CRYSTALLINE CYTOCHROME b_2

Thesis submitted for the

degree of

Doctor of Philosophy

by

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from

The Department of Biochemistry

University of Adelaide

July 1967.

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ACKNOWLEDGEMENTS

This work was supported by a grant from the Nuffield Foundation.

I thank Dr. R.H. Symons for his advice, encouragement and criticism during the experimental studies and preparation of the manuscript.

Dr. Symons also synthesised the α-[^{32}P] labelled UTP used in CHAPTER VI.

Professor M.R. Atkinson at the Department of Biology, Flinders University, supplied some materials (stated in text) and his advice and comments have been much appreciated.

The electron microscope studies reported in CHAPTER IV were made possible by the assistance of Miss P.Y. Dyer who operated the electron microscope and carried out much of the fixation and preparation.

I should like to thank Dr. G.E. Rogers for his general advice related to the electron microscope work, and Dr. D.B. Keech for advice on a large number of questions related to carbohydrate metabolism.

I should like to acknowledge the efforts of Mrs. K. Warnaby who typed the final draft of this thesis.

The interest and encouragement from the Head of the Department, Professor W.H. Elliott and other members of the Department has been invaluable.

INDEX

ABBREVIATIONS

ATP	Adenosine triphosphate.
$\underline{b}_2$-DNA	This is the DNA found in the crystals of Type I cytochrome $\underline{b}_2$ (see INTRODUCTION of thesis).
cm.	Centimeters.
CPM	The number of counts or pulses per min. as recorded by a Geiger-Muller, gas-flow or Scintillation counter.
CTP	Cytidine triphosphate.
DNA	Deoxyribonucleic acid.
E	This is the optical density of a solution with a 1.0 cm. light path at the wavelength stated in the subscript.
EDTA	Ethylene diamine tetraacetate.
FMN	Flavin mononucleotide.
g.	Gram.
GM	Geiger-Muller.
GTP	Guanosine triphosphate.
M	Molar.
mCi	Millicurie.
mg.	Milligram.
ml.	Millilitre.
mM.	Millimolar.
PEP	Phosphoenol pyruvate.
RNA	Ribose nucleic acid.
TCA	Trichloroacetic acid.
tris	Tris (hydroxymethyl) amino methane.
UTP	Uridine triphosphate.

SUMMARY

(1) It has been shown that the flavo-haemo-protein of cytochrome $\underline{b}_2$ must undergo modification before it will crystallise under the conditions of the first crystallisation step in the Appleby and Morton procedure.

(2) Air drying or ageing of the yeast is necessary for the release of $\underline{b}_2$-DNA. Preparations of crystalline Type I cytochrome $\underline{b}_2$ from fresh, freeze-dried yeast contained less than the usual amounts of $\underline{b}_2$-DNA.

(3) The protein and DNA of the oxidised Type I cytochrome $\underline{b}_2$ have been shown to be largely dissociated when the enzyme is in solution.

(4) The specificity of the association between the DNA component and the enzyme of crystalline Type I cytochrome $\underline{b}_2$ has been investigated by testing the ability of various nucleic acid preparations to form crystalline complexes with DNA-free (Type II) cytochrome $\underline{b}_2$. It was found that only double-stranded DNA molecules with a molecular weight of roughly 2×10^5 produced the square plate crystals that are characteristic of normal preparations of Type I cytochrome $\underline{b}_2$. High molecular weight DNA and single-stranded DNA, either native or denatured, produced either amorphous

precipitates or various semi-crystalline forms. These effects were independent of the base composition of the samples used. Polyacrylate, a linear, non-cross linked, polyanion produced square plate crystals with Type II cytochrome $\underline{b}_2$.

(5) The ability of $\underline{b}_2$-DNA to anneal extensively with all samples of labelled yeast RNA collected after centrifugation on a sucrose density gradient, even with RNA up to 10 times its own size, has been taken as proof that $\underline{b}_2$-DNA is a breakdown product of higher molecular weight yeast DNA.

(6) The structure of the two crystalline forms of cytochrome $\underline{b}_2$ has been studied by electron microscopy. From the results, the approximate dimensions of a single enzyme molecule of molecular weight 170,000 were determined as 92 x 82 x 26 A^{o}.

(7) Sections of the hexagonal bipyramid crystals (Type II cytochrome $\underline{b}_2$) at right angles to the $\underline{c}$ axis showed a regular hexagonal network with, apparently, one protein molecule forming the side of each hexagon. Sections parallel to the $\underline{c}$ axis showed that the empty, hexagonal tubes of protein ran right through the crystal. The structure deduced from the sections was

in agreement with that observed in negatively stained, sonicated fragments of the same crystal type.

(8) The flat, square plate crystals of Type I cytochrome $\underline{b}_2$ were seen as parallel rows of protein molecules arranged as layers which were stacked on top of each other to form the crystal. It appeared that alternate layers of protein were arranged at right angles to the ones in between. It has not been possible to obtain satisfactory side views of the structure of this crystal type, nor to locate visually the position of the DNA. However, a structure of these nucleoprotein crystals consistent with available data has been proposed.

(9) A yeast protein solution has been prepared that had DNA-dependent RNA polymerase activity that was Actinomycin D sensitive. The enzyme appeared to be producing a hetero-polymer of ribonucleotides and some of its other properties have been briefly described.

INTRODUCTION

The programme of work described in this thesis was carried out because of an apparent specific association between a DNA and one of the crystalline forms of cytochrome $\underline{b}_2$ (the L (+) lactate: cytochrome $\underline{c}$ oxido-reductase of bakers' yeast EC 1.1.2.3.).

The aim of this work was to elucidate the relationship between the enzyme and the DNA and to look for the possible *in vivo* function of such a nucleoprotein.

This introduction will give a brief history of cytochrome $\underline{b}_2$ and then consider in detail the evidence and theories that formed the basis of this work when it was started in 1964. However, where relevant, references appearing after 1964 will also be included.

Some of the work described in this thesis has already been published (see list of publications). One of the publications (Nicholls, Atkinson, Burgoyne and Symons, 1966) includes work that was carried out by this author and is described in this thesis. Whenever necessary, work of the other authors will be referred to as Nicholls *et al.* (1966).

A. HISTORICAL

(1) Yeast lactate dehydrogenases.

Interpretation of early studies of yeast lactate-dehydrogenases is difficult as it was not until recently that the presence of at least three, and probably four, yeast lactate dehydrogenases with differing substrate and acceptor specificities was recognised.

These are as follows:

(i) A D(-) lactate-dehydrogenase that was repressed by aerobic conditions and which used ferricyanide and 2,6-dichlorophenol indophenol as acceptors but not cytochrome c (Slonimski and Tysarowski, 1958; Labeyrie, Slonimski and Naslin, 1959; Nygaard, 1960).

(ii) A D(-) lactate-dehydrogenase that was repressed by aerobic conditions and used cytochrome c as an acceptor but not ferricyanide or 2,6-dichlorophenol indophenol (Nygaard, 1960, 1961).

(iii) Possibly another D(-) lactate-dehydrogenase with similar substrate and acceptor specificities, although different kinetic constants, to the anaerobic D(-) lactate-dehydrogenase described in (i).

It is thought, however, that this enzyme may have been a derivative of the D(-) lactate, cytochrome c oxido-reductase described in (ii) (Nygaard, 1961).

(iv) A L(+) lactate-dehydrogenase. This was induced by aerobic conditions, (Slonimski and Tysarowski, 1958; Labeyrie, Slonimski and Naslin, 1959; Nygaard, 1960) ferricyanide, 2,6-dichlorophenol indophenol and cytochrome c were acceptors and preparations that contained only this L (+) lactate specific enzyme have been demonstrated (Mahler and Huennekins, 1953; Boeri, Cutolo, Luzzati and Tosi, 1955). It is the crystalline form of this enzyme that is the subject of this thesis.

Thus, in many reports of yeast lactate-dehydrogenase activity prior to about 1942 it is difficult to be sure what enzymes were being studied because the substrate lactate was racemic and in the cruder preparations the nature of the primary electron acceptor was often doubtful. However, one of the first reports of lactate-dehydrogenase activity in bakers' yeast was that of Harden and Norris (1915) who found that dried yeast would reduce methylene blue when provided with lactate or lactic acid. A number of yeast preparations with lactate-dehydrogenase activity were found to be independent of free co-enzymes (Bernheim, 1928; Boyland and Boyland, 1934; Adler and Michaelis, 1935) and none of the yeast lactate-dehydrogenases has yet been shown to require a soluble co-enzyme.

(2) Yeast L(+) lactate-dehydrogenase (cytochrome b_2).

There seems to be little doubt that the preparations

of Bach, Dixon and Kielen (1942), Bach, Dixon and Zerfas (1942), and Bach, Dixon and Zerfas (1946) contained the L (+) lactate-dehydrogenase because similar preparations by Boeri *et al.* (1955) were specific for L (+) lactate. The Bach, Dixon, Keilen and Zerfas group was the first to suggest that a lactate-dehydrogenase was also a cytochrome. This group found that preparations from bakers' yeast contained a cytochrome which they designated "cytochrome b_2", and that it contained protohaem. This cytochrome was reduced by lactate and circumstantial evidence suggested that the cytochrome and lactate-dehydrogenase activity were either on one protein or were closely associated in some other way. This circumstantial evidence was as follows:

(a) In the latter stages of the purification procedures that they used the haemochrome and enzymic activity were found to maintain a relatively constant ratio.

(b) Loss of lactate-dehydrogenase activity due to aging of the preparations caused a loss of the ability to use lactate to reduce cytochrome b_2.

(c) A modified preparation procedure with a different strain of yeast still gave preparations that had a similar ratio of cytochrome b_2 as to lactate-dehydrogenase activity as was observed in the first strain.

However, Appleby and Morton (1954) succeeded in crystallising cytochrome $\underline{b}_2$ which allowed them to obtain much more definitive information (Appleby and Morton, 1959a,b; 1960) about the prosthetic groups and nucleic acids associated with the enzyme. These workers showed that not only did the enzyme contain the cytochrome $\underline{b}_2$ haem group of Bach *et al.* (1946), but that it also contained equimolar FMN with respect to the haem.

As will be discussed more fully below, Morton's group showed that both of these prosthetic groups were necessary for enzymic activity and both were integral parts of the one enzyme. Appleby and Morton (1959a,b; 1960) also showed that these crystals contained a constant 5-6% of a low molecular weight DNA with no known function. The enzyme, when it contained this "$\underline{b}_2$-DNA", was designated Type I cytochrome $\underline{b}_2$ and when it had the DNA removed was designated Type II cytochrome $\underline{b}_2$ (Morton, 1961; Morton and Shepley, 1961; 1963). This notation will be generally used throughout this thesis to describe the forms of the enzyme which contain the $\underline{b}_2$-DNA and which are free of DNA. However, when, for various reasons, this notation is not strictly applicable, the protein moiety may be referred to as the "flavo-haemo-protein".

B. A DESCRIPTION OF THE COMPONENTS OF CRYSTALLINE TYPE I CYTOCHROME b_2 OF YEAST

(1) The physical properties of the enzyme.

Armstrong et al. (1963a) obtained an appreciable experimental variation when they attempted to estimate the molecular weight of this protein by equilibrium sedimentation or by using diffusion and sedimentation coefficients but the minimum molecular weight, based on dry weight per haem, (Appleby and Morton, 1959b) has been estimated as 80,000 Daltons. Assuming that the molecular weight is an integral multiple of this value, then the ultracentrifuge results indicated that the protein molecule was a dimer with a molecular weight of 160,000 Daltons. There has been no evidence that the enzyme ever exists in the hypothetical monomeric state while the work of Morton and Sturtevant (1964) has indicated that either the dimer is stable or the monomer had identical kinetic properties in all the studies to date. This would mean that each molecule had two haems and two FMN groups per molecule suggesting that the enzyme may be a simple homodimer.

During electrophoresis in free solution the protein behaves as an anion over the pH range 5.0 to 8.5 (Armstrong et al. 1963a) which is in agreement with the amino acid composition reported by Appleby, Morton and Simmonds (1960). However, in starch gels it has been possible to

separate the protein into approximately five enzymically active species differing slightly in their mobility. These "isoenzymes" appear during the purification of the enzyme via the Appleby and Morton (1959a) procedure and they have been shown to be derivatives of a single unmodified species that existed before the purification (Nicholls *et al.* 1966). Although these electrophoretic changes in the enzyme protein are not large, they are accompanied by changes in the kinetic parameters of the enzyme (Labeyrie and Slonimski, 1964; Nicholls *et al.* 1966) and with other changes in behaviour that are reported in this thesis.

The b_2-DNA did affect the sedimentation coefficient of the enzyme protein and particularly its concentration dependence. Type II cytochrome b_2 was found to have a $S^o_{20,w}$ of 8.56 (Armstrong *et al.* 1963b) and Type I cytochrome b_2 had a $S^o_{20,w}$ of 9.17 (Armstrong *et al.* 1963a).

Type I and Type II cytochrome b_2 showed a precipitous decrease in solubility as ionic strength was lowered from approximately 0.2M to 0.05M lactate (Appleby, 1957; Appleby and Morton, 1959a; Morton and Shepley, 1961; 1963b; Symons and Burgoyne, 1966) an effect used to induce the enzyme to crystallise. As both the Type II and the Type I forms showed a lowered

solubility with lowered ionic strength, the effect was probably a property of the flavo-haemo-protein rather than being due to the formation of DNA-protein links. However, this does not mean that DNA-protein links should be entirely discounted when this solubility change is considered.

The $\underline{b}_2$-DNA was observed to have a most marked effect on the crystalline form that appeared when the ionic strength was lowered. While the enzyme was still in its Type I form (containing 5% $\underline{b}_2$-DNA) it crystallised as small square plates (Appleby and Morton, 1954, 1959a) or, as has been suggested (Appleby and Morton, 1959a) shallow tetragonal bipyramids. However, when the $\underline{b}_2$-DNA was removed from the enzyme the crystals that formed belonged to the hexagonal system and were pyramids or prisms capped with bipyramids (Morton and Shepley, 1961; 1963b). As this sharp decrease in solubility with ionic strength has been used to select active Type I cytochrome $\underline{b}_2$ from the inactive Type I cytochrome $\underline{b}_2$ that is found in aged preparations, it is probably a property of the native enzyme.

(2) Homogeneity of the flavo-haemo-protein.

When crystalline Type I cytochrome $\underline{b}_2$ was freshly prepared it was not observed to contain any contaminating proteins in studies that included electrophoresis in free solution (Appleby and Morton, 1960; Armstrong, Coates and Morton, 1963a,b), elution behaviour on DEAE-cellulose

(Symons, 1965) and electrophoresis in starch gels (Nicholls et al. 1966).

Armstrong, Coates and Morton (1960) showed that inactive proteins appeared on exposing the preparations to air or oxygen but, as they were virtually absent in fresh enzyme it appeared that they were derivatives of the active enzyme. Although the fresh enzyme did not contain enzymically inactive components it was possible to show, by electrophoresis on starch gels, that this preparation could be separated into a number of enzymically active species that differed slightly in their mobility (Nicholls et al. 1966). However, the significance of this finding will be discussed further in CHAPTER II.

(3) The haem and flavin group.

Appleby and Morton (1964) showed that the haem and flavin group in crystalline Type I cytochrome b_2 were protohaem and riboflavin phosphate (FMN). Both these groups are essential for enzymic activity as the removal of either of them from the enzyme caused loss of enzymic activity. However, this loss was not irreversible as both prosthetic groups have been replaced with concomitant recovery of some enzymic activity (Morton, 1961; Morton and Shepley, 1963a; Baudras, 1965; Motohiro and Di Franco, 1965).

During these studies, Morton (1961) observed that

the apo-enzyme recovered some of the enzymic activity if incubated with both prosthetic groups but if it was incubated in two separate lots, each with one of the prosthetic groups and the two separate lots mixed, it failed to regain activity. This showed that the two prosthetic groups were probably on one protein molecule, a conclusion supported by the observation that quite different methods of purifying yeast L-LDH gave preparations with a molar ratio of one haem to one FMN (Appleby and Morton, 1954, 1959a; Boeri, Cutolo, Luzzati and Tosi, 1955; Nygaard, 1959).

Inactivation of the enzyme with heat, oxygen, or sulphydryl-specific inhibitors resulted in the release of the FMN with simultaneous appearance of the FMN fluorescence that had previously been quenched by association of the FMN with the enzyme. However, the haem group was not released as readily from the protein as the FMN group but required low temperature precipitation with acetone in the presence of KCN (Morton, 1961; Morton and Shepley, 1963a). The spectral properties of the haem group prompted Morton, Armstrong and Appleby (1961) to suggest that positions 5 and 6 of the iron group were co-ordinated with strongly basic groups such as the imidazole nitrogen of histidine.

(4) <u>The mechanism of the enzymic reaction</u>.

The details of the reaction mechanism are not, as

yet, well established. However, it appears that the FMN group is first reduced and then reducing equivalents are transferred to an external acceptor either directly or via the haem group (Appleby and Morton, 1954; Morton and Sturtevant, 1964). Hiromi and Sturtevant (1965) have shown that the enzyme can be obtained with its FMN in the semiquinone state and it seems quite possible that this is an important reaction intermediate.

There is no evidence for the $\underline{b}_2$-DNA having any effect on, or involvement in, the reaction mechanism.

(5) The DNA component ($\underline{b}_2$-DNA).

(a) Composition. $\underline{b}_2$-DNA was found to contain the four bases adenine, guanine, cytosine and thymine in a ratio of 34, 15, 15, 36 mol. % respectively as well as deoxyribose and phosphorus (Appleby and Morton, 1960; Montague and Morton, 1960; Pereira and Mahler, 1962). It was analysed for ribose and uracil but both of these were found to be essentially absent (Appleby and Morton, 1960). It should be noted that these base ratios are in agreement with those expected for a typical "Watson and Crick" double helix.

(b) Secondary structure. Pereira and Mahler (1962) and Mahler and Pereira (1962) found that the $\underline{b}_2$-DNA had a number of properties usually associated with single stranded nucleic acids. These were the markedly ionic strength-dependent hyperchromicity, the

partial reactivity with formaldehyde, an abnormally low melting point, and susceptibility to a nuclease which is specific for single stranded nucleic acids (the nuclease is that described by Lehman, 1960). Mahler and Pereira's general conclusions received support from Bollum (1963) who found that this polynucleotide would act as a primer for a DNA polymerase that was not primed by double stranded DNA but was primed by single stranded DNA or DNA that had suffered some nuclease damage.

However, it is known that this $\underline{b}_2$-DNA has a very low molecular weight (see below) and it is possible that these unusual properties reflect a change in the average properties of the "Watson and Crick" double helix due to a high proportion of chain end regions and are not due to the presence of single stranded sections.

(c) Size of $\underline{b}_2$-DNA. Both the ultracentrifuge studies (Armstrong et al. 1963b) and the fractionation of $\underline{b}_2$-DNA on substituted cellulose anion exchangers (Montague and Morton, 1960; Symons, 1965), failed to detect gross size heterogeneity.

Early estimates of the molecular weight of the DNA, based on the proportion of DNA in the enzyme and preliminary end group studies (Montague and Morton, 1960), suggested that the molecular weight of the polynucleotide was as low as 10^4 Daltons but the ultracentrifuge

results of Armstrong *et al.* (1963b) indicated a particle weight of 1.2×10^5 Daltons. Although it was suggested by these workers that this particle might represent an aggregate of many units, attempts to demonstrate this using urea, high salt, chelating agents and para-amino-salycylate as de-aggregants were not successful (Burgoyne, 1962). An aggregate was suggested by Armstrong *et al.* (1963b) as this higher molecular weight of 1.2×10^5 was difficult to accept because it did not agree with the results of Montague and Morton (1960) and it implied that the nucleoprotein was not associated while in solution. This latter implication arose when it was realised that if the Type I cytochrome $\underline{b}_2$ was only 5-6% $\underline{b}_2$-DNA and if the DNA had a molecular weight of 1.2×10^5 then a complete protein-DNA unit would have a minimum molecular weight greater than 2×10^6 which is incompatible with the ultracentrifuge results of Armstrong *et al.* (1963a). However, Symons (1965) has shown that the reduced nucleoprotein was dissociated to a large extent while in solution so the aggregation hypothesis has thus been made unnecessary and the higher molecular weight (1.2×10^5) will be taken as the correct molecular weight for $\underline{b}_2$-DNA for the rest of this thesis.

(6) The specificity of the association between cytochrome b_2 and the DNA.

Although the DNA was not necessary for the catalytic activity of the enzyme (Morton et al. 1961), there was a large amount of circumstantial evidence that indicated a specific association between the enzyme and polynucleotide. A brief summary of the relevant evidence is presented.

(a) The DNA was found to be essential for the crystallisation of cytochrome b_2 in the form of square plates (Type I) as, on its removal, the enzyme crystallised as hexagonal bipyramids and hexagonal prisms capped with pyramids (Type II) (Appleby and Morton, 1960; Morton and Shepley, 1961; Morton and Shepley, 1963b).

(b) Although the Type I crystals were formed out of mother liquors containing a large excess of RNA over DNA, they contained DNA with only small amounts of contaminating RNA that was readily removed by recrystallisation (Appleby and Morton, 1960; Pereira and Mahler, 1962; Mahler and Pereira, 1962).

(c) The DNA was found to be present in a constant amount (5-6% by weight) in Type I crystals and to have a constant base composition different to that of bulk yeast DNA (Appleby and Morton, 1960; Morton et al. 1961; Pereira and Mahler, 1962; Mahler and Pereira, 1962).

(d) The DNA did not show heterogeneity of size

in the analytical ultracentrifuge (Armstrong *et al.* 1963b) or on salt gradient elution from ECTEOLA- or DEAE-cellulose (Montague and Morton, 1960; Symons, 1965).

(e) The pattern of pyrimidine sequences obtained on diphenylamine degradation (Burton and Peterson, 1957) of b_2-DNA was reproducible and significantly different from that of high molecular weight yeast DNA (Symons and Ellery, 1967).

(f) The protein was known to be anionic from pH 5 to 8.5 (Appleby and Morton, 1960; Armstrong *et al.* 1963a) and crystallisation of an insoluble nucleoprotein was therefore not simply explainable as the formation of a nucleohistone type of complex.

Although all this evidence implied a specific association, counter evidence has been recently presented by Jackson, Kornberg, Berg, Rajbhandary, Stuart, Khorana and Kornberg, (1965). They showed that the DNA must either have a much higher molecular weight than is presently accepted or be heterogenous with respect to base sequence. These conclusions were based on end group studies, nearest neighbour studies and the production of oligonucleotide fragments complementary to b_2-DNA using the ability of *E. coli* DNA polymerase to substitute one ribonucleotide for a deoxyribonucleotide when the polymerisation was carried out in the presence of manganese. Symons and Ellery (1967) degraded b_2-DNA

directly into oligonucleotides by diphenylamine-catalysed acid hydrolysis and hydrazinolysis and their study of the resultant oligonucleotides confirmed the conclusions of Jackson et al. (1965). Thus it would appear that the population of DNA molecules is heterogenous with respect to base sequence and this is an important piece of circumstantial evidence that weighs against a specific association between the nucleic acid and the protein.

However, the overall weight of evidence indicating a specific association, as well as the unusual nature of this nucleoprotein, was considered to make the nucleoprotein worthwhile investigating closely.

C. THE BIOLOGICAL IMPLICATIONS OF THE EXISTENCE OF TYPE I CYTOCHROME b_2

(1) The implications of Type I cytochrome b_2 with respect to nucleoproteins.

In the living cell there are many types of interactions between nucleic acids but it is not possible to give a comprehensive account of them here. However, some examples illustrating the broad groups of nucleic acid-protein interactions will be considered.

Firstly, there is the nucleohistone type in which the anionic phosphate groups are neutralised by basic amino acids in polypeptides and, in vivo, this type of

association seems to have some specificity as the protein appears to cover some sections of the genome but not others (Paul and Gilmour, 1966). Then there are the associations, albeit transient ones, between the enzymes that attach the acyl amino acid to S-RNA and the S-RNA molecule. In this case the enzyme protein is recognising a specific S-RNA molecule (Chapeville et al. 1962) and this seems likely to be an example of an association in which a protein is recognising a definite base sequence on a nucleic acid. Possibly another type of association again is the ribosome and when the virus is assembled the exclusion of "host" nucleic acid from the viral particle probably involves the protein recognising and complexing with only the viral nucleic acid.

In some of these cases the protein is probably recognising base sequences but it seems likely that proteins could recognise nucleic acids by other properties such as size, secondary structure and tertiary structure. Examples of nucleic acid tertiary structure are twisted, coiled forms of viral RNA (Vinograd, Lebowitz, Radloff, Watson and Laipis, 1965) and possibly the folds described by Hershey, Burgi and Ingraham (1963).

It was hoped that a close study of the crystalline nucleoprotein, Type I cytochrome b_2, would give information about one type of specificity between a nucleic

acid and protein that might have a more general application.

(2) The possible implications of the Type I cytochrome b_2 with respect to cytoplasmic determinants.

(a) Cytoplasmic determinants. Besides the problems directly related to the structure and organisation of the Type I cytochrome b_2, there was the question of its significance *in vivo*. This association between a presumably cytoplasmic cytochrome and a DNA originally suggested that this complex was an extrachromosomal deoxyribonucleoprotein. It was hoped that a study of this aspect could give some evidence on the physical basis of the well-known extrachromosomal genetic determinants found in yeast.

Genetic evidence has long suggested that plastids such as mitochondria, centrioles, chloroplasts, and kinetosomes have some sort of limited autonomy. Non-chromosomal, presumed cytoplasmic, determinants have long been known and these are characterised by patterns of non-Mendelian inheritance. A very common class of these determinants showed the loss of some biochemical function and this "loss" failed to segregate in the progeny of a cross between the defective organisms and normal organisms. An example of this type is the "neutral cytoplasmic petite" induced in *S. cerevisiae*

by the acridine dyes (e.g. Ephrussi and Hottinguer, 1951). It appeared that extrachromosomal determinants either were, or were carried by, chloroplasts, mitochondria, kinetosomes, virus-like infective particles and centrioles (for general discussions of the evidence for this see the reviews by Jinks, 1964; and Wilkie, 1964).

Although there are precedents for a type of inheritance in which a change of organisation of cytoplasmic units was transmitted to succeeding generations without the structure of any nucleic acid being lost or changed (Beisson and Sonneborn, 1965; Nanny, 1966), it is becoming accepted that many of these cytoplasmic determinants are nucleic acids.

(b) Cytoplasmic DNA. Before DNA found in organelles such as mitochondria, chloroplasts and kinetosomes could be accepted as organelle-specific, there had to be strong proof, in addition to mere observation of DNA in organelle preparations, that the DNA was not a contaminant from the nucleus or other sources. Satisfactory proof fell into three main categories:

1. Whole, healthy cells, free of contaminants such as bacteria, were rapidly killed and the bulk DNA rapidly extracted from them. This was then allowed to equilibrate in a caesium chloride density gradient so

that any minor components with different base ratios were detected. After the detection of these components in whole cell DNA the organelle that was suspected of containing one of these minor DNA species was separated from the other cell debris in the cell brei and as the fractions became enriched in the organelle concerned the DNA in this organelle became enriched with the minor DNA species in a parallel fashion. The initial step of establishing the presence of the minor species in whole cells is an essential part of the argument because there was the possibility that the minor species was from a micro-organism contaminant or was a modified DNA and the increase in proportion of the minor species of DNA during the organelle purification merely reflected the longer time there had been for micro-organism growth or for modifications to take place. The establishment of the presence of the minor component in quickly extracted "bulk" DNA made it unlikely to have arisen as a post-disruption artifact.

2. Light and electron microscope observations - These studies had the advantage of gaining information from undisrupted cells so that DNA identified in an organelle was not likely to be derived from a nucleus or micro-organism contamination. However, this method had the problem of unequivocally identifying the suspected material as DNA. The specificity of staining reactions

cannot be trusted entirely and even labelled thymidine has been known to be attached to other polymers besides DNA (Counts and Flamm, 1966). Hence, for an identification to be satisfactory the suspected material (stained threads or thymidine labelled zones) must be shown to be DNAase susceptible and RNAase resistant. The control experiment in which RNAase resistance is checked is to ensure that any apparent digestion by DNAase was not due to the common, albeit slight, contamination of this enzyme with RNAase.

3. Purification and analysis of an organelle after enucleation of the cell - A quick and reliable enucleation procedure allows residual DNA in a cell to be reliably assigned to the cytoplasm and thus, so long as reasonable precautions were taken to avoid microbial contamination, there was no reason to doubt that the DNA was truly organelle-specific as the cells had been shown to contain non-nuclear DNA before the fractionation procedures were begun.

The main problem in this approach is to obtain a satisfactory enucleation procedure. However, this has been possible in the marine algae Acetabularia, which has large cells and the nucleus is separated from the bulk of the cytoplasm by a long stalk. Thus, enucleation has been simply carried out by cutting the stalk and collecting the enucleate halves (Gibor and Izawa, 1963). (For the

structure of Acetabularia, see Brachet 1965). Enucleation was also used to establish that sea urchin eggs contain cytoplasmic DNA (Bibring, Brachet, Gaeta and Graziosi, 1965). These workers made the observation that when the eggs were centrifuged in a solution slightly denser than themselves a portion of the egg containing the nucleus broke away from the rest of the egg.

A number of references reporting extranuclear DNA, using one or more of the three procedures described above are summarised in TABLE 1.

The organelle DNAs studied to date appear to be relatively conventional double stranded DNA (e.g. Ray and Hanawalt, 1964) which has been shown, in some cases to be circular (Nass, 1966; Sinclair and Stevens, 1966; Brugger, Borst, Ruttenberg, Gruber and Kroon, 1966). There have been reports of DNA polymerase activity in some organelles (Parsons and Simpson, 1967; Wintersberger, 1966) and in some cases organelle DNA has been shown to have a different synthesis cycle to that of nuclear DNA (Green and Gordon, 1966; Cook, 1966).

Mitochondrial DNA in Neurospora had physical continuity through several cycles of vegetative growth, showed a maternal pattern of inheritance (Reich and Luck, 1966) and aplastidic mutants of Euglena have been shown

to lack the organelle DNA (Leff, Mandell, Epstein and Schiff, 1963) so that circumstantial evidence exists to link organelle DNA with extrachromosomal determinants.

The fact that cytoplasmic organelles have, in many cases, an organelle-specific DNA with relatively conventional properties, together with the circumstantial evidence of aplastidic mutants suggested that this DNA was responsible for non-chromosomal genetic determinants. However, it should be noted that in one case an organelle-localised DNA appeared to be originally derived from the nucleus. Bell and co-workers (Bell, 1961; Bell and Muhlethaler, 1964b) showed that eggs of the fern, Pteridium aquilinum, contain an extranuclear DNA but that the organelles containing this DNA are originally produced by "blebbing off the nucleus" (Bell, Frey-Wyssling and Muhlethaler, 1966; Bell and Muhlethaler, 1964a).

TABLE 1 shows the wide range of species of organisms that contain organelle DNA and the work of Nass, Nass and Afzelius (1965) has indicated that mitochondrial DNA is common and possibly present in all mitochondria throughout the plant and animal kingdoms. These workers used the electron microscope to examine mitochondria and, although they did not use the nuclease controls for all species, they found material like DNA in the mitochondria of a wide variety of organisms.

(c) The expression of cytoplasmic DNA. If this extra-nuclear, organelle DNA is to explain some non-chromosomal genetic determinants it must be able to express itself in some way. Thus it seemed very likely that the organelles concerned contained at least a DNA dependent RNA-polymerase and possibly the system necessary for the transcription of the RNA into polypeptide chains.

Although the evidence is far from being complete, there are papers beginning to appear that have indicated that this is, indeed, the case. A satisfactory proof that an enzyme such as RNA polymerase, or a biochemical system, such as that necessary to produce protein, is specific for a given extra-nuclear organelle has to eliminate contamination from other sources such as the rest of the cytoplasm and bacteria. Mere detection of an incorporating activity, such as amino acid or nucleotide incorporation, is of little significance unless it is accompanied by objective evidence that shows that the activity concerned is specific for the particular organelle being considered. However, in yeast (S. cerevisiae) it was possible to obtain objective proof of a mitochondrial localised protein synthesising system because the normal, non-mitochondrial cytoplasmic synthesising system was found to have a different pattern of antibiotic inhibition to that of the organelle

localised system (see below). These observations of differential inhibition were made *in vitro* and then confirmed by *in vivo* studies. The *in vitro* studies showed that mitochondrial preparations contained a protein synthesising system susceptible to chloramphenicol (Wintersberger, 1965) whereas the normal cytoplasmic system was resistant to chloramphenicol (Lucas, Schurrs and Simpson, 1964; So and Davie, 1963). If it was accepted that these results reflected a difference in susceptibility that existed *in vivo* then it would be anticipated that chloramphenicol would stop the cell from synthesising one class of proteins, probably largely mitochondrial localised, but not stop synthesis of most other cell proteins. This prediction was confirmed as chloramphenicol stopped the respiratory adaption of facultative yeasts (such as *S. cerevisiae*) to aerobic growth; it prevented the synthesis of cytochromes a and a_3 and the yeast continued to ferment anaerobically under conditions that should have induced aerobic respiration (Huang, Biggs, Clark-Walker and Linnane, 1966; Clark-Walker and Linnane, 1966).

There is, also, an example of an extranuclear RNA polymerase system that appears to be reasonably well established. Enucleated cells of the giant marine algae, *Acetabularia*, were used for the preparation of chloroplast enriched fractions which synthesised RNA *in vitro*

(Schweiger and Berger, 1964) and as the enucleated, but otherwise intact, cells synthesised RNA (Brachet, Chantrenne and Vanderhaege, 1955) it is an unnecessary complication to assume that the particle polymerase is of bacterial origin.

Because of its association with a cytochrome it was thought that $\underline{b}_2$-DNA might represent an organelle DNA and it was this assumption that was the basis of the study of yeast RNA polymerases initiated in CHAPTER VI of this thesis. The first aim of this work was the conclusive identification and localisation of any extranuclear RNA polymerase parallel with a study of the localisation of $\underline{b}_2$-DNA within the cell. If both $\underline{b}_2$-DNA and an RNA polymerase were found to be localised in any organelle the product of the RNA polymerase would have been compared to $\underline{b}_2$-DNA. However, as will be seen later, these two separate studies failed to converge in the expected fashion and many of the above predictions about $\underline{b}_2$-DNA were found to be based on misconceptions. To allow a clear understanding of the rationale behind the structure of this thesis the misconceptions have been presented here as the author saw them at the beginning of this project.

TABLE 1: PAPERS REPORTING THE EXISTENCE OF CYTOPLASMIC-ORGANELLE DNA.

Organism	Organelle	Method used (see above)	Reference
Acetabularia mediterranea	Chloroplast	3	Gibor & Izawa, 1963
Euglena gracilis	Chloroplast	1	Brawerman & Eisenstadt, 1964
Euglena gracilis	Chloroplast	1	Edelman, Epstein and Schiff, 1966
Neurospora crassa	Mitochondria	1	Luck & Reich, 1964
Chick embryo	Mitochondria	2	Nass & Nass, 1963
Chick embryo	Mitochondria	1 and 2	Rabinowitz, Sinclair, De Salle, Haselkorn and Swift, 1965
Wheat	Unspecified[1] plastids	1	Hotta, Bassel and Stern, 1965
Sheep	Mitochondria	1	Kalf & Grece, 1966
Physarum polycephalum	Mitochondria	2	Guttes & Guttes, 1964
Chlamydomonas reinhardi	Chloroplast	1	Sager & Ishida, 1963
Chlamydomonas moewusii	Chloroplast	2	Riss & Plaut, 1962
Nicotiana tabacum	Chloroplast	1	Ship, Kieras and Haselkorn, 1965
Trypanosoma mega	Kinetosome[2]	2	Steinert, Firket and Steinert, 1958
Leishmania enriettii	Kinetosome[3]	1	DuBuy, Mattern and Riley, 1965
Didymium nigripes	Mitochondria	2	Schuster, 1965
Dictyota	Chloroplast	2	Steffenson & Sheridan, 1965
Pteridium aquilinum	Mitochondria and other plastids	2	Bell & Muhlethaler, 1964b, Bell, 1961

TABLE 1 (Continued)

Organism	Organelle	Method used (see above)	Reference
Amoeba	Unspecified particles	2	Rabinowich & Plaut, 1962
Arbacia lixula	Unspecified	3	Bibring, Brachet, Gaeta and Grazi-osi, 1965
Saccharomyces cereviseae	Mitochondria(4)	1	Tewari, Jayaraman and Mahler, 1965 Monolou, Jacob and Slonimski, 1966. Corneo, Moore, Sanadi, Grossman and Marmur, 1966. Tewari, Votch, Mahler and Mackler, 1966
Tetrahymena pyriformis	Mitochondria(5)	1	Suyama & Preer, 1965
Paramecium aurelia			
Beta vulgaris	Chloroplast	1 and 2	Kislev, Swift and Bogorad, 1965.
Beta vulgaris	Chloroplast	1	Chun, Vaughan and Rich, 1963
Spinacea oleracea			

CHAPTER ONE

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

In order to avoid unnecessary repetition, those materials and methods that are used in more than one of the chapters of this thesis are described below.

Buffer 1. 0.025M sodium DL-lactate, 0.02M triethanolamine chloride, 0.01mM EDTA, pH 6.8.

Buffer 2. 0.3M sodium DL-lactate, 0.05M tetrasodium pyrophosphate, 0.1mM EDTA, adjusted to pH 6.8 with HCl.

Sucrose purification. Sucrose from the Colonial Sugar Refineries was refluxed in 80% ethanol, 0.5mM sodium EDTA, pH 6.8. The solution was centrifuged, the supernatant concentrated under vacuum and the resultant syrup was induced to crystallise by the slow addition of 95% ethanol. The crystals were collected by filtration and washed on the filter paper with 80% and then 95% ethanol before drying.

Scintillation fluid. This was a solution of 0.3% (w/v) 2,5 diphenyloxazole (PPO) and 0.03% (w/v) 1,4-bis-2-(4 methyl-5-phenyl oxazolyl benzene) (dimethyl POPOP) in toluene. Both PPO and dimethylPOPOP were Scintillation grade and were obtained from Packard Instrument Co.

Yeast strains. A typical colony was selected from a number of colonies derived from a block of commercial, pressed bakers' yeast (Pinnacle brand, Yeast Company of

Australia, Melbourne, Vic., Australia. The culture derived from this colony was designated Stock 164. The ploidy state of this culture was not determined.

The yeast strain αTr 39163, a gift from Dr. C. Lindegren, was a haploid, tryptophane dependent strain of S. cereviseae. A respiratory deficient strain was derived from it using the commercial preparation Acriflavin (obtained from B.D.H.) as described by Ephrussi and Hottinguer (1951). The resultant respiratory deficient strain was designated αTr 39163 P_1.

Sephadex G-75. (bead form) This was obtained from Pharmacia, Uppsala, Sweden, and was allowed to imbibe in water or dilute sodium EDTA at pH 7.0 for at least 24 hr. before use.

DEAE-cellulose. Whatman DEAE-cellulose (DE 50) was washed with 1.0M KOH until the washings were colourless, then with water, briefly with 0.5M HCl, water again, 0.1M tris-HCl, pH 7.5, and finally with water. It was stored as a suspension in water or as a freeze-dried powder.

DNA estimation. DNA concentrations were estimated by the method of Burton (1956). The solution of native calf thymus DNA in 0.1M NaCl used as a standard had an E_{260} of 4.0 and was taken as containing 200μg DNA per ml.

The flavo-haemo-protein of cytochrome b_2 severely

interfered with the Burton (1956) reaction even after it had been freed of FMN by a TCA precipitation. Thus, solutions containing cytochrome b_2 and b_2-DNA, were loaded onto a small column of DEAE-cellulose (usually 1 cm. by 1 cm.), the enzyme eluted with 0.4M NaCl, 0.01M tris-HCl, pH 7.5, and then the DNA eluted with 1.0M NaCl, 0.01M tris-HCl, pH 7.5. The DNA was then estimated directly.

RNA estimation. RNA was determined spectrophotometrically on the assumption that native RNA had an E_{260} of 24 at a concentration of 1 mg./ml. while estimations on alkaline hydrolysates assumed a hydrolysis hyperchromicity of 20%.

Preparation of whole yeast DNA. DNA from bakers' yeast was prepared from cells that had been freeze-dried and ground in a ball mill. The disrupted cells were suspended in a solution of 0.1M sodium citrate, 1mM EDTA, pH 8.0, and then solid sodium dodecyl sulphate was added to 0.5% and NaCl to 1.5M. The suspension was then extracted by shaking with an equal volume of water-saturated phenol for 20 min. followed by centrifuging at 10°. The nucleic acids in the aqueous phase were precipitated with two volumes of ethanol and the RNA present hydrolysed with a mixture of pancreatic ribonuclease (EC 2.7.7.16.) and Taka-diastase T_1 ribonuclease (EC 2.7.7.26.) in 0.1M tris-HCl, 0.01M EDTA, pH 7.5.

Further purification usually involved high speed centrifugation to remove denatured protein, repeated ethanol precipitation to remove hydrolysis products, and removal of residual protein by digestion with pronase in the presence of EDTA. The pronase and its hydrolysis products were then removed by phenol extraction and ethanol precipitation of the DNA.

Calf thymus DNA. Calf thymus DNA was prepared by the method of Kay, Simmons and Dounce (1952).

Type I and Type II cytochrome b_2. Type I cytochrome b_2, three times crystallised, was prepared by the method of Appleby and Morton (1959a) as modified by Symons and Burgoyne (1966). Type II cytochrome b_2 was prepared by removing the b_2-DNA from three times crystallised Type I cytochrome b_2 using a DEAE-cellulose column as described by Symons and Burgoyne (1966).

Preparation of b_2-DNA. The b_2-DNA was adsorbed onto DEAE-cellulose from solutions of three times crystallised Type I cytochrome b_2 (Symons and Burgoyne, 1966). The short DEAE-cellulose column was washed with 0.4M NaCl, 0.01M tris-HCl, pH 7.5, and the b_2-DNA eluted with 1.0M NaCl, 0.01M tris-HCl, pH 7.5. It was then transferred into the appropriate buffers by dialysis.

Estimation of enzyme. Enzyme concentration was determined from the E_{423} of solutions of the reduced enzyme; 1 mg. per ml. was taken to have an E_{423} of 2.9

(calculated from a haem millimolar extinction coefficient of 232 x 10^3 and 82,000 g. enzyme per mole of haem (Morton and Shepley, 1963)). Lactic dehydrogenase activity was estimated as described by Symons and Burgoyne (1966).

CHAPTER TWO

A STUDY OF THE EVENTS THAT OCCUR DURING THE STANDARD PREPARATION OF CRYSTALLINE CYTOCHROME $\underline{b}_2$

A STUDY OF THE EVENTS THAT OCCUR DURING THE STANDARD PREPARATION OF CRYSTALLINE CYTOCHROME b_2.

INTRODUCTION

The preparation procedure of crystalline Type I cytochrome b_2 was the modified Appleby and Morton procedure (Appleby and Morton, 1959b; Symons and Burgoyne, 1967), hereafter referred to as the "Appleby and Morton procedure". It was considered that a study of the events that occurred during this procedure would indicate whether the nucleic acid-protein association of the Type I crystalline enzyme was formed before or during the preparation procedure.

Labeyrie and Slonimski (1964) had shown that the kinetic parameters of the enzyme were modified in some way during, or just prior to, the crystallisation step of the Appleby and Morton procedure but the effect of these changes on the nature of the DNA-enzyme association, if any, was not studied. The work described in this chapter was carried out so as to further clarify the nature of these changes on the DNA-enzyme association as well as to look for any changes that occurred in the nucleic acid component.

A. CHANGES IN NUCLEIC ACIDS DURING THE PREPARATION PROCEDURE

MATERIALS AND METHODS

The details of the procedures for the estimation of DNA and RNA are given in CHAPTER I. One of the most important nucleic acid fractions described in the experimental section that follows was the fraction eluted from DEAE-cellulose between 0.4 and 1.0M NaCl, pH 7.5. Although this fraction contained large amounts of E_{260} absorbing material, the levels of DNA determined by the Burton (1956) reaction were low. However, because the Burton positive material was DNAase digestible and alkali stable, the DNA estimation procedure was considered to be satisfactory even in the presence of the large amounts of other material found in these preparations.

RESULTS

The main steps in the Appleby and Morton procedure are as follows:

Air dried yeast is ground at -15^{o} in ball mills so as to rupture the highly resistant cell wall, an important pre-requisite for the extraction of the enzyme. The milled yeast is then dispersed in n-butanol and extracted with a lactate buffer which becomes saturated by the excess of n-butanol so that, after

centrifugation, the mixture separates into a precipitate and two phases. The aqueous phase, which contains the extracted enzyme, is twice fractionated by acetone and the final precipitate represents the material precipitated between 25% and 35% (v/v) acetone. The precipitate is dissolved and dialysed for 40 hr. against low ionic strength lactate buffers and, despite the fact that the 25% to 35% acetone precipitate is known to be less than 1% cytochrome $\underline{b}_2$, the enzyme crystallises out of the mass of other proteins and nucleic acids. The enzyme is usually recrystallised twice before use and these third crystals are the usual starting point for most experiments that use purified enzyme.

A summary of the main steps of the Appleby and Morton procedure with the temperature and times for each step is set out in TABLE 2 and a detailed account of the procedure as used in this thesis can be found in Symons and Burgoyne (1966).

(1) Post-extraction changes in the population of nucleic acid molecules as studied by chromatography on DEAE-cellulose.

DEAE-cellulose was known to fractionate nucleic acids largely on the basis of their total charge (i.e. size) as has been shown by the behaviour of relatively small oligonucleotides on this resin (Tener, Khorana, Markham and Pol, 1958). Although it is an oversimplifi-

cation to state that the higher the salt concentration necessary to elute a nucleic acid the higher its molecular weight, this behaviour is predicted from the results with oligonucleotides by Tener et al. (1958). During the course of this work it has also been observed that, under conditions where degradation of nucleic acids was known to be occurring, the nucleic acids could be eluted from DEAE-cellulose at progressively decreasing salt concentrations.

As yeast contains very little DNA, and the nucleic acids found throughout the Appleby and Morton procedure are almost entirely RNA with only traces of DNA, it was technicially too difficult to estimate the DNA in fractions obtained from a continuous gradient elution off DEAE-cellulose. Thus the nucleic acids were fractionated by stepwise elution into three size classes as follows.

To the material that was to be fractionated, urea, stock NaCl solution, stock buffer solution and water were added to give a final composition of 8M urea, 0.1M NaCl, 0.1M phosphate, pH 7.0. Each sample contained approximately 100 mg. of nucleic acids which was adsorbed onto a column of DEAE-cellulose (chloride) (6 cm. long by 2.5 cm. diameter). These short columns were washed with 8M urea, 0.1M NaCl, 0.1M sodium phosphate, pH 7.0 and then various elutions with NaCl

buffered with 0.01M sodium phosphate were carried out. It should be noted here that phosphate is an anionic buffer and therefore is not completely suitable for use with an anion exchanger as the column cannot be kept in equilibrium with the buffer when salt concentrations change. However, the elution procedure was standardised and was reproducible.

The material adsorbed to DEAE-cellulose was eluted in three fractions or classes:

(a) Class 1. Nucleic acids eluted from DEAE-cellulose between 0.1M and 0.4M NaCl. This class of polynucleotides would be expected to vary from oligo-nucleotides with only a few charges up to polynucleotides slightly smaller than S-RNA (molecular weight approx. 25,000).

(b) Class 2. Nucleic acids eluted from DEAE-cellulose between 0.4M and 1.0M NaCl. This class would be expected to contain b_2-DNA and any other nucleic acids of an approximately similar size. The lower size of this class of nucleic acids would be approx. the size of S-RNA and, although definite information is not available, the upper size limit was assumed to be a molecular weight in the region of 1×10^6.

(c) Class 3. Nucleic acids that were not eluted by 1.0M NaCl but were eluted by 1.0M KOH. These were, presumably, of higher molecular weight than the nucleic

acids in Class 2.

The rather vague molecular weight limits for these classes have been given merely for convenience as the actual quantitative values of these limits are not relevant to the arguments based on the experiments presented here. It is quite sufficient if Class 2 is regarded as nucleic acid fragments approx. the size of $\underline{b}_2$-DNA, Class 1 is regarded as fragments smaller than $\underline{b}_2$-DNA and Class 3 is regarded as fragments larger than $\underline{b}_2$-DNA.

The results of these studies (see TABLE 3 and discussion below) showed that the RNA was being extensively degraded or otherwise depleted in higher molecular weight species by the acetone fractionations. These changes were indicated by the loss of Class 3 RNA relative to the lower molecular weight classes (see TABLE 3 and compare the RNA figures in Experiment 1 to those in the "Before dialysis" column of Experiment 2). Similarly, it can be seen that the RNA was being extensively degraded during the 40 hr. dialysis period (see TABLE 3 and compare the RNA figures before and after dialysis; Class 3 RNA is reduced with respect to the other classes and there is an overall loss of total RNA over the dialysis period). This degradation of RNA was confirmed by the appearance of material with the spectrum of nucleic acid fragments

in the dialysis fluid (TABLE 2, step e).

By comparison with the apparently rapid breakdown of RNA, the small traces of DNA found at the dialysis stage of the Appleby and Morton preparation were not undergoing extensive degradation although a slight transfer of DNA from Class 2 to Class 1 over the dialysis period (see TABLE 3, Experiment 2) indicated the presence of slight DNAase activity at this stage. Very high molecular weight (Class 3 DNA) was not detected at any stage in the Appleby and Morton procedure and was apparently not solubilised by the n-butanol-lactate ~~saturated~~ buffer. However, extraction of ball-milled yeast with 8.0M urea, 0.1M NaCl, 0.01M tris-HCl, pH 7.5, did extract some DNA of high enough molecular weight to fall into Class 3.

Thus, the b_2-DNA in the Appleby and Morton procedure was not arising from highly polymerised DNA during the post-extraction steps of the procedure because most of the extracted DNA was already of relatively low molecular weight and there was only a very small amount of effective DNAase activity in the preparation. Moreover, the low molecular weight DNA found in the first lactate-n-butanol extract was already quite capable of acting as b_2-DNA and this was demonstrated as follows:

The Class 2 nucleic acids found in the first

extract (TABLE 2, step c) were tested for the presence of $\underline{b}_2$-DNA by the conversion of Type II cytochrome $\underline{b}_2$ into the crystalline deoxyribonucleoprotein Type I cytochrome $\underline{b}_2$ using the method described in CHAPTER III. As crystals of Type I cytochrome $\underline{b}_2$ were obtained it was concluded that the nucleic acids in the first lactate-n-butanol extract contained $\underline{b}_2$-DNA.

However, parallel work described later in this thesis indicated that it was unlikely that the $\underline{b}_2$-DNA existed as a low molecular weight nucleic acid *in vivo*. Thus, it seemed probable that a degradation was occurring during the air drying, the milling, or very early in the extraction step. The following experiments were designed to test the air drying step.

(2) *The effect of different yeast handling and drying procedures on the subsequent events in the preparation procedure.*

Fresh blocks of commercial pressed yeast were sliced and freeze-dried. The dried yeast was then milled and carried as usual through the rest of the Appleby and Morton procedure. In contrast to the constant 5-6% by weight of DNA found in enzyme prepared from air-dried yeast, the crystalline enzyme prepared from freeze-dried yeast had a very low DNA content which varied from batch to batch of yeast and was often too low to detect.

Although this type of crystal, as it first appeared, had the crystalline form of Type I cytochrome $\underline{b}_2$ it contained largely RNA and recrystallisation caused it to lose the RNA and crystallise in the hexagonal system characteristic of Type II cytochrome $\underline{b}_2$.

The variation in DNA composition between batches of crystals from freeze-dried yeast could be explained by the variable age of the commercial yeast used for these experiments. To prove this point one batch of fresh commercial yeast was carefully divided into two equal samples by slicing the blocks and alloting the slices alternately to the two samples. One sample was frozen while the other was allowed to age at 25^{o} for 70 hr. in a humid atmosphere and then both were freeze-dried. 170 g. of each of the freeze-dried yeast samples were then carried through the Appleby and Morton procedure as usual and the resultant crystals were harvested, washed, and analysed for nucleic acid content using the methods described in CHAPTER I. The results of this experiment are summarised in TABLE 4. It can be seen that the crystals from the fresh, freeze-dried yeast contained largely RNA instead of the usual DNA but the aging treatment caused the crystal DNA levels to rise towards the normal level of 5-6% with a corresponding decrease in the RNA level.

The general conclusion from this work was the the degradation processes leading to the production of $\underline{b}$-DNA

occur during the aging or air drying of the yeast.

B. CHANGES IN THE PROTEIN DURING THE PREPARATION OF CRYSTALLINE ENZYME

INTRODUCTION

Yeast lactate dehydrogenase has been shown to be at least partly attached to particles in breis of freshly disrupted yeast cells (Somlo, 1962; Mahler, Mackler, Grandchamp and Slonimski, 1964; Vitols and Linnane, 1961) and it was found that the particle bound enzyme had different kinetic properties to the free enzyme (Somlo, 1962). However, the particle-bound enzyme had the same properties as the soluble enzyme after it had been released by a detergent (Somlo, 1962). Thus, if the particle-bound enzyme represented the state of the enzyme *in vivo*, then even the act of solubilisation of the enzyme causes the first observed change in its properties. Another change in the properties of the enzyme was first described by Labeyrie and Slonimski (1964) who detected a change in the kinetic parameters from those characteristic of the freshly extracted enzyme to those characteristic of the crystalline preparation. Nicholls *et al*. (1966) have shown that these changes were accompanied by the appearance of multiple bands of enzyme activity on starch gel electrophoresis together with an overall increase of mobility in the gels. It seemed quite possible that these

changes had a considerable effect on the cytochrome b-DNA association and the experimental work of this section was designed to show that one such effect of this type did exist.

The work of Symons (1965) showed that the Type I cytochrome b_2 was only an associated nucleoprotein at relatively low ionic strengths under which conditions it crystallises. The results presented in CHAPTERS III and IV, together with this fact, strongly indicated that the process of crystallisation of Type I cytochrome b_2 is identical with the process of association for this nucleoprotein, i.e. observing the growth of the crystals is just another way of observing the process of association of the nucleoprotein.

MATERIALS AND METHODS

(1) Preparation of [^{35}S] enzyme.

The aim of this preparation procedure was to obtain the best recovery possible of [^{35}S] Type I cytochrome b_2 while avoiding the production of large volumes of radioactive wastes that would occur if the simple Appleby and Morton procedure was used. Basically, a very small amount of highly radioactive yeast was grown, harvested, and extracted. The extract was then acetone fractionated in a small volume containing large amounts of carrier enzyme with other yeast proteins (see details below) and

put through the crystallisation procedure. For the major experiment described in this section (see TABLE 5) the details of the [^{35}S] enzyme preparation were as follows:

Four mCi of [^{35}S] carrier-free sulphate was added to a minerals-vitamins medium that contained per litre - 0.5 g. $MgCl_2.7H_2O$, 2.0 g. NH_4Cl, 0.5 g. $CaCl_2$, (note that a mixture of these first three components was autoclaved separately and added to the medium after it had cooled), 1.5 g. KH_2PO_4, 2 mg. $MgSO_4.10H_2O$, 30 µg. $FeCl_3$, 3.0 µg. $KMnO_4$, 42 µg. $ZnCl_2$, 5.0 µg. $Co(NO_3)_2.6H_2O$, 10 µg. KI, 50 µg. biotin, 4 mg. calcium pantothenate, 20 µg. folic acid, 20 mg. inositol, 4 mg. nicotinic acid, 2 mg. paraamino benzoic acid, 4 mg. pyridoxine hydrochloride, 2 mg. riboflavin, adjusted to a final pH of 5.7. The carbon sources for this experiment were 1% glucose and 1% sodium DL-lactate and the cells were grown aerobically at 30° for 48 hr. Under these conditions 67% of the [^{35}S] sulphate was taken up by the yeast cells. The labelled cells were centrifuged out, washed in a solution of 0.5% KCl, 0.5% NaCl and then mixed with a slurry of 1 g. of commercial bakers' yeast in 0.5% NaCl, 0.5% KCl. The slurry was freeze-dried, ground at -15° under nitrogen in a sealed glass ball mill and was dispersed in 15 ml. of n-butanol followed by 50 ml. of 0.1M sodium DL-lactate, 1mM $MgCl_2$, 0.1mM EDTA, 0.2 mg.

per ml. cysteine, and 4 mg. per ml. of $Na_2SO_4.10H_2O$, pH 6.8. The glutathione, cysteine and sulphate were added so as to dilute out as rapidly as possible [^{35}S] in these forms. In particular it was feared that the labelled sulphydryl compounds might become attached to proteins by oxidation and it was preferred that all [^{35}S] compounds present in the enzyme should be attached by peptide bonds.

The suspension of yeast in n-butanol-saturated buffer then had added one g. of the final 35% acetone precipitate (TABLE 2, end of stage d) from the Appleby and Morton preparations as the first lot of carrier. The mixture was centrifuged (5,000 x gravity for 10 min. at -5°) and the aqueous layer kept. In the major experiment (Experiment 4, TABLE 5) a microscopic examination showed an unusually poor cell breakage at this stage and so the precipitates were further disrupted in the Nossal shaker (2 by 30 sec.) with Ballotini beads and re-extracted as above with 25 ml. of extraction mixture.

To the combined aqueous layers was then added all the final 35% acetone precipitate (TABLE 2, end of stage d) from 500 g. of yeast that had been carried through the Appleby and Morton procedure. The solution was then fractionated by acetone and the material precipitated

between 15% and 35% v/v acetone was collected. This was then carried through the rest of the Appleby and Morton procedure (TABLE 2, steps e and f) with the addition of crystalline carrier Type I cytochrome $\underline{b}_2$ to the first crystals before the two recrystallisations. The final product consisted of 20 mg. of Type I cytochrome $\underline{b}_2$ with a specific activity of 3660 CPM/mg.

(2) Radioactive counting of cytochrome $\underline{b}_2$.

A number of different methods were used with varying success.

(a) Method 1. This was the least efficient of all and consisted of G.M. counting of wet paper discs which were maintained at constant weight to give constant self absorption. Extremely long counting times were necessary to get satisfactory results but it was used until the more efficient liquid scintillation methods had been made reproducible. Specific activities with this method were stated as counts/1,000 sec./mg.

(b) Method 2. Liquid scintillation counting using the ECKO N683A scaler with an ECKO N664A counting head. With this method the problem of accurate estimation of quenching was the main difficulty. The method first adopted to estimate quenching was as follows. The sample was loaded onto a 2.54 cm. diam. disc of Whatman 3 mm. paper in a known volume, dried, washed in cold 5% TCA,

ethanol and ether. The dry paper was counted in a toluene solution of 0.3% PPO, 0.03% dimethyl POPOP and then washed with toluene. The washed paper was then loaded with a standard sample of [^{35}S] sulphate in the same volume as had been the original load. This was then dried and recounted to estimate the quenching. Although this method gave satisfactory results it was tedious and was soon abandoned for the much more satisfactory Method 3.

(c) Method 3. To avoid the quenching problem only purified enzyme was counted and for any given experiment a standard mass (200 µg) of this was loaded in a standard volume (0.05 ml.) onto a 2.54 cm. diameter disc of Whatman 3MM chromatography paper. This was then dried, washed in cold 5% TCA, ethanol, ether and finally dried. The papers were counted in a toluene solution of 0.3% PPO, 0.03% dimethyl POPOP in a Packard liquid scintillation spectrometer which had recently become available. As the material being counted was a relatively pure enzyme and the protein load and load volume were standardised, the quenching factor was constant and could be neglected.

Except where it is stated otherwise, specific radioactivities were estimated by this method.

(3) Radiochemical purity of the radioactive enzyme.

During the checking of radiochemical purity, count-

ing method 2 was used and a different batch of enzyme to that described above. However, this material served to demonstrate that the enzyme effectively reached a constant specific activity after two recrystallisations. With this batch of enzyme the first crystals had a specific activity of 1680 CPM/mg., the second 1200 CPM/mg., the third 960 CPM/mg. and the fourth 960 CPM/mg. The fourth crystals were then adsorbed onto a column of DEAE-cellulose, the column was washed in a solution of Buffer 2 and water (1:2) (see CHAPTER I for Buffer 2) and eluted with a solution of buffer 2 and water (1:1). After this treatment the enzyme was found to have an unchanged specific radioactivity. As the crystalline preparation of Appleby and Morton had previously been shown to be free of contaminating proteins (see general thesis INTRODUCTION) the above evidence was considered a sufficient check of the radiochemical purity of the enzyme produced by this technique and third crystals were used as starting material for all subsequent experiments.

RESULTS

The first crystallisation stage (TABLE 2, step e) of the Appleby and Morton procedure took an average of approx. 40 hr. to complete despite the known fact that the attainment of low ionic strength during the dialysis

step took 4 to 8 hr. and enzyme that had previously been crystallised one or more times was known to crystallise as soon as low ionic strength was achieved. This point suggested that there was something anomalous about the first crystallisation step and the following observations confirmed this.

The crude yeast protein, containing cytochrome $\underline{b}_2$, found at the end of the acetone fractionations (TABLE 2, stage d) of the Appleby and Morton preparation was transferred quickly into low ionic strength buffer by dissolving the protein in Buffer 1 (see CHAPTER I for Buffer 1), precipitating it with acetone and redissolving it in Buffer 1. Despite the fact that low ionic strength had been attained quite quickly, crystals of Type I cytochrome $\underline{b}_2$ did not appear until the solution had been stored for 40 hr. at 4°. Thus the delay in the first crystallisation was not due to a slow rate of ionic strength reduction during dialysis. Also, if third crystals of Type I cytochrome $\underline{b}_2$ were dissolved in the solution just before the dialysis step, some crystals appeared as soon as the ionic strength had dropped low enough (in 4 to 8 hr.) and still another lot appeared after the usual 40 hr.

These experiments <u>inferred</u> that the enzyme, before the dialysis step, was not capable of forming the Type I cytochrome $\underline{b}_2$ nucleoprotein under these conditions and that it was modified over a period of dialysis into a

form that was capable of crystallisation. The subsequent work in this section consisted of one basic experimental design that was to test this hypothesis.

The experimental plan was simply to add [^{35}S] labelled, crystalline, Type I cytochrome $\underline{b}_2$ to an unlabelled batch of material at the beginning of the dialysis stage (TABLE 2, step e) of the Appleby and Morton preparation and then find whether the specific radioactivity of the enzyme that had crystallised after 8 hr. was the same as the specific radioactivity of the crystals that appeared later at 40 hr.

In Experiment 4 most of the technical difficulties had been largely resolved and so this experiment has been taken as the type example. The other experiments (summarised in TABLE 5) are technically less satisfactory but nevertheless, confirm the result of Experiment 4.

(1) Experiment 4. A normal Appleby and Morton preparation was carried out starting from 500 g. of air dried and milled yeast. At the beginning of the dialysis step (TABLE 2, step e) the final 35% acetone precipitate was dissolved in a relatively high ionic strength lactate buffer (Buffer 2) and to this was then added 4.6 mg. of [^{35}S] labelled Type I cytochrome $\underline{b}_2$ with a specific activity of 3660 CPM/mg. The high ionic strength lactate buffer ensured that the labelled enzyme thoroughly

dissolved and freely mingled with the unlabelled enzyme that was present at this stage of the Appleby and Morton preparation. The whole solution, containing labelled and unlabelled enzyme, was then dialysed as usual for the Appleby and Morton procedure. One batch of crystals began to appear in the dialysis bag after 4 hr. and at 16 hr. these crystals were centrifuged out, washed briefly in Buffer 1, dissolved in Buffer 2, and were found to consist of 3.6 mg. of enzyme, with a specific activity of 3060 CPM/mg. Another batch of crystals was harvested after 40 hr. and this was estimated to contain 3.6 mg. of enzyme with specific activity of 240 CPM/mg. Thus, nearly all of the radioactive enzyme that was recovered was found in the 16 hr. batch and not the 40 hr. batch. Crystalline radioactive enzyme obviously had a different ability to form crystals as compared with the enzyme that had never been crystallised before.

DISCUSSION

The results presented here, in conjunction with those of CHAPTER V, have shown that b_2-DNA was a degradation product that arose during the aging of the yeast cells before and during the drying step of the Appleby and Morton preparation. It is of interest to note that while investigating the metabolism of DNA in yeast, Sa&kar and Poddar (1965) noted that yeast cells begin to

degrade their DNA as they become senescent and they drew attention to the fact that this could have something to do with the origin of $\underline{b}_2$-DNA.

Thus the crystals obtained by Rippa (1961), who disrupted yeast with Ballotini beads, could be the normal Type I cytochrome $\underline{b}_2$ if the yeast he used was not completely fresh. If the yeast was completely fresh then the crystals he obtained would probably have contained RNA instead of DNA. Either of these explanations would account for the crystals he obtained having the spectrum of Type I cytochrome $\underline{b}_2$.

The changes in the properties of the enzyme protein that occur at the first crystallisation step are in agreement with the work of Labeyrie and Slonimski (1964) who showed that the K_m for L-lactate of the enzyme, as well as other kinetic parameters, changed during the crystallisation step of the Appleby and Morton procedure. These results were further confirmed by the work of Nicholls *et al.* (1966) who observed changes in electrophoretic and kinetic behaviour of the enzyme at the crystallisation step and found that these changes could be prevented by high concentrations of n-butanol.

In addition, the experiments described in this chapter have shown that changes in the protein not only accompany the crystallisation but are a pre-requisite for it. As crystallisation appeared to be actually the

process of association of the nucleoprotein (see CHAPTERS III and IV) it seemed that these changes in the protein were necessary for the formation of crystalline Type I cytochrome $\underline{b}_2$.

TABLE 2: THE APPLEBY AND MORTON PROCEDURE

A brief summary of the times and temperatures for the major operations in the Appleby and Morton preparation as described in Symons and Burgoyne (1967) and based on the preparation of Appleby and Morton (1959b).

Step	Temp. °C.	Time hr.	Description of step or steps
a	-15	48	Air drying the yeast.
b	-15	48	Mechanical rupture (milling) of the dry yeast.
c	10	0.5	Extraction with n-butanol-saturated lactate buffer.
d	-6 to 4	3	Acetone fractionations to give a final 25% to 35% v/v fraction.
e	4	40	First crystallisation by dialysis of the final acetone fraction against low ionic strength lactate buffer. (Buffer 1.)
f	4	6	Recrystallisations (usually two).

TABLE 3: THE DIVISION OF NUCLEIC ACIDS INTO THREE CLASSES BY STEPWISE CHROMATOGRAPHY ON DEAE-CELLULOSE

Experiment 1. The nucleic acids from 52 ml. of the n-butanol-lactate extract (TABLE 2, step c) of the Appleby and Morton preparation.

DEAE-cellulose class (see text)	mg. RNA	µg. DNA
1	2.7	40
2	10.0	310
3	30.0	<40*

Experiment 2. The nucleic acids in a 5 ml. sample of material from the Appleby and Morton preparation, just prior to and just after the dialysis step (TABLE 2, step e).

DEAE-cellulose class (see text)	mg. RNA		µg. DNA	
	Before dialysis	After dialysis	Before dialysis	After dialysis
1	1.7	5.5	<40*	60
2	47.0	33.0	380	360
3	16.0	6.3	<40*	<40*
TOTAL	64.7	44.8	-	-

*After allowance was made for dilution factors the lowest amount of DNA that could be estimated with any degree of reliability was a total of 40 µg. per class. Thus, <40 µg. represents an amount of DNA too low to estimate.

TABLE 4: THE EFFECT OF AGING THE YEAST CELLS ON THE NUCLEIC ACID CONTENT OF THE FIRST CRYSTALS OF CYTOCHROME b_2 PREPARED FROM FREEZE-DRIED YEAST

Treatment	Yield of enzyme (mg) per g of freeze-dried yeast	Nucleic acid composition of first crystals		
		% DNA*	% RNA*	% Total nucleic acids*
Fresh freeze-dried yeast	0.03	1.1	4.7	5.8
Aged freeze-dried yeast (70 hr at 25°)	0.02	4.6	1.8	6.4

*The nucleic acid percentages are % (w/w) nucleic acid in the total cytochrome b_2 crystalline precipitate. Cytochrome b_2 (the flavohaemoprotein) was determined from its E_{423} as described in CHAPTER I.

TABLE 5: CYTOCHROME b_2 MOLECULES THAT HAVE NOT BEEN CRYSTALLISED ARE DIFFERENT WITH RESPECT TO THEIR CRYSTALLISATION PROPERTIES TO MOLECULES THAT HAVE BEEN CRYSTALLISED

This Table represents a summary of Experiment 4 (see text) and a summary of a number of supporting experiments of the same design. The other experiments were carried out with enzyme of lower specific activity and with less satisfactory counting techniques. Despite their technical shortcomings, these experiments demonstrated that the result obtained in Experiment 4 was quite clearly repeatable. The specific activities in Experiments 1 and 2 are in counts/1,000 sec./mg. and the specific activities in Experiments 3 and 4 are in CPM/mg.

Expt. number	Original radioactive enzyme		Crystals at 16 hr.		Crystals at 40 hr.	
	mg.	Specific activity	mg.	Specific activity	mg.	Specific activity
1	17	265	13	208	26	21
2	15	455	12	253	23	30
3	12	192	6	72	4.3	21
4	4.6	3660	3.6	3060	3.6	240

NOTES.

Experiment 1. The $[^{35}S]$ yeast was grown on 2% DL-lactate as a carbon source instead of 1% DL-lactate, 1% glucose. The treatment with the Nossal shaker was not in the preparation procedure and counting Method 1 was used.

Experiment 2. The treatment with the Nossal shaker was not in the preparation procedure and counting Method 1 was used.

Experiment 3. The treatment with the Nossal shaker was not in the preparation procedure and counting Method 2 was used. There has been an extensive loss of enzyme in this experiment that may have been due to a contaminant or some other variation in experimental conditions that interfered with crystallisation.

Experiment 4. This method is the type experiment described in detail in the text. Counting Method 3 was used.

CHAPTER THREE

THE FACTORS DETERMINING THE TYPE OF ASSOCIATION BETWEEN POLYANIONS AND TYPE II CYTOCHROME $\underline{b}_2$

THE FACTORS DETERMINING THE TYPE OF ASSOCIATION BETWEEN POLYANIONS AND TYPE II CYTOCHROME b_2

INTRODUCTION

The work in this chapter comprises part of an attempt to explain the observed specificity of the association between the DNA and the enzyme in Type I crystalline cytochrome b_2 (see the thesis INTRODUCTION). The other part of this attempt is contained in the following chapter.

At first, the most attractive hypothesis explaining the specificity was that the Type I cytochrome b_2 was assembled inside the cell as a unit and then fractionated and recrystallised as a non-dissociating unit. However, the complex did readily dissociate in solution because the DNA could be separated from the reduced flavo-haemo-protein by centrifuging through a sucrose density gradient (Symons, 1965) and, as explained in the thesis INTRODUCTION, the known molecular weights of the reduced Type I and Type II cytochrome b were incompatible with the existence of a non-dissociating complex.

However, before further studies were initiated, the association between the oxidised flavo-haemo-

protein and the $\underline{b}_2$-DNA was studied on a sucrose gradient so as to ensure that the nature of the Type I cytochrome $\underline{b}_2$ association did not drastically change its character according to the oxidation state of the flavo-haemo-protein.

A. THE ASSOCIATION OF THE OXIDISED FLAVO-HAEMO-PROTEIN AND b_2-DNA IN SOLUTION.

Although the flavo-haemo-protein is more unstable in the oxidised state than in the reduced state (Appleby, 1957; Armstrong *et al.*, 1960) it can be stabilised to some extent by glutathione which does not reduce the haem (Morton and Shepley, 1963). Even so there was still a substantial activity loss (20% over 32 hr.) if the enzyme was kept under the usual working conditions of 0° in the presence of air. A procedure was developed that used a column of Sephadex G-75 to transfer the enzyme from lactate-buffer (reducing) to acetate-glutathione buffer containing $K_3Fe(CN)_6$ (oxidising conditions) and then into acetate-glutathione buffer. This method (described in detail below) allowed reduced enzyme in lactate buffer to be oxidised and transferred into acetate buffers in a total time of approx. 5 min.

MATERIALS AND METHODS

The lactate buffer referred to here is Buffer 2. The acetate-glutathione buffer was 0.3M Na acetate, 0.05M tetrasodium pyrophosphate, 0.1 mg./ml. glutathione, 0.1mM EDTA adjusted to pH 6.8 with HCl.

(1) Preparation of oxidised enzyme.

A small column of Sephadex G-75 (10 cm. by 0.5 cm. diameter) was equilibrated with the acetate-glutathione buffer. A solution of 0.1M $K_3Fe(CN)_6$ (0.1 ml.) was loaded and allowed to run into the column and then washed in with approx. 0.1 ml. of the acetate-glutathione buffer. The Type I cytochrome b_2 was then loaded onto the column in 0.1 ml. Buffer 2 and the elution started with the acetate-glutathione buffer. The Type I cytochrome b_2 was oxidised as it passed through the ferricyanide zone and emerged from the bottom of the column in the acetate-glutathione buffer. Immediately after this treatment the Type I cytochrome b_2 was found to have an unchanged specific activity. If the ferricyanide was omitted from this procedure the enzyme was oxidised by air dissolved in the acetate-glutathione buffer. However, ferricyanide was used so as to minimise any possibility of damage to the enzyme that might occur during oxidation with free oxygen and to ensure that oxidation was complete.

RESULTS

Oxidised Type I cytochrome b_2 was centrifuged through a sucrose gradient as described by Symons

(1965) for reduced Type I cytochrome $\underline{b}_2$. As shown in FIGURE 1, the $\underline{b}_2$-DNA sedimented more slowly than the flavo-haemo-protein and more or less independently of the flavo-haemo-protein although slight interactions cannot be discounted.

Moreover, when a solution of the oxidised Type I cytochrome $\underline{b}_2$ in the acetate-glutathione buffer was passed down a short column (5 x 1 cm.) of DEAE-cellulose equilibrated with the acetate-glutathione buffer, the oxidised flavo-haemo-protein (oxidised Type II cytochrome $\underline{b}_2$) passed through without any apparent adsorption. A spectrophotometric examination of the enzyme in the effluent showed a quantitative adsorption of the $\underline{b}_2$-DNA.

Thus, the oxidation of the enzyme did not cause any observed changes from the behaviour of the reduced enzyme in similar experiments described by Symons (1965) and it appeared that the oxidised enzyme was also largely dissociated from the $\underline{b}_2$-DNA when the complex was in solution.

B. THE FACTORS THAT DETERMINE WHETHER A NUCLEIC ACID CAN ENTER INTO A CRYSTALLINE ASSOCIATION WITH TYPE II CYTOCHROME b_2 THAT HAS THE FORM OF TYPE I CYTOCHROME b_2

From the material discussed in the INTRODUCTION together with the results presented in CHAPTER II it was known that Type II cytochrome b_2 could select a DNA with certain definite properties out of a mixture containing large amounts of other nucleic acids, but the mechanism by which it accomplished this was not known. In the studies described here, Type II cytochrome b_2 was added to relatively simple and much more defined mixtures of polyanions in an attempt to elucidate the basis of this unusual selection process.

MATERIALS AND METHODS

Type II cytochrome b_2 was mixed with polyanions from various sources that had been given various treatments. These mixtures were then put through the normal crystallisation procedure as described in the legend to TABLE 7. The nature of the resultant insoluble complexes was then studied under the light microscope and they were then classified into two groups. The first group was crystals and the second group consisted of any material that had any other form.

Although the second group was designated "non-crystalline" it was often composed of needles and electron microscope studies of non-crystalline precipitates in CHAPTER IV indicated that some of these had a considerable degree of internal order.

Type II cytochrome $\underline{b}_2$ was prepared as a solution in Buffer 2 by step-wise chromatography of Type I cytochrome $\underline{b}_2$ on DEAE-cellulose (see CHAPTER I).

Single stranded DNA was prepared by phenol extraction of the purified phage AE-2 (Panter and Symons, 1966), Micrococcus lysodeikticus DNA was prepared from cells that had been lysed with lysozyme and phenol extracted as described for yeast DNA in CHAPTER I, and calf thymus DNA was prepared by the method of Kay, Simmons and Dounce (1952).

The yeast RNA used in this chapter was a low molecular weight RNA containing traces of DNA and is the DEAE-cellulose fraction, Class two, described in CHAPTER II.

The rat liver S-RNA was prepared by the method of Holley, Apgar, Doctor, Farrow, Marini and Merrill (1961). However, before the elution of the S-RNA from DEAE-cellulose with 1.0M NaCl, 0.01 tris-HCl, pH 7.5, as described by these workers, the column was washed with 0.4M NaCl, 0.01M tris-HCl, pH 7.5. Both

the yeast RNA and the rat liver RNA were found to be contaminated with DNA when prepared by the above procedures. However, no attempt was made to remedy this as a DNA contaminant was desired for the experiments in which they were to be used.

Nucleic acids were sonicated in 3 ml. of solution using a Dawe Type 1130 A Soniprobe at full power and a cumulative time of approx. 1 min. at 0°. The sonication and heat denaturation processes were carried out in Buffer 2 with the exception of M. lysodeikticus DNA, which could not be heat denatured unless Buffer 2 was diluted with three volumes of water. Heat denaturation was carried out at 100° for 10 min. followed by cooling in ice.

The molecular weight of the sonicated fragments was estimated from their sedimentation coefficients using the UV optics of a Beckman Model E analytical ultracentrifuge. For double-stranded fragments of DNA, the sedimentation coefficient-molecular weight correlation of Doty, McGill and Rice (1958) was used and for the single stranded fragments derived from phage AE-2, the sedimentation coefficient-molecular weight correlation of Eigner and Doty (1965). After the sonication both the double and single-stranded DNA samples were found to have an average molecular

weight of approx. 0.3×10^6.

The DNA to be fragmented with DNAase was dissolved in 0.01M tris-HCl, 1.0mM EDTA, pH 7.5, to give a viscous solution containing 4 mg. per ml. A small amount of solid DNAase I (EC 3.1.4.5., obtained from Calbiochem., B grade) was added and thoroughly dispersed through the viscous mass. The reaction was initiated by shaking 0.1 ml. of 1.0M $MgSO_4$ with 11 ml. of the DNA solution. At the point where viscosity had been reduced to approx. that of water but no hyperchromicity had developed, the reaction was terminated by the addition of 0.5 ml. of 0.5M Na EDTA, pH 7.0, and the DNAase removed by shaking with chloroform.

Polyacrylic acid was obtained from K and K Laboratories, Plain View, New York, and was purified as follows. A solution of the polymer in water was added to three volumes of cold, concentrated HCl and the mixture cooled to -15°. The precipitated polymer was dissolved and reprecipitated twice more and then the HCl removed *in vacuo*. The polyacrylic acid was then made up as a solution of its sodium salt at pH 6.8. Although the molecular weight of the product was not known, dilute alkaline solutions were quite viscous which indicated a relatively high degree of polymerisation.

RESULTS

(1) Preferential selection of DNA in the presence of RNA.

The first process studied was the preferential selection of DNA out of a mixture of DNA and RNA fragments of similar sizes. The two nucleic acid samples, one from yeast and one from rat liver, had been fractionated on DEAE-cellulose to reduce their size heterogeneity (see MATERIALS AND METHODS) and the elution fraction used (DEAE-cellulose fraction 2), see CHAPTER II) was chosen because it was the class that would contain fragments the size of $\underline{b}_2$-DNA.

The results shown in TABLE 6 showed that, in all cases, the crystals showed a marked tendency to select DNA in preference to RNA. As this effect occurred with the nucleic acids derived from rat liver as well as with yeast nucleic acids, it seemed unlikely that cytochrome $\underline{b}_2$ was recognising a specific DNA but that it was selecting on the basis of some more general differences between DNA and RNA fragments. It is of interest to note that as the Type II cytochrome $\underline{b}_2$ was presented with increased total amounts of nucleic acid with a fixed ratio of DNA as to RNA the ratio of DNA as to RNA in the crystals rapidly increased (see TABLE 6). The results obtained in this experiment

satisfactorily explained the high DNA as to RNA ratios that were found in the crystalline Type I cytochrome $\underline{b}_2$ obtained by the method of Appleby and Morton (1960). Moreover, the marked selection would be repeated at each recrystallisation step which would explain the observed rapid elimination of all RNA from these crystals (Appleby and Morton, 1960).

(2) <u>The effect of size and secondary structure of DNA on its ability to be incorporated into crystals</u>.

TABLE 7 is a summary of an experiment in which the crystalline complexes formed between Type II cytochrome $\underline{b}_2$ and various polyanions were studied. In all the cases tested (see TABLE 7) DNA did not support the formation of crystals unless it was native, double stranded DNA of relatively low molecular weight (approx. 0.3×10^6). The molecular weight was usually reduced by sonicating at 0^o but brief treatment of DNA with traces of DNAase also gave fragments that supported crystal formation. The base ratio of the DNA concerned did not appear to have any bearing on the ability of the DNA to support the formation of crystals as shown in TABLE 7. However, these particular experiments cannot rule out the possibility that Type II cytochrome $\underline{b}_2$ might show a slight preference for fragments of one

base ratio with respect to other base ratios. The synthetic polyanion, sodium polyacrylate, was found to be a most effective substitute for DNA and would support the formation of large amounts of well formed Type I-form crystals (TABLE 7 and FIGURE 4b).

The significance of this result, in relation to the enzyme's observed behaviour with the various polynucleotides, became clear after the studies reported in the next chapter (CHAPTER IV) of this thesis.

TABLE 6: SELECTION OF DNA BY PURIFIED TYPE II CYTOCHROME b_2 FROM LOW MOLECULAR WEIGHT NUCLEIC ACIDS ISOLATED FROM YEAST AND RAT LIVER.

Source of Nucleic Acids	$\frac{\text{Weight nucleic acid}}{\text{Weight enzyme}} \times 100$		DNA as % of total nucleic acids		% of Total DNA found in crystals	% of Total enzyme as crystals
	Initial solution	Crystals	Initial solution	Crystals		
Yeast	50	4.2	0.72	4.6	16	30
	100	4.6	0.72	15	30	36
	400	6.0	0.72	27	11	19
Rat liver	28	7.3	22	86	17	17

Nucleic acids from either source were added to a solution of purified, Type II enzyme at the stated ratios of nucleic acid as to enzyme. This solution in Buffer 2 was dialysed against degassed Buffer 1 under oxygen-free nitrogen. The resultant crystals were washed thoroughly and the nucleic acids estimated by the procedure described in CHAPTER I. The enzyme recovery was estimated from E423, as described in CHAPTER I.

TABLE 7: THE ABILITY OF POLYANION PREPARATIONS TO FORM CRYSTALLINE COMPLEXES WITH TYPE II CYTOCHROME b_2.

In the standard test 60 µg. of the appropriate DNA preparation was added to 1.0 mg. of Type II cytochrome b_2 in a final volume of 0.7 to 1.4 ml. of Buffer 2. The mixture was dialysed overnight at 4° against degassed Buffer 1 under O_2-free nitrogen (as for the normal recrystallisation procedure described in Symons and Burgoyne (1966)) and the insoluble material examined under the light microscope. The recovery of enzyme as insoluble material varied but was usually about 80%. In the case of the polyacrylate test, 4.5 mg. of Type II cytochrome b_2 was added to 0.125 mg. polyacrylic acid as its sodium salt. The complete mixture was in 1.0 ml. of Buffer 2 and was dialysed against anaerobic Buffer 1 as described above.

Photographs of some of these precipitates are presented in FIGURES 2,3 and 4 as noted in this Table.

The details of the DNA sources and treatments are given in the MATERIALS AND METHODS of this chapter and this section (CHAPTER III, SECTION B).

TABLE 7: THE ABILITY OF POLYANION PREPARATIONS TO FORM CRYSTALLINE COMPLEXES WITH TYPE II CYTOCHROME b_2.

Nucleic acid preparation	Treatment	Formation of square plate crystals. (Type 1)	Comments	Photographs
DNA from* Type I Cyt. b_2 (%G+C=30)	None	+	Standard square plate crystals.	Fig.2C and 2D.
	Heated	-	Various semi-crystalline forms.	-
Whole Yeast DNA (%G+C=36)	None	-	Various semi-crystalline forms.	-
	Sonicated**	+	Standard square plate crystals.	Fig.3C.
	Sonicated then heated	-	Feathery ppt.	Fig.3D.
Calf thymus DNA (%G+C=42)	None	-	Amorphous ppt.	-
	Sonicated**	+	Standard square plate crystals.	Fig.3A.
	Sonicated then heated	-	Needle-like precipitate.	Fig.3B.
	Partly DNA-ase digested	+	Poor quality square plate crystals.	Fig.4A.
M. lysodeikticus DNA. (%G+C=72)	None	-	Amorphous ppt.	-
	Sonicated**	+	Standard square crystals	Fig.4C.
	Sonicated then heated	-	Amorphous ppt.	-

TABLE 7 (cont'd.)

Nucleic acid preparation	Treatment	Formation of square plate crystals. (Type 1)	Comments	Photographs
Phage AE2 DNA (single-stranded) Moles % G=21, A=26, C=21, T=32.	None	-	Amorphous ppt.	-
	Sonicated**	-	Small ill-defined needles.	Fig.4D.
Sodium polyacrylate	None	+	Standard square plate crystals.	Fig.4B.

*b_2-DNA has a molecular weight of 1.2×10^5 (Armstrong et al., 1963b).

**The sonicated DNA preparations all had an average molecular weight of 3×10^6. (see MATERIALS AND METHODS).

FIGURE I: SEPARATION OF b_2-DNA AND THE FLAVO-HAEMO-PROTEIN ON A SUCROSE GRADIENT.

A two chamber mixer was used to produce a linear sucrose gradient from 20 to 5% (w/v) sucrose in the acetate-glutathione buffer. The gradient volume was 4.4 ml. and was overlaid with a solution of 1.72 mg. Type I cytochrome b_2 (oxidised) in 0.15 ml. acetate-glutathione buffer. This was centrifuged for 15 hr. at 35,000 revs./min. in the SW-39 head at 2°. The centrifuge tube was removed and three drop fractions collected (approx. 0.07 ml. each). To each fraction was added 0.5 ml. of the acetate-glutathione buffer and the E_{413} and E_{260} read.

The E_{413} (indicated by crosses on the graph) is due to the Soret peak of the oxidised flavo-haemo-protein and, as there is no contribution from the DNA at this wavelength, the E_{413} was a direct measure of the amount of flavo-haemo-protein at a point.

The E_{260} (indicated by filled circles on the graph) due to DNA was calculated by deducting the E_{260} due to the flavo-haemo-protein (based on the relationship of $E_{260}/E_{413} = 0.48$) from the observed E_{260}.

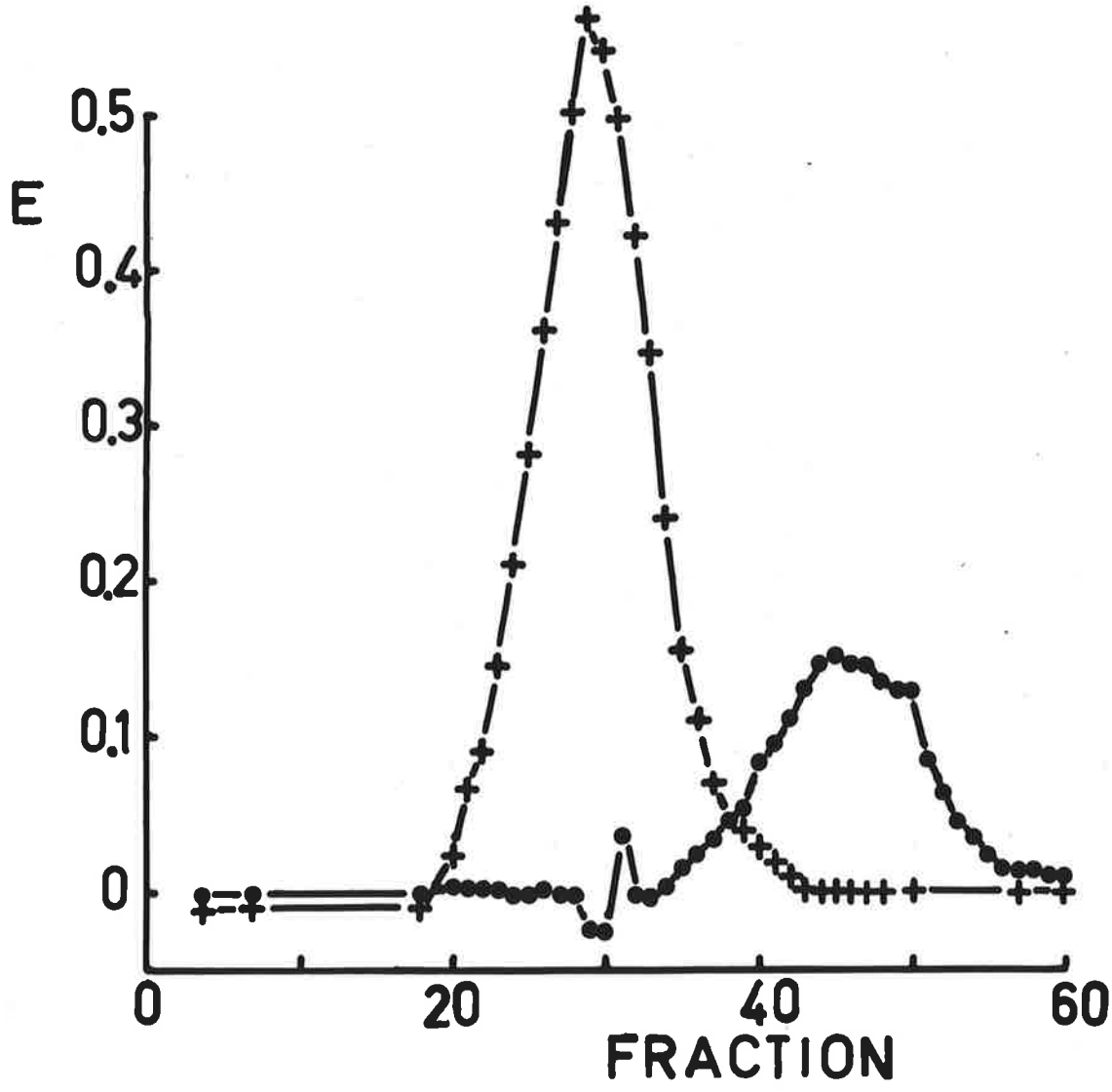

E
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
FRACTION

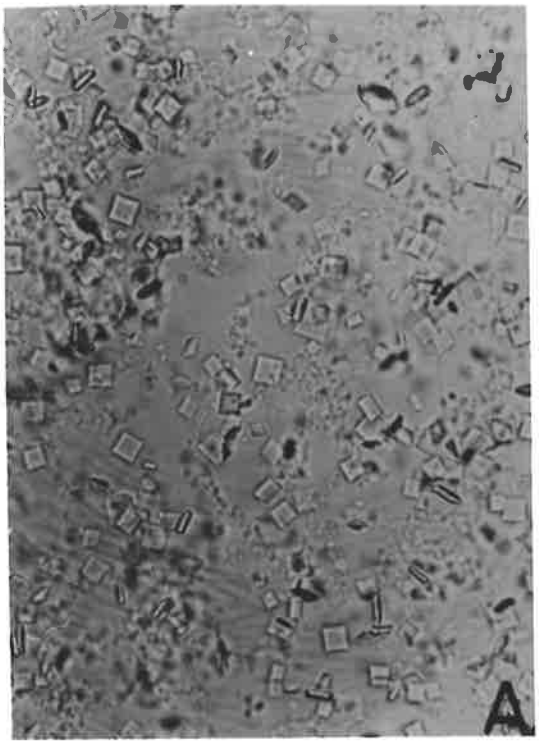
FIGURE 2: CRYSTALLINE PREPARATIONS OF TYPE I CYTOCHROME b_2.

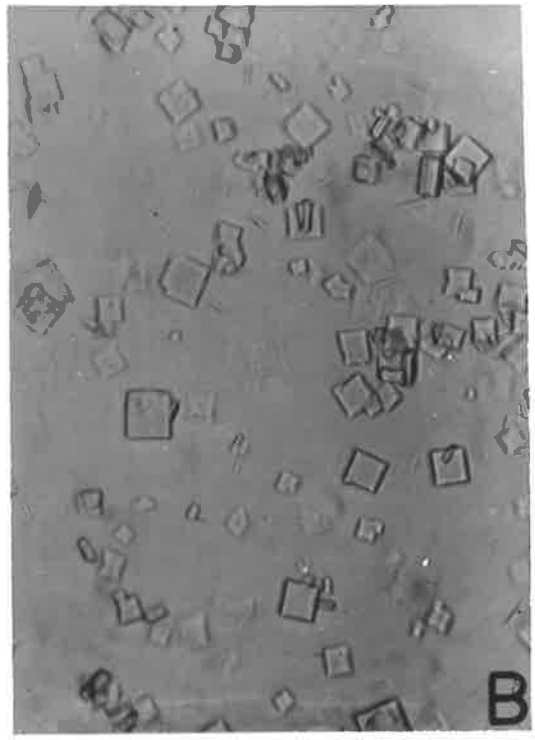
(A) The first crystals obtained from the Appleby and Morton preparation procedure photographed in their mother liquor. Mag. x 600.

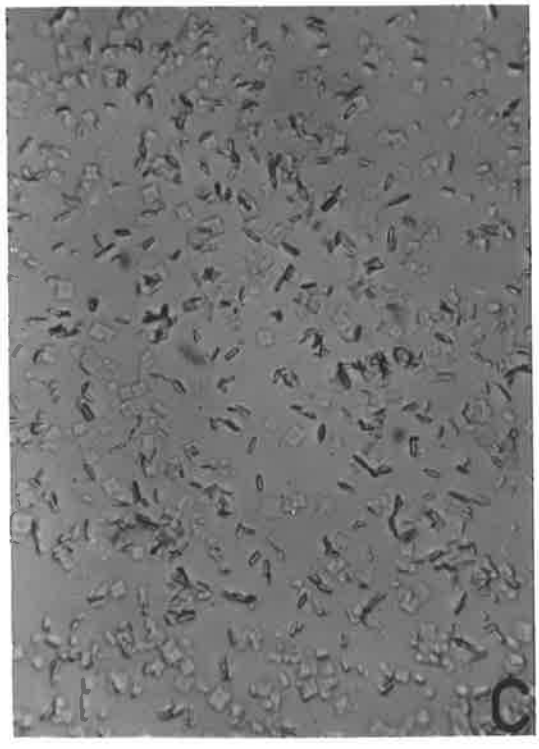
(B) The crystals from A (above) recrystallised once. Mag. x 600.

(C) Type I cytochrome b_2 from which the DNA was removed (see CHAPTER I) and replaced to demonstrate the reversibility of the removal process. Re-association was effected by mixing the appropriate amount of b_2-DNA with DNA-free enzyme and then carrying out the crystallisation procedure (see legend to TABLE 7). Mag. x 445.

(D) A repeat experiment of C (above). Mag. x 500.


A


B


C


D

FIGURE 3: THE INSOLUBLE COMPLEXES FORMED BETWEEN TYPE II CYTOCHROME b_2 AND VARIOUS DNA FRAGMENTS.

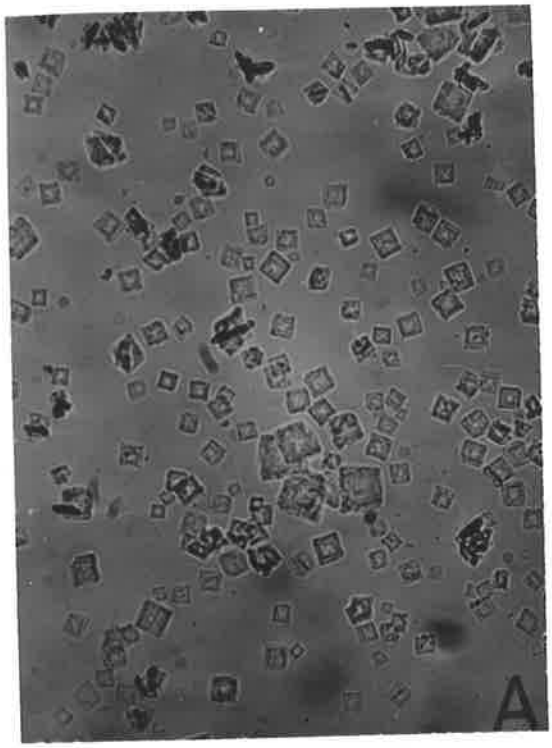
The complexes were formed by mixing the Type II cytochrome b_2 with the appropriate nucleic fragments and putting the mixture through the crystallisation procedure as described in the legend to FIGURE 7. The DNA source sonication and heat denaturation are described in the MATERIALS AND METHODS of this section (CHAPTER III, SECTION B).

(A) Crystalline preparations containing sonicated calf thymus DNA in the Type I crystalline form. Mag. x 250.

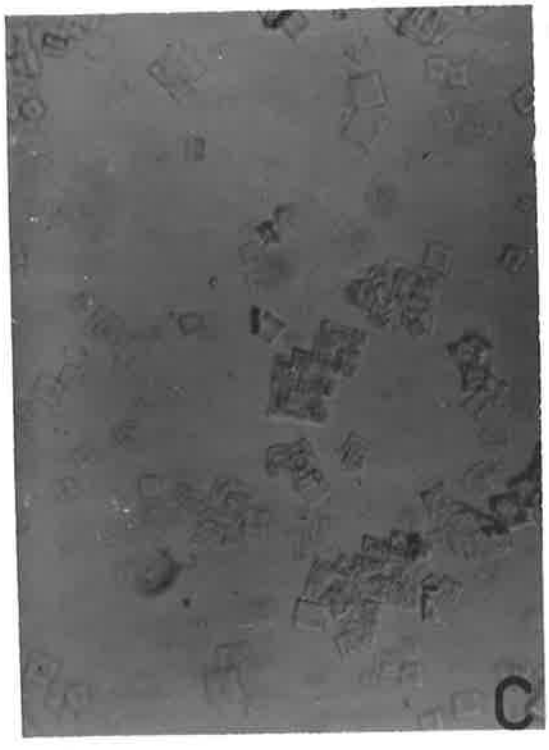
(B) Non-crystalline preparation containing calf thymus DNA that had been sonicated and then heat denatured.

(C) Crystalline preparation containing sonicated bulk yeast DNA. The complex had the Type I crystalline form. Mag. x 225.

(D) Non-crystalline precipitate, containing bulk yeast DNA that had been sonicated and then heat denatured. Mag. x 225.


A


B


C


D

FIGURE 4: COMPLEXES BETWEEN VARIOUS DNA FRAGMENTS, POLYACRYLATE AND TYPE II CYTOCHROME b_2.

The nucleic acid or polyacrylate was mixed with the Type II cytochrome b_2 and taken through the crystallisation procedure (see legend to TABLE 7).

(A) A crystalline precipitate containing fragments of calf thymus DNA prepared by briefly exposing the DNA to pancreatic DNAase (see MATERIALS AND METHODS of this section) and then selecting the material that was desorbed from DEAE-cellulose between 0.4 and 1.0M NaCl, pH 7.5. The complex had the Type I crystalline form. Mag. x 460.

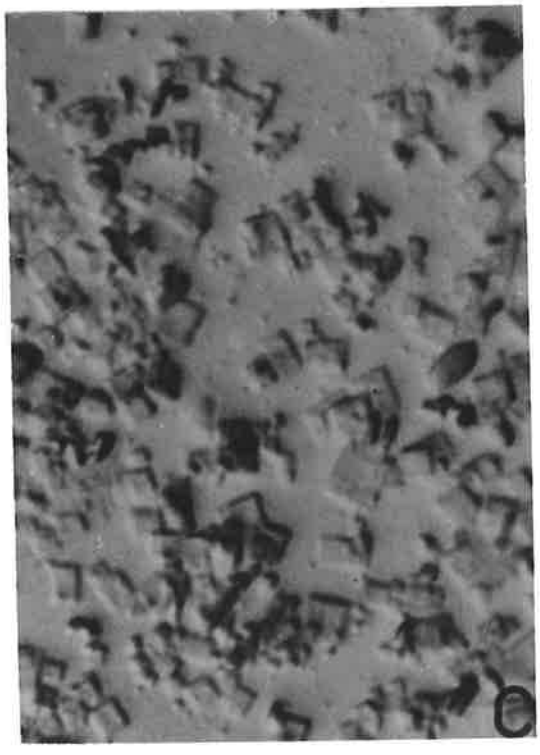
(B) Crystalline complex of the Type I-form formed between Type II cytochrome b_2 and polyacrylate. Mag. x 400.

(C) Crystalline preparation of the Type I-form containing sonicated M. lysodeikticus DNA. Mag. x 800.

(D) Non-crystalline precipitate formed when sonicated, single-stranded coli-phage AE-2 DNA was complexed with Type II cytochrome b_2. Mag. x 445.


A


B


C


D

CHAPTER FOUR

THE STRUCTURE OF THE CRYSTALS OF TYPE I AND II CYTOCHROME $\underline{b}_2$

THE STRUCTURE OF THE CRYSTALS OF TYPE I AND II CYTOCHROME $\underline{b}_2$

INTRODUCTION

The previous chapter (CHAPTER III) showed that, during the formation of crystalline Type I cytochrome $\underline{b}_2$, nucleic acid molecules that satisfied certain criteria of size and secondary structure were selectively incorporated into the crystal.

In this chapter the electron microscope has been used in an attempt to show how the presence of nucleic acid caused the marked change in crystalline form from the Type II crystals, which belonged to the hexagonal system, to the small square plates of the Type I crystals. It was also considered that a study of the structure of these two crystalline forms might explain why nucleic acids that are double stranded and of low molecular weight are preferentially bound into the Type I crystals.

MATERIALS AND METHODS

A Siemens Elmiskop I electron microscope was operated at 80 KV with a 50 μ objective aperture.

(1) Preparation of the carbon films for the grids.

A clean glass slide was coated with a perforated collodion film by gently breathing a coating of water droplets onto a film of 0.5% (w/v) collodion (Gurr's histological grade) as it dried.

The collodion film was detached from the glass slide on a water surface, deposited on electron microscope grids and then coated with a thin film of evaporated carbon (Spectrographically standardised, from Society Carbonne-Lorraine-Paris). The collodion was removed by leaving the grids in contact with an amyl acetate surface for 15 min., a procedure which left grids covered with a thin film of perforated carbon.

(2) Preparation of solutions of Type I or II cytochrome b_2 for electron microscopy.

Enzyme preparations were negatively stained with 2% uranyl acetate, pH 4.5, or sodium phosphotungstate, pH 3.5, after the molecules had been deposited on a carbon film from solution. For shadowing, the enzyme was dissolved in 0.2M ammonium acetate, pH 6.8, and sprayed onto a freshly cleaved mica surface. After shadowing with platinum (from Matthey Garrett Pty. Ltd.)

the surface was coated with a carbon film which was then stripped off the mica for study in the electron microscope.

(3) Preparation of DNA-free (Type II cytochrome b_2) crystals for electron microscopy.

Small hexagonal bipyramids of the Type II cytochrome b_2 were prepared by transferring a concentrated solution of the enzyme from Buffer 2 to 1/10 x Buffer 2 on small columns of Sephadex G-75 and then inducing crystallisation in the supersaturated solution by freezing and thawing. These crystals were then used for the preparation of sonicated fragments or sections as follows.

(a) Sonicated fragments. A suspension of the small Type II cytochrome b_2 crystals in 1/10 x Buffer 2 was sonicated at 0° for approx. 1 min. by a Dawe Type 1130 A Soniprobe at half power.

(b) Crystal sections. To a suspension of Type II cytochrome b_2 crystals in 1/10 x Buffer 2 was added a solution of neutralised glutaraldehyde to give a final concentration of 6% and the mixture was allowed to fix for one hour at 4°. The fixed crystals were then washed in 1/10 x Buffer 2 and transferred into Araldite via acetone-water and acetone. The Araldite was allowed to set at 64° for 48 hr. and ultra-thin sections were cut with a glass knife on a LKB Ultrotome.

(4) Preparation of Type I cytochrome b_2 crystals for electron microscopy.

Small crystals of the Type I cytochrome b_2, suitable for electron microscopy, were prepared by slowly adding Buffer 1 to a solution of the Type I cytochrome b_2 in Buffer 2. When a fine suspension of the crystals first appeared, the mixture was allowed to stand for 10 min. and the smallest crystals were concentrated by differential centrifugation. The crystals were put on the electron microscope grids with Buffer 1 and the surface of the carbon film was briefly washed with the same buffer before staining with a 2% solution of sodium phosphotungstate, pH 3.5, or a 2% solution of uranyl acetate, pH 4.5.

RESULTS

(1) Studies of single protein molecules.

The protein molecules of cytochrome b_2 (either Type I or Type II) were clearly observed when negatively stained with 2% uranyl acetate, pH 4.5, or 2% sodium phosphotungstate, pH 3.5, and they were observed to be approximately isometric objects with an average width of 90 A^{o}. FIGURES 5A, 5B and 5D show negatively stained protein molecules which appear to be composed of 2 or 4 subunits. However, no clear details of

molecular sub-structure were observed.

A solution of Type I cytochrome $\underline{b}_2$ was deposited out of 0.2M ammonium acetate and shadowed so that the height of the molecules could be estimated and the DNA fragments observed (FIGURE 5C). The shadows produced were 180 A^{o} long so that, with a ratio of shadow length to object height of 3:1, the height of the protein molecule was calculated to be 60 A^{o}. There were reasons for considering that this 60 A^{o} was an over estimate of molecule height. First, there was the fact that the platinum layer had a depth in the same order as the depth of the objects being shadowed (platinum depth approx. 27 A^{o}) so the actual height of the molecules would be smaller than 60 A^{o} but not smaller than approx. 30 A^{o}. In addition, this shadow length was determined by the length of the highest parts of the molecule so that irregularities in the shape of the molecule or differences in the orientation of the molecules on the grid surface, would all tend to cause over-estimations of the average height. This situation is further complicated by the possibility of surface tension effects and shrinkage on dehydration which would tend to reduce the observed heights of the molecules.

Thus, although the conclusions drawn from the shadowing studies were necessarily qualified by the above

considerations, they did indicate that the height was considerably less than the other two dimensions of the protein molecules.

The shadowed preparation allowed the observation of strands, presumably DNA, in the preparation of Type I cytochrome $\underline{b}_2$. The strands were present in approx. the amount and had approx. the length predicted from the composition of Type I cytochrome $\underline{b}_2$ and the molecular weight of $\underline{b}_2$-DNA. However, the strands did not appear to be all exactly the same length and it was of interest to note that most of the strands appeared to be attached to at least one protein molecule (FIGURE 5C).

(2) <u>The crystals of Type I and Type II cytochrome $\underline{b}_2$ as observed by the light microscope</u>.

The crystals of Type I and Type II cytochrome $\underline{b}_2$ have been thoroughly described by Appleby and Morton (1959a, and Morton and Shepley, 1961, 1963). However, preparatory to an examination of the crystals with the electron microscope, a short light microscope study was carried out.

(a) <u>Type I cytochrome $\underline{b}_2$</u>. A photograph of the characteristic flat, square plates of this form of the enzyme has been given earlier in FIGURE 2. In agreement with Morton and Shepley (1961), these crystals were found

to be apparently isotropic when viewed through the large square faces of the plates but anisotropic when viewed edge on.

Many crystals had markings on their large square surface and these markings looked like grooves or ledges running parallel to the crystal edges. Occasionally, crystals were seen with diagonal markings which suggested that they were tetragonal bipyramids (Morton and Shepley, 1961; Appleby and Morton, 1959a). However, the evidence from the electron microscope studies indicated that the actual shape of the crystals is a flat square plate (see later).

(b) Type II cytochrome b_2. DNA-free enzyme always crystallised in the hexagonal system as described by Morton and Shepley (1961, 1963) but the actual form of the crystals showed a considerable variation. The two main forms observed were hexagonal bipyramids and hexagonal prisms capped with pyramids. Although very large crystals with these forms could sometimes be grown, attempts to produce large crystals usually gave crystals that had grown unevenly (see FIGURE 6). However, when large crystals were observed between crossed polarising filters they were found to be isotropic when viewed parallel to their C axis (the hexagonal axis) but anisotropic when viewed at a right

angle to it (see FIGURE 6).

Anisotropy of these crystals was first reported by Morton and Shepley (1961).

The micro-crystals of Type II cytochrome $\underline{b}_2$ that were prepare for study in the electron microscope, were almost entirely hexagonal bipyramids with the shape shown in FIGURE 7. Because they were extremely small and uniform in shape, they were the most suitable for the purposes of fixation and study in the electron microscope.

(3) Study of the hexagonal bipyramid crystals of Type II cytochrome b_2 in the electron microscope.

The hexagonal bipyramid crystals were found to have an extremely porous structure consisting of open hexagonal tubes which gave the appearance of a honeycomb when cut across the C axis (FIGURE 8) and parallel lines of protein alternating with empty spaces when cut parallel to the C axis (FIGURE 9). In both these types of sections the approximate relationship of the plane of cut to the C axis was deduced from the overall shape of the crystal slice and the fact that the crystals were hexagonal bipyramids. However, for sections cut across the C axis a more accurate estimation of the section angle was obtained from the variation of the observed pattern from one composed of hexagons. In the case of

sections across the C axis (e.g. FIGURE 8) objective estimates of the side length of the individual hexagons were obtained from four separate crystals but in the case of the longitudinal sections (e.g. FIGURE 9) the spacings observed depended on the relationship of the section plane to the hexagonal tubes as well as on the dimensions of the individual hexagons. Thus, although the spacings in the longitudinal sections agreed approximately with that predicted from the cross sections, close agreement could not be objectively proved as the observed spacing depended on the orientation of the section plane to the hexagonal tubes.

When Type II cytochrome $\underline{b}_2$ crystals were sonicated and negatively stained with uranyl acetate, the broken pieces allowed simultaneous observation of both the cross and longitudinal section of the crystal structure (FIGURES 10A and B) and the results confirmed those obtained from crystal sections. Examination of a number of similar electron micrographs showed that the structure appeared to break at the corners of the hexagons which indicated that each side of a hexagon was one molecule. Thus, it appeared that the corners of a hexagon represented the junction of three molecules at 120° so that the length of a molecule,

measured from hexagon corner to hexagon corner, was 82 A°. The thickness of the walls of the hexagonal pattern seen in sections across the C axis (FIGURE 8) was approx. 30 A° and was presumably the narrowest dimension of the molecule.

(4) Electron microscope studies of the Type I cytochrome b_2 crystals.

When negatively stained, these flat, square, crystals gave the appearance of a mesh (FIGURES 11A and B). Examination and measurement of a number of similar electron micrographs showed that the smallest structural unit was about 90 A° by 30 A° which presumably corresponded to a single protein molecule. These single units were arranged side by side in pairs which were themselves arranged lengthwise in twin rows. As can be seen from FIGURES 11A and B these twin rows were arranged side by side to form a layer of protein and from these and other micrographs, these layers of protein appeared stacked on top of each other to form the crystal. Close examination of micrographs, especially around the edges of crystals, indicated that each protein layer was orientated at 90° to the one above it. The twin rows of molecules seen in FIGURES 11A and B repeated every 92 A°; it can be seen that the space between these twin

rows was wider than the space down the centre of each twin row.

Since these crystals were flat, orientation of the crystal was necessary to obtain a side view. However, despite numerous attempts to embed and section these crystals, it has not been possible to obtain a satisfactory side view of the crystal structure. This lack of success has been due to the instability of these crystals towards the dehydration and embedding procedures and to the failure to obtain satisfactory contrast even when imbedding had been partially successful.

The DNA, although known to be present in the Type I cytochrome $\underline{b}_2$ crystals, could not be directly observed due to lack of a suitable stain, however, evidence on its probable location in the crystal is given below.

(5) Proposed shape of the protein molecule and the structure of the Type II cytochrome b_2 crystals.

The proposed shape and size of a single enzyme molecule of molecular weight 170,000 is showed in FIGURE 12. The dimension of 82 A^{o} was obtained from studies of the Type II cytochrome $\underline{b}_2$ crystals as described above. Measurement of negatively-stained free molecules showed that a second dimension was approx. 90A^{o}, a value taken to be 92 A^{o} as this allows each molecule, in the Type I

cytochrome $\underline{b}_2$ crystal, to span exactly two rows (one twin row) of molecules orientated at right angles to it as described below and shown in FIGURE 14. The third dimension was known to be approx. 30 A° from sections of the Type II cytochrome $\underline{b}_2$ crystals (FIGURE 8), a value corrected to 26 A° so as to fit this model to the expected molecular volume of 200,000 cubic A°. (These calculations assumed a partial specific volume of 0.71; Armstrong et al., 1963a.)

It is proposed that these single protein molecules are arranged in the hexagonal bipyramid crystals as shown in the model of FIGURE 13A and B. The 120° angle at the top and bottom of the protein molecule of FIGURE 12 has been assumed so as to allow a perfect junction of three molecules in the hexagonal pattern. In FIGURE 13A the molecular junctions along the C axis of the Type II crystals have been shown as being in phase (i.e. the junctions all occur at junction planes which are at 92 A° intervals and perpendicular to the C axis). This need not be so and it is possible to propose a regular structure, similar to that shown in FIGURE 13A, in which the molecular junctions are not in phase so that there are no definite junction planes. A small part of such a structure is shown in FIGURE 13B.

(6) Proposed structure of the Type I cytochrome b_2 crystals.

The proposed arrangement of DNA and protein molecules in the Type I cytochrome b_2 crystal is shown diagrammatically in FIGURE 14. The top view is looking at the flat face of the square, flat crystal (cf. FIGURES 7 and 11) while the side view represents the crystal edge on; as discussed above, it has not been possible to view the crystal directly from this angle. The top view shows two layers of the crystal with rows of protein molecules in one layer running at right angles to the rows in the other layer. The DNA strands (black in FIGURE 14) are located in the wider spaces between the protein molecules while the narrower spaces are empty. The two rows of protein molecules bordering the narrow space make up the twin rows of protein molecules seen in FIGURES 11A and B. The side view in FIGURE 14 further illustrates the postulated relative arrangement of the protein and DNA molecules.

Although DNA makes up 5-6% by weight of the Type I cytochrome b_2 crystal, none of the staining methods used in the present work have shown its location in the crystal. However, the most likely space for the DNA molecules to occupy is in the grooves between the

parallel rows of protein as predicted in FIGURE 14. If we assume that the wide grooves contain the double-stranded DNA and the narrow grooves are empty, then the DNA composition of 4.9% calculated on this model is in very good agreement with the 5-6% by weight of DNA found in these crystals. Since the average molecular weight of this DNA is 1.2×10^5 (Armstrong et al., 1959b), each DNA molecule will be 650 A^o long and will therefore span about 14 single rows of protein (see FIGURE 14).

Experimental evidence in support of the proposed relative positions of DNA and protein in FIGURE 14 was provided when large amounts of high molecular weight DNA were added to Type II cytochrome b_2 in Buffer 1. The large skeins of DNA adsorbed the enzyme molecules which were arranged in imperfect systems of short parallel rows orientated at 90^o to the long axis of the DNA skein (FIGURE 15). In the electron micrograph of FIGURE 15 and a number of others showing the same result, the actual DNA strands were not visible but there seems to be little doubt that the pattern of enzyme molecules traced out the DNA skeins because enzyme molecules deposited on carbon films without DNA did not show such organised structures.

(7) Possibility of artifacts in the crystal studies.

The ultrastructure of the hexagonal bipyramids of the Type II cytochrome b_2 crystals was studied by negatively staining sonicated fragments of the crystals with uranyl acetate and by sectioning suspensions of the microcrystals embedded in Araldite. The two different techniques showed the same microstructure and seemed to agree in all respects so that it would appear unlikely that fixation or artifacts from other sources need to be considered in this case. In the case of the Type I cytochrome b_2 crystals, the ultrastructure was the same on negatively staining with uranyl acetate or sodium phosphotungstate, although the former gave better definition, thus reducing the likelihood of any major structures being staining artifacts.

DISCUSSION

The evidence obtained in the electron microscope studies has allowed the building of models of the structure of the Type I and Type II cytochrome b_2 crystals which are represented in FIGURES 13 and 14. Although it would seem that the structure of the hexagonal bipyramid crystals has been reasonably well

described, it is unfortunate that the absence of satisfactory side views of the flat, square plate Type I crystals has prevented a more complete description of this complex structure.

It is of interest to note that one site on the protein molecule that is considered to bond to the DNA is the 120° edge (see FIGURE 14 and consider implications of FIGURE 15). Traces of DNA would therefore interfere with any attempt to assemble hexagonal bipyramid crystals, thus providing a reasonable explanation for why the square plate crystal form is preferred to the hexagonal bipyramid form when the enzyme is crystallised in the presence of polyanions.

Although most of the evidence suggested that Type II crystals had a structure of the type shown in FIGURE 13A it seemed quite likely that some details of this structure were incorrect. For example, the proposed structure for the Type I crystal (FIGURE 14) implied that the protein molecules had a positive charge somewhere on their 120° edges that attaches to the polyanion in the Type I crystal (if FIGURE 14 was correct it would be expected to be at the centre of the molecule). Consequently, the structure of the Type II crystals proposed in FIGURE 13A would be expected to force a

number of positive charges close together. One possible variation of the (FIGURE 13A) proposed structure for Type II crystals is shown in FIGURE 13B. This variation of the proposed Type II structure would allow the postulated positive sites to be spaced apart and would also be in accord with the apparent absence of a cleavage plane at right angles to the C axis of the Type II crystals. The final elucidation of this, and other details of the Type I and Type II crystal structures may require X-ray diffraction studies.

The tests in which polynucleotides of various types were allowed to associate with Type II cytochrome $\underline{b}_2$ indicated that the enzyme showed a marked ability to select double stranded nucleic acids of relatively low molecular weight. However, although DNA was preferentially incorporated into Type I crystals (see TABLE 6), crystals that contained predominantly yeast RNA were often quite perfect in form. In this case, the enzyme may have been accepting helical yeast RNA of the Type described by Spencer and Poole (1965).

The proposed structure for the Type I form crystal (see FIGURE 14) suggested that any linear, non-crosslinked polyanion that was not over about 25 A° wide would support the formation of Type I-form crystals. Thus, the preference of cytochrome $\underline{b}_2$ for double-stranded nucleic

acids probably reflects the fact that only linear nucleic acids are incorporated into the crystal while single-stranded nucleic acids tend to cross link with themselves by local hydrogen bonding. This conclusion was supported by the successful substitution of sodium polyacrylate for DNA in the crystalline complex. Similarly, high molecular nucleic acids will become entangled and this would have the same effect as cross linkage, thus explaining why the crystal preparations are found to contain a relatively low molecular weight DNA.

The information gained, from the electron microscope studies, about the shape and dimensions of the protein itself, must be viewed with care as it is known that the native protein is modified during the preparation procedure and will not form crystals until so modified. (This thesis and Nicholls et al., 1966.) However, these modifications are believed to be relatively minor ones and so the information may be applicable to the unmodified protein.

The structure of these nucleoprotein crystals seemed to bear little or no relation to any other similarly studied nucleoprotein. Although this association of nucleic acid and protein is a preparation artifact

(see CHAPTERS II, III and V) it nevertheless represents a class of nucleoprotein different to the nuclechistones and viruses. It is conceivable that associations of this type may have biological significance.

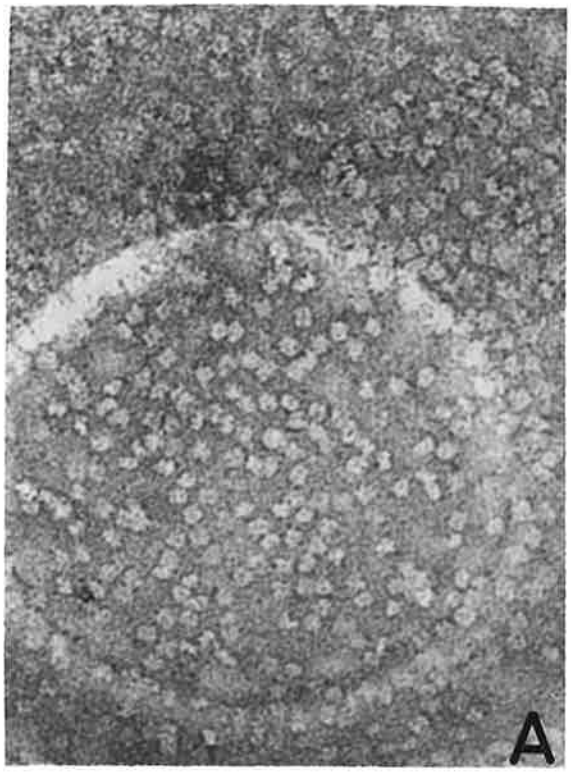
FIGURE 5: ELECTRON MICROGRAPHS OF FREE ENZYME MOLECULES.

(A) File No.3090. A dilute solution of Type I cytochrome $\underline{b}_2$ in Buffer 2 was fixed with 5% formaldehyde, deposited on a carbon film and stained with 2% uranyl acetate, pH 4.5. Part of this field is over a hole in the carbon film which reduces the background interference. The DNA fragments are not observable with this stain. Mag. x 240,000.

(B) File No.3090. Same material as in field (A) (above) but on a normal carbon film area and at half the magnification. Mag. x 120,000.

(C) File No.3278. Type I cytochrome $\underline{b}_2$ in Buffer 2 was diluted into a large vol. of 0.2$\underline{M}$ ammonium acetate, pH 6.8, and immediately sprayed onto a freshly cleaved mica surface which was then shadowed with platinum to a depth of 27 A^{o} so as to give a shadow length as to height ratio of 3:1. The platinum film was then stabilised with a carbon film and the composite film stripped off for study. Mag. x120,000.

(D) File No.3143. Type II cytochrome $\underline{b}_2$ deposited on the carbon film without fixing, washed with KCl and negatively stained with 2% uranyl acetate, pH 4.5. Despite the high overall magnification no consistent molecular substructure could be seen. Mag. x 400,000.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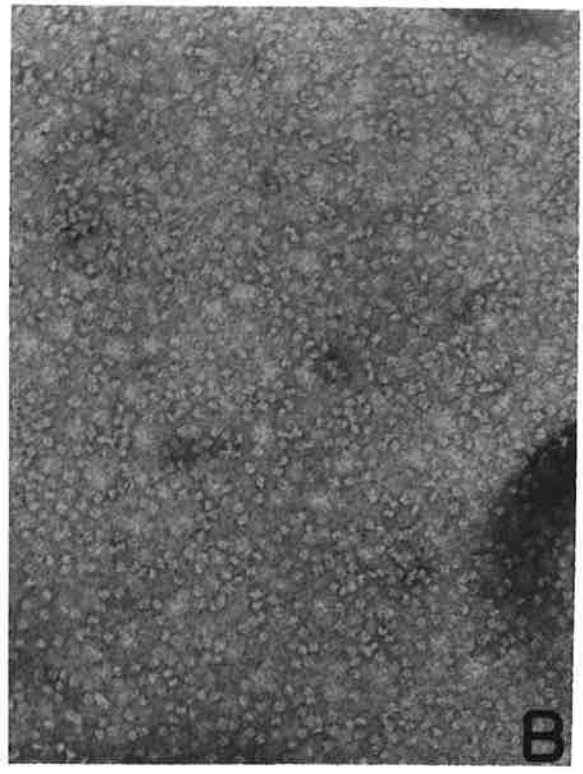
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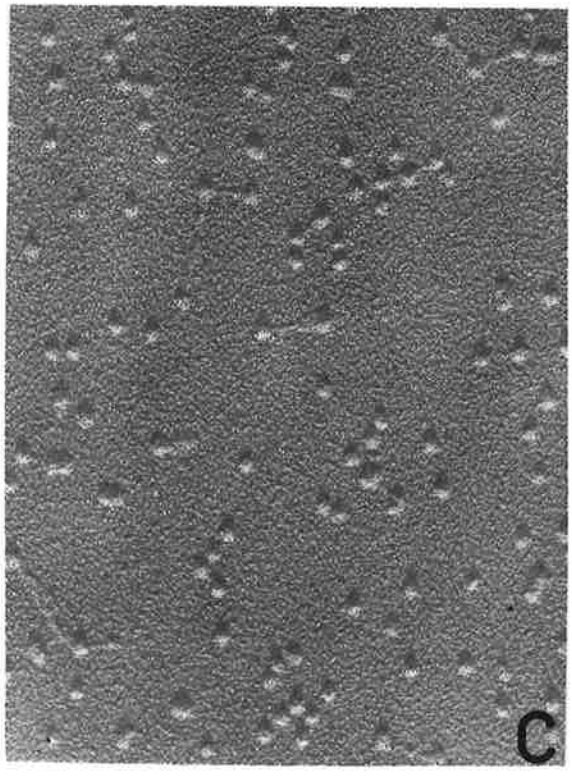
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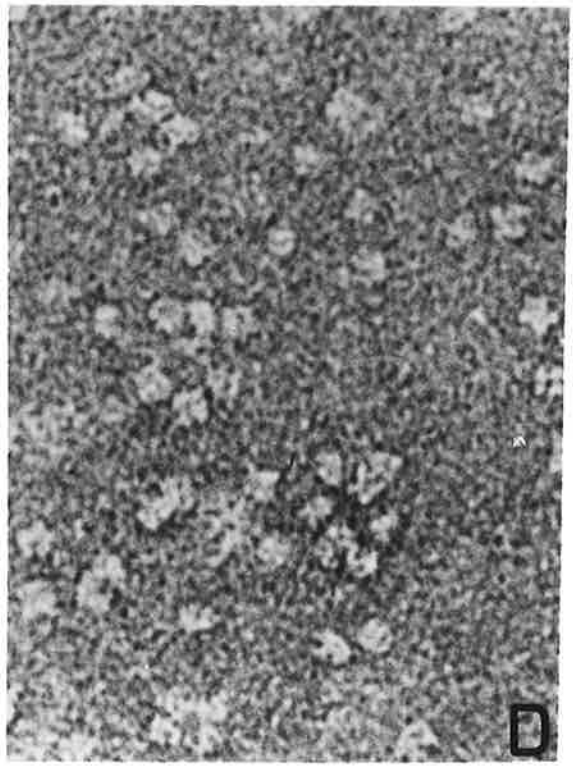

D

FIGURE 6: ANISOTROPY IN CRYSTALLINE TYPE II CYTOCHROME $\underline{b}_2$

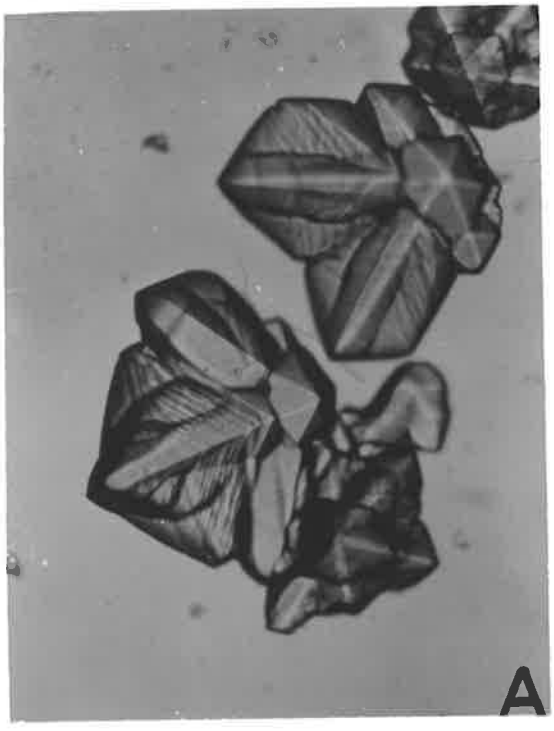
(A) Type II cytochrome $\underline{b}_2$ crystals photographed with normal illumination. Mag. x 51.

(B) Identical material, field and magnification to that in (A) but viewed between crossed polarising filters.

(C) Type II cytochrome $\underline{b}_2$ crystals photographed with normal illumination. A different field and sample to that used in (A) (above). Mag. x 124.

(D) Identical material, field and magnification to that in C above but viewed between crossed polarising filters.

Anisotropy of these crystals was first reported by Morton and Shepley (1961).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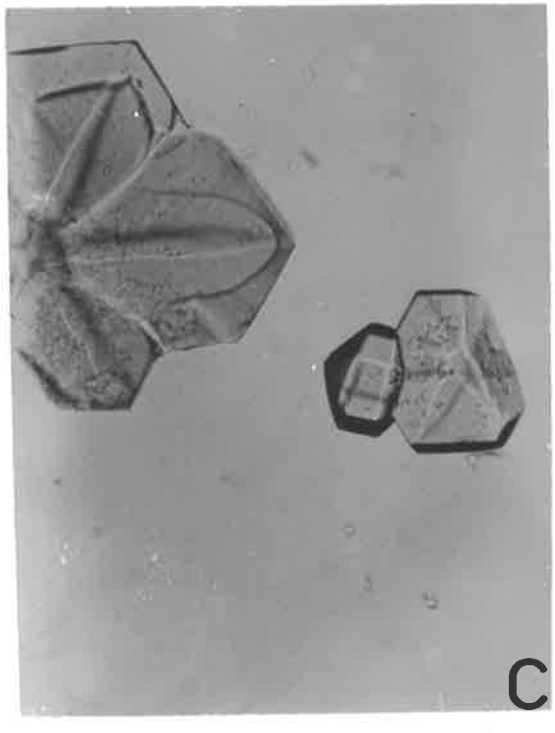
B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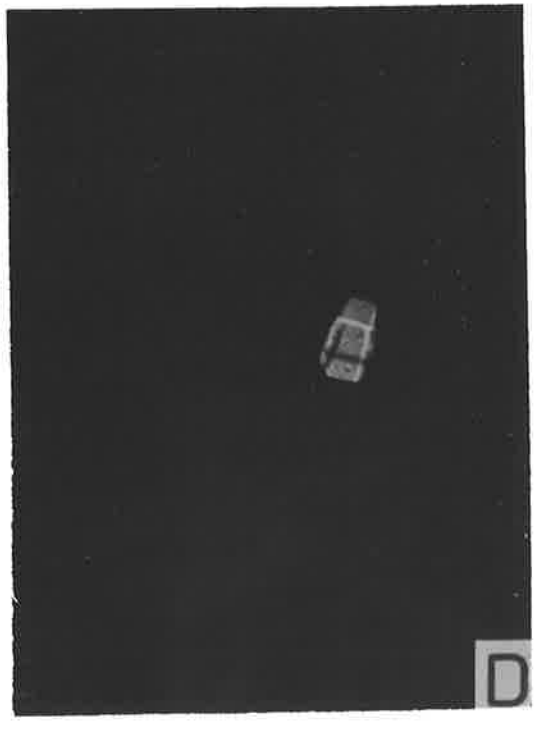

D

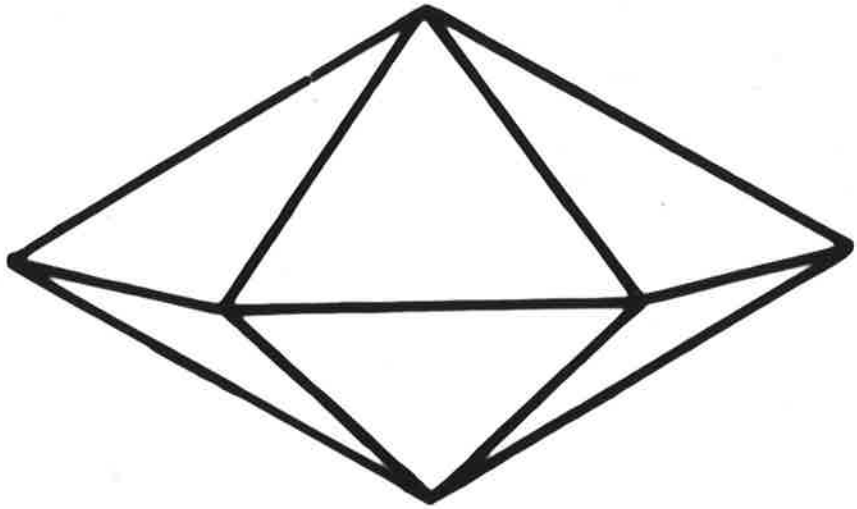
FIGURE 7: DRAWINGS SHOWING THE SHAPE OF TYPICAL TYPE I AND II CRYSTALS AS USED IN THE ELECTRON MICROSCOPE INVESTIGATION.

Type I-form crystals. The general shape of these is depicted in the lower diagram. Most of the observed crystals were square, flat plates although the plates often varied in thickness across the crystal.

Type II-form crystals. One of the common shapes of this crystalline form is depicted in the upper diagram.

All the above crystalline forms were first described by Appleby and Morton (1954, 1959a and Morton and Shepley (1961, 1963).

Type II



Type I

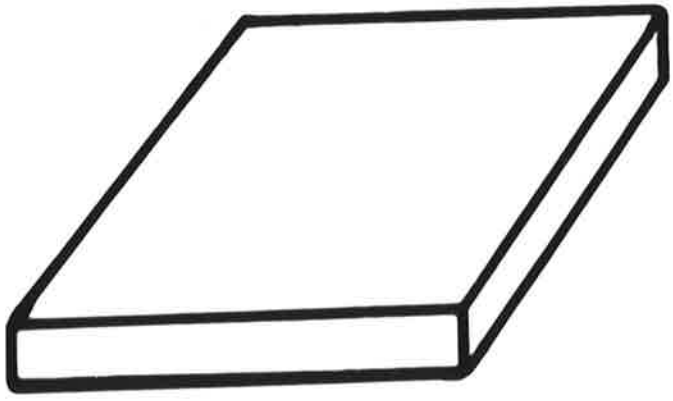


FIGURE 8: SECTION ACROSS THE C AXIS OF A CRYSTAL OF TYPE II CYTOCHROME b_2.

File No.3484. A suspension of small crystals of Type II cytochrome b_2 was fixed, embedded and sectioned as described in MATERIALS AND METHODS of this chapter. The sections were stained with 2% $KMnO_4$, pH 7.2.

The distortion of this pattern from a perfect hexagonal array indicated that the section angle was inclined at 56° to the C axis. After correcting for this distortion the side length of each small hexagon was calculated to be 79 A°.

Note: The average obtained from a total of four such estimations was 82 A°. Mag. x 200,000.

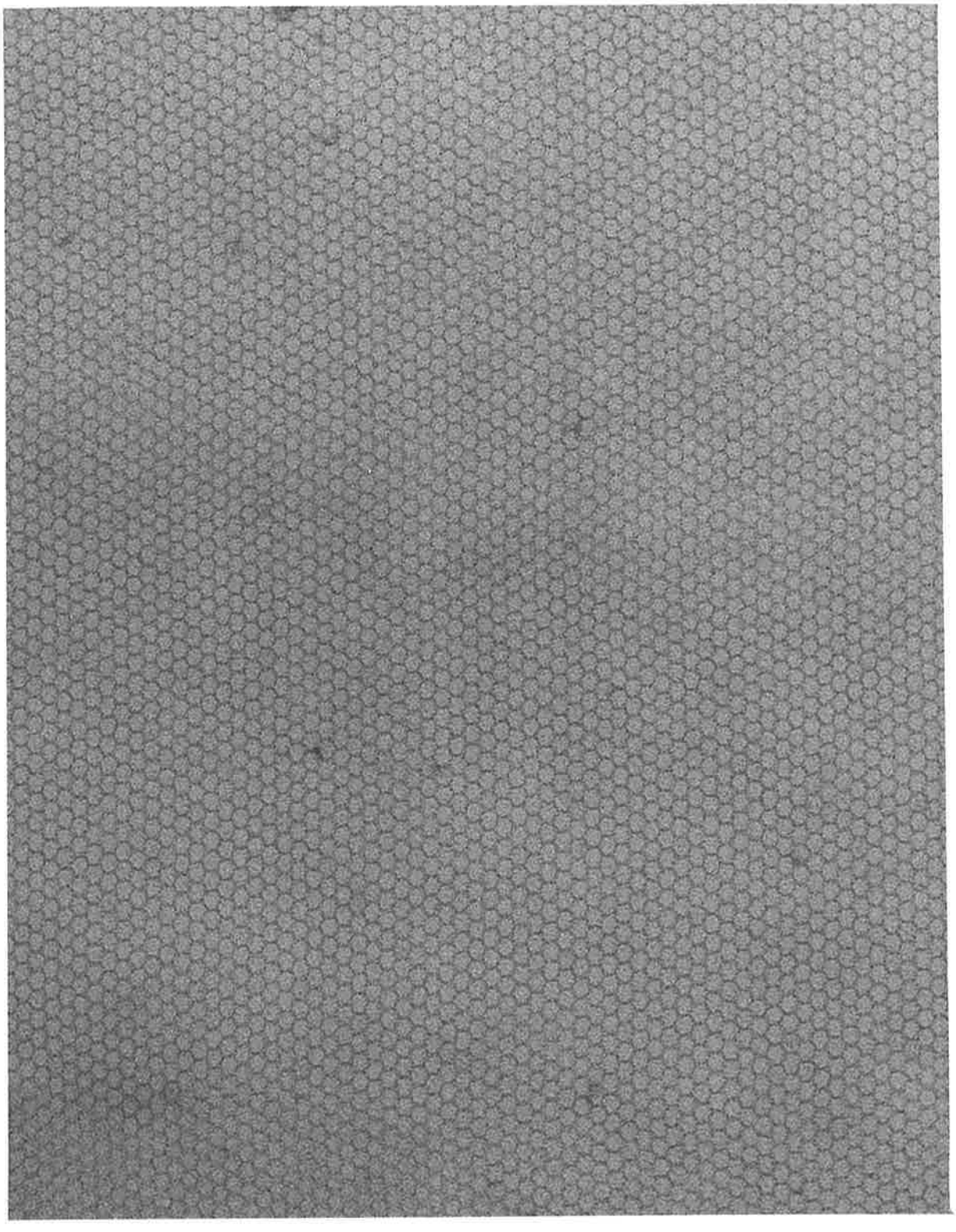


FIGURE 9: A SECTION PARALLEL TO THE C AXIS OF A CRYSTAL OF TYPE II CYTOCHROME b_2.

File No. 3489. Fixation, embedding, sectioning and staining with $KMnO_4$ as for FIGURE 8. This particular angle of section through the longitudinal, hexagonal tubes that made up the Type II crystal has given a spacing of 122 Å. Mag. x 60,000.

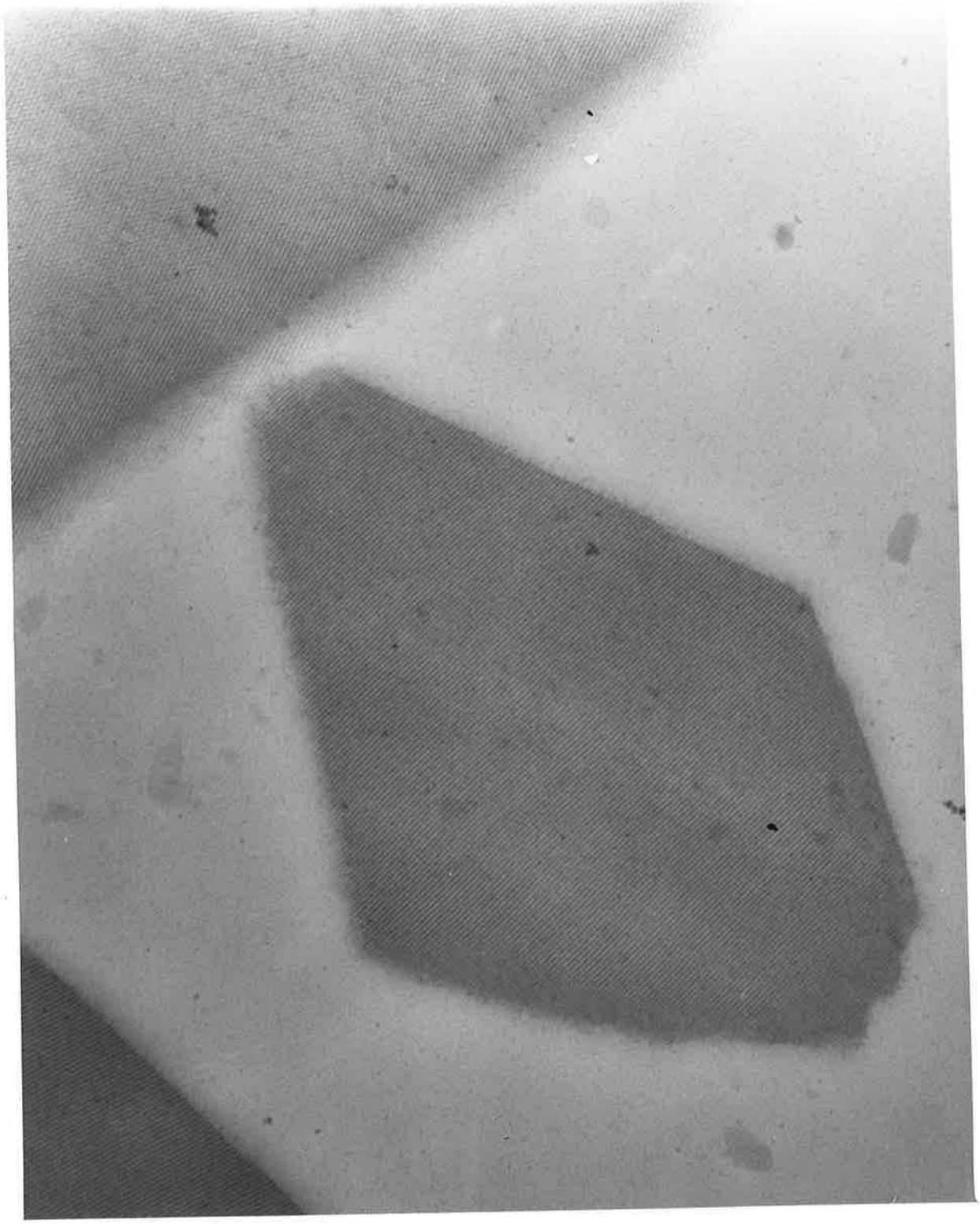
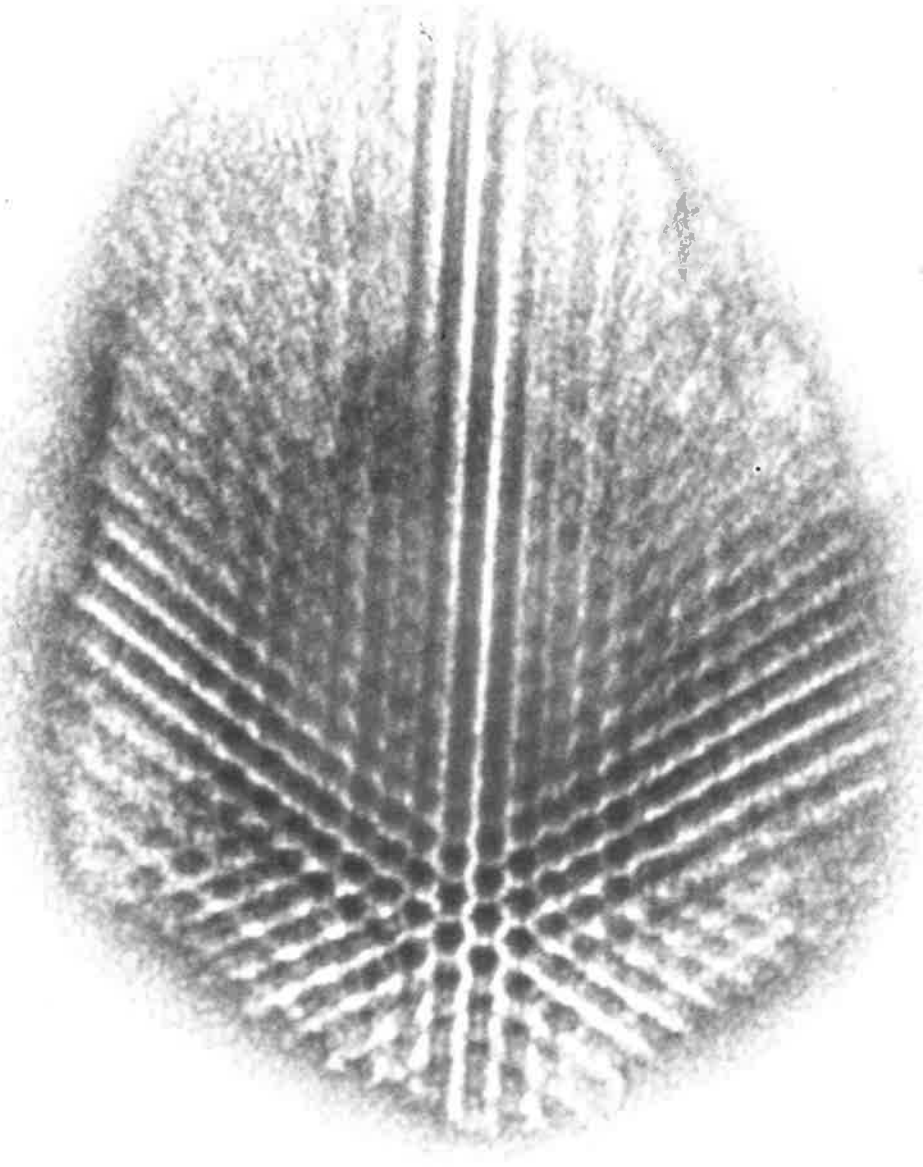


FIGURE 10: FRAGMENTS OF TYPE II CRYSTALS PRODUCED BY SONIC SHATTERING.

Small Type II crystals were produced and then sonicated as described in the MATERIALS AND METHODS of this chapter. The suspension of fragments was then spread on a carbon film and negatively stained with 2% uranyl acetate, pH 4.5.

A. (The plate facing the legend). File No.3246. This fragment shows both the hexagonal arrays, observed in sections across the C axis (cf. FIGURE 8) and the longitudinal array seen in section parallel to the C axis (cf. FIGURE 9). Mag. x 430,000.

B. (This is the second plate). Selections from File Nos. 3233, 3237, 3239 and 3249. Same material as in plate A (above). Mag. x 120,000.



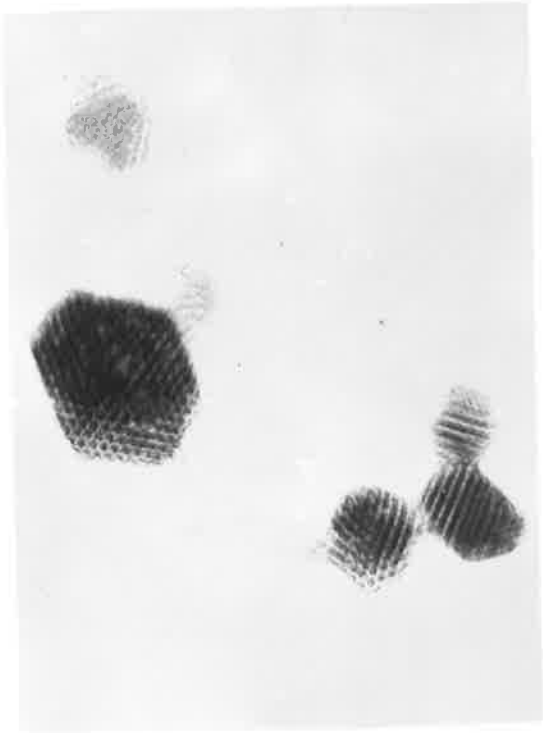


FIGURE 11: NEGATIVELY STAINED CRYSTALS OF TYPE I CYTOCHROME b_2.

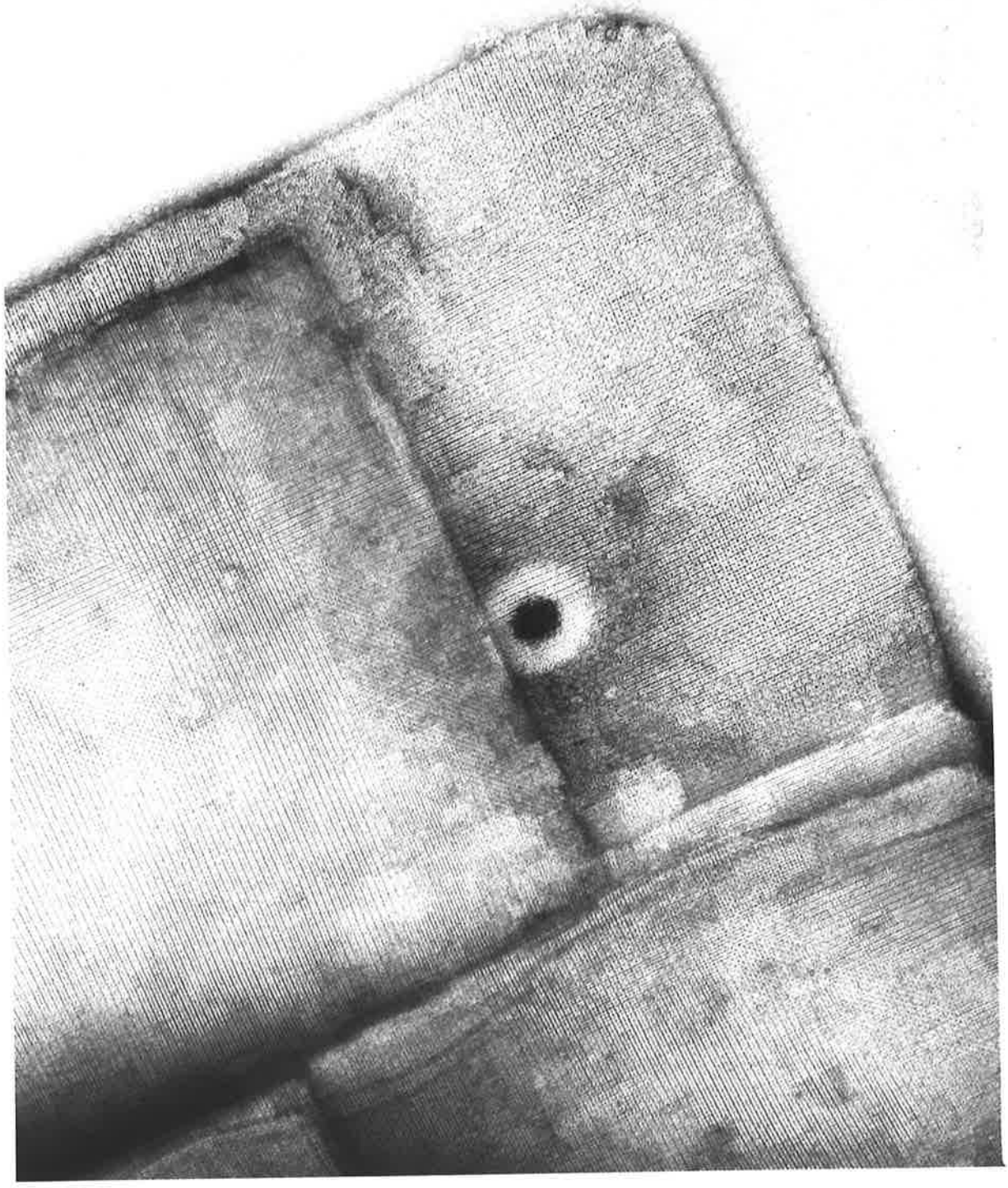
Small crystals of Type I cytochrome b_2 were deposited on a carbon film out of their mother liquor (Buffer 1) and negatively stained with 2% uranyl acetate, pH 4.5.

A. (The plate facing the legend). File No. 3114. Mag. x 133,000.

B. (This is the second plate). File No.3119. Mag. x 120,000.

The following can be observed:

(a) The layered structure of the crystal.
(b) Areas where a simple pattern of paired rows of protein molecule can be seen.
(c) Areas where the superimposition of sheets of protein molecules orientated at right angles to each other give the impression of close packed cubes with a hole in the centre.
(d) Some areas where very faint lines run at 45° to the major pattern of rows at right angles to each other. These lines may be an illusion and not represent rows of protein molecules orientated in this direction or they may have been caused by relatively rare sheets of protein molecules that orientated themselves at 45° to the usual two alternative directions. Mag. x 133,000.



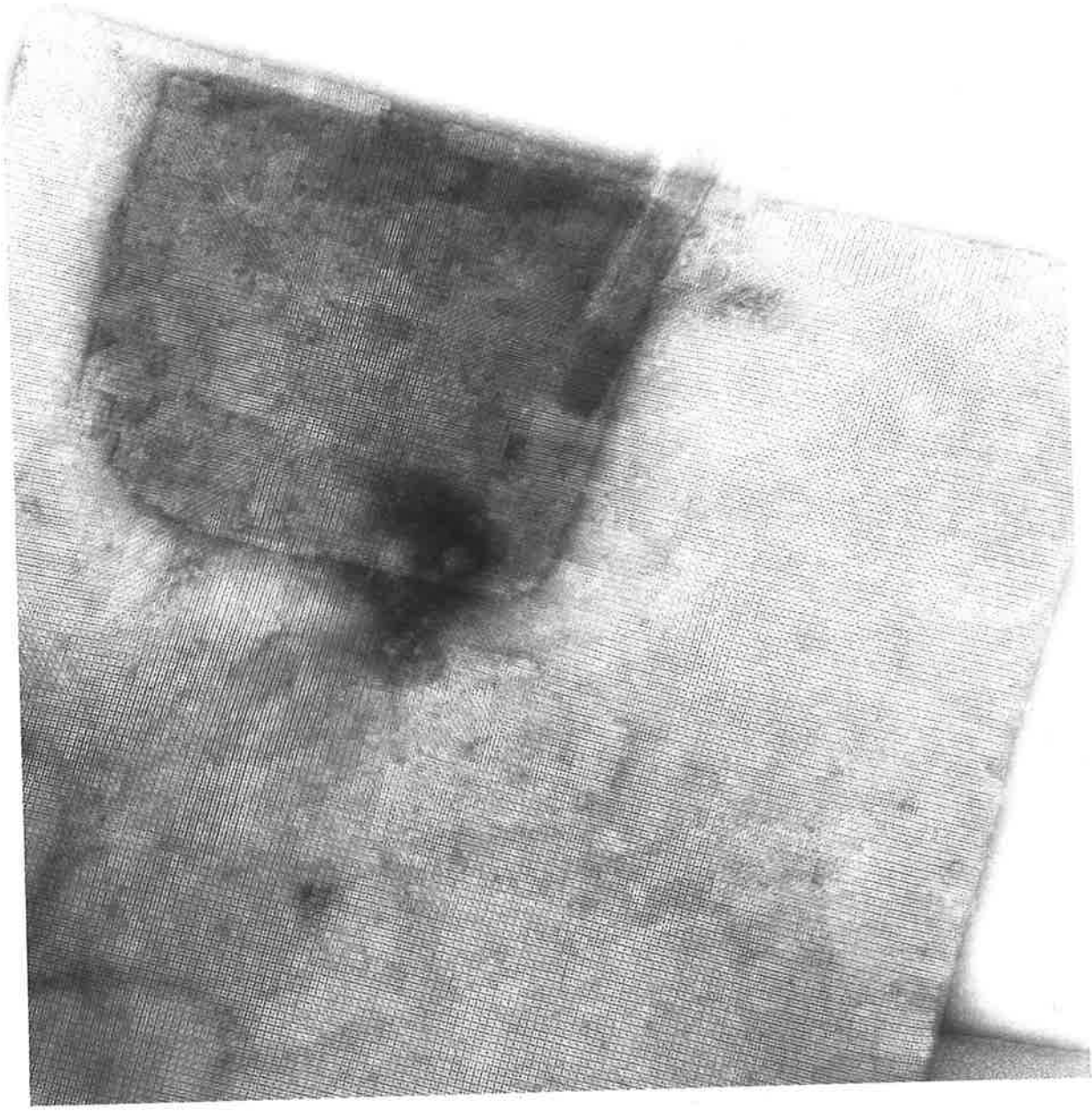


FIGURE 12: A MODEL SHOWING THE PROPOSED EFFECTIVE SHAPE OF THE CYTOCHROME b_2 PROTEIN MOLECULE.

This model has been constructed so as to be compatible with the following evidence:

(a) The hexagonal pattern of the Type II crystals has a side length of 82 A° and joins at 120 A° without noticeable joint imperfections.

(b) The repeat distance of the Type I crystal along the horizontol plane is 92 A°.

(c) The molecules look approx. 30 A° thick when observed in sections across the C axis of Type II crystals.

(d) The calculated molecular volume of the protein molecule is approx. 200,000 cubic A°.

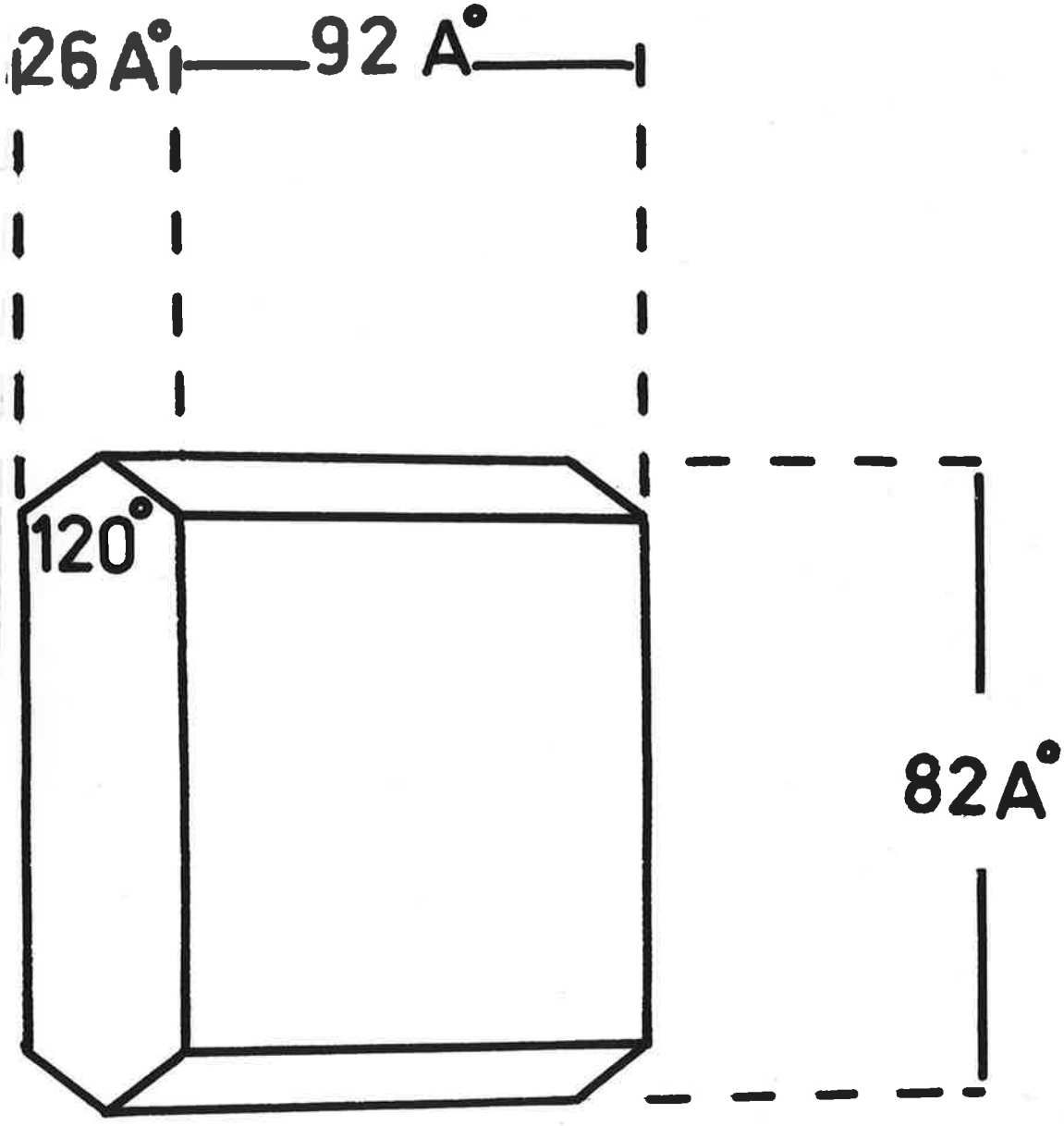
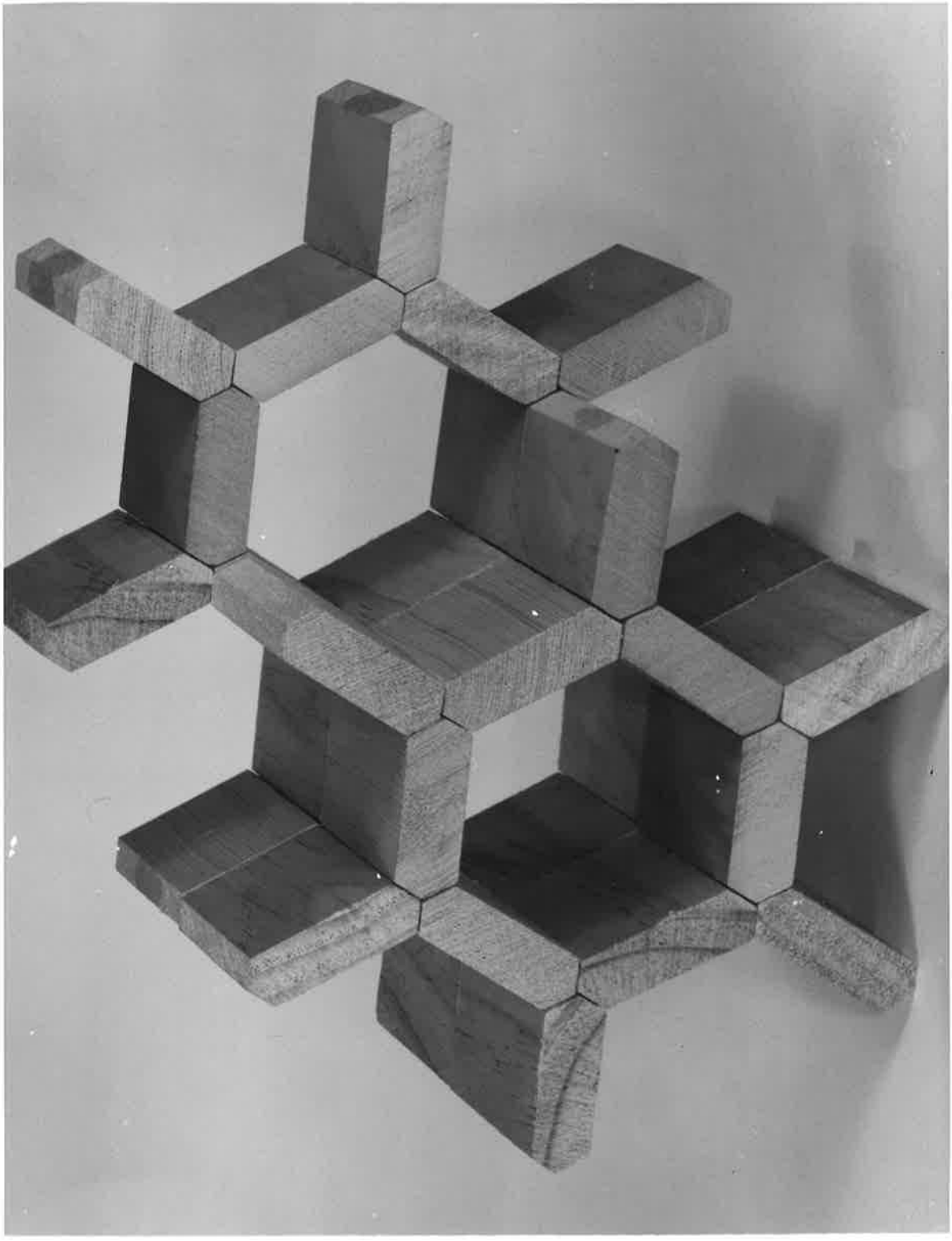

26 A°
92 A°
120°
82 A°

FIGURE 13: WOODEN BLOCK MODELS OF THE PROPOSED ARRANGEMENTS OF PROTEIN MOLECULES IN CRYSTALS OF TYPE II CYTOCHROME b_2.

The wooden blocks were made with the shape and relative dimensions of the proposed effective molecular shape (see FIGURE 12) and assembled in these models.

(A) (The plate facing the legend). A proposed structure for the crystal of Type II cytochrome b_2.

(B) (This is the second plate). The lower model represents a modification of the structure shown in (A) (above). This slightly different alternative model is considered in the DISCUSSION of this chapter. The upper model is provided as a comparison and represents a small piece of the same structure as in (A) (above).



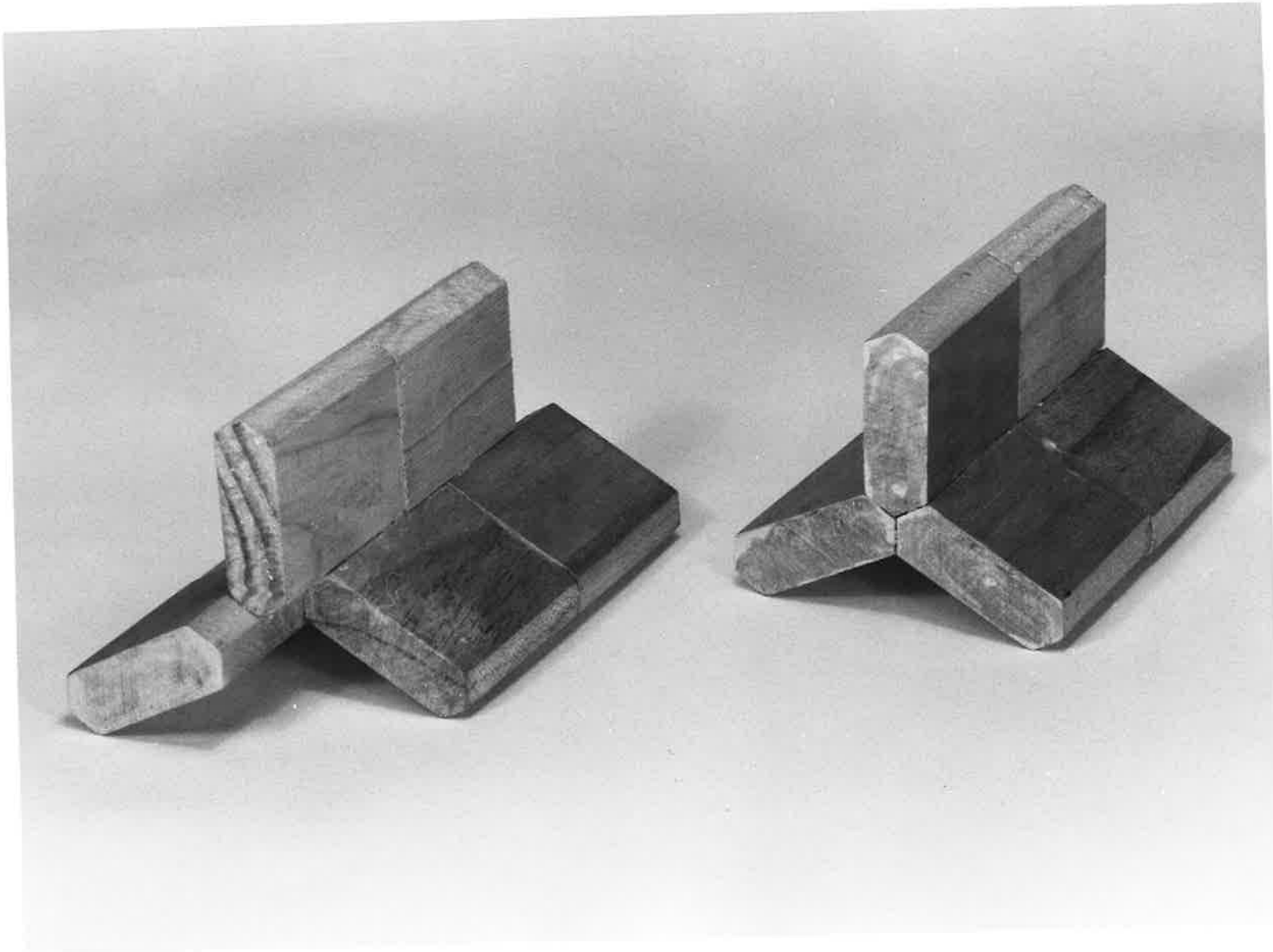
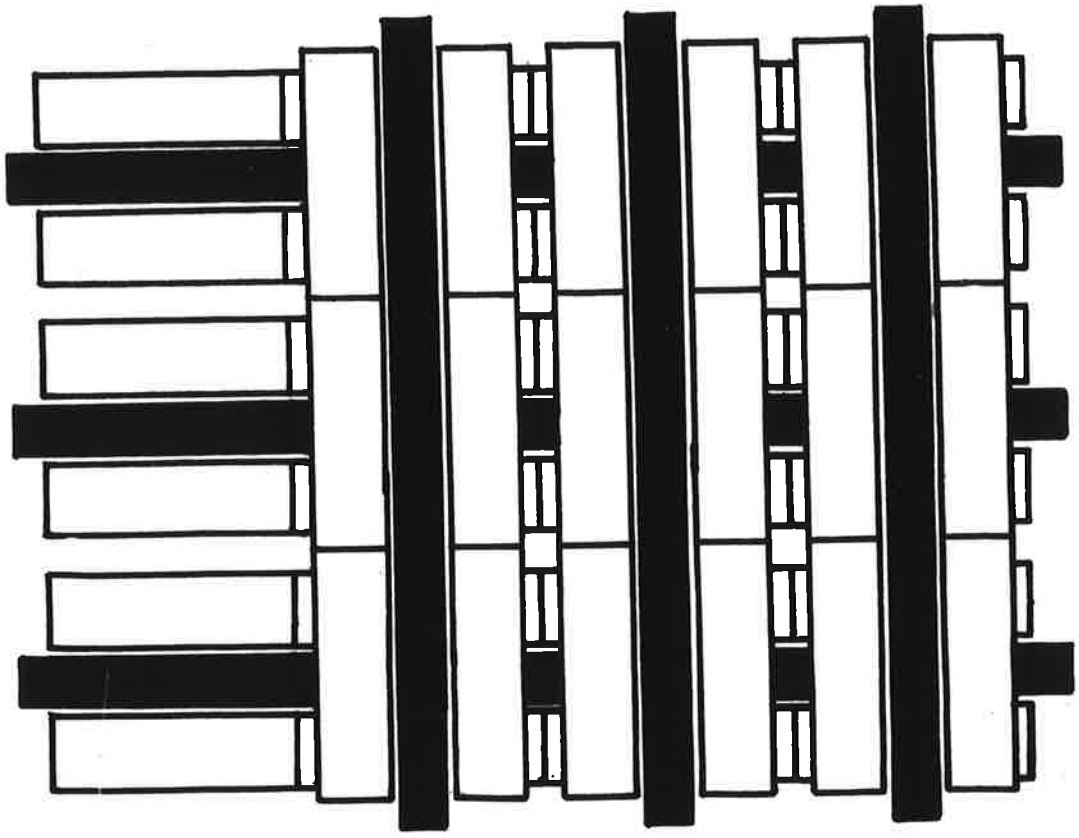


FIGURE 14: A MODEL OF THE ULTRASTRUCTURE OF CRYSTALLINE TYPE I CYTOCHROME b_2.

The filled (black) areas represent DNA and the open areas protein. The scale is approx. 3.5 cm. per 100 A°. The top view corresponds to the view of the structure seen in FIGURES 11A and B.

The rows of protein have been presumed to consist of molecules with a molecule-molecule junction every 92 A° but the relation of this junction to the rest of the structure cannot be seen and so the junctions have been arbitrarily placed under the narrow grooves.

TOP VIEW



SIDE VIEW

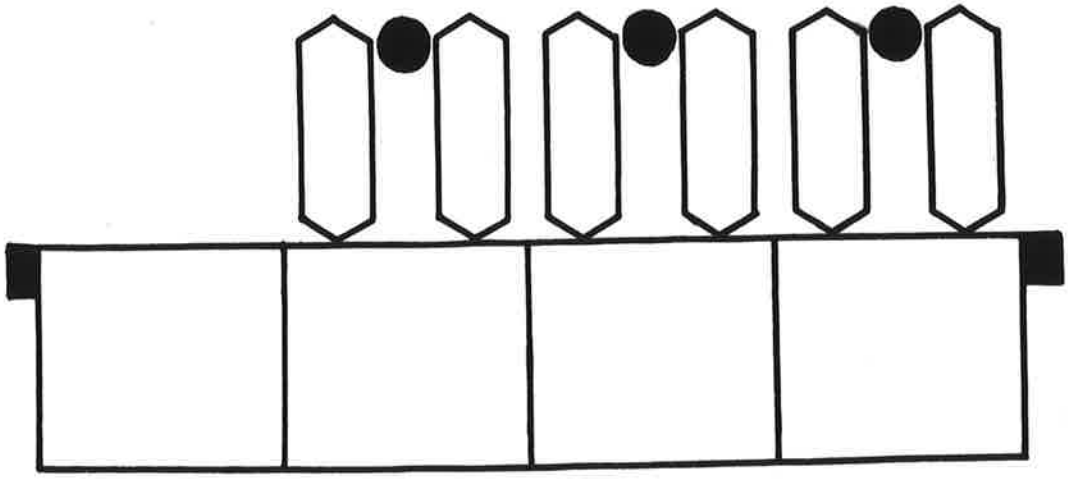
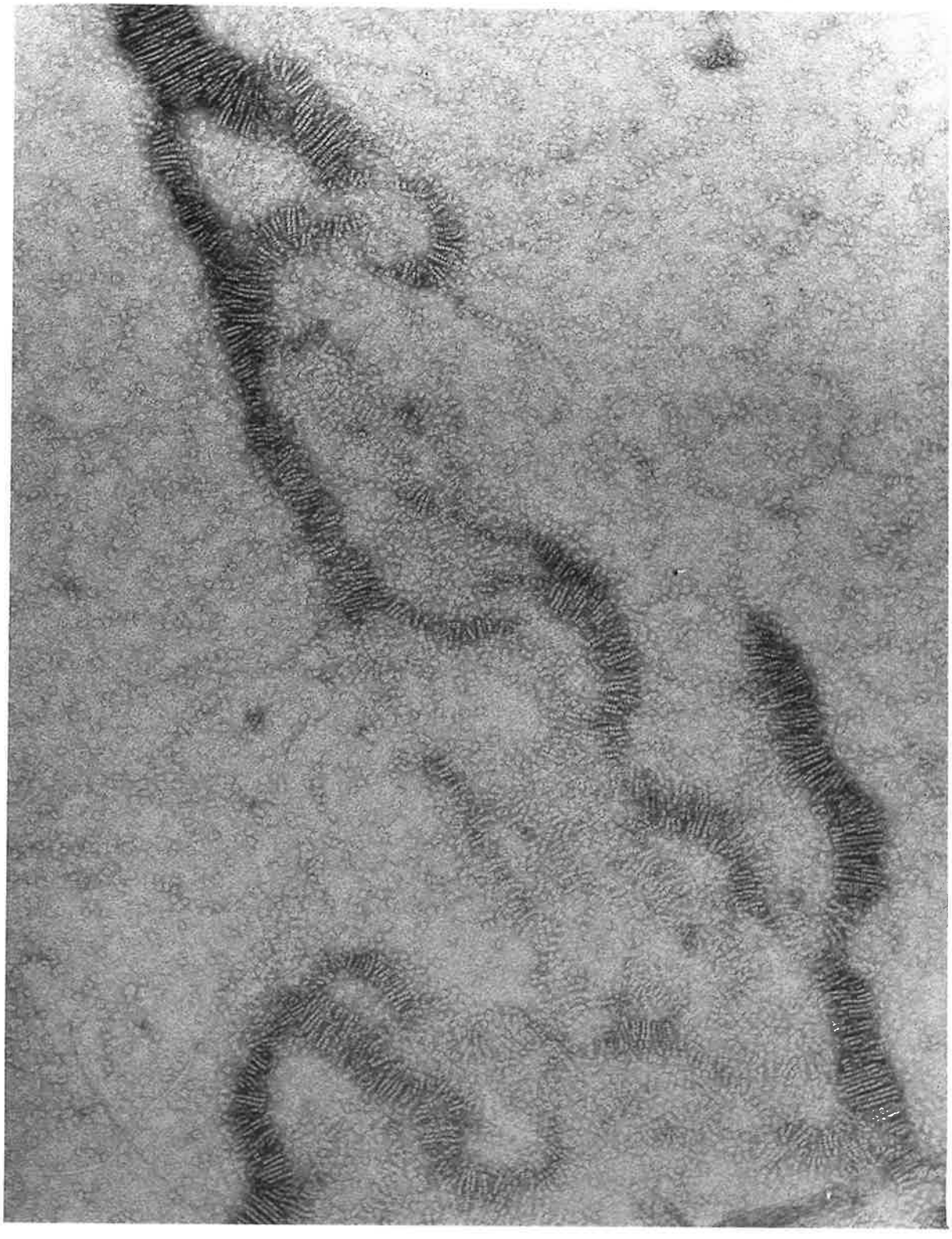


FIGURE 15: ORGANISATION OF CYTOCHROME b_2 PROTEIN MOLECULES BY SKEINS OF HIGH MOLECULAR WEIGHT DNA.

File No.3290. A large excess of calf thymus DNA was added to Type II cytochrome b_2 in Buffer 1. The development of a slight opalescence in the mixture indicated the formation of a complex. The mixture containing this complex was then deposited on a carbon film and negatively stained with 2% uranyl acetate, pH 4.5. Mag. x 120,000.



CHAPTER FIVE

A STUDY OF THE ORIGIN OF THE $\underline{b}_2$-DNA IN THE YEAST CELL BY AN ANALYSIS OF THE RNA SPECIES THAT ARE COMPLEMENTARY TO $\underline{b}_2$-DNA

A STUDY OF THE ORIGIN OF THE b_2-DNA IN THE YEAST CELL BY AN ANALYSIS OF THE RNA SPECIES THAT ARE COMPLEMENTARY TO b_2-DNA

INTRODUCTION

The currently accepted theories of DNA-primed RNA synthesis would require that the RNA produced on a template the same size as b_2-DNA would be the same size or smaller than the template. Thus, it was proposed to identify any RNA in yeast with base sequences complementary to b_2-DNA by hybridisation techniques and to study the size distribution of this RNA. The maximum size of RNA that is complementary to b_2-DNA would indicate the minimum size for b_2-DNA *in vivo*.

There are a number of techniques available for estimating the amount of RNA that has hybridised with DNA. However, the techniques based on immobilising DNA on agar columns or nitrocellulose filters (Bolton and McCarthy, 1962; Cowie and McCarthy, 1963; Gillespie and Spiegelman, 1965) or on selective adsorption by nitrocellulose filters (Nygaard and Hall, 1963) were unsuitable because of the low molecular weight of b_2-DNA (cf. Hayashi *et al.*, 1965; Gillespie and Spiegelman, 1965). The technique finally adopted was that of Robinson, Hsu, Fox and Weiss (1964) in which the DNA-RNA hybrids are precipitated by cold TCA after all the free RNA has

been digested by T_1 and pancreatic ribonucleases (EC 2.7.7.26. and EC 2.7.7.16. respectively). This ribonuclease mixture will digest single stranded RNA but not RNA that is in a double helical structure. Non-hybridised regions of partly hybridised RNA chains are destroyed by the RNAases so that falsely high estimates of the amount of RNA hybridised are not obtained. This method is probably suitable for detecting hybridisation involving RNA regions as small as 12 to 16 nucleotides long (Niyogi and Thomas, 1967).

MATERIALS AND METHODS

The preparation of yeast DNA is described in CHAPTER I. Calf thymus DNA was prepared by the method of Kay *et al.* (1952). When yeast DNA was required free of b_2-DNA or fragments in the size class of b_2-DNA, it was adsorbed onto DEAE-cellulose (chloride), the column was washed with 1.0M NaCl, 0.01M tris-HCl, pH 7.5, and then the DNA eluted with 0.3M KOH. This denatured DNA was transferred into the appropriate buffers by dialysis and throughout this section of the work it will be referred to as "b_2-DNA-free yeast DNA".

The column of cross-linked polyacrylamide used in this section was Biogel P-300 (50-150 mesh, from Calbiochem.) that has a molecular weight exclusion limit of approx. 300,000. It was allowed to imbibe buffer for 24 hr. before use. One standard column (27 cm. high, 1 cm. diam.) with a flow rate of approx. 3-4 ml. per hr. was used for all these studies. The buffer was 0.3M NaCl, 0.01M tris-HCl, pH 7.5.

(1) Preparation of radioactive yeast RNA.

Yeast strain 164 (see CHAPTER I) was grown aerobically to early stationary phase at 30° in a medium that contained 1% peptone, 1% sodium DL-lactate, 1% glucose, pH 4.5, and carrier-free [^{32}P] phosphate (usually 1 mCi per ml.). The cells were washed in a

solution of 0.5% KCl, 0.5% NaCl, freeze-dried and then disrupted in cold, dry, peroxide-free diethyl ether with glass beads in a Nossal shaker. The mixture of beads and cells in diethyl ether was shaken inside a sealed plastic tube that was fixed inside a steel shaking vial. The vial was shaken for 100 sec. with 4 pauses for cooling in dry ice. The suspension of cells and beads was centrifuged and the pellet dispersed in 0.4M NaCl, 0.01M tris-HCl, 1.0mM EDTA, 1% sodium dodecyl sulphate, pH 7.5, and then the suspension extracted by shaking with an equal volume of water-saturated phenol. After centrifugation, phenol was removed from the aqueous phase by shaking with ether. The residual ether was then removed with a stream of nitrogen and the solution used directly for centrifugation on a sucrose gradient.

When unfractionated RNA was required, the RNA was precipitated from the phenol extract with two vol. of ethanol, dissolved in a small vol. of 0.1M sodium EDTA, pH 7.0, and transferred into 0.2M NaCl, 0.01M tris-HCl, pH 7.5, on a column of Sephadex G-75. This procedure gave RNA free of low molecular weight phosphorus compounds and it contained no material that was hydrolysed by pancreatic DNAase (EC 3.1.4.6.) but not by the mixed RNAases. Inorganic polyphosphates appeared to be the only nuclease-resistant, TCA-insoluble, contaminant that

contained phosphorus. (For further information on yeast polyphosphates see Ebel et al., 1963.)

(2) Formation and estimation of DNA-RNA hybrids.

The method used was essentially that of Robinson et al. (1964). Nucleic acid samples dissolved in 0.63 ml. of 0.2M NaCl, 0.01M tris-HCl, pH 7.5, were sealed in glass tubes and annealed by placing them in a vacuum flask of water that was allowed to cool from 95° to 45° over 24 hr. The vacuum flask was kept in a 37° incubator during the cooling period so as to make the cooling curve as repeatable as possible. As it was quite possible that the nucleic acids involved in these experiments were highly heterogenous with different optimal annealing temperatures for each species, this continuous cooling method was used in preference to annealing at one fixed temperature. After the annealing the contents of the annealing tubes were transferred into digestion tubes with 0.2M NaCl, 0.01M tris-HCl pH 7.5 to give a volume of 0.9 ml. To the nucleic acid solution was then added 0.1 ml. of a solution containing 100 µg per ml. pancreatic RNAase and 1 µg per ml. T_1 RNAase in 0.05M $MgCl_2$, 0.01M tris-HCl, pH 7.5, and the mixture was incubated at 37° for one hr. The tubes were then cooled briefly in ice, 1.0 mg.

of carrier yeast RNA was added (in 0.05 ml.) and the nucleic acids precipitated with 0.1 ml. of a cold solution of TCA (final concentration 5%). The TCA precipitate was then filtered onto Oxoid discs with the aid of 5 ml. 5% TCA, washed with two lots of cold 5% TCA and then with 5 ml. of ethanol. It was necessary to carry out the ethanol wash cautiously to avoid detaching the RNA precipitate from the membrane. The membranes were then counted in a Packard scintillation spectrometer using the liquid phosphor described in CHAPTER I.

(3) Sucrose gradients.

Gradients of 3%-20% (w/v) sucrose in a solution of 0.2M NaCl, 0.01M tris-HCl, pH 7.5, were made by means of a two-reservoir mixer in Spinco SW-25 cellulose nitrate tubes. Centrifugation was carried out at 8° in the SW-25 rotor of the Beckman L2 ultracentrifuge at 25000 revs./min. for 19 hr.

RESULTS

A. DETECTION OF DNA-RNA HYBRIDS

The first experiment, described below, demonstrated that under the conditions stated in MATERIALS AND METHODS, yeast RNA did hybridise with yeast DNA. Initially, the main difficulty was material in the $[^{32}P]$ RNA, later

identified as TCA-insoluble polyphosphates, which caused a high background after the RNAase digestion. This RNAase resistant material was found to have a relatively low molecular weight as it could readily enter grains of Biogel P-300. Thus, DNA samples were annealed with yeast RNA and the mixture digested with RNAases, as described in MATERIALS AND METHODS, and the digest passed down the standard gel column (see FIGURE 16). The distribution of TCA-insoluble [^{32}P] in the fractions from the column was then compared to the elution pattern of the DNA alone as followed by optical methods. When yeast RNA had been annealed with $\underline{b}_2$-DNA or $\underline{b}_2$-DNA-free yeast RNA, a [^{32}P] containing peak was found in the elution pattern coinciding with the position of the corresponding DNA (FIGURE 16). However, when yeast RNA was annealed with calf thymus DNA no such radioactive peak was obtained. The relatively large amounts of TCA-insoluble polyphosphates, that had previously caused large backgrounds, penetrated the gel grains and were eluted in a larger volume than were the DNA-RNA hybrids. In the experiment, shown in FIGURE 16, it was apparent that much of the TCA-insoluble polyphosphate had been rendered TCA-soluble by the annealing procedure although polyphosphates are relatively stable under the conditions of annealing. The stability of the polyphosphates in

the 0.2M NaCl, 0.01M tris-HCl, pH 7.5, buffer used for annealing was studied briefly, and they were found to be rendered TCA-soluble with a half life of 20 min. at 100°. Thus, in subsequent experiments where yeast RNA containing these polyphosphates was used, the samples were kept in a boiling water bath for one hour before the annealing was carried out. The polyphosphates left after one hour at 100° were then almost completely destroyed by the prolonged high temperatures of the annealing procedure.

Polyphosphates were not present in yeast RNA derived from the stationary phase yeast cultures, under the conditions of culture used here, so RNA from stationary phase cells was used for all subsequent work unless otherwise stated.

B. EFFECT OF DNA-RNA RATIO AND THE NUCLEIC ACID CONCENTRATION ON THE EFFICIENCY OF HYBRIDISATION

Any particular mixture of DNA and RNA can be described in terms of two independent variables, e.g., the DNA:RNA ratio and the concentration of either the DNA or RNA. A satisfactory description of the hybridisation behaviour of two nucleic acids would thus require a study of how the amount of hybridisation varied with the DNA (or RNA) concentration carried out at a large number of DNA:RNA ratios. However, not only was this impractical

in this case but, as the nucleic acids were highly heterogenous, a detailed interpretation of the results would not be possible. Thus, no such broad and detailed investigation was attempted with either the crude nucleic acids or with yeast RNA that had been fractionated into various size classes. However, to obtain some understanding of how the hybridisation behaviour of yeast RNA and $\underline{b}_2$-DNA varied with DNA:RNA ratio, two experiments were carried out.

In the first of these a fixed amount of unfractionated yeast RNA was annealed with increasing amounts of yeast DNA, and in the second a mixture with a fixed ratio of DNA:RNA was annealed at a series of different DNA (or RNA) concentrations.

0.1 µg. of unfractionated, stationary phase yeast RNA (32,000 CPM. per µg.) was annealed with increasing amounts of $\underline{b}_2$-DNA and $\underline{b}_2$-DNA-free yeast DNA. Both DNA samples gave a sharp rise in the amount of RNA annealed up to a DNA:RNA ratio of approx. 100:1 but after this the slope of the curves began to rapidly decrease (see FIGURE 17a). This type of curve seems to be generally representative of the way RNA hybridises with double stranded DNA because others have obtained similar relationships with different systems (e.g. Colvill *et al.*, 1965; Armstrong and Boezi, 1965). The important implication of the shape of this curve is that when a large amount of heterogenous

DNA is annealed with a small amount of heterogenous RNA, relatively rare DNA species in the DNA molecule population would form a much greater proportion of the hybrids than would be expected on the basis of hybridisation being simply proportional to the relative frequency of any given DNA species. Thus, any DNA contaminant in a DNA could form a disproportionately large proportion of the hybrids.

In the next experiment, one mixture of [^{32}P] RNA and $\underline{b}_2$-DNA (1 µg. RNA per 200 µg. DNA) was annealed at a range of different concentrations and the proportion of RNA hybridised plotted against the DNA concentration in FIGURE 17b.

Under the particular conditions of this experiment the per cent of RNA hybridised was independent of the nucleic acid concentrations between 30 and 130 µg. DNA per ml.

C. THE SIZE DISTRIBUTION OF YEAST RNA THAT IS HOMOLOGOUS TO $\underline{b}_2$-DNA

Approximately 70 µg. of freshly prepared, stationary phase yeast [^{32}P] RNA was loaded onto a sucrose gradient in a volume of 1.5 ml. (see MATERIALS AND METHODS for the details of the sucrose gradient). This was then centrifuged at 25,000 revs./min. at 8° for 19 hr. and 14 drop fractions collected. The distribution of TCA-insoluble

[^{32}P] throughout these fractions is shown in FIGURE 18. In this particular experiment there were neglible amounts of TCA-insoluble polyphosphates or other ribonuclease resistant material detected.

The RNA was resolved into three main classes; 28S, 19S and 4S (the S values are those of Maeda, 1960). Other studies of yeast RNA which have given a similar distribution have been published by Stahl *et al.* (1964) and Weill *et al.* (1964). Twelve RNA fractions from this gradient were selected for examination of their ability to hybridise with b_2-DNA. 0.2 µg. of RNA was taken from each of the twelve selected fractions and annealed with 40 µg. of b_2-DNA. The percentage (with respect to the original 0.2 µg. RNA) of RNA hybridised was then plotted against the position of the RNA sample in the original sucrose gradient (see FIGURE 18). As can be seen in FIGURE 18, yeast RNA of very high sedimentation coefficient (in the region of 28S) hybridised with b_2-DNA at least as readily as yeast RNA with sedimentation coefficients of 6S or less. This complete experiment was repeated with RNA from yeast at a slightly earlier growth stage and the results of this second experiment agreed with the results of the first in all major respects. However, as the second experiment used yeast at an earlier growth stage, the

TCA-insoluble polyphosphates were hydrolysed by heating the RNA-$\underline{b}_2$-DNA mixtures at 100° for one hour before the annealing (see MATERIALS AND METHODS).

As will be discussed later, this result is incompatible with $\underline{b}_2$-DNA having the same molecular weight *in vivo* as Armstrong *et al.* (1959b) showed it to have *in vitro* (1.2×10^5). However, from the high ratio of $\underline{b}_2$-DNA:RNA (200:1) used in the two experiments of the type shown in FIGURE 18, and knowing the approximate relationship between hybridisation and DNA levels (FIGURE 17a), it must be concluded that trace DNA contaminants in the $\underline{b}_2$-DNA could be responsible for a disproportionately large amount of the observed hybridisation. To counter this criticism, $\underline{b}_2$-DNA was saturated with the 28S peak RNA and the saturation level compared to that for unfractionated yeast DNA and $\underline{b}_2$-DNA-free yeast DNA. The results for this experiment are shown in TABLE 9. The experimental variation is considerable so that it cannot objectively be stated that unfractionated yeast DNA or $\underline{b}_2$-DNA-free yeast DNA had a different saturation level to $\underline{b}_2$-DNA. However, it is obvious that $\underline{b}_2$-DNA was saturated by 28S RNA at a level which was in the same order as that for the other two DNA samples. Thus, the ability of $\underline{b}_2$-DNA to hybridise with 28S RNA is not due to *minor* contamination with other yeast DNA.

DISCUSSION

It has been shown that over 30% of whole, stationary phase yeast RNA could be hybridised with $\underline{b}_2$-DNA-free-yeast DNA. While it could be argued that $\underline{b}_2$-DNA is a very active DNA *in vivo*, these results were also compatible with $\underline{b}_2$-DNA being a sample (albeit slightly non-random) of the bulk yeast DNA. However, the observation that $\underline{b}_2$-DNA hybridised as readily with 28S RNA as it did with RNA of 6S or less, indicated that $\underline{b}_2$-DNA had a molecular weight of at least 1×10^6 *in vivo*. If the current theories of DNA-primed RNA synthesis are accepted, then for $\underline{b}_2$-DNA to have the same molecular weight *in vivo* as it had in the preparations studied *in vitro* (1.2×10^5; $S^{o}_{20,w} = 5.8$ Armstrong *et al.*, 1959b), the RNA that it coded for should have a molecular weight similar to, or less than, that of the $\underline{b}_2$-DNA that was its template. This would require RNA complementary to $\underline{b}_2$-DNA to have a sedimentation coefficient equal to, or less than about 6S. (The 6S is based on the molecular weight of 1.2×10^5 and the relationship of Fresco and Doty (1957) for random coil poly A). In fact, the RNA which hybridised with $\underline{b}_2$-DNA was found to have sedimentation coefficients over the whole range up to 28S and the experiment summarised in TABLE 9 showed that this result could not be explained by postulating a trace contaminant of

other DNA in the $\underline{b}_2$-DNA.

Thus, collectively, this evidence argues for $\underline{b}_2$-DNA being a degradation product of higher molecular weight DNA and this conclusion was strongly supported by the results presented in CHAPTER II which showed that $\underline{b}_2$-DNA is not produced by a yeast cell unless it is aged or air dried.

These conclusions caused attempts to fractionate organelles and study their content of RNA complementary to $\underline{b}_2$-DNA to be abandoned.

TABLE 9: SATURATION OF DNA SAMPLES BY ANNEALING WITH 28S YEAST RNA.

The 28S yeast RNA was prepared from stationary phase yeast RNA that had been fractionated on a sucrose gradient as described in the MATERIALS AND METHODS and RESULTS (see FIGURE 18) of this chapter.

The annealing is as described in MATERIALS AND METHODS but it was necessary to wash the TCA-insoluble precipitates thoroughly with 10% TCA containing an unlabelled enzymic hydrolysate of RNA in order to reduce the background radioactivity to a low level.

Saturation curves for each DNA sample were plotted by annealing a constant amount of DNA with increasing amounts of labelled RNA until a plateau level of annealed RNA was reached. After one curve had been plotted for a given sample, replicate estimations were made from single points known to be in the plateau region.

Expt. No.	% of total DNA saturated by annealing with 28S RNA		
	Unfractionated yeast DNA	b_2-DNA-free yeast DNA	b_2-DNA
1	-	1.1	0.53
2	0.60	0.62	0.26
3	0.54	0.78	0.27

FIGURE 16: USE OF A GEL FILTRATION COLUMN TO DETECT DNA-RNA HYBRIDS FORMED BETWEEN WHOLE YEAST RNA AND b_2-DNA OR b_2-DNA-FREE YEAST DNA.

The annealing procedure and the preparation of all nucleic acid samples is described in the MATERIALS AND METHODS of this section or in CHAPTER I. All mixtures to be annealed were heated in a boiling water bath for 10 min. before being placed in the annealing flask.

The lower graph shows the elution pattern of b_2-DNA and b_2-DNA-free yeast DNA followed optically at 260 mμ.

The vertical dashed line indicates the void volume of the column as indicated by the elution volume of calf thymus DNA. The elution pattern of b_2-DNA is indicated by the filled circles. The elution pattern of the b_2-DNA-free yeast DNA is indicated by the crosses.

The upper graph shows the distribution of TCA-insoluble radioactive material after annealed mixtures were digested with ribonucleases.

The filled circles show the radioactivity distribution after 70 μg. b_2-DNA was annealed with 3.5 μg. [^{32}P] RNA (133,000 CPM./μg.). The crosses show the radioactivity distribution after 70 μg. b_2-DNA-free yeast DNA was annealed with 3.5 μg. [^{32}P] RNA (120,000 CPM./μg.). The open circles show the radioactivity distribution after 70 μg. of calf thymus DNA was annealed with 3.5 μg. [^{32}P] RNA (105,000 CPM./μg.). The closed triangles show the radioactivity distribution after 3.5 μg. of [^{32}P] RNA was digested, as usual, with the ribonucleases mixture. This sample had not been heated in any way prior to the digestion.

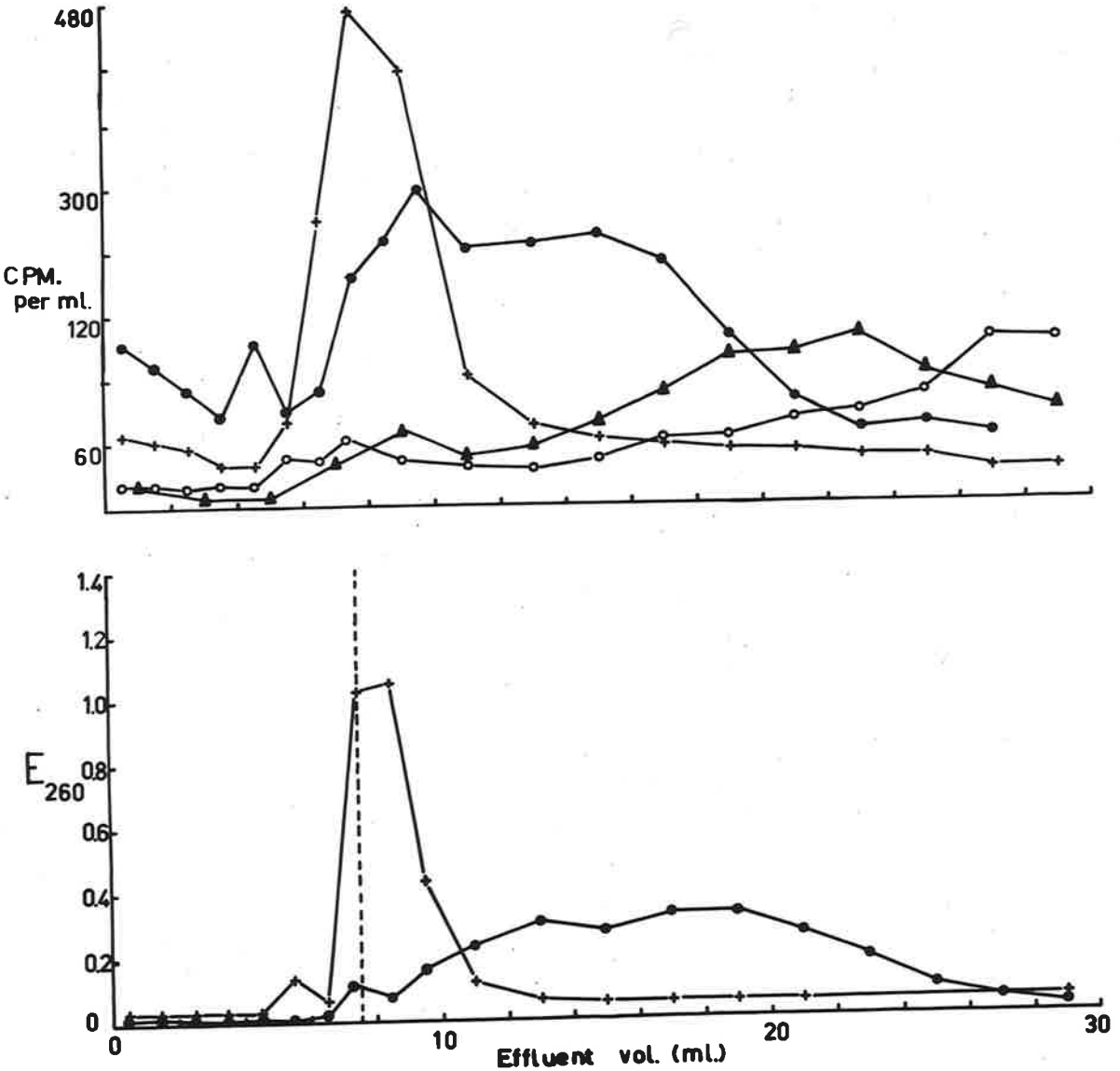

480
300
120
60
CPM.
per ml.
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
E_{260}
0
10
20
30
Effluent vol. (ml.)

FIGURE 17: A SHORT STUDY OF THE EFFECT OF DNA:RNA RATIO AND THE EFFECT OF NUCLEIC ACID CONCENTRATION ON THE % RNA INCORPORATED INTO DNA-RNA HYBRIDS.

The preparation of $\underline{b}_2$-DNA is described in CHAPTER I. The preparation of $\underline{b}_2$-DNA-free yeast DNA and [^{32}P] RNA from yeast is described in the MATERIALS AND METHODS of this section.

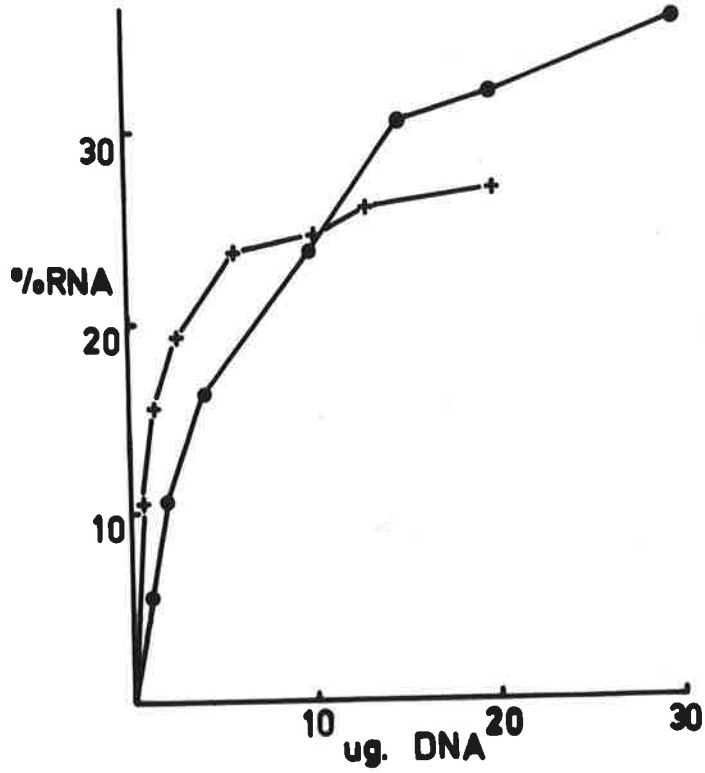
(a) The upper graph: Unfractionated stationary phase yeast DNA and [^{32}P] RNA from yeast (0.01 µg.) was annealed with varying amounts of $\underline{b}_2$-DNA and $\underline{b}_2$-DNA-free yeast DNA. The annealing conditions and method of estimation of the amount of RNA hybridised are described in the MATERIALS AND METHODS of this section (RNA spec. act. 32,000 CPM per µg.)

The filled circles show the way the % RNA hybridised changes with the level of $\underline{b}_2$-DNA.

The crosses show the way the % RNA hybridised changes with the level of $\underline{b}_2$-DNA-free yeast DNA.

(b) The lower graph: 1.0 µg. of unfractionated, stationary phase yeast, [^{32}P] RNA (spec. act. 184,000 CPM per µg.) was added to 200 µg. of $\underline{b}_2$-DNA. This nucleic acid solution was then diluted to the various stated nucleic acid concentrations.

The graph shows the amount of RNA hybridised expressed as a percentage of the RNA originally in the 0.63 ml. samples, plotted against the concentration of DNA in the 0.63 ml. sample. Note that as the RNA:DNA is constant any particular DNA concentration corresponds to an RNA concentration 1/200 of the stated DNA concentration.


30
%RNA
20
10
10
20
30
ug. DNA

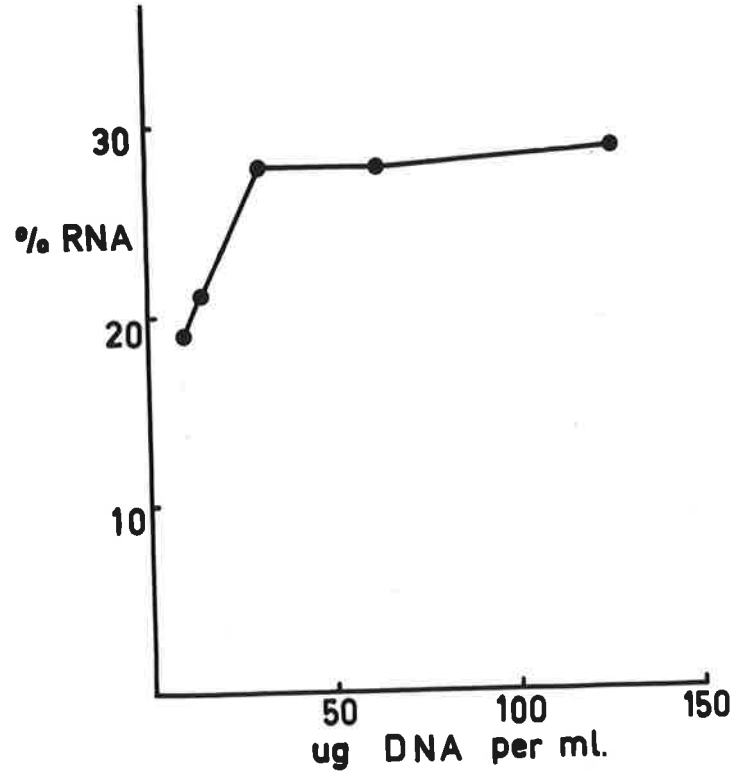

30
% RNA
20
10
50
100
150
ug DNA per ml.

FIGURE 18: THE RELATION BETWEEN THE SEDIMENTATION COEFFICIENTS OF YEAST RNA AND THE % OF THE RNA THAT HYBRIDISED WITH b_2-DNA.

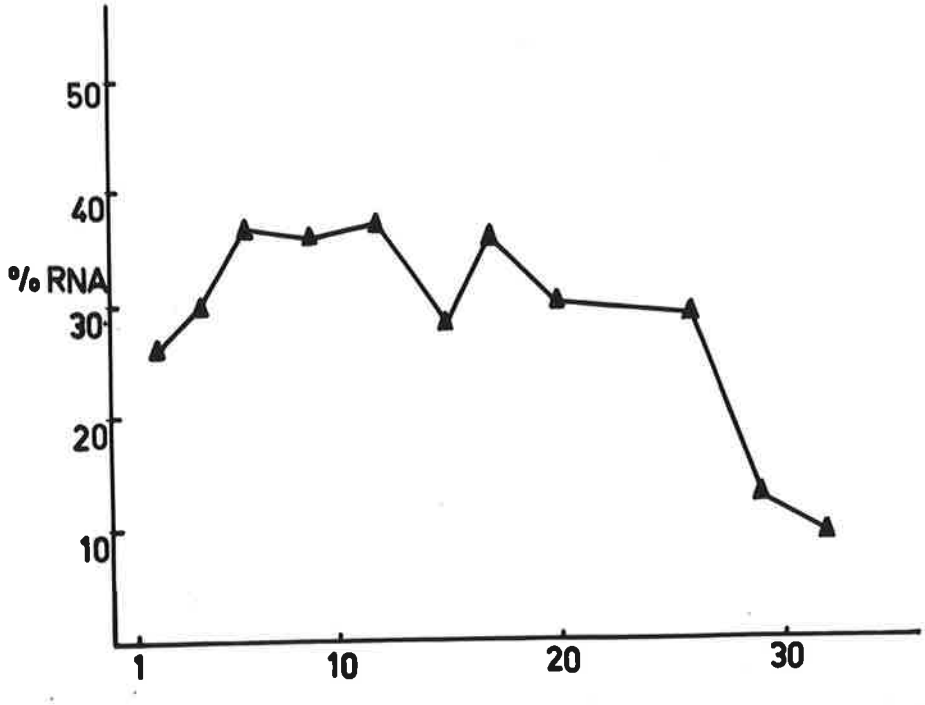
The following preparations or procedures are described in the MATERIALS AND METHODS of this section: Stationary phase yeast [^{32}P] RNA (Spec. act. of 6.5 x 10^5 CPM per µg.), the 3% - 20% sucrose gradient in the SW-25 tubes of the Spinco L ultracentrifuge, the annealing procedure and the method for estimation of the degree of hybridisation. The preparation of b_2-DNA is described in CHAPTER I.

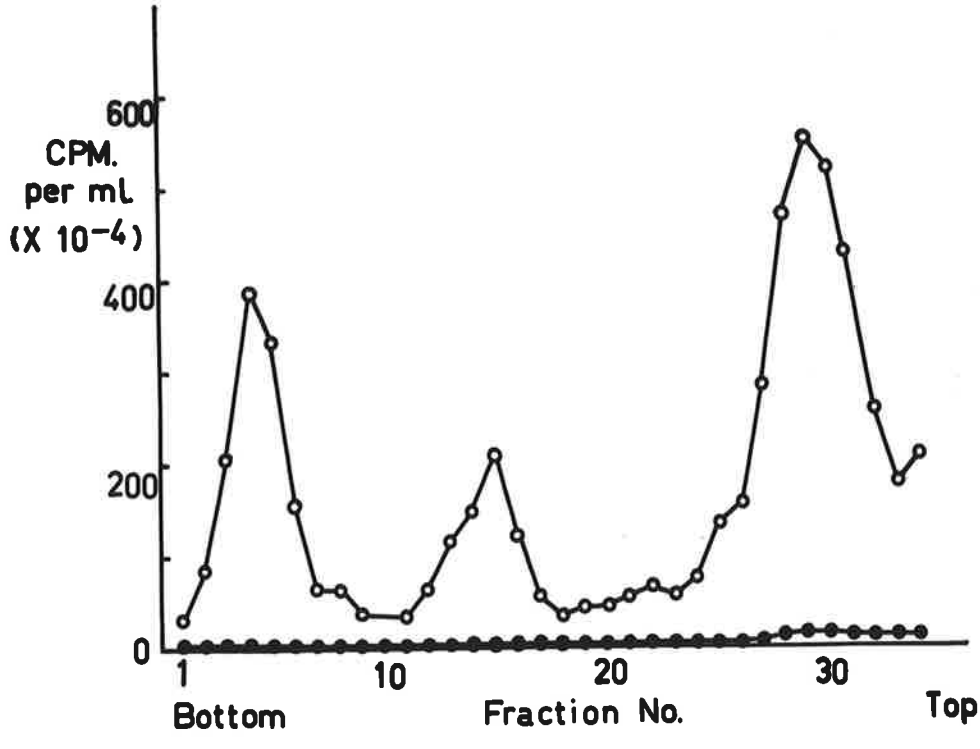
The sucrose gradient was loaded with 70 µg. of [^{32}P] RNA and centrifuged at 25,000 revs./min. for 19 hr. at 8°. The volume of the gradient plus load was 31 ml. and 34 fractions were collected from it each containing 14 drops.

The lower graph: The open circles represent the distribution of 5% TCA-insoluble [^{32}P] down the sucrose gradient.

The closed circles represent the distribution of RNAases resistant, 5% TCA-insoluble [^{32}P] down the gradient. As the value of this never rose far above zero it was concluded that RNA was virtually the only material present in this gradient that contained [^{32}P] and was insoluble in 5% TCA.

The upper graph: 0.2 µg. samples of the RNA from selected fractions of the sucrose gradient were each annealed with 40 µg. of b_2-DNA and the amount of RNA hybridised estimated. The values obtained were then expressed as a percentage of the original 0.2 µg. and plotted (closed triangles) against the fraction number.


50
40
% RNA
30
20
10
1
10
20
30


600
CPM.
per mL
(X 10⁻⁴)
400
200
0
1
10
20
30
Bottom
Fraction No.
Top

CHAPTER SIX

YEAST DNA-DEPENDENT RNA POLYMERASES

YEAST DNA-DEPENDENT RNA POLYMERASES

INTRODUCTION

The work described in this chapter was begun when the author still believed that $\underline{b}_2$-DNA was possibly a cytoplasmic DNA that functioned outside of the nucleus. It was hoped also that a study of yeast DNA-dependent RNA polymerases would provide another approach to the question of organelle autonomy. However, even after it became known that $\underline{b}_2$-DNA was a preparation artifact the primary aim of studying organelle autonomy remained and consequently this work was continued.

The long term aim of the work described in this chapter was to identify all the different types (assuming there is more than one type) of DNA-dependent RNA polymerase and then identify the intracellular site where each type of polymerase is localised.

It was predicted that the main difficulty of this programme of work would be the artificial associations and rearrangements that occur after cell disruption. This meant that before polymerase activity that was detected in any particular organelle preparation could be claimed to be specific for that organelle there would have to be independent evidence to show that this

was not a contaminant from another part of the cell. One suitable approach was to study the properties of RNA polymerases (e.g. kinetic, electrophoretic, and response to inhibitors) in two organelles such as the nucleus and mitochondria and look for differences indicating that the organelles have two different polymerases. Even this approach would have to establish that any differences observed were not just due to a different environment provided by the two organelles, or due to changes that have been induced in the polymerases by the different preparation procedures. Changes of this latter type have been observed in DNA-dependent RNA polymerases by Fuchs, Zillig, Hofschneider and Preuss (1964).

The work presented in this chapter is a description of a study that was intended to be the first part of an approach to the problem of demonstrating the localisation of yeast DNA-dependent RNA polymerases.

MATERIALS AND METHODS

(1) Assay buffers. The sodium-based buffer consisted of 0.1M NaCl, 0.01M tris-HCl, 4mM $MgCl_2$, 0.1mM EDTA, 10mM 2-mercaptoethanol, pH 7.4. The potassium-based buffer consisted of 0.1M KCl, 0.01M tris-HCl, 4mM $MgCl_2$, 0.1mM EDTA, 10mM 2-mercapto-ethanol, pH 7.4.

(2) Assay method. Each assay tube had four solutions added to it in the following sequence:

(a) 0.025 ml. of either assay buffer containing 25 mμ mole each of GTP, CTP, and ATP, 2.5 mμ mole UTP (α-[^{32}P] labelled) 0.2 μ mole $MnSO_4$ and 0.4 μ mole spermidine chloride. In the absence of PEP and pyruvate kinase this solution was made up in the sodium based assay buffer. When PEP (125 mμ mole) and pyruvate kinase (5 μg.) were incorporated into this solution the solution was made up in the potassium based assay buffer.

(b) 0.025 ml. of either assay buffer containing 5 μg. calf thymus DNA. This solution was always made up in the same assay buffer as solution (a) above.

(c) 0.01 ml. of water containing potential effectors of various types.

(d) 0.05 ml. of yeast protein dissolved in the same assay buffer as solution (a) above.

This mixture was incubated at 30° for 20 min., cooled in ice for 30 sec. and then dispersed in 5 ml. of cold 5% TCA. The precipitate was centrifuged out, washed twice by resuspension and centrifugation out of cold 5% TCA and then deposited on Oxoid membranes with 5 ml. of cold 5% TCA. The membranes were air-dried and counted in a Nuclear-Chicago automatic gas flow counter.

The pyruvate kinase was 'A' grade from Calbiochem., α-[^{32}P] UTP was a gift from Dr. R.H. Symons, Biochemistry Department, University of Adelaide, and the calf thymus DNA was prepared as described in CHAPTER I. Actinomycin D was a gift from Merck and Co. Inc., and 6-N-methyl adenosine 5'-monophosphate was a gift from Professor M.R. Atkinson.

RESULTS

(1) The extraction procedure. The assay conditions in the early crude extracts were varied considerably in attempts to get measurable incorporations of radioactive uridine triphosphate. However, in the case of the preliminary assays, the composition of the assay mixtures was similar to assay mixture (a) (described

in the MATERIALS AND METHODS of this chapter) but the volumes were much larger.

The first crude extract that had detectable activity was prepared by dispersing freeze-dried and ground yeast in 0.1M tris-HCl, pH 7.9, and then adding sequentially 1/50 vol. 0.05M tris-EDTA, pH 8.5, 1/50 vol. 0.5M $MgCl_2$ and solid NaCl to give a final concentration of 0.3M. This was then centrifuged at 30,000 revs./min. in a Spinco-40 head for 1 hr. at 2^{o}. Increased activity was obtained if early log phase cells were used and if dry DEAE-cellulose (chloride) (1 g. per 2 g. yeast) was added to the extraction mixture between the addition of EDTA and the addition of $MgCl_2$ (above). As this sequential addition of materials during the extraction procedure gave satisfactory results it was used in all subsequent extractions and no attempts were made to simplify the extraction procedure. The addition of thiol reagents (2-mercaptoethanol or 2-mercaptoethylamine) at this stage appeared to reduce rather than increase the enzymic activity extracted.

The RNA polymerase activity in the crude extracts was concentrated as follows: All operations were carried out at $0-4^{o}$ and, unless specified, the term

activity and total activity of the enzyme during the above procedures could not be followed. However, the above procedures did appear to give approximately a ten fold concentration of enzymic activity.

(2) The general properties of the enzyme preparation.

(a) Assay variation. TABLE 10 allows comparison of the replicates of a typical assay.

(b) Specific activity. The specific activity of 118μmole UTP per hr. per mg. protein shown in TABLE 10 is a representative example but in the absence of PEP and pyruvate kinase this specific activity was approximately halved.

(c) Requirements. If all four triphosphates, except the UTP, were omitted activity was reduced to low levels. However, omission of only one of the other three triphosphates caused only a partial loss of activity, an effect that may be due to traces of contaminating nucleotides from various sources. The system showed a variable dependence on the addition of exogenous primer as preparations varied between 70% and near 100% dependence on primer. The preparation example shown in TABLE 10 was 77% primer-dependent. The small amount of primer-independent activity that

converted the [^{32}P] in the UTP to a TCA insoluble form was, apparently, not due to the presence of endogenous primer as it was only slighly inhibited by levels of Actinomycin D that almost completely inhibited the DNA-stimulated activity (see TABLE 10). Thus this DNA-independent activity may be due to some other enzyme.

The enzyme was inhibited approx. 30 to 40% by 30% glycerol but over a 48 hr. period of storage at 4° the preparations in 30% glycerol only lost 30% of their activity compared to a 60% loss by the enzyme without glycerol. Thus, although glycerol inhibited the enzyme, it appeared to protect it in the same way as it protected the rat testis RNA polymerase of Ballard and Williams-Ashman (1966).

Native DNA was a better primer than denatured DNA and, as the incorporation with denatured DNA was prevented by RNAases (see TABLE 10), it does not appear that the RNA product was extensively hybridised with the DNA primer, as described by Chamberlin and Berg (1964) and Warner *et al.* (1963), since it would be expected to be resistant to hydrolysis (as the hybrids were in CHAPTER V). However, it is possible that the RNAases were inhibiting the polymerase directly rather than destroying the product and this

possibility has not been checked.

(d) Properties of the product. The product was TCA insoluble and the presence of RNAases in the incubation mixture prevented its detection, presumably by hydrolysing it (see TABLE 10). The TCA-insoluble precipitate was hydrolysed by M diethylamine (48 hr. at 60°) to give a mixture of the four nucleotides (2' and 3' uridine, cytidine, adenosine and guanosine monophosphates) with [^{32}P] in all four of the nucleotides. The nucleotides were identified from their electrophoretic mobility on paper with 0.025M sodium citrate, pH 3.5, as a buffer. Thus, it seems most likely that the product was largely a heteropolyribonucleotide as would be expected from conventional DNA-primed RNA synthesis.

(e) Inhibitors. Actinomycin D was the most effective inhibitor found. 6-N-methyl adenosine monophosphate seemed to have a slight inhibitory action on the enzyme at 0.5mM and phosphate caused a 50% inhibition at the relatively high concentration of 25mM. A detailed study of the inhibition by 6-N-methyl adenosine monophosphate and phosphate might be useful in any future attempts to characterise the enzyme.

Although many other common substances were tested for inhibitory activity none were found that had a

large effect at a concentration of 0.5mM. Some of the substances tested were: all the common amino acids, the common 2',3' ribonucleotides, lactate, pyruvate, succinate, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate and ethanol at 1% (v/v).

DISCUSSION

Most of the potential RNA polymerase effectors tested here were chosen because they would be relatively common within the cell and because the main aim of the inhibition studies was to differentiate between a nuclear and any non-nuclear RNA polymerase. While both these polymerases might be expected to have similar active centres, with similar responses to inhibitors that have relatively direct effects on the action of their catalytic centres, it seemed reasonable to hope that during the process of separate evolution they may have developed different control or allosteric centres. However, no inhibitors have yet been identified that have high enough activity to warrant further investigation at this stage.

While it should be possible to characterise the presumed nuclear enzyme by purifying it and then

using other techniques, such as its antigenic properties, its molecular weight, and its various kinetic parameters, this would be technically difficult until larger amounts of more purified enzyme were available.

Although this programme of work is far from the long term goals discussed in the INTRODUCTION to this chapter, a conventional DNA-dependent, RNA polymerase activity has been extracted from yeast and its properties briefly examined.

It should be noted that Wintersberger (1966) has demonstrated the presence of an RNA polymerase in yeast mitochondria. The mitochondrial RNA polymerase was inhibited by Actinomycin D but it was not susceptible to DNAase so that it was presumed to be a DNA-dependent RNA polymerase that was enclosed by a membrane.

TABLE 10: AN ASSAY OF ONE BATCH OF RNA POLYMERASE

The assay mixture was the potassium based assay mixture containing PEP and pyruvate kinase as described in the MATERIALS AND METHODS of this chapter.

Each tube contained 1.25 mg. of yeast (αTr 39163 P_1) protein and in the case of other additions the following amounts were added per tube (vol. 0.11 ml.): DNA native or denatured, 5.0 µg.; RNAases, a mixture of 1.0 µg. pancreatic and 0.01 µg. T_1; Actinomycin D, 5.0 µg.

The background count of the gas flow counter (31 CPM) has been deducted from all counts.

Additions	CPM in TCA precipitate (duplicate assays)		µµmole α-[^{32}P] UTP in the TCA precipitate
Native DNA	2865	2391	49.2
Denatured DNA	1625	1485	29.1
Native DNA plus Actinomycin D	766	858	15.2
Native DNA plus RNAases	165	138	2.8
No DNA	620	593	11.4
No DNA plus Actinomycin D	494	426	8.6
Native DNA (stopped at zero time)	32	29	0.6

GENERAL DISCUSSION

GENERAL DISCUSSION

A. A DESCRIPTION OF THE MAJOR EVENTS IN THE APPLEBY AND MORTON PROCEDURE

When the results, described in this thesis, were considered with the facts that were already known, it became possible to give a description of how and when the Appleby and Morton procedure modifies the DNA and flavo-haemo-protein components of Type I cytochrome $\underline{b}_2$. Although the nature and time of occurrence of the major changes is known with reasonable confidence, many interesting aspects of these changes that are still matters for speculation are also considered below.

It is now thought that, during the Appleby and Morton procedure, the following sequence of events occurs:

(a) During the air drying or ageing of the yeast, a slow, nuclease-dependent degradation of the yeast DNA begins. This degradation is not random so that the DNA fragments produced do not have a random sample of the available DNA base sequences. There is a precedent for this type of behaviour with yeast DNA as Symons and Ellery (1967) found a non-random extraction of DNA from yeast by the Chargaff and Zamenhof (1948) procedure.

The mechanism of this non-random breakdown is not known but a number of explanations can be visualised.

It could be due to the specificity of a nuclease but there are other explanations. If a nuclease showed little base specificity then the rupture of hydrogen bonded zones between two near, single chain cleavages, on separate strands of a double helix would be expected to occur more frequently at high adenine-thymine (weakly hydrogen bonded) regions. Thus, as the base composition of small fragments is appreciably affected by the base compositions of its end regions, the mode of DNA degradation would be expected to affect the base composition of the low molecular weight fragments that first appear. Still another possibility is the suggestion of Symons and Ellery (1967) that mitochondrial DNA may be degraded at a different rate to nuclear DNA so that the mitochondrial DNA with a high mol. % adenine plus thymine (Tewari et al., 1966) makes a disproportionately large contribution to the low molecular weight fragments.

During these air drying and milling steps, the flavo-haemo-protein undergoes no known modifications.

(b) During the extraction of the dried and ground yeast the n-butanol release some cytochrome b_2 from particles and releases a factor (Nicholls, 1966) that causes the enzyme to undergo the modifications that are

observed later in the preparation procedure. Only relatively low molecular weight DNA is released into the aqueous phase during this extraction and the acetone fractionations cause changes in the relative amounts of the various nucleic acids and proteins.

(c) During the 40 hr. dialysis required for crystallisation, the cytochrome $\underline{b}_2$ flavo-haemo-protein undergoes changes that affect its kinetic, electrophoretic and crystallisation behaviour (Labeyrie and Slonimski, 1964; Nicholls *et al.*, 1966). The modified enzyme molecules select linear polyanions with a certain length optimum (in the region of 650 A^o) out of those available and this results in the Type I-form crystal containing predominantly small DNA fragments, because these satisfy certain size and shape requirements. During the recrystallisations some cytochrome $\underline{b}_2$ is inevitably inactivated thus leaving a slight excess of polyanions with respect to active, crystallisable, enzyme. Hence the selection process is again repeated with, effectively, an excess of polyanions so that the DNA in the final crystals is a result of a number of selections. The difference between the base ratio of $\underline{b}_2$-DNA and bulk yeast DNA is explained by the non-random distribution of base ratio with respect to the

size distribution of the degraded DNA fragments.

B. FACTORS DETERMININING THE CRYSTAL FORM

In the absence of DNA or any other polyanion, Type II cytochrome b_2 forms the arrays of hexagonal tubes that make up the Type II crystal and under these conditions the much more closely packed Type I structure is apparently unstable. However, as soon as a polyanion is introduced the reverse becomes true. While a complete explanation of this behaviour is not yet possible, it seems likely that the polyanion attaches to the 120^{o} edge of the flavo-haemo-protein molecule. This attachment would prevent the formation of the Type II structure because there would be created a large number of sites where it was impossible for three molecules to join at 120^{o}. However, in the Type I structure the polyanion would lie in grooves and its position in the Type I crystal may allow it to neutralise charges on the flavo-haemo protein that would have otherwise made the Type I crystal unstable.

Any polyanion that tended to cause imperfections in the Type I structure might be expected to hinder the growth of the crystal and thus polyanions that allowed the formation of the undistorted Type I

structure would be expected to be rapidly incorporated into a crystal, in preference to the others. Thus, the most suitable molecules would be linear polyanions that were not cross linked, were long enough to attach to two or more protein molecules in the Type I crystal but not so long that they tended to cause crystal imperfections. Long molecules could cause crystal imperfections if they were entangled with each other or if they were long enough for the two separate ends of the molecules to become incorporated into different layers of the crystal.

C. THE BIOLOGICAL IMPLICATIONS OF THE TYPE I CYTOCHROME b_2

As the crystalline Type I cytochrome b_2 is a preparation artifact the direct implications of the DNA protein association to any *in vivo* situation are neglible. However, the existence of this nucleoprotein did cause the initiation of studies of yeast DNA-dependent RNA polymerases described in CHAPTER VI. During these studies a DNA-dependent-RNA polymerase was extracted from yeast and partially characterised. This polymerase activity was extracted from a respiratory deficient yeast grown under essentially anaerobic conditions so that mitochondria should be both defective and

repressed; consequently, it seemed quite likely that it was the nuclear polymerase. However, during the course of these RNA polymerase studies it became apparent that this was going to be a much larger project than was originally anticipated. It was considered that such a large project was beyond the scope of this thesis and so the work on DNA-dependent yeast RNA polymerases was terminated.

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