ABSTRACT OF THESIS

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Title of Thesis Studies on the Nucleic Acids of Fractionated rat Nucleoprotein

1. Two methods of fractionating chromatin were compared by measuring differences in the specific radioactivities of DNA and RNA extracted from the condensed and dispersed fractions of chromatin from regenerating rat liver.

2. Only one method of fractionation showed a clear difference in labelling of the DNA and RNA between the fractions. This method was used to prepare large amounts of pure DNA from three fractions of chromatin: condensed, intermediate and dispersed.

3. Evidence of changes in the distribution of fast, intermediate and slow renaturating sequences of DNA during liver regeneration was sought by an investigation of the kinetics of renaturation of normal and regenerating rat liver DNA. Studies of DNA renaturation kinetics were also used to investigate differences in sequence distribution in the DNA of the three chromatin fractions from regenerating liver.

4. The buoyant densities of DNAs prepared from fractionated chromatin of both normal and regenerating rat liver were measured by analytical ultracentrifugation in an attempt to detect differences in base composition.

5. RNA was transcribed *in vitro* from templates of unsheared condensed and dispersed chromatin DNA prepared from normal and regenerating rat liver. This highly labelled RNA was hybridised with a vast excess of DNA to detect the reiteration frequencies of sequences in the DNA of the different chromatin fractions complementary to the RNA.

6. The technique of hybridisation in DNA excess was also used to investigate the complementarity of DNA sequences in the chromatin fractions with *in vivo* nuclear heterogeneous RNA prepared from regenerating rat liver.

7. The results are discussed with reference to:-

i) the transcriptional activity of different fractions of the genome.

ii) changes in sequences transcribed during liver regeneration (as a model of a developmental process).

iii) the organisation of the mammalian genome.

Use other side if necessary.

STUDIES ON THE NUCLEIC ACIDS OF FRACTIONATED RAT NUCLEOPROTEIN

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INTRODUCTION

The problems which arise in studying the nucleic acids of higher organisms are many times greater than those encountered in bacteria. The bacterial chromosome consists of a simple circular DNA molecule not complexed with protein, and the ways in which co-ordinated gene expression and repression are achieved are fairly well understood. The size and complexity of the eukaryotic genome make its analysis difficult; whereas each genome of the bacterium *E.coli* has 2.7×10^9 daltons of DNA, in the rat, each cell nucleus contains 3.6×10^{12} daltons of DNA (Melli *et al*, 1971) which is complexed with protein and distributed amongst 42 separate chromosomes.

During the process of development and differentiation of mammalian cells, regulatory functions of a different kind from those common to both prokaryotic and eukaryotic cells must be effected. However, it is still difficult to account for the 1,000 times greater amount of DNA in eukaryotic cells. The DNA of higher organisms differs from that of bacteria and viruses in its kinetic complexity. When eukaryotic DNA is denatured and its renaturation studied, a proportion of the genome renatures much more rapidly than would be expected upon the basis of the size of the genome, a second fraction renatures very slowly and some DNA renatures at a rate intermediate between these two (Waring and Britten, 1966; Britten and Kohne, 1968; Wetmur and Davidson, 1968).

Under the light microscope, each eukaryotic chromosome appears to be a relatively featureless structure having a centromere and perhaps a secondary constriction, although some differentiation can be detected by the use of specialised staining techniques. Whilst

it is known that the chromosomes contain most of the information necessary to dictate structure and function of the organism, attempts to elucidate the mechanisms of transcription, translation, organisation and control of differentiation in higher organisms have met with many difficulties. It is unfortunate that at mitosis when the chromosomes are most easily observed because they are highly condensed and stain darkly, they are inactive with respect to DNA and RNA synthesis.

It is known that there are certain fractions of the mammalian genome which are not transcribed (such as mouse satellite DNA, Walker, 1971). The distinction between actively transcribed regions and those which appear to be genetically inert has been a focus of much attention since this may provide an insight into the mechanism of regulation of gene action and the processes of development and differentiation in higher organisms.

Cytological observations

A cell nucleus during interphase contains prominent darkly staining granules termed 'chromatin' granules. At the beginning of mitosis, these dots can be seen as part of the condensing chromosomes. After observing division cycles of the chromosomes and the chromatin granules of interphase nuclei, Heitz (1928, 1931, 1933, 1934, 1935) defined two classes of chromosomal material: euchromatin, which underwent a typical cycle of condensation and unravelling and heterochromatin which maintained its compactness and remained condensed and visible during interphase as large blocks of Feulgen positive material. Heterochromatin tends to occur in

similar regions of the different chromosomes of a species, usually near the centromere (centric heterochromatin) at the ends of chromosomes and in the vicinity of the nucleolar organiser. It is frequently a major constituent of the sex chromosomes.

The heterochromatin of mammalian somatic cells has been divided into two classes, constitutive and facultative (Brown, 1966; Yasmineh and Yunis, 1969). The first class, constitutive heterochromatin, occurs in both maternal and paternal chromosomes at the same locus during interphase and during condensation of the chromosome during mitosis; it has no known function. Brown (1966) and Lima-de-Faria (1959, 1968) consider heterochromatin to be a state of chromatin rather than a specific substance, because it does not occur at all stages of development and is thought to have variable expression in adult tissues since different types of cell in a mature animal have different interphase chromatin patterns. In contrast with this view, Lee and Yunis (1971) suggest that constitutive heterochromatin should be regarded as a specific substance. Their work on Microtus agrestis shows that the constitutive heterochromatin is located in specific homologous chromosomes, can be seen even in early embryogenesis and is present in the same chromosomes throughout development.

Heterochromatin is described as facultative or functional when one chromosome of a homologous pair is condensed and the other euchromatic; this is often associated with the turning off of the genes involved. The most striking example of facultative heterochromatin occurs in the X chromosome of female mammals. One of the two X chromosomes becomes heterochromatic in all somatic cells

except those which go to form the germ cells. In humans with multiple X chromosomes only one of the X chromosomes is euchromatic; all the remainder become heterochromatic and are visible as multiple Barr bodies or sex chromatin granules in interphase nuclei. Such observations, together with the evidence of genetic inactivity of the X chromosome, were correlated by Russel and formulated into a hypothesis by Lyon (1961). According to the inactive X hypothesis of dosage compensation, the genes of one or other of the two X chromosomes become inactivated at an early stage of embryonic development. The choice of which X becomes inactive in each cell is random. As a result, clones of cells arise within an individual female, some having the maternally derived X, others the paternal X in an active condition. Recently, Cohen and Rattuzi, (1971) and Hamerton and Gianelli (1971) have made an impressive inverse correlation between late replication of horse and donkey X chromosomes and the expression of the X-borne gene G6PD. The similar cytological appearance of facultative and constitutive heterochromatin suggests that there may also be a correlation of genetic inactivity with the heterochromatic state in constitutive heterochromatin although there is as yet no evidence for this.

DNA sequence repetition, staining and *in situ* hybridisation associated with heterochromatin

The definition of heterochromatin given earlier was related to its behaviour during the cell cycle, and the staining properties associated with these tightly coiled regions of chromosomes. Whilst the facultative heterochromatin appears to be associated with the inactivation of genes, the constitutive heterochromatin is thought

to consist of material which is genetically inert (Yasmineh and Yunis 1969, 1970). The association of heterochromatin and satellite sequences may suggest some similarity of function. Cooper (1959) suggested several possible functions of heterochromatin mainly concerned with the regulation of gene activity but he also placed emphasis on the functions of heterochromatin at centromeres and telomeres, involving such processes as breakage and rejoining of chromosomes and pairing or association of the terminal heterochromatic regions.

Although mouse satellite DNA does not seem to code for a corresponding fraction of RNA *in vivo* (Flamm, Walker, McCallum, 1969) it was thought that it might be possible to determine chromosomal locations for sequences of satellite DNA by hybridising single stranded satellite DNA to cytological chromosome preparations which had been denatured. Jones (1970) and Pardue and Gall (1970) prepared pure tritium-labelled mouse satellite DNA and complementary RNA. These groups showed that both the satellite DNA and its cRNA hybridised *in situ* to cytological preparations of mouse metaphase chromosomes bound only to the centromeric heterochromatin. During interphase, satellite DNA bound to the chromocentres, dense chromatin and nucleoli. Pardue and Gall (1970) showed that although both X and Y chromosomes are heterochromatic at various times satellite DNA only binds to the centromeric regions of the X chromosome, not to the Y, and it has therefore been suggested that there is another class of heterochromatin in the Y chromosome, distinct from those of constitutive and facultative heterochromatin. The resolution of the technique of hybridisation *in situ* to

cytological preparations has been greatly advanced by studies using the insect giant salivary gland chromosomes (Hennig, Hennig and Stein; 1970).

The function of centric heterochromatin and associated satellite is controversial. Cooper (1959) suggested that it was a neutral or genetically inert material acting either as an anchorage or dispensable substance during processes such as translocation or centric fusion. Non-homologous association of the telocentric chromosomes is known to occur in vertebrates (Ohno, Stenius, Faisst and Zenzes, 1965). During the process of fusion of the centromeres (Robertsonian transformation, Robertson; 1916) some of the pericentromeric DNA may be lost. Mattoccia and Comings (1971) suggest that it would be advantageous to have genetically inert DNA, or DNA consisting of highly repeated sequences (which might be considered relatively expendable) collected near the centromere. This view however does not receive universal support. Walker (1971) considers several possible functions for repeated satellite sequencies and heterochromatin in the chromosomal housekeeping functions (Walker, Flamm and McLaren; 1969). The centric heterochromatin may be directly associated with the properties of the centromeres themselves and affects pairing and disjunction (Novitski, 1955; Sandler and Novitski, 1957; Lindley and Novitski, 1958). Chromosomes with a common origin tend to segregate together (Michie, 1953; Wallace, 1953) and this is associated with the presence of a special distribution of heterochromatic material causing similarities of segregation behaviour. The actual relationship between heterochromatin and satellite DNA is still

not known. Satellite DNA itself may be heterochromatic, or satellite and heterochromatin may be intimately associated because the chromosomal condensation of heterochromatin involves the satellite sequences. A third possibility is that satellite sequences and heterochromatin have a similar chromosomal distribution because of some association of function.

It was reported by Pardue and Gall (1970) that centromeric heterochromatin is preferentially stained by Giemsa stain. It therefore appeared probable that regions staining darkly with Giemsa after denaturation and reannealing indicated the presence of highly reiterated DNA. Examinations of human metaphase preparations were undertaken (Sumner, Evans and Buckland, 1971; Gagné, Tanguay and Larberge, 1971; Yunis, Roldan and Yasmineh, 1971) and attempts were made to correlate the dark staining regions with the established areas of heterochromatin and late replicating DNA (Ohno, Kaplan and Kinosita, 1957; Church, 1965). By analogy with the _in situ_ hybridisation studies in mouse, it was assumed that the dark staining centromeric region in man represents a region of highly repeated sequences. However, mouse satellite represents 10% of the genome whereas present studies indicate that human satellites represent only 2.5% of the genome (Corneo, Ginelli and Polli, 1968, 1970). There is however a moderately repetitive fraction of main band DNA which forms 8% (Southern, pers. comm.) to 15% (Corneo _et al_, 1970) of the genome. This still cannot account for the 50% of the genome which is observed to stain darkly. A further problem is that the nucleolar organiser region which might be expected to contain DNA which renatures rapidly (Birnstiel,

Spiers, Purdom, Jones and Loening, 1968; Attardi, Hwang and Kabat, 1965; Ritossa and Spoigelman, 1965) is very pale by this technique.

The cytological techniques outlined are of the greatest importance in clarifying the relationship between chromosomes and chromosomal components. However, there are at present many unresolved problems, not least of which is the relationship between the results of the cytological investigations and those of the biophysical and biochemical studies to be described next.

Isolation of chromatin fractions

Cooper (1959); Frenster et al (1963); Grumbach, Morishawa and Taylor (1963) have suggested that heterochromatin and euchromatin differ in gene expression and that heterochromatin represents the part of the genome which is repressed. The nature of heterochromatin is a problem of great importance, and in order to study its properties attempts have been made to separate it physically from the euchromatic portion of the genome.

Frenster, Allfrey and Mirsky (1963) estimated that approximately 80% of the chromatin in the differentiated lymphocytes obtained from calf thymus was visible as condensed Feulgen-positive masses. It was postulated (Allfrey and Mirsky, 1962) that much of the interphase lymphocyte DNA is inactive with regard to messenger RNA synthesis. The gene inactivation hypothesis of cell differentiation postulates that as a cell differentiates those genes not involved in this process are inactivated by some form of repression mechanism. The differentiated state of the cells seemed to correlate with the amount of heterochromatin observed. Frenster et al therefore attempted to separate these condensed chromosomal segments of

heterochromatin from the more extended euchromatin fibrils using a technique of brief sonication of the nuclei followed by differential centrifugation. Incubation of nuclei with isotopic precursors indicated that the DNA, RNA and protein synthesis of the "heterochromatin" fraction was decreased with respect to that of the "euchromatin" fraction.

Yasmineh and Yunis (1969) isolated three chromatin fractions from mouse liver and brain nuclei by a modification of the method of Frenster et al (1963). Caesium chloride density gradient patterns of the DNA from the "heterochromatin" intermediate and "euchromatin" fractions indicated that the "heterochromatin" contained about 70% of the satellite DNA. Although mouse satellite DNA had been described several years previously and it had been thoroughly investigated (Kit, 1961; Chun and Littlefield, 1963; Waring and Britten, 1966; Flamm, McCallum and Walker, 1967) neither its function nor its cytological location were known. In mouse, heterochromatin appeared to be fully condensed in brain nuclei, rather more diffuse in liver and uncertain in fibroblasts. Hill and Yunis (1967) and Pflueger and Yunis (1966) found that condensation of constitutive heterochromatin was not always the same in all tissues of an organism, although the late replication of DNA is thought to be constant in the constitutive heterochromatin of all somatic tissues. The unusual features of mouse satellite DNA (high AT content, tandem repetition of sequences and specific proportions in all mouse cells) suggested that the specific association of satellite sequences with the "heterochromatin" fraction might be peculiar to the mouse. Accordingly, fractionation procedures

were attempted in other animals.

Fractionation of guinea pig chromatin showed that the "heterochromatin" fraction had a fourfold enrichment of both a heavy and a light satellite component whilst the "euchromatin" was almost devoid of satellite. An alternative method of fractionation was developed by McCarthy and Querkson (1970) who fractionated chromatin after shearing in a homogeniser instead of sonicating the nuclei. The pellet fraction of mouse hepatoma chromatin, sheared by this method, contained an enrichment of satellite DNA. The same method of shearing was applied to crab DNA and it was found that the proportion of crab satellite of poly-dAT (about 14% of the genome) and a crab GC-rich satellite were enriched in the pellet or "heterochromatin" fraction. The most extreme example of the association of a satellite component with a dense chromatin fraction is that given by Nagrimas and Hatch (1970) in their studies on the kangaroo rat. In this animal, about 43% of the total DNA is present as satellites and this can be isolated with the heterochromatin DNA which comprises 60% of the total DNA.

These fractionation procedures revealed that a specific association of the satellite components and "heterochromatin" seemed to exist. A difference was observed between the "heterochromatin" fractions of mouse and guinea pig, in that the latter contained a considerable amount of main band DNA which could not be separated from the "heterochromatin" fraction even by very vigorous sonication (Yasmineh and Yunis, 1969, 1970). A comparison of the fractionation of calf chromatin by researchers with different criteria for defining their "heterochromatin" fractions shows that very different

separations can be effected. Frenster, Allfrey and Mirsky (1963) based their fractionation of calf thymus lymphocytes on the morphology in both light and electron microscopes and by differences in isotopic incorporation, whilst the technique of Yasmineh and Yunis (1971) for calf liver was adjusted to restrict the amount of satellite in the "euchromatin" fraction. Whilst the "heterochromatin" fraction of Frenster et al comprised 60-80% of the total DNA and appeared to be slightly contaminated by "euchromatin" (judged by the electron microscope studies) the "euchromatin" (10-30% of the total DNA) was in a very pure state and showed an isotopic incorporation three to fourfold greater than that of the "heterochromatin" fraction. The "heterochromatin" of Yasmineh and Yunis contained about 36% of the total DNA and nearly all of the heavy and light calf satellite DNAs, whilst the "euchromatin" (28% of the total DNA) was almost completely devoid of satellite. The amount of DNA in the dense fraction differs widely between the two groups. Even though the two tissues may differ in the amount of constitutive heterochromatin or their response to the extraction methods, this is still not sufficient to explain the differences.

The presence of satellite DNA in the heterochromatin tends to support the theory that constitutive heterochromatin is composed at least in part of a specific substance, (Yasmineh and Yunis, 1969, 1970, 1971) rather than being a variable state of chromatin (Brown, 1966; Lima-de-Faria, 1969). (The presence of facultative heterochromatin was excluded by using heterogametic animals). In early experiments, attempts were made to isolate chromatin fractions corresponding both in proportion and activity to cytological

heterochromatin and euchromatin. No hesitation was shown in using the cytological terms for the separated chromatin preparations. However, a variety of isolation techniques have now been used on different tissues from many species and numerous modifications of the isolating solutions, sonication procedures and centrifugal speeds have been introduced. An example of the differences which now occur was given for the fractionation of calf chromatin and this shows the way in which confusion can arise, for both fractions are called "heterochromatin", although they differ very significantly in the proportions and amounts of nucleic acids they contain.

In view of the fact there is little if any evidence to associate these fractions with the cytological entities it is misleading to attempt to equate the two. A system of nomenclature which would convey meaningful information about a method of chromatin fractionation would define the organism and detailed conditions of isolations. This system would be rather cumbersome, but confusion could be avoided simply by retaining the terms euchromatin and heterochromatin solely as a cytological definition of chromatin, and using terms such as condensed and dispersed for isolated fractions. Further experimental evidence like that described in this thesis will help to establish whether such fractionations are meaningful and whether the heterogeneity of chromatin in cells corresponds to the differences found in isolated fractions.

The Relationship between Heterochromatin and Late Replicating DNA

Autoradiographic evidence that the DNA of heterochromatic regions is replicated later than that of euchromatin has been provided by several groups. The first experiments were those on the large heterochromatic X chromosome of the grasshopper and the heterochromatin of Secale cereale (Lima-de-Faria, 1969). Late replication of the heterochromatin in root and leaf nuclei of plants has been extensively studied (Darlington and Haque 1966; Evans, 1964; Kusamagi, 1966) as has that in insects (Lima-de-Faria, 1969; Barrigozzi et al 1966; Baer, 1965) and mammals (Taylor, 1960; Hsu, 1964; Fraccaro et al, 1965). The most detailed investigations of the late replication of heterochromatin have been made in man in individuals with multiple X chromosomes where cytogenetic autoradiography has shown the number of late labelling X chromosomes to be correlated with the number of Barr bodies (Morishawa, Grumbach and Taylor, 1962; Rowley et al 1963; Gianelli, 1964; Hsu and Lockhardt, 1964; Lima-de-Faria, 1965).

Whilst it appears to be a rule that any region of heterochromatin is late labelling, it is not true that all regions showing later DNA replication are heterochromatic. Recent experiments show that main band DNA synthesised at the beginning of S phase has a higher GC content and buoyant density than that synthesised at the end of S phase. This was observed in several different mammalian cell lines; in mouse L cells and HeLa cells by Tobia, Schildkraut and Maio (1970) in L cells and Chinese hamster cells by Bostock and Prescott (1971a & b) and in mouse lymphoma cells by Flamm, Bernheim and Brubaker (1971). The change from synthesis of GC-rich DNA

early in the synthetic period to AT-rich DNA later in the S phase could be explained by the hypothesis that euchromatin was GC rich and heterochromatin main band DNA (known to replicate late in S phase) AT-rich.

Two groups have attempted to resolve this problem by the analysis of the buoyant density of fractionated chromatin. Mattoccia and Comings (1971) found that mouse liver "euchromatin" DNA had a higher buoyant density than that of main band DNA. Whilst there was no difference between the buoyant density of heterochromatin and total mainband DNA from nuclei, a nucleolar fraction was found to have a slightly lower buoyant density than total mainband DNA. Bostock and Prescott (1971) found no difference between the fractions and total mainband DNA of rabbit, chinese hamster and mouse. These attempts to show differences in buoyant density between condensed and dispersed mainband DNA analogous to those between early and late replicating DNA have had somewhat inconclusive results. It is possible that the base composition of mainband heterochromatin and euchromatin is very similar but that the AT-rich sequences of the former replicate late in S phase. In autoradiographic experiments this might seem as if the whole of heterochromatin was replicated later than euchromatin.

On the other hand, there may be a genuine difference in base composition, but the methods of fractionation used may have concealed it. In each case, the condensed fraction was adjusted so that it contained most of the satellite, although the object was to detect differences in the buoyant density of main band DNA. If the shift in buoyant density is due to differential replication of euchromatin

and heterochromatin, a difference might be detected even in an impure fractionation. It seemed that species differences might be important in these studies, and in the rat, in which no distinct satellite fraction has been found, differences between the buoyant densities of the condensed and dispersed fractions might be visible. In this work, the buoyant densities of DNA from dispersed and condensed chromatin from both normal and regenerating liver were examined.

Properties of Chromatin

One of the most difficult problems is to attempt to reconcile the cytological observations that large regions of the chromosomes are heterochromatic and the evidence that these regions are genetically inactive, with any of the current models of chromosome and chromatin structure. The physical separation of chromatin fractions has involved two different concepts: Frenster (1963) postulated that heterochromatin was genetically repressed and that the repression was probably mediated by nuclear polycations (Frenster, 1965). Yasmineh and Yunis (1969, 1971) thought that heterochromatin was a specific substance, one component of which was satellite DNA, although no particular method of effecting the heterochromatisation was postulated.

There is evidence to indicate that the metaphase chromosome consists of a tortuously wound fibre (Du Praw, 1968). The dimensions of such fibres are extremely controversial, as is the interpretation of the data about the substructure and number of fibres per chromatid (Taylor, 1960; Cairns, 1963; Huberman and Riggs, 1966).

The structure of chromosomes is an unresolved problem. The most commonly observed element in chromatin is a 100 Å diameter fibre. X-ray analyses (Wilkins, Zubay and Wilson, 1959; Pardon, Wilkins and Richards, 1967) have indicated that the primary nucleoprotein fibre has a diameter of 35 Å and it is postulated that this has a helical structure with a repeat distance of 110 Å. Biophysical studies imply that chromatin has a single duplex of DNA running along its axis (Brahm and Ris, 1971).

Frenster (1965) examined the ultrastructure of "repressed" and "active" calf thymus chromatin during the chromatin isolation procedure when the nuclei were swollen. The condensed masses of "repressed" chromatin tended to be situated at the periphery of the nucleus whilst the extended microfibrils of "active" chromatin were distributed between these masses in the centre of the nucleus and were structurally continuous with them, although there was a sharp transition between the two. The difference was explained by Frenster in terms of repression of the heterochromatin by polycations such as histones and derepression of the euchromatin by polyanions (phosphoproteins or RNA). McCarthy and Duerkson (1970) melted both the pellet and supernatant fractions of mouse hepatoma chromatin and found that the Tm of the pellet fractions was much higher than that of the supernatant (euchromatin) fraction. This finding suggested that the pellet fraction contains the most condensed portions of the interphase chromosomes in which the DNA and protein are tightly bound. (It has been shown that both the amount and types of protein which are associated with DNA in chromatin affect the temperature at which the chromatin reveals a hyperchromic effect

(Bonner et al, 1968).

Chromosomes contain approximately equal proportions of DNA and protein and a smaller amount of RNA. The largest fraction of these proteins is the basic histones, although about 20% of the proteins are acidic. One possible control mechanism in eukaryotes is that transcription by RNA polymerase might be controlled by protein bound to the DNA. The specific masking and unmasking of the DNA is thought to be the mechanism which is responsible for the development and final differentiation which is achieved in eukaryotic cells (Bonner and Hwang, 1963; Marushige and Bonner, 1966; Paul and Gilmour, 1966; Bonner, Dahmus, Fambrough, Huang, Marushige, Tuan, 1968). It is possible to transcribe the DNA from chromatin using a bacterial DNA dependent RNA polymerase. When the proteins are removed from the DNA, much more of the DNA can be transcribed. This hypothesis is by no means universally accepted, and recent work by Clark and Felsenfeld (1971) and Itzaki (1970) suggests that much more of the DNA in chromatin may be open to transcription than has been thought. The interpretation of experiments using DNA in chromatin as a template for bacterial DNA-dependent RNA polymerase presents problems. Restriction of RNA polymerase activity may only require that the transcriptional starting point of each unit should be blocked. An obvious corollary of any speculations upon transcription involving the DNA dependent RNA polymerase is that in E.coli the specificity of transcription is regulated by factors which interact with the polymerase core enzyme (Burgess, Travers, Dunmand Bautz, 1969; Bautz and Bautz, 1970). The complexity of the situation in E.coli indicates that the regulation of transcription in higher organisms may not only be more complex still, but involve quite different mechanisms.

Features of rodents and the rodent genome

In viruses and bacteria, the DNA consists for the most part of unique nucleotide sequences, whereas the DNA of higher organisms is more complex. A large portion of the genome consists of different families of repeated nucleotide sequences (Britten and Kohne, 1968). Bolton et al (1965) suggest that the families of nucleotide sequences comprising the large repetitious fractions have arisen from a many-fold duplication of an existing nucleotide sequence. Britten and Kohne (1968) consider that a family is the result of a relatively rapidly occuring saltatory replication. They suggest that this phenomenon of excess replication of particular sequences is probably responsible for the divergence between species.

In some organisms there are nucleotide sequences whose base composition differs from that of most of the DNA so that upon caesium chloride density gradient centrifugation, satellites of the mainband DNA can be seen (Kit, 1961). The sub-family Muridae of the class Rodentia (Simplicidentata) contains the species Rattus, Mus, Acomys and Apodemus. Whilst these animals are closely related and the buoyant density (GC content) of the mainband DNA is almost identical in all species, none of them have common satellites (Hennig and Walker, 1970). This suggests that the satellites have arisen since these species diverged from each other.

In the rat, there appears to be no fraction comparable to mouse satellite DNA. Steele (1968) purified nucleolar DNA from rat and found in addition to mainband DNA with a buoyant density of 1.700 g cm^{-3}, a light satellite of 1.692 g cm^{-3} and a heavy satellite at 1.708 g cm^{-3}. Whilst the heavy satellite contained 8% of the

total nucleolar DNA the proportion of nucleolar DNA in the total DNA was only 3%. These rat satellites therefore comprise less than 1% of the genome. It is clear that the considerable amounts of "fast" and "intermediate" fraction DNA in the rat (estimated by Hennig and Walker (1970) as 10% and 4% respectively) cannot be accounted for in terms of satellite DNA and yet are discrete portions of the genome with respect to renaturation behaviour. It was therefore of interest to investigate whether an isolated chromatin fraction in rat would have any enrichment for repeated or rapidly renaturing sequences.

The possible functions of the tandemly repeated sequences of mouse satellite DNA have been the subject of several different interpretations. Britten and Kohne (1968, a & b) see these sequences in an evolutionary context as an end product of certain replicative evolutionary events whereas Walker (1969, 1971) suggests that they may have some functional role as for example in the regulation and maintainance of chromosomal organisation, the housekeeping functions of chromosomes. Southern's studies on the satellites of guinea pig (Southern, 1970) and mouse (Carr-Brown, Southern and Walker unpublished; quoted in Southern, 1970) indicate that the basic nucleotide sequence of satellite DNA is very short, but that it has been subject to random mutations at a rate not less than that of the remainder of the genome. This suggests that gradual multiplication with an accumulation of mutations is more likely than saltatory replication as a method of origin of satellite DNA, and that the sequence is not involved in the satellites function, since it does not appear subject to conservation or selection.

The fractionations achieved by Yasmineh and Yunis (1969, 1970,

1971) showed an association of satellite DNA with the "heterochromatin" fraction and according to their hypothesis, constitutive heterochromatin is present in all animals at all developmental stages. However Frenster et al (1963) had argued that the heterochromatin represents the repressed portion of the genome and in a non-differentiated developing cell this fraction should be derepressed. If it is possible to isolate a fraction corresponding to cytological heterochromatin, then it is of particular importance to see if the condensed fraction is constant throughout development. The use of male rats excludes the presence of facultative heterochromatin in the condensed chromatin fraction, and the study of regenerating liver in male rats provides a suitable developmental system in which the method of preparation is relatively simple and the yield of material adequate.

Higgins and Anderson (1932) reported that the removal of two-thirds of the liver of rat was followed within 24 hours by very rapid regeneration of the liver. The proliferative response is indicated by a burst of DNA synthesis which can be measured by the incorporation of radioactive precursors into DNA. This begins to occur about 15-18 hours after partial hepatectomy and rises to a peak at 20-24 hours. Mitotic activity follows the DNA labelling by about 6-8 hours (Harkness 1952; Grisham 1960, 1962). In regeneration (or more specifically, compensatory hypertrophy and hyperplasia) all the cellular elements are not restored in parallel. Conspicuous early changes and enlargement occur in the parenchymal cells which constitute 90-95% of the total hepatic cell mass but only 60-65% of the population. After partial hepatectomy, the

synchronisation of the cells is such that approximately 60% of the cells will enter mitosis together. (Harkness 1957; Daost 1958; Kosterlitz 1958; Leong et al 1959; Brestwick 1964). Few systems with synchronisation of this magnitude can be achieved in vivo. The mitotic activity of normal adult rat liver is in the range of 0.005% to 0.01% of the total parenchymatal cell population, (Blues and Marble 1937; Abercrombie and Harkness 1951). Consequently, these cells have a minimal rate of DNA synthesis.

In contrast to the relatively delayed changes noted in the synthesis of DNA in regenerating liver, alterations in the RNA precursor incorporation are observed as early as 2 hours after hepatectomy (even in normal rat liver, RNA turns over at an appreciable rate). There are several reports of an acceleration of incorporation between 3-6 hours (Bucher and Swaffield 1969; Schneider and Potter 1957; Hecht and Potter 1958). The maximum rate of RNA synthesis is observed from 12-30 hours postoperatively (Ericsson et al 1951; Hammerston 1951; Nygaard and Rusch 1955).

Using RNA-DNA hybridisation and competition, Church and McCarthy (1967) found that the increase in the level of hybridisation was consistent with a dramatic increase in the number of active sites after partial hepatectomy, but that as regeneration proceeds, the synthesis of the different molecules is gradually discontinued at different stages. The new RNA species, synthesised in response to partial hepatectomy were compared with those formed during embryonic development, and it was found that embryonic liver RNA can compete with RNA produced during regeneration. Their conclusion from these experiments was that in adult liver, many genes are

repressed but that during liver regeneration there is a reactivation of genes similar to the process occuring during normal development. Under the conditions used in these experiments only RNA complementary to the highly reiterated DNA would hybridise (Melli and Bishop, 1969), and it is unlikely that specific messenger RNAs involved in regeneration would be detected.

Paul (1970) reports experiments which demonstrate the marked alteration of masking of the DNA in the chromatin of regenerating rat liver and kidney. These experiments show that by measuring the transcription of RNA *in vitro* of isolated chromatin fractions, the DNA appears to remain masked in a way which reflects the masking of the same sequences in the animal. These investigations were performed in conditions in which only the repetitious sequences were hybridised, but it is possible that the situation which was observed with regard to these sequences might reflect the behaviour of some of the non-repeated sequences.

In the absence of a satellite DNA fraction in rat, the question of whether a significant chromatin fractionation could be achieved was central to the investigation, and an extension of this question was whether a condensed chromatin fraction would contain an enrichment of repeated sequences. Mouse satellite DNA appears not to be transcribed *in vivo* (Flamm, Walker, McCallum 1969) and it was therefore of interest to see whether the condensed chromatin fraction of rat was transcribed *in vivo* by doing hybridisation studies in conditions of DNA excess with cellular RNA.

The existence of a unique fraction of RNA in the nucleus of eukaryotic cells was demonstrated in duck erythroblasts by Scherrer,

Harcaud, Lajdela, Loudon and Gross (1966); Attardi, Parnas, Hwang and Attardi, (1966) and in HeLa cells by Warner, Sociro, Birnboim and Darnell, (1966), Penman Smith and Holtzman (1966). The RNA is heterogeneous in sedimentation behaviour with S values from 10-200S. It has a DNA-like base composition and hybridises rapidly with cellular DNA (Perry, Srinvasan and Kelly, 1964; Birnboim, Pene and Darnell, 1967; Sociro, Birnboim and Darnell, 1966; Yoshikawa Fukada, Fukada and Kawade, 1965; Attardi et al 1966). It is probable that this RNA is the rapidly turning over RNA whose presence was inferred by Harris (1963) and which has variously been referred to as nucleoplasmic RNA, giant RNA, DNA-like-RNA, and heterogeneous nuclear RNA.

The nuclear heterogeneous RNA (HnRNA) represents a considerable proportion of the total RNA produced in a cell. The rapid turnover of this RNA is indicated by the fact that during short periods of incorporation of radioisotopes it reaches a high specific radioactivity and constitutes the major part of cellular RNA labelled during a short pulse. From the kinetics of formation and turnover, Attardi et al (1966) thought that HnRNA was not related to messenger RNA, and according to Penman, Vesco and Penman, (1968) the two species could not be regarded as having a precursor product relationship. Later experiments by Penman, Rosbach and Penman (1970) used the drug cordycepin (3'-deoxyadenosine) which completely inhibits the synthesis of mitochondrial RNA and messenger RNA and causes premature termination and release of nascent RNA in the nucleolus. The synthesis of 4S,5S and HnRNA does not seem to be affected by Cordycepin. Since Penman et al found that Cordycepin had no effect

on the labelling of HnRNA, they suggested that messenger RNA and HnRNA are synthesised separately.

Evidence is now accumulating which indicates that messenger RNA may be related to HnRNA. It is now known that Cordycepin blocks the synthesis of poly A sequences. These sequences are thought to be involved either in the maturation of long transcriptional units into their cleavage products (Edmonds, Vaughan and Nakazato 1971) or in the transport of mRNA from the nucleus (Lee, Mendecki and Brawerman, 1971). Experiments by Wall and Darnell (1971) have shown that in SV 40 transformed mouse 3T3 cells, SV40 specific RNA is found both in the HnRNA and cytoplasmic RNA, and that the latter is derived from the HnRNA precursor. Since the sedimentation values of HnRNA (from 200S to 10S) are much greater than those of the messenger RNA on cytoplasmic polyribosomes, it has been postulated that cleavage of the large molecules occurs. (Several RNA species in eukaryotic cells are known to pass through maturation processes which change their size and properties. Both ribosomal and transfer RNA are synthesised first as large molecules and then undergo a cleavage process, or in the case of ribosomal RNA several successive degradations which yield the two final ribosomal molecules 18S and 28S RNA). The hypothesis that mRNA might be produced by a messenger precursor is supported by Ryshov and Georgiev (1970). Indeed the theory of gene regulation proposed by Georgiev (1969) is dependent upon the cleavage of a large precursor molecule to yield both a transcribed but non-conserved portion (which is involved in regulation and degraded in the nucleus) and messenger RNA. It was therefore of importance to examine the hybridisation of HnRNA with

the fractionated chromatin DNA since this would enable me to identify the fraction of chromatin from which particular sequences in the HnRNA were transcribed.

Studies on *in vivo* RNA would only detect differences in the sequences from which that RNA was transcribed. In order to examine differences in the total sequences of the fractionation, RNA was synthesised *in vitro* with a bacterial DNA-dependent RNA polymerase using fractionated chromatin DNA as template. Hybridisation of the enzymically synthesised *in vitro* RNA with the condensed and dispersed DNA fractions in conditions of DNA excess, might reveal differences in the template DNA and the cRNA transcribed from it. Both normal and regenerating condensed and dispersed DNA fractions were used as templates for cRNA synthesis in order to detect any change in the distribution of sequences in regenerating tissue.

ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DNase	Deoxyribonuclease
RNase pancreatic	Ribonuclease from bovine pancreas
RNase T1	Ribonuclease from Aspergillus oryzae
HnRNA	Heterogeneous nuclear RNA
t-RNA	Transfer RNA
r-RNA	Ribosomal RNA
m-RNA	Messenger RNA
c-RNA	Complementary (enzymic) RNA
ATP	Adenosine 5' triphosphate
CTP	Cytidine 5' triphosphate
GTP	Guanosine 5' triphosphate
UTP	Uridine 5' triphosphate
TTP	Thymidine 5' triphosphate
Pi	Inorganic orthophosphate
PPi	Inorganic pyrophosphate
poly dAT	Polymer of the deoxynucleotides adenosine and thymidine
GC ratio	The ratio of the bases G + C to that of the bases A + T in a given nucleic acid
Cot	The product of the concentration of nucleotide sequences of the renaturing DNA (Co, in moles per litre) and the time of incubation (t, in seconds) Britten and Kohne (1968)
$Cot^{d}_{\frac{1}{2}}$	The Cot at which half the DNA is renatured
Tm	The temperature at which half the DNA is denatured.

EDTA	Ethylene diamine tetracetic acid
SLS	Sodium lauryl sulphate
SSC	Standard Saline citrate
Tris	Tris (hydroxymethyl) aminomethane
TCA	Trichloracetic acid
PCA	Perchloric acid
HAP	Hydroxylapatite (calcium phosphate)
PPO	2-5-Diphenyloxazole
Dimethyl POPOP	1, 4 bis-2- (4-methyl-5-Phenyloxazole)-Benzene

BUFFERS AND SOLUTIONS

Column Buffer

0.3M NaCl; 0.01M Na acetate pH 5.0. Prepared as a concentrated 3M sodium chloride, 0.1M sodium acetate solution.

Standard Saline Citrate

0.15M sodium chloride, 0.015 tri-sodium citrate. The pH is adjusted to either 7.5 or 5.0 with concentrated HCl.

Pancreatic RNase

Ribonuclease A from bovine pancreas, 5 times crystallised. This is dissolved in 2 x SSC pH 4.5 at 10mg/ml and boiled for 5 minutes in a boiling water bath to inactivate any contaminating DNase. The solution is chilled and the pH adjusted to 7.5 with 0.1NaOH.

T1 RNase

Ribonuclease T1 from Aspergillus oryzae in 2 x SSC at a concentration of either 10,000 or 1,000 units per ml.

DNase

Deoxyribonuclease 1 from bovine pancreas, electrophoretically purified to free it from contaminating RNase. This is dissolved in 0.1M potassium acetate pH 5.0 at a concentration of 2mg/ml.

Toluene-PPO POPOP counting fluid for ^{32}P and ^{14}C

Contains 12.5g PPO (2, 5-Diphenyloxazole) Scintillation Grade and 750mg dimethyl POPOP (Scintillation Grade) in 2.5 litres of Analar Toluene.

Butyl PBD-Toluene-2 ethoxyethanol counting fluid for ^{3}H

8g of Butyl PBD in 1 litre of Analar Toluene. 600ml of Butyl PBD-toluene + 400ml 2-ethoxyethanol.

Zinc Chloride treated Visking Dialysis Tubing

Dialysis tubing placed in a 64% $ZnCl_2$ solution and left for 15'. The tubing was washed three times with distilled water, three times with dilute (0.5N) HCl and three times again with distilled water. The tubing was then boiled in 0.01M EDTA pH 7.0 and washed thoroughly with distilled water.

Bovine Serum Albumen

Albumen (fraction V) from bovine serum was dissolved at a concentration of 5mg/ml in 100ml distilled water.

1.2M Phosphate Buffer

1.2M NaH_2PO_4 and 1.2M Na_2HPO_4 mixed in equal volumes.

MATERIALS

DNase I	
Pancreatic RNase	Sigma Biochem. Inc. London.
T1 RNase	
Protease	
Tris	
Bovine Serum Albumen	
HAP (Calcium phosphate)	Kodak
Diethyloxidoformate	
^{32}P orthophosphate	
^{3}H uridine triphosphate	Radiochemical Centre, Amersham.
^{3}H thymidine	
^{14}C adenosine triphosphate	
Male albino rats	Small Animal Breeding Station, Bush House, Edinburgh.
Oxoid Filters	
Dulbecco Saline	Oxoid Ltd. London
Sephadex	Pharmacia
GF/C glassfibre	Whatman
Filter paper	

MATERIALS (cont.)

Butyl PBD	Ciba Ltd
POP	Packard Ltd.
POPOP (Scintillation)	

With the exception of the materials above, all chemicals were obtained as 'Analar' grade reagents from British Drug Houses Ltd.

(1) Preparation of Nuclear DNA

1. Male rats, body weight 170 gram (± 15g) were starved overnight, and killed by decapitation.

2. Nuclei were prepared from the rat livers by a modification of the method of Chauveau, Moule and Roviller (1956). The livers were removed and placed in ice-cold 0.25M sucrose; 0.025M KCl; 0.05M Tris-HCl pH 7.5; 4mM $MgCl_2$, blotted, weighed and homogenised at 4°C in 10 volumes 2.2M sucrose; 3mM $MgCl_2$ using a glass teflon homogeniser.

3. The homogenate was spun for 1 hour at 0°C in the 8 x 50 rotor of the MSE Superspeed 65 Ultracentrifuge at 32,000rpm.

4. DNA was prepared from the nuclei by a modification of the method of Birnstiel, Wallace and Fischberg (1966) which is derived from that of Marmur (1961). The nuclei were lysed in 1% SLS; 0.1M NaCl; 0.01M Tris-HCl pH 7.5 at 20°C; 10mM EDTA. One-tenth volume 3M Tris pH 8.3 and one quarter volume 5M $NaClO_4$ were added and the DNA was deproteinised by shaking with an equal volume of 1:1 chloroform: redistilled phenol.

5. The suspension was cleared by centrifugation and the interphase and phenol phase re-extracted with an equal volume of 1M sodium perchlorate, 0.25M Tris.

6. The pooled aqueous phases were then extracted with an equal volume of 1:1 chloroform: phenol and centrifuged at 8,000rpm in the GSA rotor of the Sorvall for 10 minutes.

7. The aqueous phase was placed in $ZnCl_2$ treated Visking dialysis bags and dialysed against 3 changes of 0.1 x SSC and then against 2 x SSC.

8. The dialysed solution was then treated with 50µg/ml of pancreatic RNase and 4 units/ml of T1 RNase at 37°C for 2 hours. 400µg/ml of Pronase were added and the incubation continued for a further 2 hours.

9. The solution was deproteinised with an equal volume of 1:1 chloroform phenol. Shaken by hand for 10 minutes and centrifuged at 8,000rpm in the GSA rotor of the Sorval. The aqueous phase was re-extracted with an equal volume of 1:1 chloroform: phenol.

10. The aqueous phase was dialysed exhaustively against 2 x SSC to remove nucleotides, chloroform and phenol.

11. The DNA solution was then treated with a second cycle of RNase pronase digestion (repeat of steps 8-10).

12. The solution was then dialysed exhaustively against 0.1 x SSC and the DNA concentrated by centrifugation at 32,000rpm for 16 hours in the 8 x 50 rotor of the MSE Superspeed 65 Ultra-centrifuge. The pellets were resuspended in a small volume of 0.1 x SSC pH 7.0.

(2) Preparation of Nuclear RNA

1. 5 male rats, body weight 170 gram (± 15g) were starved overnight and killed by decapitation.

2. The livers were removed and nuclei purified as described in Section 1 steps 2 and 3.

3. The nuclei were lysed in 1% SLS, 0.1M NaCl; 0.01M Tris-HCl pH 7.5; 10mM EDTA and the solution deproteinised by extraction with an equal volume of chloroform; redistilled phenol. The solution was shaken by hand for 10 minutes and centrifuged at 8,000rpm in the GSA rotor of the Sorvall.

4. The phenol phase and interphase were re-extracted with 0.1M NaCl, 0.01M Tris-HCl 10mM EDTA. Shaken for 10 minutes and centrifuged at 8,000rpm.

5. The pooled aqueous phases were re-extracted with an equal volume of 1:1 chloroform: phenol.

6. The nucleic acids were precipitated from the aqueous phase by the addition of one tenth volume of 2M sodium acetate pH 5.0 and 2 volumes of absolute alcohol. The solution was placed at -20°C for a minimum of 1 hour. The nucleic acids were pelleted by centrifuging at 10,000rpm for 10 minutes in the Sorvall.

7. The pellet was resuspended in 50mM Tris-HCl pH 7.5, 7mM $MgCl_2$. 50μg/ml of electrophoretically pure DNase was added and the solution incubated for 30 minutes at 37°C.

8. The solution was extracted with 1:1 chloroform: phenol, shaken for 10 minutes by hand and centrifuged at 10,000rpm for 10 minutes. The aqueous phase was re-extracted with 1:1 chloroform: phenol.

9. The RNA was precipitated from the aqueous phase by the addition of 1/10 volume 2M sodium acetate pH 5.0 and 2 volumes of absolute alcohol.

10. The centrifugal pellet was dissolved in 2ml of 0.3M NaCl; 0.01M sodium acetate and passed through a 2.5cm x 45cm (bed volume 200ml) column of Sephadex SPc 50 to free the RNA from nucleotides and oligonucleotides. Fractions were eluted with 0.3M NaCl, 0.01M sodium acetate.

11. The UV absorbancy of each fraction was examined in the Beckman DB Spectrophotometer at 260, 280 and 320 nanometers.

12. The peak fractions from the column were precipitated with 2 volumes of absolute alcohol placed at $-20^{\circ}C$ for 1 hour and centrifuged at 10,000rpm for 15 minutes. The pellets were redissolved in a small volume of 0.1 x SSC pH 7.0.

(3) Tests for purity of DNA

1. Initially, the purity of the DNA was checked by examining its ultraviolet absorption spectrum in a spectrophotometer

$$E_{260}/E_{280} = 1.66$$

$$E_{260}/E_{230} \quad 2.$$

A. Method 1 for radioactively labelled DNA

2. The DNA was tested by digestion with DNase and RNase. Four aliquots of DNA in 50mM Tris; 7mM $MgCl_2$ were prepared. To two of these aliquots, 20μg/ml of electrophoretically pure DNase were added and all the samples were incubated at 37°C for 20 minutes.

3. 4 aliquots of the DNA in 2 x SSC were prepared and the samples were placed in a boiling water bath for 6 minutes and quenched ice. Two of the samples were digested with 20μg/ml of pancreatic RNase and the remaining tubes served as controls. All the samples were incubated at 37°C for 30 minutes.

4. All the samples were precipitated by the addition of 500μg of Bovine Serum Albumen and then 50% TCA to a final concentration of 10%. The samples were allowed to stand for 20 minutes and filtered onto GF/C glassfibre filters, each tube washed 5 times with 5% TCA and filtered, and the filters were dried in a vacuum oven.

5. Samples in which the RNA was labelled with ^{3}H were digested with 0.4ml of 0.88 s.g. ammonia for 2 hours and then counted in 10ml Butyl PBD-Toluene-2 ethocyethanol. To samples labelled with ^{32}P or ^{14}C, 10ml of Toluene based scintillation fluid were added. All samples were counted in a Packard Tricarb Liquid Scintillation Counter 3320.

6. As an alternative to RNase and DNase digestion, 4 aliquots of the solution were prepared, and 2 were made 0.3M with KOH. All the samples were taken to the same volume and incubated at 37°C for 1 hour. The samples were then precipitated with BSA and TCA as in Sections 3.4 and 3.5.

B. For non-isotopically labelled DNA

7. The optical density of duplicate samples containing approximately 50µg/ml of DNA was measured in the Beckman DB Spectrophotometer at 260 and 320nm.

8. One tenth volume of 6N KOH was added to each DNA sample and to the water blanks. The samples were incubated at 37°C for 1 hour. A volume of 70% perchloric acid equal to that of the KOH was added to each sample. The samples were mixed and allowed to stand for 20 minutes in ice.

9. The precipitate of potassium perchlorate and DNA was centrifuged in the HB4 rotor of the Sorvall at 10,000rpm for 15 minutes. The supernatants were carefully decanted into clean tubes and the absorption spectrum of the supernatants at 260nm were read against that of the water blanks.

10. Only DNA samples having negligible contamination by alkali-labile or cold acid-soluble material were used in experiments.

(4) Methods for testing the contamination of RNA by DNA and RNase sensitivity of RNA

1. The ultraviolet absorption spectrum of the rat liver RNA solution was examined

$$E_{260}/E_{280} = 2.$$

A. Contamination by alkali - resistant radioactively labelled material tested by KOH digestion

2. Four aliquots of the RNA solution were taken, and two samples made with 0.3N with KOH. All the samples were adjusted to the same volume and incubated at 37°C for 1 hour.

3. After the incubation 500µg of yeast carrier RNA or 500µg of bovine serum albumen were added and all the samples were adjusted to 10% TCA with 50% TCA. The precipitated samples were allowed to stand for 20 minutes and were collected by filtering through 3cm oxoid filters or GF/C glassfibre discs under gentle vacuum. Each tube was rinsed 5 times with 5% TCA and the filters then washed with 20mls of 5% TCA. Oxoid filters were dried with 1:1 alcohol ether, and ether and glassfibre discs dried in a vacuum oven for 30 minutes. The discs were counted as described in Section 3.5.

B. RNase and DNase digestion of isotopically labelled RNA

4. An estimate of the purity of an RNA preparation and the amount of RNA in a double stranded state was gained by pancreatic RNase digestion of the samples with heating and quenching.

Heating of double stranded RNA denatures it and if rapidly cooled and then subjected to RNase digestion, all the RNA is susceptible to digestion in contrast with undenatured double stranded RNA which is resistant to pancreatic RNase.

5. A solution of the RNA to be tested was divided into 12 aliquots. 8 aliquots were adjusted to a salt concentration of 2 x SSC and 4 of these samples were placed in a boiling water bath for 6 minutes, then quenched in ice. 2 of the boiled samples were digested with 20µg/ml of heat treated pancreatic RNase and the remaining 2 samples served as treated control tubes. 2 of the 4 untreated tubes were treated with RNase and the 2 remaining tubes as their controls. All samples were incubated at 37°C for 30 minutes.

6. The remaining 4 aliquots were made 7mM with respect to $MgCl_2$ and 50mM with Tris-HCl pH 7.5 at 30°C. 2 samples were treated with electrophoretically pure DNase and the two samples and 2 controls were incubated at 37°C for 20 minutes.

7. All the samples were precipitated with BSA and TCA at the end of the incubation period as described in Section 3.4 and prepared for liquid scintillation counting as described in Section 3.5.

8. The presence of TCA precipitable counts in the denatured RNase treated samples indicated contamination of the RNA, whilst the proportion of counts in the unboiled RNase heated samples as a percentage of the total number of counts indicated the amount of double stranded RNA in the original solution.

(5) Sonication and Purification of DNA for Renaturation and Hybridisation

1. DNA prepared by two cycles of RNase - pronase treatment and in which there was negligible contamination by alkali-labile material (Materials and Methods, Sections 1 and 3) was suspended at a concentration of 500μg/ml in 0.1 x SSC.

2. 15ml aliquots of this solution were placed in glass vials, and the vials placed in ice baths. Each aliquot was sonicated for pulses of 15 seconds with intervals of 2 minutes between pulses to a total of 1.5 minutes sonication with a Dawe sonicator set at position 8.

3. After sonication, the DNA was precipitated by the addition of 2 volumes of absolute alcohol and $^{1}/_{10}$ volume of 2M sodium acetate pH 5.0 and placed at -20°C for a minimum period of 2 hours.

4. The precipitated DNA was centrifuged at 10,000rpm for 15 minutes in the HB4 rotor of the Sorvall. The pellet of DNA was taken up in 2ml of column buffer (0.3M NaCl, 0.01M sodium acetate).

5. 20mg of DNA were loaded onto each 2.5 x 45cm column of SPc50 Sephadex. The DNA was eluted with column buffer and the optical density of each 2.5ml fraction collected was examined at 260, 280 and 320 nanometers in the Beckman DB Spectrophotometer. The peak fractions were pooled and precipitated with 2 volumes of absolute alcohol and stored at -20°C.

(6) Assays for DNA, RNA and Protein in the chromatin fractions

1. DNA Diphenylamine reaction

DNA was assayed by the diphenylamine reaction derived from that of Giles and Myers (1965) which in turn is a modification of the method of Burton 1952; 1956.

2. RNA _ Orcinol reaction

RNA was assayed by methods of Schneider (1957) and Volkin and Cohne (1954).

3. Protein Lowry method for protein determination

The method of Lowry (1951) was used.

(7) Melting Curves of DNA samples (Optical Denaturation)

1. 2.5ml samples of the DNA solutions containing about 50μg/ml of DNA (1 $OD_{(260)}$) of the test DNA in a salt concentration of 0.1 x SSC (Na^+ equivalent = 0.0195M) were placed in quartz cuvettes with a 10mm light path.

2. The melting experiments were performed in a Unicam SP800 spectrophotometer fitted with an SP825 sample changer and thermostatically controlled heat block. The cuvettes were placed in the block and the ultraviolet absorption spectrum of each sample recorded from 320 to 230nm at 30°C. (The temperature in the block was measured by means of a thermister placed in the 4th cuvette containing a solution of 0.1 x SSC).

3. The extinction of each sample at 260nm was recorded on a chart three times at 5 minute intervals. After the third reading the temperature in the block was raised. (The temperature was controlled by means of a Julabo Paratherm mercury contact thermometer). The rise in temperature was 3°C every 15 minutes.

4. The ultraviolet spectrum of each sample at wavelengths from 320-230nm was drawn at 15°C intervals to ensure that there was no change in the background reading (320nm). In some cases the whole melt of the DNA was measured by recording the spectra of each DNA instead of measuring the extinction at 260nm.

5. The melting curve was plotted by calculating the extinction at 260nm for each temperature as a percentage of the final denatured E_{260} value.

(8) Fractionation of rat liver chromatin by a method modified from that of Frenster et al (1964)

1. Male albino rats body weight 170 gram (± 15g) were partially hepatectomised according to the method of Higgins and Anderson (1931).

2. 19 hours after hepatectomy, the animals were killed by decapitation, the livers removed and any necrotic tissue discarded. Nuclei were prepared from the livers by the method described in materials and methods Section 1.2 and 1.3.

3. The purified nuclei were washed twice with a solution containing 0.25M sucrose 0.003M $MgCl_2$ and the nuclei were pelleted by centrifugation at 600xg in the SS34 rotor of the Sorvall between each wash.

4. The nuclei were then washed three times with a solution containing 0.1875M sucrose, 0.02M glucose, 0.025M Tris-HCl pH 7.1, 0.128M NaCl, 0.0033MgCl_2 and then with two washes of 0.01M Tris pH 7.1, 0.0033M $MgCl_2$ and finally 1 wash with 0.01M Tris ph 7.1, 3mM $CaCl_2$.

5. The nuclei were suspended in 0.25M cation free sucrose and allowed to swell for 10 minutes. 15ml aliquots were placed in glass vials and the vials placed in ice baths, and the nuclei were sonicated with a Dawe sonicator.

6. After sonication for the required period of time, the solution was filtered through cheesecloth and then spun at 100xg for 5 minutes in the Sorvall to pellet whole nuclei and membranes.

7. The supernatant from the 100xg spin was centrifuged at 1,000xg for 10 minutes in the SS34 rotor of the Sorvall. The pellet from this fraction was termed 'heterochromatin' by Frenster et al, but in these experiments it is termed the '1,000xg condensed fraction'.

8. The supernatant from the 1,000xg spin was centrifuged at 3,000xg for 30 minutes. The pellet was the intermediate fraction of chromatin.

9. The supernatant from the 3,000xg spin was centrifuged at 78,000xg in the Spinco Model L Ultracentrifuge in the # 30 rotor. The pellet from this centrifugation was termed the 78,000xg 'dispersed' chromatin (euchromatin of Frenster).

10. The supernatant was made 10mM with respect to either $CaCl_2$ or $MgCl_2$, and the particles produced were recovered by centrifuging at 1,000xg for 10 minutes.

11. The pellets of each fraction were resuspended in a medium suitable for the subsequent procedure. For example, 1% SLS 0.1M NaCl 0.01M Tris 0.01M EDTA for the purification of DNA from the chromatin fractions.

(9) Fractionation of chromatin by a modification of the method of Yasmineh and Yunis (1969)

1. For experiments upon normal animals, male rats were starved overnight and killed by decapitation. Alternatively, in experiments involving regenerating liver, animals were partially hepatectomised according to the method of Higgins and Anderson (1931) and killed 19 hours after hepatectomy.

2. The livers were removed, placed in 0.25M sucrose, 0.025M KCl, 0.05M Tris-HCl, 4mM $MgCl_2$. Nuclei were then purified from the livers by the method described in Materials and Methods Sections 1.2 and 1.3.

3. The purified nuclei were washed three times with 0.01M Tris pH 7.1, 0.0015M $CaCl_2$ and recovered by centrifugation at 700xg between each wash.

4. The washed nuclei were then carefully resuspended in 0.25M cation free sucrose and allowed to swell for 10 minutes. The solution of swollen nuclei was adjusted to an OD of 1.0 at 425 nanometers.

5. The nuclei were sonicated by placing 15ml aliquots in glass vials, which were immersed in an ice bath to prevent the sonicate from overheating and sonicating with a Dawe sonicator set at position 6 for short pulses of 15 seconds to a total of 15, 30 or 60 seconds.

6. The solution was spun at 100xg for 5 minutes in the SS34 rotor of the Sorvall to pellet any whole nuclei or clumps of nuclei.

7. The supernatant was spun at 3,500xg for 20 minutes and the pellet from this fraction constituted the 3,500xg condensed chromatin fraction.

8. The supernatant was spun at 78,000xg in the MSE 8 x 50 rotor in the Superspeed 65 Ultracentrifuge. The pellet from this fraction formed the intermediate fraction.

9. The post 78,000xg supernatant was precipitated with 2 volumes of absolute alcohol. The pellet obtained by centrifuging this precipitate is the post 78,000xg dispersed chromatin fraction.

10. The chromatin pellets were immediately resuspended in a medium suitable for the subsequent extraction procedure i.e. 0.2% SLS for RNA or 1% SLS, 0.1M NaCl, 0.01M Tris, 0.001M EDTA for DNA.

(10) The fractionation and specific radioactivity of ^{32}P labelled RNA from regenerating liver

1. For each experiment, 10 rats were partially hepatectomised according to the method of Higgins and Anderson (1931).

2. 18 hours after hepatectomy each rat was injected with 3mCi of Phosphorus-32 orthophosphate (Radiochemical Centre, Amersham) at high specific activity in neutralised solution.

3. The animals were sacrificed 1 hour after the administration of the isotope, the livers removed and nuclei purified as described in sections 1.2 and 1.3 of Materials and Methods.

4. Fractions of chromatin were prepared from the purified nuclei either by the method derived from that of Freuster et al described in Section 8 of Materials and Methods, or by the method based upon that of Yasmineh and Yunis given in Section 9.

5. Experiments were undertaken in which the nuclei were sonicated for a total of either 5, 15, 30 or 60 seconds in the chromatin fractionation.

6. The pellets from each individual fraction (isolated by either method) were resuspended in 2ml of 0.1% SLS and stirred with a

glass rod in an ice-bath until suspended.

7. The solutions were made 7mM with respect to $MgCl_2$ and mixed. 50μg/ml of electrophoretically pure DNase was added to each solution, and the solutions incubated at 30°C for 10 minutes.

8. 1/10 volume of 3M Tris pH 8.3 and 1/4 volume of 5M $NaClO_4$ were added, the solutions mixed and a volume of 1:1 chloroform: phenol equal to the total volume was added to each fraction. The mixtures were shaken for 5 minutes and centrifuged for 10 minutes at 10,000rpm in the HB4 rotor of the Sorvall.

9. The phenol phase and interphase from each fraction were extracted with an equal volume of 1M $NaClO_4$ and 0.3M Tris. The aqueous phases of each fraction were pooled and extracted with an equal volume of 1:1 chloroform: phenol.

10. The RNA from each fraction was precipitated from the aqueous phase with 2 volumes of absolute alcohol. The solution was then placed at -20°C for a minimum of 2 hours.

11. The RNAs were pelleted by centrifugation and resuspended in 1ml of column buffer and passed through 1.5 x 30cm columns of Sephadex SPc50 at 4°C. 0.5ml fractions were eluted from each column, and 10μl aliquots from each fraction were spotted on Whatman filter paper discs, dried and placed in 5ml of Toluene based scintillation fluid and counted in a Packard Tricarb Liquid Scintillation Spectrometer.

12. The peak tubes of radioactivity eluted from each column were pooled and precipitated with 2 volumes of absolute alcohol and placed at -20°C.

13. 5-30% sucrose gradients containing 0.1M NaCl and 0.05M Tris pH 7.5 were prepared. The RNA was resuspended in 1ml of 0.1M NaCl, 0.05M Tris pH 7.5 and layered on the top of a 28ml gradient. The gradients were centrifuged at 24,000rpm for 20 hours in the 25.1 swinging bucket rotor of the Spinco Model L2 Ultracentrifuge.

14. 0.8ml fractions were collected from each of the gradients. Aliquots from each fraction were spotted on Whatman filter paper discs and counted as in 12.11. The optical density of each fraction of the gradients was read at 260, 280 and 320 nanometers in a Beckman DB Spectrophotometer.

(11) Experiments to determine the specific radioactivity of rat liver nuclear DNA (both normal and regenerating) labelled with ^{3}H thymidine

1. Normal rat liver tissue was obtained after starving normal male albino rats overnight. Regenerating rat liver was obtained by partially hepatectomising male rats by the method of Higgins and Anderson.

2. 16 hours after hepatectomy, 2 hepatectomised animals were each injected intraperitoneally with 100μCi of (Methyl-T) thymidine

at high specific activity. Normal rats were each injected with 100μCi of ^{3}H thymidine.

3. 1 hour after the administration of the isotope the animals were sacrificed and the livers removed. Nuclei were purified from normal and regenerating liver, and DNA prepared as described in Materials and Methods Section 1.2 to 1.12.

(12) Preparation of ^{32}P labelled nuclear heterogeneous RNA from regenerating rat liver

1. 6 male albino rats body weight 170 gram were partially hepatectomised according to the method of Higgins and Anderson.

2. 18 hours after operation, each animal was injected intraperitoneally with 5mCi of Phosphorus-32 orthophosphate (at a specific activity of 100 curies per milligram phosphorus).

3. 2 hours after the administration of the isotope the animals were sacrificed and the livers removed. Any necrotic tissue was discarded, and all isolation procedures were carried out at 0-4°C. Nuclei were purified as described in Section 1.2 and 1.3.

4. The purified nuclei were resuspended in 20ml of 0.2% SLS and allowed to lyse whilst the solution was stirred gently in an ice bath for 5 minutes. This solution was made 7mM with respect to Mg^{++} and 50μg/ml of electrophoretically pure DNase was added. The mixture was stirred very gently with a glass rod and left for 20 minutes in ice.

5. SLS solution was added to a final concentration of 1% and 1/10 volume of 2M sodium acetate pH 5.0 was added. To this solution an equal volume of water saturated phenol was added and the mixture shaken by hand for 5 minutes. After centrifugation at 10,000rpm for 10 minutes in the HB4 rotor of the Sorvall, the aqueous phase was removed and re-extracted with an equal volume of water saturated phenol.

6. The phenol extraction of the aqueous phase was repeated twice more, and the final aqueous phase was precipitated with 2 volumes of absolute alcohol and placed at -20°C for 16 hours.

7. After centrifugation of the precipitate, the RNA pellet was resuspended in 1ml of column buffer and passed through a 1.5 x 30cm column of Sephadex G200. The column was equilibrated at 4°C and the loading elution and collection of fractions was performed at 4°C.

8. Two aliquots of 5 microlitres were removed from each of the 0.5ml fractions (eluted from the column with 0.3M NaCl, 0.01M sodium acetate) and spotted on Whatman filter paper discs. One disc from each fraction was washed five times in 5% TCA dehydrated with 3 changes of 1:1 alcohol:ether and dried in ether. All discs were then placed in 5ml Toluene Scintillation fluid and counted in a Packard Liquid Scintillation Spectrometer.

9. The tubes containing the peak of TCA precipitable radioactivity were pooled and the RNA precipitated with 2 volumes of absolute alcohol and placed at -20°C.

10. The precipitated RNA was centrifuged in the HB4 rotor of the Sorvall at 10,000rpm for 20 minutes and resuspended in 3ml of 0.005M Tris pH 7.5 at 0°C, 0.01M NaCl, 0.001M EDTA. 1ml aliquots of this RNA solution were loaded on three 28ml 5-30% sucrose gradients containing 0.1M NaCl, 0.005M Tris, 0.001M EDTA and the gradients were centrifuged in the # 25.1 rotor of the Beckman Model L2 Ultracentrifuge at 20,000rpm for 10 hours at 0°C.

11. 0.7ml fractions were collected from each gradient and 10µl aliquots from each fraction pipetted onto Whatman paper discs and counted in 5ml toluene based counting fluid in the Beckman Liquid Scintillation Spectrometer. The absorbance of each fraction was examined at 260, 280 and 320 nanometers. The 'pellet' from each tube was recovered by resuspending it in 0.3ml of 0.1% SLS.

12. Having checked from the profiles of optical density and counts that there was no RNA breakage on the gradients all the RNA fractions having a sedimentation value greater than 80S were pooled and precipitated with 2 volumes of alcohol from a solution containing 0.2M sodium acetate and 0.5% SLS and placed at -20°C.

13. The precipitated RNA was centrifuged at 10,000rpm for 30 minutes in the HB4 rotor of the Sorvall and resuspended in 0.5ml of 0.01M NaCl. The RNA was passed through a small Sephadex G100 column to desalt it and was eluted with 0.01M NaCl.

14. 2 drop fractions were collected in shallow plastic cups.and the radioactivity of the fractions monitored. The excluded peak fractions were pooled and an aliquot removed and tested for resistance to RNase and DNase with TCA precipitation of the digested samples as described in Materials and Methods Section 4.

(13) Renaturation of DNA

1. DNA was purified from normal or regenerating rat liver as described in Section 1. Only DNA in which there was negligible contamination by cold alkali soluble material (tested as in Section 3)was used.

2. The DNA was sonicated to a molecular weight of about 100,000 and purified from contamination by heavy metal ions by passage through a Sephadex column (details of this procedure are given in Materials and Methods Section 5).

3. Each DNA was suspended at a concentration of about 30mg/ml in a solution of $^{1}/_{10}$ SSC. The concentration of the solution was determined both in neutral solution (0.1 x SSC) to determine the native value (Extinction coefficient E_{260} for DNA = 20) and in alkaline (0.6NKOH) to determine the fully denatured value (Alkali denatured extinction coefficient = 27).

4. An aliquot was removed from each DNA solution at 30D/ml to determine the molecular weight of the DNA by alkaline sedimentation in the Beckman Model E analytical Ultracentrifuge.

5. The renaturation of each DNA was examined over a range of Cot values from Cot 0.005 to a Cot of 30,000. The reaction mixtures contained either 400µg/ml, 10mg/ml or 20mg/ml of DNA Points were repeated at the same Cot values but with different concentrations of DNA incubated for the appropriate period of time to ensure that there was no differences due to the transition from one concentration to another.

6. Renaturation from Cots 0.005 to Cot 10 were generally undertaken with a DNA concentration of 400µg/ml from Cot 3 to a Cot 10,000 at 10mg/ml and from Cot 300 to 30,000 at 20mg/DNA per ml.

7. The experiments were carried out in very small tightly stoppered pyrex tubes. The salt concentration in each mixture yielded a final concentration of either 1 x SSC or 2 x SSC (1 x SSC = 0.15M NaCl 0.015M Na citrate). The DNA was denatured by heating in a boiling bath for 7 minutes in low salt (0.1 x SSC). The tubes were transferred to a paraffin annealing bath at 65°C and after 30 seconds equilibration (zero time) the appropriate amounts of concentrated (20 x SSC pH 5.0) were added to the renaturation mixture to adjust the salt concentration to either 1 x SSC or 2 x SSC, and the contents of the tube mixed thoroughly. Zero time controls were taken.

8. The renaturation reaction was stopped either by placing the dilute (400µg/ml) sample tubes in an acetone-ice bath at -15°C or by diluting the sample into a large volume of cold dilute salt solution such that the final DNA concentration in the solution was 50µg/ml (OD_{260} = 1) in a solution whose final

salt concentration was 0.1 x SSC.

9. Duplicate samples for each point of the renaturation curve were examined in a Pye Unicam SP800 Spectrophotometer. The ultraviolet spectrum of each sample was recorded from a wavelength of 325 to 220nm at a temperature of 53°C. The temperature was then increased to 90°C and the spectra of all the samples again recorded.

10. The renaturation of normal and regenerating rat liver DNA could be followed by measuring the change in extinction at 260 nanometers. At 90°C the DNA is fully denatured and above 53°C, single stranded DNA shows little or no hyperchromism. The rise in extinction at 260nm of the sample, between 53°C and 90°C is due to the hyperchromicity of the double stranded DNA and this is therefore a measure of the amount of renaturation which has taken place.

11. It is therefore possible to plot

$$\frac{E_{260(90)} - E_{260(53)}}{E_{260(90)}}$$ as a measure of renaturation

Fully denatured DNA has 138% of the E_{260} of native DNA (for rat liver DNA) i.e. it has a hyperchromicity of 38%. From the $E_{260(90)}$ it is therefore possible to calculate the E_{260} native value.

$$E_{260} \text{ native} = \frac{E_{260(90^\circ)} \times 100}{138}$$

The amount of renaturation which has taken place at a given

Cot value can thus be expressed as a percentage of the total possible renaturation by:

$$\text{Renaturation} = \frac{E_{260(90^\circ)} - E_{260(53^\circ)} \times 100}{E_{260(90^\circ)} - \frac{E_{260(90^\circ)} \times 100}{138}}$$

(14) Preparation of Complementary RNA

1. DNA was purified from both normal and regenerating condensed and dispersed chromatin fractions and from normal and regenerating liver by the methods given in Sections 1 and 3.

2. Since the nuclei had been sonicated during the fractionation of the chromatin, aliquots of each DNA were taken in order to do melting profiles of the DNA in the Unicam SP800 (Section 7) to see if the melting curve of the DNA was altered by the presence of single stranded tails. Samples were also taken to determine the molecular weight of each template DNA by alkaline sedimentation in the Beckman Model E analytical ultracentrifuge.

3. Each DNA, normal condensed, normal dispersed, regenerating condensed and regenerating dispersed was used as a primer for the synthesis of the corresponding cRNA using RNA polymerase from E.coli strain NRE 600. The polymerase was prepared by the method described by Chamberlin and Berg (1964) and purified through a glycerol gradient in low salt. (The RNA polymerase was a gift from Dr J.O. Bishop).

4. Tritium labelled UTP was obtained from the Radiochemical Centre, Amersham, 0.0769μM/ml at a specific radioactivity of 13,000. Each reaction mixture was prepared so that the incubation volume was 0.1ml. An aliquot of 0.15ml of the ^{3}H UTP was placed in the preparation tube and lyophilised. Unlabelled UTP solution was added. The final reaction mixture contained 0.4mM UTP, 0.8mM GTP, ATP, OTP; 2.5mM $MnCl_2$; 10mM Tris-HCl (pH 7.5 at 30°C); 1.6mM spermidine, 10μg of DNA template and 10μl of E.coli polymerase.

5. A 2μl sample was removed from each mixture at zero time and spotted on a filter paper disc. The mixture was incubated at 32°C for 30 minutes and the reaction stopped by placing the tube in an ice bath. A second 2μl aliquot was pipetted onto a filter paper disc for each reaction mix and the discs were washed with 5% TCA x 5, 1:1 alcohol:ether x 3 and dried with ether. All discs were counted in Toluene based liquid scintillation fluid to give an approximate measure of the incorporation over the 30 minute period.

6. Each mix was diluted with 0.315ml of 0.01M Tris 0.01M $MgCl_2$ after the addition of 0.05ml of a 10μg/ml solution of E.coli RNA. 50μg/ml of electrophoretically pure DNase was added to each reaction mixture and the solutions were incubated for 15 minutes at 37°C. After the DNase treatment 2M sodium acetate pH 5.0 was added to 0.2M and SLS was added to 0.5%. An equal volume of redistilled phenol was added to each mix, and the solution shaken by hand for 5 minutes before centrifuging at

10,000rpm for 10 minutes in the HB4 rotor of the Sorvall.

7. The aqueous phases were removed and each was re-extracted with an equal volume of phenol, shaken for 5 minutes and centrifuged at 10,000rpm for 10 minutes.

8. The RNA in the aqueous phase was precipitated with 2 volumes of absolute alcohol and resuspended in a small volume of 0.3M NaCl 0.01M sodium acetate. Each cRNA was passed through a 1.5 x 30cm column of Sephadex SPc50.

9. 0.5ml fractions were collected from each column and a 2µl aliquot from each fraction pipetted into 5ml of Butyl PBD-2-ethoxyethanol-Toluene counting fluid. Each peak fraction of radioactivity was precipitated with 2 volumes of absolute alcohol.

10. The RNA in each fraction was pelleted by centrifugation, re-dissolved in 0.1 x SSC, and tested for RNase and DNase sensitivity and TCA precipitability as described in Materials and Methods Section 4.

11. The RNA was stored at -20°C and used in the hybridisation mixture for hybridisation with normal and regenerating chromatin DNA in conditions of excess DNA.

(15) Hybridisation of ^{3}H cRNA and ^{32}P nuclear heterogeneous RNA with fractionated chromatin DNA in conditions of DNA excess

1. These experiments utilised either the ^{32}P labelled nuclear heterogeneous RNA with a sedimentation value greater than 80S

(whose preparation was described in Section 12) or cDNAs synthesised from fractionated chromatin (primer) DNA (described in Section 14).

2. DNA was purified (from total normal or regenerating rat liver nuclei, or from chromatin fractions prepared from normal and regenerating rat liver by the method derived from that of Yasmineh and Yunis described in Section 9) by the method described in Section 1, tested for purity (as described in Section 3) and sonicated to a molecular weight of about 100,000 (following the method given in Section 5).

3. The DNAs were resuspended in a small volume of 0.1 x SSC at a concentration of approximately 30mg/ml and the concentrations were checked both in neutral and alkaline solutions. Each RNA was hybridised to the different DNAs in vast excesses of DNA at Cot values from 0.005 to 20,000. At low Cot values (when the product of the concentration and time had a low value) this required both a short time of incubation and low DNA concentration.

4. Each incubation mixture contained either 400µg/ml, 10mg/ml or 20mg/ml of DNA in 0.1 x SSC, 0.2% to 2% SLS and a trace amount of the highly labelled RNA species (less than 0.1µg).

5. The mixtures were placed in small tightly stoppered pyrex tubes and heated in a boiling bath for 7 minutes to denature the DNA. They were then transferred to a 65°C paraffin annealing bath and after 30 seconds equilibration the appropriate amounts of

SSC solution were added to each mix to adjust the concentration of salt to the equivalent of 2 x SSC (0.39M sodium ion) and maintain the pH at 5.5. Zero time controls were taken.

6. The hybridisation reaction of the dilute samples, (400μg/ml) was stopped by immediately transferring the tubes to an acetone ice bath at -15°C. The more concentrated incubation mixtures (containing 10mg and 20mg of DNA per ml) which were incubated for much longer periods were overlaid by a small drop of liquid paraffin to prevent evaporation. Aliquots were removed from these hybridisation mixes at the required times for each Cot value, immediately diluted into a large volume of 2 x SSC at 4°C and placed at -20°C.

7. From each of the hybridisation samples, four 5ml aliquots were prepared. To two of these samples, 20μg/ml of pancreatic RNase were added and the duplicate RNase samples and controls were incubated at 37°C for 30 minutes.

8. The four aliquots of each sample were transferred to an ice bath, 500μg of bovine serum albumen were added to each tube as carrier and all the samples were precipitated by the addition of 50% TCA to a final concentration of 10%. The samples were allowed to stand for 20 minutes and then collected by filtration upon 2.5cm glassfibre (GF/C) discs washing each tube 5 times with 5% TCA and then washing each filter with 30ml of 5% TCA.

9. The glassfibre filters were dried in a vacuum oven for 30 minutes. For hybridisation reactions in which the RNA was

isotopically labelled with phosphorus-32, 10ml of Toluene POPOP-PPO counting fluid were added. For tritium labelled RNA the filters were digested with 0.4ml of specific gravity 0.88 ammonia for 2 hours, and then 10ml of Butyl PBD-Toluene-2- ethoxyethanol counting fluid were added. All the samples were then counted in a Packard Tricarb Liquid Scintillation Spectrometer Model 320.

10. The extent of hybrid or duplex formation is measured by the proportion of RNase resistant counts in the duplicate RNase treated samples expressed as a percentage of the total counts in the untreated control samples, and can be plotted against the product of the initial DNA concentration and the time (Cot). It is usually more convenient to plot the percentage hybridisation against the logarithm of the Cot according to the convention suggested by Britten and Kohne (1968).

(16) Analytical caesium chloride equilibrium density centrifugation of DNA derived from fractionated rat liver chromatin

1. Chromatin was fractionated by the derivation of Yasmineh and Yunis' method (following the procedure described in Section 9). DNA was purified from each fraction by the method described in Section 1 of Materials and Methods and tested for RNA contamination by the method given in Section 3.

2. Solid caesium chloride (B.D.H. grade for ultracentrifuge work) was added to 0.7ml of each DNA solution containing 5μg/ml of

the fractionated chromatin DNA with 1μg of Micrococcus lysodiekticus DNA as reference. The density was brought to 1.700g/ml and checked with a refractometer (Ifft, Voet and Vinograd, 1961).

3. The solutions were centrifuged to equilibrium (22 hours) in a Beckman Model E analytical ultracentrifuge at 44,000rpm at 25°C. The banding patterns were recorded photographically and traced from the photographs with a Joyce-Loebl microdensitometer and the buoyant density of each peak was calculated (Mandel, Schildkraut and Marmur, 1968).

RESULTS

Fractionation of Chromatin

a) Method of Frenster et al (1963)

Frenster, Allfrey and Mirsky (1963) developed their method of chromatin fractionation using calf thymus lymphocytes. When this method was applied directly to rat liver, the purified nuclei were refractory to sonication. This was remedied by substituting magnesium for calcium during the purification of the nuclei. Washing the nuclei after purification with small amounts of calcium in the washing solution (3mM) also affected the fractionation. Experiments were carried out in which nuclei were prepared in the presence of magnesium, but the final washes of the nuclei before sonication varied from three washes with magnesium to three calcium washes. The samples were then sonicated and fractionated in the usual way. Washing with calcium increased the amount of material in the condensed fraction and reduced that in the dispersed fraction. With two or three calcium washes a great deal of material could be precipitated by very gentle centrifugation (100 x g) indicating that the nuclei had been poorly disrupted.

In a second series of experiments, I investigated the effect of varying the time of sonication. Typical results are shown in Table 1. As the sonication time was increased, the amount of material in the condensed fraction fell and the amounts on the other fractions increased accordingly. The results obtained by this method were rather variable. In an attempt to increase reproducibility, I introduced a final calcium wash before sonicating the nuclei. This treatment had the effect of

Table 1

The effect of sonication on the fractionation of rat liver chromatin isolated by a method derived from that of Frenster et al (1963), using magnesium washes only

Time of Sonication	Measurement	Condensed 1,000 x g	Intermediate 3,000 x g	Dispersed 78,000 x g	Particles
5 seconds	DNA in µg	1,280	720	1,160	1,760
	% DNA	26%	15%	23%	36%
	RNA in µg	290	130	170	360
	% RNA	30%	14%	18%	38%
	Protein in µg	2,005	1,160	2,000	2,942
	% Protein	25%	14%	25%	36%
30 seconds	DNA in µg	380	900	2,400	1,730
	% DNA	7%	17%	44%	32%
	RNA in µg	190	110	440	380
	% RNA	17%	10%	39%	34%
	Protein in µg	780	1,060	3,840	3,780
	% Protein	8%	11%	41%	40%
90 seconds	DNA in µg	120	590	3,560	3,280
	% DNA	2%	8%	47%	43%
	RNA in µg	80	55	380	560
	% RNA	8%	5%	35%	52%
	Protein in µg	440	920	6,600	4,870
	% Protein	3%	7%	52%	38%

eliminating the particle fractions.

Breakage by shearing was investigated as a possible alternative to sonication. Even at a high rate of shear and after one hour of treatment most of the material remained in the condensed chromatin fraction (Table 2). A long time of treatment is undesirable because of the degradative changes which occur. The sonication procedure was therefore adopted despite the variability of the results obtained, and in succeeding experiments using the Frenster method, a sonication period of 5 seconds (as described by Frenster) was used.

Specific activities of DNAs from chromatin fractionated by Frenster methods

Frenster et al (1963) reported that when isolated calf thymus lymphocyte nuclei were incubated in vitro with radioactive nucleic acid precursors, and chromatin fractionation subsequently carried out, the "euchromatin" fraction contained a significantly greater number of counts in both DNA and RNA than the "heterochromatin" fraction. Frenster's explanation of this difference is that the "euchromatin" is the 'active' fraction in contrast to the repressed fraction or "heterochromatin".

I examined the specific activities of the condensed and dispersed chromatin DNA from rat liver prepared by the modification of Frenster's method to see whether similar marked differences in specific activity could be obtained. All the experiments reported here used in vivo labelling of the rat liver DNA and RNA. Initially, normal rats were used for study and injected with ^{32}P- orthophosphate in amounts from 1.0 to 3.5 millicuries per rat for varying periods of time. In normal rats, even when labelled with these large amounts of ^{32}P, specific radioactivities of only 2 to 10 counts per minute per

Table 2

The effects of sonication and stirring on the fractionation of rat liver chromatin isolated by a method derived from that of Frenster et al (1963) using an additional calcium wash

Time of treatment	Measurement	100 x g fraction	Condensed 1,000 x g	Intermediate 3,000 x g	Dispersed 78,000 x g
Sonication for 5 secs position 6	DNA in µg % DNA	2,875 69%	900 22%	275 7%	87.5 2%
	RNA in µg % RNA	787 71%	175 16%	100 9%	52 4%
	Protein in µg % Protein	3,450 63%	1,388 25%	415 8%	220 4%
Sonication for 30 secs position 6	DNA in µg % DNA	2,112 48%	1,000 23%	725 17%	550 12%
	RNA in µg % RNA	650 38%	675 39%	140 8%	255 15%
	Protein in µg % Protein	2,350 40%	1,900 33%	810 14%	790 13%
Stirred 1 hour (in ice) at 20µamp	DNA in µg % DNA	4,000 90%	331 8%	62.5 1%	62.5 1%
	RNA in µg % RNA	1,187 88%	112 8%	25 2%	25 2%
	Protein in µg % Protein	4,225 83%	650 13%	92.5 2%	102 2%

microgram of DNA were obtained (see Table 3). There were no apparent differences between the fractions and attempts were therefore made to resolve this problem by changing certain conditions of the system.

Regenerating liver is much more active in both DNA and RNA synthesis than normal liver. A preliminary investigation of this system was made by determining the specific radioactivity of the regenerating liver DNA 1 to 2 hours after administering ^{32}P- orthophosphate. The orthophosphate was injected at times varying from 13 to 19 hours after partial hepatectomy. At times up to 17 hours of regeneration with 1 to 2 millicurie per rat there was rather little incorporation, but at 19 hours of regeneration, a much greater amount of incorporation was found if each animal was given 5mCi of ^{32}P (see Table 4), 100μCi tritiated thymidine (at very high specific activity) was administered to both normal and hepatectomised animals to see if sufficiently high specific activities could be reached using ^{3}H as an alternative to the large quantities of ^{32}P. It was found that the total nuclear DNA extracted from normal liver had a specific activity of only 2.5 counts per minute per microgram and for 19 hour regenerating liver total nuclear DNA the specific radioactivity was only 3.5 counts per minute per microgram, thus excluding the use of tritiated thymidine for this purpose.

The minimum number of hepatectomised animals used in each fractionation experiment was 6, and the amount of isotope and number of animals which could be handled were limiting factors in the experiments. Fractionation according to the method derived from that of Frenster et al (1963) (Materials and Methods, Section 8) yielded a condensed fraction which was the pellet from a 1,000 x g

Table 3

Investigations upon normal rat liver DNA and RNA fractionated by the Frenster procedure after labelling with ^{32}P orthophosphate 3.5mCi per animal for one hour

	Measurement	100 x g fraction	Condensed 1,000 x g	Int. 3,000 x g	Dispersed 78,000 x g
DNA	Specific activity cpm μg^{-1}	2.3	2.2	2.2	2.6
	% DNA	31.2	32.7	15.2	20.9
RNA	Specific activity cpm μg^{-1}	90.8	85.1	77.9	103.3
	% RNA	29.3	30.4	18.1	22.2
PROTEIN	% Protein	31.1	25.2	21.0	22.7

Table 4

The incorporation of ^{32}P orthophosphate into DNA of regenerating rat liver chromatin fractionated according to the method derived from that of Frenster et al (1963)

Regeneration Time	Label	100 x g	Condensed 1,000 x g	Interm. 3,000 x g	Dispersed 78,000 x g	Measurement
Expt. 1	1 hour	0.17	0.23	0.27	0.49	Specific activity cpm μg^{-1}
13-14hrs	1mCi/rat	44.7%	28.4%	8.9%	18.0%	% DNA
Expt. 2	1 hour	2.8	2.4	2.3	2.9	Specific activity cpm μg^{-1}
15-16hrs	1mCi/rat	35.9%	33.8%	10.2%	20.1%	% DNA
Expt. 3	2 hours	2.3	2.2	2.4	2.6	Specific activity cpm μg^{-1}
15-17hrs	1mCi/rat	42%	24%	12%	22%	% DNA
Expt. 4	1 hour	11.5	10.9	10.0	10.1	Specific activity cpm μg^{-1}
16-17hrs	2mCi/rat	59.3%	20.3%	7.6%	12.8%	% DNA
Expt. 5	1 hour	80.8	94.1	81.6	80.9	Specific activity cpm μg^{-1}
18-19hrs	5mCi/rat	65.5%	22.2%	5.3%	7.0%	% DNA

spin and a dispersed chromatin fraction which was the pellet resulting from 60 minutes centrifugation at 78,000 x g. The values for the specific radioactivities of the DNA of the chromatin fractions can be seen in Table 4. There is very little difference between the specific activities of the condensed and dispersed chromatin fractions at any stage of regeneration up to 19 hours.

Specific activity of RNA from fractionated chromatin

The specific radioactivity of the RNA associated with each chromatin fraction was examined. The purity of the RNA preparations was checked by KoH digestion and all the counts were found to be alkali labile. The specific activities of the RNA of different fractions can be seen in Table 5. The difference in specific radioactivities between the condensed and dispersed fractions was negligible and it was concluded that this procedure did not effectively separate condensed and dispersed chromatin RNA.

b) The method of Yasmineh and Yunis

Specific activity of DNA from chromatin fractionated by a modification of the method of Yasmineh and Yunis (1969)

An alternative method of fractionation was attempted, based upon that used by Yasmineh and Yunis (1969) described in Materials and Methods, Section 9. In the initial experiments with this technique, 5mCi of ^{32}P were administered to each animal 18 hours after partial hepatectomy, and at 19 hours after operation the livers were removed. The nuclei were sonicated for 15 or 30 seconds prior to fractionation. With 15 seconds sonication, there was a considerable difference between the condensed fraction (3,500 x g fraction, corresponding to the

Table 5

Specific radioactivity of RNA from normal rat liver chromatin fractionated by the method derived from Frenster et al (1963)

Measurement	100 x g	Condensed 1,000 x g	Intermediate 3,000 x g	Dispersed 78,000 x g
Specific activity of RNA in cpm μg^{-1}	204	186	128	199
% RNA in each fraction	22.3%	45.6%	14.6%	17.5%

Each animal was injected with 5mCi of ^{32}P- orthophosphate and sacrificed one hour later.

"heterochromatin" of Yasmineh and Yunis) and the DNA of the dispersed fraction (Supernatant from the 78,000 x g centrifugation precipitated with alcohol - the"euchromatin" fraction). After 30 seconds sonication, the difference was even more marked, and with a sonication time of 60 seconds, the difference appeared to be increased slightly. The amounts of DNA in each fraction and the specific activities of each DNA were measured. The results are shown in Table 6. An obvious disadvantage of the sonication procedure is that it causes degradation of the DNA. There is a compromise state which can be achieved in chromatin fractionation in which the sonication of the DNA is sufficient to effect separation of the condensed from the dispersed fractions without causing complete degradation of the nucleic acids. Obviously in certain conditions of sonication, the fractionation is only partial. With respect to the specific activity of the DNA, the criterion of fractionation is an observable difference whilst the DNA still remains TCA precipitable. For experiments on the specific activity of DNA, sonication of the nuclei could be continued for a total of several minutes and TCA precipitable DNA still obtained.

Studies on the RNA from chromatin fractionated by the method derived from Yasmineh and Yunis

When the fractionation of chromatin is considered in terms of the association of RNA with the fractions, different conditions are required since the sonication causes the breakage of RNA and its dissociation from the chromatin. The succeeding experiments not only investigated the specific radioactivity of the RNA associated with each fraction,

Table 6

The incorporation of ^{32}P orthophosphate into DNA of 19 hour regenerating rat liver chromatin fractionated by the method derived from that of Yasmineh and Yunis (1969). Each animal labelled with 5mCi ^{32}P from 18-19 hours after hepatectomy

Time of Sonication	Measurement	Condensed 3,500 x g	Int. 78,000 x g	Dispersed Post 78,000 x g
15 seconds	Specific activity (cpm μg^{-1})	74.3	88.5	105
	% DNA	29.2	48.0%	22.8%
30 seconds	Specific activity (cpm μg^{-1})	66.9	86.5	106
	% DNA	22.2%	54.5%	23.3%
30 seconds	Specific activity (cpm μg^{-1})	69.6	100.6	91.1
	% DNA	26.4%	47.9%	25.7%
60 seconds	Specific activity (cpm μg^{-1})	64.9	98.2	95
	% DNA	17.3%	47.1%	35.6%

but also provided information about the sedimentation properties of the different species of RNA associated with each chromatin fraction. The DNA specific activity experiments indicated that the use of hepatectomised rats labelled with 5mCi of ^{32}P per animal 18 to 19 hours after operation should give sufficient incorporation. The purified RNA from each fraction was examined by centrifugation on sucrose gradients. The amount of RNA and the specific radioactivity of each fraction of the gradient was determined.

In the first experiments, nuclei were sonicated for 30 seconds prior to fractionation and purification of the RNA. The profiles (both in OD_{260} and counts) of the gradients from the condensed, intermediate and dispersed chromatin fractions are shown in Figures 1, 2 and 3. The condensed chromatin was associated with RNA which had sedimentation characteristics very similar to those observed for ribosomal RNA, the two major peaks having sedimentation values of approximately 28S and 18S. These peaks could not be observed in the sedimentation profile of the RNA from the dispersed chromatin fraction and it appeared that a major portion of the radioactivity in this dispersed fraction was contributed by a species of RNA which had a very high sedimentation value (calculated as greater than 80S). This is thought to correspond to the nuclear heterogeneous or giant RNA. The specific radioactivities of the gradient from each fraction of RNA are plotted together in Figure 4 to allow comparisons of the three fractions to be made. The intermediate fraction appears to be a mixture of the RNA species present in the condensed and dispersed fractions.

Legend to Figure 1

Profile of sucrose gradient of ^{32}P labelled RNA from the 3,500 x g condensed chromatin fraction (with 30 seconds nuclear sonication)

6 male rats were each labelled with 5mCi of ^{32}P, 18 hours after hepatectomy and sacrificed 19 hours after operation. Chromatin was fractionated from the regenerating rat liver according to the method derived from that of Yasmineh and Yunis (1969) as described in Materials and Methods Section 9. The nuclei were sonicated for 30 seconds prior to fractionation. RNA was purified from the condensed fraction (see Materials and Methods Section 10) and centrifuged on a 5-30% sucrose gradient in the #25.1 rotor of the Beckman Model L2 ultracentrifuge for 20 hours at 0°C. 0.8ml fractions were collected and 0.4ml of H_2O was added to each fraction. Radioactivity was measured by spotting 0.1ml aliquots on filter paper discs which were TCA washed, dried and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Absorption at 260nm was read in a Beckman DB Spectrophotometer.

O——O counts per minute/ml

●——● OD_{260}

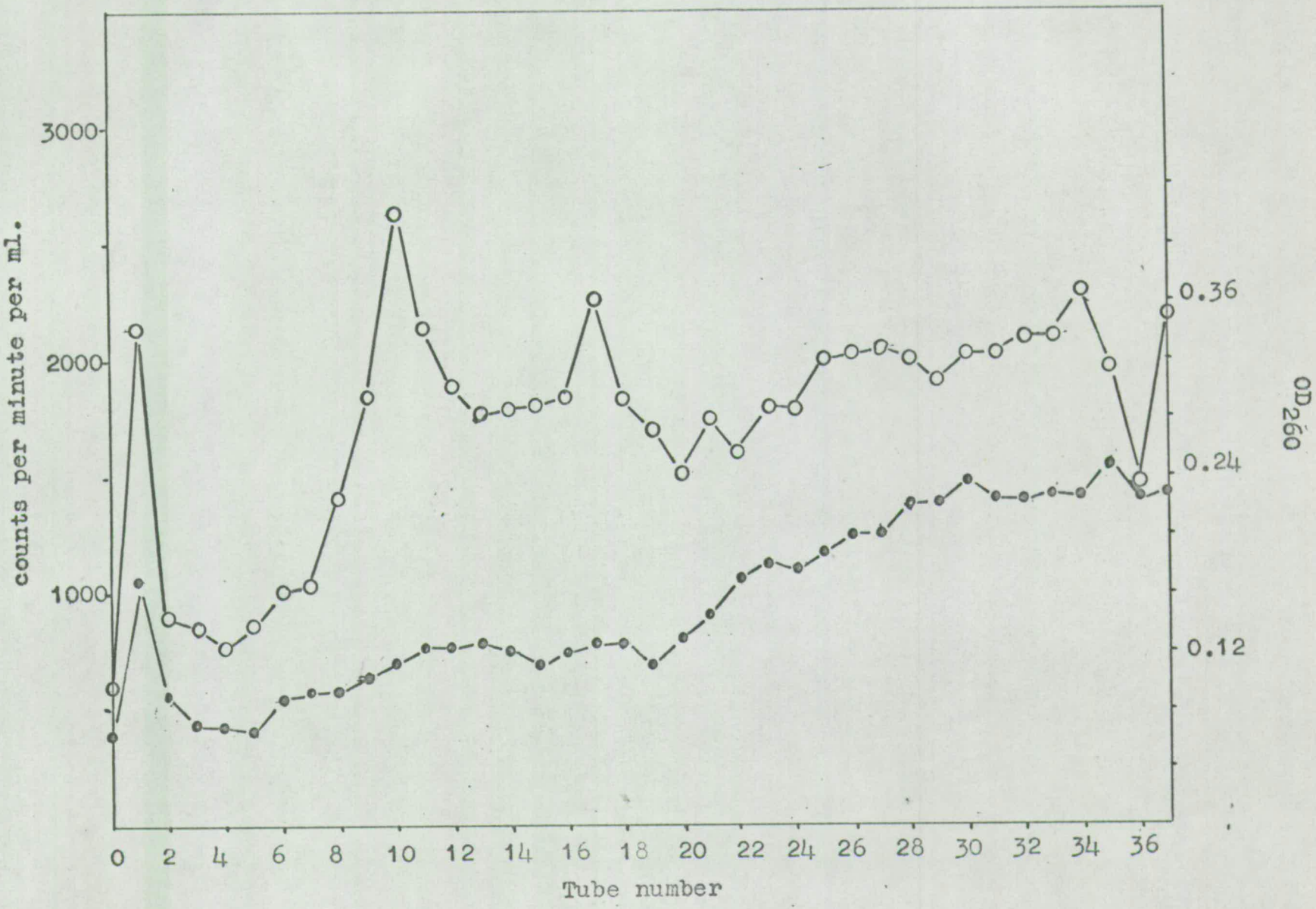

counts per minute per ml.
3000
2000
1000
0.36
0.24
0.12
OD260
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Tube number

Figure 1

Legend to Figure 2

Profile of sucrose gradient of ^{32}P labelled RNA from the 78,000 x g intermediate chromatin fraction (30 seconds nuclear sonication)

Chromatin was prepared from ^{32}P labelled 19 hour regenerating rat liver. RNA was purified from the intermediate fraction of chromatin prepared as described in the legend to Figure 1. Details of centrifugation and collection of fractions were identical.

0.1ml aliquots were spotted on filter paper discs which were TCA washed, dried and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Absorption at 200nm was read in a Beckman DB Spectrophotometer.

○——○ counts per minute/ml

●——● OD_{260}

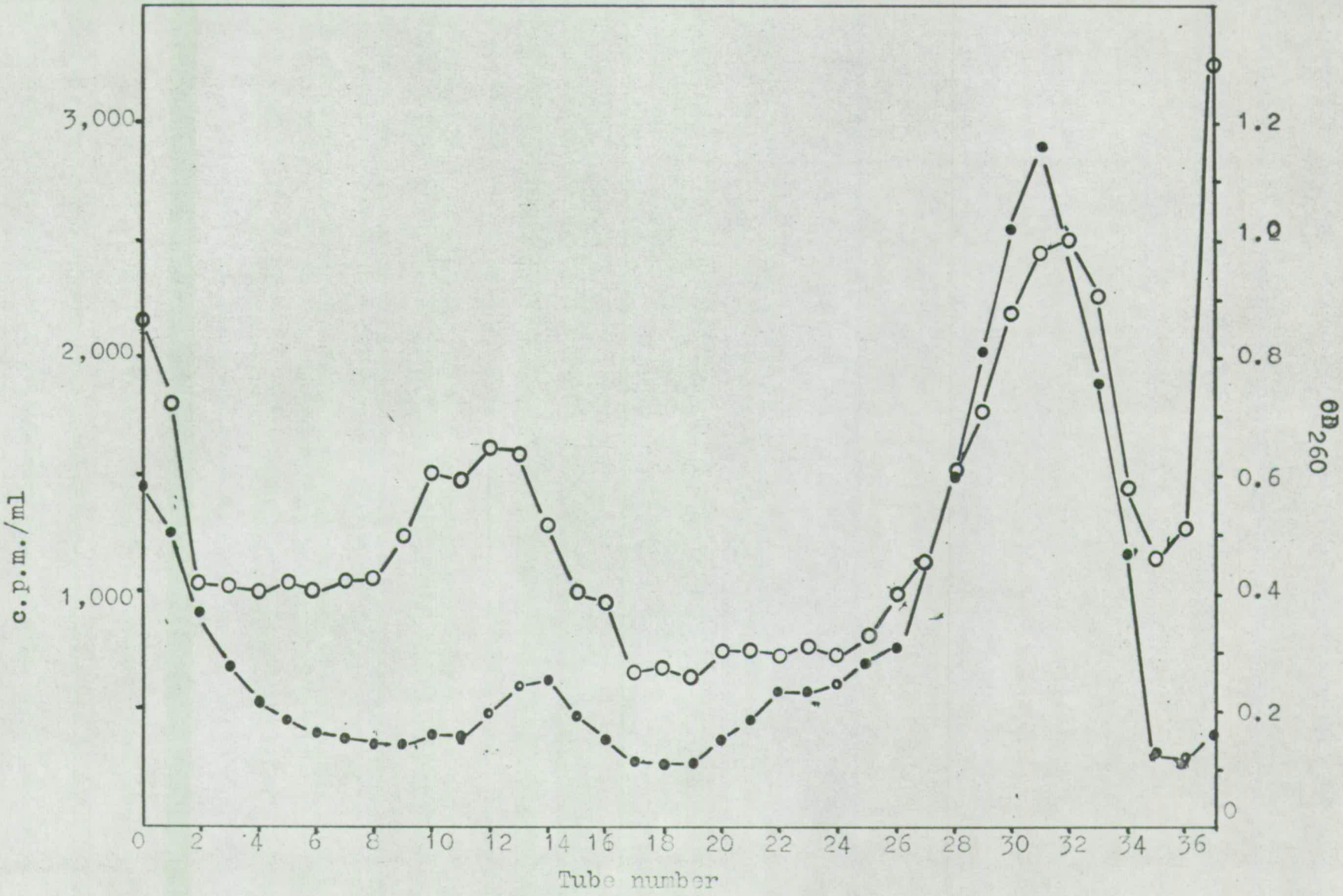

3,000
2,000
1,000
c.p.m./ml
1.2
1.0
0.8
0.6
0.4
0.2
0
OD260
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Tube number

Figure 2.

Legend to Figure 3

Profile of sucrose gradient of ^{32}P labelled RNA from the dispersed chromatin fraction (30 seconds nuclear sonication)

Chromatin was prepared from ^{32}P labelled 19 hour regenerating rat liver. RNA was purified from the dispersed fraction (post 78,000 x *g* supernatant precipitated with alcohol) of chromatin prepared as described in the Legend to Figure 1. Details of centrifugation and collection of fractions were identical.

0.1ml aliquots were spotted on filter paper discs which were TCA washed, dried and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Absorption at 260nm was read in a Beckman DB Spectrophotometer.

○——○ counts per minute per ml

●——● OD_{260}

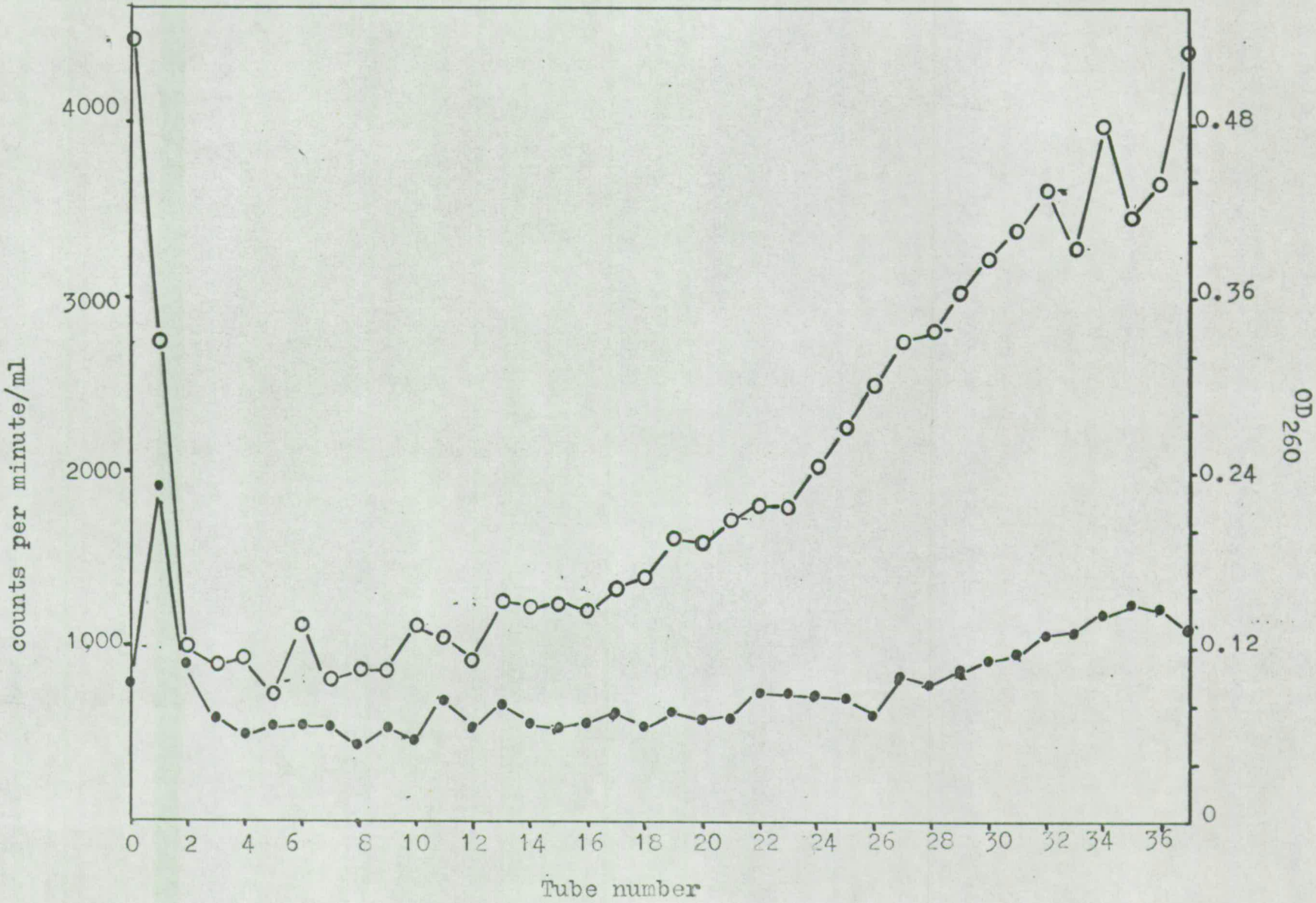

counts per minute/ml
4000
3000
2000
1000
0.48
0.36
0.24
0.12
0
OD260
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Tube number

Figure 3

Legend to Figure 4

Profiles of the sucrose gradients of the ^{32}P labelled RNA from the condensed, intermediate and dispersed chromatin fractions (30 seconds nuclear sonication)

The specific radioactivities (in counts per minute per microgram of RNA) of each tube of the condensed, intermediate and dispersed chromatin fractions (whose profiles in counts and OD_{260} were shown individually in Figures 1, 2 and 3) are shown.

●——● condensed fraction RNA

○——○ intermediate fraction RNA

□——□ dispersed fraction RNA

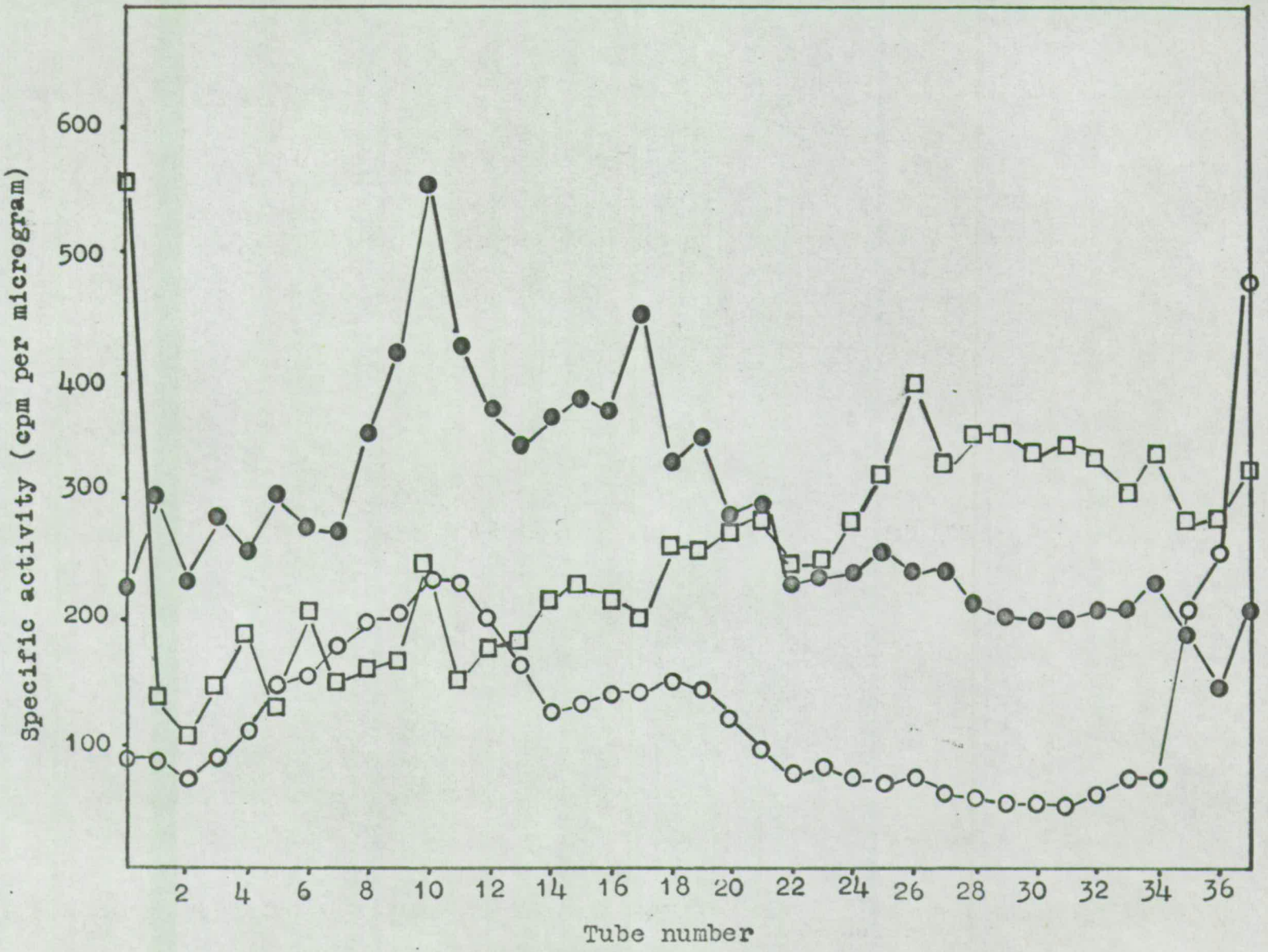

Specific activity (cpm per microgram)
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Tube number

Figure 4.

A further series of experiments increasing the sonication time to 60 seconds was performed. The profiles from the gradients of the three fractions can be seen in Figures 5, 6 and 7. If the profiles of the fractions separated after 30 seconds sonication are compared with those of 60 seconds treated RNA, the breakage effects of the greater period of sonication can be seen. There is a small peak in the 60 second condensed chromatin fraction RNA which may correspond to the 28S ribosomal RNA. However, the whole gradient is much more distorted than that of the 30 second sonicated fractions. The 60 second dispersed fraction, (the 'euchromatin' fraction of Yasmineh and Yunis), lacks the RNA with a very high sedimentation value which was seen in the sucrose gradient profile of the 30 second dispersed fraction. These are effects which are most probably attributable to degradation with the increased sonication.

Choice of fractionation method

The separation achieved by the two methods was directly compared by fractionating chromatin from the same sample of labelled nuclei, both by the adaptation of Fronster's method and by the modification of Yasmineh's procedure. The results of this experiment can be seen in Table 7. The specific radioactivity of the DNA from the condensed and dispersed fractions by Yasmineh's method show a very clear fractionation effect. The specific activity of the condensed chromatin DNA (64 counts per minute per microgram) is much lower than that of the dispersed fraction DNA at 95 cpm μg^{-1} but the fractionation by Frenster's method on the same labelled preparation showed very little difference between samples; the specific activity of all the

Legend to Figure 5

Profile of sucrose gradient of ^{32}P labelled RNA from the 3,500 x g condensed chromatin fraction (with 60 seconds nuclear sonication)

6 male rats were each labelled with 5mCi of ^{32}P 18 hours after hepatectomy and sacrificed 19 hours after operation. Chromatin was fractionated by the method of Yasmineh and Yunis with 60 seconds nuclear sonication prior to fractionation. RNA was purified from the condensed chromatin fraction (Materials and Methods Section 10) and centrifuged on a 5-30 sucrose gradient in the # 25.1 Rotor of the Beckman Model L2 ultracentrifuge for 20 hours at 0°C. 0.8ml fractions were collected, 0.4ml of H_2O added to each fraction. Radioactivity was measured by spotting 0.1ml aliquots on filter paper discs which were TCA washed, dried and counted in a Packard Scintillation Spectrometer. Absorption at 260nm was read in a Beckman DB Spectrophotometer.

O—O counts per minute per ml

●—● OD_{260}

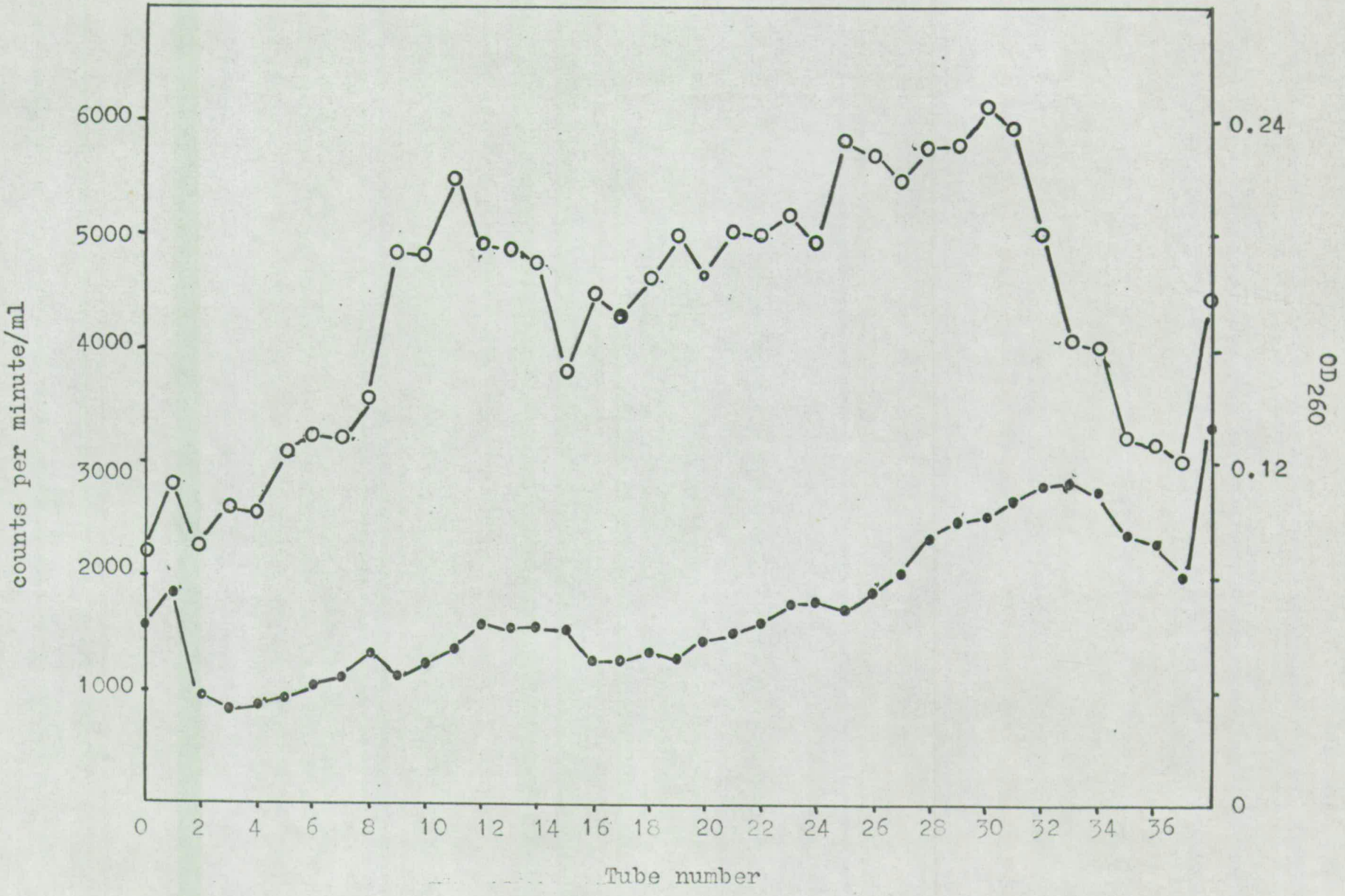

counts per minute/ml
6000
5000
4000
3000
2000
1000
0.24
0.12
0
OD260
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Tube number

Figure 5

Legend to Figure 6

Profile of sucrose gradient of ^{32}P labelled RNA from the 78,000 x g intermediate chromatin fraction (60 seconds nuclear sonication)

Chromatin was prepared from ^{32}P labelled 19 hour regenerating rat liver. RNA was purified from the intermediate fraction, prepared as described in the Legend to Figure 5. Details of centrifugation and collection of fractions were identical.

0.1ml aliquots were spotted on filter paper discs which were TCA washed, dried and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Absorption at 260nm was read in a Beckman DB Spectrophotometer.

○——○ counts per minute/ml

●——● OD_{260}

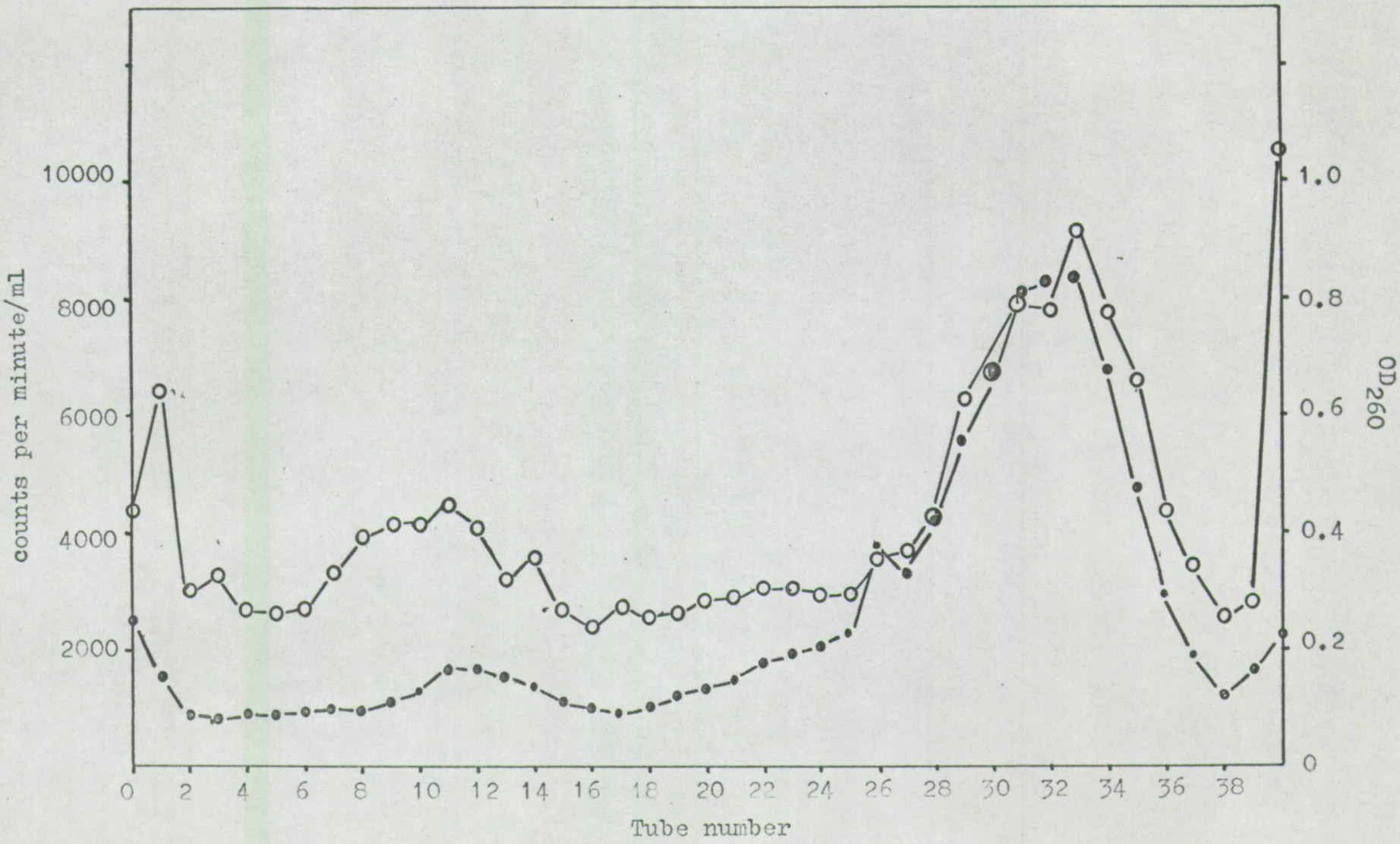

counts per minute/ml
10000
8000
6000
4000
2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
Tube number
1.0
0.8
0.6
0.4
0.2
0
OD260

Figure 6

Legend to Figure 7

Profile of sucrose gradient of ^{32}P labelled RNA from the dispersed chromatin fraction (60 seconds nuclear sonication)

Chromatin was prepared from ^{32}P labelled 19 hour regenerating rat liver. RNA was purified from the dispersed (post 78,000 x g) supernatant precipitated with alcohol) fraction of chromatin prepared as described in the Legend to Figure 5. Details of centrifugation and collection of fractions were identical.

0.1ml aliquots were spotted on filter paper discs which were TCA washed, dried and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Absorption at 260nm was read in a Beckman DB Spectrophotometer.

O——O counts per minute/ml

●——● OD_{260}

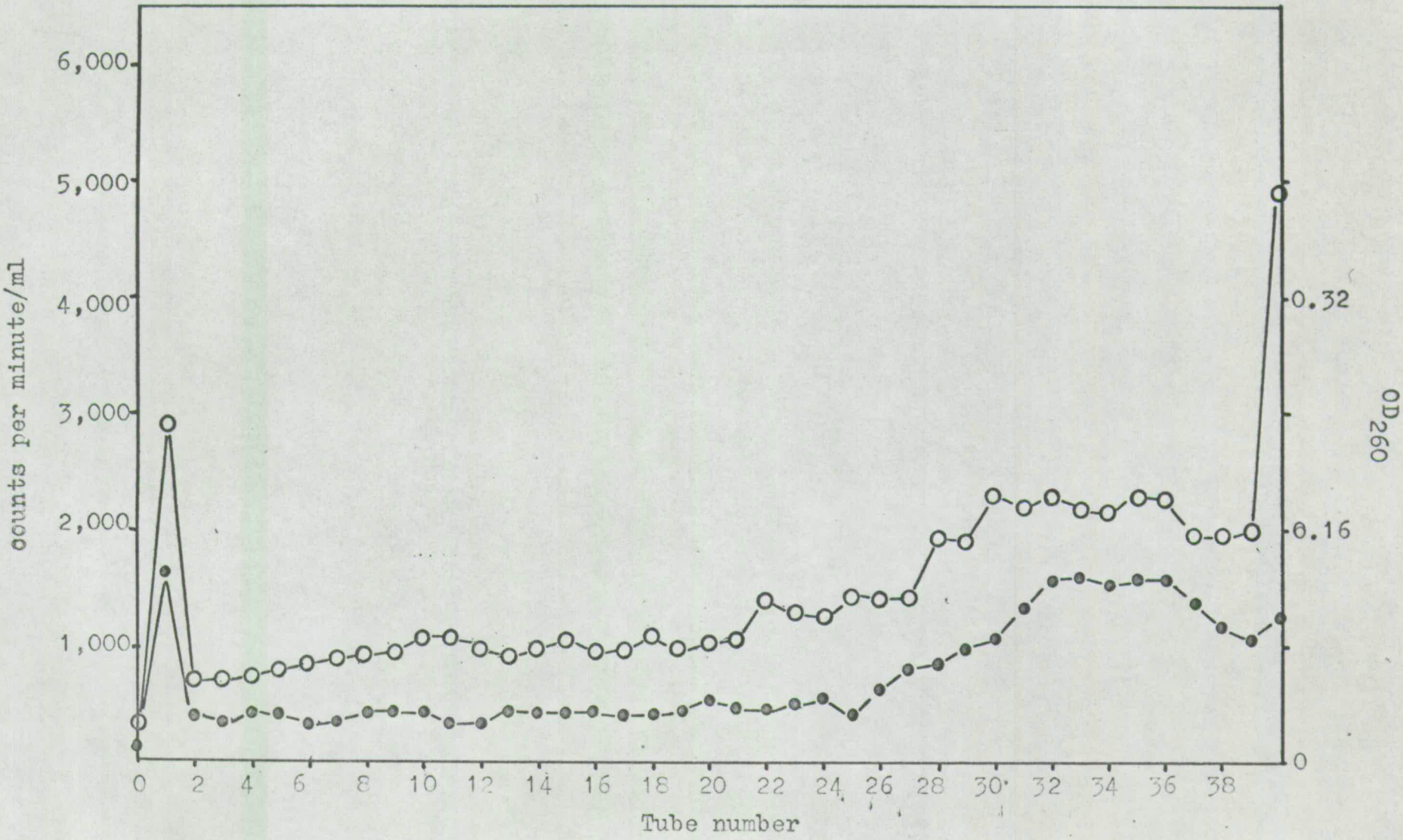

6,000
5,000
4,000
3,000
2,000
1,000
counts per minute/ml
0.32
0.16
0
OD260
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Tube number

Figure 7

Table 7

Fractionation of ^{32}P labelled regenerating rat liver chromatin by both methods

1) derived from Yasmineh & Yunis (1969)

2) derived from Frenster et al (1963)

(Each rat labelled with 5mCi ^{32}P, from 18-19 hours after hepatectomy)

1. Yasmineh and Yunis' method

Time of sonication	Measurement	Condensed 3,500 x g	Intermed. 78,000 x g	Dispersed Post 78,000 x g
30 seconds	Sp. act. DNA (cpm μg^{-1})	69.6	100.6	91.1
	% DNA	26.4%	47.9%	25.7%
60 seconds	Sp. act. DNA (cpm μg^{-1})	64.9	98.2	95.0
	% DNA	17.3%	47.1%	35.6%

2. Frenster et al method

Time of sonication	Measurement	100 x g	Condensed 1,000 x g	Intermed. 78,000 x g	Dispersed 78,000 x g
5 seconds	Sp. act. DNA (cpm μg^{-1})	80.8	94.1	81.6	80.9
	% DNA	65.5%	22.2%	5.3%	7.0%

fractions being about 80 cpm μg^{-1} of DNA. The Yasmineh and Yunis method also achieved a significant fractionation of the RNA associated with the chromatin (see Figures 1 to 4). In all subsequent experiments the fractionation method derived from that of Yasmineh and Yunis was used.

Determination of the buoyant densities of DNA's purified from fractionated rat liver chromatin

The buoyant densities of both the condensed and dispersed chromatin DNAs together with those of the total nuclear DNAs as controls were determined both for normal liver DNA and for rat liver 19 hours after partial hepatectomy.

Using Micrococcus lysodiekticus DNA(with a buoyant density of 1.731 g. cm^{-3}) as a marker, the six DNAs, normal total, condensed, dispersed and regenerating total, condensed and dispersed all banded in the same position with reference to the marker DNA. The value for the buoyant density of rat mainband DNA determined by Steele (1968) is 1.700 g. cm^{-3}. There was no evidence of any satellite components in the condensed fraction of either normal or regenerating rat liver chromatin DNA and no deviations from the value of the buoyant density of mainband DNA from that of the total nuclear DNA were found in either the condensed or dispersed fractions.

The optical renaturation of nuclear DNAs prepared from normal rat liver and 19 hour regenerating liver

The renaturation of total nuclear DNA from rat liver and total nuclear DNA from regenerating liver was examined. Data from studies

of liver regeneration (Swift, 1953; Bollum and Potter, 1959; Looney, 1960; Looney et al 1967; Bucher, 1963;) show that rat liver 19 hours after partial hepatectomy is a very active tissue and evidence of preferential amplification of either the reiterated or unique sequences was therefore sought. The time of renaturation was chosen so that these investigations could be made in parallel with the in vivo labelling studies of the incorporation of radioisotopes during the S period prior to the first synchronised mitotic division of the regenerating liver cells. The optimum temperature for DNA-DNA renaturation and DNA-RNA hybridisation is closely related to the mean melting temperature (Tm) of the DNA (Marmur and Doty, 1961; Nygaard and Hall, 1964). The optimum temperature for renaturation of T_4 DNA in 2 x SSC is about 25°C below the Tm (Wetmur and Davidson, 1968). The Tm of rat liver DNA in 2 x SSC is 96°C and the renaturation experiments were performed at 70°C.

The plots of the percentage renaturation against the logarithm of the Cot values for both normal and regenerating DNAs are shown in Figure 8. The fast and intermediate fractions are taken as those which renature at Cot values less than 100 whilst the slow or 'unique' fraction renatures above this Cot. An alternative method of estimating the renaturation of DNA utilises a hydroxylapatite column (HAP) which can separate reassociated double stranded DNA from single stranded DNA. Bishop (pers. comm.) estimates that 12½% of the apparent optical renaturation is due to single stranded base stacking and folding. This is probably responsible for the observed differences between optical renaturation experiments and those with HAP, and for the apparent renaturation of the zero time samples of the optical

Legend to Figure 8

The renaturation of total rat liver nuclear DNA and total 19 hour regenerating liver nuclear DNA

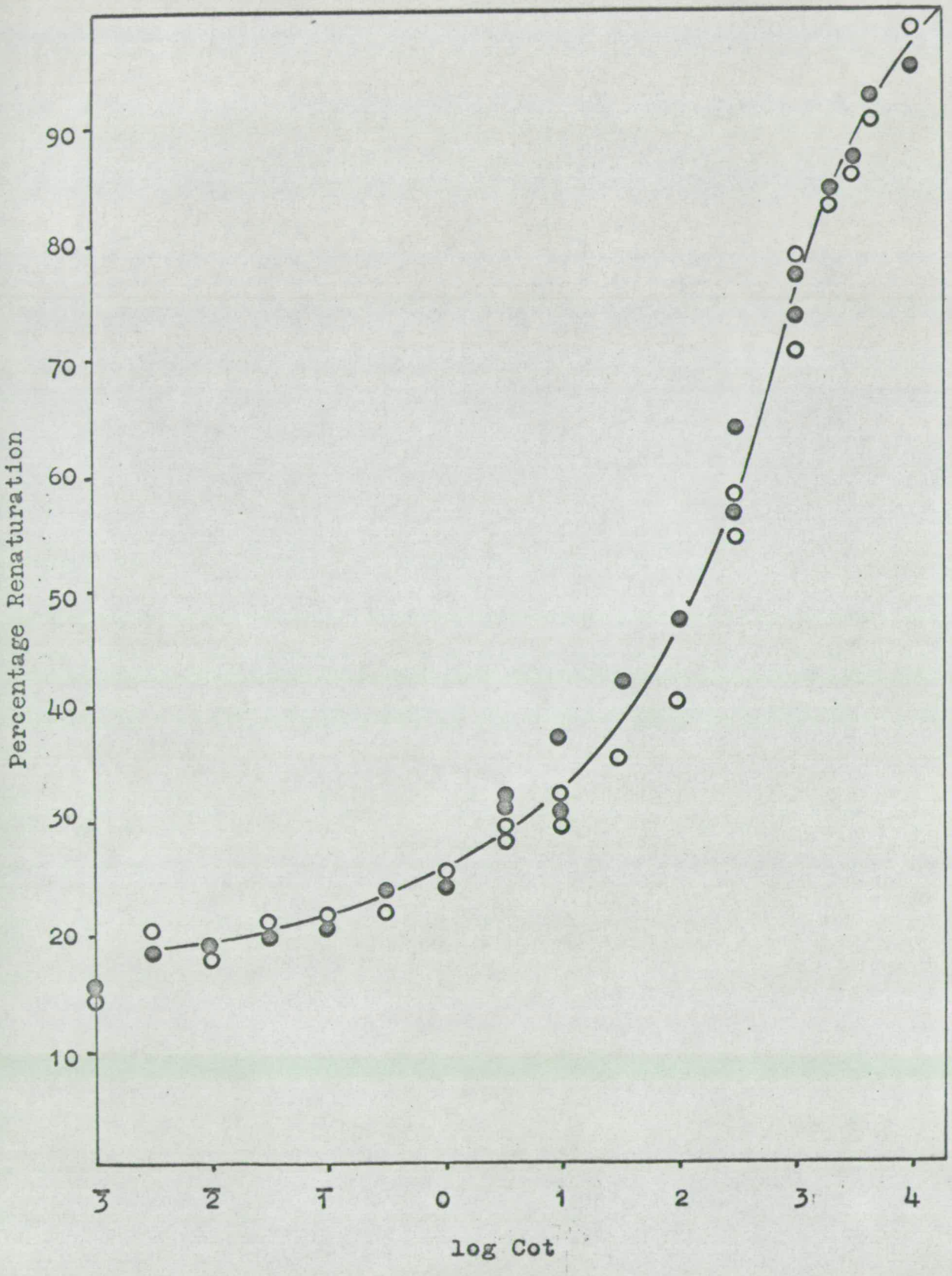
DNA was purified from the nuclei of normal male rat livers and from regenerating rat liver obtained 19 hours after hepatectomy (as described in Materials and Methods Section 1). The DNA was sonicated and passed through Sephadex columns as described in Materials and Methods Section 5.

The DNA was denatured by boiling and allowed to reanneal in a salt concentration of 2 x SSC at a temperature of 70°C, (for details see Materials and Methods Section 13).

●——● regenerating liver DNA

○——○ normal liver DNA

Figure 8


Percentage Renaturation
90
80
70
60
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

renaturations (Bishop, 1972; Studier, 1969).

The renaturation curves of normal and regenerating rat liver DNA appear to be identical. The process of regeneration does not therefore involve any preferential amplification of repeated or non-repeated sequences which can be detected by this method.

Renaturation of fractionated regenerating rat liver chromatin DNA

The length of time for which the nuclei were sonicated prior to separating the fractions was determined by the results of the specific radioactivities of the RNA and DNA. While increasing amounts of sonication improved the fractionation with regard to DNA specific radioactivity, long sonication times had a deleterious effect on the chromatin structure. A sonication time of 60 seconds for the regenerating nuclei was chosen as a compromise. The difficulties of using regenerating rat liver were much greater than those with normal liver. At 19 hours after hepatectomy, the mass of the liver is only 25% - 30% that of a normal liver. For fractionated chromatin, where a fraction consists of only 20% of the total DNA, purified DNA must be prepared from 15 hepatectomised rats to yield the amount of DNA equivalent to that from one normal rat. Preparative fractionations were repeated many times to provide sufficient DNA.

The DNA was sonicated and passed through columns of Sephadex to remove any contaminating heavy metal ions which Melli et al (1971) suggest may reduce the rate of renaturation. The optical renaturations of the purified condensed, intermediate and dispersed chromatin DNAs were followed over a range of Cot values from 0.005 to 10,000. The renaturation at each Cot plotted as a percentage

against the log Cot can be seen in Figure 9. Using renaturation conditions of a salt concentration of 1 x SSC and a temperature of 65°C with DNA prepared by the fractionation method already described, there was no apparent difference between the renaturation curves of DNA from the three fractions, condensed, intermediate or dispersed chromatin from regenerating liver.

This would appear to suggest that no fractionation of repeated or unique sequences has been achieved, but the results of the hybridisation experiments (described later) indicate that this conclusion is incorrect, and that optical measurement of renaturation is not sensitive enough to detect the differences which exist. This will be discussed more fully in a later section.

The theory of hybridisation of DNA with RNA in conditions of vast DNA excess

When RNA and sonicated denatured DNA are incubated together in solution, both DNA/DNA renaturation and DNA/RNA hybridisation take place. Melli et al (1971) showed that when similar concentrations of the DNA and RNA sequences are present, analysis of the reaction is very complex, but under conditions of vast DNA excess, that is, when very small amounts of highly labelled RNA and very large concentrations of DNA are present, the DNA drawn into the DNA/RNA hybrid is negligible in comparison with the remaining concentration of DNA. This reaction may be considered as follows:-

$$\text{DNA} + \text{DNA} \xrightarrow{k_2^d} \text{duplex} \qquad (1)$$

$$\text{DNA} + \text{RNA} \xrightarrow{k_2^h} \text{hybrid} \qquad (2)$$

Legend to Figure 9

The renaturation of DNA prepared from fractionated chromatin from regenerating rat liver

DNA was purified from the 3,500 x g (condensed), the 78,000 x g (intermediate) and the post 78,000 x g alcohol precipitate (dispersed) chromatin fractions. These were prepared by the method derived from that of Yasmineh and Yunis (1969) described in Materials and Methods Section 9, with 60 seconds nuclear sonication prior to fractionation. The proportions of DNA in the three fractions were:-

17% in the condensed fraction (3,500 x g)

50% in the intermediate fraction (78,000 x g)

33% in the dispersed fraction (post 78,000 x g)

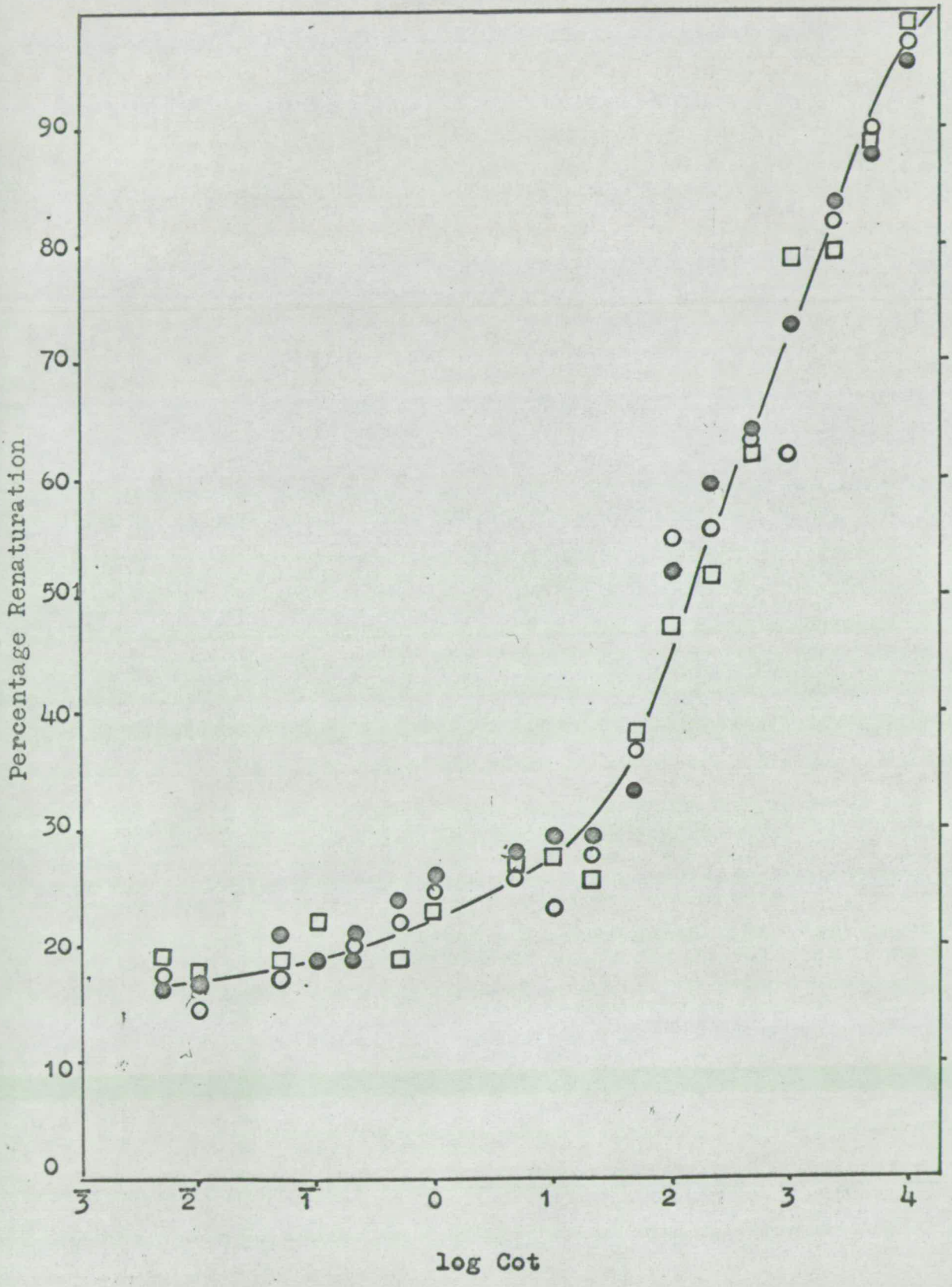
The DNA was purified until it contained no alkali labile contaminating material, and was then sonicated (see Materials and Methods Sections 1, 3 and 5). The DNA from the three fractions was denatured by boiling and allowed to renature in a salt concentration of 1 x SSC at a temperature of 65°C (as described in Materials and Methods Section 13). The percentage renaturation at each Cot value was calculated for each fraction and is plotted against the logarithum of the Cot value.

●——● condensed fraction DNA

○——○ intermediate fraction DNA

□——□ dispersed fraction DNA

Figure 9


Percentage Renaturation
90
80
70
60
50
40
30
20
10
0
3̄
2̄
1̄
0
1
2
3
4
log Cot

Where k_2^d is the rate constant for the DNA/DNA renaturation and k_2^h is the rate constant for the DNA/RNA hybridisation.

Since the DNA/DNA reaction is independent of the DNA/RNA reaction:-

Rate equation for the DNA reaction (renaturation)

$$dD/dt = -k_2^d D^2 \qquad (3)$$

Where D is the concentration of the bases in single stranded sequences at any time.

Integration equation (3) so that t = 0, D = Co

$$D/Co = \frac{1}{(k_2^d \, Cot + 1)} \qquad (4)$$

Rate equation for the RNA reaction (hybridisation)

$$dR/dt = -k_2^h D.R \qquad (5)$$

Where R is the concentration of RNA bases in single stranded sequences.

Using the value of D from equation (4) and integrating equation (5) with the condition that at t = 0, R = Ro

$$\frac{R}{Ro} = \frac{1}{(k_2^d \, Cot + 1)^{k_2^h/k_2^d}}$$

Experiments with a model system of a bacterial DNA sheared to constant size and its homologous cRNA indicated that the rate constant for the DNA-RNA reaction was lower than that for the DNA-RNA renaturation, (Melli et al 1971; Bishop, 1972). The analytical complexity of the DNA can be estimated from its hybridisation with complementary RNA and on the assumption that the ratio k_2^h/k_2^d is the same for the system under consideration as for the model reaction, the reiteration frequency of multiple DNA sequences (such as ribosomal DNA) can be

deduced by hybridising total DNA with the fraction of RNA complementary to those sequences.

In experiments using HnRNA and cRNA, the hybridisation of only about 50% of the RNA is observed. There are several possible reasons for the failure of the hybridisation reaction to proceed to 100%. Thermal breakage of the cRNA may contribute significantly to the incompleteness of the reaction, for if the RNA becomes too small, the hybridisation reaction will not take place. The thermal scission effect is likely to be constant for all three fractions since all hybridisation reactions were performed with the same salt concentration and temperature for the same incubation periods. Part of the hybrid may be susceptible to RNase digestion thus reducing the apparent value of hybridisation. However, Bishop (1972) found no correlation between base composition in bacteria of widely differing GC content and the ribonuclease resistance of the hybridised cRNAs transcribed from the various bacterial templates, although Bishop et al (1972) have suggested that DNA-RNA duplex from vertebrate nucleic acids may be partially sensitive to ribonuclease. In any case, this incompleteness of the reaction is not caused by failure to achieve the conditions of DNA excess because Bishop (1972) showed that varying the DNA-RNA ratio over a hundredfold range does not affect the result. Hybridisation in conditions of excess DNA provides a simple direct way of measuring the reiteration frequency of DNA sequences with which the RNA sequences hybridise.

Hybridisation of ^{32}P labelled nuclear heterogeneous RNA and fractionated chromatin DNA from regenerating rat liver in conditions of DNA excess

HnRNA was prepared from 6 hepatectomised rats labelled for 2

hours with 5mCi ^{32}P per rat (as described in Materials and Methods Section 14). Thus RNA was hybridised in conditions of DNA excess with 3 different DNAs, condensed and dispersed fractionated chromatin DNA (prepared by the method modified from that of Yasmineh and Yunis) and total nuclear DNA. All the DNA was obtained from rat liver 19 hours after hepatectomy. Aliquots of the same DNA solutions were used both for the hybridisation of the *in vivo* HnRNA and the *in vitro* cRNA, (the greatest drawback being that 200 rats had to be hepatectomised and fractionated to obtain sufficient DNA). The molecular weights of the purified sonicated DNAs were determined by Band Sedimentation in the Beckman Model E analytical ultracentrifuge and are given in Table 8.

The final specific radioactivity of the RNA with a sedimentation coefficient greater than 80S was 80,000 cpm/µg. 0.0125µg of regenerating HnRNA were used for each 100µg of fractionated or control DNA, giving a DNA : RNA ratio of 8,000 to 1. The percentage hybridisation at each Cot was plotted against the log Cot. Figure 10 shows the hybridisation of labelled HnRNA with total nuclear DNA from regenerating rat liver. Very little hybridisation can be seen in the very fast region, most being observable in the intermediate and slow regions. The final value for the percentage hybridisation for regenerating total DNA at a Cot of 20,000 is 46% and this hybridisation curve has a $Cot_{\frac{1}{2}}^{h}$ of approximately 220 (see Table 9).

The hybridisation of the HnRNA with regenerating condensed chromatin DNA (Figure 11) shows very marked differences from the total curve. Whilst at low Cots (Cot 1) very little hybridisation occurs with either DNA, there is a marked inflexion of the condensed

Table 8

The Molecular Weights of the sonicated fractionated chromatin DNAs used in hybridisation experiments determined by alkaline analytical density gradient centrifugation

DNA	Molecular Weight
Normal condensed	99,900
dispersed	98,000
total	133,000
Regenerating condensed	88,600
dispersed	80,000
total	99,000

Legend to Figure 10

Hybridisation of ^{32}P labelled HnRNA with total nuclear DNA from regenerating rat liver in conditions of DNA excess

^{32}P labelled HnRNA from 20 hour regenerating rat liver was prepared as described in Materials and Methods Section 12. Purified RNA with a sedimentation value greater than 80S and a specific radioactivity of 80,000 cpm/μg was hybridised with total nuclear DNA prepared from rat liver 19 hours after hepatectomy. DNA was extracted, sonicated and purified (Materials and Methods Sections 1, 3 and 5) and the molecular weight, as determined by band sedimentation in the Beckman Model E analytical ultracentrifuge was 99,000.

Hybridisation was performed over a range of Cot values from 0.005 to 20,000 using DNA concentrations of 400μg/ml, 10mg/ml and 20mg/ml. 0.0125μg of RNA were hybridised with each 100μg of DNA (the DNA/RNA ratio was 8,000 : 1) using a salt concentration of 2 x SSC at a temperature of 65°C. (Details of the hybridisation technique are given in Materials and Methods Section 15). The percentage hybridisation at each Cot was calculated from the RNase resistant counts in each sample as a proportion of the total TCA precipatable counts in that sample.

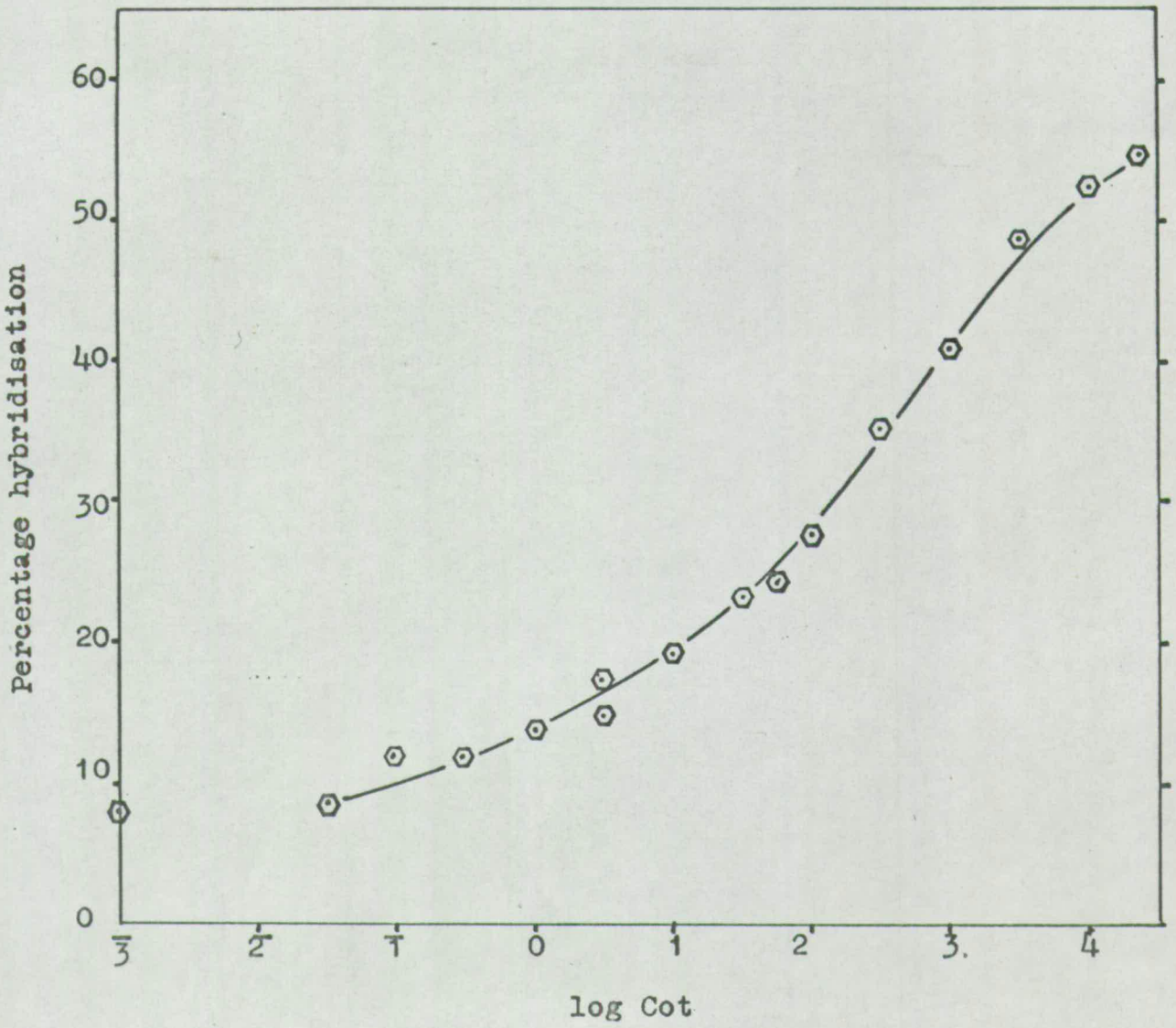

Percentage hybridisation
60
50
40
30
20
10
0
3
2
1
0
1
2
3
4
log Cot

Figure 10

Table 9

Hybridisation of regenerating fractionated chromatin DNA with ^{32}P labelled nuclear heterogeneous RNA from regenerating liver.

Values of $Cot^{h}_{\frac{1}{2}}$

DNA	RNA	$Cot^{h}_{\frac{1}{2}}$
Regenerating Condensed (RC)	^{32}P HnRNA	450
Regenerating Dispersed (RD)	^{32}P HnRNA	50
Regenerating Total (RT)	^{32}P HnRNA	220

Legend to Figure 11

Hybridisation of ^{32}P labelled HnRNA with fractionated condensed chromatin DNA from regenerating rat liver in conditions of DNA excess

^{32}P labelled HnRNA (as used in the experiment shown in Figure 10) was hybridised with DNA purified from chromatin from rat liver at 19 hours after hepatectomy, fractionated by the method of Yasmineh and Yunis (described in Materials and Methods Section 9). Condensed (3,500 x g) fraction DNA was purified until it contained no alkali-labile material, sonicated and passed through a column of Sephadex (described in Materials and Methods Sections 1, 3 and 5). The molecular weight of the condensed fraction DNA determined by alkaline band sedimentation in the Beckman Model E analytical ultracentrifuge was 88,600.

Hybridisation was performed as described in the Legend to Figure 10 with a DNA/RNA ratio of 8,000 to 1.

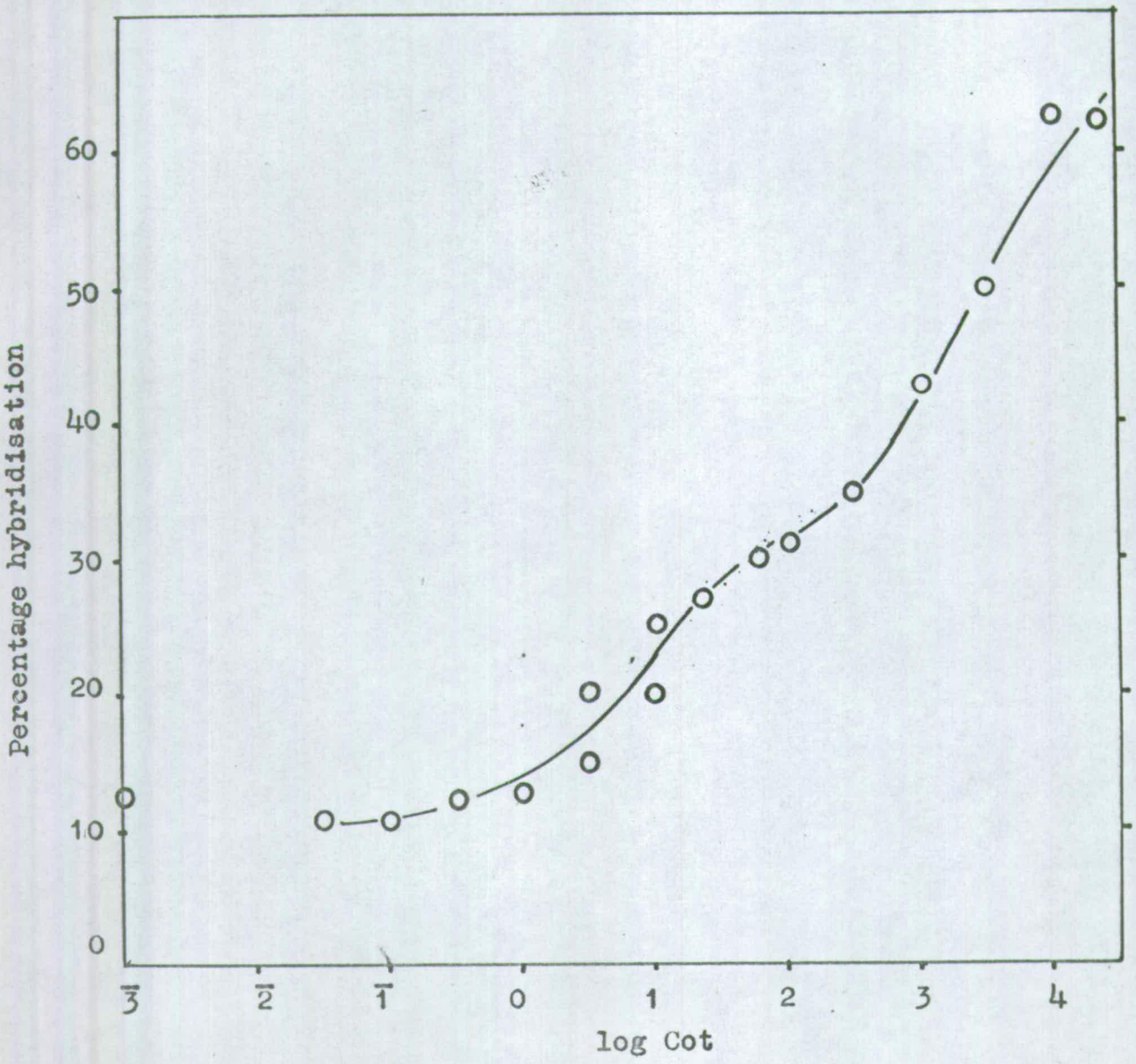

Percentage hybridisation
60
50
40
30
20
10
0
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 11

DNA curve from a Cot of 1 to a Cot of 100. There is a very distinct component of hybridisation in this region with a $Cot_{\frac{1}{2}}^{h}$ of 15 which is not found in the dispersed chromatin DNA but can be seen to a lesser extent in the total. M.S. Campo (pers. comm.) has shown that, in conditions of hybridisation of excess normal rat liver DNA with HnRNA, this characteristic component can be competed out with increasing amounts of unlabelled purified 18S and 28S ribosomal RNA. The value for the final percentage hybridisation (Cot = 20,000) is 54% and is thus greater than that of the total regenerating DNA curve. This increase is due to the amount of hybridisation occurring in the region of Cots 1 - 100, since the presence of this distinct component of hybridisation contributes in an additive manner to the total hybridisation. The $Cot_{\frac{1}{2}}^{h}$ of the whole curve of condensed chromatin DNA has an approximate value of 450 which is much higher than the $Cot_{\frac{1}{2}}^{h}$ of the total regenerating DNA at 220.

The profile of the hybridisation curve of the dispersed chromatin DNA with HnRNA (seen in Figure 12) differs very significantly from both the total and the condensed chromatin DNA curves. (The combined curves of the three fractions can be seen in Figure 13). Until a Cot of one is reached there is no apparent increase in hybridisation and the values for all three DNAs are very similar to each other and to the zero time samples. At Cot values greater than 10 the hybridisation with the different DNAs begins to rise much faster than the condensed DNA, although this fraction appears to lack the "bump" sequences. This agrees with the fact that some of the bump is ribosomal RNA and ribosomal DNA is probably in heterochromatin. Although the final value reached by the dispersed fraction is only

Legend to Figure 12

Hybridisation of ^{32}P labelled HnRNA with fractionated dispersed chromatin DNA from regenerating rat liver

^{32}P labelled HnRNA (as used in the experiments shown in Figures 10 and 11) was hybridised with DNA purified from 19 hour regenerating rat liver chromatin fractionated by the method of Yasmineh and Yunis (Materials and Methods Section 9). The dispersed fraction (alcohol precipitated post 78,000 x *g* fraction) was purified until it contained no alkali labile material and passed through a column of Sephadex (Materials and Methods Sections 1, 3 and 5). The molecular weight of the dispersed fraction DNA determined by band sedimentation in the Beckman Model E analytical ultracentrifuge was 80,000.

Hybridisation was performed as described in the Legend to Figure 10, with a DNA/RNA ratio of 8,000 to 1.

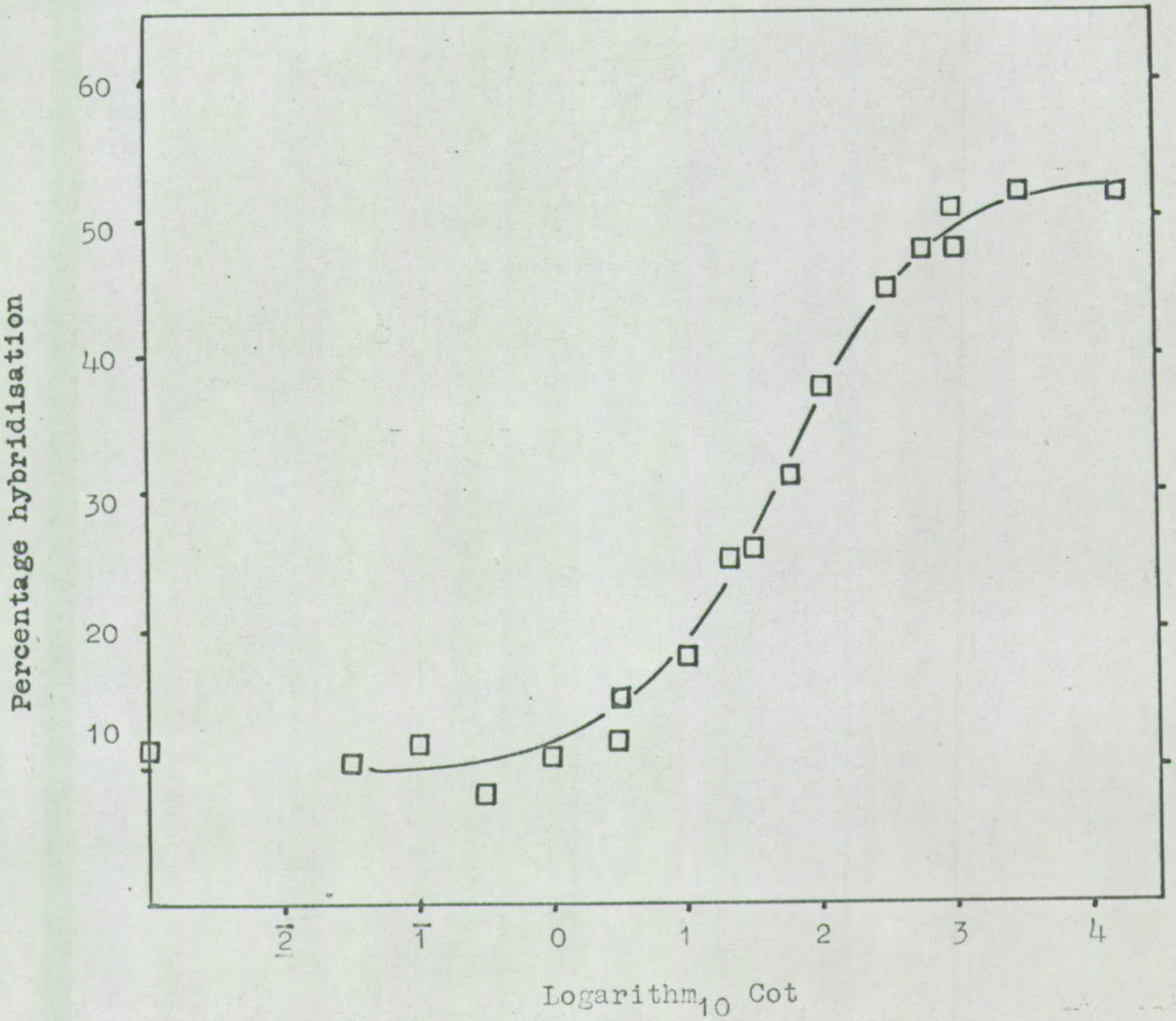

Percentage hybridisation
60
50
40
30
20
10
2̄
1̄
0
1
2
3
4
Logarithm10 Cot

Figure 12

Legend to Figure 13

Hybridisation of ^{32}P labelled HnRNA with condensed and dispersed chromatin DNA and total nuclear DNA from regenerating liver

The three hybridisation curves shown individually in Figures 10, 11 and 12 are shown together in this figure, for comparison. The conditions of hybridisation were a salt concentration of 2 x SSC and an annealing bath temperature of 65°C.

— — — —	condensed fraction DNA
— . — . — . —	dispersed fraction DNA
———————	total nuclear regenerating liver DNA

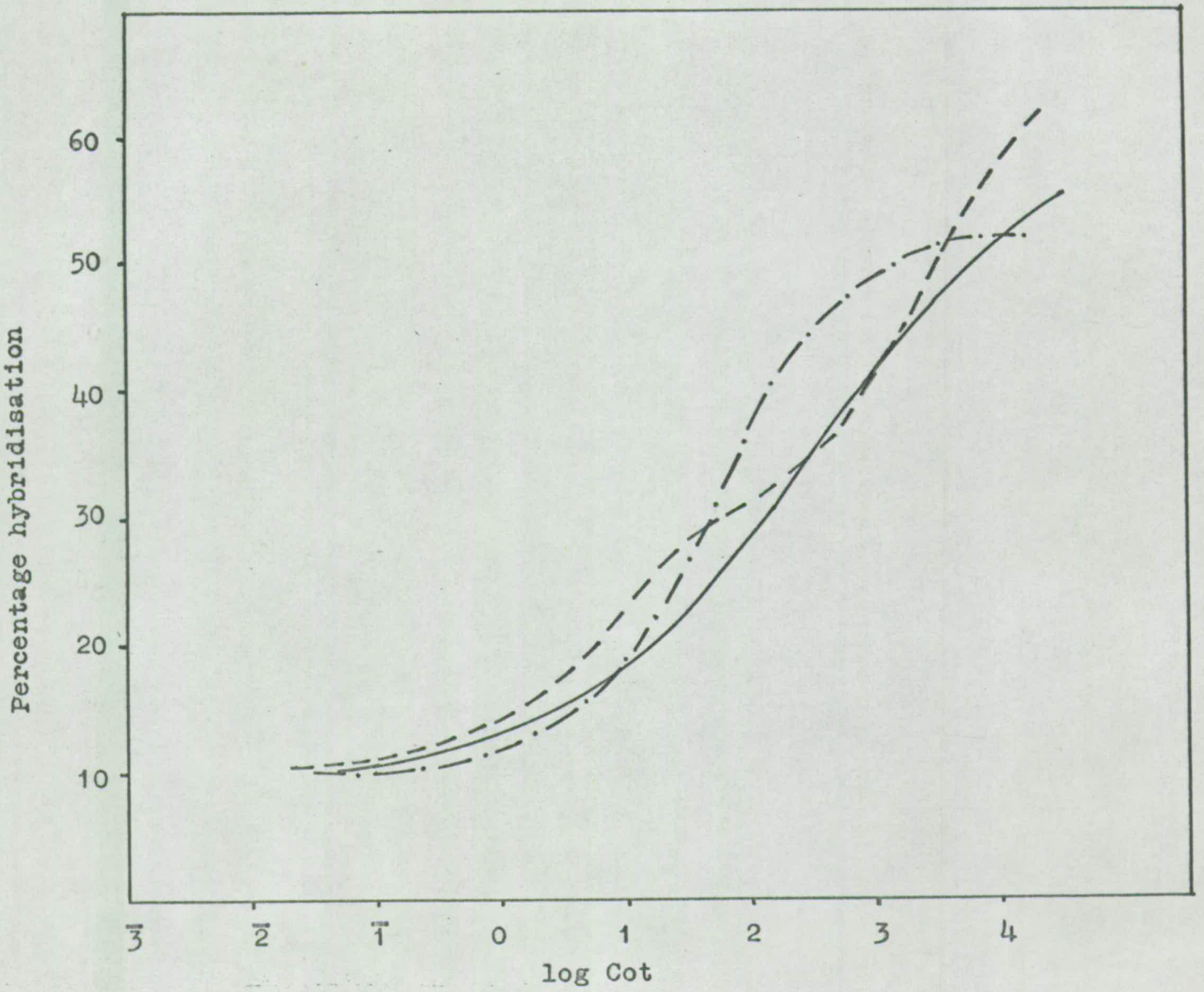

Percentage hybridisation
60
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 13

42% the faster rise between Cot = 10 and Cot = 1,000 indicates an enrichment of the unique sequences. The value of the $Cot^{h}_{\frac{1}{2}}$ of the dispersed curve is approximately 50.

With respect to their hybridisation with HnRNA, there is a true fractionation of sequences between condensed and dispersed regenerating DNA. The dispersed fraction is enriched for 'slow' sequences (i.e. slow transcribed sequences) by a factor of three to four. This is quite possible in terms of the observed recovery. The condensed is enriched for 'bump' sequences and depleted of slow sequences.

Hybridisation of cRNA with DNA from fractionated rat liver chromatin in conditions of DNA excess

^{3}H labelled complementary RNAs (cRNA) were enzymically synthesised on templates of purified DNA from both normal and 19 hour regenerating fractionated rat liver chromatin. The DNA primer for the cRNA synthesis was not sonicated after purification from the fractionated chromatin, but the nuclei had been sonicated for 60 seconds during the fractionation procedure. It was feared that the fractionation method might have partially denatured the DNA or caused single stranded ends. To examine this possibility, samples of each template DNA and controls of unfractionated normal and regenerating nuclear DNA were subjected to increasing temperature in a UV spectrophotometer. The melting curves of the normal fractions and their control are shown in Figure 14A and those of the regenerating fractions and their control DNA in Figure 14B. The melts of the fractionated chromatin DNA superimpose exactly on the curves of the untreated DNA and have the same final percentage hyperchromicity, indicating that there is

Legend to Figure 14

Melting curves of fractionated chromatin DNA used as templates for cRNA synthesis

14a. Normal rat liver DNA

DNA was purified from both condensed (3,500 x g) and dispersed (post 78,000 x g alcohol precipitate) fractions of chromatin prepared from normal rat liver by the method of Yasmineh and Yunis using 60 seconds nuclear sonication prior to the fractionation procedure. (See Materials and Methods Section 9) samples of condensed and dispersed chromatin DNA together with a control sample of purified total nuclear DNA from rat liver at concentrations of 1.0 OD_{260}/ml in 0.1 x SSC were subject to thermal denaturation from 40°C to 95°C in an ultraviolet spectrophotometer. The optical melting profiles of the DNA are shown in Figure 14a.

●—● Normal condensed (3,500 x g) fraction DNA

⊡—⊡ Normal dispersed (post 78,000 x g) fraction DNA

⊙—⊙ Normal total nuclear DNA.

14b. Regenerating rat liver DNA

Fractionated chromatin was prepared as above, but from 19 hour regenerating rat liver. Samples of purified regenerating condensed (3,500 x g) fraction DNA and dispersed (post 78,000 x g) fraction DNA together with total nuclear DNA from 19 hour regenerating rat liver were thermally denatured as above. Optical melting profiles are shown in Figure 14b.

○—○ Regenerating condensed (3,500 x g) fraction DNA

□—□ Regenerating dispersed (post 78,000 x g) fraction DNA

⬡—⬡ Regenerating total nuclear DNA

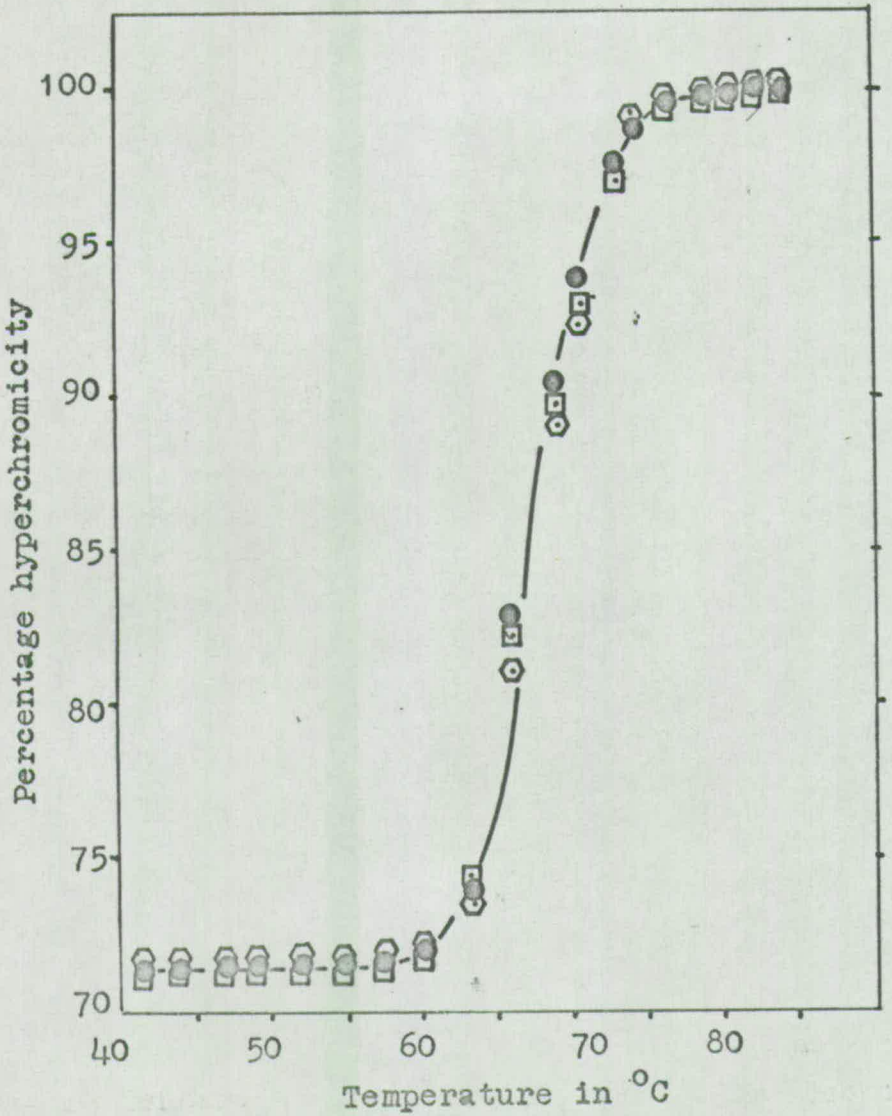


Figure 14a

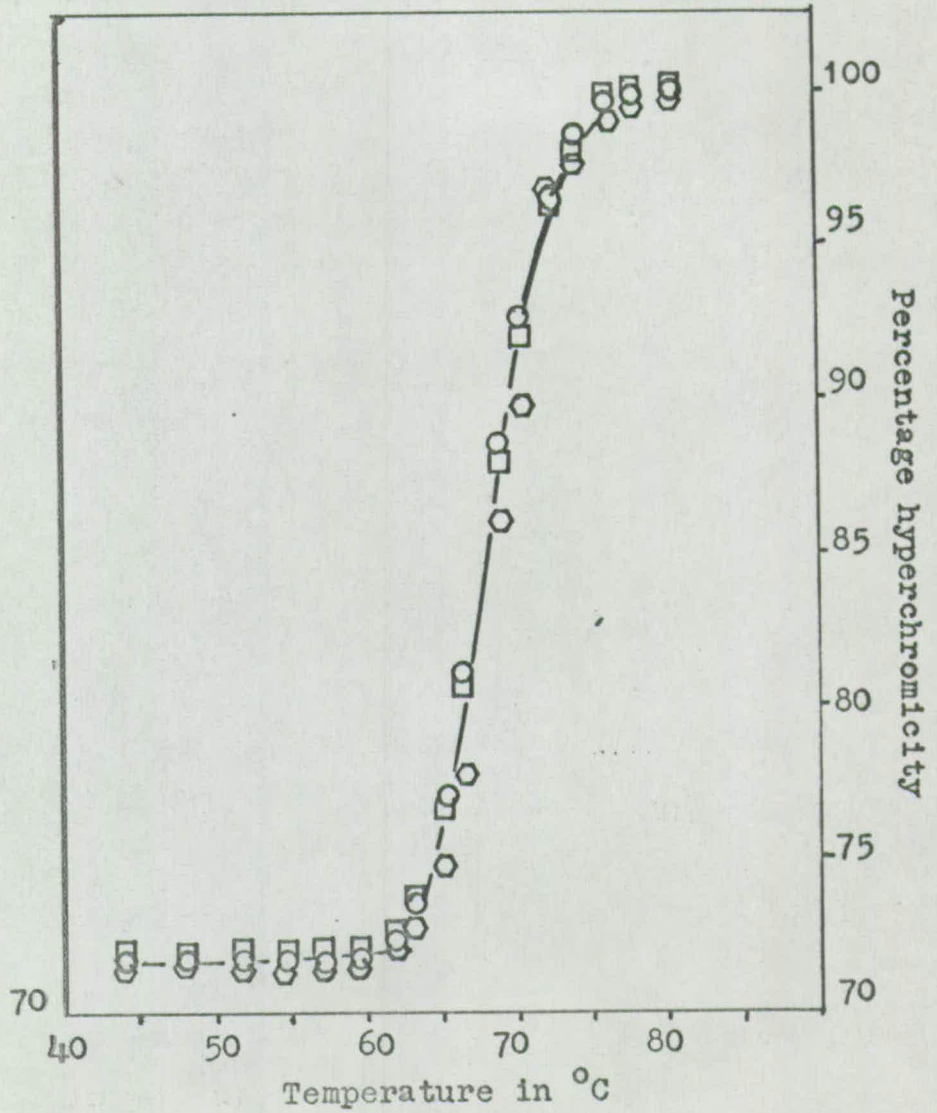


Figure 14b

no significant proportion of single stranded regions in the fractionated chromatin DNA.

The molecular weight of a sample of each template DNA was measured by band sedimentation in caesium chloride in an analytical ultracentrifuge. The molecular weights of these unsheared primer DNAs were found to be approximately half a million so that sonication during preparation had not broken the DNA into small fragments. The system of nomenclature of the cRNAs and DNAs is given in Table 10. The fractionated chromatin DNAs used for hybridisation with the cRNAs were sonicated and passed through a Sephadex column (as described in Materials and Methods Section 5) and their molecular weights were also determined by band sedimentation in the Beckman Model E. The values for each DNA are given in Table 8.

Each cRNA species was hybridised with 3 DNAs: its homologous DNA(e.g. nc cRNA and NC DNA) the DNA from the heterologous chromatin fraction (e.g. nc cRNA and ND DNA) and also with the total nuclear control DNA (e.g. nc cRNA and NT DNA). That is, each of the six DNAs was hybridised with the two cRNAs transcribed from either the normal condensed and dispersed or regenerating condensed and dispersed chromatin DNA templates. The results of the hybridisation reactions over a range of Cot values from 0.005 to 20,000 (plotted on the log Cot scale) of the cRNAs with the fractionated chromatin DNAs are drawn so that comparisons can be made (Figures 15-23). Firstly, the hybridisation curves of each DNA with the two different cRNAs are plotted. (Thus, in the curves using the same DNA, no differences can be attributed to such factors as DNA size since the same DNA is used for both hybridisation curves and the cRNA is the only variable).

Table 10

The preparation of cRNA from fractionated chromatin template DNA

Chromatin fraction	Preparation of chromatin	DNA primer	cRNA produced
normal condensed	3,500 x g for 20 minutes	NC	nc
normal dispersed	post 78,000 x g sn alcohol precipitated	ND	nd
Regenerating condensed (19 hour regenerating liver)	3,500 x g for 20 minutes	RC	rc
Regenerating dispersed (19 hour regenerating liver)	post 78,000 x g sn alcohol precipitated	RD	rd

Secondly, the results are plotted to show the same cRNA hybridised with each of the different DNAs.

Results of the hybridisation experiments using cRNAs made on normal rat liver chromatin DNA template

The hybridisation curves of reactions using normal liver DNA are shown in Figures 15-19. The hybridisation of NC DNA with nc cRNA displays features which are quite distinct from those with nd cRNA (Figure 15). The reaction with the nc cRNA reaches a much higher level over the Cot range 1 to 100 which indicates that NC DNA is enriched for a reiterated fraction of DNA which is absent from (or not transcribed from) ND DNA. The $Cot^{h}_{\frac{1}{2}}$ of this reaction is 30. With nd cRNA the reaction is generally slower except for the final points Cots 1,000 to 20,000 and reaches a lower level of hybridisation (45% hybridisation compared with 53% for NC DNA - nc cRNA). The $Cot^{h}_{\frac{1}{2}}$ is 560. The estimated values of the $Cot^{h}_{\frac{1}{2}}$ of each hybridisation reaction with fractionated chromatin DNA and total nuclear DNA from normal liver can be seen in Table 12.

Figure 16 shows the hybridisation of ND DNA with nd and nc cRNAs. These reactions both proceed more slowly than that of nc cRNA with NC DNA, and reach a final value of 46% hybridisation. The $Cot^{h}_{\frac{1}{2}}$ of the NDnd reaction is 200 and that of the NDnc reaction is 400. The slope of the ND-nd curve is much steeper over the Cot range from 3 to 100, but that of the NDnc reaction is faster over the Cot range from 100 to 1,000 indicating a slight enrichment for sequences which renature at these Cot values.

The hybridisation of total normal DNA (NT) with both cRNAs

Legend to Figure 15

Hybridisation of normal condensed chromatin DNA in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of normal condensed and dispersed DNA

^{3}H cRNAs were synthesised using condensed and dispersed DNA from fractionated normal liver chromatin as templates (described in Materials and Methods Section 14).

The DNA for hybridisation was prepared from rat liver chromatin also fractionated by the method derived from that of Yasmineh and Yunis (1969) (Materials and Methods Section 9). The DNA was purified, sonicated and passed through a Sephadex column (Materials and Methods Sections 1, 3 and 5). The Molecular Weight of the condensed (3,500 x g) fraction DNA determined by band sedimentation in the Beckman Model E analytical ultracentrifuge was 99,900.

Hybridisation of the cRNAs with the condensed fraction DNA was performed as described in Materials and Methods Section 15 using DNA concentrations of 400µg/ml, 10mg/ml and 20mg/ml.

●——● normal condensed ^{3}H cRNA and normal condensed DNA (nc - NC)

○——○ normal dispersed ^{3}H cRNA and normal condensed DNA (nd - NC)

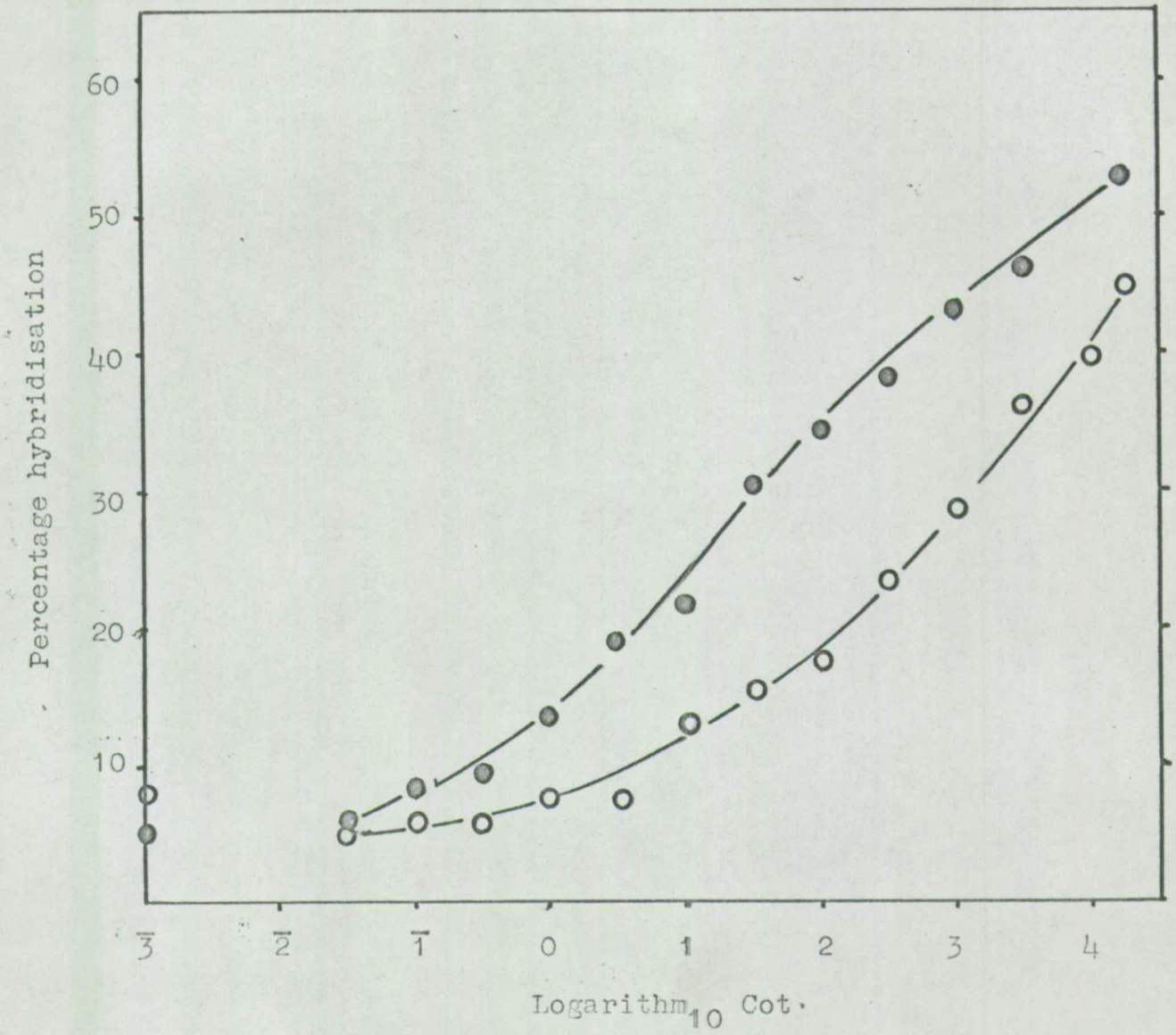

Percentage hybridisation
60
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
Logarithm$_{10}$ Cot.

Figure 15

Table 11

Hybridisation of DNA with cRNA in conditions of DNA excess.

Estimated values for $Cot^{h}_{\frac{1}{2}}$ of each hybridisation reaction

NORMAL RAT LIVER

DNA	cRNA	$Cot^{h}_{\frac{1}{2}}$
Normal condensed (NC)	nc	30
Normal condensed (NC)	nd	560
Normal dispersed (ND)	nc	400
Normal dispersed (ND)	nd	200
Normal Total (NT)	nc	350
Normal Total (NT)	nd	500

Legend to Figure 16

Hybridisation of normal dispersed chromatin DNA in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of normal, condensed and dispersed DNA

^{3}H cRNAs were synthesised using condensed and dispersed DNA from fractionated normal liver chromatin as templates (described in Materials and Methods Section 14).

The DNA for hybridisation was prepared from rat liver chromatin also fractionated by the method of Yasmineh and Yunis (1969) (Materials and Methods Section 9). The DNA was purified, sonicated and passed through a Sephadex column (see Materials and Methods Sections 1, 3 and 5). The Molecular Weight of the dispersed (alcohol precipitated post 78,000 x *g* supernatant) fraction DNA determined by band sedimentation in the Beckman Model E analytical ultracentrifuge was 98,000.

Hybridisation of the cRNAs with the condensed fraction DNA was performed as described in Materials and Methods Section 15.

■ —— ■ normal dispersed ^{3}H cRNA and normal dispersed DNA (nd - ND)

□ —— □ normal condensed ^{3}H cRNA and normal dispersed DNA (nc - ND)

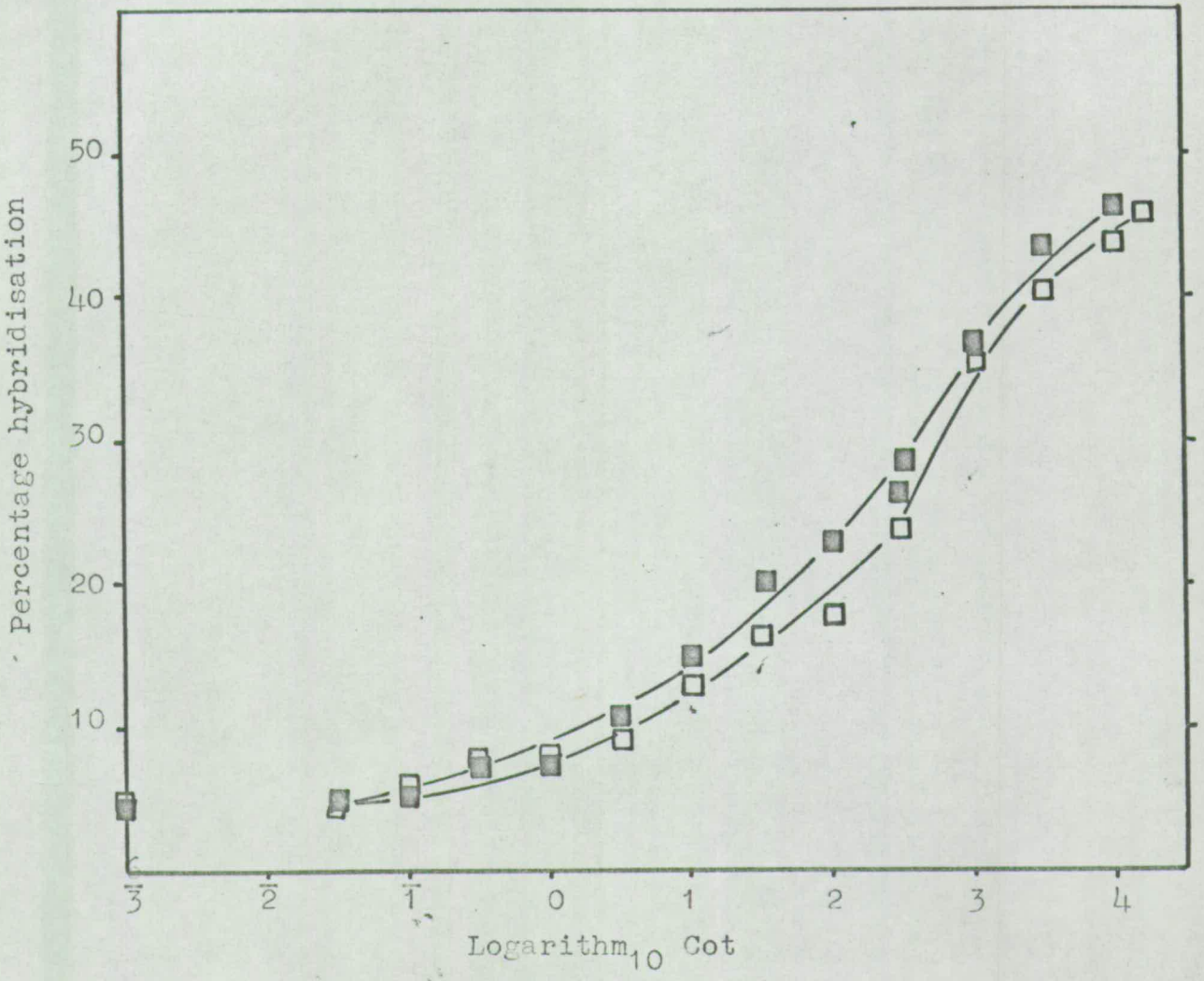

Percentage hybridisation
50
40
30
20
10
3̄
2̄
1̄
0
1
2
3
4
Logarithm10 Cot

Figure 16

(shown in Figure 17) is different from either of the fractions. (In Figures 18 and 19, the hybridisations of all three DNAs with the same cRNA are given for ease of comparison). It will be seen that both with nc cRNA (Figure 18) and nd cRNA (Figure 19) the reaction with NT DNA is intermediate between those with the DNA fractions. A comparison of the reactions of the three DNAs with nc cRNA indicates that the homologous NC-nc reaction, has the lowest $Cot^{h}_{\frac{1}{2}}$ (30) whereas the values for NTnc ($Cot^{h}_{\frac{1}{2}}$ = 350) and NDnd ($Cot^{h}_{\frac{1}{2}}$ = 400) are much higher (see Table 11). In the reactions with nd cRNA the homologous reaction (ND-nd) again has the lowest $Cot^{h}_{\frac{1}{2}}$ (200) although the difference between this and NT-nd ($Cot^{h}_{\frac{1}{2}}$ = 500) and NCnd ($Cot^{h}_{\frac{1}{2}}$ = 560) reactions is not as pronounced as that observed in the hybridisation of nc cRNA with its homologous and heterologous DNA.

Results of hybridisation experiments using fractionated regenerating liver chromatin DNA with cRNAs made on regenerating DNA templates

The data from the hybridisation of the fractionated regenerating DNA with the cRNAs are presented in exactly the same way as those for normal DNA and cRNA. The results however are very different.

Figure 20 shows the hybridisation of RC DNA with rc and rd cRNAs. The reaction with rc cRNA, while lacking the extremely rapid rise between Cot 1 and 100 found with NC DNA and nc cRNA, is considerably faster over this region than that with nd cRNA, indicating that there is an enrichment for these moderately reiterated DNA sequences in this fraction. In contrast, the reaction with rd cRNA shows its greatest rise between Cots 300 and 3,000. The $Cot^{h}_{\frac{1}{2}}$ (800) compared with 150 for the RC rc reaction also indicates the differing courses of the

Legend to Figure 17

Hybridisation of normal total nuclear DNA in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of normal condensed and dispersed DNA

^{3}H cRNAs were synthesised using condensed and dispersed DNA from normal liver chromatin fractionated by the method derived from that of Yasmineh and Yunis (described in Materials and Methods Sections 9 and 14).

The DNA for hybridisation was prepared from rat liver nuclei and was purified, sonicated and passed through a Sephadex column (Materials and Methods Sections 1, 3 and 5). The Molecular Weight of the total nuclear DNA determined by band sedimentation in the Beckman Model E ultracentrifuge was 133,000.

Hybridisation of the cRNAs with the total nuclear DNA was performed as described in Materials and Methods Section 15.

○——○ normal condensed ^{3}H cRNA and normal total DNA (nc - NT)

⊙——⊙ normal dispersed ^{3}H cRNA and normal total DNA (nd - NT)

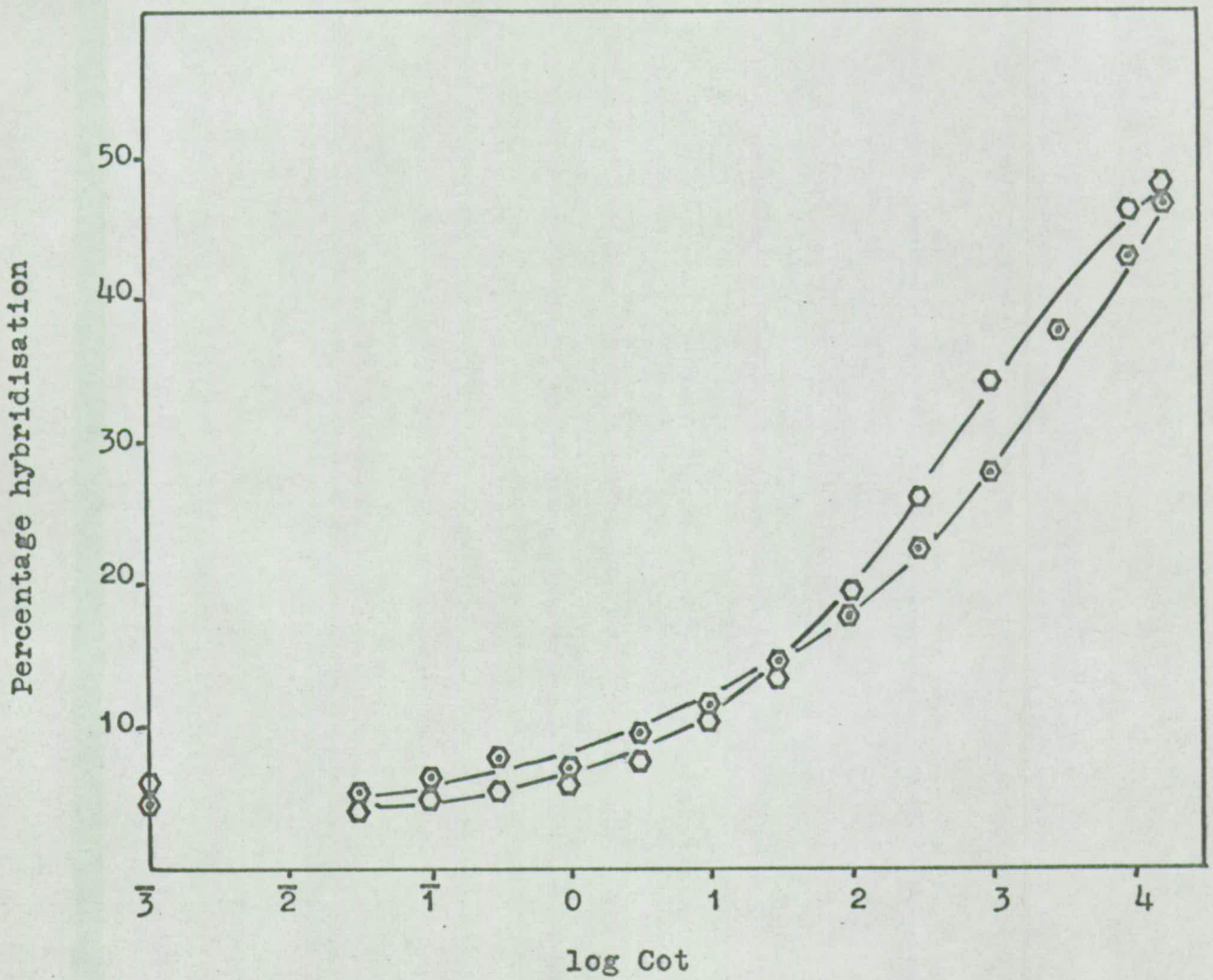

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 17

Legend to Figure 18

Hybridisations of normal condensed and dispersed chromatin DNAs and total nuclear DNA in conditions of DNA excess with ^{3}H cRNA synthesised on a template of normal condensed DNA

The hybridisation curves of nc ^{3}H cRNA with NC, ND and NT DNA which were seen separately in Figures 15, 16 and 17 are shown together for ease of comparison.

●—● normal condensed cRNA and normal condensed DNA (nc - NC)

□—□ normal condensed cRNA and normal dispersed DNA (nc - ND)

○—○ normal condensed cRNA and normal total DNA (nc - NT)

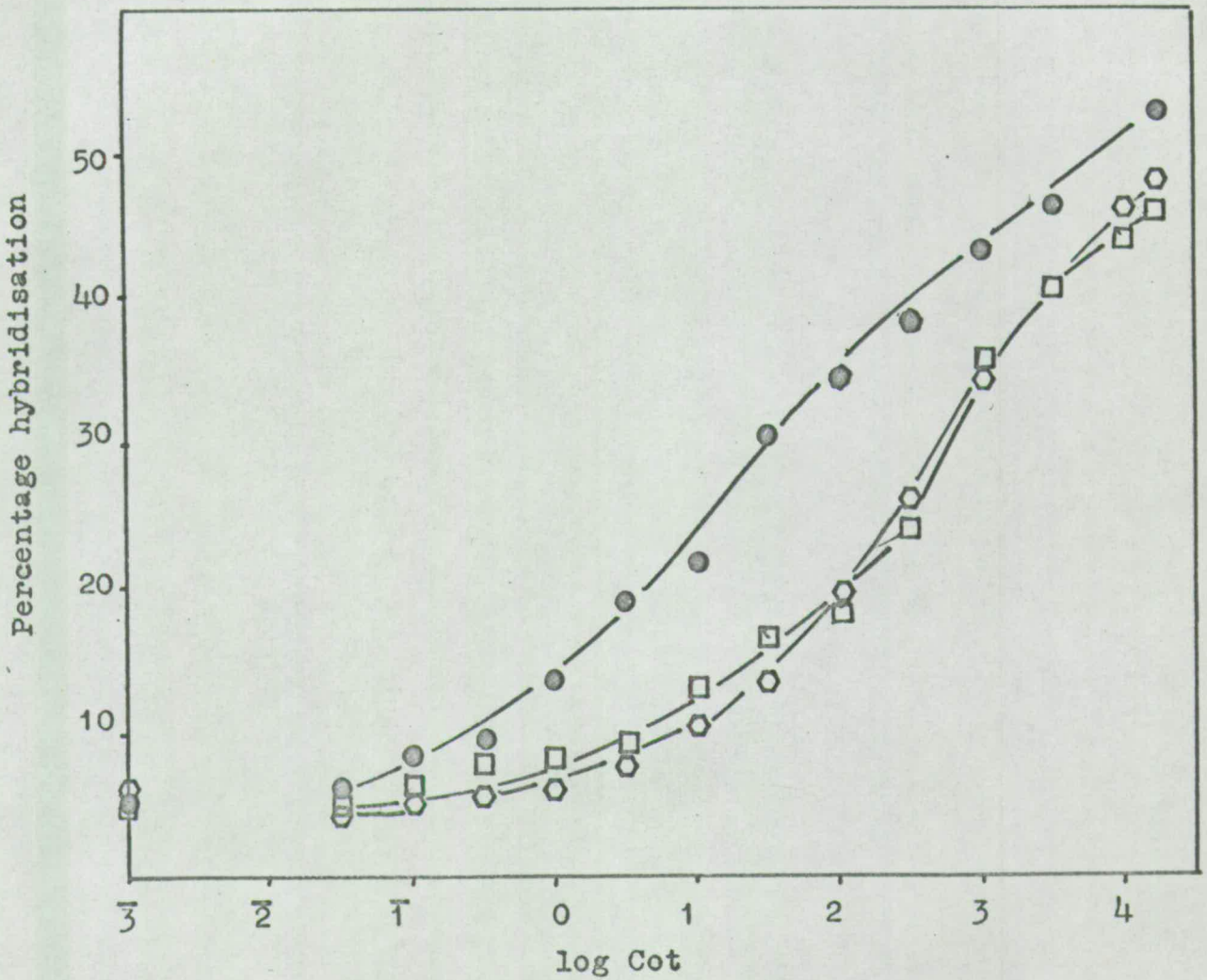

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 18

Legend to Figure 19

Hybridisations of normal condensed and dispersed chromatin DNAs and total nuclear DNA in conditions of DNA excess with ^{3}H cRNA synthesised on a template of normal dispersed DNA

The hybridisation curves of nd ^{3}H cRNA with NC, ND and NT DNA which were seen separately in Figures 15, 16 and 17 are shown together for ease of comparison.

○—○ normal dispersed cRNA and normal condensed DNA (nd - NC)

■—■ normal dispersed cRNA and normal dispersed DNA (nd - ND)

⊙—⊙ normal dispersed cRNA and normal total DNA (nd - NT)

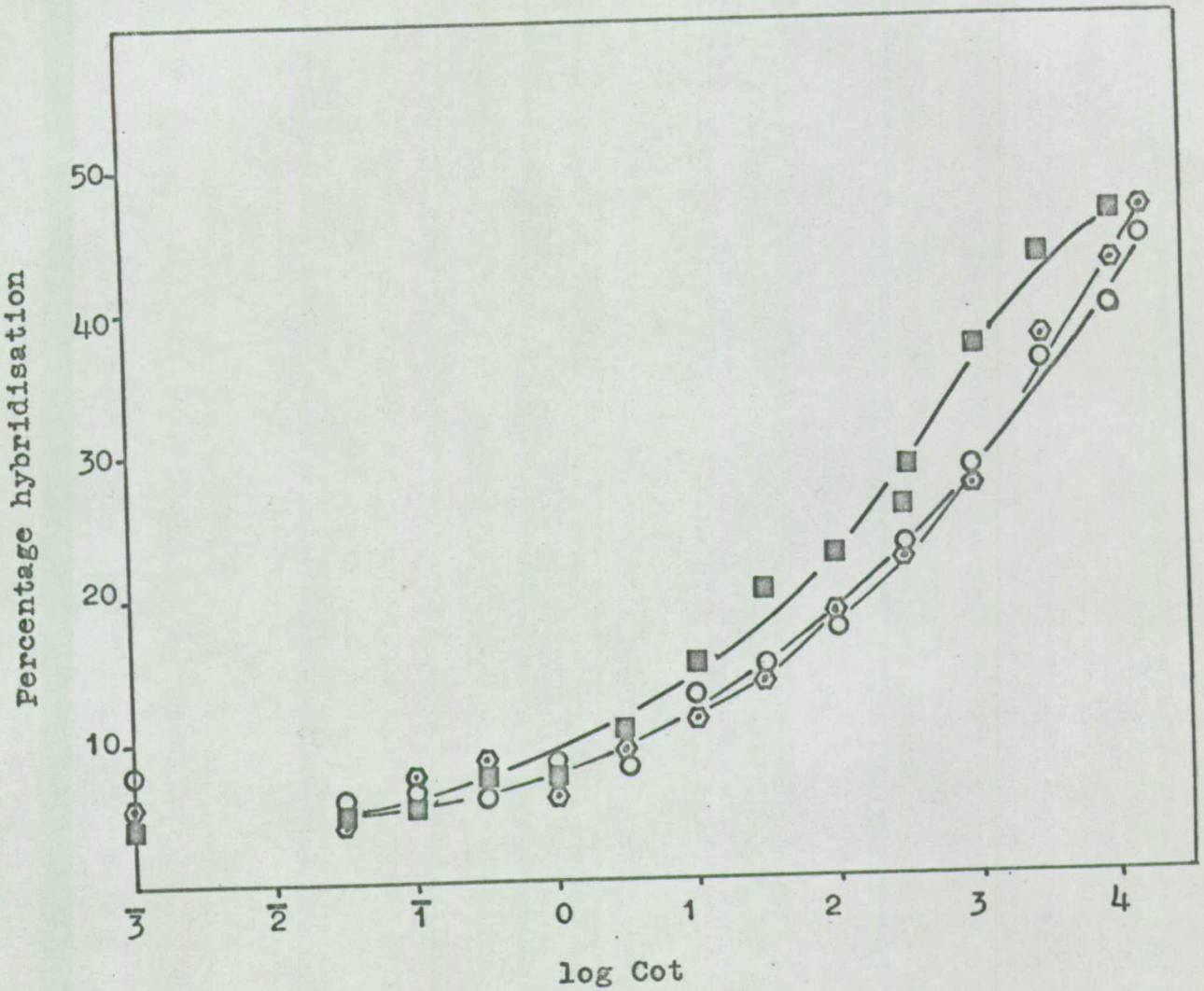


Figure 19

Legend to Figure 20

Hybridisation of condensed chromatin DNA from regenerating rat liver in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of condensed and dispersed chromatin DNA from regenerating liver

^{3}H cRNAs were synthesised using condensed and dispersed DNA from fractionated liver chromatin (obtained from rat liver 19 hours after partial hepatectomy) as templates, as described in Materials and Methods Section 14.

The DNA for hybridisation was prepared from rat liver chromatin from 19 hour regenerating liver also fractionated by the method of Yasmineh and Yunis (Materials and Methods Section 9). The DNA was purified, sonicated and passed through a Sephadex column (see Materials and Methods Sections 1, 3 and 5).

The Molecular Weight of the condensed (3,500 x g) fraction DNA determined by band sedimentation in the Beckman Model E ultracentrifuge was 88,600.

Hybridisation of the cRNAs with the condensed fraction DNA was performed as described in Materials and Methods Section 15.

●——● regenerating condensed ^{3}H cRNA and regenerating condensed DNA (rc - RC)

○——○ regenerating dispersed ^{3}H cRNA and regenerating condensed DNA (rd - RC)

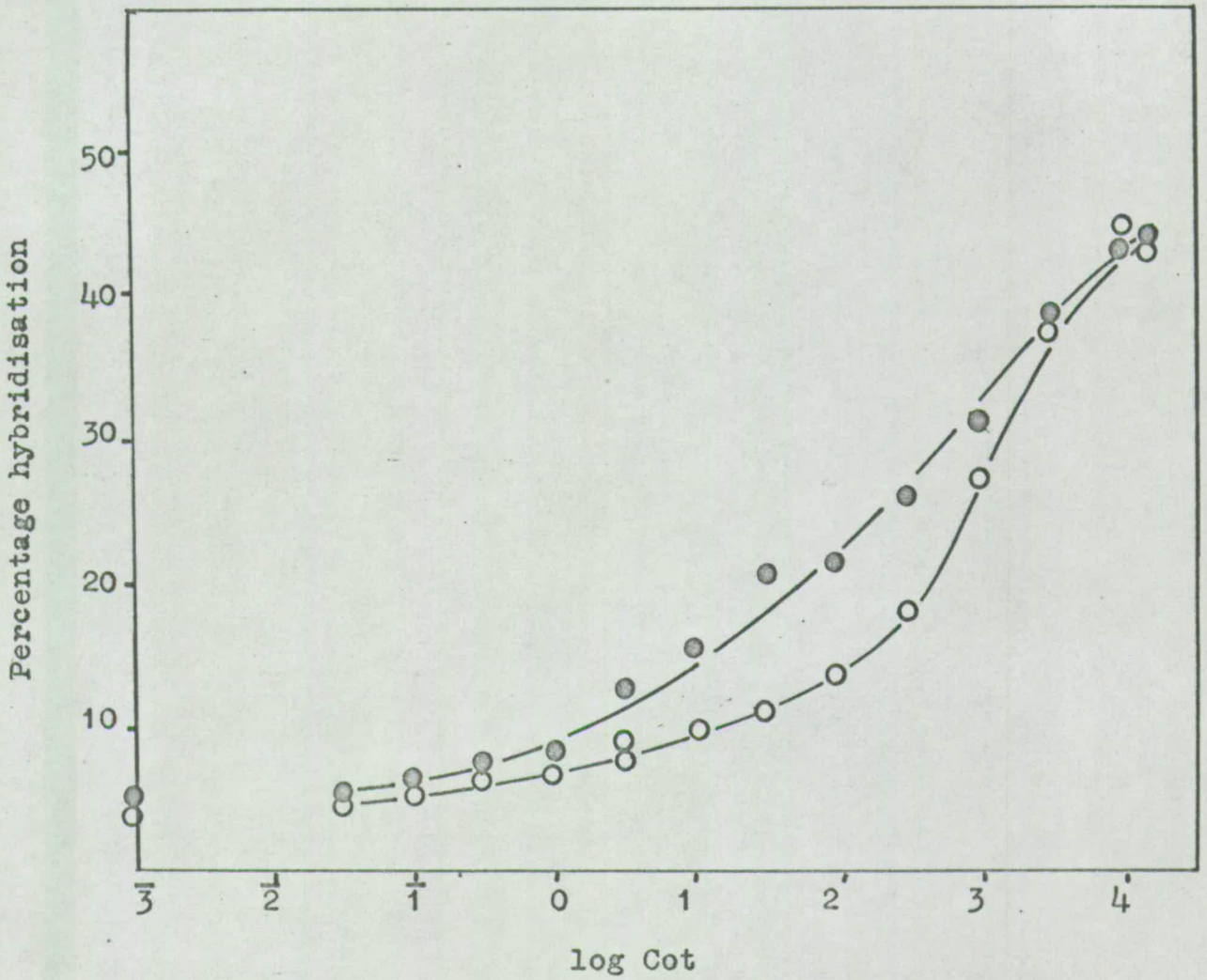


Figure 20

reactions (see Table 12).

The reactions of RD DNA with the same cRNAs (shown in Figure 21) differ less than those of RC DNA (as the reactions of nc and nd cRNA with ND DNA were closer than those with NC DNA). In this case however, the reaction with rc cRNA is rather more rapid in the early points, (up to Cot = 300) than that with rd cRNA (the reverse of the result in the normal cRNA/DNA reaction). This suggests that the moderately reiterated DNA sequences are almost completely absent from rd cRNA, although their presence in RD DNA is indicated by the reaction with rc cRNA.

The hybridisation of regenerating total DNA (RT) with the two cRNAs (Figure 22) shows that until a Cot of about 300 is reached, there is a greater percentage hybridisation at each Cot with the rc cRNA than with rd cRNA; only at Cot values greater than 300 does the slope of the rd hybridisation reaction become steeper than the RTrc reaction. The values of the $Cot^{h}_{\frac{1}{2}}$ of the two reactions (rc = 200 and rd = 500) indicate the difference between the two hybridisation curves.

In Figures 23 and 24 where the reactions of all three DNAs with each cRNA are given, the demonstration that the hybridisation of RT DNA with nc and nd cRNAs comes between those of the fractionated condensed and dispersed DNAs, similar to the effect in the normal system is again given. With rc cRNA, the homologous RC-rc reaction again has a lower $Cot^{h}_{\frac{1}{2}}$ than the RTrc and RDrc reactions ($Cot^{h}_{\frac{1}{2}}$ 150, 200 and 250 respectively). Whilst the RDrd reaction $Cot^{h}_{\frac{1}{2}}$ of 500 is lower than that of the RCrd reaction ($Cot^{h}_{\frac{1}{2}}$ = 800) there is a very notable difference between the reactions with rc cRNA and rd cRNA.

Table 12

Hybridisation of DNA with cRNA in conditions of DNA excess.

Estimated values for $Cot^{h}_{\frac{1}{2}}$ of each hybridisation reaction

REGENERATING RAT LIVER

DNA	cRNA	$Cot^{h}_{\frac{1}{2}}$
Regenerating Condensed	rc	150
Regenerating Condensed	rd	800
Regenerating Dispersed	rc	250
Regenerating Dispersed	rd	500
Regenerating Total	rc	200
Regenerating Total	rd	500

Legend to Figure 21

Hybridisation of dispersed chromatin DNA from regenerating rat liver in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of condensed and dispersed chromatin DNA from regenerating liver

^{3}H cRNAs were synthesised using condensed and dispersed DNA from fractionated liver chromatin (obtained from rat liver 19 hours after partial hepatectomy) as templates (described in Materials and Methods Section 14).

The DNA for hybridisation was prepared from rat liver chromatin from 19 hour regenerating liver also fractionated by the method of Yasmineh and Yunis (Materials and Methods Section 9). The DNA was purified and sonicated (Materials and Methods Sections 1, 3 and 5).

The Molecular Weight of the dispersed (alcohol precipitated post 78,000 x g) fraction DNA determined by band sedimentation in the Beckman Model E ultracentrifuge was 98,000. Hybridisation of the cRNAs with the dispersed fraction DNA was performed as described in Materials and Methods Section 15.

■——■ regenerating dispersed ^{3}H cRNA and regenerating dispersed DNA.

□——□ regenerating condensed ^{3}H cRNA and regenerating dispersed DNA

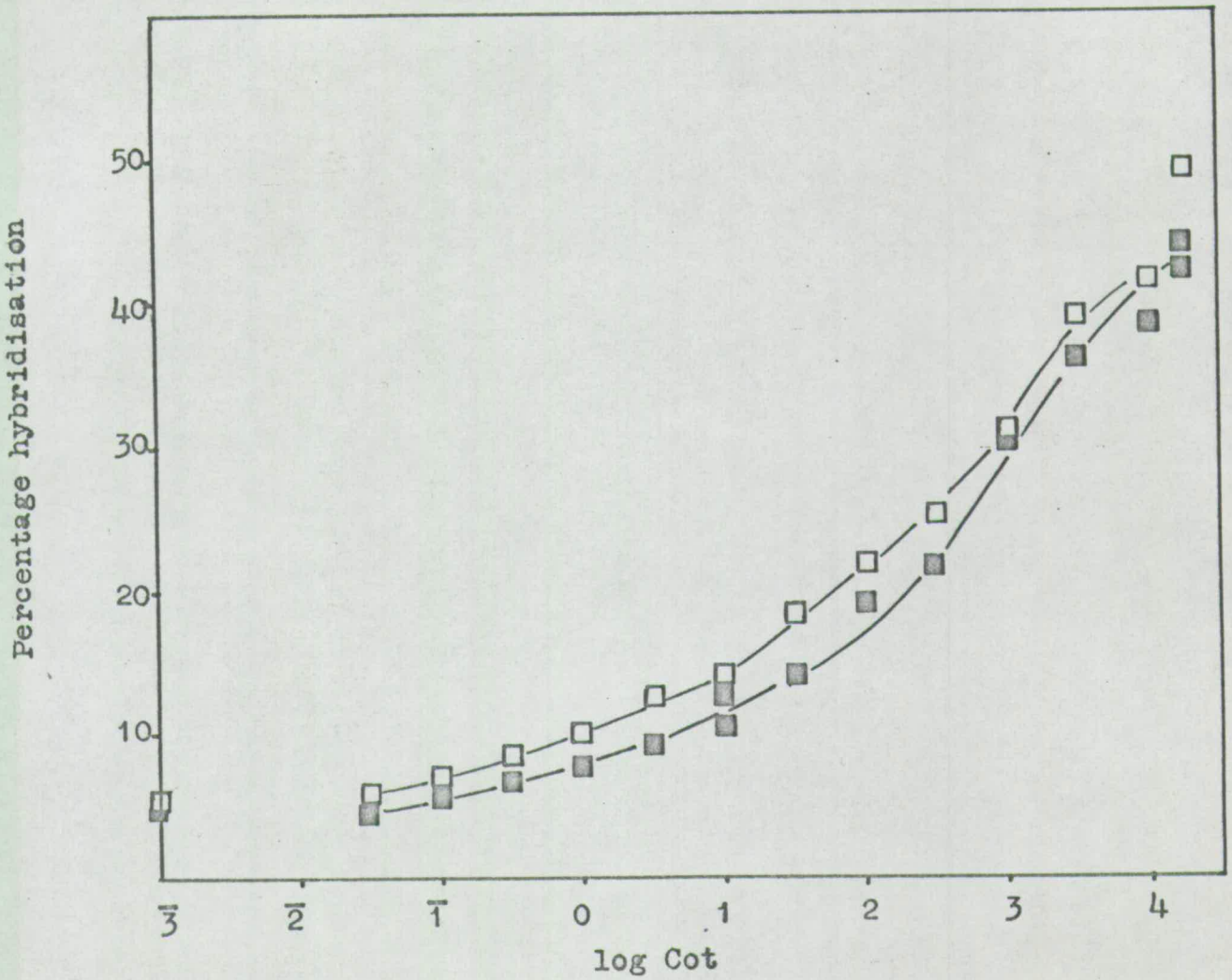

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 21

Legend to Figure 22

Hybridisation of total nuclear DNA from regenerating rat liver in conditions of DNA excess with ^{3}H cRNAs synthesised on templates of condensed and dispersed chromatin DNA from regenerating liver

^{3}H cRNAs were synthesised using condensed and dispersed DNA from liver chromatin (from rat liver 19 hours after hepatectomy fractionated by the method derived from that of Yasmineh and Yunis) as templates (see Materials and Methods Sections 1, 9 and 14).

The DNA for hybridisation was prepared from rat liver nuclei (obtained from regenerating liver, 19 hours after partial hepatectomy), purified and sonicated as described in Materials and Methods Sections 1, 3 and 5. The Molecular Weight of the total regenerating nuclear DNA, determined by band sedimentation in the Beckman Model E analytical ultracentrifuge was 99,000.

Hybridisation of the cRNAs with total nuclear DNA was performed as described in Materials and Methods Section 15.

○—○ regenerating condensed cRNA and regenerating total DNA (rc - RT)

⊙—⊙ regenerating dispersed cRNA and regenerating total DNA (rd - RT)

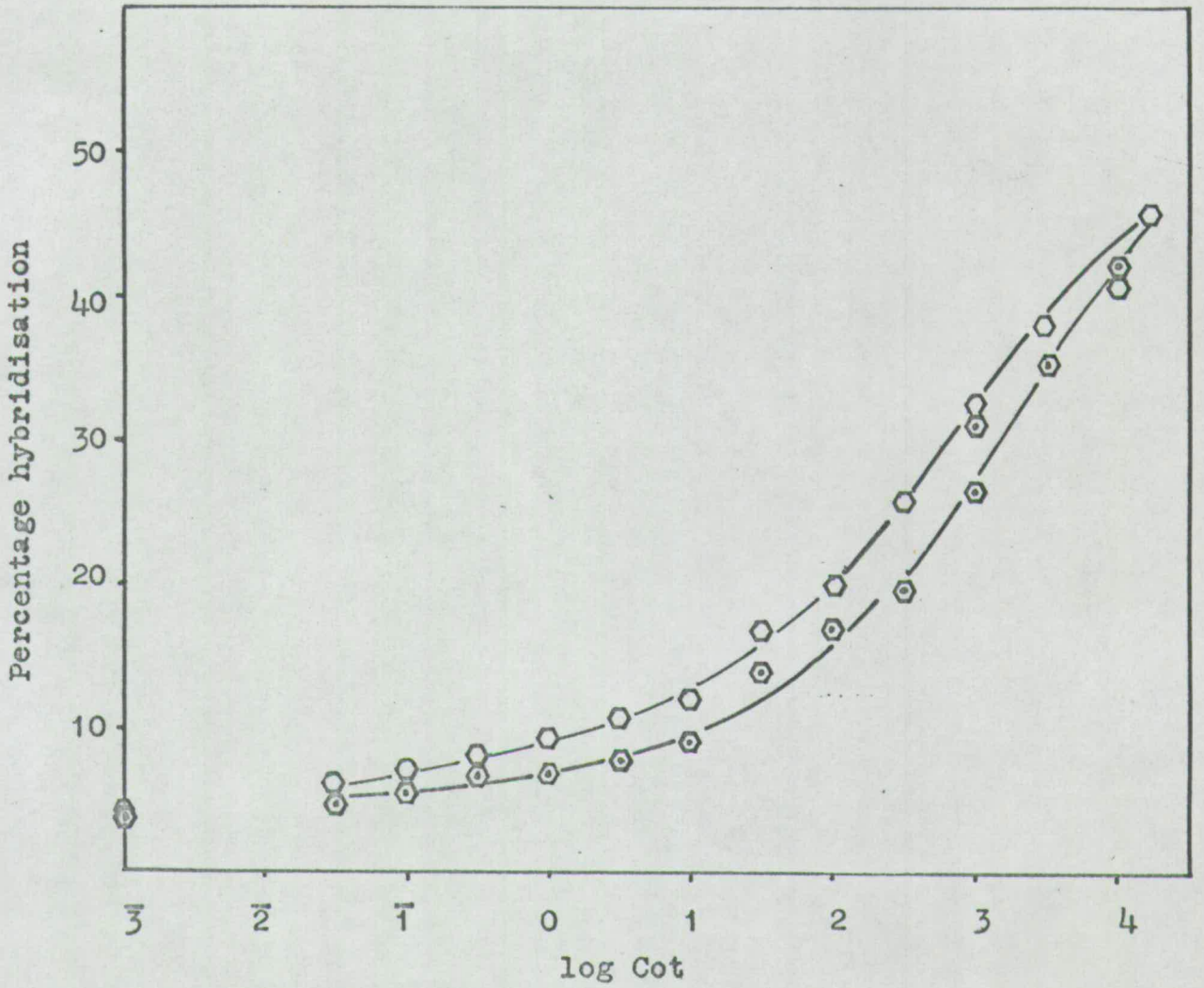

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 22

Legend to Figure 23

Hybridisations of condensed and dispersed chromatin DNA and total nuclear DNA from regenerating rat liver in conditions of DNA excess with ^{3}H cRNA synthesised on a template of condensed chromatin DNA prepared from regenerating rat liver

The hybridisation curves of rc ^{3}H with RC, RD and RT DNA which were seen separately in Figures 20, 21 and 22 are shown together for ease of comparison.

●—● regenerating condensed cRNA and regenerating condensed DNA (rc - RC)

□—□ regenerating condensed cRNA and regenerating dispersed DNA (rc - RD)

○—○ regenerating condensed cRNA and regenerating total DNA (rc - RT)

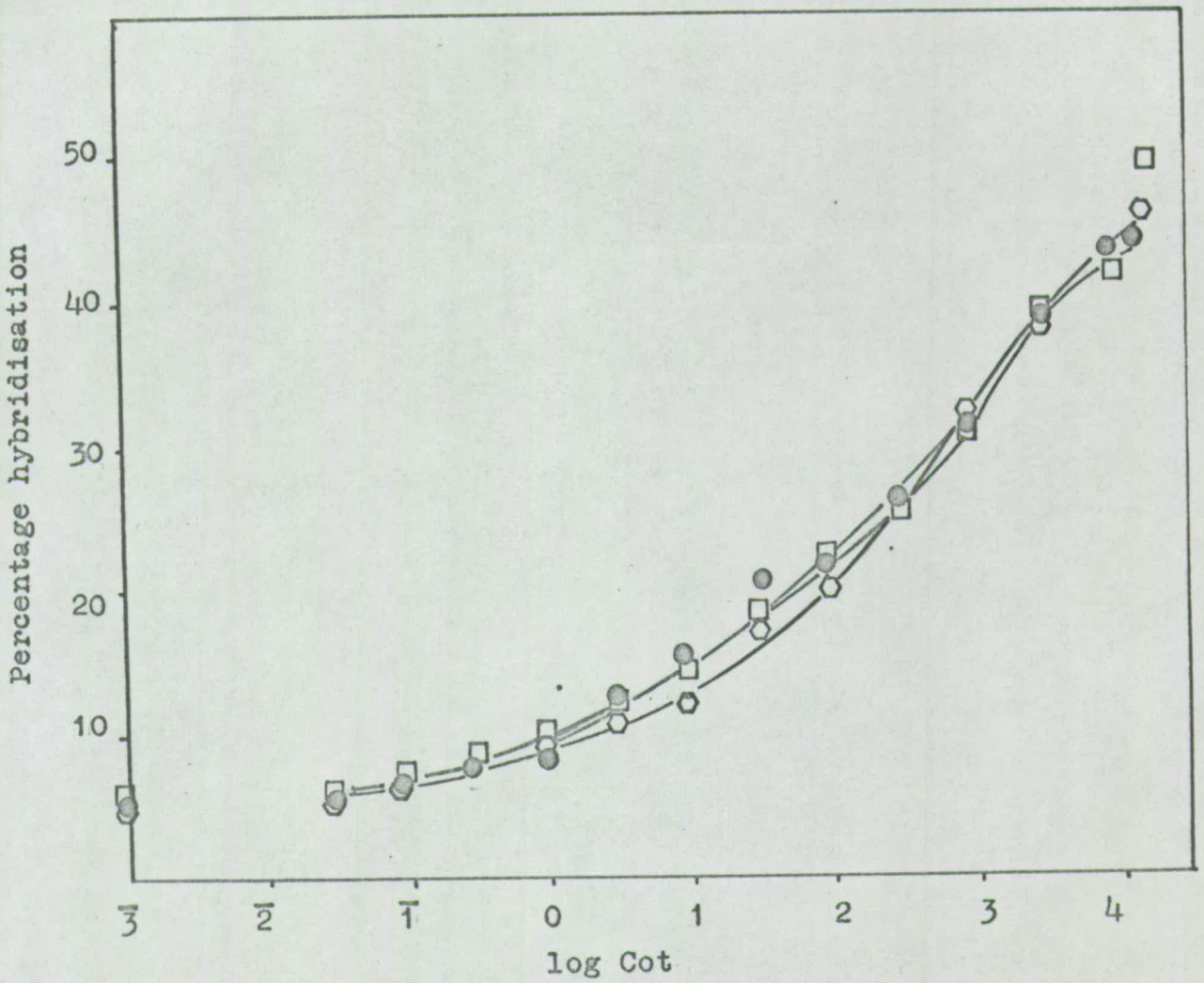

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 23

Legend to Figure 24

Hybridisations of condonced and dispersed chromatin DNA and total nuclear DNA from regenerating rat liver in conditions of DNA excess with ^{3}H cRNA synthesised on a template of dispersed chromatin DNA prepared from regenerating rat liver

The hybridisation curves of rd ^{3}H cRNA with RC, RD and RT DNA which were shown separately in Figures 20, 21 and 22 are shown together for ease of comparison

O—O regenerating dispersed cRNA and regenerating condonced DNA (rd - RC)

■—■ regenerating dispersed cRNA and regenerating dispersed DNA (rd - RD)

⊙—⊙ regenerating dispersed cRNA and regenerating total DNA (rd - RT)

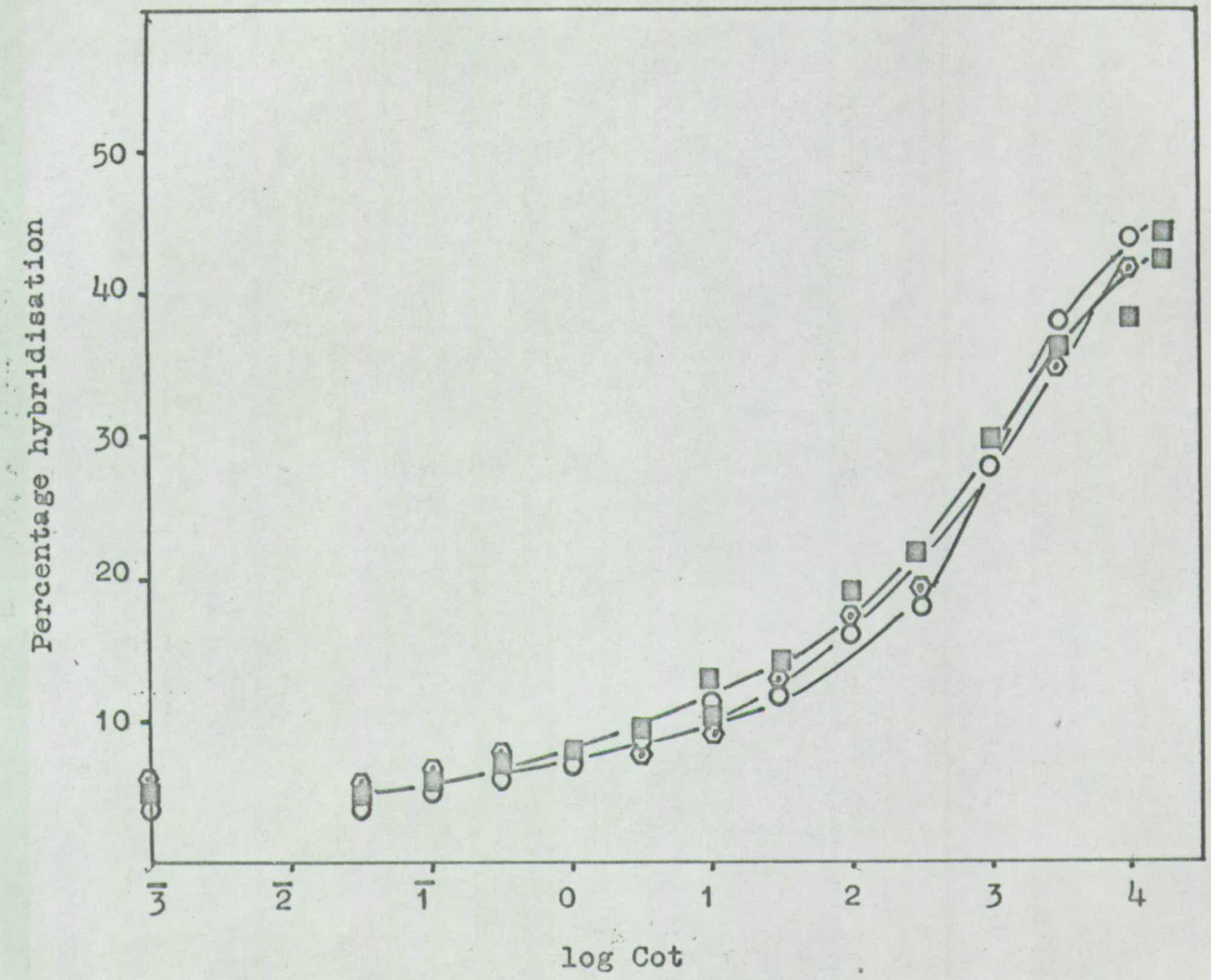

Percentage hybridisation
50
40
30
20
10
$\bar{3}$
$\bar{2}$
$\bar{1}$
0
1
2
3
4
log Cot

Figure 24

All the reactions with rc cRNA proceed much faster i.e. have lower $Cot_{\frac{1}{2}}^{h}$ s than those with rd cRNA, although all the reactions reach the same level of hybridisation (about 44% at a Cot of 20,000).

The template properties of the DNAs are shown in the hybridisation of the condensed and dispersed RNAs with total DNA. The hybridisation of nc and nd cRNAs with normal total DNA indicates that the RNA transcribed from condensed DNA is enriched for reiterated sequences when compared with the RNA transcribed from dispersed DNA, but that there is little or no partition of the highly reiterated sequences. Hybridisation of rc and rd cRNAs with regenerating total DNAs shows the same effect.

The partition of sequences in the fractionated chromatin DNAs can be seen by comparing them with total DNA. Comparison of the NC reactions with those of NT indicates that the former contains more reiterated sequences complementary to those in nc than does NT, but that it contains fewer sequences complementary to nd cRNA than does NT. ND has an enrichment of sequences complementary to nd compared with NT DNA. Reactions with RC DNA differ from those of normal dispersed DNA in that the homologous reaction RDrd has a higher $Cot_{\frac{1}{2}}^{h}$ than RDrc. Hybridisation of regenerating condensed DNA is similar to NC DNA indicating an enrichment of reiterated sequences in RC.

It appears that there are definite sequence differences between the fractions, the dispersed fraction containing the slower sequences from which some of the HnRNA is transcribed and the condensed chromatin containing a greater proportion of reiterated sequences. Hybridisation of <u>in vivo</u> RNA detects only those sequences in the DNA complementary to that RNA. Hybridisation of the cRNA is expected to show far

more of the sequence composition of the DNA. However, the use of both methods of investigation provides more information than either method alone.

DISCUSSION

The Concept of Fractionation

Previous studies of isolated chromatin fractions attempted to relate the properties of the isolated dispersed and condensed chromatin to the cytological euchromatin and heterochromatin by differential labelling and microscopic appearance (Frenster et al, 1963; Frenster 1965) and by the amounts of satellite DNA contained in the heterochromatin fraction (Yasmineh and Yunis, 1969, 1971; Yunis and Yasmineh, 1970). Heterochromatin, originally defined as a chromosomal material, the condensation of which was out of phase with the other nuclear material (so that it was heteropycnotic), is now also considered to be a genetically inert substance. The discovery that mouse satellite DNA was associated both with cytological heterochromatin and with isolated condensed chromatin has created a situation in which the method of fractionation is influenced by the necessity of maintaining this association.

However, the important questions of transcriptional activity or potential of the fractions and the differential distribution of sequences other than satellite DNA have not been explored. In this thesis, the problems under consideration are:-

a) In the rat, an animal with no significant satellite fraction, cytological heterochromatin is clearly visible. The initial criterion for fractionation was based on Frenster's finding (Frenster et al 1963) that the dispersed fraction from calf thymus lymphocytes in interphase incorporated DNA precursors more rapidly than the condensed fraction.

Could such a difference be observed in fractionated rat liver chromatin?

b) The differential replication of GC rich and AT rich DNA may be correlated with that of euchromatin and heterochromatin.

Is it possible to detect buoyant density differences between isolated condensed and dispersed chromatin DNA in rat?

c) Can sequence differences between the condensed and dispersed fractions be detected -

i) by optical renaturation?

ii) by hybridisation of cRNAs transcribed from the DNA of chromatin fractions?

iii) If sequence differences are found in normal DNA by hybridisation of the cRNAs are these the same in normal and regenerating fractionated chromatin DNA?

(i.e. Are there changes in the distribution of DNA in condensed and dispersed chromatin in regeneration?)

d) Is the condensed fraction transcriptionally inert or less active than the dispersed fraction?

i) are these differences in the RNA associated with each chromatin fraction and is this RNA transcribed from the fraction with which it was isolated?

ii) Is there a significant difference between the hybridisation patterns of condensed and dispersed chromatin DNA with *in vivo* HnRNA?

a. Differential labelling of the condensed and dispersed fractions

Frenster *et al* (1963) found great differences in the labelling properties of 'heterochromatin' and 'euchromatin' prepared from interphase lymphocytes after incubation with DNA precursors *in vitro*.

In the experiments reported in this thesis, fractionation after *in vivo* labelling was first attempted in normal rat liver. No significant differences in specific radioactivity were found in this normal tissue, but two problems were associated with these studies; a) normal tissue has a very low rate of incorporation of precursors so that the resulting specific activity of the DNA obtained was very low in all the fractions; b) this was a study of a random cell population. Different specific radioactivities of DNA in the normal system would therefore only disclose differences if the newly synthesised DNA were associated with the dispersed fraction.

Using regenerating liver (which is a tissue in which about 60% of the cells are in synchrony at the first mitosis (Brues and Marble, 1937; Cater *et al*, 1956; Harkness, 1957; Grisham, 1960; Bucher, 1963) fractionation and labelling by Yasmineh and Yunis' method during the S phase before the first synchronised division showed a much greater uptake of precursors and a clear differential uptake of ^{32}P between the condensed and dispersed fractions. Two possible explanations of this phenomenon are:

I) that the time of labelling and killing selected, coincides with the time at which dispersed chromatin is being synthesised;

II) that all the newly synthesised DNA (due to some unwinding process during replication) is in a dispersed state and is fractionated with the dispersed chromatin.

Hypothesis I could be tested by pulse labelling synchronised cells in culture at different times during S phase and fractionating chromatin. If this hypothesis is correct, one would find mostly dispersed chromatin DNA labelled at one time and condensed chromatin

DNA at another. An experiment to test hypothesis II could be designed as follows:- cells in culture could be given a short radioactive pulse followed by a chase period. Fractionation of chromatin could be carried out at intervals during the chase. If the hypothesis is correct, initially most of the labelled DNA would be found in the dispersed fraction, but during the chase period the amount of label in the condensed fraction would increase at the expense of that in the dispersed fraction.

In either case, the method of Yasmineh and Yunis does effect a fractionation which cannot be achieved by Frenster's method.

b. The buoyant densities of fractionated chromatin DNA

The phenomenon of replication early in S phase of main band DNA of higher buoyant density (GC rich) and late synthesis of AT rich low buoyant density material (Tobia, Schildkraut and Maio, 1970; Bostock and Prescott, 1971; Flamm, Bernheim and Brubaker, 1971) was thought possibly to have a relationship with the replication of DNA in euchromatin and heterochromatin, although there was no evidence about base composition differences in the cytological chromatin fractions. Previous investigations were carried out on fractions in which the criterion of purity was the absence of satellite from the "euchromatin" and its presence in the "heterochromatin". No fractionation of main band DNA was demonstrated.

In the present studies, a true fractionation of main band sequences has been shown by the results of the hybridisation experiments described on pages 74 -83, and the isotope incorporation experiments described on pages 65 to 69. Although there is no definite evidence that

the isolated chromatin fractions are related to cytological euchromatin and heterochromatin, if any difference in buoyant density existed it should have been possible to detect it. However, the banding patterns of the DNA extracted from the rat chromatin fractions showed no differences between normal and regenerating systems or between condensed and dispersed fractions and no rat satellite fractions were seen. The buoyant density data presented on page 69 are in agreement with those of Bostock and Prescott (1971) and Mattoccia and Comings (1971) in that they found no significant difference between the buoyant density of "heterochromatin" main band DNA and that of total nuclear main band DNA in mouse, rabbit and chinese hamster. However, Mattoccia and Comings found that mouse euchromatin DNA had a slightly higher buoyant density than mouse main band. Bostock and Prescott did not detect such a difference. Two interpretations of this are possible: Mattoccia and Comings consider that the difference is significant; Bostock and Prescott suggest that if it is real then it is probably significant only for the mouse.

The findings here for the rat suggest that even if this difference exists in mouse it is not generally applicable.

c. Renaturation studies on the DNA of fractionated chromatin

The hypothesis that the rat genome is susceptible to fractionation in a way similar to that of mouse, guinea pig and calf (as performed by Yasmineh and Yunis, 1969, 1971; Yunis and Yasmineh, 1970) was tested by investigating the distribution of reiterated sequences or sequences with similarities to satellite DNA. A comparison of the

optical renaturation curves of the condensed and dispersed chromatin DNAs shows no differences between the fractions with regard to repeated or non-repeated sequences. It is not possible to state without qualification that there is no enrichment of repeated sequences in the condensed chromatin fraction. A small significant difference may exist between fractions but it could not be detected under the conditions used here. This result does not exclude the possibility that differences may be present at stages of liver regeneration either earlier or later than those investigated in this study.

On the basis of the hypothesis that euchromatin is genetically active it might be expected that the dispersed fraction would contain a greater proportion of the genes from which messenger RNA is transcribed. The presence of reiterated DNA in this fraction is not unexpected if Georgiev's hypothesis (Georgiev, 1969) of genetic control is true. This postulates that portions of repeated sequences are transcribed in a manner analogous to that of the bacterial operator regions. Britten and Kohne (1968) suggest that repeated sequences occur commonly between structural genes. With regard to the condensed fraction, the situation is a little more complex. The isolation method was expected to separate nucleoli with the condensed fraction. (The preparation of this fraction is very similar to the method used by Steele (1968) to prepare rat nucleolar DNA). Several estimations have been made of the number of ribosomal cistrons in the rat. These vary from those of Quincey and Wilson (1969) who estimated that the amount of ribosomal DNA was 0.14×10^9 daltons and Mohan, Dunn and Casola (1968) (0.46×10^9 daltons) to that of Steele (1968) of

0.53×10^9 daltons. The figures for the multiplicity of the ribosomal cistrons from these results are 280, 950 and 1,100 respectively. Taking the size of the rat genome to be 5.8×10^{12} daltons DNA per cell these figures for the ribosomal RNA mean that between 0.02% and 0.094% of the total DNA of the rat cell is involved in coding for ribosomal RNA. (The discrepancy between estimates may be explained either by differences in the hybridisation methods and the interpretation of the data, or the possibility that the ribosomal RNA used in the experiments was not pure).

In addition to the several hundred copies of the cistrons coding for ribosomal RNA, there are other minor classes of DNA coding for such short sequences as 4S and 5S RNA. Evidence has recently been presented for the clustering of the genes for the putative histone messenger (Kedes and Birnstiel, 1971). The DNA represented by these sequences is probably less than 1% of the whole genome. At the level of discrimination of optical renaturation studies, the presence of only one of these components in only one of the three fractions could not be seen.

The next question is whether the condensed fraction is expected to be associated with any satellite sequences. To date, only Steele (1968) has described satellites in rat. He found satellites with a buoyant density of 1.692 g. cm^{-3} and 1.708 g. cm^{-3} in the nucleolar DNA. The heavy satellite comprised 0.24% of the total DNA and the light satellite 0.12% of the total DNA and thus even if located exclusively in the condensed fraction it is probable that they would not be seen in the renaturation curves. In the absence of a significant satellite fraction in the rat to monitor fractionation,

and with no apparent differences in sequence distribution by renaturation studies, it was necessary to look further afield for methods to demonstrate differences in the sequence distribution between fractions.

d. Hybridisation of cRNA with fractionated chromatin DNA

These hybridisation reactions examine the fractionation which has been effected in the DNA using a highly labelled tracer RNA synthesised on the fractionated chromatin DNA template. Nearest neighbour analysis (Weiss and Nakamoto, 1961) showed good agreement between the cRNA and primer DNA, although it cannot be assumed from this that all sequences are equally transcribed. The technique of cRNA transcription from DNA using a bacterial polymerase has been used very frequently as a method for the evaluation of genomic differences in higher organisms (Robertson, Chipchase and Man, 1969; Hennig, Hennig and Stein, 1970; Melli and Bishop, 1969; Paul and Gilmour, 1968).

i) Normal liver DNA

The NC-nc hybridisation has a low value for the $Cot^{h}_{\frac{1}{2}}$. This is similar to the value reported by Melli et al for ribosomal RNA hybridisation to total rat liver DNA. It thus appears that in the condensed fraction there is a transcription of DNA which has a similar reiteration frequency to ribosomal DNA. Steele's experiments (Steele, 1968) on the isolation of nucleoli suggest that this DNA might be found in association with a condensed fraction.

Less of the nc RNA hybridises with ND DNA (46% compared with 53% for NC DNA) and the $Cot^{h}_{\frac{1}{2}}$ is much greater. This suggests that

the ND DNA lacks certain reiterated sequences found in the NC DNA. Hybridisation between nc cRNA and ND DNA does indicate that there are sequences in common between ND and NC DNAs but the high transition between Cots 300 and 1,000 in the ND-nc reaction indicates that those sequences which both DNAs have in common have only a low degree of reiteration in ND DNA compared with that in NC DNA.

The RNA transcribed from ND DNA hybridises to the same extent with both ND and NC RNA although the $Cot_{\frac{1}{2}}^{h}$ from the reaction with ND (200) is lower than that for the reaction with NC DNA. The difference in the shapes of the curves indicates that NC DNA has more unique sequences than ND. Sequences with a low degree of reiteration in ND and transcribed from it are also present in NC DNA, but hybridise at higher Cots (1,000 to 20,000) and are therefore presumably represented only once. Normal condensed DNA therefore contains reiterated sequences which are not detected in ND DNA. This is a fractionation of sequences in NC and ND DNA which is consistent with the original expectation that some separation of reiterated sequences in the condensed fraction of rat (similar to that in mouse) might be achieved.

ii) Regenerating liver DNA

In normal liver, the proportions of DNA in the condensed, intermediate and dispersed fractionswere 30: 50: 20 whilst in regenerating liver chromatin this changed to 20: 50: 30. This suggested that the distribution of sequences in fractions from regenerating liver may differ from that in fractions from normal liver, and was confirmed by the results of the *in vitro* RNA hybridisation experiments.

The very fast NC DNA-nc cRNA hybridisation with a $Cot_{\frac{1}{2}}^{h}$ of 30 had no exact parallel with the DNA-cRNA reaction in regenerating liver. There is an enrichment of reiterated sequences in normal condensed DNA which does not appear to be present to the same extent in the regenerating condensed fraction.

The RNA transcribed from regenerating condensed DNA contains a high proportion of moderately reiterated sequences. This RNA hybridises with RD DNA almost as well as with RC DNA (its template). The RNA transcribed from the dispersed fraction of regenerating liver chromatin hybridises mainly with the unique sequences of both RC and RD DNA. The renaturation of fractions of DNA from regenerating liver indicates that there were no detectable sequence differences between the fractions. These data are not inconsistent with the hybridisation of regenerating DNA-cRNA where the differences between hybridisation of the two fractionated DNAs with the same cRNAs are only small.

The main difference in the regenerating chromatin fraction DNAs lies in the sequences transcribed by the RNA polymerase. It is probable that in the mammalian genome there are special sequences which initiate transcription and it is therefore likely that the fractionation of such sequences has occurred.

The difference between the normal and regenerating systems may be due to an uncoiling of condensed chromatin, making more sequences available for transcription or to a reorganisation of the intermediate fraction. Certainly the process of regeneration involves changes in the state of chromatin. Sequences which in normal liver are largely restricted to condensed or dispersed chromatin, in regenerating liver

seem to be more uniformly distributed (though there is still a higher proportion of the reiterated sequences in condensed chromatin DNA). Such a change is to be expected if regeneration requires an increase in total cellular activity and if condensation of chromatin is associated with a reduction in activity. This is therefore consistent with the considerable differences found between cRNA-DNA hybridisations from normal and regenerating liver.

e. The transcriptional activity of chromatin fractions

There is a considerable body of evidence to suggest that the DNA in cytological heterochromatin is not transcribed. Certainly, facultative heterochromatin appears to be genetically inactive (Lyon, 1961). Constitutive heterochromatin appears identical to facultative heterochromatin, and the coiling which makes it stain heterochromatically is thought to be the mechanism which inactivates facultative heterochromatin. Satellite DNA is associated with heterochromatin (Pardue and Gall, 1970; Jones, 1970) and with isolated condensed chromatin (Yasmineh and Yunis, 1969) and, at least in mouse, does not appear to be transcribed *in vivo* (Flamm, Walker and McCallum, 1969). Studies on Actinomycin D binding to chromatin are inconclusive (Ringertz and Bolund, 1969; Carmago and Plaut, 1967; Ebstein, 1967; Desai and Tencer, 1968) but Sieger, Garweg and Schwarzacher (1971) showed by studies on ^{3}H-uridine incorporation that the constitutive heterochromatin of *Microtus agrestis* is transcriptionally inactive.

It is therefore probable that much of the constitutive heterochromatin is not transcribed *in vivo*. Is this true of the isolated condensed fraction in rat? The labelling studies of the RNA in the

condensed fraction (discussed in the results pages 66 to 68) indicated that RNA having very similar properties to ribosomal RNA was associated mainly with the condensed fraction and to a lesser extent with the intermediate fraction. The isolated condensed fraction DNA also hybridises readily with the in vivo nuclear heterogeneous RNA and the hybridisation curve has a prominent inflexion with a $Cot^{h}_{\frac{1}{2}}$ of about 15 which is most probably due to the presence of ribosomal sequences. The sedimentation value of the largest precursor molecule for ribosomal RNA is 45S (Girard, Penman and Darnell, 1964; Penman, 1966; Scherrer, Latham and Darnell, 1963). It is possible that in normal mammalian tissues there are precursor molecules or aggregates of even greater size than 45S and those having a sedimentation value greater than 80S would be isolated with the HnRNA. Those sequences in the HnRNA homologous with the ribosomal cistrons in the condensed DNA would show the characteristic hybridisation at the intermediate Cot value.

The method of preparation of the condensed fraction suggested that nucleoli would be isolated with this fraction (Steele, 1968). Taking the results of the RNA labelling experiments in conjunction with the presence of the ribosomal 'bump' in the hybridisation of condensed DNA and cellular RNA indicates that at least the greater portion of the ribosomal cistrons are located in the condensed chromatin fraction.

In the studies of the RNA associated with the chromatin fractions, most of the HnRNA was found associated with the dispersed fraction. While it is possible that the nascent HnRNA may be released into the nucleoplasm, and in the fractionation method isolated with the dispersed fraction, the very significant hybridisation of the HnRNA

with dispersed fraction DNA over the slow (unique) sequence range of Cots, suggests that the HnRNA is probably transcribed from the fraction with which it is associated. It is in the high Cot range that one would expect to find the classical genes and their messengers. The finding that nuclear heterogeneous RNA (which probably includes the precursor molecules of messenger RNA (Georgiev, 1969; Ryshov and Georgiev, 1970; Melli and Pemberton, 1972) hybridises with the slower sequences in the condensed chromatin fraction indicates that there is a partition of the slow sequences complementary to HnRNA in the dispersed fraction. (This finding is complemented by the cRNA hybridisation experiments which show an enrichment of slow sequences in the dispersed fraction). This suggests that the dispersed fraction of chromatin contains the classical 'structural genes' those from which messenger RNA is transcribed.

Apart from the ribosomal component of hybridisation of the HnRNA with condensed fraction DNA, the only hybridisation with HnRNA occurs at very high Cots. While it is possible that this RNA is the product of unique sequences which may form a small fraction of this DNA, it is equally possible and consistent with current theories that this hybridisation is the result of some dispersed sequences being isolated with the condensed fraction. Although it is probable that heterochromatin is more condensed than euchromatin, it is doubtful whether pure preparations can be obtained by the mechanical means used here. However, the fact that ribosomal sequences are found only in the condensed fraction and that there is an enrichment of unique sequences in the dispersed fraction indicates that the method used in this work has achieved some degree of purity of partition.

CONCLUSION

The total DNA of a cell may be divided into three functional classes:-

a) the classical genes, that this, that class of DNA which is transcribed into RNA at least a part of which is transported to the cytoplasm there to be involved in the synthesis of protein.

b) structural DNA, which is involved in such functions as maintainance of the physical integrity of the chromosomes, correct association of chromosome pairs during meiosis etc.

c) control DNA. Whichever current model of differentiation and development is accepted, some DNA is probably involved in the synthesis of regulatory molecules and more must form the sites upon which these molecules act.

Speculation about the possible distribution of these classes of DNA within the genome involves consideration of the degree of constancy of heterochromatin and euchromatin. Yunis and Yasmineh (1971) consider that constitutive heterochromatin is constant throughout the development of an organism. The alternative is that there is progressive inactivation by a process of heterochromatisation as differentiation continues (Brown, 1966). In the first case, one would expect to find all the classical genes in euchromatin, while in the second, distribution would change during the course of development, and in the adult one would expect to find a considerable proportion of the classical genes in the heterochromatin.

The results of the experiments presented here indicate an enrichment of the unique sequences in the dispersed fraction and a depletion in the condensed fraction. It is among these unique

sequences that one would expect to find the classical genes and it would therefore appear that there is little heterochromatinisation of the unique sequences. However, both in the hybridisation with cRNA and the proportions of material isolated in each fraction, there were differences between normal and regenerating rat liver and this may tend to support the alternative hypothesis. These differences may not be due to changes in condensation of chromatin as a result of differentiation but to other factors such as the state of the nuclei, which may affect the fractionation process.

It is not particularly profitable to speculate about the distribution of control DNA because there is little or no evidence. However, one may find it among reiterated sequences since that part of HnRNA not exported to the cytoplasm appears to be reiterated, and this may be involved in the regulation of synthesis of messenger.

Satellite DNA, in organisms which have it (mouse is the best studied) appears not to be transcribed, and may be an example of structural DNA. Since satellite is always intimately associated with heterochromatin it is probable that in those organisms which do not have satellite (e.g. rat) at least part of the constitutive heterochromatin may be involved in these functions. The condensed fraction which may, or may in part, be homologous with constitutive heterochromatin was enriched for reiterated sequences which suggests that one of its function may be analogous to that of satellite.

Possible extensions of this work

These studies have investigated only the nucleic acids of fractionated chromatin, but it is the association of protein with nucleic

acids which confers on the material the characteristics of chromatin. One of the most important questions to be investigated with regard to the association of protein and nucleic acids is whether this restricts the availability of the DNA for transcription or in any way alters the pattern between fractions. There are however several difficulties involved in the synthesis of cRNA from a template of fractionated 'chromatin'. The dispersed or 'euchromatin' fraction was obtained (in the experiments reported here) by alcohol precipitation and is relatively insoluble in most solutions. The pellets of the condensed and intermediate fractions are not appreciably soluble in low salt solutions (such as 0.1 x SSC or dilute phosphate buffer) suitable for transcriptional experiments involving the use of a DNA dependent RNA polymerase. To facilitate such studies, an alternative isolation method would be required, but this would necessitate studies on the fractionation achieved by the new technique.

The association of RNA with the specific fractions might cause problems. Kanehisa, Fujitani, Sano and Tanaka (1971) showed that a species of RNA in chick liver which had a sedimentation coefficient of 7-10S inhibited RNA synthesis in a cell free system. They thought that the RNA had a higher affinity for chromatin isolated from the same tissue and probably modified its structure with regard to template properties. The problems of removing the RNA are considerable. The difficulty involved in the use of RNase is its removal from the preparation prior to the synthesis of cRNA. Washing fractionated chromatin to remove RNA is not without problems, since 'chromatin' fractionated after sonication unlike normal chromatin has already been subjected to disruptive proceedures. If the RNA was not completely

removed from the preparation, then it would be isolated in conjunction with the cRNA and add to the complexity of the hybridisation reaction in addition to its interference with the actual transcription.

Melting curves of the fractionated chromatin would also be of great interest to demonstrate the association of different proteins with the chromatin.

However, these experiments suffer from the same disadvantages as those involving the transcription of cRNA, notably the insolubility in solvents suitable for melting curves.

The relationship of the cytological preparations to the isolated chromatin fractions could be investigated by the technique of <u>in situ</u> hybridisation. This would help to relate the biophysical properties of isolated fractions with the cytological hybridisation patterns of cRNA transcribed from chromatin fractions with interphase cells and cytological chromosome preparations from the rat.

The method of fractionation reported here suggests that it may be possible to separate a chromatin fraction enriched for sequences from which messenger RNA is transcribed. One would expect to find an equal enrichment for protein initiation sequences in this fraction. Experiments to measure these sequences would provide an independent assessment of the validity of the work described here.

SUMMARY

1. Two methods of fractionating chromatin were compared by measuring differences in the specific radioactivities of DNA and RNA extracted from the condensed and dispersed fractions of chromatin from regenerating rat liver.

2. Only one method of fractionation showed a clear difference in labelling of the DNA and RNA between the fractions. This method was used to prepare large amounts of pure DNA from three fractions of chromatin: condensed, intermediate and dispersed.

3. Evidence of changes in the distribution of fast intermediate and slow renaturating sequences of DNA during liver regeneration was sought by an investigation of the kinetics of renaturation of normal and regenerating rat liver DNA. Studies of DNA renaturation kinetics were also used to investigate differences in sequence distribution in the DNA of the three chromatin fractions from regenerating liver.

4. The buoyant densities of DNAs prepared from fractionated chromatin of both normal and regenerating rat liver were measured by analytical ultracentrifugation in an attempt to detect differences in base composition.

5. RNA was transcribed *in vitro* from templates of unsheared condensed and dispersed chromatin DNA prepared from normal and regenerating rat liver. This highly labelled RNA was hybridised with a vast excess of DNA to detect the reiteration frequencies of sequences in the DNA of the different chromatin fractions complementary to the RNA.

6. The technique of hybridisation in DNA excess was also used to investigate the complementarity of DNA sequences in the chromatin fractions with *in vivo* nuclear heterogeneous RNA prepared from regenerating rat liver.

7. The results are discussed with reference to:-

i) the transcriptional activity of different fractions of the genome.

ii) changes in sequences transcribed during liver regeneration (as a model of a developmental process).

iii) the organisation of the mammalian genome.

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