ABSTRACT OF THESIS

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Title of Thesis A New Approach to the Study of DNA-Protein Interrelationships in Calf Thymus Nuclei

Observations correlating changes in chromatin structure with genetic events in eukaryotic organisms have stimulated interest in the chromatin proteins as controllers of gene structure and function. Studies on the chromatin proteins are hampered, however, by their inaccessibility within the cell nucleus and by the extreme chemical procedures necessary for their preparation and analysis. This research was undertaken in order to examine and exploit the advantages of a continuous flow system for chromatin fractionation.

A nuclear extraction cell was designed within which whole cell nuclei were isolated by suspension in a sephadex gel, trapped between filters and exposed to extraction by a sequence of eluants. The cell and accoutrements were designed to minimize dead space, successfully permitting high sensitivity and good resolution in detecting and recovering the extracted fractions. The major drawback to the system was the difficulty of achieving and maintaining adequate rates of eluant flow.

The extraction cell was used to fractionate nuclei prepared with either acetic acid or sucrose solutions as the isolation media. In each case the nuclei yielded a 'wash' fraction, a nuclear digest fraction produced by elution with Deoxyribonuclease I, and an SDS-soluble fraction produced by extracting the enzyme-resistant residue with 2% SDS. These fractions contained RNA plus protein, DNA plus protein, and DNA plus protein, respectively.

The deoxyribonucleoproteins of the nuclear digest and of the SDS-soluble nuclear residue were subjected to secondary fractionation by exclusion chromatography, and the resulting fractions were further analyzed by polyacrylamide disc gel electrophoresis and amino acid analysis. Both digest and residue contained all the histone fractions, a degraded DNA fraction, and a nucleoprotein fraction whose protein was possibly non-histone in character and bound to the DNA by a firm, non-ionic linkage.

The failure to achieve complete DNA extraction with Deoxyribonuclease I in spite of the continuous removal of hydrolysis products was surprising. Limitations may have been imposed on digestion either by the enzyme's

Use other side if necessary.

specificity or by structural features of the DNA/protein complex. The influence of divalent cation concentration was particularly interesting in this respect. The parallel fractionation of the nuclear proteins was also striking and suggests that further distinctions between the proteins of the enzymic digest and the enzyme resistant nuclear residue should be sought.

Although extensive comparisons between the two types of nuclei did not prove possible, the limited comparisons achieved showed remarkable consistency in the behaviour of nuclei prepared in very different ways.

A NEW APPROACH TO THE STUDY OF DNA-PROTEIN INTERRELATIONSHIPS IN CALF THYMUS NUCLEI

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1972

I declare that this thesis is my own composition and that the work it describes is mine, apart from the amino acid analyses which were carried out by Mr. J. McGowan.

SUMMARY

Observations correlating changes in chromatin structure with genetic events in eukaryotic organisms have stimulated interest in the chromatin proteins as controllers of gene structure and function. Studies on the chromatin proteins are hampered, however, by their inaccessibility within the cell nucleus and by the extreme chemical procedures necessary for their preparation and analysis. This research was undertaken in order to examine and exploit the advantages of a continuous flow system for chromatin fractionation.

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INTRODUCTION

A 1 Preface

Recent advances in biochemical research have confirmed man's ability to understand biological phenomena in terms of molecular structures and interactions. The key to our current molecular model for gene expression and inheritance was provided by F. H. C. Crick and J. D. Watson's proposal (1953) for the structure of deoxyribonucleic acid (DNA), the molecule which had been implicated by other workers (particularly O. T. Avery, C. M. MacLeod and M. McCarty, 1944) as the biological information store. Research is now focused on the details of the molecular mechanisms and the controls exerted on the rates and rhythms of life processes.

Advances in molecular genetics have been limited almost entirely to the analysis of prokaryotic oranisms. The emphasis on prokaryotes rather than on eukaryotes is a consequence of their much greater susceptibility to experimentation. Unicellular or colonial in form and undifferentiated, their short generation times and structural simplicity facilitate the correlation of molecular, cellular and environmental events. Eukaryotes, on the other hand, present severe technical difficulties because of their structural and genetic complexity. Not only are the differentiated cells of eukaryotes often difficult to isolate and disrupt in a controlled manner, but their DNA is isolated from the cytoplasm by the nuclear membrane as a nucleoprotein complex called chromatin, and involved in the remarkable cycles of mitosis and meiosis. All of these factors conspire to limit our rate of progress in eukaryote molecular genetics.

In spite of these technical difficulties, interest in the eukaryotes continues to be stimulated by our desire to understand

the molecular basis of such phenomena as cancer, cell differentiation and the immune response. The key to these processes lies in the controls exerted over expression of the genetic information store and many experiments suggest correlations between structural change and genetic activity in eukaryotic chromatin. The most obvious of these are the chromosome changes during mitosis and meiosis whose correlation with classical breeding experiments provided the first link between genetic events at the sub-cellular and the whole organism levels. These have been followed, however, by observations of more subtle changes. Developmental events, either naturally occurring or stimulated by hormone administration, are accompanied by 'puffing' of the polytene chromosomes of certain Dipteran cells. This puffing is further related to gene transcription by radioactive labelling experiments indicating high RNA synthetic activity in the structurally diffuse puff regions of the chromosomes (W. Beerman, 1952; and W. Beerman and U. Clever, 1964). Amphibian oocytes and many other cells of wide distribution contain 'Lampbrush' chromosomes, so named because of their many lateral loops of DNA-containing material. Once again, RNA synthetic activity is localized in the structurally specialized loop regions of the chromosome (J. G. Gall and H. G. Callan, 1962). Finally, variations in the staining reaction of chromatin thought to be related to its degree of condensation or coiling are observed in many cells at various stages of the life cycle. The effect has been specifically related to gene expression in several species, the information in condensed or heterochromatin remaining unexpressed while that of dispersed or euchromatin is expressed (L. Berlowitz, 1965 and E. E. Beutler, M. Yeh and V. F. Fairbanks, 1962). In a more general way it has been suggested that heterochromatinization reflects progressive repression of the genome during cell differentiation, the high degree of condensation in spermatozoa representing the terminal stage of the process (S. W. Brown, 1966). Assuming that the process of

transcription in eukaryotes is basically the same as that established for prokaryotes, all of these observations suggest that transcription of eukaryotic DNA can be prevented by alterations in its physical state which render it inaccessible to the transcriptional machinery. Eukaryotic chromatin is a complex of DNA with many proteins, and interest therefore centres on the structure of this nucleoprotein complex as a possible key to the control of eukaryote gene expression.

FIGURE A1

TABLE A1

THE COMPOSITION OF CALF THYMUS CHROMATIN

(Results are given as weight ratios referred to DNA.)

Source	Preparation	Histone	Non-Histone Protein	RNA
a	Sucrose homogenate, differential centrifugation.	1.14	0.33	0.007
b	0.14 M. NaCl extracted of tissue homogenate.	1.15	0.25	
c	NaCl extracted of tissue homogenate.	0.95	0.33	<0.005
d	Sucrose homogenate, differential centrifugation.	1.15	0.50	
e	0.15 M. NaCl extracted of citric acid nuclei.	0.90	0.53	0.05
f	Dried acetic acid nuclei.	0.69	0.79	0.08
f	Dried sucrose nuclei.	0.72	0.40	

Sources:

a J. Bonner, M. E. Dahmus, D. Fambrough, R. C. Huang, K. Marushige and D. Y. H. Tuan (1968).

b E. W. Johns and S. Forrester (1969a).

c R. Chalkley and R. H. Jensen (1968).

d H. H. Ohlenbusch, B. M. Olivera, D. Tuan and N. Davidson (1967).

e J. Paul and R. S. Gilmour (1968).

f Current work.

A 2 Chromatin Composition

Before the structure of chromatin can be determined, its composition and the nature of its components must be assessed. Measurements of chromatin composition are hampered, however, by the technical difficulties inherent in its isolation. The nuclear membrane is disrupted by all nuclear isolation procedures, so that exchange of nuclear and cytoplasmic components is likely and the identity of native and isolated nuclei doubtful. This difficulty, and that of successfully extracting each chromatin component from the others, is reflected in the variability of the values for chromatin composition shown in Table A1 which is adapted from E. Fredericq's thorough discussion of this problem (1971). In spite of the variability in the quantitative data, chromatin can be considered, qualitatively at least, as including DNA, a small amount of RNA, and two major protein classes. An important role in recognition of specific DNA base sequences has been attributed to the RNA components (R. C. Huang and J. Bonner, 1965; W. Benjamin, O. A. Levander, A. Gellhorn and R. H. Debellis, 1966; I. Bekhor, G. M. Kung and J. Bonner, 1969; T. Y. Shih and J. Bonner, 1969; H. W. von Heyden and H. G. Zachau, 1971 and T. Kanehisa, H. Fujitani, M. Sano and T. Tanaka, 1971) but other workers have been unable to reproduce these results (S. L. Commerford and N. Delihas, 1966; J. E. Loeb, 1967; and M. Artman and J. S. Roth, 1971). It thus seems safest to assume no major structural role for this component. The chromatin proteins can be classed as histones ~~or basic nuclear proteins~~ or as non-histone nuclear proteins.

A 3 The Histones

Although they were first identified by A. Kossell in 1884, extensive study of the histones did not begin until interest in them was revived by E. Stedman in 1944. Since then their distribution, structure and function have been thoroughly examined and progress in the field has been catalogued in a series of review articles and books (D. M. P. Phillips, 1962; J. Bonner and P. Ts'o, ed., 1964; K. Murray, 1965; H. Busch, 1965; A. V. S. de Reuck and J. Knight, ed., 1966; L. S. Hnilica, 1967; R. H. Stellwagen and R. D. Cole, 1969; E. L. Smith, R. J. Delange and J. Bonner, 1970; R. J. Delange and E. L. Smith, 1971; and D. M. P. Phillips, 1971), the last of which was published at the end of last year. As this recent and comprehensive review of the literature concerning the histones is available, no attempt will be made here to provide a comparable review. The available information will be summarized giving only representative literature citations, selected where possible for their support of varying viewpoints.

The histones, then, are defined as basic proteins which spend at least part of their lifetime in the nucleus of a eukaryotic cell. Following the Stedmans' (1951) initial fractionation employing ethanol precipitation, they have been fractionated on a preparative scale by a wide variety of methods including column chromatography on Amberlite IRC-50 (P. S. Rasmussen, K. Murray and J. M. Luck, 1962), carboxymethylcellulose (E. W. Johns, D. M. P. Phillips, P. Simson and J. A. V. Butler, 1960) and Sephadex G-75 (H. J. Cruft, 1961) and by selective extraction from chromatin with salts such as NaCl (H. H. Ohlenbusch, B. M. Olivera, D. Tuan and N. Davidson, 1967), acid (K. Murray, 1966 and 1969), and organic solvents (E. W. Johns, 1964 and 1967). Improved fractionation and characterization of the histone fractions has been achieved on an analytical scale by a variety of electrophoretic methods including moving boundary electrophoresis (H. J. Cruft, 1953), and zone

electrophoresis in starch (J. M. Neelin and G. F. Connell, 1959) and polyacrylamide gels (H. J. Cruft, 1962). Polyacrylamide gel electrophoresis has yielded the clearest resolution of histone components, and recently S. Panyim and R. Chalkley (1969 a and b) have produced elegant separations in this system using acetic acid containing 6.2 M. urea as electrophoresis buffer. During the development of these fractionation methods a variety of nomenclatures for the histone fractions have been used and these have been summarized by J. A. V. Butler, E. W. Johns and D. M. P. Phillips, (1968). It is now generally accepted that there are only five main histone fractions and these will be referred to here according to the nomenclature of E. W. Johns, D. M. P. Phillips, P. Simson and J. A. V. Butler (1961) who numbered them in order of elution from CM-cellulose. In Panyim and Chalkley's electrophoretic system, they are, in order of increasing mobility, F1, F3, F2B, F2A2, and F2A1.

The histones have also been characterized by amino acid analysis and, to some extent, by primary structure analysis. These show that they contain no tryptophan and little tyrosine or phenylalanine, no cystine or cysteine with the exception of fraction F3, and a high proportion of basic amino acids. The individual fractions are distinguished from each other by further differences in amino acid composition, particularly with respect to their contents of lysine and arginine (E. W. Johns, 1971). The main C-terminal amino acids are lysine, glycine and alanine and the N-terminal groups are alanine, proline and acetyl (D. M. P. Phillips, 1963). The histones are small proteins with molecular weights in the range of 10,000 to 20,000 (P. A. Edwards and K. V. Shooter, 1969). The primary sequences have been completed for fractions F2A1 (R. J. Delange, D. M. Fambrough, E. L. Smith and J. Bonner, 1969 a and b; Q. Quagliarotti, Y. Ogawa, C. W. Taylor, P. Sautiere, J. J. Jordan, W. C. Starbuck and H. Busch, 1969) and F2B (K. Iwai, K. Ishikawa and H. Hayashi, 1969 and L. S. Hnilica, H. A. Kappler and

J. J. Jordan, 1970) and partial sequences are available for fractions F1 (R. D. Cole et al., 1971), F2A2 (D. M. P. Phillips, 1968 and D. M. P. Phillips and P. Simson, 1969) and F3 (K. Yokotsuka and K. Shimura, 1969 and D. M. Fambrough and J. Bonner, 1968; J. A. Hooper, R. J. Delange and E. L. Smith, 1972). These studies show that in each fraction the basic residues are grouped to give the molecule polar and apolar regions.

All of the histones are subject to a variety of post-synthetic side chain and terminal substitution reactions which lead to marked microheterogeneity (M. Greenaway and K. Murray, 1971). These include methylation of lysine (K. Murray, 1964), arginine (W. K. Paik and S. Kim, 1970), histidine (E. L. Gershey, G. W. Haslett, G. Vidali and V. G. Allfrey, 1969) and, in vitro at least, carboxyl groups (S. Kim and W. K. Paik, 1970 and 1971). Both lysine (V. G. Allfrey, R. Faulkner and A. E. Mirsky, 1964) and some N-termini (D. M. P. Phillips, 1963 and 1968) are enzymically acetylated and deacetylated (V. G. Allfrey, 1964). O-phosphorylated serine has been identified (L. J. Kleinsmith, V. G. Allfrey and A. E. Mirsky, 1966a) and T. A. Langan (1969) has shown this process to be enzymic.

A 4 The Chromatin Non-Histone Proteins

The non-histone proteins of chromatin are less well characterized than are the histones, but the available information regarding these proteins has been reviewed by several authors (H. Busch, 1965; L.S. Hnilica, 1967; R.H. Stellwagen and R.D. Cole, 1969; E. Fredericq, 1971; V.G. Allfrey, 1971; and A.J. MacGillivray, J. Paul and G. Threlfall, 1972). Again, this information will be summarized here giving only representative literature citations.

The non-histone proteins have usually been prepared by salt dissociation (T.C. Spelsberg and L.S. Hnilica, 1969; E.W. Johns and S. Forrester, 1969 a; R.S. Gilmour and J. Paul, 1970; F.X. Wilhelm and M. Champagne, 1969) or detergent extraction K. Marushige, D. Brutlag and J. Bonner, 1968) of chromatin, sometimes accompanied by exclusion (J.E. Loeb, 1968) or ion exchange (R. Hacha and E. Fredericq, 1968; A.J. MacGillivray, D. Carroll and J. Paul, 1971) chromatographic techniques. Total extraction of all non-histone protein has seldom been achieved, and the significance of the 'residual' protein fraction, the subject of a longstanding controversy, remains unexplained (P.M. Frearson and K.S. Kirby, 1964; J.E. Leveson and A.R. Peacocke, 1966; E. Fredericq and C. Houssier, 1967; J.E. Loeb, 1968; J. Sonnenbichler and P. Nobis, 1968 and J. Sonnenbichler, 1969). The uncertainty is based on the variable yield of these proteins according to the chromatin preparation method employed (see Table A1) and on the severity of chromatin fractionation methods which may induce the formation of artifactually intractable nucleoprotein complexes.

The non-histone proteins are acidic in overall amino acid composition (R. Hacha and E. Fredericq, 1968; K. Marushige, D. Brutlag and J. Bonner, 1968) and include a major phosphoprotein component (E.L. Gershey and L.J. Kleinsmith, 1969; L.J. Kleinsmith and V.G. Allfrey, 1969 a and b), phosphorylation

and dephosphorylation again occurring after protein biosynthesis. Electrophoresis shows that they are very heterogeneous (A. J. MacGillivray, D. Carroll and J. Paul, 1971), but no detailed fractionation or characterization of these proteins has yet been achieved.

A 5 The Functions of Nuclear Proteins

In 1951 Edgar and Ellen Stedman suggested that

> "the basic proteins of cell nuclei are gene inhibitors, each histone or protamine being capable of inhibiting the activities of certain groups of genes".

This postulate is supported by such observations as the reduction in RNA synthetic activity in the lateral loops of lampbrush chromosomes caused by the addition of histone (V. G. Allfrey and A. E. Mirsky, 1963), the increased binding of Actinomycin D to DNA in heterochromatin after histone removal (L. Berlowitz, D. Palotta and C. H. Sibley, 1969), and the correlation of histone modification reactions with developmental events in the cell (T. Tidwell, V. G. Allfrey and A. E. Mirsky, 1968; W. K. Paik and S. Kim, 1971; V. G. Allfrey, 1968 and K. Marushige, V. Ling and G. Dixon, 1969). It catalyzed much of the histone research that has been summarized above, but it must now be seen as an oversimplification of the mechanism for gene control. In spite of variations in the in vitro interactions of the various histone fractions with DNA (R. C. C. Huang, J. Bonner and K. Murray, 1964; K. Murray, 1966 and 1969; H. H. Ohlenbusch, B. M. Olivera, D. Tuan and N. Davidson, 1967 and J. E. Smart and J. Bonner, 1971 a and b), their very limited intra- and inter-species heterogeneity (see, for example, R. J. Delange, D. M. Fambrough, E. L. Smith and J. Bonner, 1969 and and b) renders them ineligible for interactions of any known type direct with specific DNA sequences (c. f. M. Leng and G. Felsenfeld, 1966 and S. Lewin, 1970), and no qualitative or quantitative variation in the histone content of euchromatin or heterochromatin of either coccids (D. C. Comings, 1967 and D. Palotta, L. Berlowitz and L. Rodruiguez, 1970) or lymphocytes (J. H. Frenster, 1965) has been found. Although abundant proof has been given that in vitro the histones do suppress RNA synthetic activity (R. C. C. Huang and J. Bonner, 1962; V. G. Allfrey, V. C. Littau and A. E. Mirsky, 1963; G. C. Barr and J. A. V. Butler, 1963; J. Hindley, 1963 and

A. Skalka, A. V. Fowler and J. Hurwitz, 1966) the biological relevance of the assay systems has been seriously questioned as they depend on artificial partial or reconstituted chromatins (which are discussed below) and, often, on non-homologous enzyme systems (B. P. Sonnenberg and G. Zubay, 1965; A. K. Roy and G. Zubay, 1966; J. Bonner and R. C. Huang, 1966; E. W. Johns and S. Forrester, 1970; E. W. Johns and T. A. Hoare, 1970; T. A. Hoare and E. W. Johns, 1971; R. J. Clark and G. Felsenfeld, 1971; J. E. Smart and J. Bonner, 1971c and V. G. Ilyin, A. Ya. Varshavsky, U. N. Mickelsaar and G. P. Georgiev, 1971).

Disenchantment with the histones and evidence such as the concentration of the acidic and phosphoproteins in the active puffs of polytene chromosomes (M. Robert and H. Kroeger, 1965) and in the euchromatin of lymphocytes (J. H. Frenster, V. G. Allfrey and A. E. Mirsky, 1963; J. H. Frenster, 1965) has led to recent emphasis on the latter proteins as possible gene regulators. The greater heterogeneity of these proteins makes them more suitable for a role in specific DNA-protein interaction (although they must include many other proteins with specific metabolic functions) and indeed both sequence (I. Bekhor, G. M. Kung and J. Bonner, 1969) and species (L. J. Kleinsmith, J. Heidema and A. Carroll, 1970; C. T. Teng, C. S. Teng and V. G. Allfrey, 1970) specific interactions between non-histone proteins and DNA have been demonstrated. Their phosphorylation has been correlated with gene activation (L. J. Kleinsmith, V. G. Allfrey and A. E. Mirsky, 1966a) and their synthesis with hormone action (D. Killander and R. Rigler, 1965 and 1969), and they have been shown to stimulate DNA-dependent RNA synthesis (J. H. Frenster, 1965; C. S. Teng and T. H. Hamilton, 1970), C. S. Teng, C. T. Teng and V. G. Allfrey, 1971 and M. Kamiyama and T. Y. Wang, 1971). DNA-RNA hybridization competition experiments have indicated that they confer transcriptional specificity on reconstituted chromatin used as a substrate for <u>in vitro</u> RNA synthesis (J. Paul and R. S. Gilmour, 1968 and J. Paul,

R. S. Gilmour, R. S. Thomou, C. Threlfall and D. Kohl, 1970). Proper evaluation of these metabolic results must, however, await derivation of more structural information about the non-histone proteins and their interactions with DNA and nucleohistone. This will allow a more stringent assessment of these assay systems.

All of these experiments serve to reinforce our impression of the complexity of the eukaryotic gene control mechanism, and of the necessity to consider the roles of all the chromatin components.

A 6 The Structure of Chromatin

In an attempt to learn more about the crucial DNA-protein interactions in chromatin, a variety of physical and chemical methods have been used to study the chromatin components and their complexes. These studies can be divided into two main categories according to the source of their substrate material. Reconstituted nucleoprotein or chromatin is an artificial complex containing DNA with some or all of the nuclear proteins (most commonly the histones) or with model polypeptides. It is prepared by mixing the components in either 2.6 M. NaCl (G. Zubay and P. Doty, 1959) or 2 M. NaCl/ 5 M. urea (R.S. Gilmour and J. Paul, 1969), where they remain dissociated, and dialyzing to low ionic strength, where they reassociate. Examination of various properties including thermal denaturation (E. Fredericq and C. Houssier, 1967), X-ray diffraction (J. Palau, J. F. Pardon and B. M. Richards, 1967), viscosity (C. F. Crampton, R. Lipshitz and E. Chargaff, 1954), chemical extractibility of components (C. F. Crampton, 1957) and optical properties (D. Y. H. Tuan and J. Bonner, 1969; J. Sponar, M. Boublik, I. Fric and Z. Sormova, 1970 and I. Fric and J. Sponar, 1971) shows that while these artificial complexes are very similar to native chromatin, the two are not identical. The differences probably result not only from the common absence of the non-histone proteins from these complexes, but from structural alterations to the components caused by the drastic chemical procedures such as high salt or urea concentrations and acid or detergent treatment required for their isolation. Certainly the histones are known to aggregate at high ionic strength (H. J. Cruft, C. M. Mauritzen and E. Stedman, 1958) and the high salt concentrations employed in reconstitution may cause such aggregation to interfere with the reconstitution process.

A wide variety of methods have been used to examine nucleoprotein complexes. The solubility of complexes between DNA and some or all of the histones has been examined by many

authors (I. P. S. Agrell, 1969; I. P. Ashmarin and P. S. Muratchatova, 1969; E. W. Johns and S. Forrester, 1969b; M. Sluyser and N. H. Snellen-Jurgens, 1970; M. C. Touvet-Poliakow, M. P. Daune and M. H. Champagne, 1970) as has that of DNA with basic (M. Leng and G. Felsenfeld, 1966; J. T. Shapiro, M. Leng and G. Felsenfeld, 1969 and K. G. Wagner, 1969) and aromatic (S. Friedman and P. O. P. Ts'O, 1971) polyamino acids. They have shown that as the lysine/arginine ratio of the histones increased, less protein was required to completely precipitate the same quantity of DNA. X-ray studies (B. M. Richards and J. F. Pardon, 1970; S. Bram and H. Ris, 1971 and S. Bram, 1971) indicate that the histones impose on the DNA a modified B structure, and they have been used to suggest higher order supercoiled or folded nucleohistone structures. The conclusions regarding DNA structure are supported by circular dichroism studies (F. X. Wilhelm, M. H. Champagne and M. P. Daune, 1970; H. J. Li, I. Isenberg and W. C. Johnson, 1971; and M. Haynes, R. A. Garrett and W. B. Gratzer, 1970) which show the DNA-histone interactions to be influenced by their environment in solution (G. D. Fasman, B. Schaffhausen, L. Goldsmith and A. Adler, 1970 and D. E. Olins and A. L. Olins, 1971) and the extent of phosphorylation of the histones (A. J. Adler, B. Schaffhausen, T. A. Langan and G. D. Fasman, 1971). Optical rotatory dispersion and circular dichroism measurements have also been used to analyze modifications to histone structure imposed by their interactions with DNA, but these are difficult to interpret conclusively (E. M. Bradbury and C. Crane-Robinson, 1971). Nuclear magnetic resonance studies (M. Boublik, E. M. Bradbury, C. Crane-Robinson and H. W. E. Rattle, 1971) suggest that the polar, basic part of each histone is bound to the DNA leaving the remaining sequence free for other interactions. Finally, the thermal stability of nucleohistone complexes has been thoroughly examined (A. T. Ansevin and B. W. Brown, 1971 and T. Y. Shih and J. Bonner, 1970) showing that the histones stabilize the DNA double helix in a way that increases with the lysine/arginine

ratio of the histone, and some authors have found biphasic melting profiles which suggest the existence of varying types of DNA-histone complex or the existence of uncomplexed DNA. These studies all yield valuable information concerning DNA-protein interactions, but their identity with the interactions critical to chromatin structure and gene control cannot be assumed, particularly as they usually include only DNA and histones. Such considerations become particularly important when reconstituted complexes are used in transcriptional experiments.

The second source of substrate material for studies of chromatin structure and function is partial nucleoprotein or chromatin, that is, chromatin from which some of the protein has been removed by selective extraction. Although the composition of such material is not as readily controlled and varied as that of reconstituted chromatin, it seems more likely to be closely related to the native complex and its preparation yields additional information concerning the relative binding strengths of the extracted components. The relatively harsh chemical procedures required during the fractionation process may, however, damage the residual complex.

Once again, many methods have been used to examine partial chromatins. Extraction of the chromatin with increasingly concentrated salt solutions (H. H. Ohlenbusch, B. M. Olivera, D. Tuan and N. Davidson, 1967) or decreasing pH (K. Murray, 1969) shows that in most systems the F1 fraction is much more readily extracted than the other histone fractions (c. f. J. E. Smart and J. Bonner, 1971a). X-ray diffraction studies of various nucleoprotein preparations (M. H. F. Wilkins, 1956; M. H. F. Wilkins, G. Zubay and H. R. Wilson, 1959 and V. Luzzatti and A. Nicolaieff, 1959) confirm the assessment of DNA structure found in reconstituted nucleohistone. Again, higher order structures are postulated from these studies. Electron microscope studies (V. C. Littau, C. J. Burdick, V. G. Allfrey and A. E. Mirsky, 1965 and H. Ris,

1966) confirm the role of histones in maintaining chromatin structure and the suggestion that the F1 fraction links complexes of DNA and the other histones. Circular dichroism studies confirm the effect of histones on DNA structure (V. I. Permogorov, V. G. Debabov, L. A. Sladkova and B. H. Rebentish, 1970; T. Y. Shih, and G. L. Fasman, 1970; R. T. Simpson and H. A. Sober, 1970; and T. Wagner and T. C. Spelsberg, 1971) but further interpretation of these results in terms of supercoiling or other structures is not conclusive (P. Henson and I. O. Walker, 1970b). Sedimentation experiments (D. Brutlag, C. Schlehuber and J. Bonner, 1969; J. D. Duerksen and B. J. McCarthy, 1971; R. A. Garrett, 1970 and P. Henson and I. O. Walker, 1971a) have fractionated various complete and partial chromatins, but no distinctive chromatin subunit has been found. Thermal denaturation studies again show stabilization of the DNA by the nuclear proteins (K. Murray and A. R. Peacocke, 1962; J. Bonner and R. C. Huang, 1963; P. Henson and I. O. Walker, 1970a; and R. A. Garrett, 1971), some preparations giving biphasic melting profiles. Finally, studies of the interactions with DNA by various molecules such as polylysine (R. F. Itzhaki, 1970), Actinomycin D. (L. Kleiman and R. C. C. Huang, 1971), Toluidine Blue (A. Miura and J. Ohba, 1967) and others (R. T. Simpson, 1970 and R. F. Itzhaki, 1971) have been made in an attempt to determine the locations at which the histones are bound to DNA. Most show, in confirmation of the biphasic melting profiles, that in spite of its interaction with proteins, some of the DNA is still accessible to interactions with other molecules.

Most studies of DNA-protein interaction have been made with DNA-histone complexes because the histones are so much better characterized and so much easier to handle experimentally than the other nuclear proteins. It is obvious, however, that the latter play a crucial role in modifying, if not in maintaining chromatin structure. Effort must therefore be expended towards an understanding of their structures and interactions.

A 7 A New Approach to the Study of Nuclear DNA-Protein Interactions

In view of the difficulties in examining chromatin structure described above, a new method was sought whereby its components and their interrelationships could be studied using native chromatin as substrate and avoiding the chemical extremes usually necessary for its fractionation. The method chosen was continuous, sequential extraction of whole cell nuclei immobilized within a flow system.

Nuclei and chromatin have conventionally been fractionated by repeated extractions involving suspension in a suitable medium followed by centrifugation. The success of such a method depends on the maintenance of a steep concentration gradient of the extracted component between the substrate material and the extraction medium, and on the persistence of the experimenter in repeating the extraction process to completion. A compromise must be reached between the use of large volumes of solution for efficient extraction and the desire for concentrated extracts whose components are readily detected and subjected to further analysis. If a series of extraction media are to be used on a single sample these difficulties are compounded. In addition, there are problems in avoiding physical damage or losses in resuspending the substrate after each sedimentation.

In contrast to the difficulties of static extraction, continuous flow extraction provides a situation which is ideal in terms of mass law considerations. The substrate is continuously exposed to a fresh supply of extraction medium or eluant, so that in theory the concentration gradient for any of its components is always infinite. Any of the common chemical extraction techniques for chromatin employing salt or acid could be used in the flow system in a manner analogous to the development of chromatographic columns. However the potential usefulness of enzymic extraction in this system is particularly striking.

Enzymic extraction of nuclei and chromatin has been employed by many workers as a probe of chromatin structure, using both nucleases (V. G. Allfrey, A. E. Mirsky and S. Osawa, 1957; P. H. Von Hippel and G. Felsenfeld, 1964; R. J. Clark and G. Felsenfeld, 1971 and J. M. Gottesfeld, M. Calvin, R. D. Cole, D. M. Igdaloff, V. Moses and W. Vaughan, 1972) and both constitutive (J. Bartley and R. Chalkley, 1970) and added (P. Henson and I. O. Walker, 1971b) proteases to examine the effect on chromatin structure of DNA and protein degradation respectively. The earlier studies of this type are very difficult to interpret since contamination of the enzyme preparations was very likely, and the experiments with proteolytic enzymes are hampered by the lack of enzymes absolutely specific for particular proteins. However the results have confirmed the roles of DNA and the histones as essential to the maintenance of chromatin structure.

Deoxyribonuclease extraction is attractive as a tool, chemically gentle to the protein components, for selective extraction of DNA and its associated proteins. In a flow system where inhibitory degradation products are continuously removed it would provide a means, not only of studying those proteins released during DNA degradation, but of producing DNA depleted nuclei whose protein-protein interactions could be further studied. Continuous flow extraction would allow monitoring of the eluate for its content of nuclear components which, with suitable design of the flow system, would be eluted at maximal concentrations for high sensitivity. Sequential extraction could then be carried out in confidence of complete extraction at each stage of the sequence. The same principles apply to metabolic studies with whole cells or organelles, where the objective is to emulate the capacity of biological systems to control the intra- and extra-cellular environment despite metabolic activity.

Although the flow system has important theoretical advantages in fractionation studies, its practical application does present some technical difficulties. The problems are similar to those faced in continuous cell culture, where liquid flow past the cells is essential to ensure removal of waste products and supply of nutrients. The current problem is more demanding, however, in that to maximize resolution of eluted materials dead space within the system must be minimized. For flow to proceed at all, the nuclei must be dispersed and fixed in position so that they will not form an impenetrable mass, but at the same time they must be in intimate contact with any eluant passed through the system. G. Sauermann (1970) has solved this problem for his studies on RNA metabolism by immobilizing nuclei in a column containing fragments of nitrocellulose membrane. Such a support is undesirable for structural studies, however, because it will itself interact with the eluted nuclear components. M. Sung and O. Smithies (1969), on the other hand, have studied the differential elution of histones from nuclei trapped in threads of polyacrylamide gel. This support, too, is undesirable because of the gel barrier between eluant and nuclei. For this work, sephadex was chosen as the supporting medium. The intention was that the nuclei, once mixed with the sephadex, would be trapped within the rather inert gel but still readily accessible to the aqueous environment. The mixture of nuclei and sephadex was to be contained between filters in a flow cell through which eluants could be forced by a pump. The design and operation of such an apparatus are described in Section C.

A 8 The Choice and Preparation of Nuclear Material

The method described above is designed to fractionate whole, isolated cell nuclei. The nuclei used for these studies were prepared from calf thymus tissue which was chosen here, as it has been frequently in the past, because its large, interphase nuclei are relatively easy to prepare free of cytoplasmic contamination. This choice is thus also supported by the large body of information already available regarding these nuclei and their chromatin.

Isolation of the nuclei from calf thymus tissue poses the problem common to all organelle preparations - the problem of preparing the organelle free of contamination from other cellular components but in a state that accurately reflects its in vivo condition. Where the organelle is to be prepared for a structural study, these conditions are particularly important.

Many methods have been employed for isolation of cell nuclei (see D. B. Roodyn, 1963 and H. Busch, 1967), each designed to prepare undamaged nuclei with a minimum of contamination. The basis of the most common technique in current research is sedimentation of the nuclei collected from a tissue homogenate in dilute sucrose through a concentrated sucrose solution of density intermediate between those of the nuclei themselves and the cytoplasmic components or whole cells (the method originated by J. Chauveau, Y. Moule and C. H. Rouiller, 1956). Nuclei prepared in this way, while they are considered to accurately reflect the in vivo condition, are very subject to microbial attack and autolysis and cannot, therefore, be stored. Their preparation requires high speed centrifugation (a centrifugal force of 40,000 xg is used in the final step of the preparation) and so cannot usually be carried out on a large scale. If the nuclear extraction method were to be developed using these nuclei, therefore, a nuclear preparation would have had to precede each extraction experiment and the quantity of nuclei available would constantly have been a limiting

factor. A more stable nuclear preparation was therefore sought which could be used to develop the extraction method.

An alternative to the preparation of nuclei in sucrose is the earlier preparation procedure employing a weak acid such as acetic acid as the isolation medium. This procedure is based on the observation that in such acids cells rupture, ejecting their nuclei which can then be collected and purified by washing in a similar medium and centrifugation at forces less than 1000 xg. Such nuclei have, indeed, been subjected to a low pH during their isolation but they are much more stable than sucrose prepared nuclei and can be prepared in large quantities. Their stability can be increased further still by extracting their lipid and water with organic solvents. This yields a dry preparation which is stable indefinitely at room temperature.

Dried acetic acid nuclei were therefore chosen, because of their stability and convenience, as the nuclear material used during the development of the extraction method. The method, once developed, could then be applied to the less convenient sucrose nuclei. Such an approach would have the added advantage of allowing a comparison to be made between the two types of nuclei, each with its own merits and drawbacks. The description of this work is given in the following sections according to the chronological order in which this plan was followed.

SECTION B

EXPERIMENTAL METHODS

SECTION B

EXPERIMENTAL METHODS

B 1. Preparative Techniques

B 1.1 Nuclei

The nuclei used in these experiments were prepared from calf thymus tissue obtained from the Edinburgh Corporation Slaughterhouse. The tissue was always chilled in crushed ice on removal from the animal and the preparation carried out immediately in the cold room at 5°C., where possible using vessels in direct contact with crushed ice. The tissue was first trimmed with a scalpel to remove connective tissue and fatty material, and then finely minced using an extrusion mincer. Nuclei were isolated from the resulting pulp using either acetic acid or sucrose solutions as the isolation medium.

Acetic Acid Method

The preparation procedure employing acetic acid was essentially that of E. Stedman and E. Stedman (1950). The tissue pulp was poured into approximately four volumes of 4% acetic acid and the mixture stirred vigorously with a mechanical stirrer for one hour. The froth was allowed to drain and lumps of tissue fibre removed by sieving through one layer and then four layers of fine muslin. The filtrate was centrifuged for twenty minutes at 800xg (2000 RPM) using the 550 ml. swinging buckets in the MSE Refrigerated Centrifuge. The supernatant was discarded and the deposit resuspended in 1% acetic acid and centrifuged. This step was repeated until all non-nuclear material had been removed from the deposit. The purity of the preparation was monitored throughout by microscopic examination of the suspended nuclei stained with methylene blue. The purified nuclei were fat extracted and dried by suspension during

24 hours in four volumes of cold ethanol, followed by resuspension in ethanol and ether, and air drying.

Sucrose Method

The preparation procedure employing sucrose solutions was a modification of that first used by J. Chauveau, Y. Moulé, and Ch. Rouiller (1956). All of the sucrose solutions were buffered to pH 7 with 0.02 M. veronal/hydrochloric acid buffer. The tissue pulp was mixed with an equal volume of 0.5 M. sucrose and squeezed through two layers of fine muslin. The filtrate was mixed with five volumes of 0.25 M. sucrose, 3.3 mM. in $CaCl_2$ and homogenized in 40 ml. aliquots using three up and down strokes of a Potter-Elvejhem type homogenizer with a motor driven teflon pestle and glass tube. The homogenate was squeezed through four layers of fine muslin and centrifuged for ten minutes at 1000xg (1900 RPM) in the 550 ml. swinging buckets of the MSE Refrigerated Centrifuge. The supernatant was discarded and the deposit resuspended in the original tissue volume of 2.2 M. sucrose containing 3.3 mM. $CaCl_2$. This suspension was squeezed through four layers of fine muslin. The filtrate was layered over three o four volumes of the concentrated sucrose solution and centrifuged for one hour at 40,000xg (18,000 RPM) in the Type 42 Head of the Beckman L2-65B Ultracentrifuge. The pellets of nuclei were used immediately in the nuclear extraction cell or kept in the deep freeze overnight before extraction the following day. To provide a basis for comparison with the dried, acetic acid nuclei, the sucrose nuclei were suspended overnight in 0.25 M. sucrose, 3.3 mM. in $CaCl_2$ and centrifuged for 30 minutes at 1000xg (2100 RPM) in the 50 ml. swinging buckets of the MSE Refrigerated Centrifuge. They were then dried by suspension and centrifugation in two portions of 70% ethanol, three portions of ethanol, and two portions of ether followed by air drying. These dried preparations were used for determinations of nucleic acid and histone content.

B 1.2 Crude Histone

The histones were extracted from dried nuclei with hydrochloric acid according to the method of E. Stedman and E. Stedman (1951). The nuclei were dampened with absolute alcohol and the resulting paste treated with two volumes of water. The suspension was centrifuged in the bench centrifuge and the pellet of dampened nuclei resuspended in ten volumes of 0.1 N. hydrochloric acid. After twenty minutes of occasional stirring, the suspension was centrifuged and the supernatant mixed with ten volumes of acetone to precipitate the histone chloride. These steps were repeated until acetone precipitated no further histone from the extract. All extracts were left overnight to flocculate and then pooled and collected by centrifugation. The pooled histone was washed with acetone and then ether, and finally stirred until air dry.

B 2. Estimation Methods

B 2.1 Ultraviolet Absorbance Measurements

This work has required the estimation of DNA, RNA and protein. All of these macromolecules absorb ultraviolet light, and this property is often used for their detection and, in some circumstances, their estimation. During the present work absorbance measurements have been used in detecting and partially identifying nucleic acid and protein material. They could not, however, be used in a quantitative manner. Although the nucleic acids have an absorption maximum at 260 nm. while that of most proteins is at 280 nm., the extinction of the nucleic acids at either of these wavelengths (expressed in terms of mg. of material per ml. of solution) is at least an order of magnitude greater than that of the proteins. This means that nucleic acid contamination of protein solutions is readily detected at very low levels, whereas small quantities of protein in the presence of nucleic acid are difficult to detect. This effect is even more pronounced for the histones whose extinction at 280 nm. is more than an order of magnitude smaller than that of most other proteins. Thus ultraviolet light absorbance could not be used for the estimation of proteins in this work where the protein absorbance was either obscured by that of the nucleic acid or too low for meaningful measurement. The nucleic acid absorbance is itself variable, the position and extinction of an absorption maximum depending on the degree of polymerization of the sample, secondary bonds in individual polynucleotide chains, and interactions between these chains and both other nucleotide chains and proteins. These factors are in turn influenced by the composition of the sample medium, in particular its pH and ionic strength. As the nucleic acid samples have usually contained a mixture of polynucleotides produced by enzymic digestion in the presence of proteins and other potentially interactive substances, their ultraviolet absorbance has provided only a very rough guide to the quantity of

nucleic acid present.

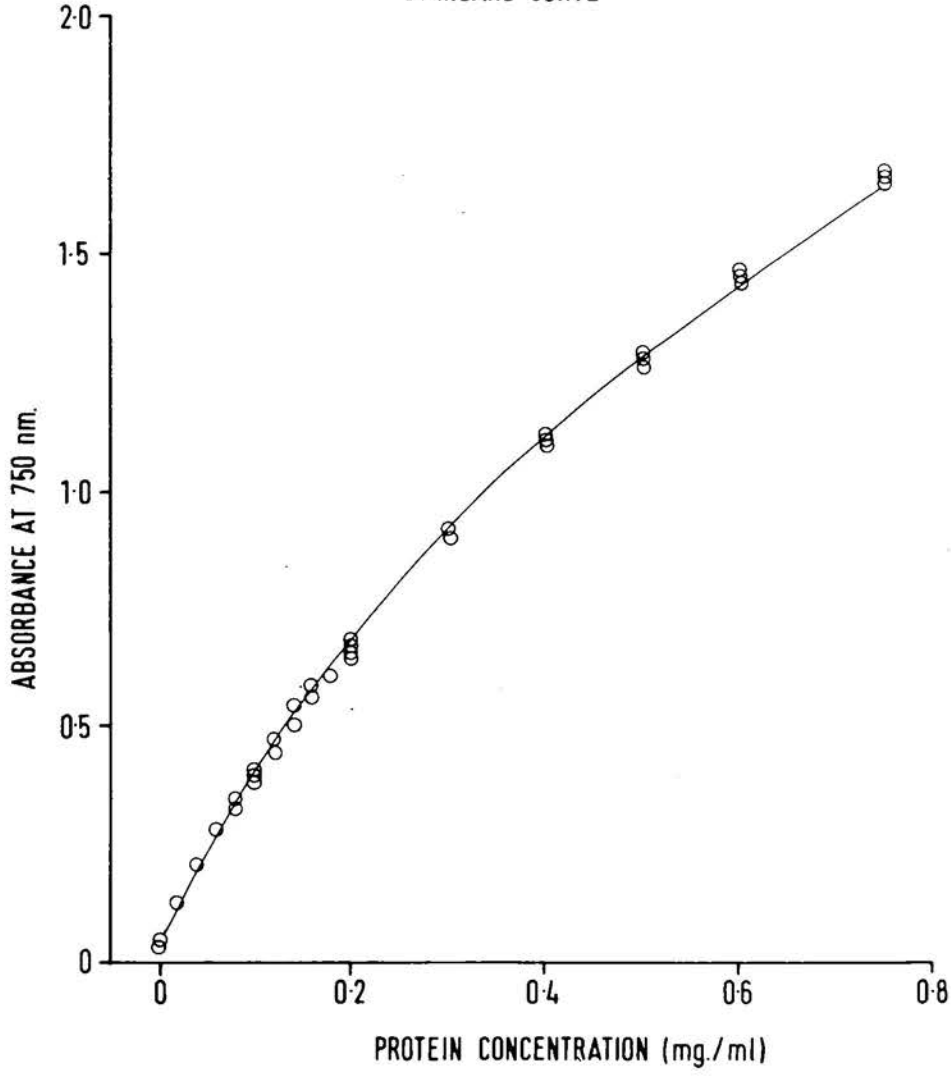
As was mentioned above, absorbance measurements have, in spite of these limitations, been used extensively for purposes of detection and identification. The eluant from the nuclear extraction cell was monitored with a Gilson Absorptiometer and Phillips Recorder using a light filter at 280 nm. This wavelength was chosen to optimize protein absorbance while minimizing the absorbance due to veronal buffers and still detecting the nucleic acids. The fractions from chromatograms of nucleic acid/protein mixtures were read at 260 nm. to detect all nucleic acid, protein and veronal. Sometimes the absorbance at 280 nm. was also measured and the ratio of absorbance at the two wavelengths determined as a first step in identifying the components of a fraction. The fractions from chromatograms of crude calf thymus histone were read at 230 nm. in order to detect these proteins whose low aromatic amino acid composition gives them a low extinction at 280 nm. All of these measurements were made with either the Hilger Uvispec or the Unicam SP 500 Spectrophotometer using quartz cuvettes with a 1 cm. light path and a distilled water reference (unless otherwise specified). Where rapid identification of a sample was required, an absorption spectrum was prepared using the Unicam SP 800 Spectrophotometer with cuvettes and reference as above.

B 2.2 Protein Estimation

The ninhydrin and Lowry methods were both tested as possible estimation techniques for protein. The Lowry Method (O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951) was found to be the simpler of the two and to give less variable results. It was equally sensitive to bovine serum albumin and to histone in spite of their widely different amino acid compositions and was therefore considered useful for estimating total protein in mixtures of unknown composition. The method is

FIGURE B1

FIGURE B1
LOWRY PROTEIN ESTIMATION
STANDARD CURVE



as follows.

Reagents:

A : 2% sodium carbonate in 0.01 N. sodium hydroxide.

B : 0.5% cupric sulphate pentahydrate in 1% sodium citrate.

C : 50 ml. of reagent A plus 1 ml. of reagent B.

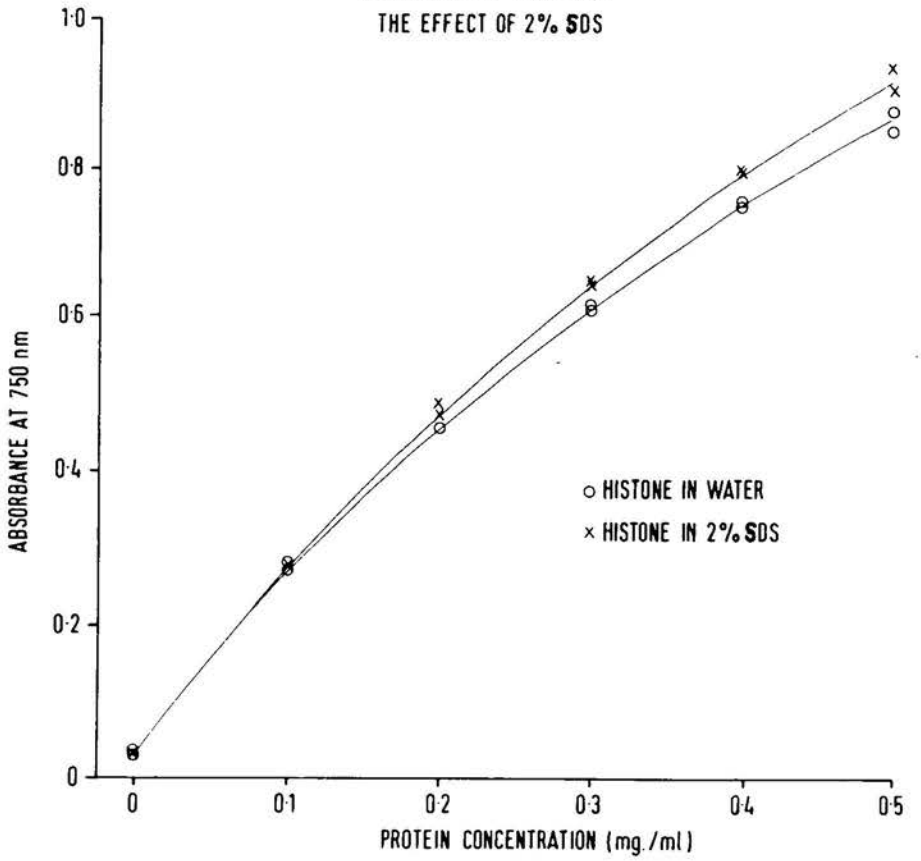
D : Diluted Folin's Reagent : Folin and Ciocalteu's Phenol Reagent was titrated with sodium hydroxide to a phenolphthalein end point. The Reagent was diluted to make it 1 N. in acid.

Reagents B and C keep for one day. The undiluted Phenol Reagent is kept refrigerated and an aliquot diluted freshly for each set of estimations.

To a sample of 25 to 500 µg. of protein in 1.0 ml. of solution, 5 ml. of reagent C were added, mixed thoroughly, and allowed to stand for ten minutes or longer at room temperature. 0.5 ml. of reagent D were added and mixed very rapidly. After at least 30 minutes the colour of the solutions was measured at 750 nm. using the Unicam SP 500 or the Unicam SP 600 Spectrophotometer. Optical glass cuvettes with a 1 cm. light path and a distilled water reference were used throughout. Where only small quantities of sample were available, determinations were made using one-half the quantities described above. This did not affect the standard determinations.

A standard curve was prepared using aqueous solutions of known weight composition of air dried crude calf thymus histone chloride, which is readily water soluble. This curve is shown in Figure B1. Determinations made in the presence of SDS showed that it slightly reduced colour formation. This effect is shown in Figure B2. Trishydroxymethylaminomethane, even after recrystallization, gave a very high interfering colour with

FIGURE B2
LOWRY PROTEIN ESTIMATION
THE EFFECT OF 2% SDS



both the Lowry and the ninhydrin methods. It was therefore replaced with veronal as the buffer employed at an early stage in the experiments. $MnCl_2$ was also found to interfere with the reaction when present at concentrations greater than 0.05 mM. It gave a brown colloidal precipitate which was slow to flocculate and a blue colour whose absorbance at 750 nm. reached 0.3 for 1 mM. $MnCl_2$. Under the same conditions $CaCl_2$ produced a heavy white precipitate but no blue colour. Each set of determinations was accompanied by a duplicate reagent blank and by duplicate standard determinations. The standard solution of calf thymus histone chloride was prepared in bulk and frozen in 5 ml. aliquots in sealed ampoules for later use.

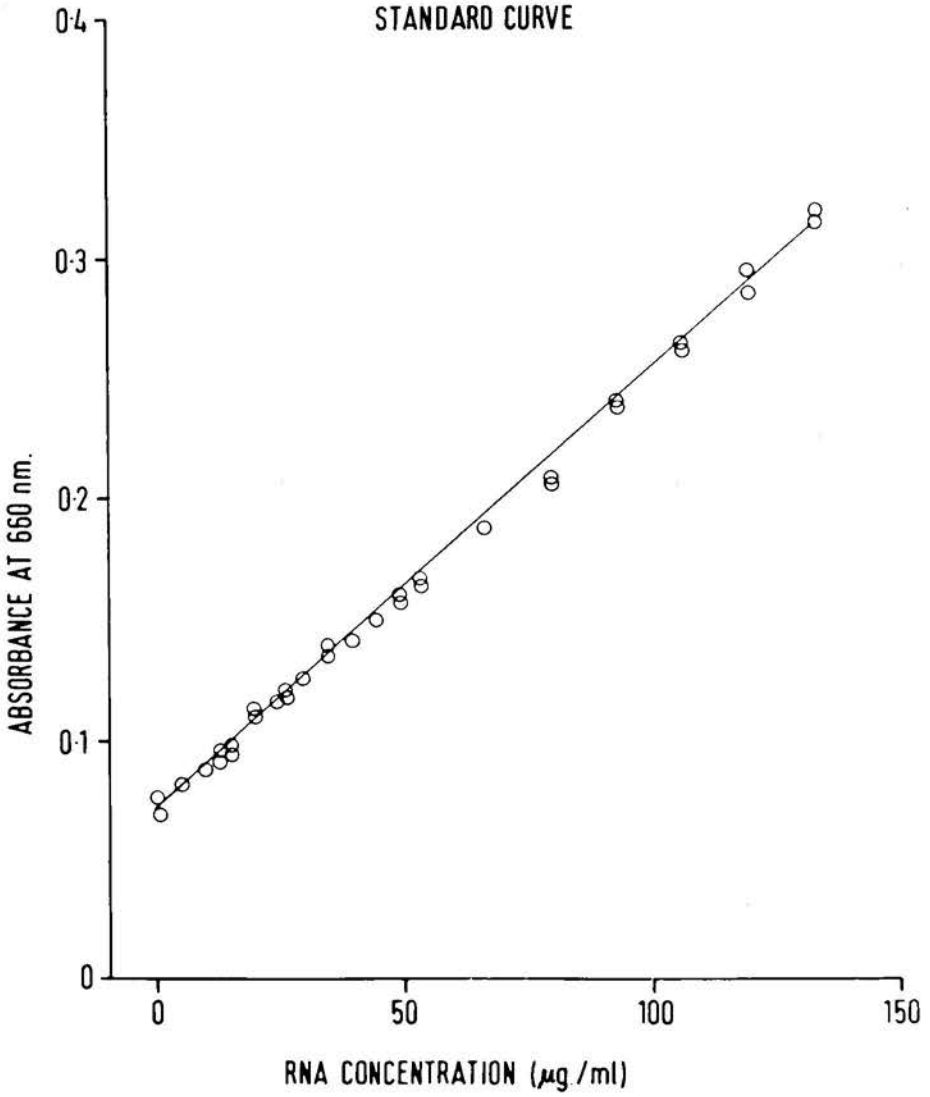
2.3 Nucleic Acid Estimation

Most estimation methods for nucleic acids depend on the reaction of a chromogenic substance with the sugar moiety of their ribose-phosphate backbones. As such these methods are subject to interference from other carbohydrate material, as well as from many chemical compounds commonly used in work with nucleic acids. (W.C. Hutchison and H.N. Munro, 1961; W.C. Hutchison, E.D. Downie and H.N. Munro, 1962; W.C. Schneider, 1945; R. Allerton, W.G. Overend and M. Stacey, 1952.) Attempts to avoid interference by selective extraction of the nucleic acid material and by correction for known interfering substances must be used together with a high degree of caution in estimating the nucleic acid composition of samples whose total composition is not known.

In order to estimate the total nucleic acid in nuclear preparations the dry, weighed nuclei were extracted three times with one-half volume of 0.5 M. perchloric acid at 70°C. for 15 minutes. DNA and RNA analyses were performed directly on the pooled extracts. The procedure of D.W. Hatcher and G. Goldstein (1969) for determining DNA and RNA freed of interfering

FIGURE B3

FIGURE B3
ORCINOL RNA ESTIMATION
STANDARD CURVE



substances by cadmium precipitation was examined at the beginning of the work. It was found to precipitate a variable proportion of the nucleic acids and was therefore rejected in favour of direct analysis using the diphenylamine reaction for DNA and the orcinol reaction for RNA. The techniques were based on those of Z. Dische (1967).

RNA Estimation

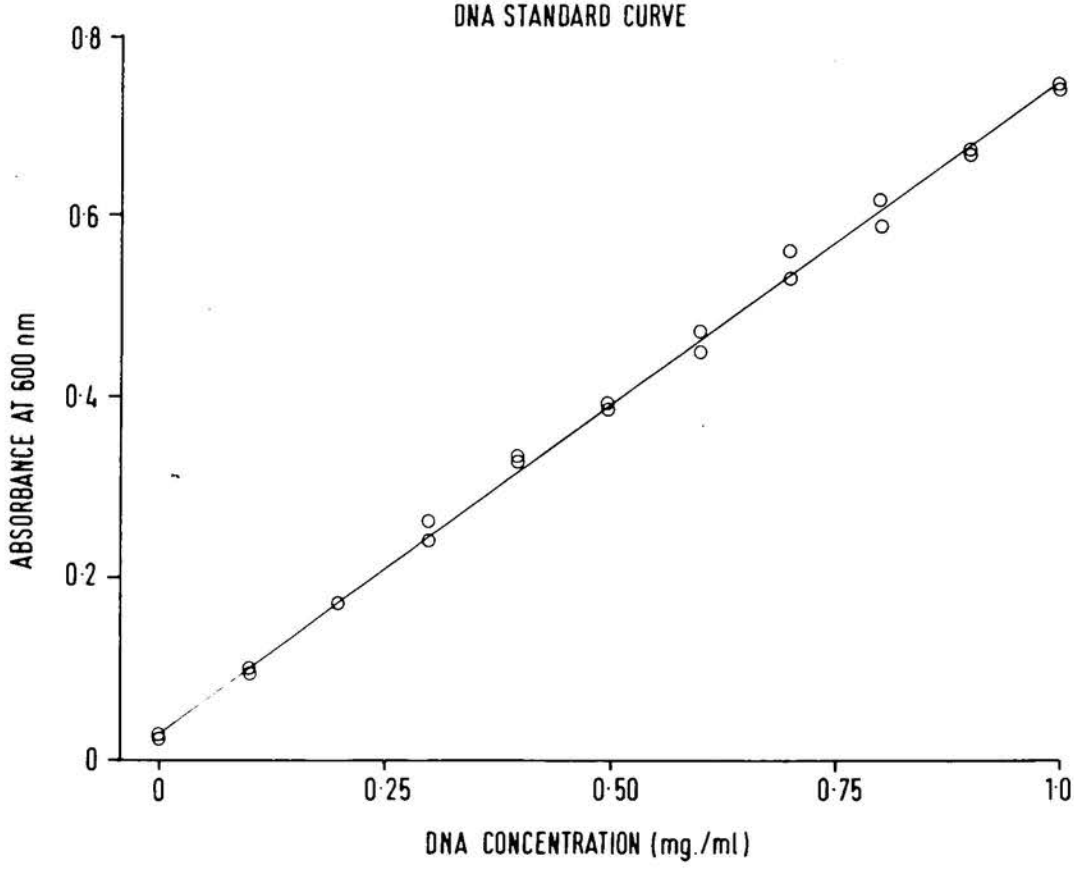
The orcinol reagent was prepared by dissolving one gram of orcinol, immediately before use, in 100 ml. of concentrated hydrochloric acid containing 0.5 g. of ferric chloride. 1.5 ml. of this reagent were mixed with 1.5 ml. of sample containing 50 to 200 μg. of RNA and heated for twenty minutes in boiling water. The samples were cooled in cold water and diluted with three ml. of a one to one mixture of concentrated hydrochloric acid and distilled water. Their green colour was read in an optical glass, 1 cm. cuvette against a distilled water reference in the Unicam SP 500 or the Unicam SP 600 Spectrophotometer at 660 nm.

A standard curve was prepared from yeast RNA as follows. 25 mg. of RNA were weighed into a 25 ml. volumetric flask, several ml. of 0.1 M. NaCl added, and the mixture left overnight in the refrigerator. The solution was diluted to the mark, and dilutions containing 20 to 200 μg. of RNA prepared and analyzed. The standard curve is shown in Figure B3. The remainder of the RNA solution was frozen as 1 ml. aliquots in sealed ampoules for later dilution and use as standard solutions. Each set of determinations was accompanied by a duplicate reagent blank and duplicate standard determinations.

The most common substance interfering with RNA estimation using orcinol is DNA. It gives a colour which is estimated at from 12.5% (W. C. Hutchison, E. D. Downie and H. N. Munro, 1962) to 20% (present work) of the colour given by an equal weight

FIGURE B4

FIGURE B4
DIPHENYLAMINE DNA ESTIMATION
DNA STANDARD CURVE



of RNA. Experiments showed that a solution of highly polymerized calf thymus DNA gave twenty percent of the colour developed by RNA in the same sample. All RNA estimations were therefore corrected using this factor and the DNA concentration of the samples estimated by the diphenylamine reaction. Most RNA estimates therefore became the difference between two large and similar numbers which must be considered as only rough estimates of the RNA present. Analyses performed in the presence of SDS showed that under the reaction conditions it yields a brown, oily substance which renders reading of the orcinol colour impossible.

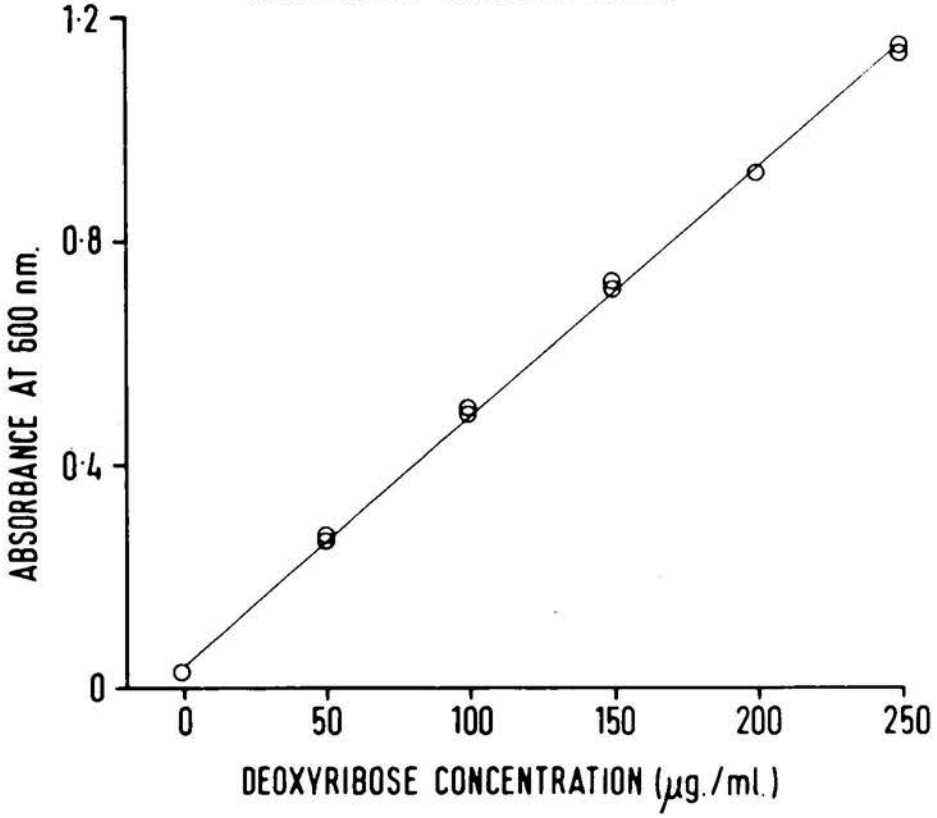
DNA Estimation

The diphenylamine reagent was prepared by dissolving one gram of diphenylamine (recrystallized from 70% ethanol) in 100 ml. of glacial acetic acid containing 2.75 ml. of concentrated sulphuric acid. 2 ml. of this reagent were mixed with 1 ml. of sample containing 50 to 1000 μg. of DNA and heated for ten minutes in boiling water. The samples were cooled in cold water and diluted with 3 ml. of a mixture of two volumes of glacial acetic acid to one volume of distilled water. Their blue colour was read using 1 cm. optical glass cuvettes against a distilled water reference in the Unicam SP 600 or the Unicam SP 500 Spectrophotometer at 600 nm.

Initially a standard curve was prepared using highly polymerized calf thymus DNA in NaCl solution as described above for the RNA standard curve. It was found, however, that the frozen DNA samples formed a gel which did not readily redissolve to give a homogeneous standard preparation. In addition, later work showed that chloride ions strongly inhibit the formation of the blue diphenylamine colour with DNA (see Appendix II) so that a standard curve prepared using a NaCl solution of DNA was not universally applicable. 25 mg. of the same DNA preparation were therefore allowed to swell in 0.5 M. perchloric acid, dissolved by heating at 70°C. for 15 minutes, and diluted to 25 ml.

FIGURE B5

FIGURE 85
DIPHENYLAMINE DNA ESTIMATION
DEOXYRIBOSE STANDARD CURVE



Dilutions of this standard solution were used to prepare the standard curve shown in Figure B4. To avoid the difficulty of preparing and preserving DNA solutions, a standard curve was also prepared using aqueous solutions containing 50 to 250 μg./ml. of crystalline 2-deoxy-D-ribose. It is shown in Figure B5. Duplicate deoxyribose standard samples were then analyzed with duplicate reagent blanks for each set of DNA determinations. Where limited quantities of sample were available, DNA estimations were performed using one-half of the reagent and sample volumes given above. This procedure did not influence the standard determinations.

Many authors (W. C. Hutchison and H. N. Munro, 1961; Z. Dische, 1967; G. Ashwell, 1957; and W. G. Overend, 1951) have shown that the intensity of the diphenylamine colour for any sample of DNA is a function of its base composition, all claiming that only purine-bound deoxyribose is chromogenic. This specificity may impose a further limitation on the analyses presented here of fractions from an enzymic digest of DNA. W. G. Overend (1951) also claims that histones and protamines give an appreciable colour with the diphenylamine reagent. This claim has not been substantiated during the present work.

B 2.4 Amino Acid Analysis

All amino acid analyses were made with a Locarte Amino Acid Analyzer using a Leeds and Northrop Recorder. The method was based on that of S. Moore, D. H. Spackman and W. H. Stein (1958).

Reagents:

A : Aristar concentrated hydrochloric acid diluted one to one with distilled water.

B : 0.2 M. sodium citrate, pH 2.20

C : 0.2 M. sodium citrate, pH 3.25, containing 0.11% thiodiglycol and 0.044% brij 35.

D : 0.2 M. sodium citrate, pH 4.25, containing 0.11% thiodiglycol and 0.044% brij 35.

E : 1.0 M. sodium citrate, pH 6.65, containing 0.044% brij 35.

F : 0.2 N. sodium hydroxide, containing 0.044% brij 35.

G : 4 M. sodium acetate, pH 5.5.

H : 3 l. methyl cellosolve plus 1 l. reagent G, plus 40 g. ninhydrin and 0.8 g. stannous chloride bubbled with nitrogen and kept in an atmosphere of oxygen free nitrogen.

The molarities quoted for the citrate buffers are given with respect to sodium ions, the buffers having been prepared from citric acid or trisodium citrate, sodium hydroxide and concentrated hydrochloric acid.

Dry samples estimated to contain 0.6 micromole of the most abundant amino acid were placed in new test tubes with 1 ml. of reagent A. The tubes were narrowed in a flame, the samples frozen with an acetone/solid CO_2 bath, evacuated to 0.05 mm. Hg, and the tubes sealed in the flame. The samples were digested for 24 hours in an oven at 105°C., and then dessicated in the opened tubes for 24 hours over concentrated sulphuric acid and caustic soda. Each sample was taken up in approximately 0.4 ml. of reagent B, centrifuged, and an aliquot of approximately 0.2 ml. applied to the 50 cm. by 1 cm. column of cross-lined polystyrene resin which had been equilibrated with reagent C. The sample

was forced into the column with oxygen-free nitrogen and then washed in with 20% redistilled methanol in reagent C. Elution was carried out at a column pressure of 100 to 150 pounds per square inch at 51°C. using buffer C for 60 minutes, buffer D for 50 minutes, and buffer E for 150 minutes. The eluate was mixed with reagent H, heated for 15 minutes at 100°C., and the colour recorded on a logarithmic scale at 440 nm. and 570 nm. A complete set of EEL standard solutions containing 0.4 micromoles of proline and 0.2 micromoles of every other amino acid was analyzed with each batch of the ninhydrin colour reagent in order to prepare a set of colour constants for that group of analyses. The column was regenerated by washing with reagent F and re-equilibrated with reagent C.

The calculation of amino acid composition was carried out as follows. A straight line was drawn through the base of as many peaks as possible in a series. The baseline usually rose a total of approximately 0.015 optical density units from aspartate to histidine, rose sharply during the elution of lysine and ammonia and fell again to a stable value during the elution of arginine. Where the baseline rose during the elution of a single amino acid the midpoint of the baseline was used for calculation purposes. The baseline value was subtracted from the peak maximum to give the peak height, and a line was drawn parallel to the baseline at one half the peak height above it. The number of spaces between recorder dots above this line was counted to one tenth of a space. The record of absorbance at 440 nm. was used for proline, and that at 570 nm. for all the other amino acids. The colour constants were calculated from the standard analysis by finding the product of peak height and spaces, and dividing it by the number of micromoles applied to the column for each amino acid. The quantity of micromoles of each amino acid in an unknown sample was then found by dividing the product of peak height and spaces by the appropriate colour constant. These

were then summed and the mole percent composition of each amino acid calculated. The calculations were performed by the Wang Calculator using a program written by Mrs. Fiona O'Brien. No corrections were made for hydrolytic losses.

Each of the traces showed a very small peak just before valine which was identified as cystéine by analyzing an appropriate standard solution. This amino acid was not included in the calculations of mole percents described above because of the likelihood of hydrolytic losses. The number of micromoles in these peaks was, however, calculated in the usual way and they were expressed as a percent of the total micromoles of the other amino acids. These results are given in the tables of amino acid composition as follows:

$0 < \%$ cystéine $\leqslant 0.5$ as +

$0.5 < \%$ cystéine $\leqslant 1.0$ as ++

$1.0 < \%$ cystéine $\leqslant 1.5$ as +++

$1.5 < \%$ cystéine $\leqslant 2.0$ as ++++

in order to distinguish them from the other values, while providing a basis for comparison between analyses.

B 3. FRACTIONATION METHODS

B 3.1 Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis, in the presence and absence of SDS and of urea, was used to fractionate and identify the nuclear proteins prepared in the course of this work.

Electrophoresis in Acetic Acid

The electrophoretic method employing acetic acid as buffer was based on that of S. Panyim and R. Chalkley (1969a). Unless otherwise stated all gels contained 15% acrylamide and were electrophoresed in 0.9 M. acetic acid. The gels were prepared as follows.

Reagents:

A : 60% acrylamide and 0.4% N,N'-bisacrylamide in deaerated distilled water.

B : 43.2% glacial acetic acid (v/v) and 4% N,N,N',N'-tetramethylethylenediamine in deaerated distilled water.

C : 0.2% ammonium persulphate in distilled water.

A and B are stable for several weeks if stored at 0°C., while C must be freshly prepared. All solutions were warmed to 25°C. before mixing.

For 7.5 cm. gels, 10 cm. lengths of 0.6 cm. diameter hydrometer tubing were cut and one end sealed with a triple layer of parafilm. These were fixed upright in a stand with pressure applied to the parafilm-sealed bottom end. The reagents were mixed in the proportions A:B:C::2:1:5 and 2 ml. poured steadily, without frothing, into each tube using a hypodermic syringe and large bore needle. Ether was layered over each gel to prevent contact with the air and they were left undisturbed for $1\frac{1}{2}$ to 2 hours. The parafilm was removed and they were stored in

0.9 M. acetic acid or treated as follows.

The gels were equilibrated by applying a voltage of 50 v. overnight or a current of 2 mA. per gel (about 80 v.) for 5 hours. Both reservoirs contained 0.9 M. acetic acid. The gels were again stored in 0.9 M. acetic acid or used for protein separations as follows.

The optimum range of sample size per gel was found to be 20 to 200 μg., depending on the number of protein species present and the desired loading. Although a very small sample volume (about 20 microlitres) is desirable to optimize band sharpness, it was found that for purposes of detection, where only dilute samples were available, volumes of up to 0.5 ml. could be used successfully. The sample was dissolved in 0.9 M. acetic acid containing 15% sucrose and an appropriate volume applied to the top of the gel with a pipette or syringe. 0.9 M. acetic acid was then layered over the sample until the tubes were full and both reservoirs were filled with this solution. The samples were electrophoresed for three to four hours at 2 mA. per gel. The gels were removed by cracking the glass tubes and stained overnight in a saturated solution of Amido Black 10B in a mixture of glacial acetic acid:methanol:distilled water in the proportions 1:5:5. They were then destained and stored in the same acetic acid:methanol:water mixture. An attempt was made early in the work to apply the ultra-micro staining technique for starch gels of M. Sung and O. Smithies (1969) to the polyacrylamide gels. The technique showed no promise after considerable examination, however, and as the original paper gave no indication of either its mechanism or its applicability to other systems it was not pursued further.

Electrophoresis in Sodium Dodecyl Sulphate

The method used for electrophoresis in the presence of SDS was based on that of K. Weber and M. Osborn (1969). The

gels contained 10% acrylamide and were electrophoresed in 0.1 M. phosphate buffer, pH 7.0, containing 0.1% SDS. On some occasions the gels and samples contained 6 M. urea in addition to the other reagents. Electrophoresis was carried out as follows.

Reagents:

A : 0.2 M. phosphate buffer, pH 7.0 and 0.2% SDS in deaerated distilled water.

B : Reagent A diluted 20 times and made 0.1% in SDS and 0.1% in 2-mercaptoethanol.

C : Reagent A diluted 20 times and made 1% in SDS and 1% in 2-mercaptoethanol.

D : Reagent A diluted one to one with distilled water.

E : 22.2% acrylamide and 0.6% N,N'-bisacrylamide in deaerated distilled water.

F : 1.5% ammonium persulphate in distilled water.

G : 10% N,N,N',N'-tetramethylethylenediamine in ethanol.

Reagent E must be stored in the dark at 4° C. while reagent F must be freshly prepared.

In order to prepare twelve gels, 10 cm. glass tubes were prepared as described above for electrophoresis in acetic acid and a mixture was prepared of 15 ml. A, 13.5 ml. E, 1.5 ml. F, and 0.045 ml. G, all at room temperature. The gels were poured and allowed to set for about 30 minutes as described above, again using ether to protect them from the air. They were stored in reagent A or used directly for electrophoresis.

Samples were prepared for electrophoresis by dissolving the protein at a concentration of 0.5 mg./ml. in reagent C and incubating the solution for 2 hours at 37°C. A mixture was then

prepared of 3 μl. of 0.05% Bromphenol Blue in water, 1 drop of glycerol, 5 μl. of 2-mercaptoethanol, 50 μl. of reagent B, and 10 to 50 μl. of the incubated protein solution. This mixture was applied to the gel tops and carefully covered with reagent D which was also used to fill the reservoirs. Electrophoresis was carried out at 5 mA. per gel for about four hours or until the Bromphenol Blue had migrated three quarters of the length of the gel. The gels were then removed from the tubes, stained and destained as described above for the acetic acid gels. Band sharpness was not found to be as good with the Coomassie Brilliant Blue stain used by Weber and Osborn as with the Amido Black.

B 3.2 Exclusion Chromatography

Exclusion chromatography has been employed extensively for the separation, fractionation, and purification of DNA-protein mixtures. The media used have included the cross-linked dextran, sephadex, in Grades G-25, G-50, G-75 and G-100, the cross-linked agarose, sepharose 6B, and beads of polyacrylamide gel, called bio-gel P10. Each of these was poured into a Pharmacia glass or acrylic plastic column according to the following procedure. The required quantity of medium was suspended in an excess of dilute (0.02 M.) NaCl solution and allowed to swell for several hours with occasional stirring. During this time the fines were repeatedly decanted from the settled gel and more suspending solution added. A thin slurry of the medium was carefully poured into the column which was partially full of saline and the gel particles were allowed to settle by gravitation until the supernatant was almost clear but a definite boundary had not yet formed between gel and supernatant. A slow drip from the column outlet was maintained throughout this procedure, but most of the supernatant was removed by siphoning. Additions of the slurry were repeated until the desired column height had been reached, when a reservoir was connected to the column input. It was arranged in relation to the 1.7 mm.

diameter teflon outlet tubing to provide sufficient hydrostatic pressure for the desired flow rate. A total column volume was always allowed to pass through the column before initial use and between runs. A sample of eluate was checked for ultraviolet absorbance before beginning each run to ensure that nothing remained on the column. All columns were maintained at room temperature and all buffer solutions were prepared with distilled water deaerated by boiling. Between runs a continuous, slow flow of eluant was maintained. No difficulty was experienced with declining flow rates except in the case of one sephadex G-100 column to which an excessive hydrostatic pressure had been applied. In this case the column was repoured. When the pouring medium and eluant were different, at least a column volume of the eluant was allowed to run through the column before it was used. In the case of elution with 2% SDS, the NaCl was first washed away with water and then the column was equilibrated with SDS.

The exclusion or void volume of each column was measured after its use as a fractionating column by measuring the elution volume of a small sample of Blue Dextran 2000 which had been allowed to swell and dissolve at a concentration of about 4 mg. per ml. in 1 M. NaCl or 2% SDS. This volume and the total column volume for each column are indicated on the figures in this thesis illustrating chromatographic separations by two downward-pointing arrows.

Samples were applied to the columns by allowing the eluant level to fall just to the surface of the gel and applying the sample slowly, dropwise, with an even distribution of drops over the gel surface. It was then washed into the column in the same manner with several small aliquots of eluant, avoiding any disturbance of the gel surface. Finally, the column was topped up with eluant and the reservoir reconnected. Fractions of appropriate size were collected with a Central, siphon-operated or a Gilson drop-

counting fraction collector.

The calculation of recoveries of the material applied to these columns presented serious difficulties. The problems inherent to the estimation of protein and nucleic acid have been discussed in conjunction with each estimation method. The molecular environment of each protein and nucleic acid molecule was very different after fractionation from that in the original sample, so that it was not feasible to assume consistency of extinction coefficient or chromogenicity in sample and fractions. In addition, the error introduced in correcting for a baseline or blank value when summing the content of a large number of fractions, often containing small quantities of material, must make a major contribution to the error of any calculated recovery. It is for these reasons that recoveries are not quoted for the chromatograms discussed in this thesis.

A further problem was the physical recovery of material from the chromatographic fractions which commonly contained 2% SDS, and sometimes veronal. The fractions containing protein or protein plus nucleic acid were recovered by precipitation with an excess of acetone according to the procedure used to recover histone from acid extracts of nuclei. Samples were made 0.2 N. in hydrochloric acid, precipitated with ten volumes of acetone and dried with washes of acetone and ether as described previously. The SDS remained in the acetone/water supernatant. The veronal/polynucleotide fractions were treated in a slightly different manner to avoid losses of low molecular weight material. Samples were acidified to pH 4 with hydrochloric acid and freeze-dried using the Edwards Freeze Drier, Model 10P. The residue was extracted with 95% acetone to remove the veronal and SDS, and the residue washed successively with acetone and ether and air dried. This procedure was practiced with a mixture of AMP, veronal and SDS in the proportions commonly found in the

chromatograms to ensure that it did not result in a loss of nucleotides.

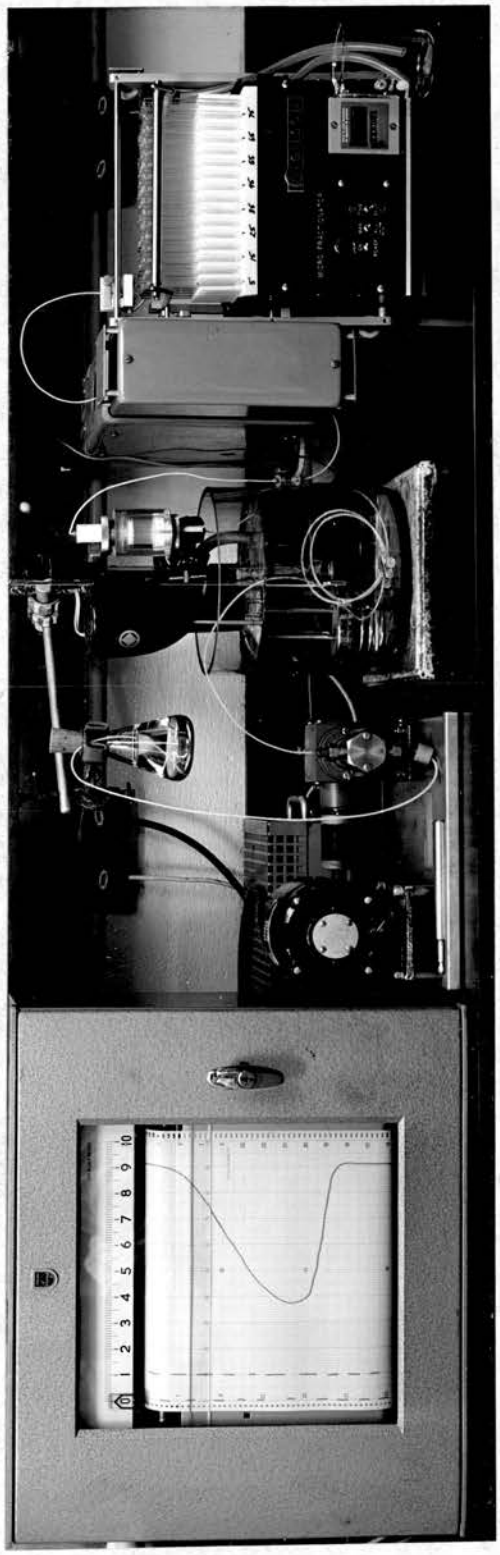
SECTION C

DESIGN AND OPERATION OF THE NUCLEAR EXTRACTION CELL - ITS PHYSICAL CHARACTERISTICS

FIGURE C1

FIGURE C1

THE EXTRACTION APPARATUS



SECTION C

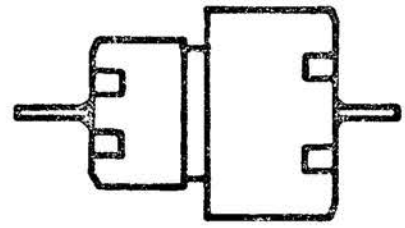
DESIGN AND OPERATION OF THE NUCLEAR EXTRACTION CELL - ITS PHYSICAL CHARACTERISTICS

C 1. THE FLOW SYSTEM

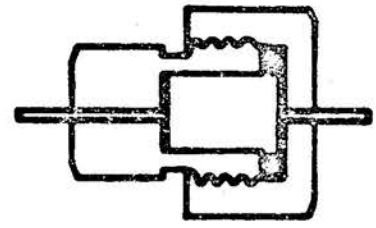
Nuclear extractions were performed using the extraction cell in conjunction with the flow system pictured in Figure C 1. Eluants were pumped by a Beckman Accu-Flow Pump through 1.7 mm. diameter teflon tubing (Beckman) from a reservoir to the extraction cell. On leaving the cell the eluate passed through similar teflon tubing to the flow cell of the Gilson Absorptiometer and from the absorptiometer to the Central Fraction Collector. The absorbance of the eluate at 280 nm. and the collection of fractions were recorded by the Phillips recorder. The extraction cell shown is the third of the three cells designed to meet the demands of the experiments as they progressed.

FIGURE C2

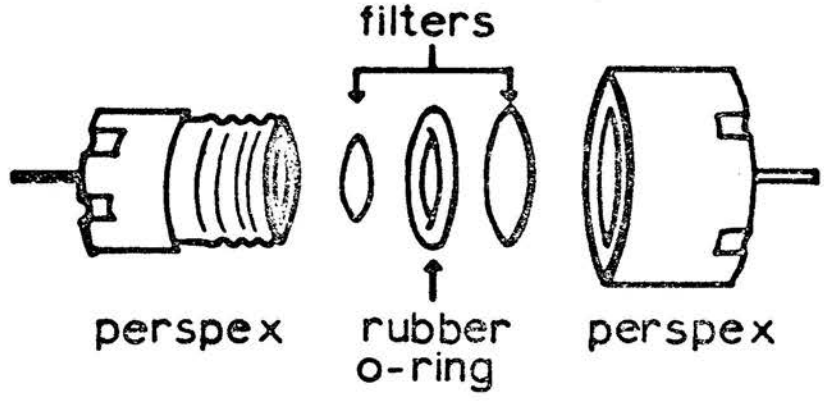
FIGURE C2 - CELL I



ASSEMBLED VIEW
(actual size)



LONGITUDINAL SECTION



EXPLODED VIEW

C 2. CELL I

The first extraction cell (Cell I) is shown diagramatically in Figure C2. It was made of perspex with a segment of 1.5 mm. diameter hypodermic needle cemented into each end. By warming the end of the teflon tubing in an alcohol flame until it became transparent and inserting the extension of hypodermic needle before the tubing could cool, the cell was integrated into the flow system. When the tubing had cooled, this gave a fairly secure push-fitting. The interior of the cell, which was about 1.5 ml. in volume, was sealed with a rubber O-ring and with a double thickness of Whatman No. 1 filter paper cut to fit each end.

Nuclei were introduced into this cell as follows. 25 mg. of dry, acetic acid nuclei were mixed thoroughly with 75 mg. of sephadex in order to isolate the nuclei. A minimum of 0.05 M. Tris buffer, pH 8, 0.1 M. in NaCl[1] was added to this mixture to give a thick slurry. The slurry was added to the input half of the cell, topped with filter paper, and sealed with the output fitting. A sequence of eluants such as pH 8 buffer, 0.1 N. HCl, and pH 8 buffer were pumped through the cell at a flow rate of about 10 ml. per hour. Complete extraction with each eluant was judged as return of the recorded ultraviolet absorbance trace to a stable baseline.

The above procedure was followed using sephadexes G-25, G-50, G-75 and G-100 as supporting medium for the nuclei. The protein/DNA/RNA distribution in the extracts did not vary with the sephadex type. The superfine grade of sephadex G-25 was

---

1. The reasons for the choice of each eluant mentioned in this Section and the properties of the eluted materials are described fully in the following Sections.

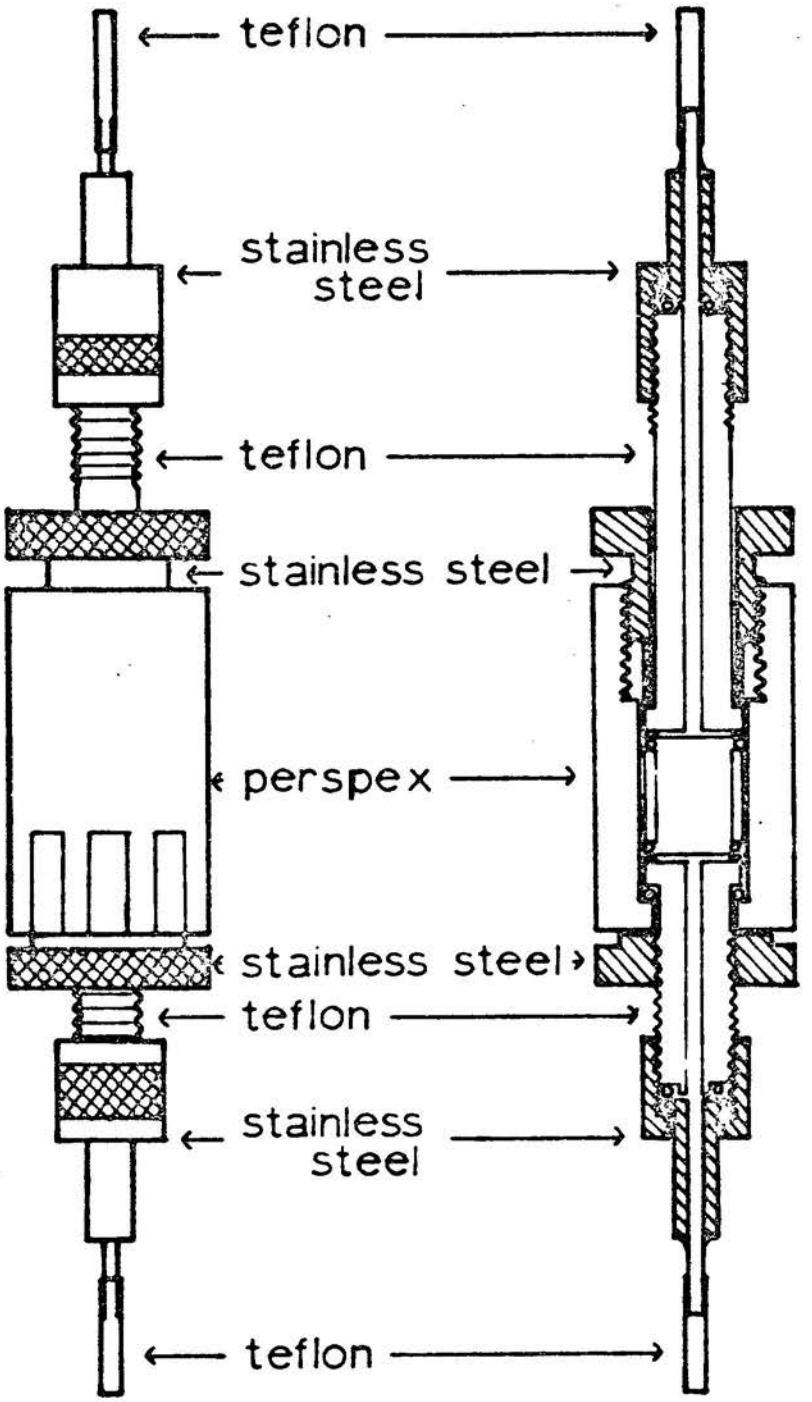
therefore chosen for further work on the basis that it would best resist the high pressures that might become necessary to produce a reasonable rate of solvent flow, that it was the closest in particle size (10 to 40 μmetres) to the nuclei themselves, and that it reached its final swollen volume very quickly in aqueous suspension. The sephadexes of lower porosity were avoided because of their high content of free carboxyl groups.

Extractions using this first cell were subject to a number of difficulties. In spite of care taken in filling the cell, air bubbles tended to become trapped inside the filters causing irregular flow pathways or a resistance to flow. It was difficult to completely transfer the wet sephadex/nuclei mixture to the cell, and once filled the cell tended to leak at the teflon/needle push fittings, the cell closing thread, and the filters, particularly when pressures were increased to overcome resistance to flow. A second extraction cell was therefore designed with a view to eliminating these problems.

FIGURE C3a

FIGURE C3a

CELL II - ASSEMBLED



(actual size)

C 3. CELL II

The second extraction cell (Cell II) is shown diagramatically in Figure C 3, where its specifications are given. Its most important features are as follows. The incorporation of millipore filter systems at the cell input and outlet effectively prevented escape of nuclei from the cell. When the stainless steel screw was tightened into the perspex casing, the interior of the cell (volume, approximately 1 ml.) was sealed by means of pressure exerted on the internal perspex cylinder, the teflon O-rings and millipore filters and the teflon plungers at each end of the cell. Finally, the modified connections with the teflon tubing enabled the cell to be introduced into and removed from the flow system without disturbing the push-fittings of teflon tubing on hypodermic needles. Thus, although the push-fittings were still present, they were not rapidly weakened by disconnection and reconnection.

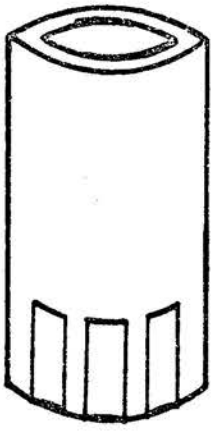
In an attempt to achieve more complete transfer of the nuclei to the cell, a new procedure was adopted for cell filling. 250 mg. of sephadex were mixed carefully with 25 mg. of dry nuclei and transferred to the interior of the partially assembled cell. Tris or veronal buffer (pH 8) was pumped very slowly up into the cell and added from a hypodermic syringe while the mixture was stirred with the hypodermic needle to prevent bubbles from becoming trapped. A little extra sephadex was sprinkled onto the top of the mixture to take up any excess liquid that had collected and the cell was sealed into the flow system by tightening the outlet filter system and teflon plunger against the perspex cylinder with the stainless steel screw.

The flow characteristics of the cell were now examined. The system was set up with sephadex only in the extraction cell and water as eluant. A series of solutions including tris buffers, HCl, and calf thymus histone solutions at various concentrations

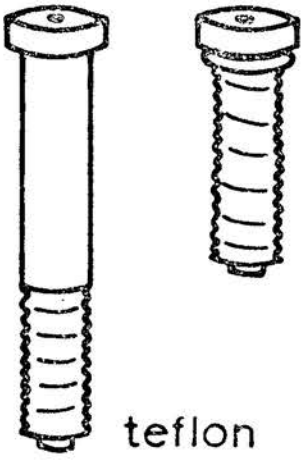
FIGURE C3b

FIGURE C3b

CELL II - COMPONENTS

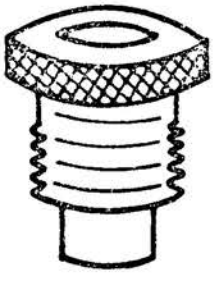


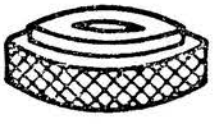
perspex casing and internal cylinder



teflon plungers

rubber o-rings 





stainless steel sealing screws and connection units

The central cylinder is sealed at each end with a 13.0 mm. millipore filter system, including teflon o-rings, stainless steel filter support and polyvinyl chloride filter.

were pumped from the reservoir through the cell. The rate at which the reservoir concentration was reached in the eluate was measured by collecting the eluate in 1 ml. fractions and measuring its Lowry colour. This showed that at least 6 ml. of solution were eluted before a new eluant concentration reached the fraction collector. In general the trace showed a lag volume of 3 to 4 ml. followed by a very rapid increase until the new concentration was reached at 6 ml. A much larger volume (10 to 20 ml.) was required to wash away the components of an eluant, the volume increasing with the concentration of solute in the system.

Exposure of the cell contents to the eluant was examined as follows. The cell was filled with sephadex and methylene blue was pumped through it as a short pulse. External observation showed uniform penetration of the dye through the cell as did examination of its contents. Elution of the dye from the cell was a very slow process, confirming the above results. In a second experiment the cell was filled as usual with nuclei and sephadex which were washed with tris buffer, a pulse of methylene blue, and more tris buffer. The central cylinder and filter systems were then removed from the cell, placed in a short, broad test tube and frozen solid by swirling the test tube in an acetone/solid CO_2 bath. The millipore filters were removed and smears made from their surfaces for microscopic examination. The solid core of nuclei and sephadex was gradully pushed from the cylinder with a 'tufnol' plunger and sectioned with a razor blade, giving a series of approximately 1/16 inch sections which were smeared onto glass slides. Microscopic examination of all the smears showed an even distribution of nuclei throughout the cell with no tendency to collect at the output filter.

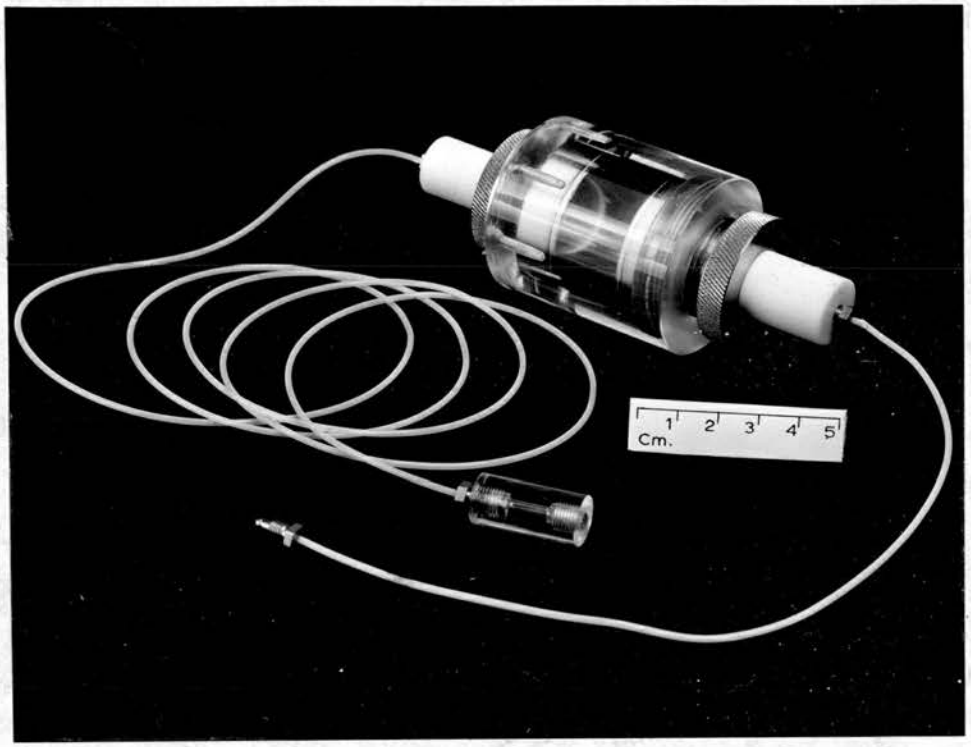
Cell II was used extensively to produce enzymic digests whose characterization is described in Section D. The problem of leakage at the cell connections remained, particularly when

increased pump rates were required to maintain high flow rates during enzymic elution. In addition, the small quantities of some digest fractions began to hamper characterization studies. Cell III was designed to deal with these problems.

FIGURE C4

FIGURE C4

CELL III - ASSEMBLED



C 4. CELL III

Cell III is shown in Figures C 4 and C 5 and in Figure C 1. The design is based on that of Cell II with the following modifications. The push-fittings of teflon tubing over hypodermic needle segments were replaced by the fittings for teflon tubing supplied by Beckman for use with the Accu-Flow Pump in chromatographic systems. These can be seen in Figures C 4 and C 5. The internal diameter of the chamber in Cell III is 2 cm., and it was anticipated that eluant from a single point source might not spread uniformly across this wider area. A cone shaped cavity was therefore made in the teflon plunger at the cell input and filled with a teflon cone channelled and tunnelled to spread the flow over the surface of the input filter. This system was tested and found to be effective as long as the cell was completely filled with sephadex. Uniform flow through the cell was partially dependent on the back pressure produced by the sephadex flow barrier, while over filling with sephadex blocked the cell. The improved cell connections now made it possible to control the elution temperature by submerging the cell and a coil of input tubing in a water bath with a Circotherm temperature control and a refrigeration unit for counter-cooling. All experiments with this cell were carried out at $15.0 \pm 0.5^{\circ}$ C.

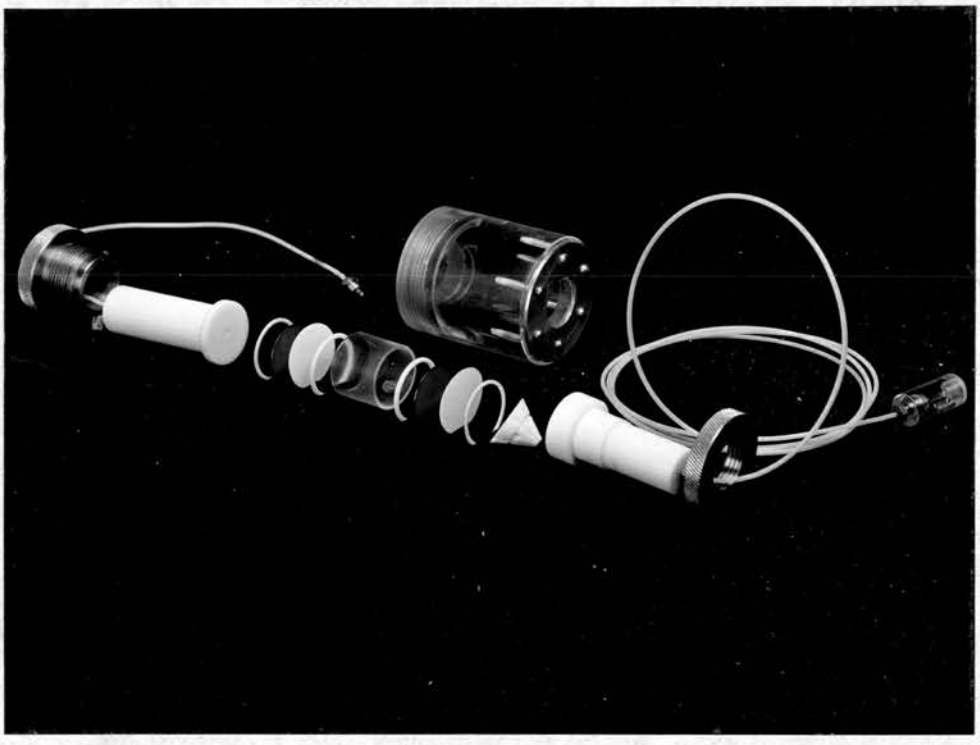
Cell III had an internal volume of about 8 ml. and it held 200 mg. of acetic acid nuclei mixed with 2 g. of sephadex. These were mixed and added to the cell as described above. Elution was begun either by wetting the dry mixture with buffer using a hypodermic syringe and needle as before, or by closing the cell, holding it vertical, and slowly pumping buffer up through the mixture which swelled as it absorbed the liquid. In either case, filling the cell was an exacting procedure which determined the success or failure of an experiment by permitting or not permitting adequate flow. The minimum flow rate required for successful

FIGURE C5

FIGURE C5

CELL III - COMPONENTS

(EXPLODED VIEW)



enzymic elution with this cell was 80 ml./hr.

All of the experiments involving sucrose nuclei were performed using Cell III. These more fragile nuclei presented special difficulties in operation of the extraction cell which are described fully in Section F. It suffices, here, to say that once again the problem was one of maintaining an adequate rate of flow through the cell.

Once again, blockage of Cell III with sephadex and nuclei was a serious problem. As pressure increased within the cell leaks developed, particularly at the connections with the teflon tubing. This weakness was largely due to wear on the threads in the teflon plungers and could be corrected by cementing them in place. The cell was then disconnected and reconnected by way of the perspex connecting pieces shown in Figures C4 and C5. The problem could also be solved by adding stainless steel inserts into which the threads for the connecting units would be cut, thereby avoiding wear on the soft teflon parts.

In summary, the major technical problems encountered during this phase of the work have been to achieve an acceptable and constant rate of flow through the extraction cell and to ensure uniform exposure of its contents to each eluant. The latter problem has been solved. The former, however, continues to be a major obstacle to successful application of the technique.

SECTION D

THE FRACTIONATION OF ACETIC ACID NUCLEI:

DEVELOPMENT OF THE METHOD

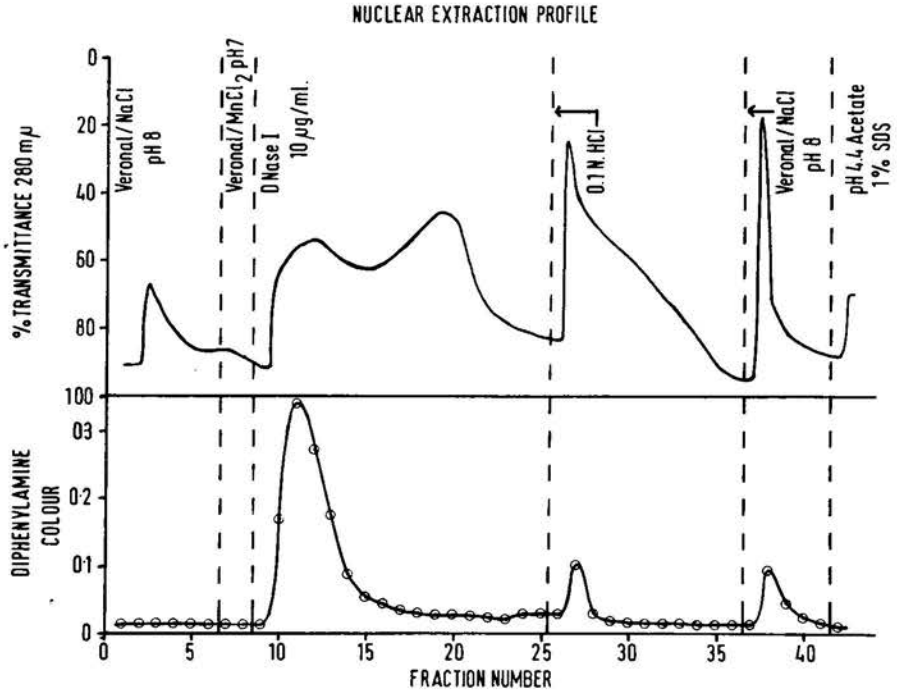
SECTION D

THE FRACTIONATION OF ACETIC ACID NUCLEI: DEVELOPMENT OF THE METHOD

D 1. INTRODUCTION

The fractionation method includes two main stages, the elution of nuclear fractions using the extraction cell, and secondary fractionation and characterization of these fractions. The operation of the extraction cell is described in the previous section. This section describes the choice and use of elution media and sequences, and the development of techniques for eluate characterization.

FIGURE D1


NUCLEAR EXTRACTION PROFILE
% TRANSMITTANCE 280 mμ
0
20
40
60
80
100
Veronal/NaCl pH 8
Veronal/MnCl2 pH7
DNase I
10 μg/ml.
0.1 N. HCl
Veronal/NaCl pH 8
pH 4.4 Acetate 1% SDS
DIPHENYLAMINE COLOUR
0
0·1
0·2
0·3
0
5
10
15
20
25
30
35
40
FRACTION NUMBER

D 2. NUCLEAR EXTRACTION SEQUENCES

D 2.1 Introduction

Each experiment began with the extraction of an aliquot of nuclei using a series of solvents according to the procedure described in Section C. The eluate was characterized initially by correlating its ultraviolet absorbance record with chemical analyses for protein and the nucleic acids. Figures D1 and D2 show the ultraviolet absorbance and analytical profiles from a typical experiment in which 25 mg. of nuclei were eluted using Cell II with the sequence of solvents shown in the figures. (The DNA profile is shown in both to allow its comparison with the absorbance profile). In later experiments the eluate produced by each extraction medium was collected and analyzed in bulk rather than as a series of fractions. Results from an experiment of this type in which 200 mg. of nuclei were eluted using Cell III are given in Table D1. The choice and use of various extraction media will be discussed with reference to these data.

D 2.2 Suspension and 'Wash'

The first step of each extraction was to suspend and wash the nuclei with a suitable buffer, thereby regenerating the dried, acetic acid nuclei. The initial composition of the wash buffer was 0.05 M. tris/HCl, pH 8.0, 0.1 M. in NaCl. The pH and ionic strength were designed to solublize ribonucleoprotein without disrupting chromatin structure. Tris was found to give a significant colour with the Lowry reagent so it was replaced by a veronal buffer with the composition 0.04 M. veronal/HCl, pH 8.0, 0.1 M. in NaCl. Both of these buffers eluted RNA and protein but no DNA.

When enzymic elution later required 0.02 M. buffer at pH 7.0 as the enzyme medium, the wash buffer composition was changed to 0.02 M. veronal/HCl, pH 7.0, 0.12 M. in NaCl. This change was designed to simplify the extraction sequence by avoiding

FIGURE D2

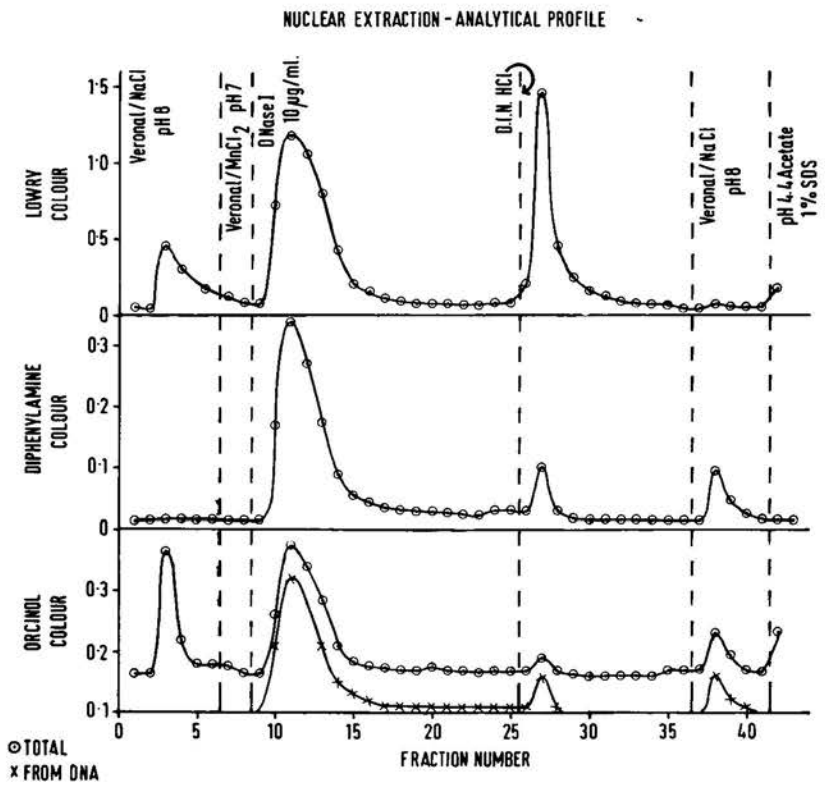

NUCLEAR EXTRACTION - ANALYTICAL PROFILE
LOWRY COLOUR
DIPHENYLAMINE COLOUR
ORCINOL COLOUR
Veronal/NaCl pH 8
Veronal/MnCl2 pH 7
DNase I 10 µg/ml.
D.I.N. HCl
Veronal/NaCl pH8
pH 4.4 Acetate 1% SDS
⊙ TOTAL
× FROM DNA
FRACTION NUMBER

TABLE D1

TABLE D 1

NUCLEAR EXTRACTION RESULTS

EXPERIMENT A IV

200 mg. of dry nuclei ⟶ pH 7 Veronal Wash + DNase I Digest + Residue Soluble in 2% SDS + ~~Unextracted~~ Insoluble Residue												
containing		containing			containing			containing			containing	
Species	X	A	B	C	A	B	C	A	B	C	A[d]	B
DNA	78	0	0	0	55	71	63	33	42	37	-	-
RNA	6	5	83	100	a	-	-	a	-	-	1	17
Histone	54	c	-	-	27[b]	50	43	35[b]	65	57	-	-
Other	62	5[c]	8	100	b	-	-	b	-	-	57	92
Total Protein	116	5	4	8	27	23	40	35	30	52	49	43

TABLE D1

COLUMN HEADINGS

X Composition of nuclei by analysis (mg. in 200 mg. of nuclei).

A Quantity in mg.

B The percent the quantity in column A represents of that in column X.

C The percent the quantity in column A represents of the total quantity in nuclear extracts.

NOTES

a Orcinol Analysis for RNA impossible because of interference by SDS.

b. Most of the protein in the digest and SDS soluble residue has been shown by electrophoresis to be histone.

c This uncharacterized protein material is arbitrarily classed as 'other'.

d These values are calculated as the difference between the quantities in column X and the summed extract analyses in columns A.

e Total Protein = Histone + Other.

a change in buffer concentration and pH. The new wash buffer (used in the experiment described by Table D1) eluted a smaller proportion of RNA than had the previous buffer. Although this did not appear to influence the quantity or type of components eluted by subsequent eluants, it did cause a more gradual release of UV absorbing material by deoxyribonuclease. Neither type of 'wash' has been further characterized.

Autolysis of the nuclei during the course of an extraction was anticipated as a major drawback to this fractionation method. In order to estimate the significance of autolysis, nuclei that had been washed were repeatedly incubated for periods of ten to thirty minutes at room temperature and then washed again with a pulse of buffer. Although some light absorbing material proportional in quantity to the time of incubation was eluted at each stage, the total quantity (by calculation, for an estimated total extraction time of 3 hours) was insignificant in comparison to that intentionally eluted by other means for comparable incubation and extraction times.

All experiments with Cells I and II were carried out at room temperature (15 to 20° C.), while those using CellIII were completed at 15.0° C. Temperature variations are known to dramatically influence the structure of chromatin components so this temperature was chosen as a compromise between limiting autolytic activity and allowing intentional enzymic elution.

D 2.3 The Choice of Deoxyribonuclease I as Eluant

Preliminary experiments with Cell I using the extraction sequence pH 8 buffer, 0.1 N. HCl, pH 8 buffer had, interestingly, yielded fractions containing RNA plus protein, protein, and DNA plus protein respectively. The object of this research was, however, to accomplish a nuclear fractionation without resorting to such chemical extremes as extraction with 0.1 N. acid. This sort of sequence was not, therefore, pursued. Instead, the next

step was to attempt an enzymic fractionation of the nuclei using a deoxyribonuclease.

The choice of enzyme was dictated by several factors. It must be active under conditions not disruptive to chromatin structure, it must be relatively non-specific in order to cause maximal degradation of all accessible DNA sequences, and it must be readily available as a highly purified preparation free of contamination by other enzymes, particularly proteases. The enzyme which best met these requirements was Worthington's electrophoretically purified preparation of bovine pancreatic deoxyribonuclease I. The available information concerning this enzyme has been thoroughly reviewed by M. Laskowski, Sr. (1967 and 1971). It is a glycoprotein of molecular weight approximately 30,000 consisting of three components separable by ion exchange chromatography. Its kinetics and specificity are complex functions of the composition of its medium, both in terms of substrate and of ions. In general, however, it is an endonuclease active at neutral pH, causing single and double-strand scissions at the 3' ribose-phosphate linkage of, preferably, double stranded DNA. It therefore shows autoretardation caused by the gradual disappearance of the preferred double stranded substrate and concomitant increase in concentration of the less susceptible single stranded substrate. It is activated by divalent cations which cause qualitative changes in its base specificity depending on the combinations and concentrations of ions used and on the stage of the hydrolysis reaction considered (see V. Desreux, R. Hacha and E. Fredericq, 1962; J. Shack and B.S. Bynum, 1964; and F.J. Bollum, 1965). Mn^{++} ions seem to confer the lowest specificity of any ion used on its own. Although there has been some controversy on this point, the limit hydrolysis products of DNase I digestion are now thought to be mono, di and trinucleotides. NaCl is thought to inhibit the enzyme at concentrations greater than 0.05 M. (M. Laskowski, Sr., 1967).

As was mentioned above, the medium chosen for enzymic extraction was 0.02 M. buffer, pH 7, μM. in $MnCl_2$. Although the ionic strength of this medium was rather low for the maintenance of chromatin structure, it was necessary to avoid inhibition of the enzyme and should, in any case, increase the accessibility of the DNA by causing the chromatin to swell. The Mn^{++} ions, on the other hand, should counteract this process. DNase I is unstable in dilute aqueous solution so all solutions used in this work were freshly prepared.

D 2.4 Enzymic Elution

Prior to enzymic extraction the washed nuclei were equilibrated with the enzyme buffer by passing a few volumes of it through the cell. Initially it was composed of 0.02 M. tris/HCl, pH 7.0, μM. in $MnCl_2$ and later of 0.02 M. veronal/HCl, pH 7.0, μM. in $MnCl_2$. This was followed by the same buffer containing 10 μg. per ml. of deoxyribonuclease I. Early experiments using 5, 10 and 20 μg. of the enzyme per ml. showed 10 μg. per ml. to degrade the DNA at the most convenient rate, yielding a digest containing both DNA and protein. Enzymic elution repeatedly produced a dual-peaked ultraviolet absorbance record which became a single peak in later absorbance or analytical profiles (see Figure D1). A nucleo-protein precipitate always appeared spontaneously in the eluate after fractions had been collected, and careful scrutiny showed that a similar precipitate was gradually forming and redispersing in the absorptiometer flow cell. It caused a non-specific light scattering effect which appeared as a second peak on the recorder trace. This precipitation effect was eliminated by maintaining the flow rate during enzymic elution above about 13 ml. per hour for Cell II and 80 ml. per hour for Cell III. Enzymic elution was continued until the absorbance trace had returned to a stable baseline. This baseline was always at a slightly higher absorbance than that prior to enzymic elution. Increasing the enzyme concentration to 20 μg. per ml. at this stage of one experiment did not cause a significant increase in

TABLE D2

TABLE D2

THE EXTENT OF DNA ELUTION WITH DEOXYRIBONUCLEASE I

(25 mg. of nuclei, containing 9.8 mg. of DNA by analysis, were extracted in each case.)

Experiment	Enzyme Concentration (μg./ml.)	DNA Content of:		% Digest Represents of:	
		Digest (mg.)	$HClO_4$ Ext. (mg.)	DNA Content	DNA in Extracts
1	20	5.5	0.6	56	90
2	10	5.5	0.5	56	92
3	5	4.8	1.6	49	75

the rate of release of UV absorbing material.

D 2.5 Extent of DNA Elution with DNase I

When the absorbance trace had returned to a stable baseline, the next step was to elute the DNase resistant nuclear residue with reagents that would release the remaining nuclear components. Before such a sequence could be developed, however, an estimate of the extent to which enzymic elution had released the nuclear contents was required. Analysis of the remaining cell contents for DNA by extraction with perchloric acid and diphenylamine analysis indicated that 90% of the DNA extracted was in the digest fraction. (A perchloric acid extract of sephadex gave no diphenylamine colour.) A similar calculation based on the DNA content of the 25 mg. of nuclei added to the cell suggested that only about 60% of the DNA was eluted by the enzyme (see Table D2). The validity of the first value was questionable because of the difficulties in obtaining precise analytical results by these methods, particularly where the quantities of material are small. On the other hand an exact knowledge of the quantity of DNA subjected to extraction was prevented by the cell filling problems described in Section C. It was possible that losses of nuclei during cell filling led to an erroneously low value for the extent of enzymic elution. In any case, elution of DNA by deoxyribonuclease did not appear to be complete, but further analyses were postponed pending development of a larger extraction cell in the use of which quantities would not be so limiting, nor losses during cell filling so significant.

2.6 The Choice of a Post-Enzymic Eluant

Eluting the DNase resistant nuclear residue with 0.1 N. HCl showed that a significant quantity of histone remained after DNase elution, while subsequent extraction with pH 8 buffer/NaCl released a small fraction of DNA, giving a pattern analogous to the behaviour of nuclei untreated with enzyme. Finally, elution with the detergent SDS, although it began to elute a final fraction, caused disconnection

of the cell and termination of the experiment (see Figures D1 and D2). Once again these chemical extremes were undesirable in their possible effects on the residual nuclear components, but further development of an extraction sequence for the DNase resistant residue was suspended at this stage pending characterization of the DNase digest. This proved to be a time consuming task, so that in fact only a rather crude analysis of the nuclear residue was possible. 2% SDS was known to dissolve chromatin, so extractions were performed using the scaled up extraction cell (Cell III) and the DNase resistant cell contents were suspended overnight in 2% SDS in an attempt to solublize the remaining nuclear components. The extract was collected by centrifugation and its nucleic acid and protein content measured, giving the results shown in Table D1. Although there is still a discrepancy between the extents of DNA elution based on nuclear DNA content and on the total extracted DNA, the two values are closer and suggest that the enzyme releases only about two-thirds of the nuclear DNA. This method also accounts better for the quantity of DNA present in the system, although it does not succeed in solublizing all of the nuclear protein. The significance of these quantitative results will be discussed further in Sections E and G.

The development of techniques for further characterizing the nuclear digests and SDS-soluble nuclear residues is described in Section D3 below. The results of their application to acetic acid and sucrose nuclei are given in Sections E and F respectively.

D 3. THE CHARACTERIZATION OF NUCLEOPROTEIN FRACTIONS

D 3.1 Introduction

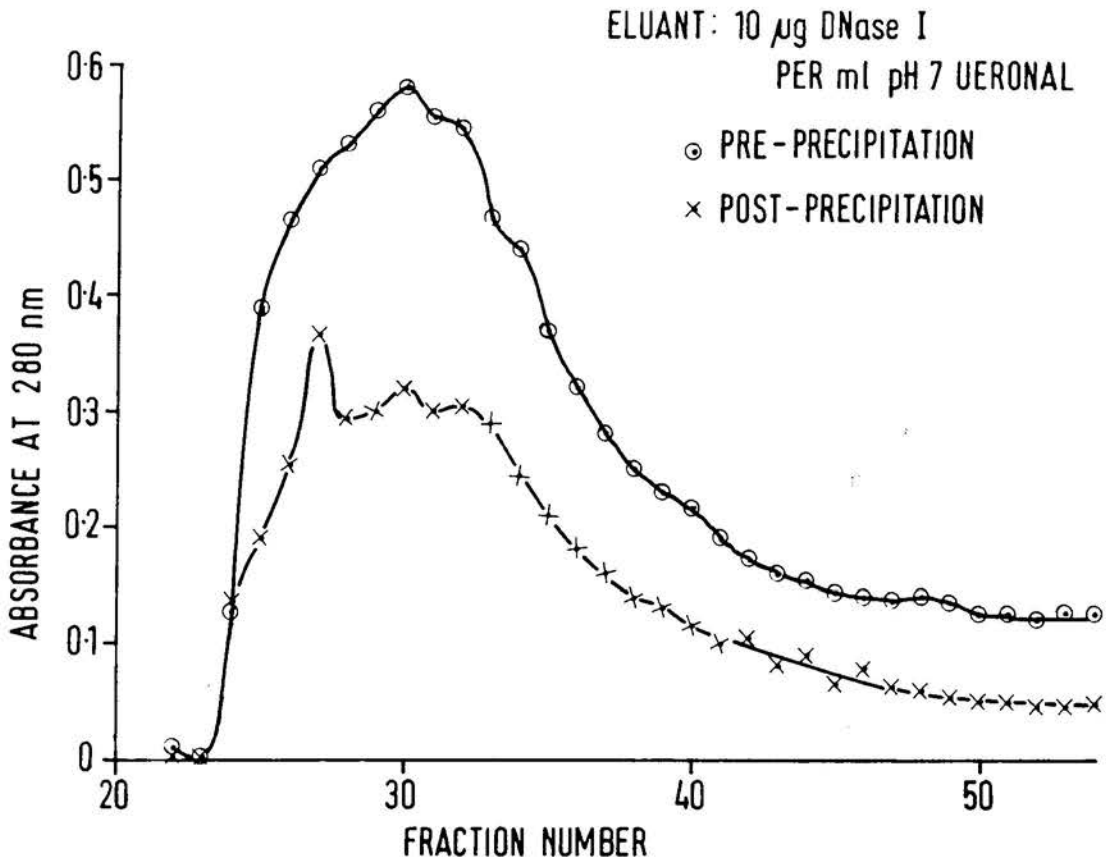
The second stage of each nuclear fractionation was to characterize the fractions eluted from the extraction cell. For fractions containing only protein this involved recovery of the protein for electrophoresis and amino acid analysis. The DNase I digest fractions, however, contained a mixture of nucleic acid and protein, so characterization was complicated by the necessity to separate these components before the usual methods could be employed. Protein characterization took priority over DNA characterization, since the dearth of knowledge regarding the structure of eukaryotic DNA would prevent interpretation of information about the DNA except as it was related to protein composition and structure. The problem became, therefore, to rid the proteins in the nuclear digest of nucleic acid while preserving them for later characterization steps.

D 3.2 Direct Electrophoresis of Fractions

During early experiments such as the one shown in Figures D1 and D2, an aliquot of each digest fraction was electrophoresed directly in a polyacrylamide gel. Using a modification to the standard electrophoresis sample application procedure, 0.4 ml. of the digest fraction were mixed on top of an acetic acid gel with 0.13 ml. of saturated aqueous urea, 2 M. in acetic acid and 0.1 N. in hydrochloric acid. The proteins were run into the gel for ten minutes using a filter paper wick as electrical connection between sample and reservoir, the sample removed, and the gel electrophoresed in 0.9 M. acetic acid as soon as eight fractions had been treated in this manner. These experiments showed the presence of all the histone fractions in the digest and suggested that there might be a continuous change from a predominance of histone fraction F1 at the beginning of digestion to its absence late in digestion.

FIGURE D3

FIGURE D3 - ENZYMIC EXTRACTION PROFILE



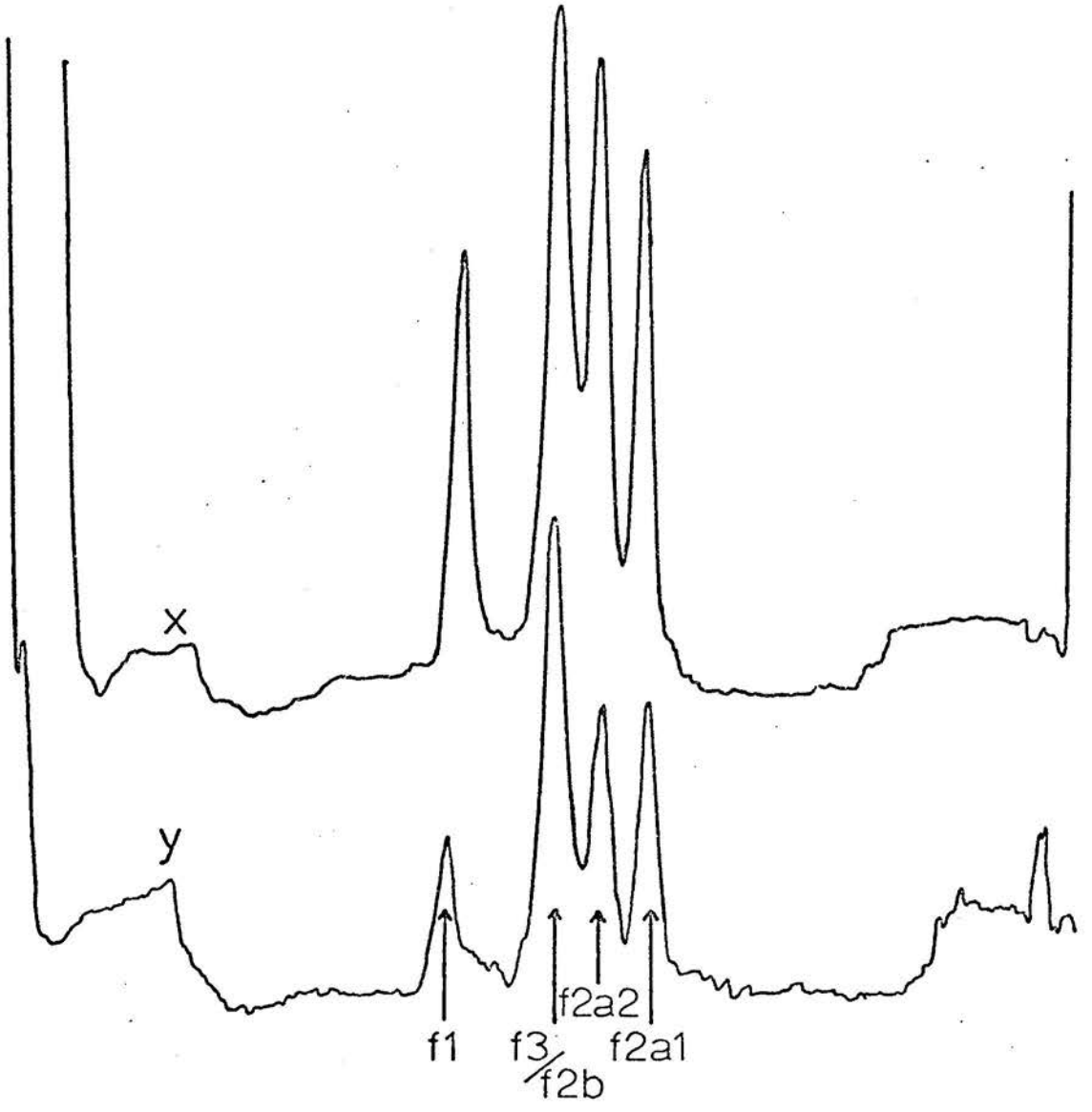
Spontaneous precipitation of nucleoprotein from the digest was mentioned above as interfering with the UV absorbance monitoring of nuclear extractions. It also complicated further analysis of these fractions. The data presented in Figures D1 and D2 were obtained by sampling each fraction prior to precipitation for each of the three colorimetric analyses and for electrophoresis. During enzymic elution of another aliquot of nuclei measurements of the light absorption at 280 nm. by each fraction before precipitation and by the supernatant from each fraction when precipitation had proceeded to completion (overnight in the cold) gave the curves shown in Figure D3. It is evident from these data that not all of the digest components were precipitated, and chemical analysis of total digest precipitates and supernatants prepared in this way suggested that each contained DNA and some protein. These results corroborated earlier tests which showed the precipitate to be insoluble in acid but soluble in base or 1 M. NaCl, and to give an absorption spectrum characteristic of nucleic acid.

The presence of nucleic acid in the digest fractions and the insolubility of the components in acid cast doubts on the electrophoretic results described above. The effect on electrophoresis of the nucleic acid is difficult to assess, but the apparent variation in histone composition of the digest fractions could reflect selective precipitation of a homogeneous histone population by a varying proportion of DNA (see, for example, T. A. Hoare and E. W. Johns, 1971). The significance of the fractionation provided by the spontaneous precipitation of the digest components was not clear, but in an attempt to elucidate this phenomenon and the electrophoretic results further separate characterization of the precipitate and supernatant was undertaken. The precipitate and supernatant from entire digestions were pooled in these experiments to provide larger quantities of material for analysis.

FIGURE D4

FIGURE D4

DISC GEL ELECTROPHORESIS



X Acid Extracted Protein of Nuclear Digest, Precipitate Fraction

y Crude Calf Thymus Histone

(Gels were scanned for their absorbance at 265 nm. by a Joyce-Loebl Gel Scanner using a Servoscribe Recorder with full scale deflection set at O. D. 5. 0.)

D 3.3 Chemical Analysis of the Digest Precipitates and Supernatants

Extraction of the pooled precipitate with 0.1 N. HCl yielded a protein fraction which, when recovered by acetone precipitation and electrophoresed, contained all of the histone fractions in roughly their usual proportions (see Figure D4). Extraction of the acid insoluble residue with 2 M. NaCl solublized only DNA, leaving a large insoluble nucleoprotein component. Analysis of the sodium chloride extract lead to discovery of the influence of inorganic ions on the diphenylamine reaction (see Appendix II).

Once again, acid treatment had rendered nuclear components insoluble and intractable to further study, so another chemical fractionation procedure that avoided acid treatment was applied to both digest and residue. The procedure is shown in Figure D5 with the quantities of material derived in this way from a single nuclear digest. The supernatant was concentrated by freeze drying and the polynucleotide fraction extracted with water from the large excess of less soluble veronal. This and other methods failed to detect the supernatant protein mentioned above. A part of this DNA component was dialyzable. 1 M. NaCl solublized most of the nucleoprotein precipitate, but a small insoluble residue was dissolved in NaOH for chemical analysis. These results show that while the DNA was divided between supernatant and residue, the protein was concentrated in the NaCl-soluble precipitate fractions. The major DNA and nucleoprotein fractions were next subjected to further analysis by exclusion chromatography.

D 3.4 Chromatographic Fractionation of the Digest Precipitate and Supernatant

The chromatogram of the water extract of a lyophilized supernatant fraction on Sephadex G-50 (Fine) is shown in Figure D6. The polynucleotides are eluted as a single broad peak almost coincident with the peak of veronal. (See the discussion of veronal light

FIGURE D5

CHEMICAL FRACTIONATION OF THE NUCLEAR DIGEST

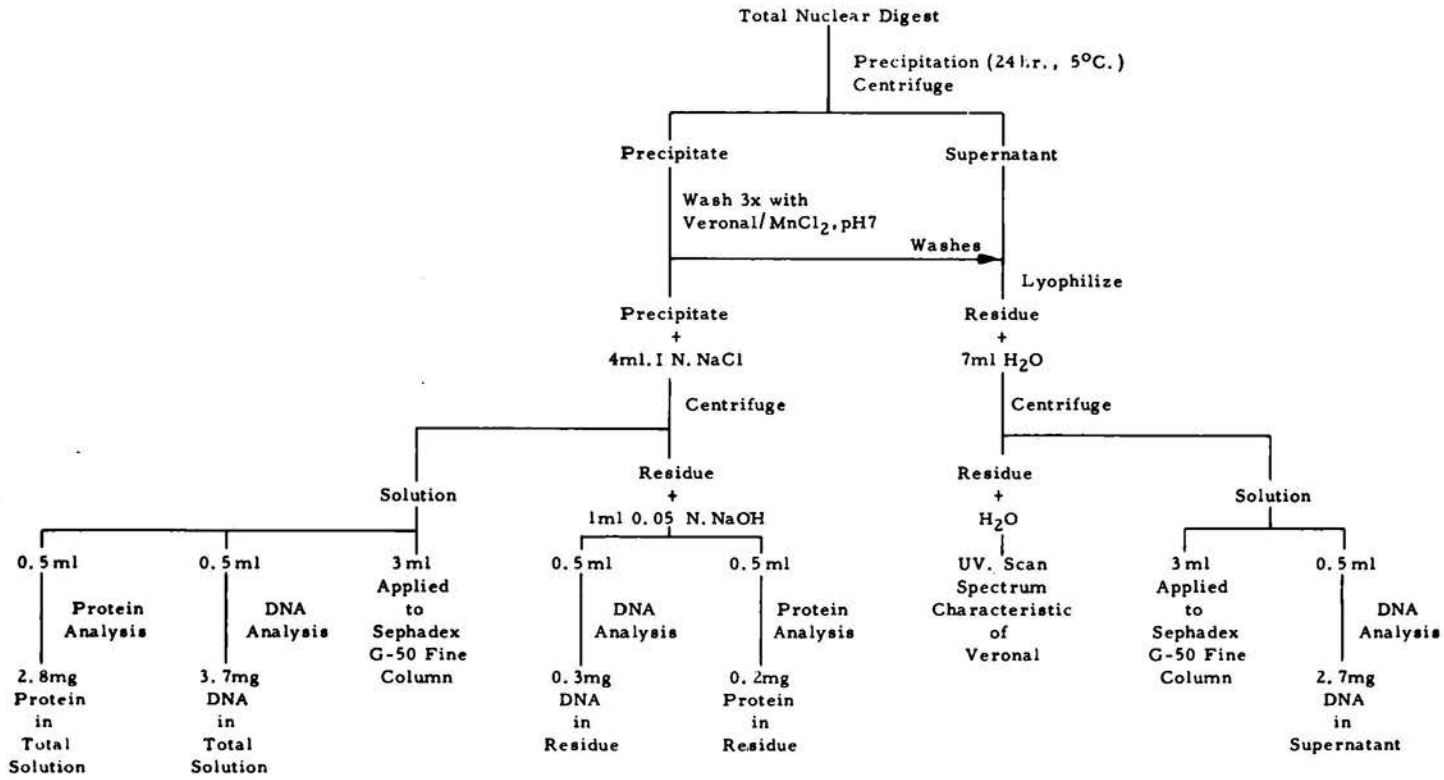


FIGURE D6

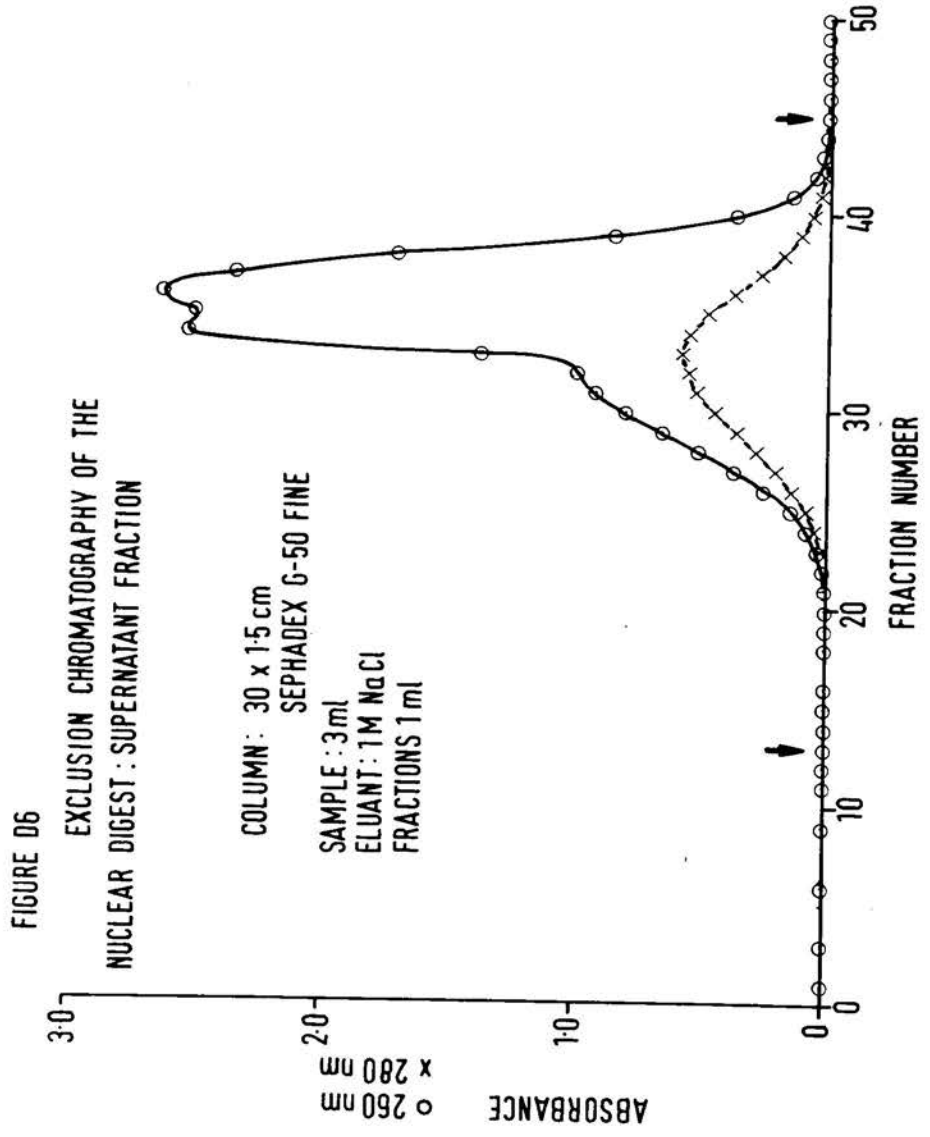


FIGURE D6
EXCLUSION CHROMATOGRAPHY OF THE
NUCLEAR DIGEST : SUPERNATANT FRACTION
COLUMN : 30 x 1·5 cm
SEPHADEX G-50 FINE
SAMPLE : 3 ml
ELUANT : 1M NaCl
FRACTIONS 1 ml
ABSORBANCE
o 260 nm
x 280 nm
3·0
2·0
1·0
0
0
10
20
30
40
50
FRACTION NUMBER

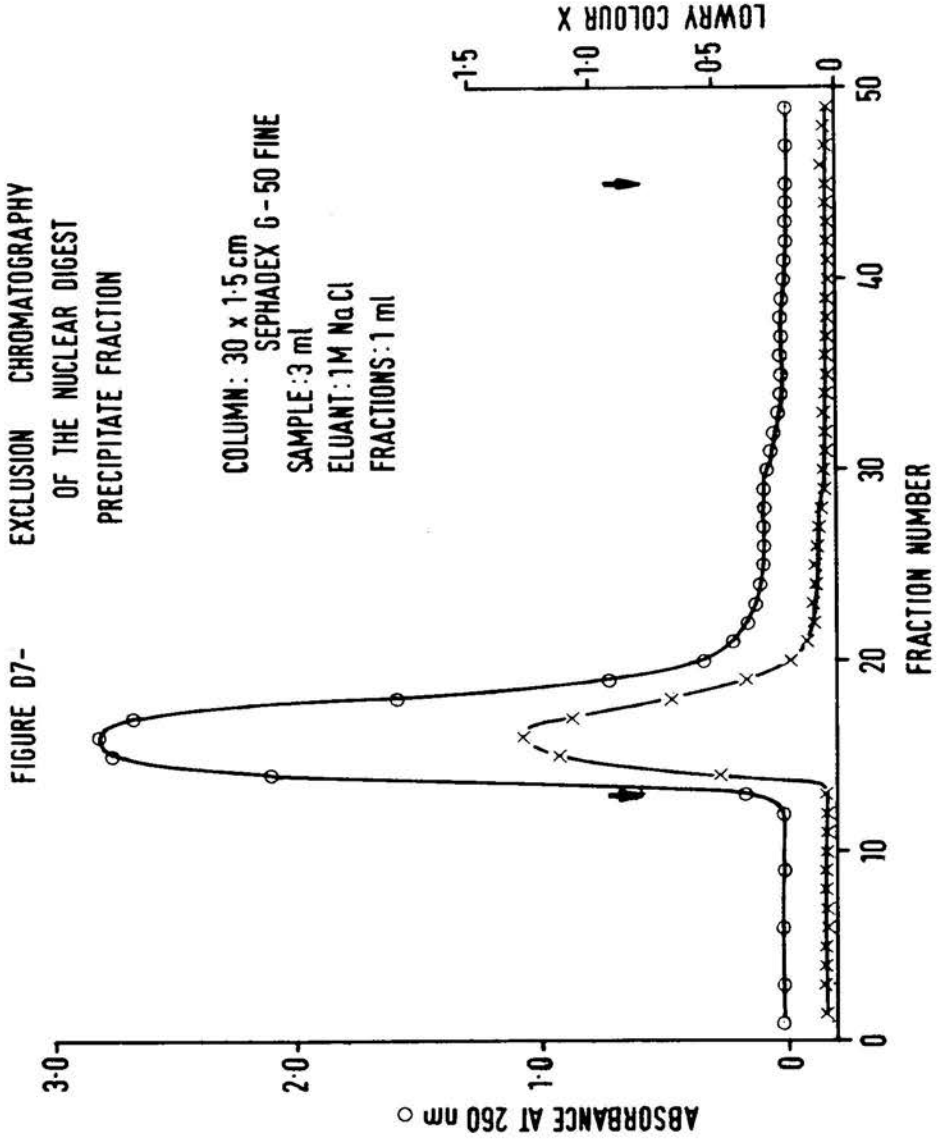


FIGURE D7- EXCLUSION CHROMATOGRAPHY
OF THE NUCLEAR DIGEST
PRECIPITATE FRACTION
COLUMN: 30 x 1·5 cm
SEPHADEX G-50 FINE
SAMPLE: 3 ml
ELUANT: 1M Na Cl
FRACTIONS: 1 ml
ABSORBANCE AT 260 nm ○
LOWRY COLOUR X
FRACTION NUMBER
3·0
2·0
1·0
0
1·5
1·0
0·5
0
10
20
30
40
50

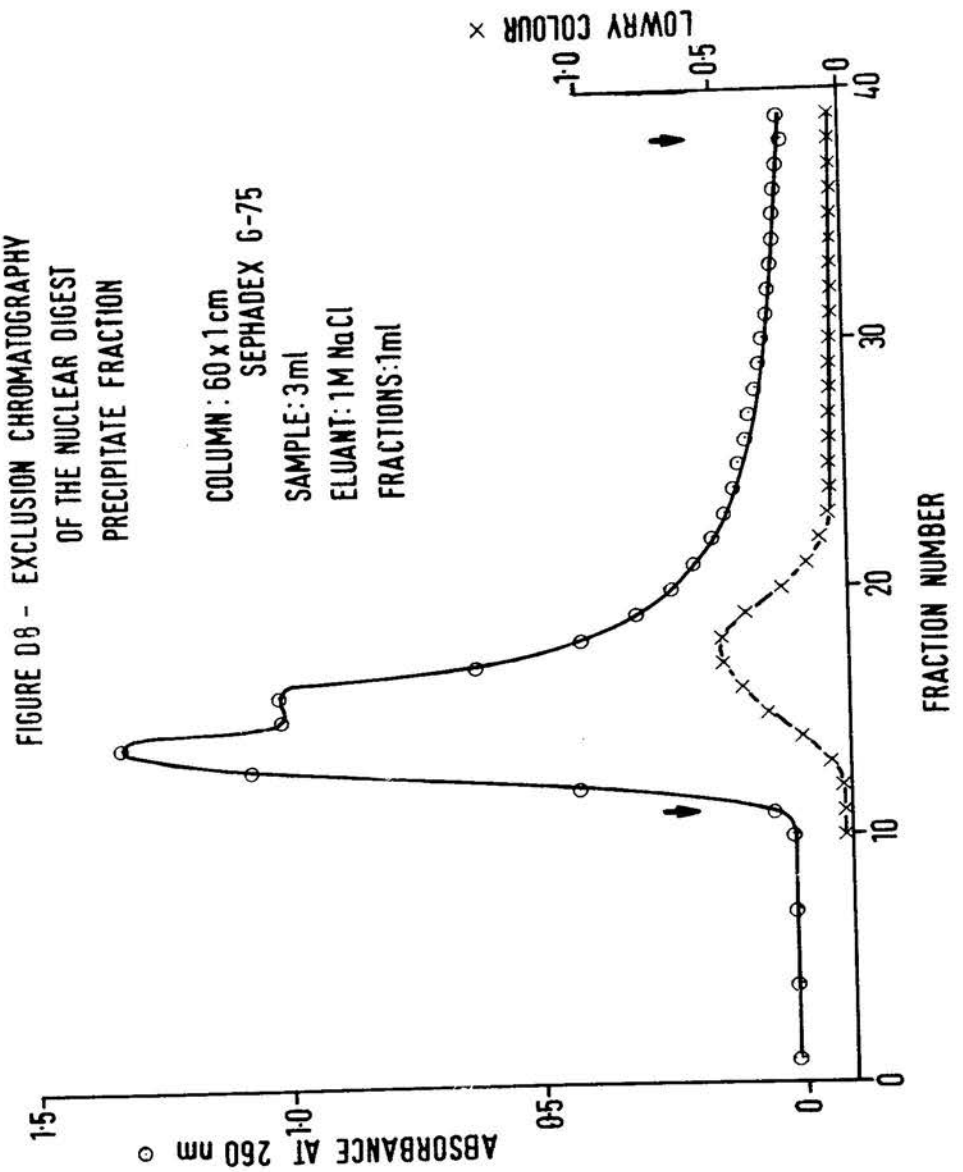


FIGURE D8 - EXCLUSION CHROMATOGRAPHY OF THE NUCLEAR DIGEST PRECIPITATE FRACTION

absorbance in section E6. 3). Chromatography of a similar fraction from which the veronal and some polynucleotides had been removed by dialysis prior to lyophilization gave a broad, symmetrical peak in the same position as that shown. Lowry analysis failed to detect protein in either case.

Chromatography of the NaCl extract of the nucleoprotein precipitate on the same column gave the results shown in Figure D7. Most protein and nucleic acid was eluted in a single, slightly asymmetrical peak at the exclusion volume of the column with a long tail of slightly Lowry positive material. The remainder of fractions 14 to 20 from this chromatogram were pooled and a sample applied to a longer column of Sephadex G-75. The nucleic acid and protein were once again eluted near the exclusion volume, as shown in Figure D8, but the asymmetry of the peak was increased by retardation of the protein component.

The chromatographic separation thus showed an interesting separation between two size classes of DNA (or DNA-containing) fragments in the supernatant and precipitate, and promise of a DNA-protein separation. No fractionation of the digest protein was afforded by the precipitation which yielded a nucleoprotein fraction that was difficult to redissolve. It was postulated that the DNA size separation might arise through long term degradation of DNA segments unprotected by complexing with protein. If precipitation and DNase action could be stopped immediately upon elution of the digest from the cell by adding NaCl to a concentration of 1 M., the total digest could then be fractionated on a longer column of more porous sephadex, testing the hypothesis regarding DNA degradation and further separating the DNA and protein.

D 3.5 Precipitation Prevention

Sodium Chloride

25 mg. of nuclei were extracted with DNase I as usual and the

FIGURE D9

FIGURE 09 - EXCLUSION CHROMATOGRAPHY
OF THE NUCLEAR DIGEST
Column : 95 x 2.5 cm
Sephadex G-100
Sample : 4 ml
Eluant : 2 M NaCl
Fractions : 3 ml
ABSORBANCE AT 260 nm ○
280 nm ×
LOWRY COLOUR
FRACTION NUMBER
A
B
C
D
I
II
III
IV
V
0
0.5
1.0
1.5
0.05
0.10
0.15
20
40
60
80
100
120
140
160
180
200

digest collected in bulk by way of a 1 ml. fraction collector siphon to which 0.25 ml. of 4M. NaCl was added for each 0.75 ml. fraction of digest. The resulting total digest solution was concentrated by freeze drying and a portion applied to a 100 cm. column of sephadex G-100 with 2 M. NaCl as eluant. The chromatogram is shown in Figure D9. Once again a large nucleoprotein peak (Peak A) was excluded from the gel, but two low, spread protein peaks (Peaks B and C) were retarded. The veronal was again eluted last (Peak D), this time without a significant quantity of polynucleotide.

The fractions containing protein components B and C were pooled and a 15 ml. portion of each applied in turn to a 30 by 1.5 cm. bio-gel P10 column for desalting by elution with distilled water. Instead of being eluted as a broad peak starting at the exclusion volume of the column, the protein appeared in each case as a single sharp peak coincident with the beginning of NaCl elution (which was detected with $AgNO_3$). The UV absorbance of the peaks was too high to be accounted for by the quantity of protein present, suggesting that aggregation may have been causing a light scattering effect. A second 15 ml. portion of each fraction was then applied to the same column which had been equilibrated with mM. NH_4HCO_3 and eluted with the same buffer. Each protein was now eluted as expected, in a broad peak beginning at the exclusion volume of the column, with a realistic level of ultraviolet absorbance. Although the protein was now ready for concentration by freeze-drying, the quantity in each case was too small for further characterization.

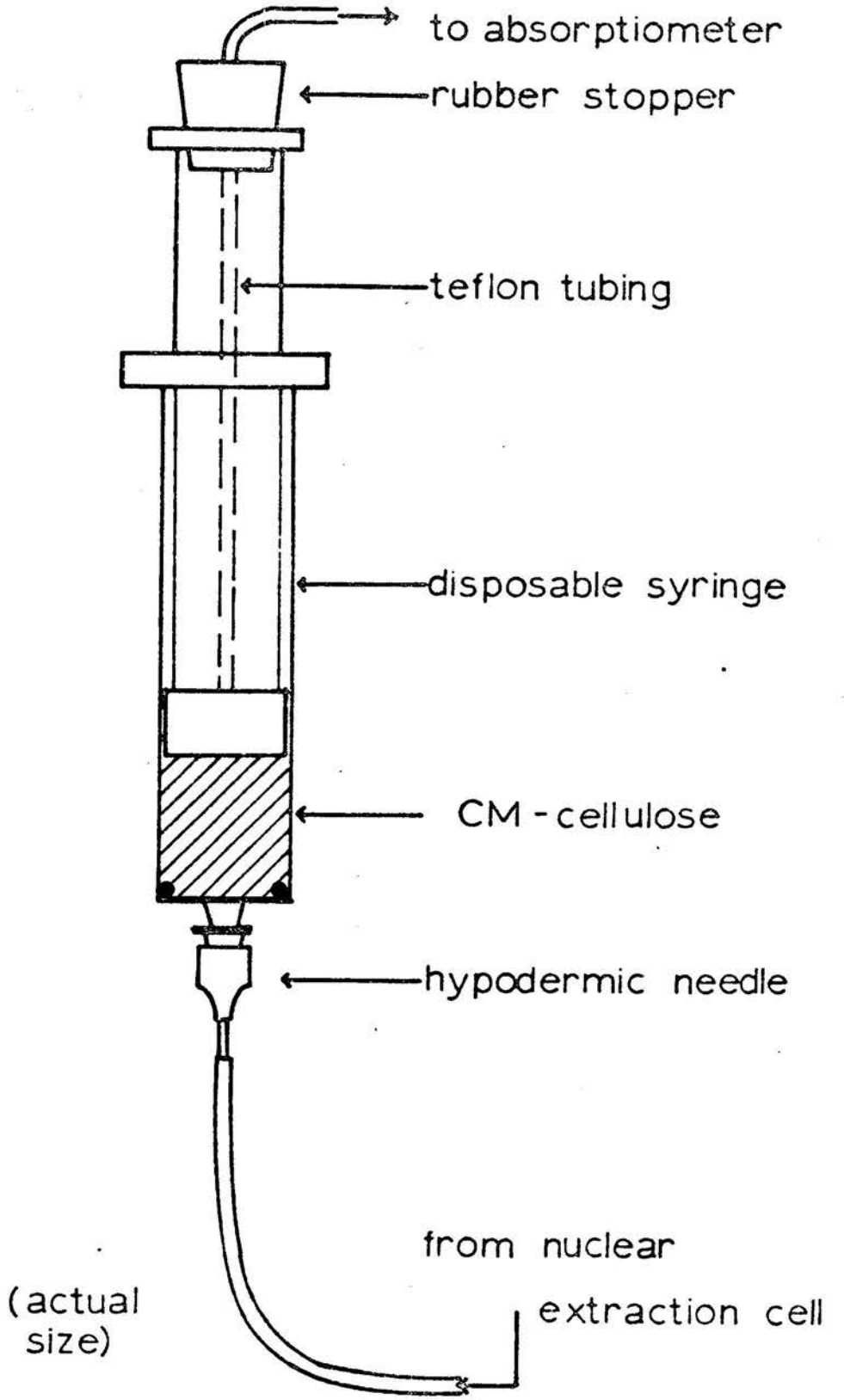
Carboxymethyl Cellulose

Although chromatography in NaCl had succeeded in freeing some protein from its complex with DNA, most remained in the excluded nucleoprotein peak and therefore inaccessible to further characterization. Another method was therefore sought which would withdraw the histones from the digest, thereby preventing

FIGURE D10

FIGURE D10

HISTONE TRAPPING COLUMN



their coprecipitation with the DNA. Adsorption chromatography using carboxymethylcellulose, as originated by D. M. P. Phillips and E. W. Johns (1959) has long been used for histone fractionation, and it was postulated that passage of the nuclear digest through a column of CM-cellulose as it was eluted from the extraction cell might achieve this objective.

A few grams of CM-cellulose that had been prepared in 0.05 M. NaCl were packed into an ion exchange column with no dead space adapted from a hypodermic syringe (the column is shown in Figure D10). The cellulose was then prepared by washing with 0.1 N. HCl, distilled water, 0.1 M. Na_2HPO_4/NaH_2PO_4 buffer (pH 7), and the DNase I buffer (0.02 M. veronal, pH 7, μM. in $MnCl_2$). 10 mg. of crude histone were applied to the column in the latter buffer, adsorbed successfully, and eluted with 0.1 N. HCl, showing that adsorption was possible under these conditions. Finally, the regenerated column was incorporated into the flow system immediately after the extraction cell which contained 25 mg. of washed nuclei and DNase I extraction was completed as usual. When extraction was complete the ion exchange column was eluted with acid to recover the adsorbed histone. Precipitation proceeded as usual in the digest fractions and chemical analysis showed that only 5% of the total digest protein had been retarded by the CM-cellulose. This approach was therefore not successful and was not pursued further.

Sodium Dodecyl Sulphate

With failure of the CM-cellulose procedure further consideration was given to the chromatographic separation using 2 M. NaCl. Although the high salt concentration had succeeded in preventing formation of a nucleoprotein precipitate, the histones have long been known to aggregate in salt solutions (H. J. Cruft, C. M. Mauritzen, and E. Stedman, 1958), and such aggregation may have accounted for the exclusion of the protein from the sephadex gel. The same

experiment was therefore repeated using 0.25 ml. additions of 4% SDS to prevent nucleoprotein precipitation in the nuclear digest, and chromatographing the concentrated digest on the sephadex G-100 column in 2% SDS. The procedure was successful in separating most of the digest protein from its nucleic acid and was therefore adopted as the standard fractionation method for such mixtures. The protein and nucleoprotein in these SDS-containing fractions was recovered according to the methods described in Section B3.2.

The chromatographic results are described more fully in the next section which gives the final fractionation of acetic acid nuclei achieved using the methods described in this and the preceding Sections.

SECTION E

THE FRACTIONATION OF ACETIC ACID NUCLEI:

RESULTS

SECTION E

THE FRACTIONATION OF ACETIC ACID NUCLEI: RESULTS

E 1. INTRODUCTION

This section gives the results obtained by applying the fractionation methods whose development was described in Sections C and D to acetic acid nuclei. Section D explained how the experiments employing the extraction cell yielded three primary nuclear fractions whose gross composition was given in Table D1 (reproduced here for convenience as Table E1). The 'wash' fraction contains RNA and protein which have not been further characterized. The deoxyribonuclease I digest and SDS-soluble residue, however, each contain DNA and protein whose secondary fractionation and characterization are detailed below.

TABLE E1

TABLE E1

NUCLEAR EXTRACTION RESULTS

EXPERIMENT A IV

200 mg. of dry nuclei ⟶		pH 7 Veronal Wash			+ DNase I Digest			+ Residue Soluble in 2% SDS			+ Unextracted Residue	
containing		containing			containing			containing			containing	
Species	X	A	B	C	A	B	C	A	B	C	A[d]	B
DNA	78	0	0	0	55	71	63	33	42	37	-	-
RNA	6	5	83	100	a	-	-	a	-	-	1	17
Histone	54	c	-	-	27[b]	50	43	35[b]	65	57	-	-
Other	62	5[c]	8	100	b	-	-	b	-	-	57	92
Total Protein	116	5	4	8	27	23	40	35	30	52	49	43

TABLE E1

COLUMN HEADINGS

X Composition of nuclei by analysis (mg. in 200 mg. of nuclei).

A Quantity in mg.

B The percent the quantity in column A represents of that in column X.

C The percent the quantity in column A represents of the total quantity in nuclear extracts.

NOTES

a Orcinol Analysis for RNA impossible because because of interference by SDS.

b Most of the protein in the digest and SDS soluble residue has been shown by electrophoresis to be histone.

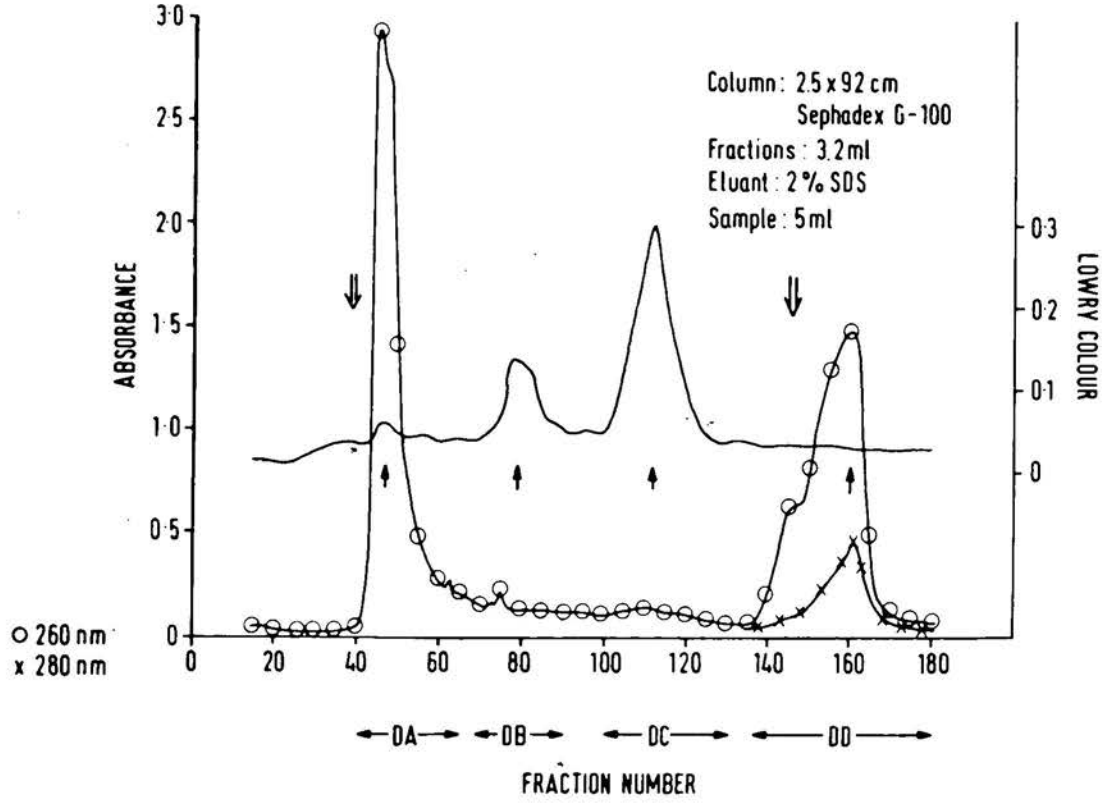
c. This uncharacterized protein material is arbitrarily classed as 'other'.

d These values are calculated as the difference between the quantities in column X and the summed extract analyses in columns A.

e Total Protein = Histone + Other.

FIGURE E1

FIGURE E1
EXCLUSION CHROMATOGRAPHY OF THE
NUCLEAR DIGEST
EXPERIMENT A1



E 2. SECONDARY FRACTIONATION

E 2.1 The Nuclear Extracts

The digests from four extraction experiments were fractionated giving several components for further characterization, two digests coming from 25 mg. samples of nuclei using Cell II (Experiments AI and AII of Table E5) and two from 200 mg. samples of nuclei using Cell III (Experiments AIII and AIV of Table E5). In each case precipitation was prevented according to the procedure described in Section D 3.5 by adding SDS to give a final concentration of 1% and the digest fractions were pooled, concentrated by freeze drying, and redissolved in a minimum of water. A part or all of this concentrated digest, which varied in volume from 5 to 40 ml., was in each case applied to the sephadex G-100 column and eluted with 2% SDS. The absorbance of the fractions was measured at 260 and sometimes at 280 nm. and selected fractions were analyzed for their DNA and protein content using the diphenylamine and Lowry analyses. Such chromatograms are shown in Figure E 1 (from Experiment AI, a small scale extraction) and Figure E 2a (from Experiment AIV, a large scale extraction). The SDS-soluble residue from Experiment AIV was concentrated and fractionated as were the digests and the chromatogram is shown in Figure E 2b.

The SDS/G-100 chromatograms were in general similar to the corresponding chromatogram performed with 2M. NaCl as eluant. The critical difference was in the distribution of protein among the peaks (referred to by letter in order of elution with the prefix D to identify them as digest fractions). Whereas chromatography in NaCl led to elution of most protein in association with the nucleic acid at the exclusion volume (fraction DA), chromatography in SDS left only a small fraction of the protein in this peak, most having been retarded and eluted in fractions DB and DC. The nucleic acid was once again eluted in two distinct regions of the chromatogram - as an excluded, protein associated fraction (fraction

FIGURE E2a

FIGURE E2a
EXCLUSION CHROMATOGRAPHY OF THE NUCLEAR DIGEST: EXPERIMENT A IV

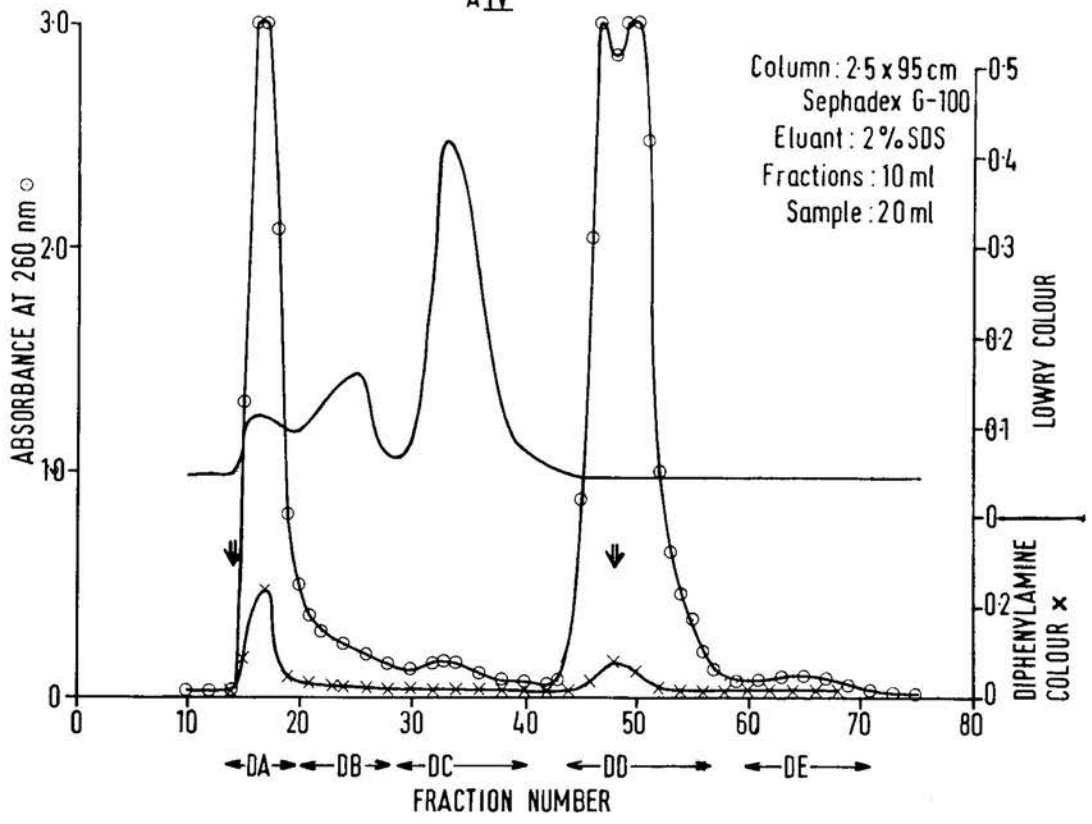


FIGURE E 2b

EXCLUSION CHROMATOGRAPHY OF THE SDS-SOLUBLE NUCLEAR RESIDUE: EXPERIMENT A IV

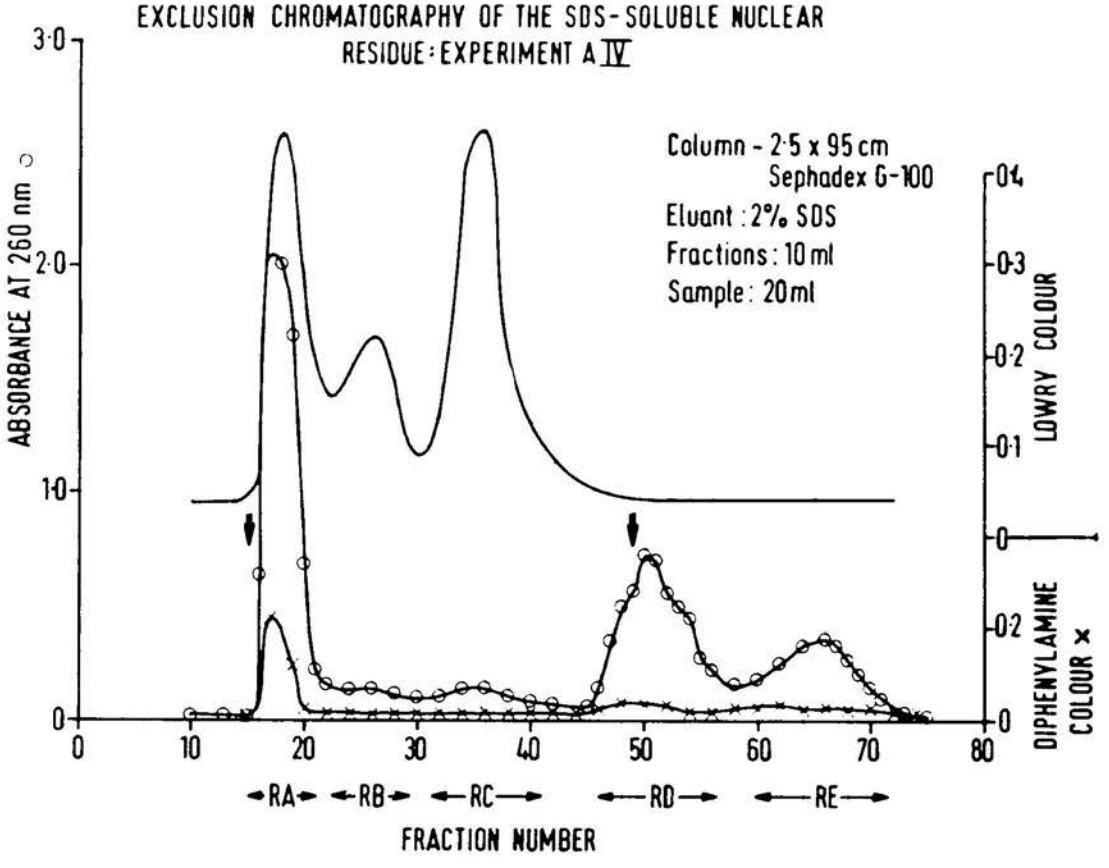
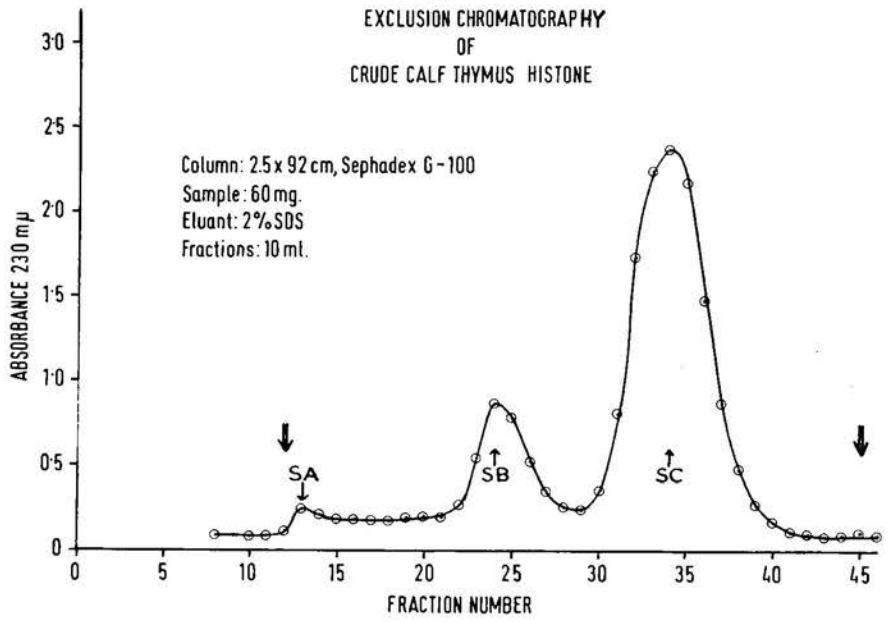


FIGURE E3

FIGURE E3

EXCLUSION CHROMATOGRAPHY
OF
CRUDE CALF THYMUS HISTONE



DA) and as a fraction eluted almost coincidentally with the veronal buffer (fraction DD). The large scale extraction experiments revealed the presence of yet another nucleic acid fraction (fraction DE) which was below the sensitivity of the detection methods in the small scale experiments (c.f. Figures E1 and E2a). Surprisingly all of these fractions, with the same qualitative composition, appeared in the residue chromatogram as well. They were labelled fractions RA to RE and are shown in Figure E2b.

E 2.2 Crude Calf Thymus Histone Chloride

For purposes of comparison and identification, 50 and 60 mg. samples of crude calf thymus histone chloride were dissolved in 4 and 5 ml. respectively of 4% SDS and chromatographed in 2% SDS on the sephadex G-100 column. Solution was achieved by dissolving the histone in 1 ml. of water and rapidly adding the concentrated SDS to achieve the desired final concentrations. The histone was detected by Lowry analysis in the first experiment and by its absorbance at 230 nm. in the second experiment (shown in Figure E 3) where maximum recovery of the material in each fraction was required. The histone was eluted in three peaks coincident with peaks DA, DB and DC of the digest chromatogram. They were therefore recovered and labelled SA, SB and SC as corresponding standard histone fractions. In an attempt to prepare fraction SA in larger quantity 500 mg. of crude histone were dissolved in 25 ml. of 4% SDS and fractionated as above, the much larger quantity of material being detected by its absorbance at 280 nm. The resolution of the fractions was very much reduced by this overloading of the column (Figure E 4), but the protein from fractions 13 to 25 (the region of the chromatogram usually occupied by fraction SA and the beginning of SB) was recovered, redissolved in 2 ml. of 4% SDS and rechromatographed on the same column. Fraction 16 of the first chromatogram was scanned for ultraviolet absorbance and showed a spectrum characteristic of protein, although the possibility of nucleic acid contamination could not be eliminated. ($A_{260} = 0.12$,

FIGURE E4

FIGURE E4
PREPARATIVE EXCLUSION CHROMATOGRAPHY
OF CRUDE CALF THYMUS HISTONE

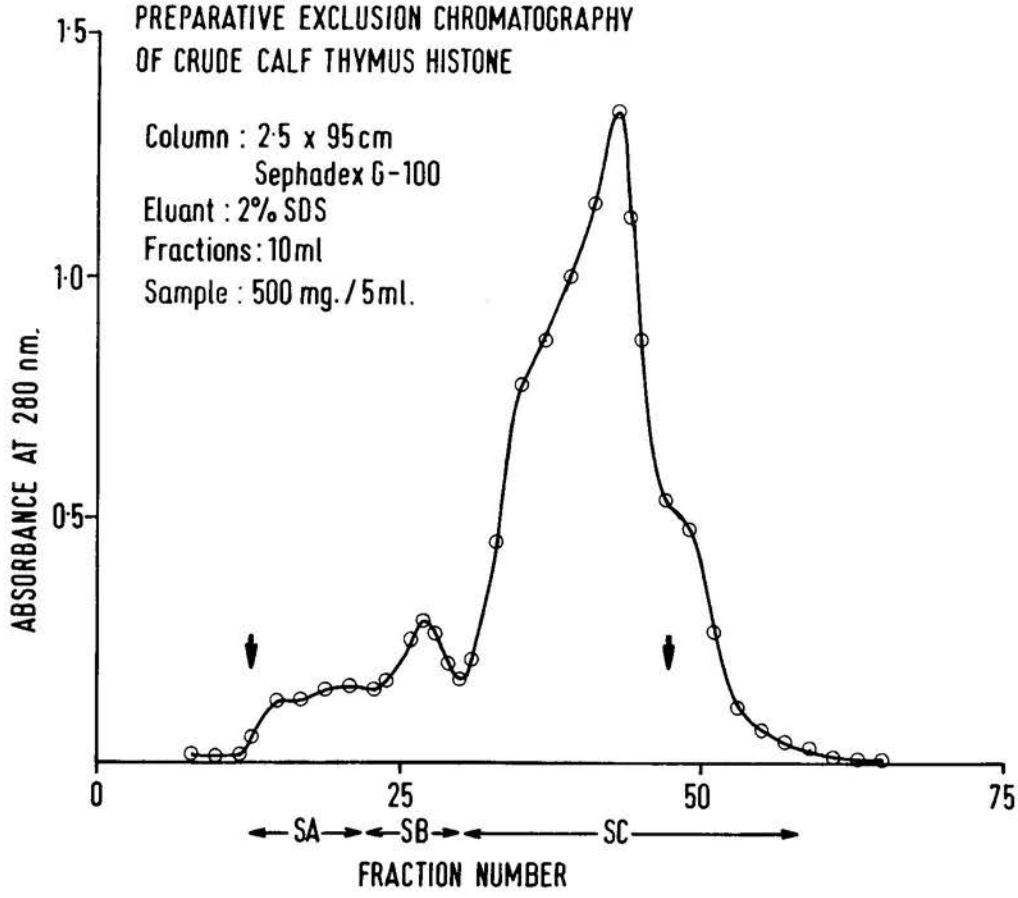
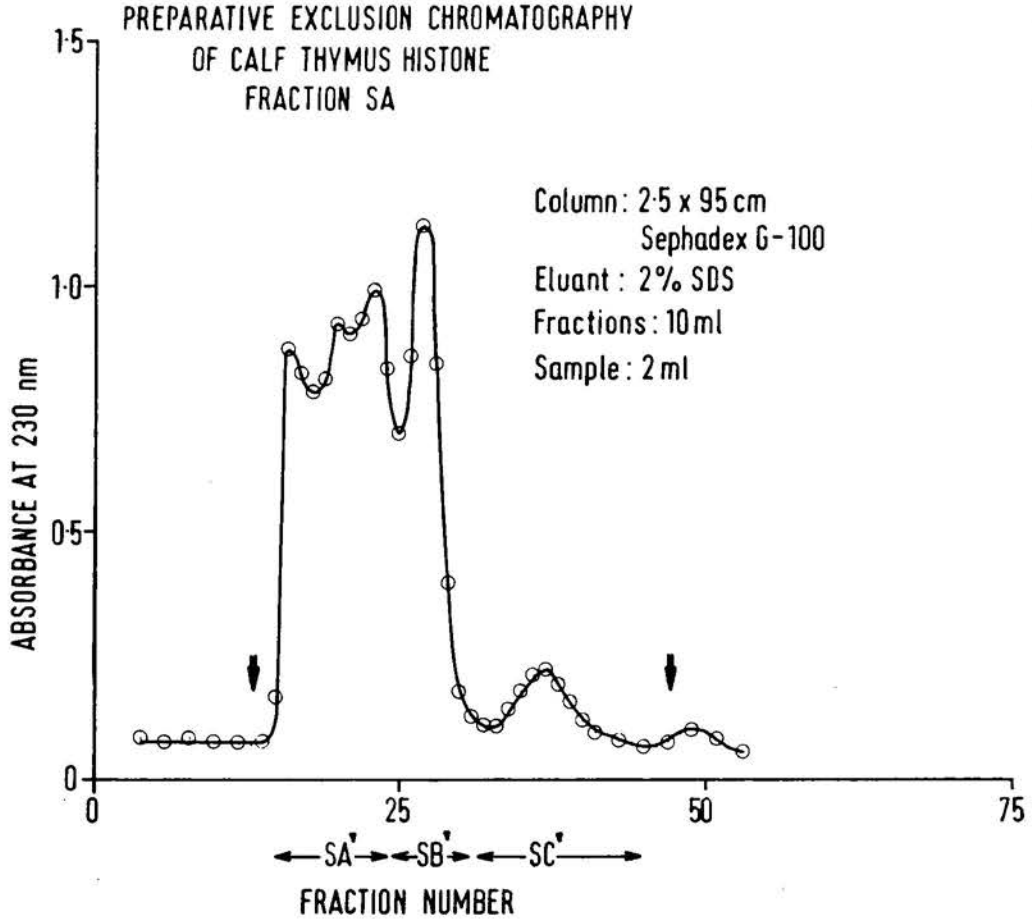


FIGURE E5
PREPARATIVE EXCLUSION CHROMATOGRAPHY
OF CALF THYMUS HISTONE
FRACTION SA



$A_{278} = 0.15$, $A_{280} = 0.15$; c.f. for 1.25 mg. crude calf thymus histone in three ml. of 2% SDS, $A_{260} = 0.09$, $A_{278} = 0.16$, $A_{280} = 0.15$).

The second chromatogram (Figure E5) shows much of the protein material again eluted near the exclusion volume, in the region usually occupied by fraction SA, as a broad and apparently heterogeneous peak. The material from fractions 15 to 24 and 25 to 31 of this chromatogram was recovered and retained as standard histone fractions SA' and SB' respectively.

E 3. PROTEIN CHARACTERIZATION: FRACTIONS A

E 3.1 Acid Extraction

The proteins from fractions RA and DA were most difficult to characterize because of their continued association with DNA. Once again removal of the DNA was a necessary prerequisite to the usual protein characterization steps. In order to detect any histone still in the form of large aggregates despite SDS treatment, fraction DA from Experiment AI was extracted with two 1.0 ml. aliquots of 0.1 N. HCl, the absorbance spectrum of the pooled extracts determined, and their contents precipitated with acetone. The resulting material was subjected to SDS-polyacrylamide gel electrophoresis along with fraction SA' and a sample of crude calf thymus histone chloride. Once again, although the spectrum showed a characteristic protein absorbance maximum at about 275 nm., the absorbance at 260 nm. was sufficiently high to suggest nucleic acid contamination. ($A_{280}/A_{260} = 0.9$). On electrophoresis the fraction DA gel remained unstained while the SA' gel stained heavily at the origin. Repeating this procedure with heavier sample loadings produced origin staining in gel DA as well as SA' and many fine, diffuse, slow-moving bands in both gels. The addition of 6 M. urea to samples and gels did not alter these patterns.

E 3.2 Sepharose Chromatography

In an attempt to separate the nucleic acid and protein in fraction DA on the basis of molecular size the fractions from Experiments A II and AIII were chromatographed on sepharose 6B in 2% SDS. Fraction DA from Experiment A II was recovered and dissolved in 3 ml. of 0.01 M. phosphate, pH 7.0, 2% in SDS and 1% in 2-mercaptoethanol, incubated for two hours at 37° C. and centrifuged. Fractions DA and DB from Experiment A III were pooled (the resolution of the chromatogram was insufficient to allow isolation of DA), recovered by acetone precipitation and redissolved in 4 ml. of 2% SDS, 0.05 M. $NaHCO_3$ (pH 6). Each of these solutions was

FIGURE E6

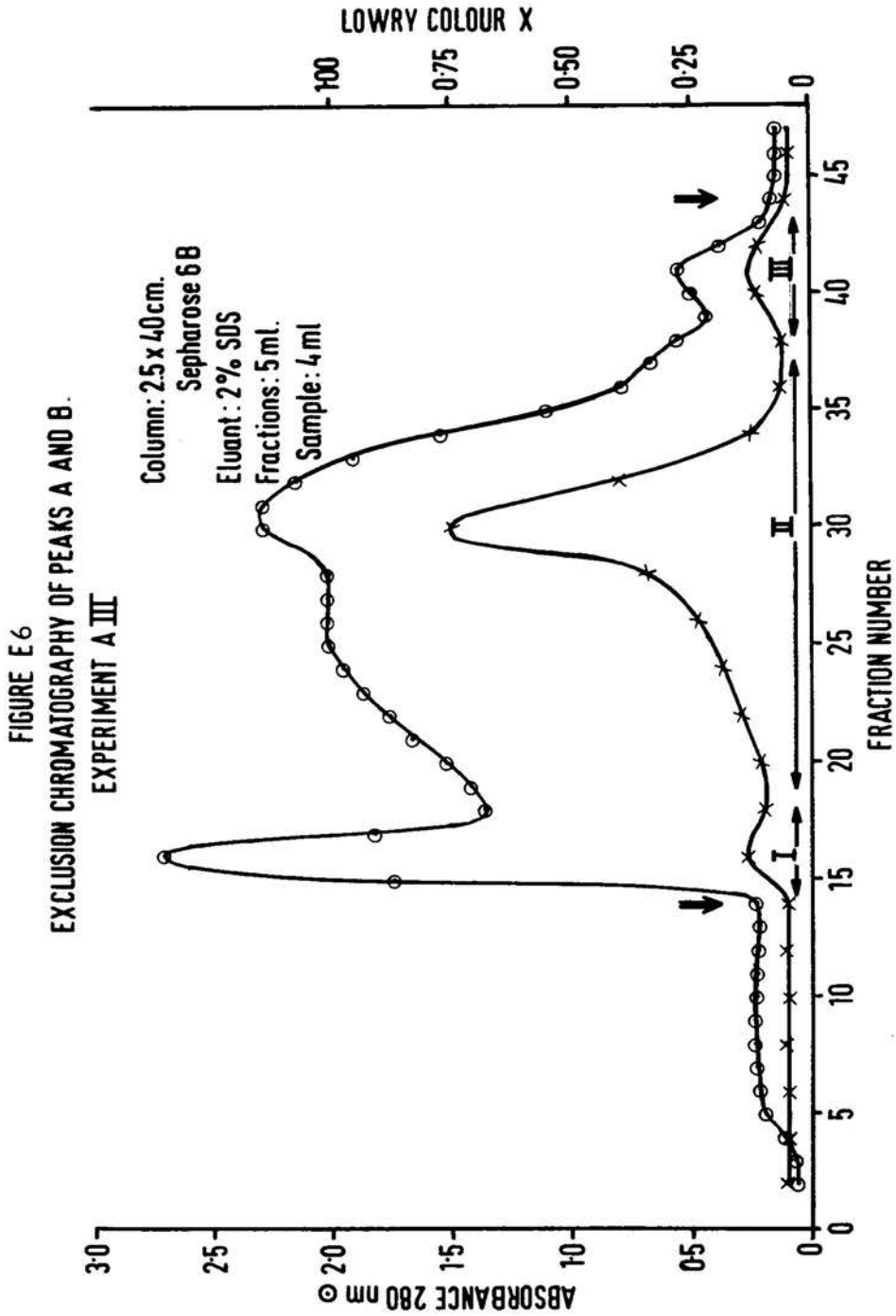


FIGURE E 6
EXCLUSION CHROMATOGRAPHY OF PEAKS A AND B.
EXPERIMENT A III

chromatographed on the sepharose 6B column in 2% SDS and the Lowry colour and absorbance of the fractions measured. The chromatogram of the second fraction is shown in Figure E6. The absorbance at 260 nm. was too high to be measured precisely so the absorbance at 280 nm. is given to reflect nucleic acid content. The absorbance at both wavelengths was too high throughout to be accounted for only on the basis of the protein concentrations detected by the Lowry analyses. The protein is eluted as three fractions; I, II, and III. Fraction II is much larger in relation to I and III in this chromatogram than in the other, suggesting that it contains the fraction DB protein (this was corroborated by later evidence). The 2-mercaptoethanol treatment of the first sample did not visibly alter the chromatographic pattern. The ~~peak of~~ background absorbance starting at fraction 3 (well before the exclusion volume of the column) and tapering to the end of the chromatogram was caused by the gradual release of sodium azide added by the manufacturers to the sepharose as an antibacterial agent and identified here by its absorbance spectrum.

This procedure was unsuccessful as a means of separating fraction DA protein and nucleic acid, and fractions I and III were too low in protein content for further analysis. Fraction II was, however, recovered for use in the next separation attempt.

E 3.3 Re-digestion and Amino Acid Analysis

The chromatographic behaviour of fractions DA, RA and SA suggested that they were composed either of very large molecules or of molecular aggregates of protein with protein or nucleic acid with protein. It seemed unlikely, however, that aggregation could persist in the presence of 2% SDS, the failure of the fractions to respond on electrophoresis or chromatography to treatment with 2-mercaptoethanol suggesting that sulphydryl bridging was not a cause of aggregation. On the assumption, therefore, that both the nucleic acid and protein in this fraction were excluded because of

FIGURE E7

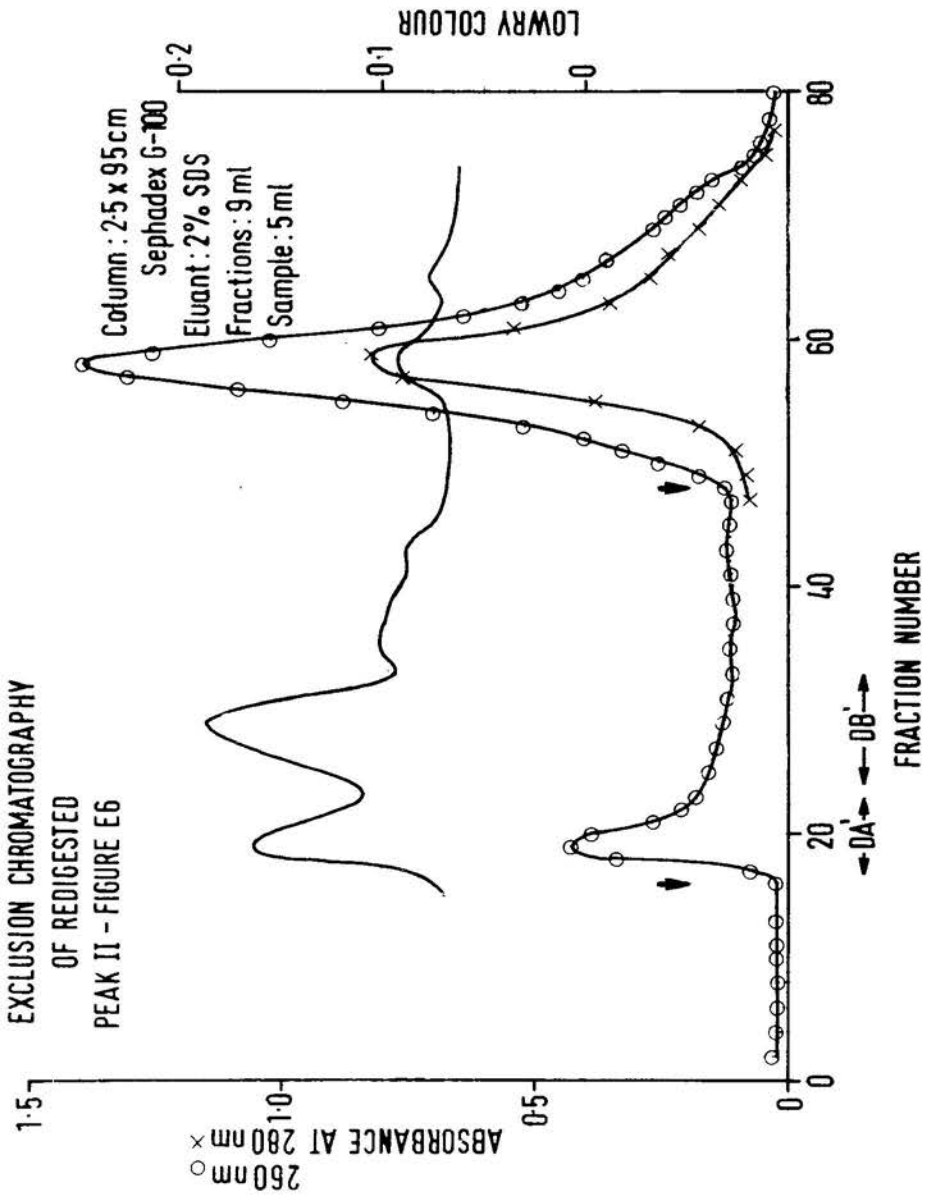


FIGURE E 7
EXCLUSION CHROMATOGRAPHY
OF REDIGESTED
PEAK II - FIGURE E6

their size and not because of aggregation, an effort was made to reduce the size of the nucleic acid fragments by a second enzymic digestion. The protein and nucleic acid could then be separated by rechromatography on sephadex G-100.

The choice of enzyme for the second digestion was limited by the nature of the substrate. For example, spleen phosphodiesterase could not be employed as its specificity makes it inactive against the products of DNase digestion. The first attempt at redigestion therefore employed DNase I again, at a higher concentration than was employed for nuclear extractions. The material recovered from fraction II of the sepharose chromatogram (Figure E6) was suspended in 4 ml. of 0.02 M. veronal, pH 7, μM. in $MnCl_2$ and containing 84 μg. of DNase I. It was incubated, with stirring, for 21.5 hours at 17° C., 100 mg. of SDS were added, and the digest was centrifuged to remove a small insoluble residue. The chromatogram of this digest on the sephadex G-100 column in 2% SDS is shown in Figure E7. This chromatogram confirms the presence of fraction DB material in fraction II. Although much of the nucleic acid formerly excluded from the gel is now eluted with the veronal-in fraction DD, a significant quantity still remains in fraction DA. The presence of Lowry positive material after fraction DB in the chromatogram, while partly attributable to Mn^{++} ions, suggests that, not surprisingly, protein degradation has occurred during the long DNase redigestion, releasing oligopeptides.

The material from fractions 16-21 and 23-32 was recovered as fractions DA' and DB' respectively. Although these were rather 'battered' protein fractions, DA' was the most nucleic acid free fraction of its type available. It was therefore subjected to amino acid analysis whose results, along with those for unfractionated histone and values from the literature for unfractionated histone and the nuclear acidic proteins are given in Table E2. DB' was also analyzed and the results are given in Table E3 (Section E 4).

TABLE E2

AMINO ACID COMPOSITIONS OF FRACTIONS DA' AND SA'

AMINO ACID	DA'	SA'	CTHC[a]	CTHC[b]	NHP[c]
Aspartic Acid	8.1	5.9	4.8	4.9	10.6
Threonine	5.2	5.3	5.5	5.3	4.3
Serine	6.4	5.8	5.1	5.0	9.0
Glutamic Acid	10.4	9.2	8.8	8.4	16.0
Proline	5.9	6.8	5.2	4.8	5.3
Glycine	9.7	7.1	8.3	8.7	15.0
Alanine	10.3	15.4	13.1	13.6	8.9
Half Cystine	+++	+++	+	-	-
Valine	6.0	5.6	5.6	6.2	4.7
Methionine	1.5	1.0	0.9	0.9	0.6
Isoleucine	4.0	3.1	4.0	4.4	2.8
Leucine	7.8	7.1	7.3	7.7	5.2
Tyrosine	2.5	1.9	2.4	2.3	1.2
Phenylalanine	3.2	2.2	1.8	1.7	2.2
Histidine	2.2	1.3	1.9	2.3	1.8
Lysine	10.8	16.3	12.8	14.9	8.4
Arginine	6.1	5.9	12.8	8.9	3.4
Basics/Acidics	1.0	1.6	2.0	2.0	0.5
Lysine/Arginine	1.8	2.8	1.0	1.7	2.5

a Calf Thymus Histone Chloride: the material fractionated to give standard fractions SA to SC.

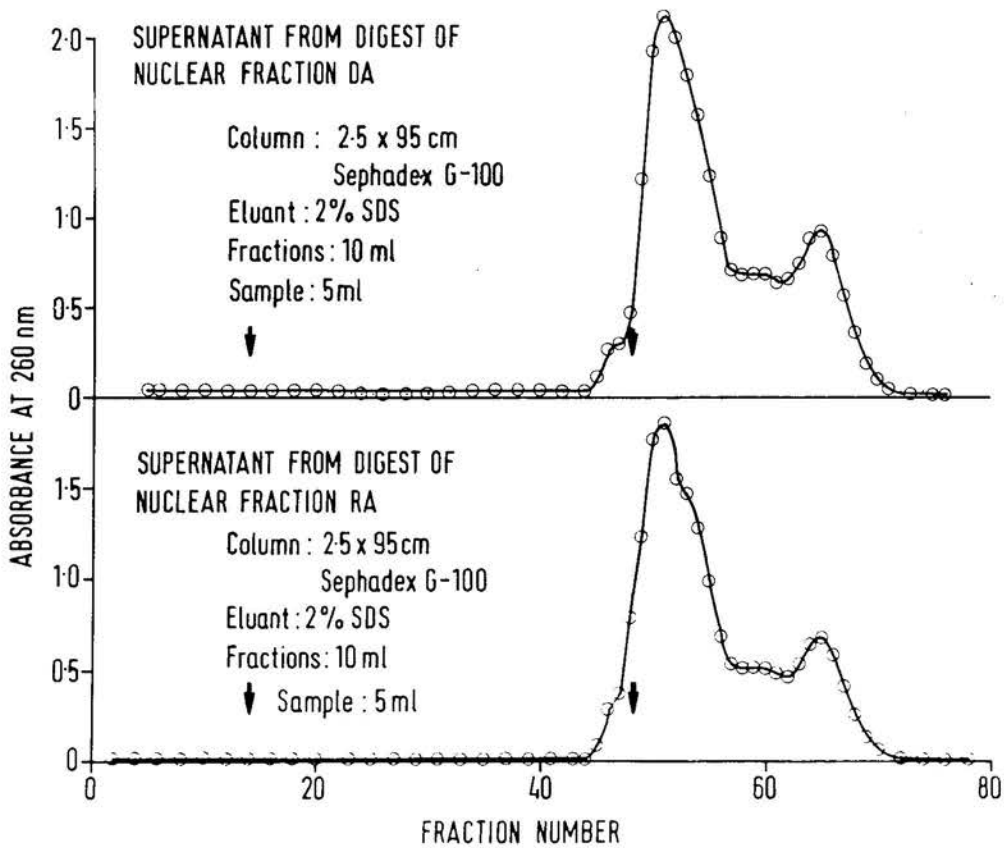
b Analysis of Calf Thymus Histone Chloride prepared by acid extraction (C. Dick and E. W. Johns, 1969).

c Analysis of Non-Histone Proteins of Calf Thymus Chromatin prepared by extraction with 0.15 M. NaCl after dissociation in M. NaCl and purified by electrophoresis (R. Hacha and E. Fredericq, 1968).

FIGURE E8

FIGURE E8

EXCLUSION CHROMATOGRAPHY - MICROCOCCAL NUCLEASE REDIGESTION OF FRACTIONS DA AND RA



Redigestion with DNase I had not completely freed fraction DA of nucleic acid contamination so a second attempt was made to redigest fractions DA and RA from experiment AIV using micrococcal nuclease. This enzyme is an endonuclease forming oligonucleotides esterified to phosphate at the 3' end. It is optimally active at pH 9.0 with calcium as cofactor and it was postulated that the fractions might dissolve at this higher pH, becoming more accessible to the enzyme and therefore more subject to degradation. The conditions chosen for the redigestion were those of P. H. Von Hippel and G. Felsenfeld (1964) who used the enzyme at pH 8.5 in 35 mM. NaCl and 1.4 mM. $CaCl_2$. 50 micromolar units of the enzyme (where, according to Sigma, one micromolar unit will produce one micromole of acid-soluble polynucleotides from DNA per minute at 37° C. at pH 8.6 based on $E^{M}_{260} = 10,000$ for the mixed nucleotides) were dissolved in 1.2 ml. of 0.02 M. veronal pH 8.5, containing 35 mM. NaCl and 1.4 mM. $CaCl_2$. 0.4 ml. of this solution was added to each of two tubes containing 4 ml. of the same buffer plus fraction DA (Figure E2a) and fraction RA (Figure E2b). The two nucleoprotein samples failed to dissolve in the nuclease buffer, but both samples were incubated with the enzyme for 3.5 hours at 30° C. and the reaction mixtures then centrifuged to remove the insoluble material. 80 mg. of SDS were added to the supernatants, which were chromatographed on the sephadex G-100 column, and 2 ml. of 2% SDS were added to the insoluble residues. The chromatograms (Figure E8) showed that some of the DNA had been solublized and appeared in the regions of fractions D and E. No protein, measurable either by Lowry analysis or by absorbance at 240 nm., was found in either supernatant. The 2% SDS failed to redissolve the undigested residues which still contained most of the DNA and all of the protein. No further steps were undertaken with this material.

E 4 PROTEIN CHARACTERIZATION: FRACTIONS B AND C

The proteins from fractions DB and DC of Experiments AI, AII and AIV were recovered and further characterized by electrophoresis and amino acid analysis in comparison with the corresponding fractions SB and SC and with crude calf thymus histone.

Electrophoresis in the standard 0.9 M. acetic acid gel system showed DB and SB to contain predominantly histone fraction F1, while DC and SC contained all the other main histone fractions (Figure E9). Fraction DB differed from SB in causing staining at the gel origin, even with small sample loadings, and in showing fewer fine bands in addition to the F1 fraction than did SB. Both fraction DC and SC totally penetrated the gels and both showed fine band patterns at high sample loadings, particularly in the regions ahead of the main histone fractions. The significance of these fine band patterns is difficult to assess as they cannot be distinguished from the fine bands common to crude histone fractions and they do not represent a quantitatively significant component of the extract protein.

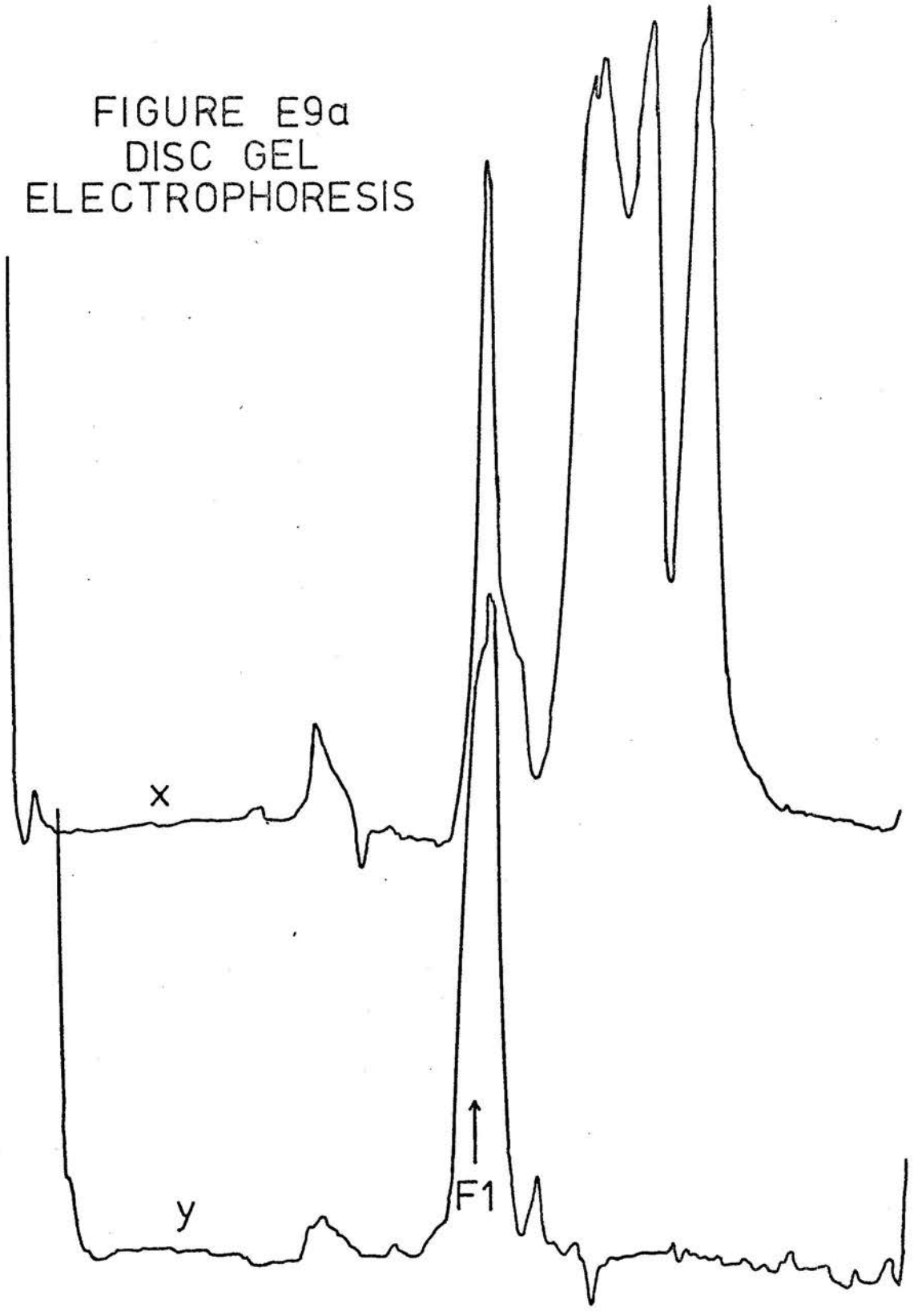
Attempts were made to achieve total penetration of fraction DB into the polyacrylamide gels by electrophoresis in the 0.9 M. acetic acid system with the gel concentration reduced from 15% to 7.5%, or with the gels and samples prepared in 10 M. urea. These methods were unsuccessful, as was electrophoresis in SDS gels, although fraction SB still penetrated each of these gel systems completely.

All of these fractions were also characterized by amino acid analysis. The analysis of the B fractions is given in Table E3 in comparison with the analysis of unfractionated histone and values from the literature for histone fraction F1 and the nuclear acidic proteins. The C fraction analyses are compared in Table E4 with the analysis of unfractionated histone.

Fractions RB and RC were also electrophoresed in the 0.9 M. acetic acid system, giving the same results as DB and DC, i.e. histone fraction F1 plus non-penetrating material in RB and all other histone fractions in RC.

The significance of the electrophoretic results and amino acid analyses will be further discussed in Section G.

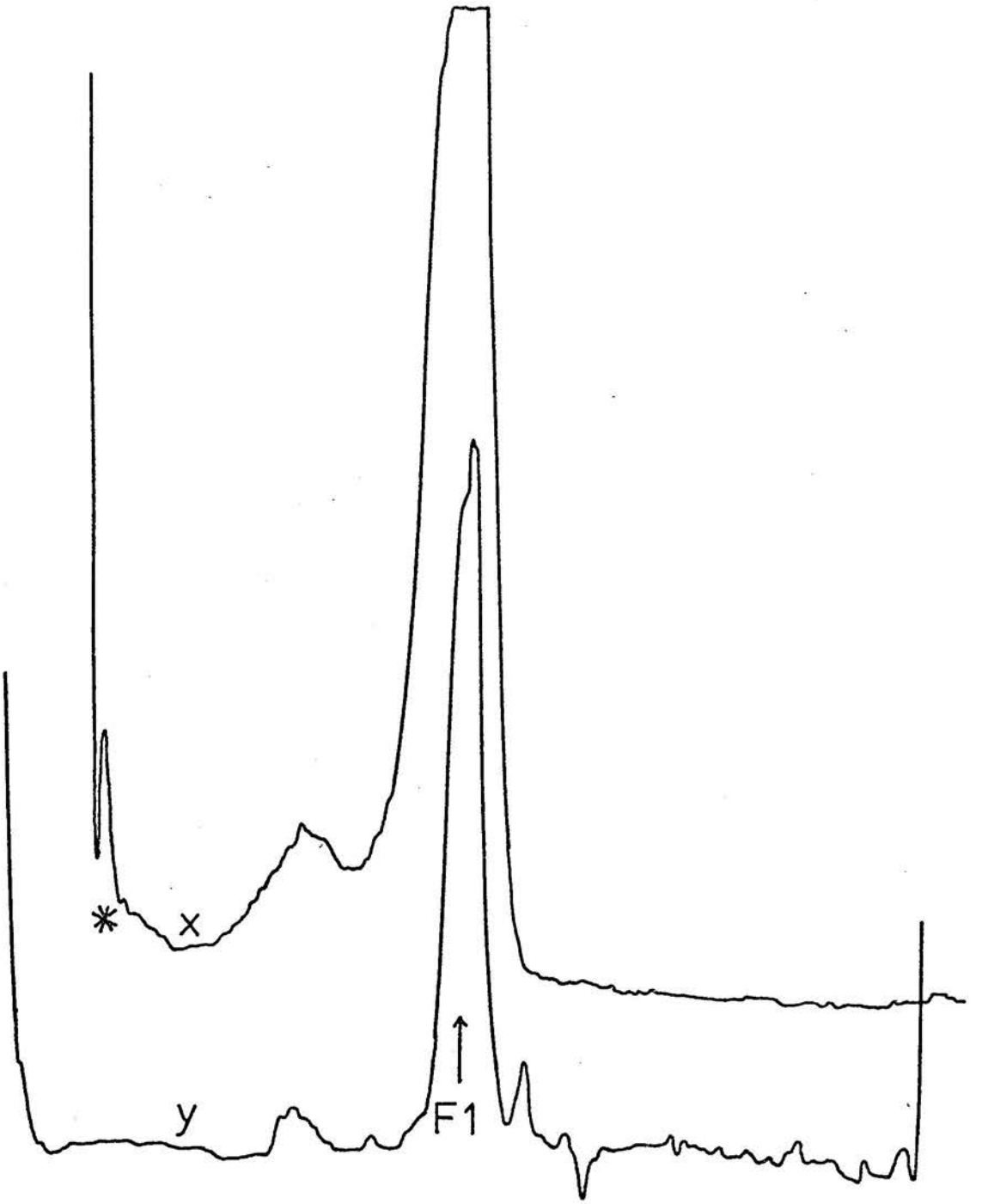
FIGURE E9a
DISC GEL
ELECTROPHORESIS



x Crude Calf Thymus Histone

y Fraction SB

FIGURE E9b
DISC GEL ELECTROPHORESIS

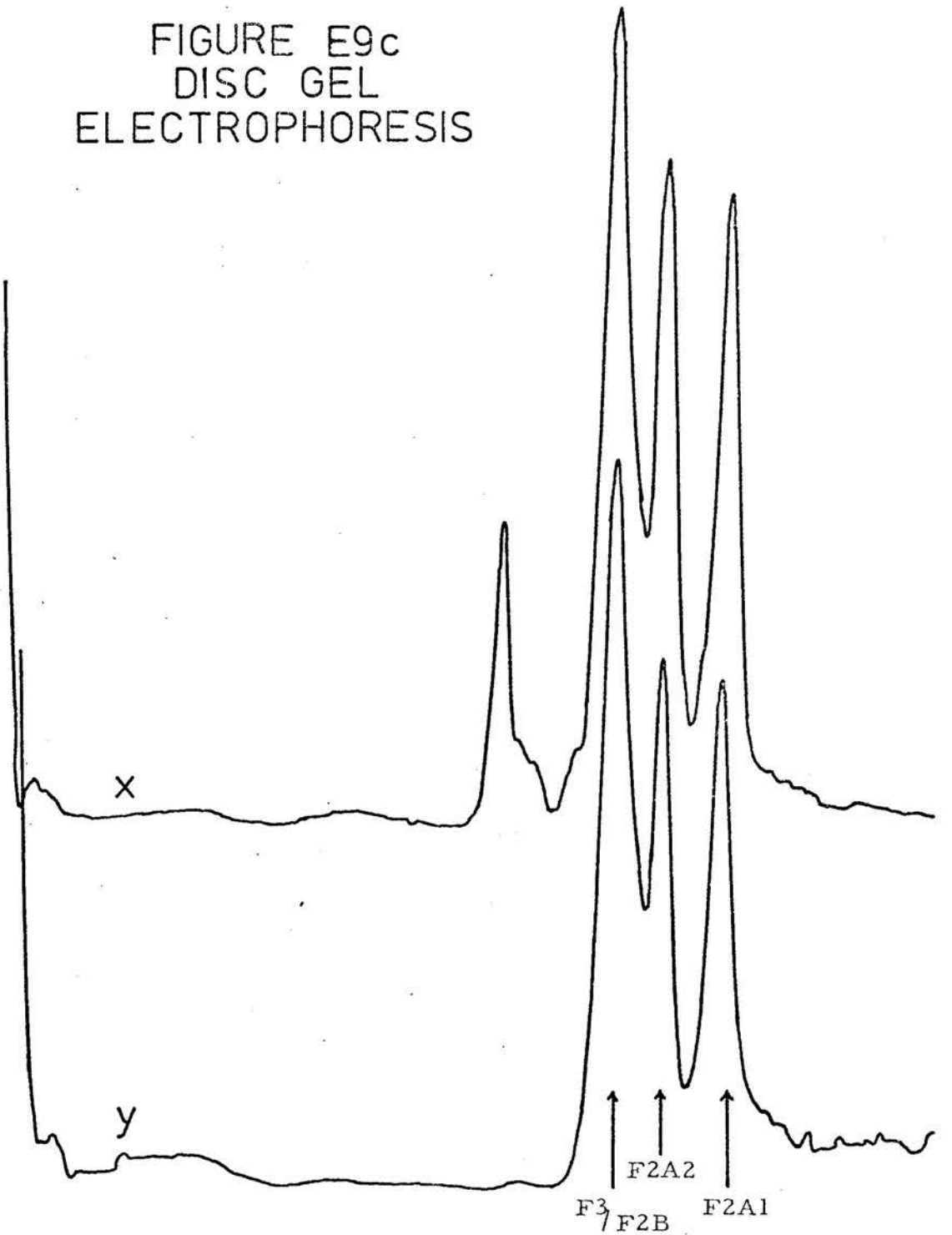


x Fraction DB

y Fraction SB

* Origin Staining

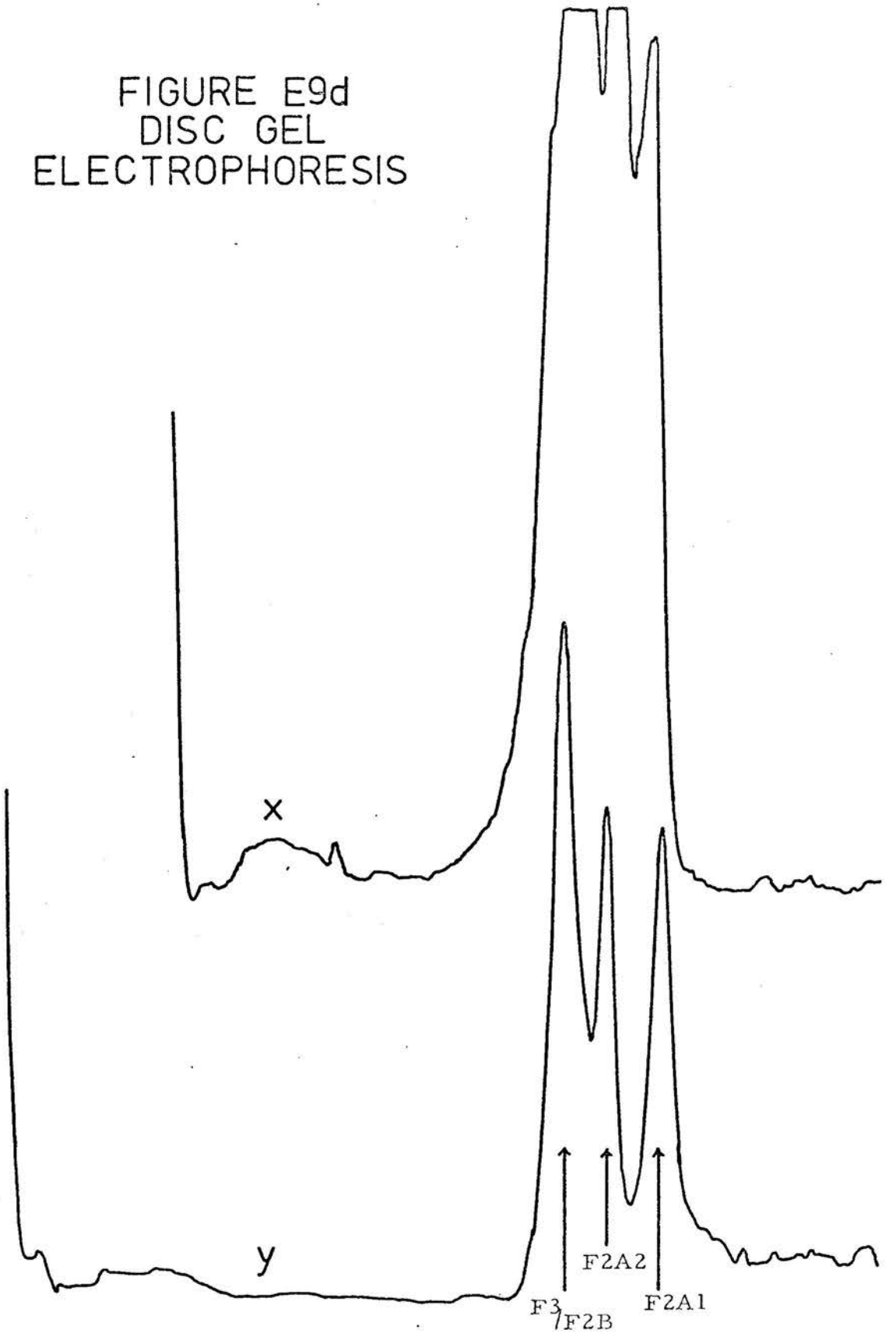
FIGURE E9c
DISC GEL
ELECTROPHORESIS



x Crude Calf Thymus Histone

y Fraction SC

FIGURE E9d
DISC GEL
ELECTROPHORESIS



x Fraction DC y Fraction SC

TABLE E3

AMINO ACID COMPOSITIONS OF FRACTIONS DB AND SB

AMINO ACID	DB	DB'[a]	SB	F1[b]	CTHC[c]	NHP[d]
Aspartic Acid	5.9	5.5	4.1	2.5	4.8	10.6
Threonine	5.5	5.3	5.7	5.6	5.5	4.3
Serine	6.5	6.1	6.0	5.6	5.1	9.0
Glutamic Acid	8.4	7.3	6.6	3.7	8.8	16.0
Proline	6.1	6.3	8.6	9.2	5.2	5.3
Glycine	9.5	8.1	7.7	7.2	8.3	15.0
Alanine	16.1	17.0	19.0	24.3	13.1	8.9
Half Cystine	++	++	+	0.0	+	-
Valine	5.9	6.1	5.4	5.4	5.6	4.7
Methionine	0.6	0.5	0.5	0.0	0.9	0.6
Isoleucine	3.2	2.7	2.2	1.5	4.0	2.8
Leucine	6.9	6.5	5.8	4.5	7.3	5.2
Tyrosine	2.0	1.8	1.3	0.9	2.4	1.2
Phenylalanine	2.2	1.8	1.4	0.9	1.8	2.2
Histidine	1.0	1.0	0.7	trace	1.9	1.8
Lysine	14.7	19.1	21.1	26.8	12.8	8.4
Arginine	5.4	4.8	4.1	1.8	12.8	3.4
Basics/Acidics	1.5	1.9	2.4	4.6	2.0	0.5
Lysine/Arginine	2.7	4.0	5.1	15.0	1.0	2.5

a For the origin of Fraction DB', see Section E 3.3.

b Analysis of Histone Fraction F1 (E.W. Johns, 1971).

c Calf Thymus Histone Chloride: the material fractionated to give standard fractions SA to SC.

d Analysis of Non-Histone Proteins of Calf Thymus Chromatin prepared by extraction with 0.15 M. NaCl after dissociation in M. NaCl and purified by electrophoresis (R. Hacha and E. Fredericq, 1968).

TABLE E4

AMINO ACID COMPOSITIONS OF FRACTIONS DC AND SC

AMINO ACID	DC	DC	SC	CTHC[a]
Aspartic Acid	5.2	5.2	5.4	4.8
Threonine	5.5	5.7	5.6	5.5
Serine	5.0	5.0	4.9	5.1
Glutamic Acid	8.4	8.7	9.0	8.8
Proline	4.9	3.9	4.5	5.2
Glycine	10.4	9.7	9.0	8.3
Alanine	11.5	12.4	11.6	13.1
Half Cystine	++	++	+	+
Valine	6.6	6.7	6.4	5.6
Methionine	0.9	1.0	1.0	0.9
Isoleucine	4.5	4.8	4.7	4.0
Leucine	7.9	8.3	8.1	7.3
Tyrosine	2.8	2.9	2.8	2.4
Phenylalanine	2.0	2.1	2.0	1.8
Histidine	2.0	1.9	2.3	1.9
Lysine	12.0	11.8	12.1	12.8
Arginine	10.7	10.1	10.6	12.8
Basics/Acidics	1.8	1.7	1.7	2.0
Lysine/Arginine	1.1	1.2	1.1	1.0

a Calf Thymus Histone Chloride: the material fractionated to give standard fractions SA to SC.

TABLE E5

TABLE E5

QUANTITATIVE ASPECTS OF PROTEIN EXTRACTION: ACETIC ACID NUCLEI

EXP. NO.	SAMPLE	RELATIVE SIZES OF PROTEIN PEAKS			
		A AV.	B AV	A+B* AV.	C
	50 mg. Histone	0.02 } 0.03	0.23 } 0.24	0.25 } 0.27	1.0
	60 mg. Histone	0.04	0.25	0.29	1.0
AI	Digest of 25 mg. of nuclei	0.06	0.39	0.45	1.0
AII	Digest of 25 mg. of nuclei	0.13 } 0.10	0.32 } 0.31	0.45 } 0.41	1.0
AIII	Digest of 200 mg. of nuclei	0.07	0.24	0.31	1.0
AIV	½ of Digest of 200 mg. of nuclei	0.13	0.30	0.43	1.0
AIV	½ of SDS-Soluble Residue from AIV	0.66 0.66	0.40 0.40	1.06 1.06	1.0

* Poor resolution of peaks A and B, particularly in experiments AIII and AIV, makes their combined estimate more reliable than the individual values.

E 5 QUANTITATIVE ASPECTS OF PROTEIN EXTRACTION

In the light of early suggestions from the electrophoretic results that the proportions of the histone fractions released might vary during enzymic extraction, a quantitative comparison was made of the protein in fractions A, B and C of crude histone, the nuclear digest and the SDS soluble residue. The area under each peak on the Lowry colour curve of each chromatogram was found by summing the colour values of the relevant fractions. The blanks for these colour values were found by drawing a baseline through the pre-A and post-C values of each curve. The sums were then expressed as fractions of the peak C value which was assigned the value 1.0. The results are given in Table E5 and will be discussed in Section G.

E 6 DNA CHARACTERIZATION

E 6.1 Introduction

As described above, DNA was found consistently in fractions A, D and E where it was coeluted with protein, veronal and no other species respectively. Although it was not an object of this work to characterize the DNA in the nuclear digests, questions arose as to the significance of the sharp distinction between the excluded and retarded DNA fractions.

Although order of elution from sephadex can be expected generally to reflect molecular size variations in DNA fractions, their precise order of elution and absolute elution volumes are functions of base composition in addition to size (see, for example, Th. Hohn and H. Schaller, 1967). In these chromatograms DNA elution was complicated, not only by these factors, but by the coelution of other molecular species.

E 6.2 Fractions A

As was discussed in Section E 3, the exclusion of fractions DA, RA and SA could have resulted either from the true molecular size of the nucleic acid and protein components or from their aggregation. Unfortunately failure to separate the two components made measurement of their sizes by such standard methods as ultracentrifugation impossible. The DNA in fractions A therefore remains uncharacterized, except in so far as its further digestion, in the presence of protein, by either DNase I or micrococcal nuclease released fragments eluted from sephadex G-100 as two peaks coincident with digest peaks DD and DE. The quantity of DNA in fractions DA and RA can be estimated with reasonable accuracy by the diphenylamine reaction.

E 6.3 Fractions D and E

The identification of veronal in fraction DD was based on its

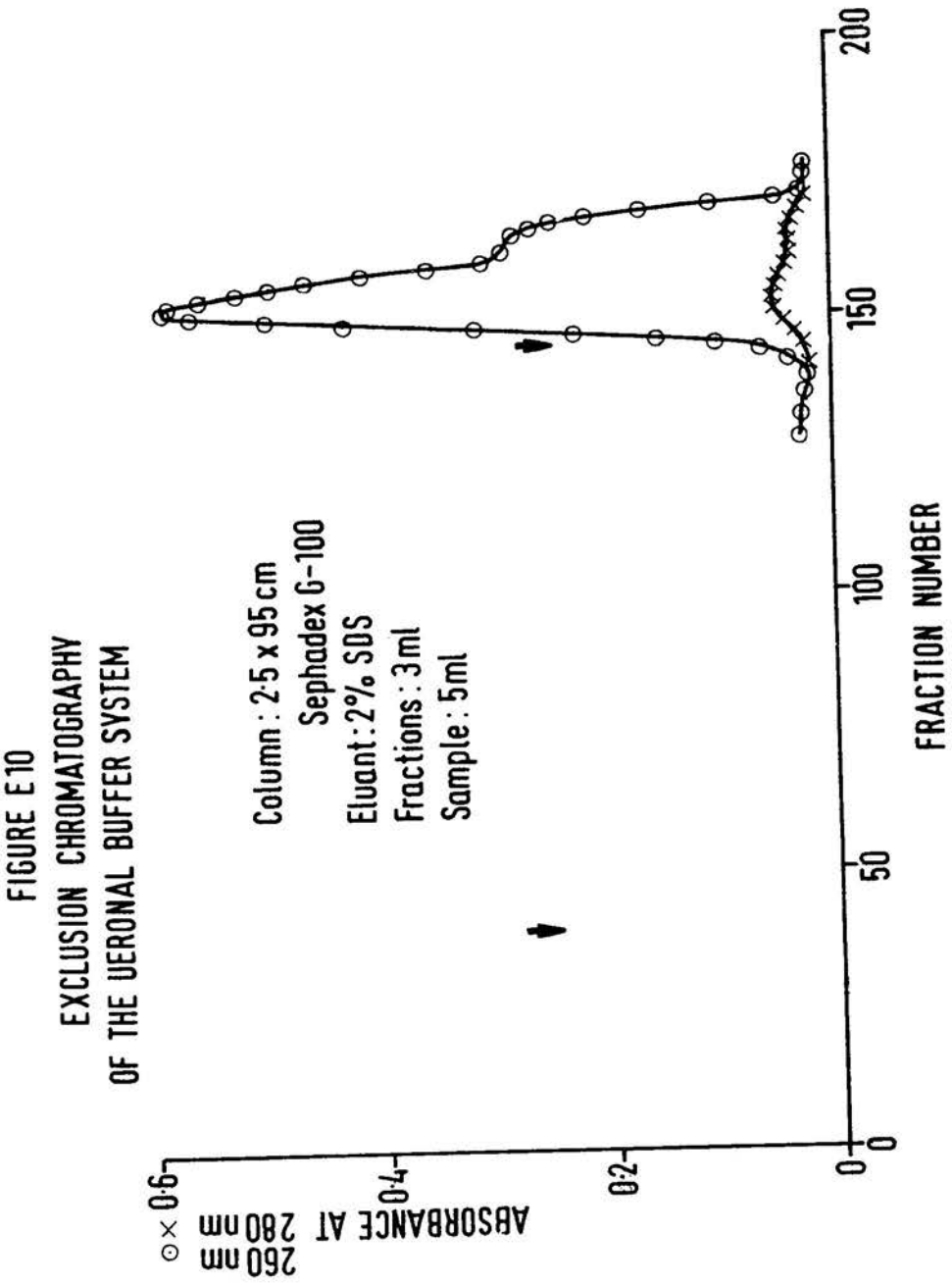


FIGURE E10
EXCLUSION CHROMATOGRAPHY
OF THE UERONAL BUFFER SYSTEM
Column : 2·5 x 95 cm
Sephadex G-100
Eluant : 2% SDS
Fractions : 3ml
Sample : 5ml
ABSORBANCE AT 280 nm
260 nm
⊙ × 0·6
0·4
0·2
0
0
50
100
150
200
FRACTION NUMBER

high absorbance at 260 nm. and low absorbance at 280 nm. In order to check its influence on the shape of this peak, however, a 5 ml. sample containing 6% SDS, 0.09 M. veronal, 4.5 micromolar $MnCl_2$ and 0.0815 M. HCl (the estimated concentrations in the concentrated DNase I digest of Experiment AI) was chromatographed on the SDS/G-100 column and the veronal was detected by its absorbance at 260 and 280 nm. The result, shown in Figure E 10, was a double peak of high absorbance at 260 nm. with a small contribution at 280 nm. The first of the two peaks was the larger, although in the digest chromatograms it usually appeared as a shoulder on the main peak which contained both DNA and veronal.

The ultraviolet absorbance pattern of veronal is complex, depending on the pH of its solutions. Spectral studies of 0.02 M. veronal, pH 8.5, 35 mM. in NaCl and 1.4 mM. in $CaCl_2$ (the micrococcal nuclease buffer) and of 0.02 M. veronal, pH 7.0, micromolar in $MnCl_2$ (the DNase I buffer) at several dilutions showed that at high dilution (i.e. 0.02 mM. in veronal) both showed an absorbance minimum at 205 nm., a maximum at 210 nm., and little absorbance above 240 nm. At ten-fold higher concentration, the pH 8.5 buffer showed a distinct minimum at 225 nm. and a maximum at 235 nm. with a shoulder at 240 nm. At the same dilution the 225 nm. minimum was absent from the spectrum of the pH 7.0 buffer, although the maximum and shoulder remained. Finally, the absolute absorbance of the pH 8.5 buffer at the higher wavelengths was always higher than that of the pH 7.0 buffer, so that for the undiluted buffers at 260 nm. the former's absorbance was greater than 2 while the latter was 1.85, while at 275 nm. they were 1.55 and 0.14 respectively. These results are not intended as a conclusive study of the absorption characteristics of veronal, but rather as an indicator of the complex way in which it will influence the absorbance measurements on peak DD fractions where many other species are present at unknown concentrations. A small contribution to the absorbance in peak RD from veronal is also

expected due to the buffer remaining in the cell at the end of enzymic extraction, and this expectation is confirmed by the shape of the absorbance curves in the chromatogram of this fraction.

Chemical estimation of the DNA in peaks D and E is also hindered by the presence in them of inorganic ions. For example chloride, which dramatically reduces the sensitivity of the diphenylamine reaction for DNA (see Appendix II), has been detected with $AgNO_3$ throughout the peak D fractions. Thus the diphenylamine reaction can be used here as a rather insensitive tool for detection but not for estimation of DNA.

The DNA in peaks DD and DE has been recovered as described in Section B 3.2 and preliminary characterization studies have been made using DEAE-cellulose chromatography. Although these studies corroborate the suggestions of earlier results that these are low molecular weight fractions, no conclusive results have yet been obtained.

SECTION F

THE FRACTIONATION OF SUCROSE NUCLEI

TABLE F1

TABLE F1

EXTRACTION SEQUENCES : SUCROSE NUCLEI

Exp.	Quantity of Nuclei	Sequence	Sephadex Chromatogram	
			Figure	Part of fraction Applied
SI	100-150 mg.	Buffer W[a] Buffer E[b] Buffer E, 10 μg./ml. DNase I	F1	$\frac{1}{2}$
SII	~100 mg.	Buffer W'[c] Buffer E Buffer E, 10 μg./ml. DNase I	F2a	1/3
		2% SDS	F2b	$\frac{1}{2}$
SIII	~25 mg.	Buffer W' Buffer E'[d] Buffer E', 10 μg./ml. DNase I 2% SDS	F4	all
SIV	~25 mg.	Buffer W' Buffer E' Buffer E', 10 μg./ml. DNase I Buffer E 2% SDS	F5	all

a Buffer W : 0.02 M. veronal/HCl, pH 7.0, 0.12 M. NaCl

b Buffer W' : Buffer W, 3.3 mM. in $CaCl_2$

c Buffer E : 0.02 M. veronal/HCl, pH 7.0, μM. in $MnCl_2$

d Buffer E' : 0.02 M. veronal/HCl, pH 7.0, 3.3 mM in $MnCl_2$

SECTION F

THE FRACTIONATION OF SUCROSE NUCLEI

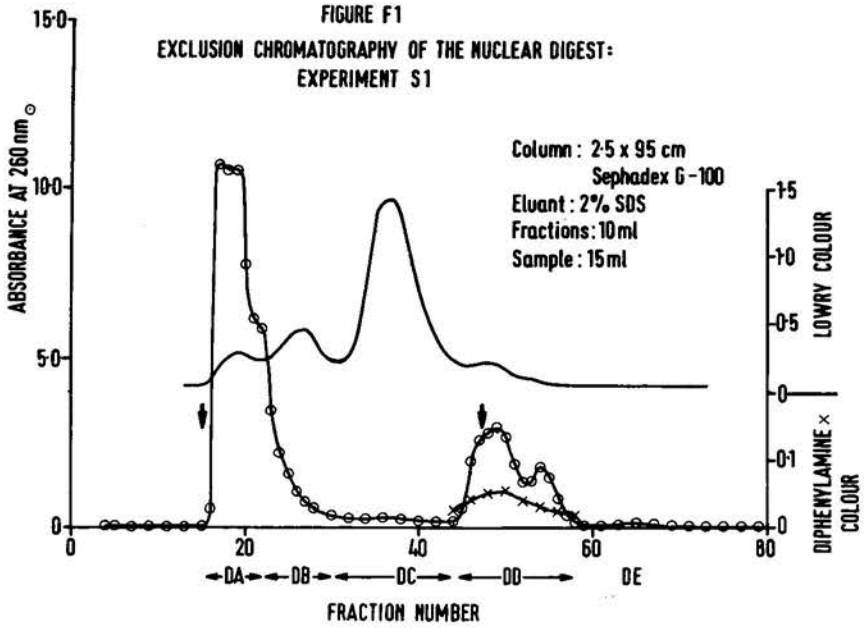
F 1 INTRODUCTION

This section gives the results obtained by applying the procedures whose development is described in Sections B through D to nuclei prepared in sucrose. They behaved differently from the acetic acid nuclei at a number of stages during the extraction, so the standard procedure was modified to adapt to these changes. Four main extractions were performed using Cell III and these will be described in chronological order to show the development of the extraction sequence. The time available for this part of the work was limited so that the results presented are not as complete as had been planned. They do, however, permit a comparison to be made between the two types of nuclei. The experiments are summarized in Table F1 which also provides a key to the Figures in this Section.

FIGURE F1

FIGURE F1

EXCLUSION CHROMATOGRAPHY OF THE NUCLEAR DIGEST:
EXPERIMENT S1



F 2 EXPERIMENT SI

For the first experiment a pellet of sucrose nuclei roughly equivalent to 100 to 150 mg. of dried nuclei was mixed with a paste of 2 g. of sephadex G-25 that had been dampened with 7 ml. of buffer W (see Table F1) and the mixture added to the preassembled extraction cell. Microscopic examination showed that while the nuclei were effectively mixed with the sephadex many were ruptured, a problem that did not arise if they were merely suspended in the same buffer. The cell was closed and the nuclei washed with buffers W and E, and then digested with a solution containing 10 μg. of DNase I per ml. of buffer E. Digestion had proceeded to an absorbance maximum comparable to one-half digestion during extraction of the acetic acid nuclei when the cell blocked, terminating the extraction sequence.

Preliminary experiments had shown that deoxyribonuclease treatment released a nucleoprotein mixture from sucrose nuclei which precipitated on standing, so during this experiment SDS was added to the digest fractions to a final concentration of 1% in order to prevent precipitation. The pooled fractions containing the partial digest were concentrated by lyophilization and fractionated on sephadex G-100, giving the result shown in Figure F1. The most striking feature of this chromatogram is its similarity to those derived from the corresponding acetic acid nuclear fractions. It included a nucleoprotein fraction, DA, two protein fractions, DB and DC, a DNA/veronal fraction, DD, and a DNA fraction, DE, the compositions of these fractions being determined as described in Section E 2.1. The small peak of Lowry colour after fraction C was due to $MnCl_2$. The resolution of these fractions was poor due to overloading of the column, so their further characterization was not undertaken.

Microscopic examination of the cell contents after this

extraction showed that nuclei had washed out of the sephadex gel, blocking the outlet filter. This factor, in addition to the formation of a nucleoprotein gel by the residual nuclei, seemed to account for the cell blockage.

F 3 EXPERIMENT SII

The cell filling and flow problems experienced during experiment SI could be attributed to two causes - physical damage to the nuclei during their mixture with the sephadex and chemical instability due to the composition of the extraction media.

To prevent physical damage a new cell filling procedure was adopted. A pellet of nuclei equivalent to about 100 mg. of dry nuclei was gently suspended in 3 ml. of buffer W' (see Table F1) by mixing with a glass rod. Approximately 1 g. of dry sephadex was gradually mixed into this suspension to form a thick paste of swollen sephadex and intact, isolated nuclei. This mixture was added to the preassembled cell, a mixture of sephadex plus buffer (approximately 1 g. plus 3 ml.) was layered over it to fill the cell, and it was sealed with the millipore filter system as usual. This procedure alleviated but did not eliminate the tendency for nuclei to escape from the sephadex gel, a situation which may have reflected more effective isolation of these nuclei during their resuspension and mixture with the sephadex than was achieved with the ~~nucleic~~ acetic acid nuclei.

The second cause of flow difficulties during the extraction of sucrose nuclei was their instability. Chromatin is well known for its tendency to swell under certain ionic conditions forming the sort of intractable gel observed in Experiment SI (see the discussion of chromatin gels by E. Fredericq, 1971). In Experiment SII, therefore, buffer W was supplemented with 3.3 mM. $CaCl_2$, the divalent cation used during the isolation of the nuclei. This buffer was effective in stabilizing the nuclei during the wash stage of the extraction.

As with the acetic acid nuclei, the wash released some light absorbing material that included protein as determined by the Lowry reaction. DNA and RNA estimation were prevented by interference of the sucrose from the nuclei with the diphenylamine and orcinol

FIGURE F2a

FIGURE F2a
EXCLUSION CHROMATOGRAPHY OF THE NUCLEAR DIGEST=EXPERIMENT SII

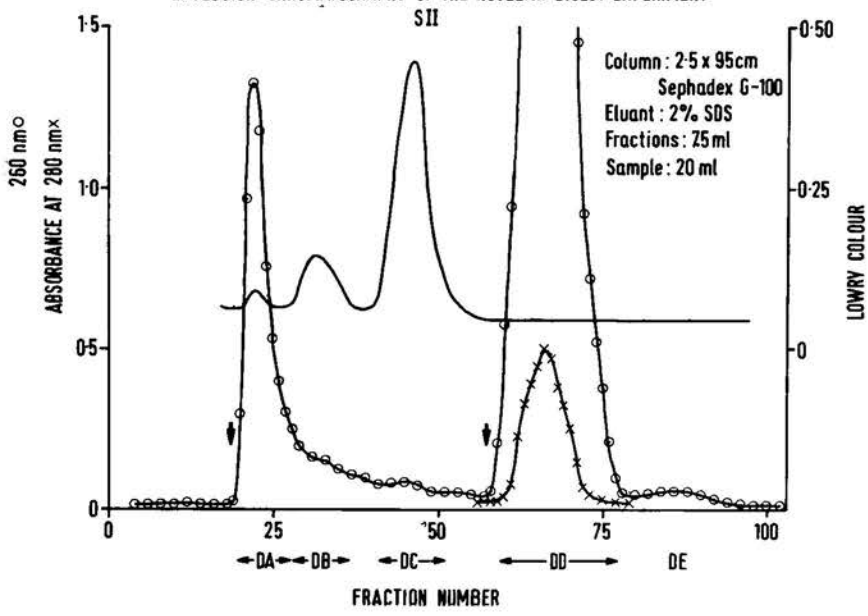
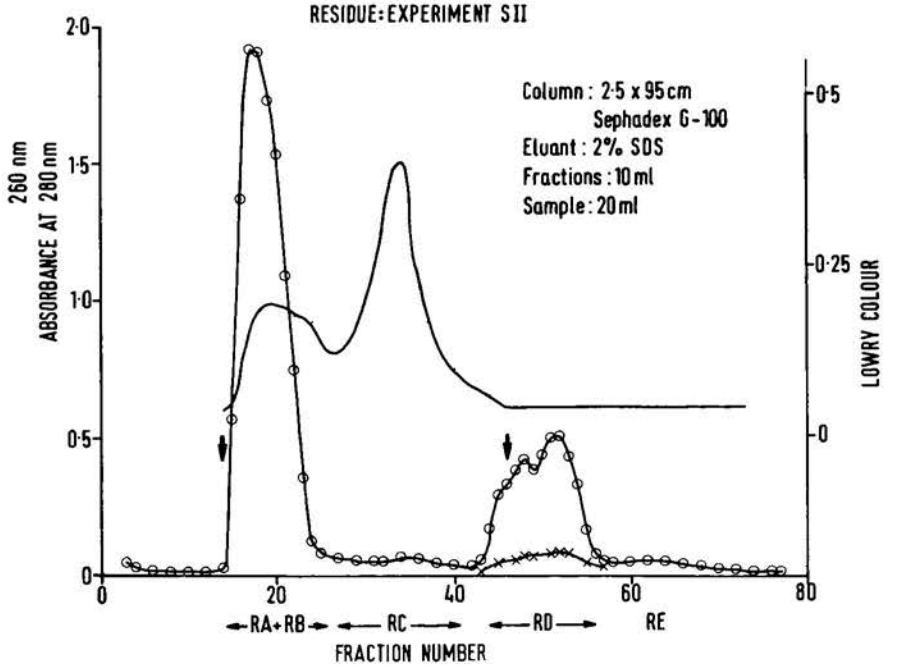


FIGURE F2b

EXCLUSION CHROMATOGRAPHY OF THE SDS - SOLUBLE NUCLEAR RESIDUE: EXPERIMENT SII

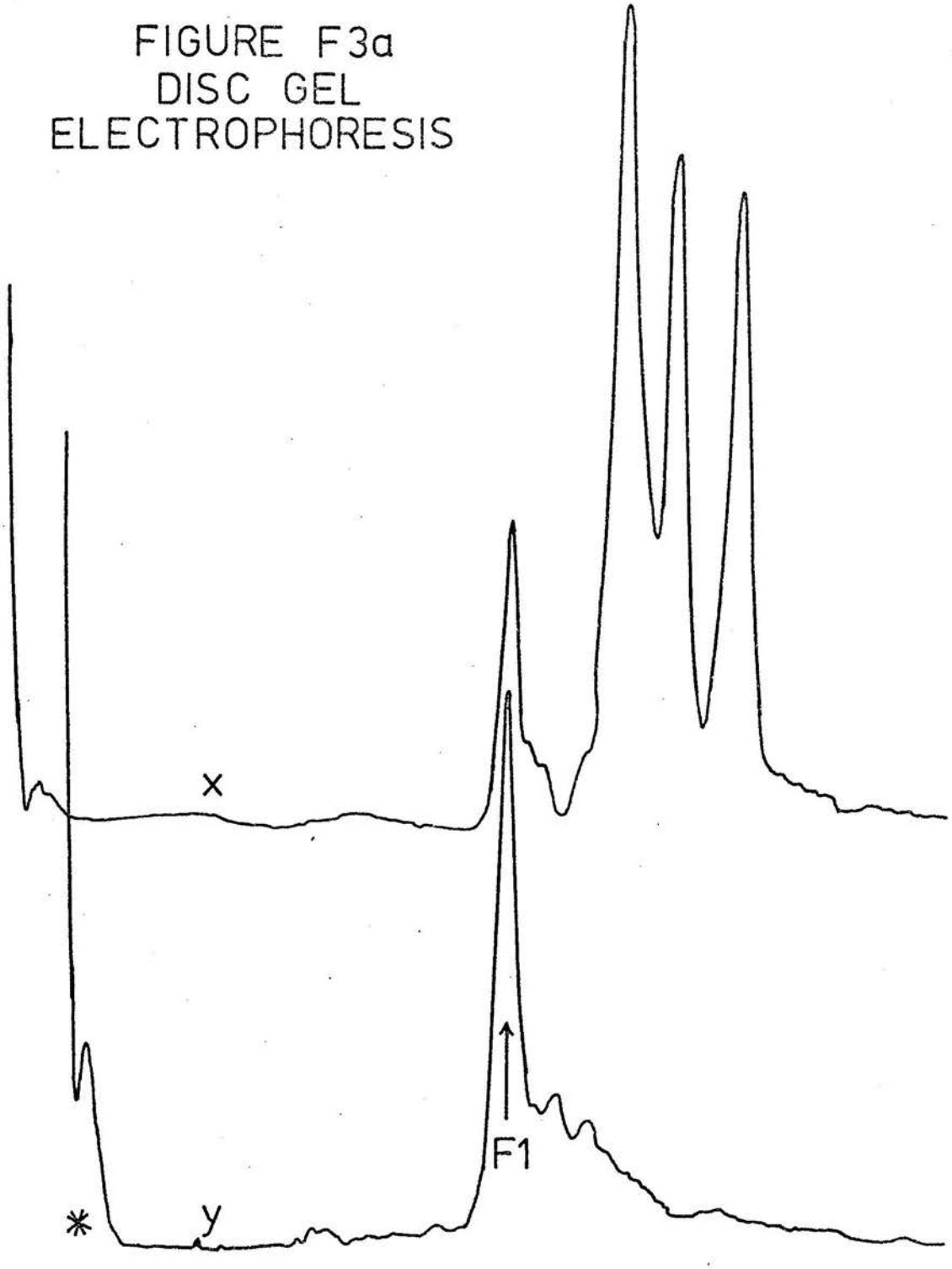


reactions, but the ultraviolet absorbance was too high to be accounted for solely on the basis of the protein content. Testing for autolysis by alternate incubation for ten minutes at 15° C. and washing suggested a rate of autolysis no greater than that of acetic acid nuclei although this was, of course, a rather crude test. To avoid the autolysis and microbial degradation to which these nuclei are particularly susceptible, they were always kept in the cold and extracted as soon as possible after preparation. No further characterization of the wash fraction was undertaken.

The washed nuclei were next extracted with 10 μg. of DNase I per ml. of buffer E. As in Experiment SI, digestion proceeded to about one-half of completion before the cell became blocked and the partial digest was fractionated by the usual methods giving the chromatogram shown in Figure F2a. Examination of the cell contents showed that a nucleoprotein gel again blocked the cell. This residue was extracted with 2% SDS and the extract chromatographed as shown in Figure F2b. The two chromatograms are very similar to comparable results from acetic acid nuclei (see Figures E2a and E2b), each yielding five main fractions with the usual compositions. The proteins from fractions DB and RB, DC and RC were recovered by acetone precipitation and electrophoresed in comparison with crude calf thymus histone chloride using the standard procedure of Panyim and Chalkley. This gave the results shown in Figure F3 - DB and RB containing histone F1 and a component which did not penetrate the gels while DC and RC contained the other histone fractions.

Cell blockage was again attributable to chemical instability of the partially digested chromatin, so an attempt was made in Experiment SIII to stabilize it during digestion.

FIGURE F3a
DISC GEL
ELECTROPHORESIS

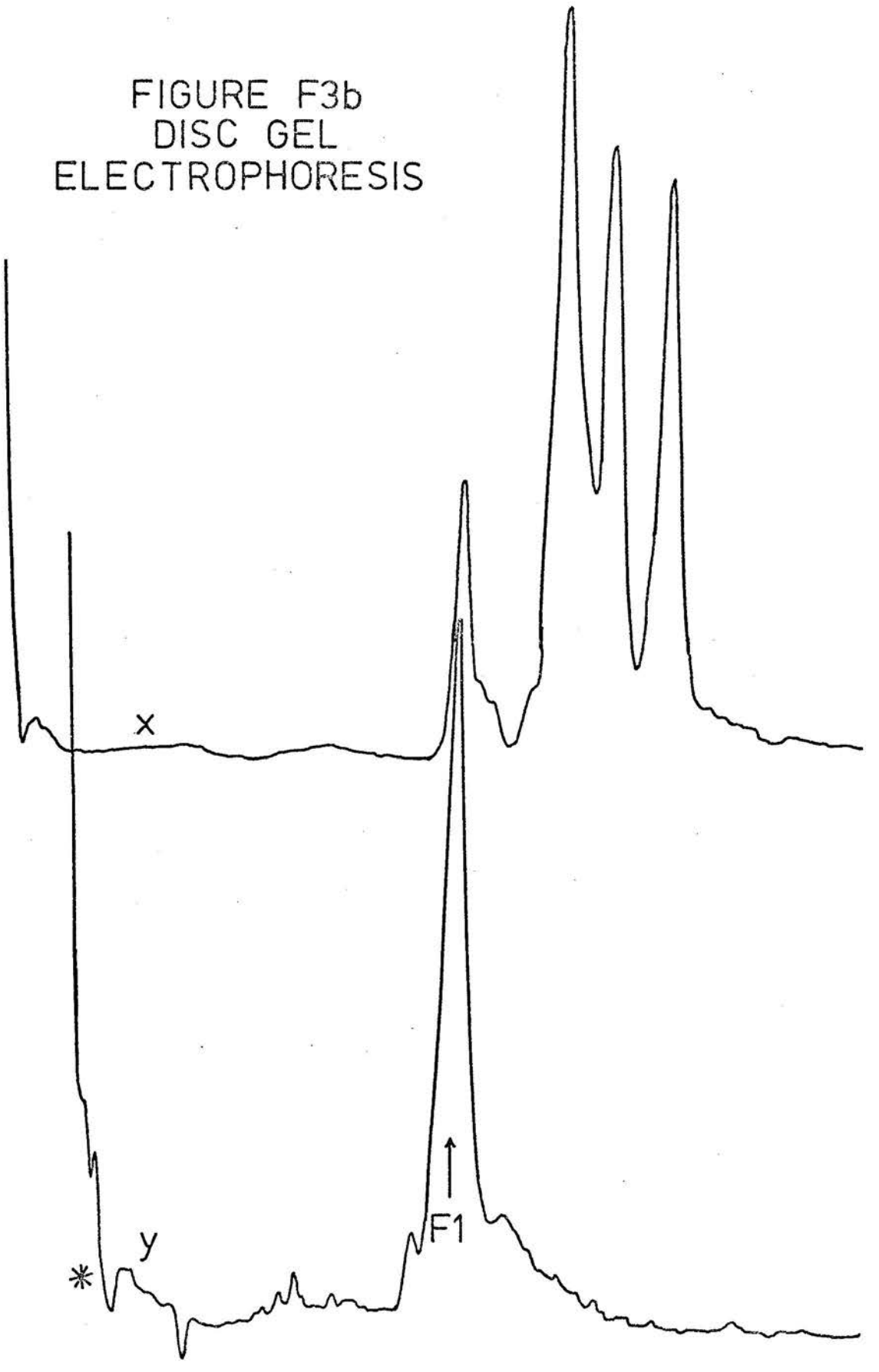


x Crude Calf Thymus Histone

y Fraction DB

* Origin staining

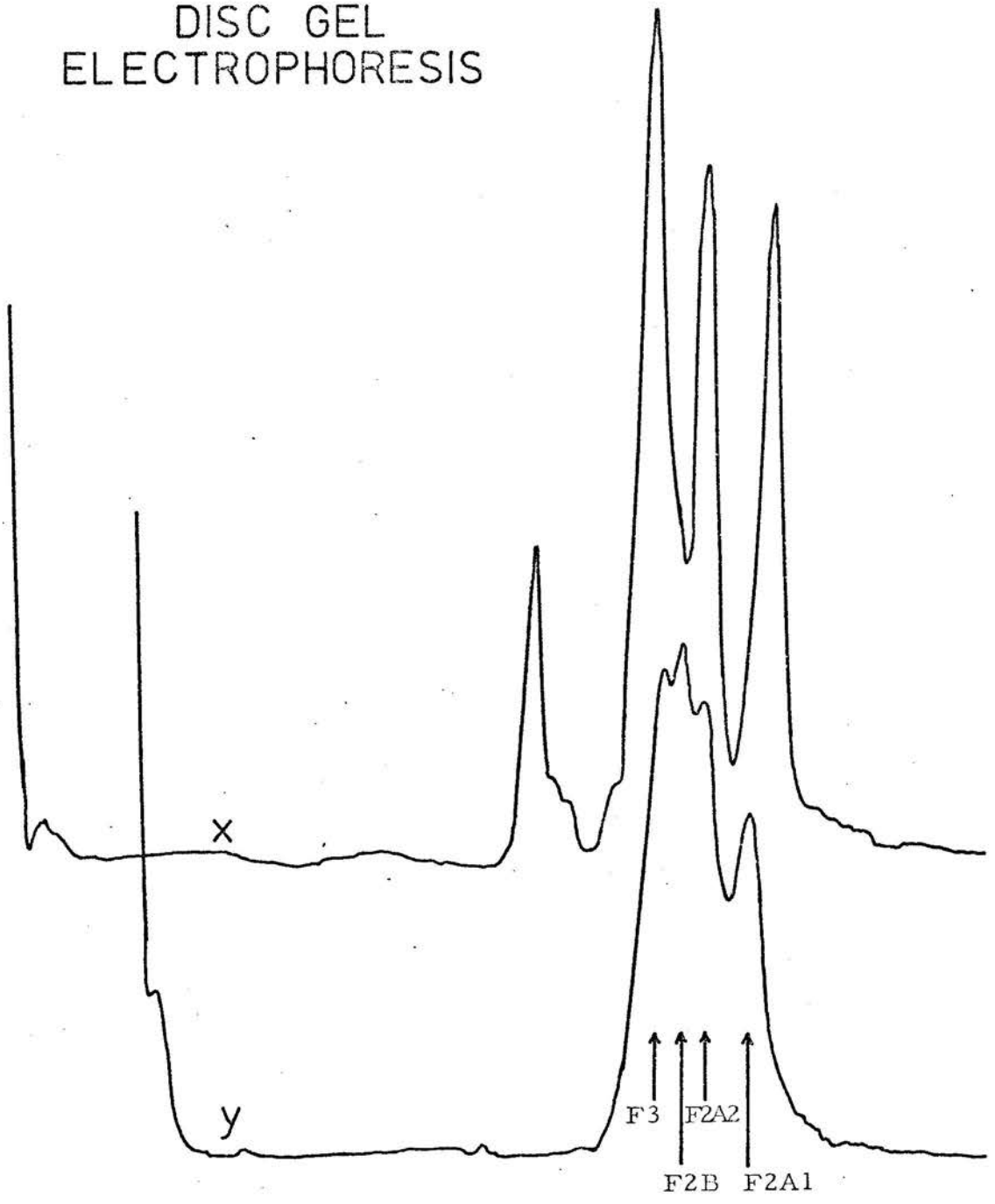
FIGURE F3b
DISC GEL
ELECTROPHORESIS



X Crude Calf Thymus Histone y Fraction RB

* Origin staining

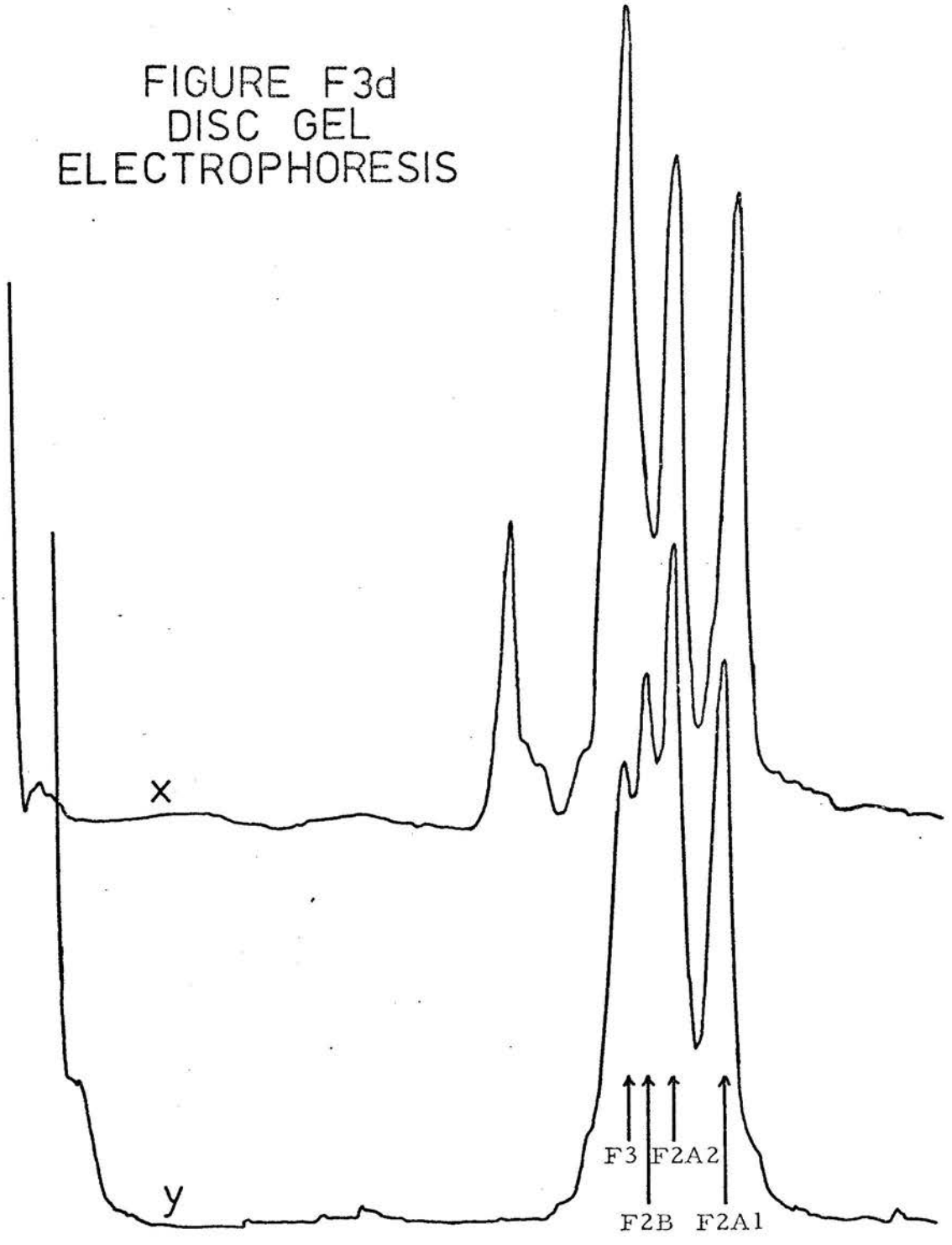
FIGURE F3c
DISC GEL
ELECTROPHORESIS



x Crude Calf Thymus Histone

y Fraction DC

FIGURE F3d
DISC GEL
ELECTROPHORESIS

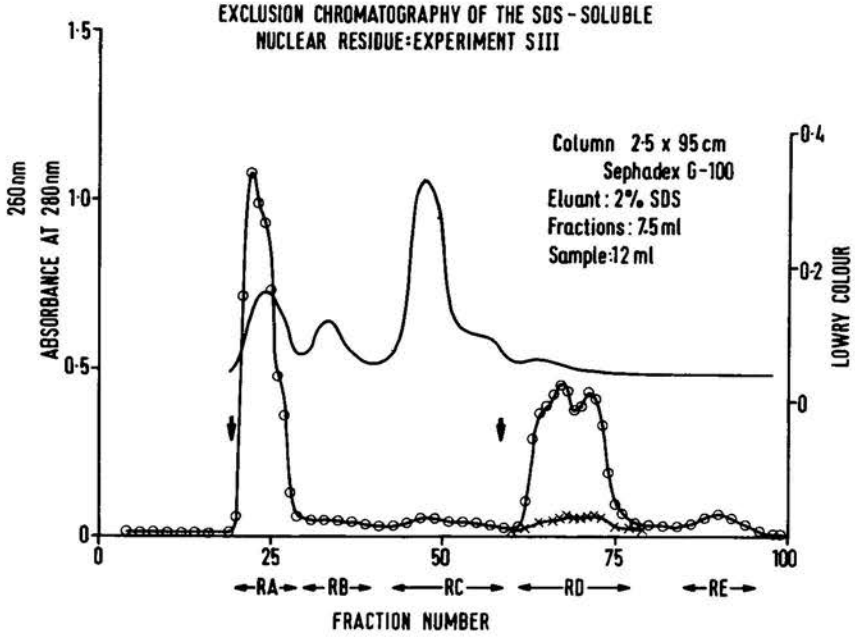


x Crude Calf Thymus Histone

y Fraction RC

FIGURE F4

FIGURE F4
EXCLUSION CHROMATOGRAPHY OF THE SDS - SOLUBLE NUCLEAR RESIDUE: EXPERIMENT SIII



F 4 EXPERIMENT SIII

Deoxyribonuclease is known to be active at higher $MnCl_2$ concentrations than that used in the studies of acetic acid nuclei (see the review on DNase I by M. Laskowski, Sr., 1971), so the $MnCl_2$ concentration of the enzyme buffer in Experiment III was increased to 3.3 mM. It was hoped that, if gel formation had again been caused by the decreasing divalent cation concentration of the eluant, this increase would stabilize the chromatin for the duration of enzymic elution. In addition, the quantity of nuclei extracted was reduced to about 25 mg. to improve the chances of maintaining flow. Flow did, indeed, persist under these conditions, but the enzyme caused a barely perceptible peak of UV absorbance in the eluate. Diphenylamine and orcinol analysis detected no DNA or RNA and the Lowry colour present was attributable to $MnCl_2$.

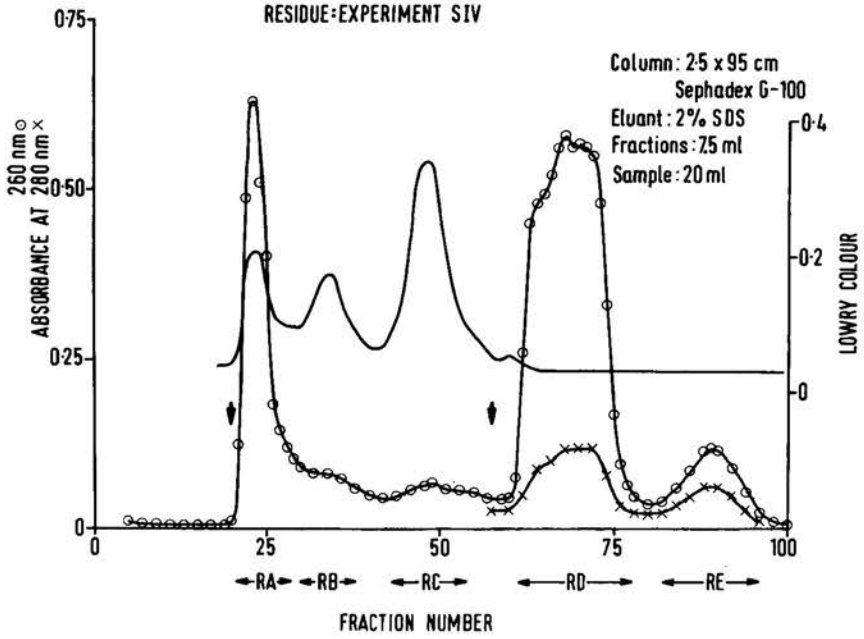
The cell residue, which included intact nuclei, was extracted with 2% SDS, which failed to dissolve all methylene blue staining material. The concentrated extract was fractionated giving the results shown in Figure F4. This chromatogram is similar in the composition and in the relative sizes of the fractions to those of the other residue extracts from both acetic acid (Figure E2b) and sucrose (Figure F2b) nuclei. The DNA is again fractionated into an excluded and two retarded fractions, the DNA content of the latter being corroborated by diphenylamine analysis.

Other workers in this laboratory have attempted to fractionate whole acetic acid nuclei by chromatography on the sephadex G-100 column in SDS and 2-mercaptoethanol. The nuclei dissolve if treated with SDS and 2-mercaptoethanol according to the procedure used to prepare samples for electrophoresis in the SDS polyacrylamide gel system (see Section B 3.1), and a sample of nuclei so prepared was applied to the sephadex column. The experiment was unsuccessful because the sample failed to enter the sephadex gel,

suggesting that some fractionation of the nuclei and/or degradation of their DNA is necessary before they can be fractionated by this method. These results, together with the presence of some DNA fragments in the SDS soluble residue from Experiment SIII, suggest that some DNA degradation must have occurred to permit chromatography of the sample, but that the degradation products were not released from the nuclei during digestion under these ionic conditions. In order to test this postulate the elution sequence was altered again in Experiment SIV.

FIGURE F5

FIGURE F5
EXCLUSION CHROMATOGRAPHY OF THE SDS - SOLUBLE NUCLEAR RESIDUE: EXPERIMENT SIV



F 5 EXPERIMENT SIV

Experiment SIII was now repeated, but a volume of enzyme solution estimated as sufficient for complete digestion was followed by the old enzyme buffer containing only μM. $MnCl_2$. This buffer did sharply release a nucleoprotein fraction, but its quantity was very small in comparison to the usual digest (too small for chromatographic fractionation). The nuclear residue was extracted with 2% SDS and chromatographed, giving the result shown in Figure F5. The proteins from fractions RB and RC of this and the previous experiment (Figure F4) were recovered by acetone precipitation and electrophoresed according to the standard acetic acid method. RB contained, in each case, histone F1 and a component that did not penetrate the gel, while RC contained the other histone fractions.

None of the procedures for dealing with the nucleoprotein fractions A developed during the experiments with acetic acid nuclei had provided conclusive analyses of these fractions, so they were not applied to the sucrose nucleoprotein fractions which were instead retained for further analysis at a later date. Possible methods for dealing with these fractions are discussed in Section G.

Apart from the identification of DNA and veronal in fractions D and of DNA in fractions E of the chromatograms no further analysis of these fractions has been undertaken for the reasons discussed in Section D 3.

TABLE F2

TABLE F2

QUANTITATIVE ASPECTS OF PROTEIN EXTRACTION: SUCROSE NUCLEI

EXP. NO.	SAMPLE	RELATIVE SIZES OF PROTEIN PEAKS								
		A	AV.	B	AV.	A+B*	AV.	C		
SI	Partial Digest	0.12	0.11	0.24	0.26	0.36	0.37	1.0		
SII	Partial Digest	0.10		0.28		0.38		1.0		
SII	SDS-Soluble Residue	Unresolved		Unresolved		0.52	0.52	1.0		
SIII	SDS-Soluble Components	0.36	0.36	0.23	0.23	0.59	0.59	1.0		
SIV	SDS-Soluble Components	0.42	0.42	0.42	0.42	0.84	0.84	1.0		
	Crude Histone (from Table E5)	0.02	0.03	0.23	0.24	0.25	0.27	1.0		
		0.04		0.25		0.29		1.0		

* Poor resolution of Peaks A and B, particularly in the chromatogram of the SDS-soluble residue from Experiment SII, makes their combined estimate more reliable than the individual values.

F 6 QUANTITATIVE ASPECTS OF THE EXTRACTIONS

The problems involved in deriving quantitative interpretations from these extraction experiments have been thoroughly discussed throughout the previous sections. The wet sucrose nuclei, prepared freshly for each experiment, were particularly difficult in this respect. The estimates of the quantities of nuclei employed in each of these experiments are based on the preparation which preceded Experiment SII, when the nuclear homogenate derived from 200 ml. of trimmed tissue was divided into four equal parts for the final centrifugation and two of the resulting pellets dried and weighed, giving 85 and 73 mg. of nuclei. The other sample sizes were then estimated by comparison with this preparation and are not, therefore, precise.

Quantitative analysis of the DNA fractions within each extraction is not possible for the reasons discussed in Section E 6, but a quantitative comparison of the protein fractions similar to that given for the experiments with acetic acid nuclei in Table E5 is given in Table F2. The failure to achieve complete digestion of the sucrose nuclei means that these results are not directly comparable with the previous ones. In particular, it cannot be assumed that the sum of digest and residue fractions in Experiment SII, or the residue fraction in Experiments SIII and SIV constitute the same fraction of the whole nuclei as did the sum of digest and residue of the acetic acid nuclei, since the portion of the nuclei solublized by these methods is very likely to be a function of the extent of DNA digestion. These results are discussed further in Section G.

SECTION G

DISCUSSION

SECTION G

DISCUSSION

The results of the experiments described in Sections C to F will be discussed under three main headings: a general assessment of the extraction method; the analysis of the nuclear fractions; and the general and comparative significance of the fractionations achieved with both acetic acid and sucrose nuclei.

G 1 THE NUCLEAR EXTRACTION METHOD

These studies were undertaken with the object of examining and exploiting the advantages of a continuous flow system in fractionating the complex aggregate of macromolecules present in the eukaryotic cell nucleus. Figures D1 and D2 (Section D 2) illustrated the success of the approach in yielding several well resolved fractions from a single aliquot of nuclei. The cell designs proved effective in minimizing dead space within the system so that each fraction was eluted sharply for high sensitivity. The tailing of some fractions hampered recognition of their complete elution but the concentrations of material involved at this stage were very low. This problem could be alleviated by replacing the broad wave-band Gilson Absorptiometer with a more precise UV monitor which would provide an immediate estimate of the absolute and relative quantities of material in the eluate.

As was described in Section D3, the small quantities of material in the nuclear digests and, more particularly, their coprecipitation, made it necessary to pool for further characterization all of the fractions produced by a single eluant. This procedure constituted a sacrifice of resolution which could be avoided where fractions contained larger quantities of more readily characterized materials. The potential value of greater resolution was indicated by the results in Section D 3. 2, where direct electrophoresis of the

nuclear digest subfractions suggested the sequential release of the histone components within one stage of the extraction. In fact this phenomenon could be re-examined, now that a fuller understanding of the digest components has been achieved, by direct electrophoresis of these fractions in the SDS-polyacrylamide gel system.

The advantages of the flow system were further demonstrated in Experiment SIV (Section F 5), where the effect of ionic environment on enzymic attack of the nuclei was examined by varying the composition of the eluting buffer. The flow system provided a rapid and sensitive assessment of events which would be difficult to detect in any other way.

Although the problem of dead space within the flow system was fairly easily eliminated, the other anticipated difficulty of achieving and maintaining adequate flow past the nuclei was not fully resolved. This difficulty stemmed from two main causes. The proclivity of chromatin to form an intractable gel that prevented flow was a problem of application of the flow system which could be resolved by modifying the eluant compositions (as was shown during the experiments with sucrose nuclei). Blockage of the outlet filter by nuclei that had escaped from the sephadex gel was, however, a problem that stemmed from the design of the system. It was partially resolved by modifying the cell filling procedure, but elimination of the problem would probably require a change in the supporting medium for the nuclei. Although a superfine grade of sephadex was used here as the supporting medium, the sephadex particles were still considerably larger than the nuclei. Thus even when closely packed the sephadex must have contained channels large enough for their passage (although the extent to which this is true depends partly on the tightness of packing of the cell, an empirical factor related to operational experience). It has been noted that acetic acid nuclei, at least, seem to stick to sephadex particles. This may be less true for sucrose nuclei, and may in

any case be overcome by the flow rates involved in these experiments, thus failing to hold the nuclei within the gel.

The permeable glass beads manufactured for exclusion chromatography might provide a replacement for the sephadex and their rigidity would be useful in avoiding compression at high hydrodynamic pressures. In view of the fragility of isolated nuclei, however, (see Sections F2 and F3), it might prove difficult to prepare a mixture of glass beads and undamaged nuclei.

Although it has not been used extensively, a procedure has been developed for the preparation of nuclei in nonaqueous media (M. Behrens, 1932). It is possible that nuclei suspended in such media, if not prepared in them,could be encapsulated in sephadex-like spheres. If sufficiently porous and rigid these should provide an ideal flow medium. The success of such a procedure would, of course, depend on the use of chemical procedures mild enough to avoid damaging nuclear structure.

In spite of these difficulties, the advantages of the flow system to this and other studies are obvious. Not only structural experiments, but studies of metabolism and permeability may be approached in this way. The critical factor in each case will be the choice of a supporting medium of appropriate permeability, rigidity and reactivity. In view of the proliferation of synthetic media for chromatographic purposes, this should not be an insoluble problem.

G 2 THE ANALYSIS OF THE NUCLEAR FRACTIONS

In this duscussion all remarks, unless otherwise stated, apply to the fractions from both acetic acid and sucrose nuclei.

G 2.1 Secondary Fractionation Procedures

Two main nuclear fractions, each containing both nucleic acid and protein, were analyzed during the course of this work. These included the DNase I nuclear digest and the SDS-soluble, post-enzymic nuclear residue. Although the former, at least, was produced by a chemically mild extraction procedure, it did not prove possible to avoid chemical extremes during the subsequent fractionation and analysis steps. As was discussed in Section D 3.5, the prevention of nucleic acid/protein coprecipitation in the digest fractions required their dissociation with such reagents as NaCl or SDS, and the latter was chosen in developing the fractionation method because it did not cause histone aggregation. SDS was chosen to solublize the enzyme-resistant nuclear components for purposes of comparison, but further development of the method would be directed to finding milder post-enzymic extraction media.

Exclusion chromatography was chosen as the secondary fractionation method for the nuclear digests in the hope that it would separate the low molecular weight DNA fragments from the higher molecular weight protein components of the mixture. Again for purposes of comparison, the SDS-soluble residue fractions were treated in the same way, and in every case chromatography yielded five fractions containing, in order of elution, DNA/protein, protein, protein, DNA/veronal, and DNA (fractions A to E respectively). Thus although the method was successful in separating most DNA and protein, they remained mixed in the excluded fraction of the chromatograms, and further fractionation attempts did not succeed in separating them (see Section E 3 and the discussion below). It is possible that a separation could be achieved by the hydroxyapatite

fractionation method for nuclear proteins of A. J. MacGillivray, D. Carroll and J. Paul (1971). This method could be applied either to the purified nucleoprotein fractions (fractions A) or to the total digest if NaCl/urea were used rather than SDS to prevent precipitation. The further characterization of the chromatographic fractions is discussed below.

G 2.2 Nucleoprotein Fraction A

An excluded nucleoprotein fraction was present in chromatograms of both the nuclear digest (Figures E1 and E2a, Sections E 2.1 and Figures F1 and F2a, Sections F2 and F3) and the SDS-soluble, post-enzymic nuclear residue (Figure E2b, Section E 2.1 and Figures F2b, F3 and F4, Sections F3, F4 and F5), and an excluded protein fraction appeared in chromatograms of crude calf thymus histone (see Section E 2.2). Throughout this thesis these fractions have been considered together, mainly because they shared common problems of fractionation and identification. It should be stressed, however, that each one is probably heterogeneous and that neither digest fractions and residue fractions nor acetic acid nuclear fractions and sucrose nuclear fractions need be identical. It seems likely, however, that the excluded fraction of crude calf thymus histone, fraction SA, was at least related to the corresponding fraction, DA, from acetic acid nuclear digests, and the analysis of these fractions that was achieved is discussed below. It is hoped that this analysis may be representative of those to be expected from the other excluded nucleoprotein fractions, but clearly their complete assessment depends on the analysis of their DNA-protein relationships and on the successful separation and characterization of the components in each fraction.

Several unsuccessful attempts were made to separate the nucleic acid and protein components of acetic acid nuclear fraction DA. Its acid soluble protein component resisted electrophoresis in that apart from some minor slow-moving components

it failed to penetrate polyacrylamide gels in SDS and 2-mercapto-ethanol, with and without urea, as did standard histone fraction SA (Section E 3.1). They were insoluble in 0.9 M. acetic acid and therefore could not be electrophoresed in this medium. The failure of both fractions to respond to 2-mercaptoethanol and SDS treatment suggested that their exclusion from the gels was not a result of protein aggregation phenomena such as sulphydryl bridging. Their ultraviolet absorbance spectra were suggestive of nucleic acid contamination, however, and if nucleic acid were present in non-ionic linkage to the proteins it could be responsible for their failure to electrophorese (see Sections E 2.2 and E 3.1).

Sepharose chromatography of the total fraction in SDS with and without 2-mercaptoethanol treatment yielded nucleoprotein subfractions (Section E 3.2), while redigestion of one of the sepharose subfractions with DNase I and of fractions DA and RA with micrococcal nuclease, each followed by exclusion chromatography, also failed to free them of nucleic acid (Section E 3.3).

The standard procedure for recovery of the material from chromatographic fractions in SDS solution which involved acidification and acetone precipitation was designed primarily for histone fractions (Section B 3.2). It is very likely that non-histone proteins present in the fractions would be damaged by such a procedure or by SDS treatment itself, and that this damage might lead to their later intractability. J. Sonnenbichler and P. Nobis (1970) and others have claimed that acid treatment causes covalent linkage of histone and DNA giving an artifactual residual protein fraction. This explanation, too, could account for the behaviour of the excluded protein after recovery from the chromatographic fractions. Its presence in the digests and SDS-soluble residues from sucrose nuclei that have never been exposed to extremes of pH, however, eliminates acid treatment as the cause for its initial behaviour on chromatography. The failure of all these separation procedures

strongly suggested an unusually firm type of bond between the excluded nucleic acid and protein.

In the absence of a purified protein preparation from this fraction, an amino acid analysis was carried out on the redigested sepharose subfraction, fraction DA' (Sections E 3.2 and E 3.3), whose nucleic acid contamination was minimal in comparison to the other available fractions. The influence of nucleic acid contamination on the amino acid analysis of proteins has been examined by several authors (for example H. Ishihara, 1960; R.H. Lindsay, W.K. Paik and R.R Cohen, 1962). They have found that under protein hydrolysis conditions the purines, particularly adenine, yield glycine and ammonia, the conversion rate from adenine to glycine being about 60 mole % for hydrolysis under the conditions used in these experiments. This factor must therefore be remembered when comparing the analysis of this fraction with those of fraction SA' (Section E 2.2), crude histone and non-histone protein, all of which were given in Table E2 (Section E 3.3). The difficulties in interpreting amino acid analyses, particularly when they are derived from mixed protein samples, are well known. However fraction DA' does show features more characteristic of non-histone than of histone proteins. These include, in the case of DA', its high content of acidic and low content of basic amino acids and its high phenylalanine and histidine contents. Most striking, however, is its high cysteine content (see Section B 3.2 for explanation), a feature which is often considered indicative of non-histone contamination. This is particularly notable in being at least twice as high as the level found in any other fraction analyzed at the same time apart from fraction SA' (see Tables E3 and E4).

In view of the above information and of the identification of all the histone components in fractions B and C (see below), it seems likely that acetic acid nuclear fraction DA, at least, contains non-histone protein.

G 2.3 Protein Fractions B and C

Protein fractions B and C appeared in all chromatograms of both nuclear digests and nuclear residues. They were characterized by electrophoresis and, in the case of the acetic acid nuclear digest, by amino acid analysis. (See Sections E4 and F3.)

Fraction B was shown in each case to contain predominantly histone F1 on electrophoresis, but both the digest and residue fractions B contained a component that did not penetrate a number of polyacrylamide gel systems and which was absent from the corresponding crude histone fraction (see Figure E9, Section E 4 and Figure F3, Section F 3). This component may have been reflected in the amino acid analysis of acetic acid nuclear fraction DB (Table E3, Section E 4) which did differ slightly from those of the corresponding standard fraction and the cited composition of histone F1. Particularly notable was its cysteine content, often indicative of non-histone contamination and particularly so in this case, as histone F1 contains no cysteine. The possible sources of this protein component were similar to those described above for the proteins of fraction DA. Indeed the chromatographic resolution of fractions A and B was often poor and this component may represent contamination of fraction B with fraction A.

Fraction C contained the non-F1 histones as shown by both electrophoresis (Figure E9, Section E 4 and Figure F3, Section F 3) and amino acid analysis (Table E4, Section E 4). No other proteins were found in significant quantities in this fraction.

G 2.4 DNA Fractions

The DNA of both the nuclear digests and the SDS-soluble residues appeared in three fractions on exclusion chromatography. These included the excluded nucleoprotein fraction and two retarded fractions. The difficulties involved in further characterizing these fractions were discussed in Section E 6.2 and it seems reasonable

to conclude only that the retarded peaks represent fairly low molecular weight degradation products of the type expected in a DNase digest. Precise interpretations of the chromatographic results in terms of molecular weight are, however, prevented by the anomalous behaviour of DNA on sephadex (see Th. Hohn and H. Schaller, 1967). The degree of polymerization of the excluded DNA and its relationship to the excluded protein remains open to question because their continued association precluded further characterization steps.

Some discussion regarding the kinetics of DNA degradation was given in Section D 3.5, where it was suggested that digestion might proceed as a two stage process: firstly, degradation of the DNA to fragments small enough to escape the extraction cell; and secondly, further degradation to the smaller fragments of fractions D and E, much of which could occur after elution from the nuclei. This model was supported by the results of the experiment during which digestion and precipitation were stopped by the addition of NaCl, and DNA was confined to fraction A (Section D 3.5). It was not supported, however, by the reappearance of DNA fractions D and E in the nuclear digest chromatograms of experiments where precipitation (and, very likely, enzyme action) was stopped with SDS (Section E2). Furthermore, the appearance of degraded DNA fractions D and E in the SDS-soluble residue fractions from both the acetic acid nuclei and the sucrose nuclei exposed to the enzyme under a variety of ionic conditions demonstrated that the release of DNA was not solely a function of its successful degradation, but depended on its release from other molecular associations. This further emphasizes the dangers inherent in interpreting these chromatographic fractionations of DNA in terms of its molecular size.

G 2.5 Summary

To summarize these results, both the nuclear digest and the SDS-soluble, enzyme resistant nuclear residue from both acetic acid and sucrose nuclei contain all the histone fractions, a degraded DNA

fraction, and a nucleo-protein fraction excluded from sephadex G-100. The excluded protein may be non-histone in character and bound to the DNA in some manner other than a simple ionic linkage.

The most notable feature of these results is the similarity, not only between fractions from the acetic acid and sucrose nuclei, but also between the chromatin fractions attacked by and resistant to DNase I. The significance of these results will be assessed below.

G 3 THE SIGNIFICANCE OF THE NUCLEAR FRACTIONATIONS

G 3.1 DNA Fractionation

Deoxyribonuclease I was chosen as the main agent of these nuclear fractionations with the objective not only of examining those proteins so intimately associated with the DNA in chromatin as to be released during its degradation, but also of producing DNA-free nuclear residues whose protein-protein interactions could be examined. While the former objective was achieved with some success, the latter was not, as was indicated by the data summarized in Tables D1 and D2 (Section D 2.5). In fact only approximately two-thirds of the DNA was released from acetic acid nuclei by DNase digestion in μM. $MnCl_2$. Failure to reach the limit of digestion with sucrose nuclei because of flow difficulties (see Sections F2 and F3) prevented direct comparison of their behaviour with that of the acetic acid nuclei. The severe limitation on attack and release of their DNA by the enzyme in the presence of an elevated $MnCl_2$ concentration was, however, equally interesting (see Sections F4 and F5).

Interpretations of these limitations on DNA release must be made within the context of the enzyme's specificity. DNase I is subject to autoretardation and exhibits a complex pattern of specificity dependent on the type and concentration of divalent cation present in the digestion medium (see M. Laskowski, Sr., 1971). In static digestion situations increased concentrations of enzyme and cation can be used to force hydrolysis to completion in the presence of inhibitory hydrolysis products. Exposure of a DNase resistant residue in these experiments to an elevated enzyme concentration did not, however, lead to a pulse of hydrolysis products in the eluate (Section D 2.4). It is difficult to assess the limitations imposed by the enzyme's specificity in the flow situation, but digestion may have been terminated by the formation of a core of DNA which the enzyme would not attack because of its specificity.

In theory this effect could be examined by exposing the DNase-resistant residue to an enzyme of differing specificity, but such an approach would require an enzyme active under identical reaction conditions to DNase I. Spleen phosphodiesterase is such an enzyme, but unfortunately it is an exonuclease specific for the hydrolysis of polydeoxyribonucleotides with 3'-phosphate end groups, the 5'phosphomonoesterified polynucleotides produced by DNase I being resistant to attack (J. F. Koerner and R. L. Sinsheimer, 1957 and W. E. Razzell and H. G. Khorana, 1958). Concurrent digestion with the two enzymes might, however, achieve further degradation than DNase I on its own.

The effect of Mn^{++} concentration must also be considered, not only as it effects the enzyme's specificity, but in its influence on chromatin structure. Divalent cations are known to cause chromatin condensation which would be expected to reduce the accessibility of DNA, and the experiments with sucrose nuclei (especially Experiments SIII and SIV of Sections F4 and F5) suggested that the extents of DNA degradation and release were both functions of Mn^{++} concentration. Unfortunately our meagre knowledge of the native ion content of nuclei makes it impossible to reproduce and examine 'native' chromatin. The effects of ion content can, however, be used as tools to modify chromatin structure. In view of the results presented here it would be interesting, for example, to digest nuclei with DNase I in the presence of a decreasing gradient of Mn^{++} concentration in order to gradually expose the chromatin to digestion without forming an impenetrable chromatin gel. The effect of other ions could also be assessed in this way.

Both these considerations and the similarity between the DNA fractions of enzymic digests and SDS-soluble residues discussed above (Section G 2.4) suggest, therefore, that digestion and release of DNA from nuclei in the flow system should be considered as interdependent functions of its molecular associations within the

nucleus. The effect of divalent cations in this respect has been discussed, but it is attractive to suggest that DNA-protein associations are also relevant. This possibility is discussed further below.

G 3.2 Protein Fractionation

Having considered the extent to which DNA was released from the nuclei during DNase I treatment, it is also relevant to consider the extent of protein release. The available quantitative information from an extraction of acetic acid nuclei was summarized in Table E1 (Section E 1). Only about one-half of the total nuclear protein was extracted by the DNase I treatment and SDS extraction which together solublized all of the DNA, each of these fractions containing about one-half of the extracted protein. Qualitative support for these results is found in the acid extraction of histone from the enzyme-resistant residues of earlier extraction experiments (see, for example, Figure D1 of Section D 2.1, and Section D 2.6). Strictly comparable results are not available for the sucrose nuclei, although histone and other protein was certainly present in both the fractions released by enzyme treatment and those extracted with SDS from the post-enzymic residues of sucrose nuclei.

The identity of the protein extracted by both methods was determined by exclusion chromatography, electrophoresis and amino acid analysis as discussed above (Section G 2.2 and 2.3). These methods showed all of the histones to be present in both the enzymic digest and the SDS-soluble residue, with no obvious deviation from their usual proportions (see further discussion below).

Quantitative analyses of the chromatographic protein subfractions from both acetic acid and sucrose nuclei (as well as from crude histone) were given in Tables E5 (Section E 5) and F2 (Section F 7) respectively. The derivation of these analyses is described in Section E 5 and the limitations imposed by the failure to achieve complete enzymic digestion of the sucrose nuclei (in the system used for the acetic acid nuclei, at least) must be recalled in

comparing the two sets of results.

The most striking feature of the quantitative analyses is the increased proportion of the total protein represented by fractions A and B in the nuclear digests and SDS-soluble residues as compared to crude histone. This effect is most marked for fraction A, and the variation in B fractions may be a result of their poor resolution from A. Comparison of the data from the acetic acid nuclear digest and SDS-soluble residue shows that the fraction A proportion is markedly higher in the latter than in the former, and the same effect is shown, although to a lesser degree, by the data from sucrose nuclear extraction Experiment SII. The peak A proportion in the SDS-soluble residues from Experiments SIII and SIV is also high.

The characterization studies discussed above (Section G 2.2) implied that the peak A fractions contain non-histone protein. Examination of Table E1 (Section E 1) supports this contention in that the total protein content of the nuclear digest and SDS-soluble residue in that experiment was greater than the histone content of the nuclei extracted. In view of the problems involved in making these estimations, however, further experiments would be necessary to prove the validity of this result.

These results, then, suggest that the DNase I digest and SDS soluble residue from acetic acid nuclei, which together contain one-half of the total protein and probably all of the histone of the nuclei, each contain all of the histone fractions and a non-histone fraction. It was suggested above that the limitation on DNA release from the nuclei during enzymic digestion was imposed by its molecular associations, those with protein being of particular interest. In view of this suggestion, a distinction between the protein of digests and SDS-soluble residues would be interesting. Superficially, at least, no such distinction exists with regard to the histones. In

view of the microheterogeneity of histones caused by side-chain modifications, however (see Section A 3), the identity of the two histone fractions cannot be assumed without examining their distributions of modified amino acids. The second possible source of distinction between digest and SDS-soluble residue proteins is provided by the non-histone proteins. Failure to characterize in detail the protein fractions from these experiments suspected of containing non-histone proteins makes it unwise to attribute to them a controlling structural role. They do, however, show quantitative variation between digest and residue, a larger proportion of this protein relative to histone being present in the less readily solublized chromatin fraction. The latter may, of course, contain nuclear proteins irrelevant to chromatin structure, as SDS is not an extraction tool of comparable selectivity to DNase I. In view of the evidence that these proteins are unusually firmly bound to the DNA, however, (see Section G 2.2) they are good candidates for a role in chromatin condensation and should therefore be examined further.

G 3.3 Summary

To summarize, the nuclear extraction cell was used here to produce two major DNA/protein fractions from both acetic acid and sucrose nuclei, one by DNase I digestion and one by SDS-extraction of the enzyme resistant residue. Each of these fractions contained DNA, histones, and a DNA-associated protein fraction which was probably non-histone in character. The two fractions may represent structurally different components of the chromatin, the structural variation being attributable to variations in protein content, or it may reflect artificial limitations imposed by the specificity of the deoxyribonuclease and the influence of the ionic environment.

One objective of this work was to compare the behaviour of acetic acid and sucrose nuclei in the extraction system, and therefore the structures of their chromatins as so reflected. The failure to achieve extraction of both types of nuclei under identical conditions

makes a direct comparison impossible. It is remarkable, however, that in so far as comparable experiments were possible the behaviour of the two types of nuclei was identical, the critical difference lying in the greater sensitivity of the sucrose nuclei to their ionic environment. Hopefully further experiments with sucrose nuclei will elucidate this observation.

SECTION H

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Section I

APPENDICES

SECTION I

APPENDICES

I 1 THE INFLUENCE OF INORGANIC SALTS ON THE REACTION OF DIPHENYLAMINE WITH DNA

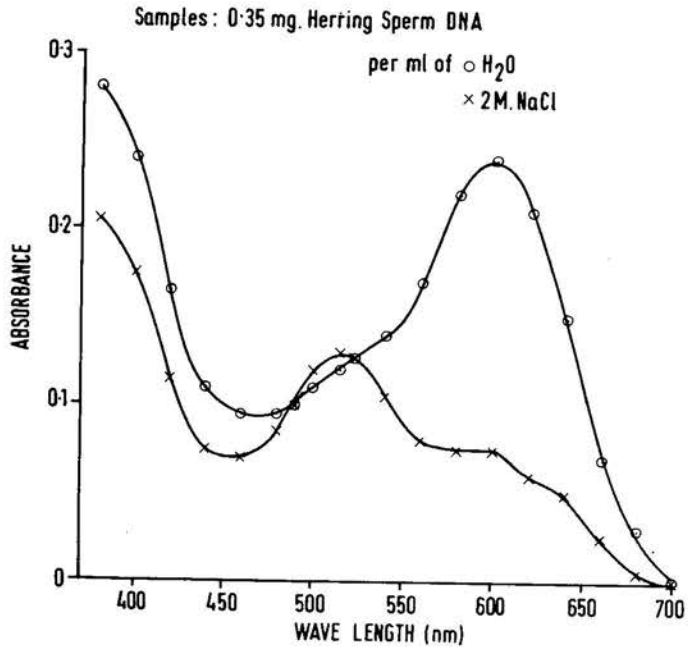
The susceptibility of the diphenylamine reaction with DNA to interference from carbohydrates and other biological materials has been discussed as a serious limitation to its use as an estimation method (Section B 2. 3). During this work, the very marked and complex effect of some inorganic salts on the reaction between DNA and diphenylamine caused considerable confusion regarding the molecular species present in the nuclear digests.

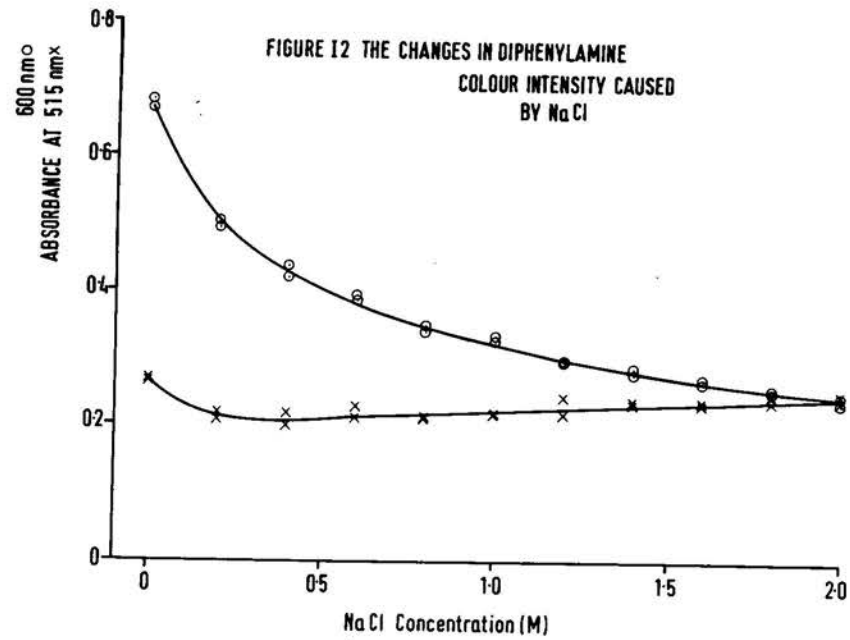
Diphenylamine is known to react with most sugars and their analogues, as well as with aliphatic and hydroxyaldehydes, producing a variety of colours (Dische, 1967). In fact the purple colour produced by human serum with the diphenylamine reagent has been suggested as a clinical index of inflammation in rheumatoid arthritis (W. Ayala, L. V. Moore, and E. L. Hess, 1951; E. Cecchi and F. Ferraris, 1955; and A. F. Coburn, R. C. Bates, J. W. Hahn and P. Murphy, 1956), in estimating urinary mucoproteins (A. J. Anderson and N. F. MacLagen, 1955) and in detecting malignancy (S. Niazi and D. State, 1948). This purple colour, with absorption maximum at 510 to 530 nm., has been generally attributed to a reaction with mucoprotein and E. L. Hess, J. W. Hahn and W. Ayala (1956) claim the deoxypentose structure of sialic acid as the active mucoprotein component.

During attempts to chemically fractionate Deoxyribonuclease I digests of calf thymus nuclei, a 2 M. NaCl extract of a nucleoprotein precipitate reacted with diphenylamine to give a purple colour with absorption maximum at 515 nm. Attempts to isolate a mucoprotein from the extract ended with the discovery that the purple colour was given by DNA in the presence of high NaCl concentrations. The

FIGURES
I1 AND I2

FIGURE I1 THE EFFECT OF NaCl ON THE COLOUR PRODUCED BY THE REACTION OF DIPHENYLAMINE WITH DNA



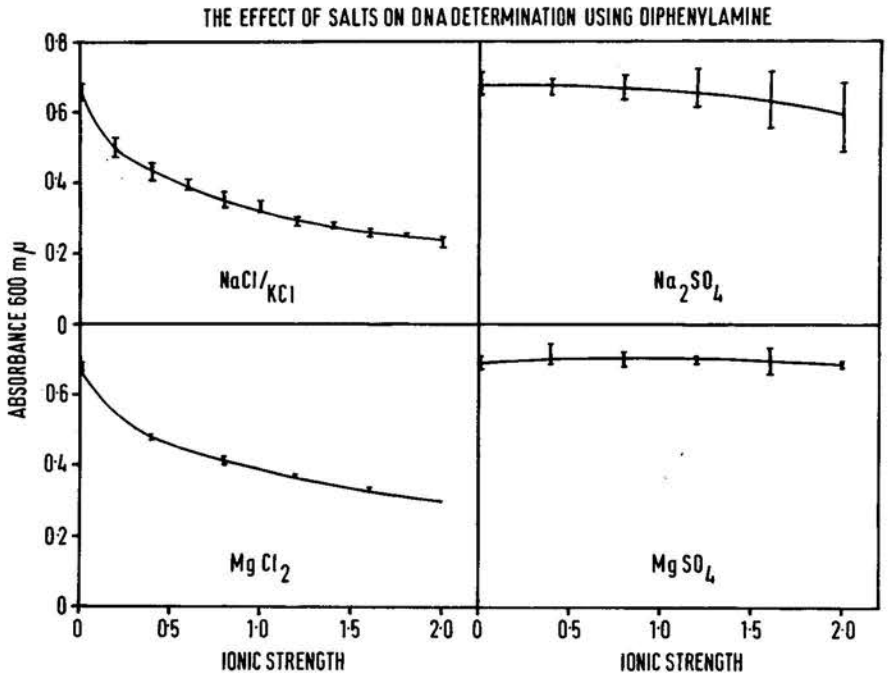


NaCl caused a dramatic reduction in the normal blue colour with absorption maximum at 600 nm., and a more gradual increase in purple colour with absorption maximum at 515 nm. This effect is illustrated in Figure I1, which shows the spectral shift and in Figure I2, which shows the changes in colour intensity at the two absorption maxima with increasing salt concentration. The effect is not limited to NaCl but seems to be a function of the chloride ion concentration (Figure I3).

This effect has been noted previously by W. W. Ackerman, F. Sokol, A. J. Brandau, (1965) and P. K. Ganguli (1967, 1970 a and b) has made extensive studies of the effects of various salts on the reaction. He has proposed an alternative estimation procedure based on these studies. Although the mechanism of the reaction between DNA and diphenylamine has been studied (W. G. Overend, F. Shafizadeh and M. Stacey, 1950; R. E. Deriaz, M. Stacey, E. G. Teece and L. F. Wiggins, 1949; and M. Stacey, R. E. Deriaz, E. G. Teece and L. F. Wiggins, 1946) no proposal has been made to account for the salt effects.

In view of the extensive use of salt solutions in work with nucleic acids, this salt effect is an important one. As was shown in Figure I2, a significant reduction in blue diphenylamine colour is caused by low NaCl concentrations which do not result in a visible purple colour. Thus the effect does not become obvious until, as in the present work, an extreme salt concentration is employed.

FIGURE I3


THE EFFECT OF SALTS ON DNA DETERMINATION USING DIPHENYLAMINE
ABSORBANCE 600 mμ
0·8
0·6
0·4
0·2
0
NaCl/KCl
Na2SO4
Mg Cl2
Mg SO4
0·5
1·0
1·5
2·0
IONIC STRENGTH

I 2 ABBREVIATIONS

REFERRING TO	ABBREVIATION	MEANING
Space	cm.	centimetre
	mm.	millimetre
	nm.	nanometre
	vol.	volume
	ml.	millilitre
	μl.	microlitre
Mass	mg.	milligram
	μg.	microgram
	ng.	nanogram
Time	min.	minute
	hr.	hour
Temperature	^{o}C.	degrees Centigrade
Concentration	N.	Normal
	M.	Molar
	mM.	millimolar
	μM.	micromolar
Electricity	v.	volts
	mA.	milliamperes
Centrifugal force	R. P. M.	revolutions per minute
Chemicals	DNA	deoxyribonucleic acid
	R NA	ribonucleic acid
	DNase I	deoxyribonuclease I
	veronal	5, 5-diethylbarbituric acid, Na salt
	tris	tris-hydroxymethylaminomethane
	SDS	sodium dodecyl sulphate
	CM-cellulose	carboxymethyl cellulose
Others	UV	ultraviolet

REFERRING TO	ABBREVIATION	MEANING
	Exp.	experiment
	Ext.	extract
	conc.	concentrated

I 3 SOURCES OF CHEMICALS

Biochemicals

Sigma Chemical Corporation	Yeast Ribonucleic Acid, Type XI
	Highly polymerized Calf Thymus Deoxyribonucleic Acid, Na Salt, Type I
	Crystalline 2-deoxy-D-ribose, Σ Grade
	Micrococcal Nuclease from Strain SA-B, Grade IV
Worthington Biochemicals	Bovine Pancreatic Deoxyribonuclease I, electrophoretically pure

Buffers

British Drug Houses	Tris-hydroxymethylaminomethane
J. F. Macfarlan and Co., Edinburgh	Veronal

Chromatographic Media

Pharmacia Fine Chemicals	Sephadex G-25
	Sephadex G-50
	Sephadex G-75
	Sephadex G-100
	Sepharose 6B
	Blue Dextran 2000
Kodak, Ltd.	Bio-Gel P-10
Whatman	Carboxymethyl Cellulose, Powder CM 70

Colour Reagents

British Drug Houses	Folin and Ciocalteu's Phenol Reagent
	Orcinol
	Diphenylamine
	Aristar Concentrated Hydrochloric Acid
Koch-Light	Ninhydrin
	Brij 35
	Thiodiglycol
Shell	Methyl Cellosolve, peroxide free

Electrophoretic Materials

Eastman Kodak	Acrylamide
Koch-Light	N,N'-bisacrylamide
	N,N,N',N'-tetramethylethylenediamine
British Drug Houses	2-mercaptoethanol
	Sodium Dodecyl Sulphate, specially pure, crystalline

Solvents

British Drug Houses	Concentrated Hydrochloric Acid (Analar)
May and Baker, Ltd.	Glacial Acetic Acid
	Concentrated Sulphuric Acid
A. & J. Beveridge, Edinburgh	Acetone
	Methanol
B. P. Chemicals International, Ltd.	Ethanol - Doubly Rectified Absolute Alcohol
Macfarlan Smith Ltd., Edinburgh	Ether, Analytical Reagent

Stains

A. H. Baird, Edinburgh	Amido Black 10B (Naphthalene Black)
Sigma Chemical Company	Coommassie Brilliant Blue
G. T. Gurr, London	Methylene Blue
British Drug Houses	Bromphenol Blue

All other chemicals were British Drug Houses' Analar Grade. All reagents were prepared using distilled water unless otherwise stated.

SECTION J

REFERENCES

SECTION J

REFERENCES

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