. The suspension was then shaken briskly but not violently on a mechanical shaker in the cold room. Even after the initial phenol treatment the DNA interface contains some DNase activity, consequently the 4-amino-salicylate extraction must be carried out in the cold although the mixture must not be allowed to freeze as this also degrades the DNA. After 20 minutes shaking the 4-amino-salicylate mixture was centrifuged at 5,000 rpm. (3000 x g) in the Serval for 10 minutes and the viscous supernatant containing the DNA withdrawn with a wide-bore pipette and kept on ice. The residue was then re-extracted with a
few ml. of L-amino-salicylate in the cold and the supernatant added to the first extract.

Purification. Since the DNA preparation still has DNase activity the first step must be the removal or inactivation of this enzyme. To this end an equal volume of ethanol was layered on the L-amino-salicylate solution and the DNA spooled. The spoolate was dissolved in a few ml. of 0.1 SSC to give a viscous solution. The solution was made up to SSC and incubated with pronase (0.5 mg. per ml.) for one hour at 37°C. The solution was then extracted three times with an equal volume of water-saturated phenol to remove the pronase and the phenol removed with four ether-extractions. The crude DNA solution was then incubated with RNase (50-100 µg. per ml.) for two hours at 37°C followed by a further incubation with pronase (0.5 mg. per ml.) for one hour at 37°C. The proteins were then removed by a further four phenol extractions followed by three ether extractions. The DNA was then spooled with an equal volume of ethanol and dissolved in 0.1 SSC, and subsequently adjusted to SSC.

The yield of DNA was approximately 1.5 mg. per 10 g. of flies, (Table I) i.e., about the same as that obtained by Hastings and Kirby (1966). DNA prepared in the method described above was contaminated with glycogen and up to 20% by ribonucleotides as determined by the difference between ultraviolet and diphenylamine estimations of the DNA content. It appears that a large fraction of the contaminating ribonucleotides may be bound to the glycogen which is known to adsorb nucleotides. If the glycogen was removed by sedimentation before the final spooling of the DNA the ribonucleotides could be eliminated (see also p. 58). Cesium chloride sedimentation analysis of DNA prepared in this way showed a relatively homogeneous peak with an S_{20}^O of about 33 S. That is, it is of a high molecular weight around 45 x 10^6 considerably larger than the 16-18 S DNA extracted by Hastings and Kirby (1966).

This is one of the best methods of preparing DNA from Drosophila but has one or two disadvantages. It cannot be used for less than 10 g. of flies and the yield is better if larger quantities are used. Furthermore, as described above the method is somewhat
laborious. This may be improved if the purification procedure of Ritossa and Spiegelman (1966) is substituted after the RNase step. Attempts to eliminate the first pronase step by substituting a second phenol extraction or proceeding directly to the RNase step resulted in a slightly lower yield. However the major disadvantage is that on certain occasions this method fails completely. This may sometimes be attributed to the phenol-NDS mixture freezing during the initial stirring, as this degrades the DNA. This freezing can be prevented if m-cresol is included with the phenol. On other occasions there was no immediately obvious reason for the failure although the method seems to be unsuccessful if the flies used are old.

**Method 2.**

This method is based on that described by Kirby (Kirby: personal communication; Kirby, 1959). This involves grinding the tissue in a mixture of 4-amino-salicylate and phenol together with SLS. The SLS and 4-amino-salicylate bring about the immediate release of DNA into solution, together with cellular RNA, and the DNA is purified from this RNA by salt extraction.

a) 20 g. of flies were ground in a Sorvall omnimix for 4 seconds together with 10 volumes of 1:1 mixture of 2% SLS in 6% 4-amino-salicylate and water-saturated phenol. The grinding period is critical: too short a period does not liberate all the DNA since not all the tissues are broken open, however; since the DNA is released directly into free solution too long a grinding period will result in shearing of the DNA. It can be seen that there is a sharp drop in viscosity if grinding is prolonged. After the homogenisation the viscous DNA solution was shaken briskly on a mechanical wrist-action shaker for $\frac{1}{2}$ hour at room temperature. The solution was then centrifuged at low speed and the aqueous phase withdrawn with a wide bore pipette. An equal volume of isopropanol was added and the fibrous precipitate collected by centrifugation. The pellet was then dissolved in 35 ml. of 0.1 SSC. 112 g. of CsCl was added to 13.5 ml. of this solution, the remainder being treated as described in procedure b) below.
The DNA solution was warmed to 30°C to dissolve the CsCl. The solution was kept overnight at 4°C. The high ionic strength of the CsCl inhibits DNase and precipitates ribosomal RNA. The precipitated RNA was sedimented at 6000 x g., the supernatant poured off, 150 μg. RNase per ml. added and the whole dialyzed against 2 x SSC at room temperature overnight. The solution was then deproteinised with phenol and the DNA precipitated with an equal volume of ethanol and collected by sedimentation. The pellet was washed three times with absolute alcohol to remove traces of phenol and dissolved in 3 ml. of 0.1 SSC.

The yield of DNA was approximately 0.7 mg. per 10 gms. of flies (Table 1) as determined by diphenylamine and was heavily contaminated, the DNA contributing only 50% to the total UV absorption of the solution. Since experience indicated that some DNase activity remained after the initial phenol treatment it seemed possible that the yield might be improved if this DNase could be eliminated. Accordingly the remainder of the DNA solution in 0.1 SSC was heat-treated to inactivate any DNase.

b) The DNA solution was brought to 2 x SSC, and the temperature raised rapidly to 60°C and kept there for 5 minutes. After cooling to room temperature 0.5 mg. RNase per ml. was added and the solution dialysed against 2 x SSC overnight. The solution was then incubated with pronase for 1 hour at 57°C and deproteinised with water-saturated phenol. After centrifugation at 6000 x g. the aqueous phase was removed and the DNA precipitated with an equal volume of ethanol. The fibrous precipitate was sedimented, washed three times with absolute alcohol and dissolved in 3 ml. of 0.1 SSC.

The yield of DNA was 1.06 mg. per 10 g. flies (Table 1) as determined by the diphenylamine reaction. However the contamination by RNA was extremely high, representing 70% of the total UV absorption of the solution. Sedimentation of the glycogen and prolonged dialysis did not greatly improve the purity.

Thus these methods give a substantially lower yield than does the phenol-NDs method. The improved yield obtained with heat treatment compared with CsCl extraction underlines the importance
of eliminating DNase activity. The very high level of contamination in both cases also shows the necessity of removing the bulk of the RNA before extracting DNA, as in the phenol-NDS method: apparently precipitation in high salt and/or RNase are not sufficient to eliminate the RNA completely.

3. Extraction from "Nuclear" Preparations

It must be stressed from the beginning that the term "nuclear" fraction is used simply as a matter of convenience to denote the DNA-containing fraction liberated by different procedures. This DNA-material is heavily contaminated and may bear little resemblance to the intact nuclei.

In 1964 Mead published a method of preparing DNA from Drosophila melanogaster which involves the preparation of a "nuclear" fraction. The flies were homogenized in the cold in SSC (the citrate acting as an inhibitor of DNase) and the homogenate centrifuged at 10,000 x g. for 30 minutes. The DNA-containing material sediments with the fragmented skeletons leaving the bulk of cellular RNA and proteins in solution. Further RNA may be removed by washing the pellet repeatedly with SSC. This step provides an initial purification of the DNA from RNA. Treatment of the pellet with Aerosol O.T. liberates DNA. The proteins are then removed by precipitation with chloroform-octyl alcohol.

DNA was prepared using this method with some minor modifications.

5 g. Drosophila were homogenised in 30 ml. SSC at 0-4°C in an MSE homogenizer for 5 seconds at maximum speed and for a further 15 seconds at half speed. Subsequent steps were all carried out at 0-4°C. The homogenate was centrifuged in the Servall for 30 minutes at 10,000xg. The supernatant was poured off and the pellet re-homogenized in 70 ml. in SSC on the MSE homogenizer for 5 seconds at maximum speed and 10 seconds at half speed and then resedimented.
This washing procedure was repeated twice more. The pellet was dispersed in 30 ml. of 0.1 M NaCl and 30 ml. of 5% Aerosol O.T. in 0.7 M sodium acetate pH 7.8 was added slowly. The mixture was left stirring on a magnetic stirrer in the cold room overnight and then made to 1 M NaCl and stirred for a further 10 minutes. After centrifugation at 10,000 x g. for 30 minutes the supernatant was decanted and 1.5 volumes of ethanol added. A flocculent precipitate was formed. This was sedimented at 3000 x g. and dissolved in 5 ml. SSC. The solution was deproteinized by shaking with 0.05 volumes of chloroform: octyl alcohol (5:1) for 30 minutes at room temperature on a mechanical shaker. The mixture was centrifuged and the upper aqueous phase withdrawn. Two volumes of ethanol were added to the aqueous phase and the flocculent precipitate sedimented at 3000 x g. and redissolved in SSC.

The yield of DNA was approximately 650 μg.=1.3 mg. per 10 g. flies (Table I) together with an equivalent amount of RNA. This is a reasonably good yield of DNA but obviously further purification is required. According to Mead (1964) his DNA preparation has a DNA:RNA ratio of 2:1 but he considered this RNA to be specifically associated with the DNA. The RNA could be removed by incubation with RNase (Mead, 1964). Vermeulen and Attwood (1965) used RNase treatment followed by fractionation on a cesium chloride density gradient to purify DNA obtained by Mead’s method. Mead (1964) reported that his DNA preparation had an S_w,20 of 11.7 (M.W. 1,360,000) before RNasing and an S_w,20 of 9.0 (M.W. 837,000) after RNase treatment. In our experience it is only rarely that the method of Mead (1964) yields DNA sufficiently large to spool. This degradation of the DNA is attributed to (1) the action of the DNase during the prolonged washing of the "nuclear" pellet, and the extraction period, and (2) to the overnight stirring of the dilute DNA solution. Since we were particularly anxious to obtain DNA of high molecular weight we decided to abandon this approach.
More recently Ritossa and Spiegelman (1965) described a method for extracting DNA from *Drosophila* in which a "nuclear" fraction was first prepared in a sucrose solution and deproteinized according to the method of Marmur (1961). For deproteinization the "nuclear" fraction is suspended in a large volume of liquid, presumably to reduce the occlusion of DNA by precipitated protein. Because of the large volumes used the DNA is in very dilute solution and consequently precipitates in a flocculent form rather than forming a spool (Ritossa and Spiegelman, 1965). In order to overcome this an alternative procedure was substituted using pronase to digest the protein from a nuclear preparation prepared according to Ritossa and Spiegelman (1965).

7 g. of *Drosophila* were ground in an ice cold mortar together with 70 ml. of cold medium (0.35 M sucrose + 0.025 M KCl + 0.005 M MgCl₂ + 0.05 tris-HCl pH 7.2). The grindate was filtered through six thicknesses of gauze and centrifuged at 3,000 rpm (Spinco SS 34 rotor) for ten minutes. The pellet was resuspended in 10 ml. saline/EDTA (0.15 M NaCl + 0.1 M EDTA pH 8.0) 2% SLS was added and the mixture was kept at 60°C for ten minutes to destroy the DNase activity. Instead of using a chloroform-amyl alcohol mixture to deproteinize as did Ritossa and Spiegelman (1965), an attempt was made to digest the protein with pronase. 500 μg. per ml. of pronase was added and the mixture was dialysed against two changes of 0.1 M NaCl + 0.05 M tris-HCl pH 7.2 for two hours in the cold to lower the concentration of EDTA (which partially inhibits pronase). The dialysate was then made 0.01 M in calcium chloride and a further 500 μg. per ml. pronase added and the incubation continued for 1½ hours at 37°C. The mixture was then dialysed overnight against 0.1 M NaCl plus 0.05 M tris-HCl pH 7.2. The remaining protein was removed by shaking the dialysate with an equal volume of water-saturated phenol. The aqueous phase was withdrawn and the phenol extracted with ether.

The yield of DNA as determined by the diphenylamine reaction was 0.700 mg. per 10 g. of flies. This is a rather poor yield. In addition the DNA produced did not spool but precipitated in a flocculent form (Table I). It was therefore decided to try and develop an alternative method of extracting DNA from a "nuclear"
fraction in which degradation of DNA was kept to a minimum.

To this end experiments were carried out to determine the best methods for (1) preparing a "nuclear" pellet, (2) minimizing DNase activity, (3) deproteinization, and (4) purification

a) Preparation of a "Nuclear" Fraction

Figure 2 shows an experiment to determine a suitable speed for sedimenting the "nuclear" fraction. Approximately 10 g. of Drosophila were ground in 40 ml. of TWEST for 15 seconds at maximum speed on an MSE homogenizer. The homogenate was then stirred at approximately 1/5th of maximum speed for 30 mins. and filtered through gauze. The temperature was kept below 4°C throughout. The filtrate was centrifuged in the Spinco No. 40 rotor for 30 mins. at 10,000 rpm (6,596 x g.). The supernatant was decanted and recentrifuged at 20,000 rpm (24,384 x g.) for 75 minutes. The supernatant was again decanted and centrifuged at 39,000 rpm (105,000 x g.) for 90 mins. The supernatant was then poured off and precipitated with 3% HC10 4 at 0°C for 1 hour. The pellets obtained at the different speeds were dispersed in water and precipitated with 3% HC10 4 in ice. The precipitates were sedimented, washed three times with cold 3% HC10 4 and the RNA and DNA determined by the modified method of Schmidt-Thannhauser described in Methods I. As shown in Figure 2 nearly all the DNA released by homogenization sediments in the 10,000 rpm pellets, only a very small fraction sedimenting at higher speeds or being released into the supernatant. The 10,000 rpm fraction contains roughly twice as much RNA as DNA but the larger part of the RNA sediments in the ribosomal fraction (39,000 rpm) or remains in the supernatant. Thus preparation of a "nuclear"
RNA and DNA content of cell fractions prepared from Drosophila:

Fraction 1 (10,000 rpm pellet) sediments at 6,596 x g
Fraction 2 (20,000 rpm pellet) sediments at 26,384 x g
Fraction 3 (39,000 rpm pellet) sediments at 105,000 x g
Fraction 4 is the 105,000 x g supernatant.

Cell fractions prepared and analysed as described in text.
Fig. 2.
fraction provides an initial purification from RNA.

A brief homogenisation of the flies is not sufficient to release all the DNA-containing material as can be seen from Figure 3. The method of Mead (1964) circumvents this by including the fragmented bodies with the nuclear fraction during the extraction procedure. However this debris is very bulky, consequently a large volume of liquid is needed for extraction. As a result the DNA liberated is in very dilute solution and therefore prone to degradation by physical means. To promote the release of DNA-containing material from the homogenised flies, Tween 80 was included in the homogenisation medium, Fisher and Harris (1962) having previously shown this to be effective in the liberation of nuclei from animal cells.

5 g. of Drosophila were homogenised with 15 ml. of SSC containing 0.1% Tween for 5 seconds at full speed on the MSE homogeniser. The temperature was kept below 4°C throughout. The homogenate was filtered through gauze and the residue retained by the gauze returned to the homogeniser together with 20 ml. of SSC + 0.1% Tween and stirred for 5 mins. at 1/5th maximum speed. Again the homogenate was filtered and the residue stirred for 10 minutes at 1/5th maximum speed. The filtration and stirring were repeated twice more. The individual filtrates were centrifuged at 10,000 rpm (12,000 x g.) for 15 mins. in the Servall SS 34 rotor, the pellets dispersed in SSC and precipitated with 3% HClO₄. The final residue left in the cheese cloth was also dispersed and precipitated in this way. After washing twice with 3% HClO₄, the pellets were extracted with 0.5 N HClO₄ for 15 mins. at 70°C. The RNA and DNA present in the
Fig. 3.

Release of DNA from a homogenate of Drosophila by repeated stirring in SSC + 0.1% Tween 80:
The Drosophila homogenate was filtered and the non-filtrable residue subjected to repeated stirring and filtration as described in the text.
**Fig. 3.**

The diagram illustrates the release of RNA and DNA over stirring time in minutes. The y-axis represents the amount of RNA and DNA released, while the x-axis shows the time in minutes. The bars indicate the amount of RNA and DNA released at different time points: 0, 5, 10, 15, 20, 25, and residue.
$\text{HClO}_4$ extract were determined by the orcinol (Mejbaur, 1939) and diphenylamine methods respectively. The results are shown in Figure 3. The initial homogenisation releases only 20% of the DNA-containing material sedimenting at 12,000 x g., however repeated stirring of the homogenate with SSC containing Tween 80 liberates nearly 90% of the DNA. This procedure was therefore adopted for the preparation of a "nuclear" fraction for the extraction of DNA. It was decided to continue the practice of filtering at 10 min. intervals and stirring the residue in fresh medium to reduce the possibility of fragmenting the liberated nuclei during a prolonged stirring period. As a routine the Drosophila were homogenised and stirred three times. Although the limited number of stirrings lowered the yield a little it also reduced the period in which the nuclei were exposed to DNase.

Table II shows that repeated washing of the "nuclear" fraction does not bring about significant purification from RNA. 10 g. of Drosophila were ground in the MSE with 40 ml. of TWEST, filtered, and the "nuclear" material sedimented at 12,000 x g. for five mins. The "nuclear" pellet was then washed by stirring for five mins. at 1/5th maximum speed and sedimented as before. The washing procedure was repeated twice more. Although nearly 60% of the RNA is removed by the washing steps there is concomitant loss of 40% of the DNA, thus the RNA:DNA ratio of the initial "nuclear" pellet is 2.3 and after 3 washing steps has only been reduced to 1.8. Since this very poor degree of purification was accompanied by a heavy loss of DNA it was decided not to wash the "nuclear" pellet before proceeding with the DNA extraction.
Table II.

Purification of "nuclear" fraction by repeated washing in
TWE ST

<table>
<thead>
<tr>
<th>Material</th>
<th>DNA content in mg.</th>
<th>RNA content in mg.</th>
<th>RNA/DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial &quot;nuclear&quot; pellet</td>
<td>3.7</td>
<td>8.7</td>
<td>2.3</td>
</tr>
<tr>
<td>&quot;Nuclear&quot; pellet after first wash</td>
<td>2.6</td>
<td>4.9</td>
<td>1.9</td>
</tr>
<tr>
<td>&quot;Nuclear&quot; pellet after second wash</td>
<td>2.3</td>
<td>4.5</td>
<td>1.9</td>
</tr>
<tr>
<td>&quot;Nuclear&quot; pellet after third wash</td>
<td>2.2</td>
<td>3.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

DNA and RNA were determined by the Schmidt-Thannhauser method as described in Methods I.
b) **Inhibition of DNase activity**

The pH optimum of purified bovine pancreatic DNase has been shown to be pH 6-7 (Kunitz, 1950) and it is known that this enzyme requires magnesium for activity and is inhibited by metal chelating agents (McCarty 1946). Consequently solutions of high pH containing metal chelating agents such as the saline/EDTA pH 8.0 of Marmur (1961) have been recommended for the extraction of DNA. However, DNase from other organisms may have somewhat different properties.

**pH effect.** Preliminary experiments showed the pH optimum of Drosophila DNase to differ from that of bovine pancreatic DNase. The pH optimum of the DNase present in Drosophila homogenate was determined over a range of pH as shown in Figure 1. It can be seen that in the homogenate the enzyme shows maximum activity at pH 7.3 - 7.5, rather higher than the optimum pH for bovine pancreatic DNase. The Drosophila homogenate itself has a pH of 6.5 in 0.01 M NaCl and appears to have quite a powerful buffering capacity. After an incubation of five mins. in 0.1 M phosphate buffer initial pH 8.0 the pH falls to around 7.1. Because of this it was considered advisable to use solution of high pH for the initial homogenisation since it seemed possible that in the Drosophila homogenate the pH might fall to around the optimum for DNase activity. For this reason a homogenisation medium of pH 6.8 was chosen as more suitable.

**Effect of inhibitors.** Figure 5 shows the effect of different concentrations of EDTA on DNase activity in a Drosophila homogenate. It can be seen that the homogenate has considerable DNase activity and that this is inhibited by the addition of EDTA.
Effect of pH (range 6-8) on DNase activity of a homogenate of Drosophila:

5g of Drosophila were homogenised in 20 ml. 0.01 NaCl at full speed on the MSE homogeniser for 15 sec. The homogenate was filtered through muslin and the filtrate homogenised briefly on a Tri-R homogeniser to give a fine suspension. To determine the rate of hydrolysis of DNA a series of incubation mixtures were set up at different pHs. Each incubation mix contained 2 ml. Drosophila homogenate, 400 μg salmon sperm DNA and 1 M sodium phosphate buffer in a total volume of 5 ml. The pH was checked and readjusted where necessary before incubation and at intervals throughout the incubation period. The samples were incubated at 37°C on a waterbath with shaking, and 1 ml. aliquots withdrawn at 5 min. intervals. The aliquots were precipitated in ice-cold 3% HClO₄ for 30 mins., centrifuged and the pellets washed twice with cold 3% HClO₄. The DNA content was determined by the diphenylamine reaction. The values shown in the figure are the initial rates of hydrolysis. These were determined by plotting the decrease in DNA content for the different incubations and taking the tangent to the origin of each curve to obtain the initial rates.
Fig. 1.

![Graph showing the hydrolysis of DNA against pH.](image-url)
Effect of EDTA on DNase activity of Drosophila homogenate:

12 g. flies were homogenised at maximum speed on an MSE homogeniser in 40 ml. of 0.01 M NaCl + 0.01 tris-HCl pH 7.4 for 20 sec. and the homogenate filtered and rehomogenised on the Tri-R as in Fig. 4. The incubation mixture (final volume 5 ml.) contained 1.5 ml. Drosophila homogenate, 110 µg. salmon sperm DNA, 0.01 M NaCl, 0.01 M tris-HCl pH 7.4 and EDTA in concentrations of 0, 0.01 molar or 0.05 molar.

The incubation was carried out at 37°C in a water bath with a shaker. 1 ml. aliquots were withdrawn at intervals and precipitated in ice-cold 3% HClO₄. The precipitates were centrifuged down, washed twice with cold 3% HClO₄ and the DNA content determined by the diphenylamine reaction.
Fig. 5.
However, even in 0.05 M EDTA some loss of DNA occurs during the 45 min. incubation period. In addition to the obvious loss of DNA seen in fig. 5 upon incubation of the Drosophila homogenate it should be pointed out that Drosophila DNase is thought to be an endonuclease specific for C-C linkages (Kirby: 1964). Since the 3% PCA used in the assay procedure precipitates quite small polynucleotides it is possible that the enzyme splits the DNA into small fragments and that this type of degradation goes undetected by the above assay method. It is possible that this resistance shown by Drosophila DNase to EDTA inhibition may be due to a certain number of intact nuclei persisting in the homogenate even after two homogenisations and that the EDTA fails to penetrate these nuclei.

Some other compounds were studied for their ability to suppress DNase activity. Citrate, a metal chelating agent, inhibits the DNase roughly 60% at 0.1 M concentration and copper and zinc ions at 10^{-2} M concentration are also strongly inhibitory. In the case of these two this is probably due to a non-specific inhibition arising from the action of heavy metal ions on the protein.

On the basis of the above findings on "nuclear" release and DNase inhibition the grinding medium TWEST was devised. This contains 0.1% Tween 80 to promote the release of nuclei, 0.05 M EDTA to inhibit DNase activity, 0.15 M NaCl to maintain ionic strength which protects DNA and 0.05 M tris-HCl pH 6.8 to prevent the pH rising to an unfavourable level. Figure 6 shows the results of an experiment to test whether TWEST completely inhibited Drosophila DNase during preparation of a "nuclear" fraction.

10 g. of Drosophila were ground in 40 ml. of TWEST as described previously (page 45) filtered and C^{14}-labelled T_4 DNA
Fig. 6.

Size of T₄ DNA before and after exposure to Drosophila homogenate in TWiST:

a) MAK elution profile of Drosophila DNA prepared as described in text. C¹⁴ T₄ DNA was added to the initial homogenate and extracted together with the Drosophila DNA as described in the text.

b) MAK elution profile of C¹⁴ T₄ DNA control. Yeast sRNA was included as a marker.

C¹⁴ DNA counts were determined as described in Methods I

o——o U.V. absorption at 260 μm
x——x C¹⁴ counts
Fig. 6.

[Diagram showing optical density against fraction number with peaks labeled for ribosomal RNA, DNA, and T4 DNA.]
(2,000 cpm total) was added to the filtrate. The Drosophila residue was stirred twice with 40 ml. of TWEST and each time half the filtrate was added to the initial homogenate. The combined filtrates were then kept in ice for 45 mins., a period roughly equivalent to the time required for centrifuging down the "nuclear" material. They were then shaken twice with water-saturated phenol to remove the bulk of the protein. The aqueous supernatant was then extracted with ether to remove the phenol. 22 ml. of this DNA-containing supernatant was then diluted with an equal volume of water and run on an MAK column (6a).

As a control a solution of C14T4DNA (initial size 60 S) and yeast sRNA marker in 0.1 M NaCl + 0.05 M potassium phosphate pH 6.9 was run on an MAK column (6a). It can be seen that exposure to the DNase present in the Drosophila homogenate results in a decrease in the size of the T4DNA. T4DNA recovered from the homogenate peaked at fraction 25 compared to fraction 26-27 in a control run. Thus the neutral pH and the EDTA present in TWEST do not completely eliminate degradation of the DNA by DNase during the preparation of a "nuclear" fraction. It should however be pointed out that the T4DNA from the homogenate and the control T4DNA elute very close to each other, consequently degradation cannot be extensive. (The smaller recovery of counts from the nuclear preparation is due to the fact that only about half of the extract was run on the column and is not due solely to loss of counts through degradation of the T4DNA). The Drosophila DNA may be less prone to attack by DNase than the T4DNA which is in solution, fully exposed to the DNase, unlike the bulk of Drosophila DNA which is bound to protein and localised in the nuclei.
where it may be protected. While some breakdown of Drosophila DNA probably occurs in TWEST homogenates, nevertheless DNA of extremely high molecular weight (up to 56.5 S) may be obtained from these homogenates. Since it had been shown previously that zinc ions inhibited Drosophila DNase an alternative grinding medium containing \( Zn^{++} \) was tested. This medium proved much less successful than TWEST in maintaining DNA integrity. 10 g. of Drosophila were ground with 40 ml. medium containing 0.15 M NaCl, 0.1 M ZnSO\(_4\) and 1% Tween 80 and a "nuclear" fraction prepared in the normal way (p. 59). The "nuclear" pellet was resuspended in 2 ml. of the grinding medium, made up to 0.5% SLS and kept at 60°C for 10 mins. (this heat treatment inactivates DNase and does not itself result in any degradation of DNA, see page 52). Calcium chloride •05 M was added and the suspension was incubated with 5 mg. pronase per ml. for 1 hour at 37°C. The mixture was then shaken with an equal volume of water-saturated phenol to eliminate the pronase and the phenol extracted with ether. The solution was then dialysed against 0.1 M NaCl and run on a MAK column together with an sRNA marker. Figure 7 shows that during this treatment extensive degradation occurs. The sRNA marker cannot be located in its usual position (fractions 16-17) on the column because of the mass of UV absorbing material which has spread into the lower fractions. A comparison of Figure 7 with Figure 6a, shows that the ribosomal RNA which in Figure 6 appears as a very distinct peak has been largely degraded and extends into the lower fractions. The presence of this degraded material completely obscures the DNA peak which can only be detected by the diphenylamine reaction. The DNA, which was recovered in very poor yield, peaks around
Fig. 7.

MAK elution profile of Drosophila DNA extracted in the presence of 0.1 M Zn SO₄:
For procedure see text.

- o—o U.V. absorption at 260 μμ
- x—x Diphenylamine O.D. 600 - O.D. 660
fractions 17-18, the region where sRNA is normally found. The DNA must therefore be extremely degraded. Since the heat treatment at 60°C destroys DNase activity (p. 52) this degradation must have taken place predominantly during the preparation of the "nuclear" fraction. Thus the zinc sulphate grinding medium is not as effective as TWEST in inhibiting DNase activity during the stage of "nuclear" preparation.

c) Deproteinisation. Pronase seemed to be the most promising method of deproteinisation since it had been used most successfully in extracting DNA of high molecular weight from several sources including Xenopus erythrocytes (M.L. Birnstiel: personal communication), mouse liver (F.W. Robertson: personal communication), higher plants and sperm (Hotta and Bassel, 1965). If all the proteins can be digested by pronase this should give a very high yield of DNA from Drosophila.

A "nuclear" fraction was prepared by grinding 8 g. of Drosophila in TWEST as described on p. 59. The 12,000 x g. pellets were collected and resuspended together with C^{14}DNA (4,000 counts per min. total) in 1.5 ml. of 0.1 M NaCl + 0.01 M tris-HCl pH 7.2. Two mg. pronase per ml. were added to the suspension together with calcium chloride in a final concentration of 0.01 M (pronase is inhibited by metal chelating agents and stimulated by calcium ions so this should counteract the inhibitory effects of any EDTA present). The suspension was incubated at 37°C for 1.5 hours. It was then shaken with an equal volume of water-saturated phenol to remove any remaining protein. The aqueous phase was withdrawn and phenol extracted with ether. 2 ml. of this DNA preparation was then diluted with 45 ml. 1 M
NaCl in 0.05 M potassium phosphate buffer, yeast sRNA added as a marker and the whole run on an MAK column. The results are shown in Figure 8.

During the incubation with pronase the added T₄ DNA is markedly degraded (8a). Thus, in the control run (8b) the T₄ DNA peaks at fraction 26 whereas after incubation with the "nuclear" preparation and pronase it peaks at fraction 22. Pronase digests the DNase present in Drosophila, consequently, the degradation of DNA observed must take place before the DNase loses its activity. If Figure 8a is compared with other MAK runs on Drosophila nucleic acids (see 1, 6a) it is immediately noticeable that the large ribosomal RNA peaks present in these runs are completely missing. Presumably the RNA was degraded before the pronase could destroy the RNase. No Drosophila DNA is visible in Figure 8a unless it is represented by the slight shoulder on the side of the sRNA peak (fractions 20-22). It is clear from this that a simple deproteinisation of the "nuclear" fraction with pronase alone cannot be used since the enzyme does not act sufficiently rapidly to destroy the DNase before it has had time to degrade the DNA.

Matters are complicated by the fact that since EDTA inhibits pronase it is necessary to saturate the EDTA with calcium chloride but this in turn reduces the extent of the EDTA inhibition of DNase. If pronase is to be used for the deproteinisation some means of inhibiting the DNase must be found. The most effective method of destroying DNase activity is by heat treatment since this enzyme is heat labile (Kunitz, 1950).

A "nuclear" fraction was prepared from 10 g. of Drosophila ground in TWIST. Labelled T₄ DNA (2,500 cpm) was added to the nuclear pellet to monitor any degradation which occurred in the
Fig. 8.

MAK elution profile of "nuclear" material treated with pronase:

a) "Nuclear" fraction and \( ^{14} \text{C} \) T\(_4\) DNA after deproteinisation with pronase. Procedures as described in text.

b) \( ^{14} \text{C} \) T\(_4\) DNA control.

Yeast sRNA was included as marker in both cases. \( ^{14} \text{C} \) DNA counts were determined as described in Methods.I

- o—o U.V. absorption at 260 mu
- x—x \( ^{14} \text{C} \) counts
Fig. 8.
subsequent steps. The "nuclear" fraction was resuspended in 3 ml. of SSC, 1% SLS was added and the mixture heated to 60°C as rapidly as possible. After maintenance at 60°C for ten minutes the mixture was cooled in ice and calcium chloride added to give a final concentration of 0.05 M. The mixture was then incubated with 2 mg. pronase per ml. for 5 hours at 37°C, cooled and shaken with water-saturated phenol to remove undigested protein. After centrifugation the aqueous phase was extracted repeatedly with ether to eliminate any phenol and the ether blown off with nitrogen. The solution was made up to 50 ml. in 0.1 M NaCl plus 0.05 M potassium phosphate buffer and run on an MAK column together with an sRNA marker (Figure 9a).

The sRNA marker cannot be detected because of the large amount of UV absorbing material contributed by Drosophila. The Drosophila DNA peak is obscured by the large ribosomal RNA mass but can be located by the diphenylamine reaction and is found to peak around fraction 25. This indicates that it must be of comparatively high molecular weight (larger than 25 S). The labelled T4 DNA also peaks at fraction 25. Comparison with the T4 control (Fig. 9b) shows that T4 DNA which has not been exposed to the Drosophila "nuclear" fraction and heat treatment peaks at fraction 26. Since repeat runs on MAK columns under apparently identical conditions may vary by one fraction this difference may not be significant. The comparison certainly shows that relatively little degradation occurs during the heat treatment and subsequent pronase extraction. Since the DNA also survives incubation at 37°C with pronase the heat treatment must have largely destroyed the DNase activity of the Drosophila "nuclear" fraction. It therefore
Fig. 2.

MAK elution profile of DNA isolated after heat treatment of "nuclear" fraction:

a) $^{14}C$ T$_4$ DNA was added to the nuclear pellet and isolated together with Drosophila DNA after heat treatment as described in the text.

b) $^{14}C$ T$_4$ DNA control.

Yeast RNA was added as a marker in both cases.

- U.V. absorption at 260 μm
- Diphenylamine reaction $O.D. 600 - O.D. 660$
- $^{14}C$ counts.
Fig. 9.
seems that this is the best method of controlling the DNase during extraction of the DNA.

In subsequent experiments using the heat treatment-pronase extraction it was found that although the molecular weight of the DNA was high (larger than 30 S) the yield was very low: 55 to 70 mg. per 10 g. of flies. Since the DNase activity has been controlled as far as possible and in view of the high molecular weight of the DNA it seems unlikely that this poor yield is due to loss of DNA from degradation but probably represents a failure in deproteinisation. A number of factors might contribute to this. (1) It is difficult to remove the SLS which may inhibit the pronase, (2) the EDTA and citrate present may inhibit the pronase in spite of added calcium ions, (3) a large amount of pronase is added in an attempt to counteract the inhibitory factors cited above. However, when phenol is added to remove remaining proteins a considerable fraction of the DNA may co-precipitate with this large amount of pronase. (4) Some of the proteins present in Drosophila may be resistant to proteolytic enzymes.

Other methods of deproteinising were tested to see if they gave a better release of DNA from nuclei. Drosophila were homogenised in TWEST and the filtrate divided into five aliquots which were centrifuged at 12,000 x g. for 40 mins. to pellet the nuclear material. The individual pellets were then treated as follows:

1. The pellet was resuspended in TWEST and made up to 1% in SLS. After heat treatment for 10 mins. at 60°C the suspension was cooled and deproteinised by addition of 1/4 volume of 5 M NaClO₄ and shaken with chloroform/isoamyl alcohol (24:1) (Marmur, 1961). The
emulsion was centrifuged at 6,000 x g. for 10 mins. and the aqueous phase withdrawn.

2. The pellet was resuspended in SSC and 1% SLS added. After heat treatment as above the suspension was made 0.02 M in CaCl₂, pronase (5 mg. per ml.) was added and the mixture incubated at 37°C for 4 hours. The solution was shaken with water-saturated phenol to precipitate the pronase, centrifuged and the aqueous phase withdrawn.

3. The pellet was heat treated as in (2), and 1 M mercaptoethanol added together with 250 µg. per ml. of pronase. (Bodenfreund et al., 1961) had previously shown that treatment with mercaptoethanol rendered resistant proteins digestible by proteolytic enzymes. (See also Hotta and Bassel, 1965). The suspension was dialysed against 0.1 M NaCl + 0.05 M tris-HCl pH 7.3 + 2% mercaptoethanol in the cold for 1½ hours. It was then transferred to a flask, made up to 0.02 M CaCl₂ and a further 250 µg. pronase per ml. added. After incubation for four hours at 37°C it was further deproteinised by shaking with water-saturated phenol, centrifuged, and the aqueous phase withdrawn.

4. The pellet was dispersed in 5 ml. of 1 M NaClO₄ and shaken with an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline. The emulsion was centrifuged at 6,000 x g. for ten mins. and the aqueous phase removed.

5. The pellet was dispersed in 5 ml. of the 6% 4-amino-salicylate and shaken with an equal volume of 90% phenol containing 0.1% 8-hydroxyquinoline. It was then centrifuged and the aqueous phase withdrawn as above.
Aliquots were taken from the five different DNA extracts and the DNA content determined (Table III). The methods involving release of DNA from protein by $\text{NaCl}_4$ or $\text{l}$-amino-salicylate and deproteinisation with phenol are the least successful (Table III). Deproteinisation with either chloroform/amyl alcohol or pronase subsequent to heat inactivation of the DNase are equally efficient. Pretreatment of the DNA-protein complex with mercaptoethanol did not improve the release of DNA.

Since the chloroform/amyl alcohol was as efficient as pronase for deproteinisation and is a considerably quicker and simpler method it was decided to use this as the deproteinising agent in subsequent experiments. Since it is no better than pronase treatment the yield of DNA is still low about $7 \text{ mg. per 10 g. of flies}$. However, further experiments showed that if the chloroform/amyl alcohol deproteinisation was carried out at alkaline pH the yield could be greatly increased (Table I).

10 gm. of Drosophila were ground with TWEST and a "nuclear" fraction prepared. The "nuclear" pellets were dispersed in 1 ml. TWEST per tube and the total volume measured. Additional TWEST was then added to give a final ratio of $1.5 \text{ ml. TWEST to 1 ml. "nuclear" fraction}$. 2% SLS was added and the solution heated at $60^\circ\text{C}$ for 10 mins. and then cooled in ice. A 1/10th volume of saturated tris-pH 8.5 was added followed by 1/4 volume 5 M NaClO$_4$. The viscous mixture was shaken with chloroform/iso-amyl alcohol (24:1) for 5 mins. and centrifuged on the Servall for 10 mins. at 6000 x g. The aqueous supernatant was withdrawn and spooled with an equal volume of cold ethanol. The precipitate was dissolved in 0.1 SSC and then purified as described in the Standard Extraction Procedure (p. 59).

The yield of DNA was approximately $1.2 \text{ mg. DNA per 10 g. of flies}$. This could be improved if a second extraction step was included. The yield of DNA obtained in this way approaches that obtained using the phenol method of Hastings and Kirby (1966).
Table III

Release of DNA from the "nuclear" fraction

<table>
<thead>
<tr>
<th>Method</th>
<th>µg. DNA released</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Heat treatment followed by chloroform/iso-amyl alcohol deproteinisation</td>
<td>232</td>
</tr>
<tr>
<td>2. Heat treatment followed by pronase digestion</td>
<td>256</td>
</tr>
<tr>
<td>3. Heat treatment followed by pronase digestion in the presence of mercaptoethanol</td>
<td>241</td>
</tr>
<tr>
<td>4. Deproteinisation with 90% phenol + 1% hydroxyquinoline in the presence of 1 M NaClO₄</td>
<td>73</td>
</tr>
<tr>
<td>5. Deproteinisation with 90% phenol + 1% hydroxyquinoline in the presence of 6% 4-amino-salicylate</td>
<td>81</td>
</tr>
</tbody>
</table>

For details of the extraction procedures see text.
The DNA content of the extracts was determined by precipitating 1 ml. aliquots of each extract in cold 75% ethanol + 2% sodium acetate pH 5.0. The precipitates were sedimtented at 2000 x g. in a MSE table model centrifuge in the cold and washed three times in cold 75% ethanol + 2% sodium acetate. The pellets were then extracted with 5 M HClO₄ for 15 mins. at 70°C, the insoluble residue sedimented and the DNA of the supernatant determined by the diphenylamine reaction.
In addition, it is very reliable. On the very rare occasions that no spoolable high molecular weight DNA has been obtained by this method the failure may be attributed to using flies which are old. For these reasons this method was ultimately adopted as the standard method of preparing DNA from Drosophila.

It was thought that the heat treatment might in itself render the DNA-protein complex resistant to deproteinisation. The experiment of deproteinising before heat treatment was therefore carried out.

20 g. of Drosophila were ground with TWEST and a "nuclear" fraction prepared and resuspended in TWEST as described in the preceding experiment. DNA was then dissociated from the protein by the addition of 1% SLS followed by a 1/10th volume of saturated tris pH 8.5 and NaClO₄ to give a final concentration of 1M. The viscous solution was then shaken briefly with an equal volume of chloroform/isoamyl alcohol. The emulsion was centrifuged and the aqueous phase withdrawn. Since it was thought unwise to proceed with the heat treatment at high pH and in the presence of a high concentration of NaClO₄, the DNA solution was first dialysed against SSC for 2 hours. The pH was then adjusted to 7.0 with citric acid and the solution was made 1% in SLS and kept at 60°C for ten mins. It was then cooled, spooled with an equal volume of ethanol and the spoolate dissolved in 1 SSC. Further purification of DNA was carried out as described for the standard procedure (page

The yield from this procedure was 1.2 mg DNA per 10 g of flies. That is, there was no improvement in the yield compared to the preceding method. Since postponing the heat treatment until after deproteinisation made the method very tedious it was not pursued further.

d) Purification of the crude DNA extract. The DNA prepared from the "nuclear" pellet by heat treatment and chloroform/isoamyl alcohol still contains protein and is heavily contaminated with RNA and glycogen. The RNA may be degraded by treatment with ribonuclease. Since it is most important for our experiments that the purified DNA contains no RNase activity, the added RNase
is destroyed by digestion with pronase and the pronase removed together with any residual proteins, by shaking with phenol or chloroform/amyl alcohol. At first considerable difficulty was experienced in purifying the DNA from RNA. Although the RNA was degraded the ribonucleotides could not be completely removed from the DNA by prolonged dialysis. Even after spooling the RNased DNA in ethanol followed by redissolving it in SSC and dialysing a persistent RNA contamination remained.

An attempt was made to eliminate the ribonucleotide contamination by passing the DNA through a Sephadex column. A sample of a DNA preparation which had been treated with RNase and pronase was run on an MAK column to obtain a picture of the nucleic acid distribution before further purification. Another sample of the same preparation was purified by passing through a Sephadex G25 column. (Figure 10).

On the MAK column (Fig. 10a) the DNA forms a distinct peak at around fraction 24 while a mass of UV absorbing material (deoxyribo- and ribonucleotides) elutes in the early fractions. All the RNA has been extensively degraded and no peaks of high molecular weight RNA can be detected.

When the crude DNA preparation is purified on a Sephadex G-25 column (Figure 10c) the DNA elutes in the early fractions and is followed by a second peak of UV absorbing material which contains the nucleotides. A similar separation is obtained if G200 Sephadex is used. When the DNA-containing fractions obtained from the Sephadex G-25 column are re-run on MAK (10b) it can be seen that the nucleotide peak present in Figure 10a has been virtually eliminated. Comparison of Figure 10a and 10b
Purification of *Drosophila* DNA on Sephadex G-25:

a) MAK elution profile of crude *Drosophila* DNA preparation

b) MAK elution profile of the same preparation after purification on Sephadex G-25. MAK runs carried out as described in Methods I

c) Sephadex elution profile of the crude DNA preparation.

A sample of the crude DNA preparation in 7 ml. 0.1M NaCl + 0.05 M potassium phosphate buffer pH 7.8 was added to the Sephadex G-25 column and eluted at a rate of 0.5 ml./min. 0.4 ml. fractions were collected.
Fig. 10.
reveals that the DNA peak elutes at the same point on the gradient. This shows that passage of DNA through Sephadex does not result in shearing of the DNA.

Purification of the DNA on Sephadex has the disadvantage that only relatively small amounts of DNA can be handled and that the DNA is recovered in a very dilute solution. Furthermore, this method does not provide a satisfactory purification from glycogen which also emerges in the early fractions eluting from the Sephadex column. Subsequent experience also showed that this procedure did not completely eliminate RNA, but that a persistent contamination of some 10-20% RNA remained. While some of this may be due to RNase-resistant RNA cores which elute with the DNA, it seems probable that a large fraction is contributed by nucleotides adsorbed to the glycogen which is recovered with the DNA. Glycogen is known to adsorb nucleotides (Cout and Flamm, 1966) and Spiegelman and Ritossa, (1965) also found that elimination of the RNA was contingent on removal of the glycogen. The DNA preparation may be purified from glycogen either by pelleting the glycogen at 78,000 x g. or by digesting it by alpha-amylase according to Ritossa and Spiegelman (1965). Both methods were found satisfactory but it is essential that the alpha-amylase used is DNase free. Pancreatic alpha-amylase (Worthington) twice recrystallized was found to be suitable. However, to eliminate all possibility of DNase activity the preferred method was to sediment the glycogen after the final deproteinisation. After the glycogen had been removed the DNA was spooled in isopropanol, dissolved in 0.1 SSC and dialysed overnight against an appropriate buffer. This procedure yielded an RNA-free DNA preparation.
Standard Procedure for DNA extraction.

10 g. or more of Drosophila were homogenized with 40-50 ml. of TWEST on an MSE homogenizer for ten seconds at maximum speed. (Everything was kept at 0-4°C during the preparation of the "nuclear" preparation). The homogenate was then filtered through two layers of gauze. If more than 20 g. of Drosophila were available the material was split into two equal batches which were homogenized and filtered separately. The non-filterable residues were then pooled. The residue retained in the gauze was returned to the homogenizer together with 40 ml. of TWEST stirred for 10 mins. at 1/5th maximum speed and filtered as before. This stirring and filtration was repeated twice more on the residue. The bulked homogenates were then centrifuged in the Servall for 30 mins. at 12,000 x g. The supernatant was decanted and the "nuclear" pellets resuspended in 1 ml. TWEST each. The suspension was collected in a measuring cylinder and the initial volume of the "nuclear" pellets calculated by subtracting the volume of TWEST added. Additional TWEST was then added to give a final ratio of TWEST: "nuclear" volume of 1:5:1.

SLS was added to the suspension to give a final concentration of 2%. This liberates DNA from the protein forming a very viscous, almost gelatinous solution. This solution was then brought rapidly to 60°C and maintained at that temperature for 10 mins. It was then cooled in ice to room temperature and a 1/10th volume of saturated tris solution (pH 8.5) was added with gentle stirring. 5 M NaClO₄ was then added to give a final concentration of 1 M. The viscous solution was then transferred to a round-bottomed flask
together with an equal volume of chloroform/isoamyl alcohol (24:1). The flask was gently swirled by hand until the two phases formed a creamy-red emulsion. The emulsion was then centrifuged in the Servall for 10 mins. at 6,000 x g. This separates the mixture into two phases - a lower chloroform phase and an upper orange-red aqueous phase containing the DNA. The two phases are separated by an interface resembling liver in colour and consistency. The aqueous phase was removed and the interface re-extracted for DNA. The interface was first dispersed by hand in 2-3 ml. of TWEEN and then 1/10th volume saturated tris and half volume 5 M NaCl added and the suspension deproteinized with chloroform/isoamyl alcohol as above. The aqueous extracts were combined and the DNA spooled by stirring with an equal volume of cold ethanol. The DNA may be stored at this point if necessary. For storage the spool was washed by agitation first in 80% ethanol then in absolute ethanol and kept under absolute ethanol in the deep-freeze. In this way several spools of DNA may be accumulated over a period of weeks and purified together. The yield of DNA was somewhat lower from stored material.

**Purification.** The pink-coloured spool was drained and dissolved in 1 SSC (it was usually necessary to leave it overnight at 4°C to get all the DNA into solution). The solution was then made up to SSC and 150 µg. RNase (boiled) per ml. added. After 4 hours incubation at 37°C 2 mg./ml. of pre-incubated pronase was added and incubation was continued for 1 hour. The solution was then deproteinized as follows: SLS was added to make a 1% final concentration followed by an equal volume of water-
saturated phenol. The solution was shaken by hand and centrifuged. Centrifugation yielded two phases: a nearly colourless aqueous phase containing the DNA and a pink phenolic phase with a thin interphase between. The aqueous phase was withdrawn, SL3 and phenol added as before and the deproteinisation repeated. The aqueous phase was then shaken for five minutes with an equal volume of chloroform/isoamyl alcohol, centrifuged, and the aqueous phase re-extracted with chloroform/isoamyl alcohol. The DNA solution, which by now was completely colourless but slightly turbid with glycogen, was then transferred to a stainless steel centrifuge tube and centrifuged in the Spinco No.30 rotor for 30 minutes at 30,000 rpm. This pelleted the glycogen and the supernatant could then be poured off cautiously. The DNA solution was then spooled after the addition of 0.54 volumes of isopropanol. The spool was washed by gentle agitation in 80% alcohol and then dissolved in 0.1 SSC and dialysed overnight against SSC. The DNA solution could then be stored in the refrigerator over a few drops of chloroform. In this form it was stable for several weeks. The yield of DNA obtained was about 1.2-1.5 mg per 10 g flies.

Characteristics of the DNA

DNA prepared by the standard procedure outlined above contained less than 1% protein as determined by the Lowry reaction (Lowry, et al. 1951). It was also RNA-free as shown by the correspondence of U.V. and diphenylamine determinations of DNA content. (This method is not sensitive enough to detect very small RNA contaminations of around 1% which may become important
when hybridisation is carried out using high DNA:RNA ratios. For highly critical work it may be necessary to include a further purification step by banding the DNA in a CsCl gradient.

Figure 11 shows the melting curve of purified *Drosophila* DNA in 0.1 SSC. The DNA melts over a narrow range of about 60-85°C with a $T_m$ of 70°C. This agrees with the value obtained under similar conditions by Hastings and Kirby (1966) for *Drosophila* DNA prepared by the phenol method. Hastings and Kirby (1966) have argued that the absence of any hypochromicity in the temperature range 20-50°C in the melting curve can be used as a criterion for freedom from RNA since any RNA present should melt in this region. In the melting curve shown in Figure 11 there is no evidence of any increase in hyperchromicity below 60°C. This confirms that DNA prepared by the standard extraction procedure is virtually free from RNA.

The G-C content of the DNA was calculated from its buoyant density in CsCl. This was determined by sedimenting the DNA in a cesium chloride gradient together with denatured DNA from *Pseudomonas aeruginosa* as a marker. A tracing of the U.V. distribution obtained with the model E is shown in Figure 12. The *Drosophila* DNA bends at a density of 1.698 ± 0.004. This gives a value of 40-42% for the G-C content calculated from published formulae (Rolfe and Meselson, 1959; Sueoka et al., 1959; Sueoka, 1961). This is in good agreement with the estimates published by Hastings and Kirby (1966), who obtained a value for the G-C content of 40-41% from base analysis and between 37-42% calculated from buoyant density measurements. It is also in agreement with previous determinations of the G-C content of
Thermal denaturation curve of purified *Drosophila* DNA:

A solution of *Drosophila* DNA (0.4 O.D. per ml.) in 0.1 SSC was placed in the measuring cell of a Beckman DK spectrophotometer equipped with a water-jacketed light chamber. The control cell was filled with 0.1 SSC. The U.V. absorption was measured at 260 μm and the temperature raised in steps over a period of two hours. The temperature was measured directly in the experimental cell and the increase in optical density recorded.
Fig. 11.
Densitometer tracing of a CsCl buoyant density centrifugation of *Drosophila* DNA:

1 μg. *Drosophila* DNA and 1 μg. denatured *Pseudomonas aeruginosa* DNA in 0.1 SSC was made up to a mean buoyant density of ca. 1.720 g.cm⁻³ with CsCl. This solution was centrifuged at 45,770 rpm in the Spinco Model E analytical centrifuge at +20°C for an equilibration time of 20 hours. The photographic record (U.V. optics) was traced in a Joyce-Loebl densitometer. The buoyant density of the *Drosophila* DNA was calculated according to Hearst and Vinograd (1962) assuming a buoyant density of 1.739 ± 1 g.cm⁻³ for the denatured *Pseudomonas* DNA.
Drosophila DNA: Agyarakis and Bessman (1963) obtained a value of 38-40% from chromatography and electrophoresis measurements. Mead (1964) also obtained a value of 38% by chromatography and Ritossa and Spiegelman (1965) estimated the G-C content as 38% from buoyant density measurements.

Hastings and Kirby (1966) reported that Drosophila DNA isolated by their method had an $S_{20,w}$ value of 16-18 S and this appears to be the highest value reported in the literature. DNA prepared by the standard procedure outlined above is invariably larger than this with an $S_0^0$, of 30-56 S. Figure 13 shows the tracings of a sedimentation analysis of a preparation of Drosophila DNA. The $S_{20,w}$, calculated from this tracing is 56.5 S, that is the DNA has a MW of 110 x 10^6 (Studier, 1965).

Thus the DNA extracted by the standard procedure reported here has the characteristics of Drosophila DNA as determined by other authors and is obtained in reasonable yield with a high degree of purity and very high molecular weight. DNA produced by this procedure was used as a primer for the RNA polymerase reaction and was also used for hybridisation studies with the polymerase-produced cRNA.
Fig. 13.

Densitometer tracing of band centrifugation of Drosophila DNA in 2 M CsCl:
The centrifugation and determination of the S value were carried out as described in Methods I.
Fig. 13.
PART II.

HYBRIDISATION STUDIES
PART II

INTRODUCTION

The next step in this research project was to use the high molecular weight Drosophila DNA as a template for the synthesis of cRNA in vitro and to use this cRNA for the study of RNA-DNA hybridisation. As explained in the General Introduction cRNA-DNA hybridisation studies can only give a fully quantitative measure of genetic relatedness if the cRNA is an exact and faithful copy of the DNA template. However, the relationship between the DNA template and its cRNA product is still not fully understood. It has been shown that the cRNA synthesized by both E. coli and M. lysodeikticus RNA-polymerase resembles the DNA template in base ratio (Furth et al., 1961a, b; Weiss and Nakamoto, 1961a), base pair frequency and nearest neighbour frequency (Weiss and Nakamoto, 1961a, b; Hurwitz et al., 1962). It has also been shown, by hybridisation techniques, that the cRNA synthesized by E. coli polymerase (Green, 1964) or M. lysodeikticus polymerase (Geiduschek et al., 1961) contains base sequences complementary to those of the DNA template.

Green (1964) found that at least 66% of the cRNA synthesized on a T₄ DNA template by E. coli polymerase could be hybridised with T₄ DNA, and concluded from this and his other results that probably more than 90% of the in vitro synthesized cRNA is complementary to the sense strand of the DNA. This would show that virtually all the cRNA is composed of faithful copies of DNA sequences, however it does not indicate how much of the DNA is copied. T₄ mRNA synthesized in vivo during multiplication
of the phage can complete to 93% with the $T_4$ crNA synthesized in vitro (Green, 1964), indicating that the two have a very similar composition. This suggests strongly that the majority of the DNA sequences are transcribed into crNA.

Whether or not all the DNA sequences are transcribed considerable evidence has accumulated recently that not all sequences are transcribed to the same extent.

Geiduscheck et al. (1966) using $T_2$ and $T_4$ DNA templates and E. coli polymerase found that the in vitro crNA competed more than 90% with the "early" mRNA synthesized in vivo in the first 4 mins. after infection, for hybridisation sites on the corresponding DNA, but competed to a much lesser extent with the "late" mRNA. On the other hand Green (1964) also using E. coli polymerase and $T_4$ DNA claimed that the "late" mRNA synthesized 4-20 mins. after infection with $T_4$ competed 93% with in vitro crNA, whereas mRNA formed in the first 4 min. after infection competed to a lesser extent. The fact that the crNA appears to resemble "early" mRNA in one series of experiments and "late" mRNA in another may be an indication of the variability of the in vitro system. However both sets of results show that not all DNA cistrons are transcribed to the same extent by the in vitro system. In addition the possibility remains that some DNA cistrons are never transcribed in vitro.

Cohen et al. (1966) working with E. coli polymerase and $\lambda$ phage DNA—also found that transcription of DNA in vitro did not occur uniformly along the genome but that there was a preferential copying of the AT-rich region of the DNA.
Bremer, et al. (1966) using \( T_4 \) DNA as a template found that a certain proportion of the E. coli polymerase molecules were "early quitters" synthesizing only short RNA chains whereas "late quitters" formed RNA chains of average length 20 S. These short RNA chains would be expected to show lowered specificity in hybridisation reaction and might interfere with the hybridisation of the larger molecules. Fortunately they form only a small proportion of the cRNA. Bremer et al. (1966) with \( T_4 \) and Richardson (1966) with \( T_7 \) concluded from their results that phage DNA contains specific sites for the attachment of RNA polymerase. Such a mechanism might well direct selective transcription of the DNA in vitro.

How far these findings with phage DNA and E. coli polymerase, which copies only the sense strand of a native DNA template (Green 1964), can be applied to a system containing Drosophila DNA and M. lysodeikticus enzyme which copies both strands of the DNA in vitro (Geiduscheck et al., 1961; Geiduscheck et al., 1962) is not known. It has previously been shown that different DNAs vary widely in their priming ability for the polymerase reaction with E. coli enzyme (Chamberlin and Berg, 1962; Hurwitz et al. 1962) suggesting that there may be difficulties of attachment between enzyme and heterologous DNA. It has also been observed that mouse satellite DNA has a greater priming ability with E. coli polymerase than does the bulk of mouse DNA (W.G. Flamm; personal communication) suggesting that selective transcription may occur with heterogeneous DNA in vitro.

Since purified M. lysodeikticus polymerase transcribes both strands of a native DNA template in vitro (Geiduscheck et al., 1961;
Geiduschek et al., (1962), unlike E. coli polymerase or the living cell where only the sense strand of the DNA is transcribed (Tocchini-Valenti et al., 1963), the M. lysodeikticus enzyme must presumably attach to both strands of the DNA in vitro; that is it shows relaxed specificity in its binding to the DNA. It is of interest that if the DNA template is denatured the E. coli polymerase transcribes both strands (Green, 19614; Chamberlin et al., 1963) and selective transcription of the AT-rich region of λ phage DNA is abolished (Cohen et al., 1966). This suggests that it is not essential for the polymerase to attach to predetermined specific DNA sites for RNA synthesis to proceed in vitro.

The ideal method of proving that M. lysodeikticus polymerase transcribes all the DNA sequences into cRNA in vitro, would be to anneal the cRNA with its DNA template and show that 100% of the DNA was bound to cRNA in a cRNA-DNA hybrid, indicating that all the sequences present in the DNA were also represented in the cRNA. In one of the few comprehensive studies made on higher organisms Paul and Gilmour (1966a) used M. lysodeikticus polymerase to synthesize cRNA on a thymus DNA template. They annealed the thymus DNA with different amounts of the cRNA and found, by extrapolation, that at infinite cRNA concentration 45-50% of the DNA would be covered with cRNA in a cRNA-DNA hybrid. Since M. lysodeikticus polymerase copies both strands of the DNA 100% coverage would be expected if transcription were complete. Failure to obtain a higher level of coverage may be due to competing RNA-DNA interactions during annealing since the cRNA synthesized by M. lysodeikticus polymerase is self-complementary and hybridises with itself. The results of Paul and Gilmour (1966a) therefore indicate that M. lysodeikticus polymerase transcribes 50% or more of the
thymus DNA cistrons into cRNA.

From the limited evidence available at the moment we cannot assume that all the base sequences present in Drosophila DNA are transcribed into cRNA by *M. lysodeikticus* polymerase. Ideally we would like to measure the extent of transcription of Drosophila DNA by annealing a small amount of DNA with a large excess of cRNA and determining the maximum coverage of the DNA. With the hybridisation techniques presently available it is never certain that all the DNA used for the hybridisation is recovered at the end of the reaction. Consequently to obtain a quantitative measure of the DNA:cRNA ratio of the hybrid it is necessary to use labelled DNA. The preparation of labelled Drosophila DNA is a major technical problem which has not yet been solved. For this reason the fraction of Drosophila DNA transcribed into cRNA has not yet been fully established.

If only a selected class of Drosophila DNA cistrons are transcribed into cRNA and hence are available for hybridisation with heterologous DNAs then, as pointed out in the General Introduction, the values obtained cannot be used as a truly quantitative measure of genetic relatedness. However a low level of transcription might be tolerable for the present objective, which is to find a quantitative measure of differences in a population, provided that some of the divergent cistrons were transcribed.

Theoretically hybridisation levels may be measured either by annealing DNA with excess RNA or vice versa. However practical problems arise if the DNA-excess RNA hybridisation system is used.
For one thing to obtain a quantitative measure it is necessary to know the exact amount of DNA present in the hybrid and this requires labelled DNA. In addition if the transcription level is poor, say 3%, only 3% of the Drosophila DNA could be covered even under saturation conditions. Since we would expect an even smaller percentage of heterologous DNAs to be covered by Drosophila cRNA comparisons between Drosophila and other DNAs would be difficult without a rather high level of labelling of the cRNA.

Because of these problems it was decided to carry out the hybridisations by annealing a small amount of RNA with an excess of DNA. It seems clear from the results of numerous workers including Geiduschek et al. (1961), Tocchini-Valenti (1963), Green (1964), Cohen et al. (1966), Geiduschek et al. (1966) and Paul and Gilmour (1966) that virtually all of the cRNA synthesized under the direction of RNA polymerase consists of base sequences complementary to the template DNA, no matter what fraction of the total genome these sequences represent. If the level of hybridisation depends only on the number of complementary sequences shared by the DNA and cRNA it should therefore be possible to hybridise 100% of the Drosophila cRNA with an excess of Drosophila DNA. From a practical standpoint therefore small differences in hybridisation obtained under different conditions or with other DNAs should be relatively easy to detect.

Hybridisation of Drosophila cRNA with an excess of Drosophila DNA was therefore used to establish the optimal conditions for hybridisation before use as a test for genetic relatedness. If transcription is 100% and uniform throughout the genome the level
of hybridisation obtained with the cRNA and excess DNA may be used directly as a measure of relatedness. If, as seems possible, transcription of the DNA is selective then the interpretation of hybridisation values becomes more difficult. However the method may still be used to detect differences within a population provided that the DNA transcribed contains some of the differing sequences.

The aim of the second part of the programme was therefore to prepare cRNA on a Drosophila DNA template, establish the conditions giving maximum hybridisation of the cRNA with an excess of Drosophila DNA and then to see what level of genetic difference the cRNA-DNA hybridisation technique could detect.
METHODS II

Chemicals and Solutions

Nitrocellulose membranes (Schleicher and Scheuell) or millipore HA filters were used to trap the DNA-RNA hybrids.

The RNase-mix used to determine the RNase-resistance of the hybrids was prepared from the following stock solutions: 1) 5000 units T1 RNase (taka-diastase RNase: Calbiochem) was dissolved in 10 ml. 0.1 M NaCl. 2) pRNase (pancreatic ribonuclease: Sigma Chemical Co.) in 0.01 SSC pH 4.5 (100 μg. pRNase per ml.) was heated for 10 mins. at 100 °C. After cooling to room temperature the pH was adjusted to 7.5 by the addition of NaOH.

The two stock RNase solutions could be stored in the deep freeze. The final RNase-mix contained 0.6 ml. T1 RNase soln. + 5.0 ml. pRNase soln. + 4.4 ml. distilled water giving a concentration of 50 μg. pRNase + 300 units T1 RNase per ml. The RNase-mix could be stored in the refrigerator for several weeks without loss of activity. Before use in an experiment the RNase-mix was tested for activity by incubating 0.03 ml. RNase-mix + 0.1 ml. C14 RNA (2000 c.p.m./ml.) in 0.4 ml. 2 x SSC for 30 mins. at 37 °C. Control incubations without any RNase-mix were also set up. The C14 RNA was precipitated with TCA, the precipitates collected on oxoid filters and counted as described in Methods I.

Formalin-RNA was prepared by heating yeast sRNA (0.5 mg. sRNA per ml.) in 0.001 M phosphate buffer pH 7.0 containing 0.5% formalin for 7 hours at 80 °C in a water bath. The formalin treated RNA was then dialysed for 2 days in the cold against 3 changes of 0.001 M phosphate buffer. The formalin RNA was then
stored in the deep-freeze.

Toluene scintillation fluid contained: 5g. PPO + 0.3 g. POPOP in 1000 ml. A.R. toluene.

Dioxane scintillation fluid contained: 8.6 g. PPO, 70 mg. dimethyl POPOP, 88 g. naphthalene, 200 ml. xylene, 1000 ml. dioxane.

Preparation of Drosophila DNA

Drosophila DNA was isolated by the standard extraction procedure outlined in Part I. The concentration of DNA was determined from its U.V. absorption. All DNA samples were routinely assayed for RNA and RNase contamination. Freedom from RNA was ascertained by the correspondence of U.V. and diphenylamine determinations of the DNA. RNase activity was tested as follows: 0.05 ml. DNA solution was incubated with 1 ml. C\textsuperscript{14}RNA (2000 cpm/ml.) in a total volume of 0.4 ml. Control incubations in which the DNA solution was omitted were also set up. All samples were run in duplicate. The samples were incubated for 16 hours at 37\degree C, chilled in ice and precipitated together with 1 ml. carrier RNA (50 mg/ml.) in 5% TCA. The precipitates were filtered, washed and the C\textsuperscript{14}DNA counts determined as described in Methods I.

Any DNA preparations contaminated with RNA or RNase were rejected. In point of fact RNase contamination was never detected in any DNA sample and RNA contamination only rarely.

Preparation of Schistocerca DNA

Adult Schistocerca which had just moulted were obtained from Fox Biological Supply Co. 24 adults were anaesthetised with ether. The heads, legs, and wings were cut off with scissors and the gut
scraped out. The carcasses were immediately dropped into 150 ml. ice-cold TWEEN and homogenised in a pre-chilled Waring blender for 5 secs. at 'slow' speed. The homogenate was filtered through two layers of gauze and the residue returned to the blender with 40 ml. TWEEN, ground for a further 2 secs. and filtered again. The combined filtrates were centrifuged for 30 min. at 12,000 x g. in the Servall, the supernatant decanted and the "nuclear" pellet taken up in TWEEN. The DNA was isolated from the "nuclear" material according to the standard extraction procedure for Drosophila DNA (part I). The DNA was tested for contamination by RNA or RNase as described for Drosophila DNA and was found to be free of this contamination. The yield of purified DNA was 135 μg. DNA per adult.

Preparation of Aedes DNA

1) Culture of the mosquitoes. Approximately 20,000 eggs of Aedes aegyptii were donated by Professor Bertram of the London School of Tropical Medicine. The eggs were cultured in chlorine-free tap-water at 25°C. When the larvae emerged they were fed on Farex (Glaxo Ltd.). After pupation the pupae were collected in small dishes of chlorine-free tap-water and transferred to population cages at 27°C and 99% humidity. The adults emerged about 48 hrs. after pupation and were maintained on a sucrose solution spread on cotton-wool pads. When all the adults had emerged they were collected by transferring the cages to a deep-freeze for 10-15 mins. This immobilised the mosquitoes which were then shaken out of the cage onto a large sheet of paper and poured into a beaker packed in ice. 17 g. of Aedes aegyptii were obtained in this way.
ii) Extraction of Aedes DNA. The homogenisation of the mosquitoes and the extraction of the DNA from the "nuclear" pellet was carried out exactly as for Drosophila. This DNA was tested for contamination by RNA or DNase as described for Drosophila and found to be free from both.

Preparation of Drosophila C14cRNA

The RNA polymerase used for this reaction was prepared from spray-dried Micrococcus lysodeikticus (Cambrian Chemicals) according to the method of Nakamoto et al. (1964) and was the gift of Dr. J. Bishop.

Drosophila DNA in SSC was dialysed against 0.01 M NaCl + 0.1 tris pH 7.5 and brought to a final concentration of about 200 μg./ml. for use as primer in the polymerase reaction. The incubation mix for the synthesis of cRNA contained 100 mM tris pH 7.5, 2.5 mM MnCl₂, 1.6 mM spermidine, 0.8 mM GTP, CTP, and UTP, 0.4 mM C14ATP (sp. act 1.5 μCi/μM), 100 μg. DNA and 0.3 ml. (approx. 300 units) polymerase preparation in a final volume of 5 ml. The mixture was incubated at 30°C for 45 mins, chilled and brought to 0.1 M NaCl. It was then shaken for 30 mins. in the cold with an equal volume of water-saturated phenol, centrifuged for 10 mins. at 6000 x g. in the Servall, and the aqueous phase withdrawn. A large fraction of the DNA primer precipitates with the protein during this phenol treatment. A DNase step was not included to eliminate any remaining DNA since the primer consists of native DNA which does not enter into the hybridisation reaction. To free the cRNA from contaminating salts and mononucleotides the solution was passed through a Sephadex (G-25) column set up in the
cold room. The cRNA was eluted from the column in 1 M NaCl + 0.01 M tris pH 7.5 at a flow rate of 3 ml./min. and 2.5 ml. fractions were collected. The distribution of the counts was determined by taking 0.1 ml. aliquots from each fraction and counting on a Nuclear Chicago scintillation counter in dioxane counting fluid. The first peak of radioactivity eluting from the column contained the cRNA. The cRNA fractions were bulked and dialysed against distilled water in the cold room for 8 hrs. with vigorous stirring. The amount of cRNA present could be calculated from the counts incorporated or determined from its absorption at 260 μm. These two estimates were in good agreement indicating negligible contamination by DNA primer.

The cRNA was assayed for RNase sensitivity as follows: 1 ml. cRNA solution was incubated with 0.04 ml. RNase-mix (2 μg.pRNase + 12 units T1RNase) in 1 ml. 2 x SSC for 16 hours at 37°C. cRNA counts were determined by precipitation with carrier RNA and TCA as described in Methods (Part I). Control samples without RNase were kept in the deep freeze and precipitated as above.

Assay of RNA-DNA hybrids

1) Hybridisation with DNA immobilised on filters. This method is based on that of Gillespie and Spiegelman (1965). Both nitrocellulose membranes and millipore HA filters (25 mm. diam.) were found to be suitable for this technique. Drosophila DNA dissolved in 0.01 SSC at a concentration of about 40μg./ml. was denatured by heating at 96-98°C for 5 mins and quenched immediately by pouring onto ½ or ⅓ volume of frozen 12 x SSC or
18 x SSC to give a final concentration of 6 x SSC. The filters were first washed with 20 ml. of 6 x SSC under gentle suction on miniature Büchner Funnels and then the denatured DNA solution (final concentration about 6 μg./ml. in 6 x SSC) was poured through under suction. DNA retention was monitored by measuring the U.V. absorption of the denatured DNA solution before and after filtration (see section on DNA retention in Results). The filters were then washed with a further 20 ml. of 6 x SSC, air-dried overnight and then baked for 2 hrs. at 80°C under full vacuum. Hybridisation was carried out in scintillation vials, the discs being trimmed to lie flat at the bottom of the vial. Unless otherwise specified each vial contained 50 μg. denatured DNA immobilised on a filter + 0.25 μg. C_{14}cRNA in 0.5 ml. 6 x SSC. The vials were screwed tight and incubated for 16 hrs. at 63°C. The filters were then removed, drained and transferred to boiling tubes filled with 6 x SSC. The tubes were stoppered and placed in a revolving rack. This ensured adequate washing of both sides of the disc. The discs washed in this way for one hour with two changes of buffer. If the discs were to be RNased they were drained and transferred to clean vials and 0.08 ml. RNase-mix (4 μg. pRNase + 24 units T_{1}RNase) in a total volume of 1 ml. 6 x SSC added. The discs were incubated for 15 mins. at 37°C, chilled in ice, drained and washed as before for 2 hrs. with two changes of buffer. They were then dried in an oven at 60-80°C and counted on a Nuclear-Chicago scintillation counter in toluene scintillation fluid.
ii) Nygaard and Hall technique. Both nitrocellulose membranes and millipore HA filters were found to be suitable for this method which is based on that of Nygaard and Hall (1963). It was found that 30 nm. nitrocellulose membranes filtered on Stephi filters were more convenient if more than 50 µg. DNA were to be filtered since this gave a much larger surface area for the retention of DNA. *Drosophila* DNA at a concentration of 250 µg./ml. was heat-denatured for 5 mins. at 96-98°C in 0.1 SSC and quenched by pouring onto ½ or ⅓ volume frozen 12 x SSC or 18 x SSC to give a final concentration of 6 x SSC. Unless otherwise specified each incubation mix contained 50 µg. denatured DNA + 0.25 µg. ^{14}C^lRNA in 5 ml. 6 x SSC. Annealing was carried out in tightly stoppered vials at 63°C for 12-16 hrs. The annealed mixture was then disaggregated by the addition of 20 volumes of 6 x SSC (pre-warmed to 63°C) followed by incubation at 63°C for 1 hr. The vials were then cooled in ice and either filtered immediately or treated with RNase. For RNasing 8 ml. RNase-mix were added per 10 ml. of the disaggregated mixture (4 µg. pRNase + 24 units T RNase per ml.) and the tubes incubated for 20 min. at 37°C. The tubes were then cooled in ice and filtered immediately.

For filtration the discs were soaked briefly in 6 x SSC and then washed with 10 ml. of the same buffer under gentle suction. 100 µg. of formalin-treated yeast sRNA in 10 ml. 6 x SSC was filtered through each disc. This supposedly saturates all sites capable of binding RNA and reduces background noise. Pretreatment of the yeast RNA with formalin inhibits any subsequent binding of ^{14}C^lRNA from the annealing mixture to the yeast RNA. After the formalin-RNA treatment the discs were again washed with 10 ml.
6 x SSC. The hybridisation mixture was filtered under gentle suction, the incubation vessel rinsed with 2 ml. 6 x SSC and the washings added to the filter and the disc finally washed with 10 ml. 6 x SSC. The discs were dried in an oven at 60-80°C and counted in toluene scintillation fluid.

iii) Precipitation of RNase-resistant hybrid. The denaturation of the DNA and hybridisation were carried out exactly as for the Nygaard and Hall technique described above. After incubation for 16 hrs. at 63°C the tubes were cooled in ice and 0.1 ml. RNase-mix added to each 0.5 ml. incubation mixture (4 µg. pRNase + 24 units T1 RNase per ml.). The tubes were then incubated for 20 mins. at 37°C to hydrolyse all the free RNA present in the solution. The DNA together with the RNase-resistant RNA-DNA hybrid was then precipitated in the cold in 2 ml. 5% TCA in the presence of 500 µg. carrier RNA. The precipitates were collected on oxoid filters and washed as described for the determination of precipitated 3H RNA counts (Methods I). The filters were dried in the oven at 60-80°C and counted in toluene scintillation fluid.
RESULTS

Synthesis of Drosophila $^{14}C$RNA

$^{14}C$RNA was prepared as described in Methods II. Figures 14 and 15 show sucrose gradient analyses of the two different preparations of $^{14}C$RNA used in the subsequent experiments. It can be seen that there are considerable differences between the two preparations. $cRNA(\text{I})$ (Fig. 14) contains quite a high proportion of material sedimenting in the size range 18 S - 28 S. As a rough estimate the bulk of the $cRNA(\text{I})$ material appears to be in the 6 - 28 S size range. On the other hand $cRNA(\text{II})$ has very little material larger than 18 S and the bulk of the $cRNA$ appears in the lightest fractions of the gradient (Fig. 15).

The $cRNA$ preparations were assayed for RNase-resistance as described in Methods II. $cRNA(\text{I})$ was found to contain only 10% of RNA resistant to overnight digestion by RNase. $cRNA(\text{II})$ however contained 30% RNase-resistant RNA. The RNase-resistant fraction consists of double-stranded RNA formed during the polymerase reaction. The RNase-resistance may be abolished by first heating the RNA to 95°C for 10 mins. to melt the RNA and then quenching rapidly so that it remains single-stranded.

Experience has shown that prolonging the incubation period for the polymerase reaction results in a decrease in the size of the $cRNA$ and an increase in the percentage of RNase-resistant RNA. The differences between $cRNA(\text{I})$ and $cRNA(\text{II})$ cannot be attributed to this since the incubation period was the same for both. The variation probably arises from the fact that different DNA and different polymerase preparations were used for the two reactions.
Sucrose gradient analysis of Drosophila $^{14} \text{C}$ cRNA (I):

Drosophila $^{14} \text{C}$ cRNA(I) (approx. 500 c.p.m.) together with reticulocyte ribosomal RNA (12 O.D. units) as marker was dissolved in 2 ml. of buffer containing 0.1 M tris pH 7.5 + 0.1 M NaCl + 5% SLS. The RNA solution was then layered over a gradient of 15-30% sucrose in the same buffer. The gradient was centrifuged for 23 hrs. at 23,000 rpm. in Spinco No.25 rotor at 21°C. The tube was pierced and 1.5 ml. fractions were collected. $^{14} \text{C}$ RNA counts were determined as described in Methods I.
Fig. 2n
Sucrose gradient analysis of a second sample of *Drosophila* C14cRNA (II):
The sucrose gradient was set up and fractions analysed as in Fig. 14.
Fig. 15.
The cRNA(I) (specific activity 1000 c.p.m/μg.) preparation was used for the experiments to determine optimum hybridisation conditions and to study the specificity of the hybridisation while cRNA(II) (specific activity 6700 c.p.m/μg.) was used in an attempt to estimate the percentage transcription of the DNA.

Denaturation and filter retention of DNA

Both nitrocellulose membranes and millipore HA filters were used to trap DNA in the experiments described here. As a preliminary it was necessary to determine how well and under what conditions these filters retained DNA. Since no labelled DNA was available retention was estimated by measuring the U.V. absorption of the solution before and after filtration. In the first experiments it was found that nitrocellulose membranes frequently contained a U.V. absorbing material (0.05 - 0.15 O.D. per ml. at 260 μm) which interfered with the determinations and that the level of this U.V. absorption varied from filter to filter within a batch. Even washing the membranes by first filtering 20-40 ml. of 6 x SSC through them did not completely remove this contaminant.

Millipore H.A. filters were also found to contain some U.V. contaminating material but this was present in only small amounts (0.005 - 0.02 O.D. per ml. at 260 μm) and this was more or less constant for all filters in a given batch. For this reason the experiments on DNA retention were carried out on millipore HA filters and this type of filter was used for all other experiments in which it was essential to check the retention of the DNA.

Table IV shows the percent retention of DNA in 6 x SSC by millipore filters. DNA heated in 0.01 SSC for five to twelve
Table IV

Retention of denatured Drosophila DNA by millipore filters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.01 SSC</td>
</tr>
<tr>
<td>Heated for 5 min.</td>
<td>87</td>
</tr>
<tr>
<td>Heated for 5 min (annealed)</td>
<td>90</td>
</tr>
<tr>
<td>Heated for 12 min.</td>
<td>89</td>
</tr>
<tr>
<td>Heated for 12 min. (annealed)</td>
<td>86</td>
</tr>
</tbody>
</table>

Millipore HA filters were first soaked in 6 x SSC and then washed on miniature buchner funnels with 30 ml. 6 x SSC. A test sample of 4 ml. 6 x SSC was then poured through each filter and the O.D. at 260 μm measured to determine the U.V. contribution of any contaminating material present in the filter.

Drosophila DNA at a concentration of 40 μg/ml. in -0.01, -1 or 6 x SSC was denatured by heating for 5 or 12 minutes at 97-98°C in a water-bath. The samples were then quenched by pouring onto an equal volume of frozen buffer, 12 x SSC in the case of the samples heated in -0.01 or -1 SSC and 6 x SSC for the samples denatured in 6 x SSC. The solutions were then adjusted to a final concentration of 8 μg. DNA per ml. in 6 x SSC. The O.D. 260 of each sample was determined and 4 ml. aliquots were filtered through the washed millipore filters under gentle suction, the filtrates collected and their O.D. 260 determined. Other 4 ml. aliquots were annealed for 16 hours at 60°C prior to filtration to assess the effect of annealing on the retention of DNA. All determinations were carried out in duplicate. The difference between the input O.D. and the O.D. of the filtrate (less any U.V. contamination from the filter) gave the measure of the DNA retained by the filter.
minutes is retained to a level of 85-90%. Although the values of the annealed DNA are slightly higher, the difference in retention of annealed and non-annealed DNA is probably not significant. Heating for 12 minutes in 0.1 SSC also gives good retention (about 80%) but DNA heated in 6 x SSC is only retained 70-80%. This is probably due to the incomplete denaturation of the DNA when heated at high ionic strength. For the subsequent hybridisation experiments therefore Drosophila DNA was denatured by heating in 0.01 SSC for 5 minutes at 96-98°C. Other experiments, using nitrocellulose membranes selected for their low background U.V. absorption, showed that heat-denatured DNA was retained to 85-90% on these membranes.

Drosophila DNA could also be denatured by treatment at high pH (Gillespie and Spiegelman, 1965). Drosophila DNA in 0.01 SSC was made up to 1 N in NaOH (pH 13). After standing for 10 mins. at room temperature the DNA solution was brought to pH 7.0 with citric acid. pH-denatured Drosophila DNA showed 90% retention on membranes and millipore filters.

It was also necessary to find suitable conditions for the denaturation of Schistocerca and Aedes DNA. Table V shows that after heating for 10 mins. at 96-98°C in 0.1 SSC both Schistocerca and Aedes DNA are retained to about 95%. Annealing of the DNA at 65°C does not significantly affect retention. In subsequent experiments Aedes and Schistocerca DNA were heat denatured in 0.1 SSC.

T4 DNA (gift of Dr. F.W. Robertson) which was used as a control in most of the hybridisation experiments was denatured either by heating for 10 mins. at 98°C in 0.01 M NaCl + 0.01 M
Table V
Retention of denatured DNA from Aedes and Schistocerca by millipore filters

<table>
<thead>
<tr>
<th></th>
<th>Aedes</th>
<th>Schistocerca</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-annealed</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>annealed</td>
<td>94</td>
<td>97</td>
</tr>
</tbody>
</table>

Millipore HA filters were washed in 6 x SSC and their U.V. contribution determined as described in Table IV. Aedes and Schistocerca DNA, each at a concentration of 30 µg./ml. in 1 SSC were heated for 10 mins. at 98°C in a water-bath. They were then quenched by pouring onto an equal volume of frozen 12 x SSC. The concentrations were adjusted to 7.5 µg. DNA per ml. in 6 x SSC. Some samples were filtered directly while others were annealed for 16 hours at 60°C before filtration. The O.D.\textsuperscript{260} of the DNA solutions was measured and 4 ml. aliquots were filtered through the filters. The filtrates were collected and the O.D.\textsuperscript{260} determined. DNA retention was then calculated as in Table IV.
tris pH 7.5 and quenched by pouring onto a half volume of frozen 12 x SSC (F.W. Robertson: personal communication) or by exposure to pH 13 as described above. Both pH-denatured and heat-denatured T₄ DNA showed 85-90% retention on membrane and millipore filters.

**Assay of hybrid**

Initially it was decided to use the method of Gillespie and Spiegelman (1965) in which the DNA is immobilized on filters by vacuum drying prior to hybridisation with RNA. This method appeared to have a number of advantages over hybridisation in free solution. Since very high DNA:RNA ratios were being used it was feared that DNA-DNA interaction might interfere with the DNA-RNA duplex formation and seriously reduce the level of hybridisation. With the Gillespie and Spiegelman technique the DNA is immobilised on the filter and therefore DNA-DNA interactions should be eliminated.

The Gillespie-Spiegelman method relies on treatment with RNase to distinguish genuine hybridisation from spurious base-pairing since true RNA-DNA hybrids have been shown to be resistant to RNase (Yankofsky and Spiegelman, 1962a, b). To find a suitable level of RNase for the identification of *Drosophila* DNA-RNA hybrids DNA-carrying filters were prepared as described in Methods II and incubated with C¹⁴cRNA(I). The discs were drained, washed and incubated in 6 x SSC together with different quantities of RNase-mix, and then washed and counted (Figure 16). In the absence of RNase a large percentage of the RNA appeared to be hybridised. However treatment with RNase showed that much of this was RNase-sensitive and therefore represented spurious base-pairing. The RNA counts decreased rapidly with increasing RNase concentration and finally reached a plateau level of 9-10% input-
Fig. 16.

RNase resistance of DNA-cRNA hybrid:

Hybridisation was carried out by the method of Gillespie and Spiegelman as described in Methods II. Denatured Drosophila DNA in 6 x SSC was filtered and dried on nitrocellulose membranes (25 μg. DNA per membrane) and each membrane incubated with *25 μg cRNA(I) in *5 ml. 6 x SSC for 16 hrs. at 63°C. The membranes were washed as described in Methods II and incubated with different concentrations of RNase-mix in 1 ml. 6 x SSC for 15 mins. at 37°C. All samples were run in duplicate. After incubation the membranes were washed and counted as described in Methods II. RNase concentration is expressed in terms of pRNase present per ml. (The original RNase-mix contains 50 μg. pRNase + 300 units T1 RNase per ml.)
Fig. 16.
RNA bound at about 0.08 ml. RNase-mix per ml. incubation medium (4 μg. pRNase + 24 units T₁ RNase/ml.).

In this experiment the RNase treatment was carried out in 6 x SSC. The stability of RNA-DNA hybrids towards RNase is increased by high ionic strength. Thus Hayashi et al. (1965) found that φ x 174 RNA-DNA hybrid was sensitive to 5 μg. RNase per ml. in 0.1 M NaCl but resistant to 30 μg. RNase per ml. in 0.3 M NaCl. Gillespie and Spiegelman (1965) recommended that RNase treatment should be carried out in 2 x SSC rather than 6 x SSC since the latter gave higher levels of background noise. However even in 6 x SSC the background noise represented only about 0.008% of the RNA input (compared to 0.003% in 2 x SSC), consequently at the low RNA inputs used in the present experiments this level of contamination is unimportant. Furthermore the fact that a plateau value is attained under the conditions employed here, and that further increases in RNase concentration do not affect the amount of hybrid recovered, indicates that RNase treatment in 6 x SSC yields a true RNase-resistant RNA-DNA hybrid.

On the basis of these findings RNase-resistant hybrids were assayed by incubating for 15 mins. at 37°C in 6 x SSC at an RNase concentration of 0.08 ml. RNase-mix (4 μg. pRNase + 24 units T₁ RNase) per ml.

Since disappointingly low levels of hybridisation were obtained in preliminary experiments with DNA immobilised on membranes it was decided to compare the efficiency of this method with hybridisation in free solution. Two series of membranes containing DNA immobilised according to the method of Gillespie
and Spiegelman were set up and annealed with cRNA. After
hybridisation one series was RNased while the other was not RNased
but was washed repeatedly with 6 x SSC. The hybridisation in free
solution was measured according to the method of Nygaard and Hall
(1963) or by precipitating the RNase-resistant RNA-DNA hybrid.
Controls containing T₄ DNA instead of Drosophila DNA were set up
for all treatments to monitor background noise. All incubations
were carried out in duplicate under identical conditions of DNA:RNA
ratio and total volume. The duplicate measurements were in good
agreement (within 5% of each other). The results are shown in
Table VI. T₄ DNA immobilised on membranes formed virtually no
RNase-resistant RNA-DNA hybrid with Drosophila cRNA. However
where the RNase step was omitted a considerable amount of non-
specific binding occurred between T₄ DNA and Drosophila cRNA.
This confirms that the RNase treatment is essential for detecting
specific RNA-DNA hybrid formation by the Gillespie-Spiegelman
technique. Drosophila DNA immobilised on filters bound some
10% of the cRNA as RNase-resistant hybrid. An apparently larger
percentage was bound in the non-RNased Drosophila series but if the
T₄ control value was subtracted it was found that the percentage of
RNA bound which could be attributed to specific Drosophila DNA-RNA
hybrid is about that measured in the RNased series.

In the samples where hybrid formation was measured by
precipitation of the RNase-resistant counts the percentage of RNA
recovered in the T₄ DNA controls was very high. This high back-
ground was largely due to the double-stranded RNase-resistant cRNA
present in the incubation mix which amounted to some 10% of the
total cRNA. The percentage of cRNA incorporated into specific
Table VI
Comparison of techniques for the estimation of DNA-RNA hybrids

<table>
<thead>
<tr>
<th>Assay method</th>
<th>% RNA retained</th>
<th>% RNA hybridised</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA annealed with DNA in free solution:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Nygaard &amp; Hall technique</td>
<td>29.9</td>
<td>1.9</td>
</tr>
<tr>
<td>b) Precipitation of RNase-resistant counts</td>
<td>35</td>
<td>19.3</td>
</tr>
<tr>
<td>RNA annealed with DNA immobilised on filters:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) No RNase treatment</td>
<td>21.2</td>
<td>12.6</td>
</tr>
<tr>
<td>d) with RNase treatment</td>
<td>13.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

All incubations were carried out under identical conditions of time, temperature and concentration. Each determination was carried out in duplicate and control incubations in which $T_4$ DNA replaced the Drosophila DNA were included for all determinations. Millipore filters were used throughout. Drosophila DNA was heat-denatured as described in Methods II. $T_4$ DNA was heat-denatured as described in the section on denaturation and filter-retention of DNA.

a), b): 50 µg. denatured Drosophila or $T_4$ DNA was incubated with 25 µg. cRNA(I) in 5 ml. 6 x SSC for 16 hours at 63°C. The amount of hybrid formed was assayed by the Nygaard and Hall technique or by precipitation of the RNase-resistant material as described in Methods II.

c), d): denatured Drosophila or $T_4$ DNA was immobilised on millipore filters as described in Methods II. Each filter, containing 50 µg. DNA was incubated with 25 µg. cRNA(I) in 5 ml. 6 x SSC for 16 hours at 63°C. The filters were then divided into two groups. One group was washed in 6 x SSC while the other group was subjected to RNase treatment (Methods II) prior to counting.
RNase-resistant *Drosophila* DNA-RNA hybrid, calculated by subtraction of the $T_4$ DNA control value was significantly higher than that measured with DNA immobilised on filters (15% compared to 10%). However the high background reduces the accuracy of this method.

When *Drosophila* DNA and cRNA were annealed in free solution and the hybrid assayed according to Nygaard and Hall (1963) a much higher percentage of the cRNA was hybridised. In this case the $T_4$ DNA control value was very low even without RNase treatment. It may be considered that the difference in the percentage of RNA bound to *Drosophila* DNA and that bound to $T_4$ DNA, measured by the Nygaard and Hall technique, represents a specific *Drosophila* DNA-RNA hybrid and in this case contains some 28% of the input cRNA. On this basis the amount of RNA hybridised by the Nygaard and Hall method is more than twice that obtained in the Gillespie and Spiegelman system for identical DNA:RNA ratios.

Most of the RNA bound to DNA as measured by the Nygaard and Hall method is present as RNase-resistant hybrid (Table VII). cRNA was annealed with DNA in free solution or immobilised on discs and one of the free solution series was treated with RNase. Again $T_4$ controls were included for all treatments. Gillespie and Spiegelman (1965) claimed that treatment of the annealing mixture with RNase before filtration led to retention of RNA counts adsorbed on the RNase. However there was no evidence of this in our experiments for either nitrocellulose membranes or millipore filters. The discrepancy is probably due to the fact that Gillespie and Spiegelman (1965) were using very much higher levels of radio-active RNA than those used in the experiments described
Table VII

Comparison of methods for the assay of specific RNA-DNA hybrids

<table>
<thead>
<tr>
<th>Method Description</th>
<th>% RNA retained</th>
<th>% RNA control</th>
<th>% RNA hybridised</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Nygaard &amp; Hall technique: No RNase step</td>
<td>21.8</td>
<td>1.3</td>
<td>20.5</td>
</tr>
<tr>
<td>b) Nygaard &amp; Hall technique: with RNase step</td>
<td>19.4</td>
<td>0.7</td>
<td>18.7</td>
</tr>
<tr>
<td>c) Hybridisation with DNA immobilised on filters</td>
<td>10.1</td>
<td>1.0</td>
<td>9.1</td>
</tr>
<tr>
<td>(including RNase step)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All samples were incubated under identical conditions of time, temperature and concentration. T₄ DNA controls were included for all determinations and all determinations were run in duplicate. Millipore filters were used throughout.

a) 50 µg. of Drosophila or T₄ DNA, denatured as described in Table VI, was incubated with 29 µg. cRNA(I) in 5 ml. 6 x SSC for 16 hours at 63°C and the hybrid assayed by the Nygaard and Hall technique as described in Methods II.

b) Incubation as in a) but after disaggregation the annealing mixture was treated with RNase (see Methods II) prior to assay by the Nygaard and Hall technique.

c) Denatured Drosophila or T₄ DNA was immobilised on millipore filters. Each filter containing 50 µg. of DNA was incubated with 29 µg. cRNA(I) in 5 ml. 6 x SSC for 16 hours at 63°C. After annealing the filters were washed and RNased (see Methods II) before counting.
here. As can be seen (Table VII) the retention of RNA counts in the T4 DNA control was extremely small whether treated with RNase or not. The cRNA-DNA hybrid measured by the Nygaard and Hall technique was 90% RNase-resistant. It therefore seems that T4 DNA provides a valid control for measurement of background noise and that only a small proportion of the RNA retained by the DNA in the Nygaard and Hall method is spuriously bound and may be removed by RNase digestion. Taking the value of the RNased samples as the most accurate measure of true hybrid formation it can be seen that the percentage of cRNA hybridised to DNA in free solution is twice that for DNA immobilised on filters. This apparently is in conflict with Gillespie and Spiegelman's claim that their immobilised DNA technique gives comparable results to hybridisation in free solution. Some possible explanations of this discrepancy will be considered in the Discussion. From a practical standpoint it was decided to use the Nygaard and Hall technique for subsequent measurements since this gave the highest and most accurate measure of hybrid formation.

Effect of DNA:RNA ratio on the percentage of cRNA bound

Figure 17 shows the effect of the DNA:RNA ratio on hybrid formation measured by the Nygaard and Hall technique. The amount of cRNA bound increased with increasing DNA:RNA ratio reaching a level of about 28% at a DNA:RNA ratio of 300:1. When the results in Fig. 17 were plotted as reciprocals

\[ y = \frac{\text{input RNA}}{\text{bound RNA}}, x = \frac{\text{RNA} \times 1000}{\text{DNA}} \]

the regression \( y = 0.341x + 3.62 \) could be calculated. It follows that when \( x = 0 \) \( y = 3.62 \), i.e. at infinitely high DNA:RNA ratios 28% of the cRNA may be bound.
Effect of DNA:RNA ratio on the percentage of cRNA hybridised.

Hybridisation carried out in free solution (method of Nygaard and Hall). For each sample denatured Drosophila DNA at the appropriate concentration was incubated with 0.25 μg. cRNA(I) in 5 ml. 6 x SSC for 16 hours at 63°C. All incubations were run in duplicate. Controls containing corresponding amounts of T₄ DNA were set up for all DNA concentrations and incubated with cRNA(I) under the same conditions. The samples were disaggregated, filtered onto 30 mm. nitrocellulose membranes and washed as described in Methods II. The counts retained by the T₄ DNA were very low and remained constant for all DNA:RNA ratios. The counts retained by the Drosophila DNA less those of the T₄ DNA controls were used to calculate the percentage of cRNA hybridised.
Fig. 17.
Thus the DNA:RNA ratios commonly used in the experiments described here are sufficiently high to give a level of hybridisation approaching the maximum which cannot be significantly improved by further increases in DNA concentration.

**Effect of time and temperature of incubation**

Figure 15 shows that the hybridisation level cannot be improved by varying the time or temperature of incubation. At all three temperatures studied the percentage of RNA bound reached a maximum within six hours, and then levelled off. There is some evidence of a decrease in the percentage of RNA hybridised after 14 hours incubation at 63° and 70° but this is not significant. The levels of hybridisation attained after incubation at 50° or 63° were virtually identical, however incubation at 70°C seems to be deleterious.

**Percentage transcription of the DNA**

Experiments were carried out to determine what percentage of the *Drosophila* DNA was transcribed into cRNA by the *M. lysodeikticus* polymerase. The aim of the experiment is to discover what percentage of the DNA may be covered by the cRNA in an RNA-DNA hybrid. A small amount of DNA was incubated with an excess of cRNA and the hybrid recovered by the method of Nygaard and Hall. Since labelled DNA was not available the exact amount of DNA retained on the filters is not known. A 100% retention was assumed. If retention is considerably less than this then the value obtained for coverage of the DNA will be too low. The results are shown in Table VIII. It can be seen that at the
Fig. 12.

Effect of time and temperature of incubation upon DNA–cRNA hybrid formation:

Hybridisation was assayed by the method of Nygaard and Hall with millipore HA filters. Samples containing 50 μg. denatured *Drosophila* DNA and 0.2 μg. cRNA (I) in 5 ml. 6 x SSC were incubated for the times and temperatures indicated in the figure. *T₄* DNA controls were set up and all incubations were run in duplicate. The DNA was disaggregated, filtered and counted as described in Methods II.
Fig. 18.
Table VIII

Percent coverage of Drosophila DNA by cRNA(II)

<table>
<thead>
<tr>
<th>µg. cRNA per incubation</th>
<th>cRNA:DNA ratio</th>
<th>µg. cRNA bound</th>
<th>% coverage of DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3</td>
<td>6.9:1</td>
<td>0.245</td>
<td>3.97</td>
</tr>
<tr>
<td>8.6</td>
<td>13.8:1</td>
<td>0.322</td>
<td>5.19</td>
</tr>
<tr>
<td>13.0</td>
<td>20.8:1</td>
<td>0.405</td>
<td>6.53</td>
</tr>
</tbody>
</table>

cRNA(II) was heated for 10 mins. at 95°C in SSC to denature the double-stranded RNA and quenched by rapid cooling in ice. Drosophila DNA and T₄ DNA were pH denatured according to the method of Gillespie and Spiegelman (1965). The hybridisation was assayed by the Nygaard and Hall technique (Methods II) using nitrocellulose membranes.

0.625 µg. of denatured Drosophila DNA was incubated with cRNA(II) in the amounts specified above in a final volume of 0.34 ml. 2 x SSC. Incubation was for 16 hrs. at 63°C. Controls containing 0.625 µg. denatured T₄ DNA were set up for all cRNA(II) concentrations and all determinations were run in duplicate. The samples were RNased and filtered as described in Methods II. After this treatment no counts were recovered in the T₄ controls so that the µg. cRNA bound could be calculated directly from the counts retained by the Drosophila DNA.
highest cRNA:DNA ratio employed only 6-7% of the DNA was hybridised with RNA. This is clearly a minimum value since the percentage of DNA covered by cRNA increases linearly with increasing cRNA input and shows no signs of levelling off at the highest RNA:DNA ratio used. It seems almost certain that at still higher RNA:DNA ratios a larger percentage of the DNA could be covered.

Specificity of DNA-cRNA hybridisation

The ultimate aim of this research is to develop a technique suitable for studying genetic variation within and between species. To obtain some idea of the level of specificity of the DNA-cRNA hybridisation reaction DNA prepared from various different organisms was annealed with Drosophila $^{14}C$ cRNA. The following were selected as examples of organisms unrelated to Drosophila: $T_4$ (bacteriophage), Xenopus (Amphibia) and Rat (Mammalia). Aedes and Schistocerca were chosen for comparison within the Class Insects. Schistocerca belongs to the Order Orthoptera whereas both Drosophila and Aedes are members of the Order Diptera. Aedes, however, belongs to the sub-order Nematocera while Drosophila belongs to the sub-order Cyclorrhapha.

DNA from these organisms was annealed with Drosophila $^{14}C$ cRNA under identical conditions of time and temperature. One series was treated with RNase to determine how much of the RNA bound was present as specific RNase-resistant RNA-DNA hybrid. The results are shown in Table IX. If the amount of RNA bound by $T_4$ DNA in the non-RNased series is taken as a measure of background we see that 24.4% of the cRNA hybridised with Drosophila DNA. However in the case of all other DNAs very little RNA was hybridised,
### Table IX

**Specificity of DNA-cRNA hybridisation**

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Non-RNased series</th>
<th>RNased series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% RNA bound</td>
<td>% RNA hybridised (less T₄ control)</td>
</tr>
<tr>
<td>Drosophila</td>
<td>29.2%</td>
<td>24.4%</td>
</tr>
<tr>
<td>Aedes</td>
<td>6.7%</td>
<td>1.9%</td>
</tr>
<tr>
<td>Schistocerca</td>
<td>7.3%</td>
<td>2.5%</td>
</tr>
<tr>
<td>Xenopus</td>
<td>8.1%</td>
<td>3.6%</td>
</tr>
<tr>
<td>Rat</td>
<td>8.4%</td>
<td></td>
</tr>
<tr>
<td>T₄</td>
<td>4.8%</td>
<td>0%</td>
</tr>
</tbody>
</table>

The DNAs used in this experiment were heat-denatured as follows:
- Drosophila DNA was heated for 5 mins. in 0.01 SSC at 98°C.
- Aedes and Schistocerca DNA were heated for 10 mins. in 0.1 SSC at 98°C.
- Xenopus DNA (gift of Dr. Max Birnstiel) was heated for 5 mins. in 0.1 SSC at 98°C.
- Rat DNA (gift of Dr. Lula Melli) was heated for 10 mins. in 0.1 SSC at 98°C.
- T₄ DNA (gift of Dr. Forbes Robertson) was heated for 10 mins. in 0.01 M NaCl + 0.01 M tris pH 7.5 at 98°C.

In the last three cases the conditions for heat-denaturation of the DNA were those recommended by the donors and have been shown by them to result in 85-90% retention of the DNA by the filters.

All samples were quenched by pouring onto an equal volume of frozen 12 x SSC.

Each incubation mixture contained 50 µg. denatured DNA + 25 µg. cRNA(I) in 0.5 ml. x 6 SSC. All samples were incubated for 12 hours at 63°C. After disaggregation one series was assayed immediately by the Nygaard and Hall technique while a second series was RNased (see Methods II) prior to assay. All determinations are the average of two duplicates.
amounting to less than 15% of the value obtained with Drosophila DNA. When we study the RNased series we find that the Drosophila DNA-cRNA hybrid was approximately 80% RNase-resistant and some 19.4% of the cRNA input was bound in a specific DNA-cRNA hybrid. Virtually no RNase-resistant RNA-DNA hybrid was formed with DNA from any other organism. Even DNA from Aedes, which is the species most closely related to Drosophila, did not bind any significant amount of Drosophila cRNA.

These results confirm the very specific nature of the hybridisation of Drosophila DNA with Drosophila cRNA. This leads one to the hope that cRNA-DNA hybrid formation may be specific even at the species or sub-species level and that the method may therefore be extended to look at differences within a population of Drosophila.
DISCUSSION

In Part I the problems of extracting the DNA from Drosophila were listed, namely (1) release of DNA from the exoskeleton, (2) the potent DNase, (3) high RNA:DNA ratio and (4) difficulties of deproteinisation. It was found that virtually complete release of DNA-containing material from the exoskeleton could be obtained by stirring in a solution of Tween 80.

Sedimentation of a "nuclear" fraction effected a purification of the DNA from RNA, particularly ribosomal RNA, reducing the RNA:DNA ratio from roughly 10:1 to 2:1. Further purification from RNA was dependent on RNase treatment and the removal of glycogen which adsorbs nucleotides. Degradation by RNase could be reduced during preparation of the "nuclear" fraction by the presence of EDTA and also by maintaining the pH at about 6.3. Thereafter the activity of the DNase was destroyed by heat treatment. The most difficult problem was that of deproteinisation. The most effective method was to dissociate the DNA from protein in high salt and at high pH and to precipitate the protein by shaking with chloroform/isooamy alcohol mixture.

The DNA obtained by the standard extraction procedure which incorporated these techniques was of high purity. It was shown to have a $T_m$ of 70°C in 0.1 SSC, a density of $1.698 \pm 1$ g.cm$^{-3}$ corresponding to a G-C content of 40-42%. It is therefore comparable to Drosophila DNA prepared by other techniques. In addition the DNA prepared by the method developed here appears to be larger than Drosophila DNA obtained by other methods having an $S_{20,w}^0$ of 30-56 S equivalent to a molecular weight of approx.
$45 \times 10^6$ to $110 \times 10^6$ daltons. Hitherto the highest S value reported for *Drosophila* DNA is approximately 18 S obtained by Hastings and Kirby (1965).

This purified *Drosophila* DNA functioned effectively as a primer for the synthesis of cRNA by *M. lysodeikticus* polymerase. cRNA of up to 28 S in size has been obtained although the cRNA product is variable, both in regard to the size distribution of the RNA molecules and to the content of double-stranded RNA. As yet there has been no investigation as to how far the size of the RNA molecules affects the hybridisation reaction.

The *Drosophila* cRNA was used to study the hybridisation reaction with *Drosophila* DNA. In the introduction to Part II it was pointed out that if the level of hybridisation depended only on the number of complementary sequences held in common by the DNA and RNA than by annealing cRNA with an excess of DNA we would expect 100% hybridisation of the cRNA. (In the present case since 10% of the cRNA is double-stranded a 90% hybridisation level might be anticipated). In fact a maximum of 28% of cRNA was bound after annealing at a DNA-RNA ratio of 300:1. By plotting the results of Figure 17 as reciprocals and calculating the regression, it may be calculated that an infinitely high DNA:RNA ratio the maximum percentage of RNA which could be bound is about 28%. Thus further increases in the DNA:RNA ratio will not improve the hybridisation level. Changes in the annealing temperature or length of incubation did not improve the level of hybridisation.

A possible explanation of the comparatively low level of hybridisation in free solution is that with the large excess of DNA commonly used (DNA:RNA 200:1) DNA-DNA interactions seriously
compete with the DNA-RNA hybridisation. Because such a
competition was anticipated the first experiments were carried out
using the Gillespie-Spiegelman technique in which the DNA is
immobilised on membranes and therefore DNA-DNA interactions are
eliminated during incubation with RNA. However it was found that
for the same DNA:RNA ratio the level of hybridisation was markedly
lower with immobilised DNA compared with DNA in free solution.

A possible explanation for the lower levels of hybridisation
obtained with immobilised DNA is that a large proportion of the
DNA was eluted from the filters during the incubation and washing
steps. Attempts to measure the DNA remaining on the discs after
these steps by hydrolysis and U.V. or diphenylamine determinations
were unsuccessful. For an accurate determination of DNA
retention it is necessary to use labelled DNA. While the
possibility cannot be dismissed that leaching of the DNA was
a contributory factor to the lower level of hybridisation observed
with immobilised DNA it must be pointed out that since DNA in
free solution binds twice as much cRNA as the immobilised DNA it
follows that in the latter case at least 50% of the DNA must
have been eluted, and this seems excessive. An alternative
possibility is that steric hindrance occurs if very large quantities
of DNA are loaded onto the membranes so that the cRNA in solution
cannot penetrate to the DNA strands embedded deep in the filter.

Bolton and McCarthy (1962) attributed the lower efficiency of
hybridisation of DNA embedded in nitrocellulose columns compared
to DNA embedded in agar as being due to the closer packing of the
DNA in the nitrocellulose. Another possibility is that because
the DNA trapped on the membranes prior to drying is at very high concentration (approximately 1 mg. per ml. in these experiments and increasing as the membrane dries) considerable non-specific "pairing" occurs between the strands even at room temperature. When the DNA is immobilised by vacuum drying these paired strands will also be immobilised as non-specific aggregates and will therefore not be available for hybridisation with cRNA. For the present the reason for the difference between the results obtained with immobilised DNA and DNA in free solution must remain a matter of speculation. From a practical standpoint hybridisation in free solution was chosen for subsequent experiments since genuinely higher levels of hybridisation were obtained with this method.

Present evidence suggests that DNA-DNA hybridisation does not occur to any significant extent in heterogeneous DNA of high molecular weight in free solution. Thus the Carnegie Institute Group (Bolton et al., 1965) found little evidence of renaturation occurring in denatured high molecular weight mouse DNA (approximately $10^7$ molecular weight) after incubation for 18 hours at 60°C. Thus in their experiments the melting curve of the high molecular weight DNA after annealing was very broad and shallow with a low hyperchromicity. In addition the density of the annealed high molecular weight DNA resembled that of denatured DNA (in contrast to DNA fragments ($5 \times 10^6$ molecular weight) which exhibit a density intermediate between native and denatured DNA after annealing). Furthermore, this group claimed they could bind 60% of denatured mouse DNA fragments to DNA networks formed by preannealing high molecular weight DNA in free solution. Since
the Drosophila DNA used here is of high molecular weight ($45 \times 10^6$ to $110 \times 10^6$ daltons) it seems probable that DNA-DNA hybridisation is relatively insignificant and should not interfere with RNA-DNA hybrid formation in free solution. Thus failure to bind more than 28% of the cRNA cannot be attributed to competing DNA-DNA interactions.

RNA-DNA hybridisation and DNA-DNA hybridisation are essentially similar reactions and proceed at the same rate under similar conditions (Nygaard and Hall, 1964). It is therefore legitimate to compare the results obtained from the Drosophila cRNA-DNA hybridisation with those obtained by other workers studying DNA-DNA hybridisation as well as with other DNA-RNA interactions. If we look at the maximum hybridisation obtained in other systems we find that under optimum conditions bacteriophage T$_2$ DNA will bind 72% of T$_2$ RNA (Nygaard and Hall, 1964); E. coli DNA-agar will bind a maximum of 30% E. coli pulse-labelled RNA and 40% (McCarthy and Bolton, 1963) or 50% (McLaren and Walker, 1965) E. coli DNA fragments. Hoyer et al. (1964) reported that at ratios of mouse DNA-in-agar:mouse DNA fragments of 100:1 or more a maximum of 25% of mouse DNA fragments was bound. Similarly McLaren and Walker (1965) found that at ratios of DNA-in-agar:DNA fragments of 10:1 or more a maximum of 30% of mouse DNA fragments could be bound by mouse DNA-agar. The level of hybridisation obtained with Drosophila is therefore comparable to the mouse-mouse system. The findings of Hoyer et al. (1964) and McLaren and Walker (1965), namely that above a minimum critical level changes in the ratio of DNA-in-agar:DNA fragments do not affect the percentage binding of DNA fragments is in accord with
the calculation made here that increasing the DNA:RNA ratio above 300:1 does not improve the binding of cRNA.

The maximum level of hybridisation obtained in the above experiments seems to be related to the heterogeneity of the DNA. Nygaard and Hall (1964) pointed out that the rate of hybridisation is governed by the concentration of one complementary sequence; thus the binding of T2 RNA by T2 DNA represents $10^5$ separate reactions. Hybridisation of bacterial DNA (approximately 1000 times more complex than phage) proceeds more slowly than the phage interaction, but mammalian DNA renatures more rapidly than would be expected if it were completely heterogeneous (Bolton et al., 1965). The Carnegie Institute Group take the view that this unexpectedly rapid renaturation is due to the presence of reiterated sequences within the genome of higher organisms and that only these reiterated sequences enter into the hybridisation reaction. Thus the level of hybridisation obtained with mouse is inherent to the complexity of the DNA. The same may well be true of Drosophila. The Drosophila genome ($10^9$ nucleotides per cell: Ritossa and Spiegelman, 1965) is less complex than the mouse genome ($10^{10}$ nucleotides per cell: Bolton et al., 1965) but much more complex than the bacterial genome ($10^7$ nucleotides per cell: Bolton et al., 1965). Maximum hybridisation between Drosophila cRNA and DNA is roughly the same as for mouse DNA-DNA and it seems probable that as in mouse the hybridisation observed with Drosophila is due to reiterated sequences. In this connection it is significant that the cRNA-DNA interaction for Drosophila is virtually complete in six hours (see Figure 18). This rapid rate of hybridisation itself indicates that relatively simple reiterated sequences are
involved in *Drosophila* cRNA-DNA interaction. Although this is highly suggestive additional evidence is required that reiterated sequences occur in *Drosophila* and that the hybridisation observed is due primarily to the interaction of these sequences. Thus studies on the renaturation of *Drosophila* DNA in the manner of Bolton *et al.* (1965) would show if rapidly renaturing fractions were present. In addition recovery of the unbound cRNA after annealing with *Drosophila* DNA followed by reincubation with a second batch of DNA would indicate whether or not the first incubation selected for a class of rapidly binding molecules contributed by reiterated sequences leaving an "unbindable" fraction. Despite the scantiness of the evidence it seems probable that reiterated sequences occur in *Drosophila* and are largely responsible for the hybridisation observed.

Theoretically it should be possible to hybridise even the non-reiterated genes if the concentrations were raised sufficiently high (approximately 100 fold), but this is a practical impossibility. At present it seems that one possibility of investigating the whole genome by the technique of cRNA-DNA hybridisation is to reduce the complexity by fractionating the DNA in some arbitrary manner and to investigate the individual fractions. It is interesting that the only truly quantitative determinations made on heterogeneous DNA are the assay of the ribosomal cistrons (Chipchase and Birnstiel, 1963; Ritossa and Spiegelman, 1965; Vermeulen and Attwood, 1965; Wallace and Birnstiel, 1966) which are just such a simplified system, representing approximately 3% of the genome.
The probability that only a limited class of DNA molecules enter into hybridisation must be borne in mind in the interpretation of the results. At present analysis of Drosophila cRNA-DNA hybrids has the further complication that it is not certain how much of the DNA is transcribed into cRNA. In experiments in which Drosophila DNA was annealed with an excess of cRNA only 7% of the DNA was covered. However this did not appear to be a plateau value and the indications were that greater coverage would be obtained with higher RNA:DNA ratios. It is also possible that since both DNA strands are transcribed by M. lysodeikticus polymerase that duplex formation may occur between the cRNA copies, reducing the effective cRNA concentration for cRNA-DNA hybridisation. For this reason E. coli polymerase which transcribes only a single strand of the DNA might be a better choice for future work. There is also a possibility that some reiterated sequences such as mouse satellite DNA are transcribed preferentially by RNA polymerase in an in vitro system (Flamm: personal communication). The effect of this would be to increase the concentration of these reiterated sequences, thus increasing the bias in favour of hybridisation of these regions.

The relationship of the cRNA product to its DNA template is of great significance and requires further study. In particular we would like to know (a) does the size of the cRNA molecules affect their capacity for hybridisation, (b) how far is the cRNA a true copy of the DNA. The possibility exists that RNA polymerases from different sources may show preferential transcription of different DNA sequences and this could be used as a tool to investigate divergence within and between species.
(c) what is the relationship between cRNA and mRNA? This might be investigated by studying the competition between cRNA and mRNA for DNA sites. If, as Bolton et al. (1965) suggest, the reiterated sequences are derived from damaged genes these sequences may not be transcribed into mRNA. An analysis of this point could clarify the nature of both cRNA and mRNA and also the mechanics of transcription of DNA within the living cell.

When *Drosophila* cRNA was challenged with DNA from various organisms RNase-resistant hybrids were formed only with *Drosophila* DNA, that is no apparent homology could be observed between *Drosophila* and any of the other organisms including other members of the Insecta. This is in sharp contrast with the findings of Hoyer et al. (1964) who found some cross reaction between DNA from animals as far apart as mouse and fish. There is no theoretical reason why the same taxonomic status should indicate the same degree of genetic relatedness within different groups such as Insecta and Vertebrata, and indeed Bolton et al. (1965) claim that the diversity within the plant Family *Leguminosae* is equivalent to the diversity between different Orders of mammals. Nevertheless if the findings of Hoyer et al. (1964) are valid we would expect at least some cross reaction between *Drosophila* and *Aedes* since they are both members of the Order Diptera. Two possible explanations of this discrepancy may be offered. The first possibility is that transcription is biased and that either only sequences peculiar to *Drosophila* are transcribed or there may be selective transcription of reiterated sequences present in *Drosophila*. In the latter case this would increase the proportion of reiterated sequences in the cRNA and thus have the same effect
as "pre-selection" of DNA fragments on homologous DNA. McLaren and Walker (1966) have already shown that the rapidly renaturating sequences of mouse DNA show the greatest discrimination against rat and that by pre-selection of mouse DNA on mouse DNA-agar this discriminating power can be increased (McLaren and Walker 1965, 1966). Thus because of biased transcription in favour of discriminatory sequences Drosophila cRNA may be unable to form duplexes with heterologous DNA. An objection to this theory is that if Drosophila cRNA does contain a disproportionate amount of rapidly renaturing sequences then we would expect a higher level of hybridisation with Drosophila DNA as has been observed between "pre-selected" mouse DNA fragments and mouse DNA-agar (McLaren and Walker, 1965).

The second possible explanation is that the RNase-resistance of Drosophila cRNA-DNA duplexes provides a stringent test for the specificity of the hybrid. No such test was employed on the DNA-DNA hybrids of Hoyer, McCarthy and co-workers (Hoyer et al., 1964; McCarthy et al., 1963). In the General Introduction it was pointed out that non-specific binding of DNA fragments to DNA-agar occurs to a considerable extent even at 60°C. Indeed the view of the Carnegie Institute Group appears to be that specific hybridisation occurs only between homologous reiterated sequences and that loops and loose ends of DNA remain held to the DNA-agar simply by virtue of the fact that they adjoin reiterated sequences. Under these conditions any estimate of DNA homology by the DNA-agar technique is liable to be too high. Schildkraut et al. (1961) who worked with DNA from bacteria of different species and used DNase to digest non-specific DNA aggregations in a manner
analogous to the RNase test were unable to detect homologies later claimed by McCarthy and Bolton (1963).

If the RNase test was omitted after annealing Drosophila cRNA with heterologous DNA there appeared to be some low level interactions with all the DNAs tested, analogous to the results of Hoyer et al. (1964). It seems probable that the "homologies" reported by Hoyer et al. (1964) are in part due to non-specific hybridisation of the DNA. It therefore seems that the discrepancy between the present results and those of Hoyer et al. (1964) are principally due to elimination of non-specific base-pairing in the cRNA-DNA system, although there may be some increased discrimination resulting from the presence of a high proportion of reiterated sequences in the cRNA.

From the evidence presented above it is considered that the cRNA-DNA hybridisation technique provides a better measure of hybridisation between polymolecules from different species than does the DNA-DNA system. The question is now: how far do such hybridisation measurements reflect DNA homology in higher organisms?

In the General Introduction it was stated that the Carnegie Institute group (Bolton et al., 1965) consider that the reiterated sequences are scattered throughout the genome, individual families being separated by heterogeneous regions 300-1000 nucleotides in length. Bolton et al., (1965) believe the reiterated sequences to be derived by duplication and deletion from damaged genes during the course of evolution. In this case they would be distinct from the functional genes controlling the constitution of the organism and these are presumably confined to the non-
hybridising heterogeneous regions. Many of the families within a genome may share sequences in common (Bolton et al., 1965). Thus different functional genes may adjoin identical reiterated stretches. Indeed we can envisage that in the extreme case two different organisms might possess identical families of reiterated sequences but that the functional genes of the species were completely different. Thus the homology measured between the reiterated sequences of two species does not supply a valid estimate of the DNA as a whole. Hoyer et al. (1965) claimed that a relationship existed between the percentage of polynucleotides held in common by different vertebrates (as determined by the DNA-agar technique) and the appearance of these different species in the evolutionary time scale. As DNA-DNA hybridisation is somewhat unspecific their values are probably too high although they may indicate the presence of homology if not its true extent. Since it appears that these homologies are basically those of reiterated sequences Bolton et al. (1965) have used these results to calculate that one new family of reiterations is produced every 100,000 years. They also imply that since the DNA homologies determined by the DNA-agar technique are those expected from the taxonomic position of species and their emergence in evolution these measurements therefore reflect the homology of the DNA as a whole, although in fact they only show the homology of certain selected sequences. However this argument is not justified since changes in the reiterated sequences might be purely random and uncontrolled and serve as markers of chronological time rather than of evolutionary change. Indeed if these sequences arise from damaged genes as proposed by Bolton et al. (1965)
and have no function in the cell, then by this very fact they are not subject to the evolutionary pressures which act on the functional genes. Thus measurements on the reiterated sequences do not necessarily indicate changes which have occurred among the functional genes.

*Drosophila* provides a very interesting system for study of the relationship between the reiterated sequences and the rest of the DNA. It would be interesting to see how well cRNA-DNA hybridisations could discriminate within the genus *Drosophila*. Since it discriminates so well against other genera it seems possible that it can also distinguish between species. (Mouse satellite DNA is specific at least to the sub-species level. Flamm: personal communication.) It is possible, if the level of discrimination is sufficiently high, that hybridisation values can be used as a marker of fluctuations within a species of *Drosophila* even if the values do not give a quantitative measure of changes in the DNA as a whole.

If the hybridisation discriminates successfully between species it could be used to investigate *Drosophila* species whose evolutionary relationships can be traced through the banding of the salivary chromosomes. Such a study could show to what extent hybridisation measurements are an index of genetic relatedness and would also throw light on the evolution of reiterated sequences.

Thus the cRNA-DNA hybridisation technique appears to be more truly specific than the DNA-DNA agar method and seems full of promise for a study of genetic relatedness within the genus *Drosophila*. In addition to this primary objective the technique
may also supply valuable information on the origin of reiterated sequences in the course of evolution and their distribution and function within *Drosophila*. This in turn could throw light on the transcription of DNA and its organisation within the chromosome.
SUMMARY

DNA was extracted from Drosophila melanogaster according to the published procedures of other workers; however the product was usually degraded and heavily contaminated. A new method was therefore developed for the extraction of DNA from Drosophila. Adult flies were homogenised in a buffer solution at neutral pH and containing EDTA. The DNA-containing fractions were sedimented at 12,000 x g. The deoxyribonuclease present was inactivated by heating at 60°C for 10 minutes. The DNA was then deproteinised and purified by treatment with ribonuclease and further deproteinisation. The DNA extracted was of high purity and of very high molecular weight (up to 110 x 10^6 daltons). This method has also been applied successfully to other insects.

The Drosophila DNA was used as a primer for the synthesis of C_{14} labelled complementary RNA (cRNA) by Micrococcus lysodeikticus RNA polymerase in vitro. When the C_{14}cRNA was annealed with denatured Drosophila DNA under conditions of high ionic strength and high temperature a molecular cRNA-DNA hybrid was formed between them. Such hybrids are formed only between DNA and its homologous complementary RNA. Different methods of assaying the cRNA-DNA hybrids were tested. The most successful procedure was to anneal the cRNA with denatured DNA in free solution and to trap the cRNA-DNA hybrid by filtration onto nitrocellulose membranes or millipore filters.

The optimum conditions for the formation of the cRNA-DNA hybrid were determined. It was found that the percentage of the cRNA which was bound by the DNA increased with increasing DNA:RNA ratios, and that a maximum of 28% of the cRNA could be bound by
the DNA at DNA:RNA ratios of 300:1 or more. It seems probable that the cRNA bound to the DNA consists mainly of reiterated nucleotide sequences.

Denatured Drosophila DNA was annealed with an excess of cRNA to determine what fraction of the DNA could be covered by cRNA in a cRNA-DNA hybrid, indicating the percentage of DNA which had been transcribed into cRNA. At an RNA:DNA ratio of 20:1 7% of the DNA was covered with cRNA. However this was not a saturation value and it is almost certain that at higher RNA:DNA ratios a larger fraction of the DNA would be covered with cRNA. Thus 7% is a minimum value for percentage transcription of the DNA.

When DNA from other organisms, a bacteriophage (T₄), an amphibian (Xenopus), a mammal (Rat) and other insects (Schistocerca and Aedes) was annealed with Drosophila cRNA no cRNA-DNA hybrids were formed. Thus virtually no homologies can be detected between Drosophila cRNA and DNA from other genera. Since the DNA-cRNA hybridisation reaction appears to be so highly specific the prospects are encouraging that it may be extended to investigate genomal differences between and perhaps within species of Drosophila. In addition it may be used to study the transcription of DNA by RNA polymerase.
REFERENCES


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