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LAWRENCE ROBERT HALEY (Name) for the MASTER OF SCIENCE (Degree)

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Histones are presumed to be of chromosomal origin and provide both structural and functional elements for chromatin. In like manner the ribosomal proteins are of ribosomal origin and have analogous roles. Considerable controversy over their cellular roles exists because of the inability to compare directly both groups of proteins. In order to compare definitively the histone with the ribosomal proteins: 1) the organelles from which they are derived must be prepared under conditions which eliminate cross contamination and 2) a preparative scale method must be developed to fractionate both histone and ribosomal protein under similar conditions.

A chromatographic method was developed which fractionated histone and ribosomal protein simultaneously. The histone and ribosomal proteins were isolated from beef and rat liver and germinating pea roots. Histone was acid extracted from exhaustively washed

chromatin prepared from purified nuclei. Ribosomal protein was extracted from purified ribosomes. Both groups of protein were adsorbed to a column of carboxymethyl cellulose and eluted with a sodium acetate gradient (0.01 M to 0.4 M, pH 5.6) in 6 M urea. This method is superior to IRC-50 chromatography for the following reasons: 1) no irreversible adsorption, 2) both histones and ribosomal proteins elute under the same conditions, 3) all fractions elute with a single uninterrupted gradient, 4) the reagents are more conveniently prepared and 5) the arginine-rich histones can be resolved into fractions III and IV.

Beef liver and pea root histones were fractionated into six peaks corresponding in appearance to the fractions Ia, Ib, IIa, IIb, IIc and III - IV of conventional IRC-50 chromatography. Rat liver histone lacked both Ib and IIc, but peak III was resolved from peak IV with a 400 ml gradient. Ribosomal proteins were fractionated into 13 peaks (rat liver), 22 peaks (beef liver) and 17 peaks (pea root) in agreement with the number obtained from polyacrylamide gel elctrophoresis. When assayed turbidimetrically some of the histones and ribosomal proteins exhibited chromatographic identity. A spectrofluorometric assay indicated differences between chromatographically identical histone and ribosomal protein fractions.

Histones and ribosomal proteins, while possessing similar physical and chemical properties, appear to have unique cellular origins and distinct roles.

A Chromatographic Method for Fractionating Histone and Ribosomal Protein

by

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APPROVED:

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Assistant Professor of Agricultural Chemistry
in charge of major

Redacted for privacy

Chairman of Department of General Science

Redacted for privacy

Dean of Graduate School

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Typed by Gwendolyn Hansen for Lawrence Robert Haley

To my wife

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INTRODUCTION

Historical Discovery of Basic Proteins

Fredierich Miescher is generally credited with the discovery of histones in 1869. Working with pus cells collected from hospital patients, he isolated a nuclear fraction with a high phosphorus content composed of nucleic acid and protein which he referred to as "nuclein." Because of the limitations of studies on pus, Miescher turned to the study of Rhine salmon sperm which is rich in nucleic acids and possesses a special protein called protamine.

In 1884 Albrecht Kossel found the histones were more complex than the protamines and that both were associated with nucleic acids. After extracting the histones with dilute hydrochloric acid Kossel found they contained a high percentage of arginine.

Lilienfeld (1893) was the first to isolate histones from calf thymus and to initiate studies of mammalian histones on a regular basis. In 1928 Kossel and Schenk isolated a protamine from carp where lysine accounted for 25% of the total amino acids. However, lysine-rich components were not recognized as important constituents of mammalian histones until the report of Daly and Mirsky (1954). Later a third category of "slightly lysine-rich histone" was recognized

by Crampton, Moore and Stein (1955) and Johns and Butler (1962).

Throughout the 1940's the proteins from the "nuclein" (later "chromosomin") were regarded as the hereditary carriers. The tetranucleotide hypothesis of Levene (1931) encouraged this idea with the mistaken report that the four nucleotide bases occurred in equimolar amounts, thereby ruling out hereditary specificity. Only with Chargaff's (1950) report did opinion shift substantially from the proteins to the nucleic acids as the hereditary carriers.

Composition of Histones

Histone fractions are identified by their amino acid composition, but their over-all similarity has led to a bewildering terminology. Histones are classified by the amount of lysine and arginine present, producing two natural groups, the lysine-rich histones and the arginine-rich histones. The lysine-rich histones are subdivided into the very lysine-rich and the moderately lysine-rich. The very lysine-rich histones include IRC-50 peaks Ia, Ib and solvent-fractionated f1. Lysine and arginine represent 22.9 and 2.7 moles percent respectively of pea root Ia and 26.2 and 2.6 moles percent in calf thymus Ib (Fambrough and Bonner, 1966; Rasmussen, Murray and Luck, 1962). The N-termini of both Ia and Ib from pea roots and calf thymus are blocked.

The moderately lysine-rich histones include IRC-50 peaks IIa

and IIb and solvent-fractionated f2a and f2b. Fractions IIa and f2a correspond and are often called the N-acetylalanine fraction after the amino terminal residue. Fraction IIb corresponds to f2b and proline is the N-terminal amino acid. The amino acid compositions of IIa and IIb from both pea root and calf thymus are quite similar, but their peptide maps are characteristically different (Busch, 1965). Most of the major peptides have been sequenced, but a complete sequence of the entire protein has not been completed.

The arginine-rich histones include the IRC-50 peak III, IV and solvent-fractionated f3. Lysine and arginine account for 10 and 13 moles percent respectively. Variation between species is slight, with the sequence of 19 carboxy terminal amino acids of calf thymus and pea embryo histone IV being identical (Fambrough and Bonner, 1968). DeLange et al. (1968) reported the complete 102 residue sequence of calf thymus histone IV and noted replacements at only three residues in pea embryo histone IV (lysine residue 20 is unmethylated, one lysine is replaced by arginine and one valine by isoleucine). The basic amino acids are grouped at the amino terminus and the hydrophobic amino acids at the carboxy terminus.

Fractionation of Histones

At present there are three major methods for the fractionation of histones on a preparative scale; column chromatography, solvent

extraction and gel electrophoresis. The oldest and most popular method utilizes a column of Amberlite IRC-50 (Crampton et al., 1955). Thirty-five percent of the histones were eluted with barium acetate. Luck et al. (1958) developed a method which employs an interrupted gradient of guanidinium chloride (8 to 12% followed by 40%) for elution and observed five major peaks, Ia, Ib, IIa, IIb and III - IV. Each peak was heterogeneous when re-chromatographed.

Several attempts have been made to substitute carboxymethyl cellulose or phosphocellulose for IRC-50. Davison (1957) was the first to use CM-cellulose[1] chromatography and separated three fractions in a sodium chloride gradient, the first being rich in dicarboxylic acids and the second containing the lysine-rich histones. Raising the pH increased the number of fractions, but simultaneously increased the probability of aggregation. An advantage of this procedure was the high recovery of histones added to the column. Davis and Busch (1959) used a dilute gradient of formic acid to elute five fractions (not correlated with IRC-50 fractions). This method has had limited use because of nonreproducibility.

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[1] The following abbreviations are used in the text: ANSA, 8-anilino-1-naphthalenesulfonic acid; CM-cellulose, carboxymethyl cellulose; DNA, deoxyribonucleic acid; DEAE cellulose, diethylaminoethyl cellulose; DOC, sodium deoxycholate; DTT, dithiothreitol; A, absorbance; NaOAc, sodium acetate; RNA, ribonucleic acid; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane adjusted with HCl; UV, ultraviolet.

Johns (1964) developed a combination extraction-fractionation (solvent-fractionation) procedure for isolating four well characterized fractions. Fraction f1 is extracted with 5% perchloric acid, fractions f2a and f3 with 0.25 N HCl in 80% ethanol and fraction f2b with 0.25 N HCl. Dialysis against 95% ethanol precipitates fraction f3 and allows for the separation of f2a and f3.

The third scheme utilizes electrophoretic mobility as a basis for fractionation. Moving boundary and starch gel electrophoresis were easily adapted to histone fractionation (Cruft, Mauritzen and Stedman, 1957; Poulik and Smithies, 1958), but have been replaced by polyacrylamide because of its increased resolving power and reproducibility. Both starch and polyacrylamide gels are analytical tools and useful for determining the number of protein components. Preparative scale electrophoresis resolved six fractions from calf thymus and pea root (Fambrough, Fujimura and Bonner, 1968). The calf thymus histone IV used for sequence analysis was prepared this way (DeLange et al., 1968). Preparative scale electrophoresis utilizes a 15% polyacrylamide separator gel and is similar to the analytical procedure except for gel size.

Function of Histones

Stedman and Stedman (1943) first proposed that histones act as regulators of cellular activity. They believed histones controlled

mitotic events by complexing with the spindle apparatus. After studying the ratio of DNA/histone in many different cells, they proposed a specific repressor model for histones (1951). Mauritzen and Stedman (1959) extended this idea to differentiation with the proposal that histones were tissue specific and thus responsible for the degree of gene expression. Although their views on differentiation have not been substantiated, their idea that histones suppress nuclear function has received considerable support.

A direct test of the repressor model became possible with the advent of systems to study the in vitro synthesis of RNA (Weiss, 1960). Such reactions require Mg^{++}, all four nucleoside triphosphates, a DNA template and the enzyme RNA polymerase.

Huang and Bonner (1962), studying the synthesis of RNA from pea seedlings, reported a stoichiometric relationship between the blocking of RNA synthesis and added histone concentration. The reaction was reversible since removal of the histones increased template activity five-fold. They calculated that 20% of the DNA is free for transcription and 80% is repressed by complexed histones (Bonner and Huang, 1963).

Ribosomes from Escherichia coli were used to study the in vitro synthesis of a particular seed (cotyledon) globulin. Only chromatin isolated from the cotyledon supported significant globulin synthesis. The removal of histones from other sources of

chromatin appeared to stimulate globulin production, but at a lower level than cotyledon chromatin (Bonner, Huang and Gilden, 1963). That histones repress DNA-dependent RNA synthesis and perhaps regulate differential gene expression follows from these experiments.

The exact mechanism of histone-DNA interaction is not clear. When histones were added to isolated lampbrush chromosomes the DNA loops (loci for RNA synthesis) contracted (Izawa, Allfrey and Mirsky, 1963). The histone fractions differ, however, in their ability to produce loop withdrawal; contraction only occurred in the presence of the arginine-rich fractions. In contrast, Huang, Bonner and Murray (1964) reported that, although the arginine-rich histones repress RNA synthesis, the lysine-rich fractions were clearly more inhibitory. The greater repression by the lysine-rich histones (Huang et al., 1964) parallels the observations by Johns and Butler (1964) that the very lysine-rich histones precipitate DNA to a greater extent than do the arginine-rich histones. Bonner dismisses the argument that histones precipitate the DNA rather than blocking the RNA polymerase by criticizing the high ratios of histone/DNA which Johns and Butler used.

Arginine-rich histones repressed RNA synthesis to a greater extent than did the lysine-rich histones when added to dehistonized thymus nuclei (Allfrey, Littau and Mirsky, 1963). By combining the observations from lampbrush chromosomes with the above data,

Allfrey et al. proposed that the arginine-rich histones have functional roles in gene expression while the lysine-rich histones have structural roles within the chromatin. The lysine-rich histones stabilized metaphase chromosomes to a greater extent than did the arginine-rich histones (Mirsky et al., 1968). The functional role assigned to the arginine-rich histones was supported by the discovery that these histones underwent acetylation, methylation and phosphorylation (Allfrey, Faulkner and Mirsky, 1964; Phillips, 1963; Kleinsmith, Allfrey and Mirsky, 1966a) whereas the lysine-rich histones did not. During the course of gene activation in phytohemagglutinin-stimulated lymphocytes the acetylation and phosphorylation reactions preceeded RNA synthesis (Pogo, Allfrey and Mirsky, 1966; Kleinsmith, Allfrey and Mirsky, 1966b).

Histones inhibit the in vitro synthesis of both DNA and protein, but these reactions may be precipitation artefacts in that any polycation will bind to a nucleic acid. The lysine-rich histones were the most effective inhibitors of DNA synthesis producing 80% inhibition at histone/DNA ratios of 2. The arginine-rich histones produced 20% inhibition at the same ratio (Gurley, Irvin and Holbrook, 1964). As for protein synthesis, histones f1 and f2b were the most inhibitory when assayed using a cell-free synthesis for hemoglobin (Kruh and Labie, 1968). Several enzymes have been assayed for inhibition by histones, but the experiments have not been convincing.

Interpretation of Heterogeneity

The extent of heterogeneity among histones is undecided. When the repressor concept was first proposed each gene was thought to have a separate repressor. This requires histones to be both tissue specific and extremely heterogeneous. While the exact number of histones has not been determined the best estimates are six to twelve for any given tissue. Heterogeneity is difficult to determine because of the possibility of 1) ribosomal protein contamination, 2) aggregation at low pH's and 3) cysteinyl dimers, particularly in fraction III. Where substantial heterogeneity is theoretically required to fit a one-gene-one-repressor protein concept it is easy to overlook the possibility of artefacts. Aggregation artefacts are also a problem when homogeneity is required, although end group analysis is useful in assaying this. Six homogeneous histones have been obtained by preparative gel electrophoresis under reducing conditions in 6 M urea and may be the most reliable estimate to date (Fambrough and Bonner, 1966).

The problem of heterogeneity induced by dimerization about the cysteinyl residues can be reduced using sulfhydryl compounds (Fambrough and Bonner, 1968). Solutions to problems involving aggregation have relied on using high concentrations of urea or guanidine hydrochloride. Even though aggregation is a known

problem, many workers, including Shepherd and Noland (1968), Shepherd and Gurley (1966) and Lindsay (1966), have not included urea in their methods.

There are three reasons for retaining the one-gene-one-repressor control mechanism. First, the specificity may reside in a small molecular weight RNA bound to the histone (Bonner and Widholm, 1967). Secondly, the specificity may result from secondary acetylation, methylation and phosphorylation (Phillips, 1963; Kleinsmith, Allfrey and Mirsky, 1966a). Thirdly, the repressors may be a class of acidic nuclear proteins which have not been studied in detail (Benjamin and Gellhorn, 1968).

Isolation and Extraction of Histones

The standard methods for histone isolation contribute to the ambiguity over heterogeneity. No method guarantees the removal of nonhistone proteins and failure to purify chromatin is common. Mauritzen et al. (1967) used whole nuclear extracts in reporting the isolation of ten arginine-rich histones as did Shepherd and Gurley (1966) who reported up to 30 histones.

Interphasic chromatin is the conventional source of histones and is subject to contamination from the nucleolus. Nucleolar contamination can be reduced by two different approaches. The first isolates histone from colchicine arrested cells in which the nucleolus

is dispersed prior to metaphase (Maio and Schildkraut, 1967). However, metaphase chromosomes are associated with increased amounts of ribosomal RNA. Presumably the chromosomes absorb ribosomes during metaphase. The anucleolate mutant of Xenopus laevis was used successfully in the second approach. No new ribosomes are synthesized by the homozygous mutant and the maternal supply of ribosomes from the oocyte is reduced by extensive cell division by Niewkoop-Faber stage 42 (Berlowitz and Birnstiel, 1967).

Dilute acid and high salt concentrations are the most common methods for extraction of histones. Both sulfuric and hydrochloric acid are used with equal effectiveness. Originally sodium chloride was used to dissociate histones from chromatin, partially because it was a necessary constituent for reconstituting a native DNA-histone complex. Now a combination of high salt in urea is used for reconstitution because the complex more closely resembles the native structure. High concentrations of cesium chloride are employed in conjunction with buoyant density centrifugation to dissociate the histones from the chromatin. Huang and Bonner (1965) used this method to extract the histone-RNA complex. Acid and salt-extracted histones band at different densities upon isopycnic centrifugation in cesium chloride which may indicate an acid catalyzed cleavage of the RNA portion. The solvent-fractionation method of Johns (1964) also involves acid extraction. Ethanol aids both the extraction and

precipitation of histones from the extracting solvent.

Organization of the Ribosome

Ribosomes contain two unequal subunits which associate reversibly during protein synthesis. They are subdivided into two classes on a size basis. Ribosomes from mammals and vascular plants have molecular weights of about 4.0 million and S values of 80. The large subunit of mammalian ribosomes contains a 28S and 5S RNA while the small subunit contains an 18S RNA. The plant ribosomes differ in the large subunit, where a 26S RNA replaces the 28S RNA. Both subunits contain approximately equal amounts of RNA and protein (Arnstein, 1963). The nucleolus is the site of ribosome formation (Brown and Gurdon, 1964).

Bacterial ribosomes are smaller having molecular weights of about 2.4 million, S values of 70 and containing 63% RNA and 37% protein (Tissières et al., 1959). The large subunit consists of a 23S and a 5S RNA and up to 40 proteins. The small subunit contains 16S RNA and 19 proteins (Traut et al., 1967).

Composition of Ribosomal Proteins

The ribosomal proteins have not been studied as extensively as have the histones. This is partly due to their more recent discovery and to their considerable heterogeneity. Most investigators have

recognized a small number of acidic ribosomal proteins, but these have not been characterized. The basic ribosomal proteins have received the most attention (particularly those isolated from Escherichia coli) and their study has been dependent on the development of adequate methods for fractionation.

Ribosomal proteins are identified according to elution order from chromatographic or electrophoretic columns. Separate peaks possess characteristic amino acid compositions and the peptide maps reveal unique primary structures (Traut et al., 1967). They contain 5.8 to 14.5 moles percent lysine and 6.0 to 13.7 moles agrinine. Aspartic and glutamic acids total 20 moles percent, but may be present as the amides, glutamine and asparagine. Tyrosine is found only in 30S proteins and occurs at low levels as does methionine and cysteine. The N-terminal residues include methionine (30_4), alanine (30_5, 50_5), serine (30_{11}) and threonine (30_3). The N-terminus of 30_7 appears to be blocked. The end groups correspond to that found for total cellular protein.

Traub et al. (1967) prepared five "synthetic" ribosomes deficient in one basic protein from the 30S subunit. The amino acid compositions of the five proteins correlated with Traut's data and the peptide maps demonstrated a unique primary structure for each protein. Protein B_4 was noteworthy in that it contained 3.6 moles of tyrosine.

The range in amino acid composition of E. coli ribosomal

proteins is similar to that found in yeast (Schmidt and Reid, 1968), but data on the composition of ribosomal proteins from the higher plants and animals is almost entirely lacking. Molecular weight determinations have received more attention and fall between 10,000 and 40,000 daltons for both rat liver and yeast (Hamilton and Ruth, 1967; Chersi et al., 1968).

Fractionation of Ribosomal Proteins

Ribosomal proteins have been fractionated on a preparative scale by CM-cellulose chromatography and polyacrylamide gel electrophoresis. In addition, both methods have been used as analytical tools and are invaluable in determining the extent of heterogeneity and the distribution of proteins between the large and small subunits.

The present status of CM-cellulose chromatography is an outgrowth of Waller's (1964) method designed to fractionate ribosomal protein from E. coli. It has been refined by Traut et al. (1967) and Otaka, Itoh and Osawa (1967). Both groups use a linear gradient of sodium acetate, pH 5.6 in 6 M urea. Otaka et al. have used it primarily as an analytical tool to compare the proteins from the 40S precursor to 50S subunit, erythromycin resistant mutant to wild type, 30S subunit to 50S subunit and E. coli ribosomal proteins with a variety of other bacteria (Otaka, Itoh and Osawa, 1968; Tanaka et al.

1968). Traut et al. (1967) have isolated and purified many of the E. coli proteins and have begun to study their physical and chemical properties (discussed under Composition).

Preparative polyacrylamide gel electrophoresis has had limited use, but in theory is an attractive method. Hamilton and Ruth (1967) adapted Duesberg and Rueckert's (1965) procedure to fractionate the proteins from the large subunit of rat liver. Nine fractions were obtained and a preliminary determination of molecular weight was made. Schmidt and Reid (1968) utilized this same procedure to fractionate the 80S ribosomal proteins of yeast, but report some difficulty in handling the method. They isolated eight proteins which proved to be homogeneous on re-electrophoresis.

Analytical gel electrophoresis has been applied extensively to a wide range of problems, many dealing with heterogeneity and the number of ribosomal proteins present and their distribution among the subunits. Leboy, Cox and Flaks (1964) first used it to show that ribosomal proteins derived from E. coli strain B differed from the proteins of strain K 12. Analytical gel electrophoresis was used to show that ribosomal proteins isolated from rat liver, kidney, heart and muscle tissue were similar and presumably coded from the same structural genes in each tissue (Low and Wool, 1967).

Function of Ribosomal Proteins

The determination of cellular roles for ribosomal proteins depends on the availability of methods for fractionation and specific tests. A priori, ribosomal proteins can be expected to have both structural and functional roles in stabilizing the ribosomes and assembling the new polypeptide.

Many laboratories have worked on the steps required to initiate and synthesize new proteins including the ribosomal subunit relationships. In addition, Nomura's laboratory has initiated a study into the roles of the ribosomal proteins. They produced biologically active E. coli ribosomes by permitting a spontaneous assembly of RNA and proteins (Traub and Nomura, 1968). By modifying this technique a series of ribosomal derivatives were produced, each deficient in a specific protein from the 30S subunit. By comparing the derivatives in an in vitro protein synthesizing system they found that two of the proteins, B_3 and B_5, and possibly B_1, were necessary for both amino acid incorporation and binding of specific aminoacyl tRNA. Protein B_4 was not required for either of these reactions, but could be involved in chain initiation. Protein B_2 had a stimulatory effect on amino acid incorporation, but no definite role for it could be assigned (Traub et al., 1967). While this work is extremely elegant it is by no means definitive and depends upon the

future development of in vitro tests with greater specificity. Investigation is proceeding on forming B protein derivatives from the large (50S) subunit.

Antibiotic resistant mutants have contributed greatly to understanding ribosome function. An erythromycin resistant mutant was isolated from E. coli in which the ribosomes were unable to bind erythromycin. Ribosomal proteins isolated from the mutant were similar to the wild type, except the chromatographic mobility of one protein was altered (Tanaka et al., 1968). Other antibiotic resistant mutations mapped adjacent to the erythromycin locus, but the electrophoretic mobility of the ribosomal proteins was not altered (Flaks et al., 1966).

One additional function has been ascribed to ribosomal proteins: the destruction of the message following translation. This has been attributed to a ribonuclease that is either closely associated with the ribosomes or is a ribosomal protein. To date, evidence for this has not been produced.

The difficulty in determining the function of ribosomal proteins exists because the necessary specific tests have not been devised. While histones have been tested in vitro for many doubtful functions the ribosomal proteins have escaped such confusion.

Comparison of Histones and Ribosomal Proteins

Although no definitive comparison has been made between ribosomal protein and histones, many investigators believe the ribosomal proteins to be less basic than the histones. These reports are influenced by the observation that ribosomal proteins are not adsorbed to IRC-50 (Berlowitz and Birnstiel, 1967; Neelin and Vidali, 1968) and are encouraged by incomplete reporting of amino acid composition. The occurrence of amide nitrogen (destroyed by acid hydrolysis) in glutamine and asparagine is often not reported and will influence the ratio of basic to acidic amino acids. When histone fraction IV was sequenced, four of the twelve acidic amino acids were found to be amides (DeLange et al., 1968). The basic amino acid composition of ribosomal protein does compare favorably with that from histone in two separate reports. Ribosomal proteins from E. coli contain 5.8 to 9.0% lysine and 6.1 to 13.7% arginine and have a total basic amino acid composition of 13.3 to 21.8%. Ribosomal proteins from yeast contain 10.8 to 13.7% lysine and 7.1 to 9.6% arginine and have a total basic amino acid composition of 21.8 to 25.5%. These values compare with 8.5 to 25.5% lysine, 2.8 to 15.6% arginine and total 21.7 to 28.3% for histones (Fambrough and Bonner, 1966). Mauritzen et al. (1967) reports comparable values for the arginine-rich histones for 9.6 to 12.2% lysine and 9.8 to

13.3% arginine and total 21.7 to 24.4%. Therefore an inspection of both basic and acidic amino acids (not cited) reveals some similarity in total basicity.

Several recent investigations, while not definitive, have reported a similarity between histones and ribosomal proteins using analytical gel electrophoresis. The most extensive work compared the intracellular distribution of basic proteins from hamster ovary (Shepherd and Noland, 1968). No attempt was made to purify the fractions and with as many as 15 to 20 proteins per fraction it is not surprising that some identity was established. Previously Lindsay (1966) reported that seven histones co-electrophoresed with seven ribosomal proteins. Again, no attempt was made to exclude nuclear ribosomal protein or prevent aggregation during electrophoresis. These objections were reduced by using more highly purified starting material and electrophoresing in 8 M urea (Beeson and Triplett, 1967). Upon electrophoresis the lysine-rich histones (Ia, Ib) possessed the same mobility as two of the more basic ribosomal proteins. In addition, two proteins from the IIa region were similar, but not identical to the histone.

A few attempts have been made to determine the relationship between histone and ribosomal protein using column chromatography. All report that ribosomal proteins do not bind to IRC-50 under the conditions observed (Berlowitz and Birnstiel, 1967; Neelin and

Vidali, 1968). One of these reports seems especially significant. Berlowitz and Birnstiel (1967) worked with the homozygous recessive mutant of Xenopus laevis which lacks nucleoli and is devoid of ribosomal RNA synthesis. They reported the mutant histones to be deficient in the Ia, Ib and IIb fractions while IIa was greatly reduced. Although the mutant does not produce new ribosomes, a clear causal relationship could not be established between loss of the nucleolus (accumulated ribosomes) and the absence of these histone fractions. The authors favored a nucleolar origin for the lysine-rich histones because ribosomal proteins isolated separately failed to bind to IRC-50. The difficulty in resuspending ribosomal protein following ethanol or acetone precipitation may be responsible for the lack of binding observed (Neelin and Vidali, 1968).

Additional evidence for histones having a ribosomal origin could be drawn from the many histochemical papers, although each can be criticized for lack of specificity in the staining agent. Kornguth and Tomasi (1968) reduced the objection to specificity by the following experiment. The f2a1 histone of Johns (1964) was isolated and purified extensively. A fluorescent immune γ-globulin was prepared against the histone and incubated with fixed tissue sections. When examined by dark-field microscopy, dorsal root ganglia sections showed an intense nucleolar reaction in 20 day fetal rats. At 2 days (post-natal) a weak reaction appeared in the

perinuclear region followed at 10 days by a reaction of the rough endoplasmic reticulum. While the initial reaction weakly implicates the nucleolus as the site of histone synthesis, it is also consistent with a nucleolar role in ribosome formation. The subsequent reactions of the outer nuclear envelope and rough endoplasmic reticulum provide evidence for a ribosomal origin. The temporal order of events resemble Sadowski and Howden's (1968) report showing the outer nuclear membrane polysomes to be of higher specific activity than the cytoplasmic polysomes. The outer membrane polysomes were thus implicated as intermediates in the transfer of newly synthesized RNA from the nucleus to the cytoplasm.

MAJOR OBJECTIVES

The cellular role of nuclear histones is unclear, as is their relationship to other basic proteins. Possibly some of these histones are linked to the nucleolus and are involved in the biogenesis of new ribosomes.

The first objective in this investigation was to isolate histones and ribosomal proteins in a highly purified condition. By rigorous purification and exclusion of cross contamination the ambiguities of previous work could be reduced.

The second and major objective was to develop a chromatographic method to simultaneously fractionate both ribosomal protein and histones. With such a method available, the relationship between histones and ribosomal protein may be determined. If identities could be established for certain histones and ribosomal proteins, then new tests should be designed to determine their function. A knowledge of the functional role of histones should lead to a clearer understanding of chromosome structure, nucleolar structure, including ribosome biogenesis, and gene expression.

EXPERIMENTAL PROCEDURES

Source of Materials

Pea Roots: Pea seeds (Pisum sativum var. Alaska) were sterilized in one kg lots by immersion in 95% ethanol for 10 minutes, followed by 10% chlorox for 10 minutes. After washing with distilled water the seeds were allowed to imbibe 0.1 mM $CaCl_2$ solution for eight hours with aeration. The seeds were planted in vermiculate soaked with 0.1 mM $CaCl_2$ solution and aerated. The etiolated seedlings were harvested after 48 hours growth, rinsed free of vermiculite and frozen in liquid nitrogen. The seedlings were shaken vigorously in a covered styrofoam ice bucket and the resulting mixture (roots and cotyledons) seived through 6 mesh stainless steel screen. The broken roots were refrozen and stored in liquid nitrogen until homogenization.

Beef Liver: An intact liver was obtained within ten minutes of the death of the animal from D. E. Nebergall Meat Co., Albany, Oregon. The median lobe was excised, sliced into small pieces, placed in plastic bags and plunged into ice. The animals were fasted 24 hours prior to slaughter to facilitate butchering.

Rat Liver: Male Wistar rats (average weight 408 g) were obtained from the Corvallis colony where they were maintained on

an ad libitum diet of Purina Lab Chow. The animals were not fasted prior to sacrifice, except in the experiment to determine the effects of fasting on ribosomal purity. For this experiment the test animals (two, average weight 408 g) were deprived of food for 24 hours prior to sacrifice, while the control group (two, average weight 435 g) was fed. The animals were sacrificed by a blow on the head and the livers removed.

Escherichia coli: Escherichia coli, a mutant HFr strain of Q 13 (originally isolated by D. Vargo in W. Gilbert's laboratory and obtained from S. Spiegelman), was selected because it lacks ribonuclease I and RNA phosphorylase. The culture was grown aerobically in 1% tryptone, 1% NaCl, 0.5% yeast extract, 0.1% glucose, 0.025% $MgCl_2$, pH 7.0 at 35°C. Fourteen liters of culture media were inoculated and grown until the $A_{660} = 0.5$; doubling time 40 minutes. The bacterial cells (31 g) were collected in the Servall continuous flow rotor, type KS 13/R, at 28,000 × g at a flow rate of 200 ml per minute. Following resuspension in 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 at 4°C., the cells were washed twice by centrifugation at 10,000 × g for 10 minutes and stored as a thick paste at -70°C.

Isolation and Purification Procedures

Isolation of Rat Liver Nuclei: The livers were excised and rinsed with homogenization buffer, HB, (250 mM sucrose, 5 mM $MgCl_2$, 0.1 mM DTT and 20 mM Tris-HCl, pH 7.6 at 4°C.) and the fresh weight determined. All subsequent procedures were carried out at 0-4°C. Five volumes of HB buffer were added to the minced liver and the cells ruptured with a Potter-Elvehjem homogenizer fitted with a teflon pestle (gap 0.0015 inches). After four full strokes the homogenate was transferred to a Tenbroeck tissue grinder and homogenization completed with an additional 3-4 strokes. The homogenate was filtered through four layers of cheesecloth followed by one layer of Miracloth (Chicopee Mills, Inc.) under vacuum. The pellet, collected by centrifugation at 350 × g for 10 minutes in the Servall refrigerated centrifuge, contained nuclei, erythrocytes and some mitochondria.

Purification of Rat Liver Nuclei: (Hymer and Kuff, 1964). The pellets were resuspended in HB buffer and purified using detergent to disperse the cytoplasm. Triton X-100 was added to a final concentration of 0.5% v/v, and red blood cells lysed by two strokes of the Tenbroeck homogenizer. The nuclei were collected by centrifugation at 350 × g for 10 minutes, treated twice with Triton X-100 as described above, washed with HB buffer and stored

at -70°C.

Isolation of Beef Liver Nuclei: Nuclei from beef liver were isolated and purified in a manner similar to rat liver nuclei. The modifications were required because of the larger volume of liver to be processed and the higher percentage of connective tissue. The median lobe was rinsed with HB buffer and 250 g lots passed through a meat grinder. All processes were carried out at 0-4°C. Five volumes of HB buffer were added and the mince homogenized in a Waring blender (Model CB5) for 10 seconds at 16,500 RPM and 10 seconds at 20,000 RPM. The homogenate was filtered successively through one layer and four layers of cheesecloth and the nuclei collected by centrifugation at 350 × g for 10 minutes. Subsequent purification was identical to that listed for rat nuclei above.

Isolation and Preparation of Chromatin: Purified nuclei were thawed and pelleted out of the storage buffer. After swelling in cation-free sucrose (250 mM) the nuclei were ruptured in a tight fitting Tenbroeck homogenizer and the chromatin collected by centrifugation at 10,000 × g for 10 minutes. The chromatin was routinely extracted twice with 140 mM NaCl, 0.1 mM DTT and 10 mM Tris-HCl at pH 8, to remove nuclear ribosomes (Pogo et al., 1962).

To determine the extent of contamination by nuclear ribonucleoprotein particles, the chromatin was extracted six times with 30 ml of SSC buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.8

at 25°C.). After each extraction the chromatin was pelleted by centrifugation at 10,000 × g for 10 minutes. The final pellet was suspended in water and extracted with acid as described below. Total acid soluble nuclear proteins served as the control and were prepared by suspending the nuclei in distilled water (15 ml), rupturing for 10 seconds at top speed in the VirTis and extracting with acid.

Isolation of Pea Root Chromatin: The liquid nitrogen was poured off and the tissue weighed. Five volumes of cold HB buffer (250 mM sucrose, 5 mM $MgCl_2$, 0.1 mM DTT, 20 mM Tris-HCl, pH 7.8 at 4°C.) were added to each gram of tissue. All subsequent procedures were carried out at 0-4°C. The tissue was homogenized in the VirTis homogenizer for one minute at top speed and filtered through one layer of Miracloth. The chromatin was collected by centrifugation at 6,000 × g for 30 minutes, resuspended in SSC buffer, washed by centrifugation until the supernatant remained clear and stored at -70°C.

Isolation and Purification of Beef and Rat Liver Ribosomes: Ribosomes from beef and rat liver were isolated using a buffer developed by Wettstein, Staehelin and Noll (1963). The liver was processed during the initial stages in the same fashion as for the nuclei isolation. When nuclei and ribosomes were isolated from the same tissue the post-mitochondrial supernatant from the nuclear isolation was used. Beef liver was washed and passed through a meat

grinder. Five volumes of cold RB buffer (250 mM sucrose, 1 mM $MgCl_2$, 0.1 mM DTT, 25 mM KCl, 50 mM Tris-HCl, pH 7.5 at 0°C.) was added to one volume of liver mince. The mince was homogenized for 20 seconds in the Waring blender at 20,000 RPM. The homogenate was strained through one and four layered cheesecloth and centrifuged at 10,000 × g for 15 minutes to remove nuclei and mitochondria. Rat liver was hand homogenized in a Potter-Elvehjem homogenizer in the same manner as the nuclear isolation. Sodium deoxycholate (Mann Research Laboratories, enzyme grade), pH 8, was added to a final concentration of 1% w/v to solubilize the ribosomes. The homogenate was centrifuged at 20,000 × g for 10 minutes to remove membranes and the ribosomes collected by centrifugation at 210,000 × g for 60 minutes in the Spinco Model L Ultracentrifuge (65 rotor). Two additional low and high speed centrifugations were made to wash the ribosomes. Finally the ribosomes were resuspended in 60 mM sucrose, 1 mM $MgCl_2$, 0.1 mM DTT, 25 mM KCl, and 10 mM Tris-HCl, pH 7.5 and stored at -70°C.

DEAE Cellulose Chromatography of Ribosomes: DEAE cellulose was cycled through 0.5 M HCl, water, 0.5 M NaOH, water, 0.5 M HCl, water to pH 6.0, 0.1 M Tris-HCl, pH 7.0, 0°C. and the fines removed. The cellulose was packed into the column (2.5 × 10 cm) with nitrogen at 20 psi and washed in situ for two hours with 1 mM $Mg(OAc)_2$, 20 mM Tris-HCl, pH 7.0 at 0°C. The ribosomes

(200 mg) were adsorbed to the column and eluted with a 200 ml increasing linear concentration of NaCl (0 to 1 M) in 1 mM $Mg(OAc)_2$, 20 mM Tris-HCl, pH 7.0, at a flow rate of 30 ml/hour. The first peak was ferritin and the second ribosomes as determined by UV absorption spectra. The above procedure was adapted from Furano (1966) by reducing the magnesium level ten-fold. Fractions containing the ribosomes were pooled, dialyzed against 1 mM $Mg(OAc)_2$, 20 mM Tris-HCl, pH 7.0, for one hour and collected by centrifugation at 210,000 × g for 60 minutes. The pellet was resuspended in 60 mM sucrose, 1 mM $MgCl_2$, 0.1 mM DTT, 20 mM Tris-HCl, pH 7.4 and stored at -70°C.

Isolation of Polysomes and Ribosomes: In order to evaluate the effects of fasting on the different ribosome fractions, the technique of Wettstein et al. (1963) was used. The nuclear ribosome fraction was prepared as described later from fasted and nonfasted male Wistar rats. Polysome and ribosome fractions were isolated from the post-mitochondrial supernatant. Sodium deoxycholate (1% v/v) was added to five ml of supernatant which was then layered on four ml of 0.5 M sucrose layered over three ml of 2 M sucrose. Following centrifugation at 210,000 × g for two hours, the polysomes were collected from the 2 M sucrose layer, ferritin from the 2 M to 0.5 M interface and the ribosomes from the 0.5 M sucrose layer. Following dilution with 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.6, the

UV absorption spectra were determined for each fraction.

Isolation of Pea Root Ribosomes: The supernatant from the 6,000 × g centrifugation was collected and centrifuged for an additional 10 minutes at 20,000 × g. Sodium deoxycholate, pH 8.0, was added to a final concentration of 1% v/v and the supernatant centrifuged for 10 minutes at 20,000 × g. The ribosomes were collected by centrifugation at 210,000 × g for 60 minutes and resuspended in 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4 at 4°C. Two additional low and high speed centrifugations were made following the addition of deoxycholate. The final pellet was resuspended in 60 mM sucrose, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4 and stored at -70°C.

Isolation of Nuclear Ribosomes: Nuclear ribosomes were isolated from purified nuclei according to Allfrey (1963). Purified nuclei were suspended in 150 mM NaCl, 1 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.6 at 4°C. and ruptured for 10 seconds at top speed in the VirTis homogenizer. The chromatin was collected by centrifugation at 28,000 × g for 10 minutes, the supernatant removed and the chromatin extracted again in the same way. The extract and washings were pooled and centrifuged at 210,000 × g for 60 minutes at 0.5°C. in the Spinco Model L Ultracentrifuge. The pellet was resuspended and stored at -70°C.

Isolation of E. coli Ribosomes: (Tissières et al., 1959) The bacterial paste was chipped into small pieces and the cells (20 g)

broken with the aid of a French press at 10,000 psi. All subsequent procedures were carried out at 0-4°C. Three volumes of CB buffer (5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) were added to one volume of ruptured cells and stirred vigorously. Deoxyribonuclease (ribonuclease free) was added to a final concentration of one μg/ml and the solution stirred for an additional 10 minutes. Following DNA digestion, the homogenate was centrifuged twice at 6,000 × g for 15 minutes and the pellets discarded. The ribosomes were collected by centrifugation at 210,000 × g for 70 minutes, resuspended in CB buffer, and centrifuged at 8,000 × g for 15 minutes to remove aggregated material. The ribosomes were washed twice by high and low speed centrifugation as described above and frozen in CB buffer at -70°C.

Criteria for Purity

Most of the controls employed in this project have been concerned with purity or homogeneity. The elimination of cross-contamination of nuclear and cytoplasmic fractions, brought about by tissue homogenization, is a strong prerequisite.

Nuclei: Nuclear preparations were examined by phase contrast microscopy at 330 diameters (blue filter). Acceptable standards for homogeneity were set at: 1) no whole cell contamination, 2) less than 5% chromatin and damaged nuclei and 3) no cytoplasmic tabs.

Cytoplasmic Ribosomes: Ferritin is the major contaminant of liver ribosomes preparations. Its presence was detected using ultraviolet absorption spectra. Purified ribosomes have $A_{260/280}$ and $A_{260/235}$ ratios of 2.0 and 1.7 respectively (Takanami, 1960), contrasted with ferritin where the $A_{260/280}$ and $A_{260/235}$ ratios are 1.2 and 0.5 respectively (Figure 2). An additional criterion was homogeneity by sucrose gradient centrifugation and an S value of 80.

Extraction of Proteins

Acid Extraction: The pellet of purified chromatin or ribosomes was resuspended in 15 ml of distilled water and titrated to pH 5.0 with 6 N sulfuric acid (Satake et al., 1960). Following 10 minutes incubation at 4°C. the acid-insoluble material was removed by centrifugation at 28,000 × g for 10 minutes. The supernatant was removed and exchanged into 0.01 M NaOAc, pH 5.6, in 6 M urea as described below.

LiCl-Urea Extraction: In contrast to histones, ribosomal protein may also be extracted with LiCl-urea (Spitnik-Elson, 1965). One volume of 6 M LiCl in 8 M urea was added to an equal volume of ribosomes (40 A_{260} units/ml). After 12 hours incubation at 4°C. the RNA was pelleted at 28,000 × g for 10 minutes and the proteins exchanged into 0.01 M NaOAc, pH 5.6 in 6 M urea as described below.

Gel Exclusion Chromatography: Regardless of the extraction

method the proteins must be exchanged into the starting buffer since both salt and acid alter adsorption to the cellulose column. For this purpose Bio-Gel P-2 (exclusion limit 2,000 daltons) was used. The Bio-Gel column (2.5 × 23 cm) was equilibrated with 0.01 M NaOAc, pH 5.6 in 6 M urea and fractions (3 ml) collected at a flow rate of 20 ml per hour (Figure 6).

Fractionation of Proteins

Carboxymethyl Cellulose Chromatography: The basic proteins were fractionated by carboxymethyl cellulose chromatography (Otaka et al., 1968). Carboxymethyl cellulose (microgranular, CM-32, Reeve Angel, 1.0 mequiv/g) was cycled through 0.5 M NaOH, water, 0.5 M HCl, water to pH 6 and 0.75 M NaOAc, pH 5.6 in 6 M urea, and the fines removed. The cellulose column (1.2 × 15 cm) was packed with nitrogen (15 psi) and washed in situ for four to six hours with 0.01 M NaOAc, pH 5.6 in 6 M urea at 10°C. Following adsorption to the cellulose in 0.01 M NaOAc, the proteins were eluted with a 200 ml linearly increasing concentration of acetate buffer (0.01 to 0.4 M NaOAc, pH 5.6 in 6 M urea) at a flow rate of 6 ml/hour.

Two experiments reduced the slope of the gradient to improve the resolution. For rat liver histones, Figure 13, the gradient had a 200 ml volume over the range 0.2 M NaOAc to 0.4 M NaOAc. For beef liver histones and ribosomal protein, Figure 9, a two step

interrupted gradient (120 ml of 0.01 M to 0.16 M NaOAc followed by 240 ml of 0.16 to 0.4 M NaOAc, all in 6 M urea at pH 5.6) was used.

Polyacrylamide Gel Electrophoresis: Chromatographic fractions were dialyzed for one hour against 0.01 M NaOAc in 6 M urea to remove the eluting salt. The fractions were electrophoresed on cylindrical polyacrylamide gels containing 6 M urea employing a reservoir buffer composed of 31.2 g of β-alanine, 8 ml of glacial acetic acid to pH 4.3 and water to 1 l. This method of Leboy et al. (1964) is Reisfeld's (1962) procedure with the addition of urea to circumvent aggregation. The analytical and stacking gels were 10% and 2.5% polyacrylamide respectively. The analytical gel was formed by mixing one volume of buffer (48.0 ml of 1 M KOH, 17.2 ml of glacial acetic acid, 4.0 ml of tetramethylethylenediamine and water to 100 ml) with two volumes of acrylamide (40 g acrylamide, recrystallized three times from chloroform, 0.4 g methylenebisacrylamide and water to 100 ml). Five volumes of urea-persulfate (0.02 g ammonium persulfate, 10 ml of freshly deionized 10 M urea) were added and the aliquots dispensed into glass tubes (0.5 × 10 cm) and allowed to polymerize for 30 minutes under a 3 M urea overlay. The overlay was then removed and the stacking gel polymerized in place. The stacking gel was formed by mixing one volume of buffer (48.0 ml of 1 M KOH, 2.87 ml of glacial acetic acid, 0.46 ml of tetramethylethylenediamine and water to 100 ml) with two volumes of acrylamide (10 g

acrylamide, 2.5 g of methylenebisacrylamide and water to 100 ml). Five volumes of urea-persulfate were added and 0.2 ml aliquots dispensed into the glass tubes, overlayed with 3 M urea, and allowed to polymerize for 30 minutes. The proteins were electrophoresed at 3 ma/tube at 25°C. until the tracking dye (pyronine Y) reached the bottom of the tube. The proteins were stained for one hour with 0.5% w/v Amido Schwarz in 10% v/v acetic acid and dialysed against 10% acetic acid to remove excess dye.

Estimation of Protein Concentration

Spectrofluorometric Analysis with ANSA: Protein concentration was estimated fluorometrically following the addition of the dye 8-anilino-1-naphthalenesulfonic acid (ANSA). To each 0.5 ml sample was added 1 ml of 1 M glycine-HCl, pH 2, containing 400 μg/ml ANSA. The sample was excited at 405 nm and the fluorescence emission measured at 500 nm in an Amico-Bowman Spectrofluorometer (Shepherd and Noland, 1965). Standard solutions of calf thymus histone, Sigma IIa (unfractionated), were prepared and measured at meter multiplier settings of ×0.01, ×0.03 ×0.1 and ×0.3. Standard protein concentration curves were drawn for each deflection scale (Figure 1).

Turbidimetric Analysis with Trichloroacetic Acid: Protein concentration was estimated turbidimetrically following the addition

of 1.6 M trichloroacetic acid (TCA). To each 0.3 ml sample was added 0.6 ml TCA (1.6 M) to give a final concentration 1.1 M. After 20 minutes development, the optical density was measured at 400 nm (Luck et al., 1958). Calf thymus histone was again used to generate the standard curve (Figure 1).

Gel Scanning of Polyacrylamide Electrophoretic Patterns: Protein concentration can be estimated following electrophoresis on polyacrylamide by staining with 0.5% Amido Schwarz in 10% v/v acetic acid. Gels were scanned using the Gilford Model 2410 Linear Transport attached to a Beckman DU Spectrophotometer. The spectrophotometer was equipped with the Gilford Model 2000 Multiple Sample Absorbance Recorder. The gels were routinely scanned at 525 nm at a rate of 2.5 cm/minute. The 0.05 mm wide by 2.3 mm high aperture plate gave the best resolution.

Miscellaneous Procedures

Purification of 8-Anilino-1-Naphthalenesulfonic Acid: The dye ANSA was obtained as the sodium salt from Eastman Organic Chemicals and purified by recrystallization of the magnesium salt from hot water.

Preparation and Purification of 10 M Urea Stock: For best results the eluting buffer was prepared prior to use by dilution from 10 M urea and 2.5 M NaOAc, pH 5.6, stock solutions. When used

immediately the pH remains at 5.6, but rises during storage at room temperature. A two liter stock solution of 10 M urea was prepared free of ammonium cyanate by adding 200 g of mixed bed deionizing resin, composed of equimolar amounts of Dowex 2 and Dowex 50, and warming the slurry for one hour at 50°C. The resin beads were removed by vacuum filtration and the solution stored at -20°C.

Sucrose Gradient Centrifugation: The homogeneity of ribosome preparations was determined by sucrose gradient centrifugation. A sucrose gradient from 10% to 35% was made in 20 mM KCl, 1 mM $MgCl_2$ in 20 mM Tris-HCl, pH 7.4. The gradient was formed using a two chambered mixing vessel by placing 2.3 ml of 35% sucrose in the mixing chamber and 2.3 ml of 10% sucrose in the reservoir chamber and layering the resulting gradient into 5 ml nitrocellulose tubes. A 0.4 ml sample of ribosomes (containing 10 to 20 A_{260} units) was layered over the sucrose gradient and centrifuged in a Spinco Model L Ultracentrifuge (SW-50 rotor) at 210,000 × g for 60 minutes. The tubes were punctured at the bottom with a needle and 0.2 ml fractions collected. The fractions, when diluted to 1.0 ml with 1 mM $MgCl_2$ in 20 mM Tris-HCl, were assayed at 260 nm in the Beckman DU.

Estimation of Salt Concentration in Column Effluent: The sodium chloride concentration of the column effluent was estimated by means of the refractive index. Standard solutions were prepared

and a curve drawn for increasing molarity (Figure 1). Refractive indexes were determined using a Bausch and Lomb refractometer. Sodium acetate concentration could not be estimated this way because of the high concentration of urea. Accordingly, a conductivity bridge was used following dilution of 0.1 ml aliquot to 20 ml to determine conductance which was referred to a standard curve (Figure 1).

Chemicals

Calf thymus histone, type IIa, unfractionated, was obtained from Sigma Chemical Company. Carboxymethyl cellulose (CM-32; microgranular) was purchased from Reeve Angel. Deoxyribonuclease, ribonuclease free, was obtained from Worthington Biochemical Corporation. Bio-Gel P-2 was obtained from Bio-Rad Laboratories. Sodium deoxycholate, enzyme grade, was obtained from Mann Research Laboratories. The fluorescent dye 8-anilino-1-naphthalenesulfonic acid, N, N-methylenebisacrylamide and N,N,N,N-tetramethylethylenediamine were obtained from Eastman Organic Chemicals. Clelands Reagent (dithiothreitol) was purchased from Calbiochem. All other chemicals were reagent grade or better.

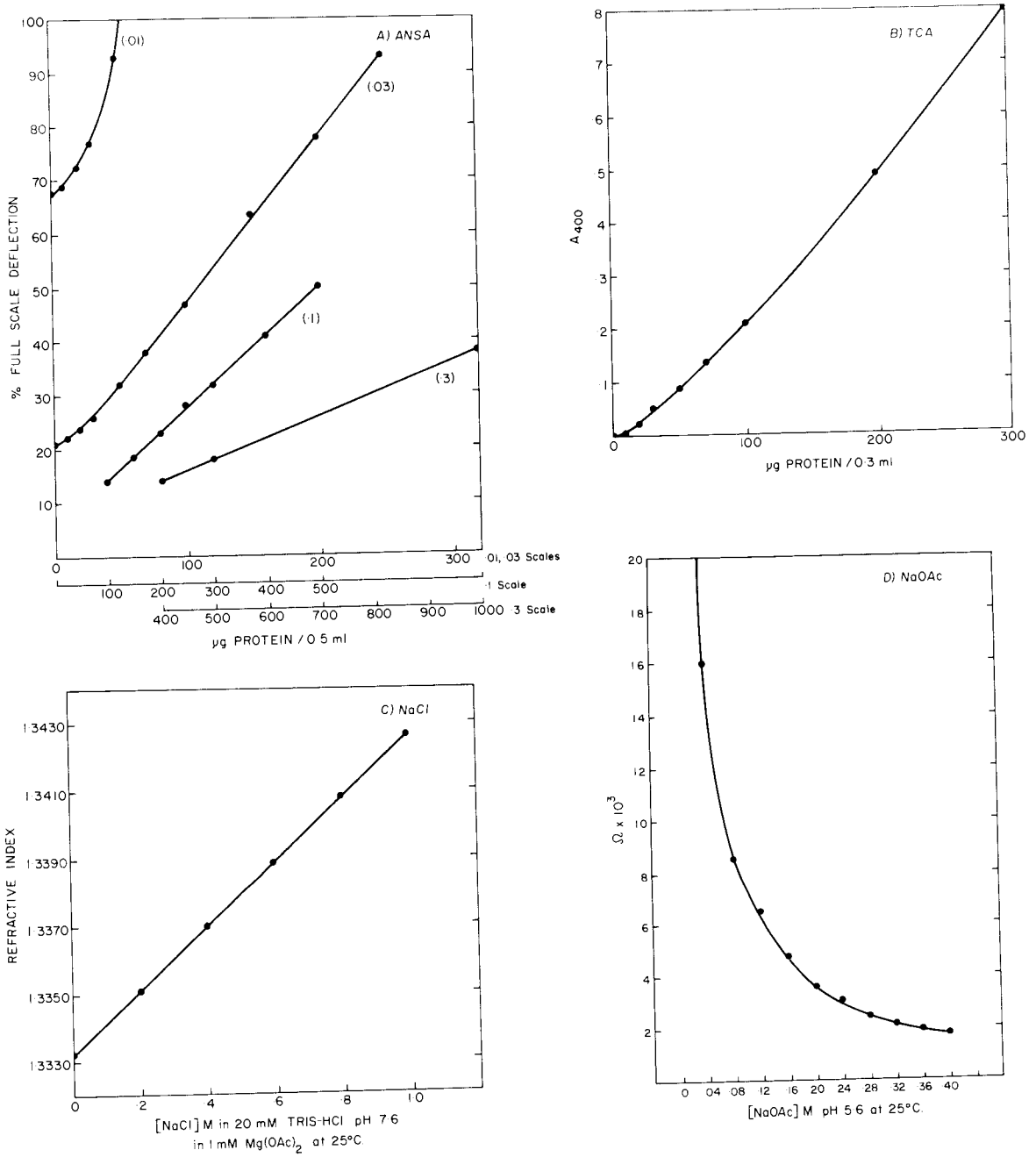

Figure 1. Standard curves for protein concentration a) ANSA and b) TCA. Meter multiplier settings are as indicated for ANSA. Standard curves for molarity c) NaCl and d) NaOAc (conductivity).

RESULTS

Establishment of Purity

Nuclei and Chromatin: Isolation of nuclei was a necessary prerequisite to the isolation of pure chromatin. Hymer and Kuff's (1964) method for isolating nuclei has proved satisfactory. High yields of morphologically intact nuclei, subject to a minimum of cross contamination, were produced by substituting a Tenbroeck homogenizer for their vigorous pipetting step. Miracloth filters were used to eliminate whole cell contamination, although extensive use reduces yield. Triton X-100 dispersed the cytoplasm and high nuclear purity was achieved by differential centrifugation. When examined by phase contrast microscopy, preparations of acceptable purity and homogeneity contained: 1) no whole cell contamination, 2) less than 5% chromatin threads and damaged nuclei and 3) no cytoplasmic tabs. Chromatin prepared from isolated nuclei must also exclude contamination from intranuclear particles such as "nuclear ribosomes." Although no standards for purity can be established for chromatin, exhaustive saline-citrate extraction reduced contamination from ribonucleoprotein particles as determined by the CM-cellulose fractionation of acid soluble proteins (Figure 11).

Ordinarily nuclei cannot be isolated from plant tissue because of the forces required for cell breakage. The usual procedure involves a direct isolation of chromatin (Huang and Bonner, 1962), but this yields a mixture of chromatin, starch grains, mitochondria and cytoplasmic remnants. Although centrifugation through 2 M sucrose was useful in excluding mitochondria and membranes, less confidence can be placed in the degree of cross contamination (particularly nuclear particles cross contaminating cytoplasmic ribosomes). Nevertheless, the chromatographic fractionation of acid extractable protein was comparable to chromatin prepared from isolated nuclei (compare Figures 8 and 9).

Ribosomes: When isolated by differential centrifugation mammalian ribosomes are contaminated by a highly aggregated iron-containing protein, ferritin, which has S values ranging from 70 to 400 and which cannot be eliminated by physical techniques (Wilson, 1965). The ultraviolet absorption spectra of crude ribosomes prepared by deoxycholate solubilization is displayed in Figure 2. In view of the high A_{230} absorption and reduced $A_{260/280}$ ratio it was clear that these ribosomes were heavily contaminated with ferritin (compare with ferritin spectra, Figure 2). Ferritin is involved with electrolyte regulation and fasting produces a stress on this mechanism which results in increased contamination of ribosomal preparations. Although all three fractions, polysomes, ribosomes and

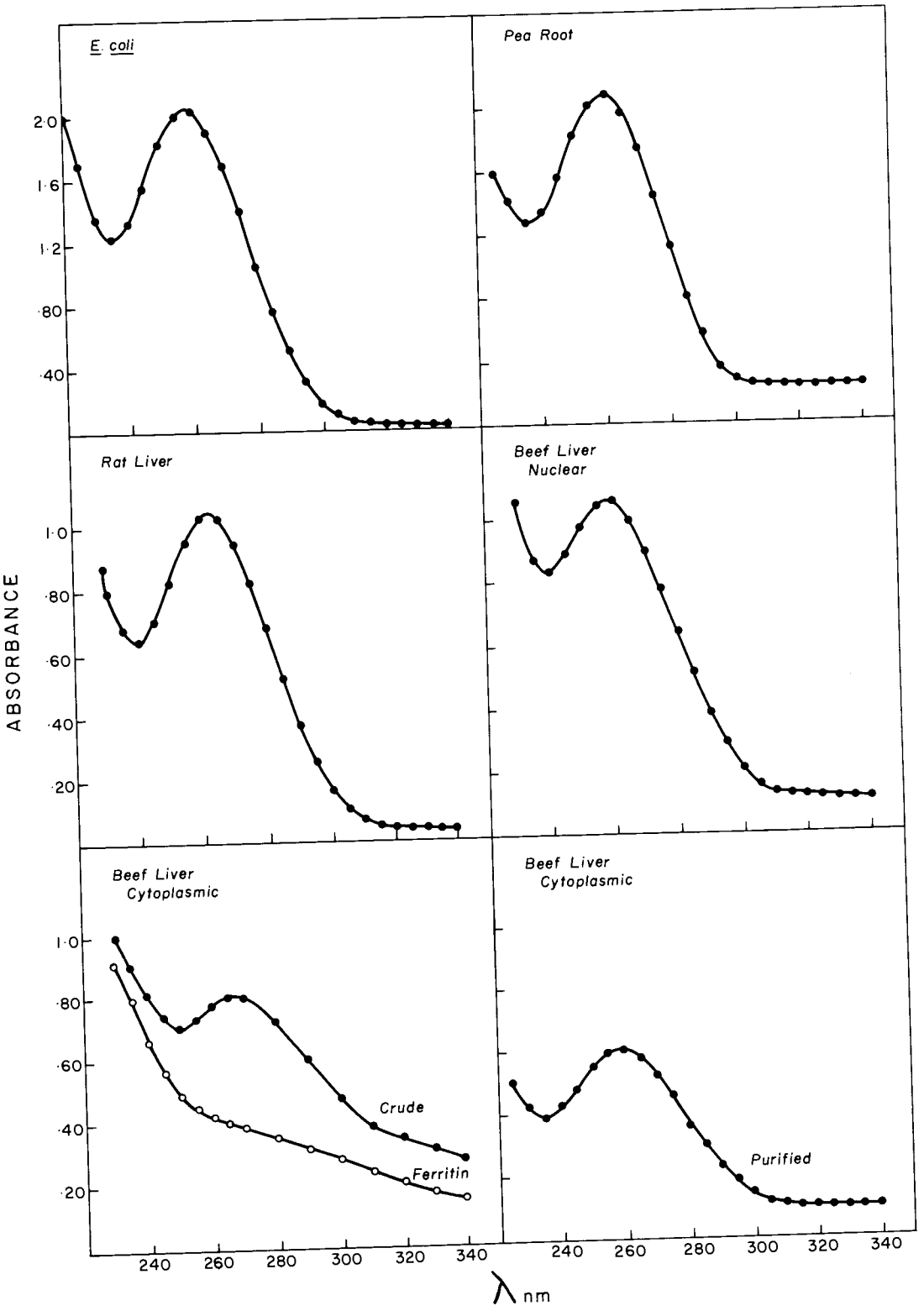

Figure 2. The ultraviolet absorption spectra for crude and purified ribosomes and ferritin. Curves were plotted from a Cary continuous recording spectrophotometer.

"nuclear ribosomes," were contaminated, the nuclear ribosomes contained the largest amount of ferritin (Figure 4). For this reason rats were fed until sacrificed, but cattle could not be obtained in that condition, the fasted animal being easier to butcher. A 1% solution of enzyme grade deoxycholate (suggested by Wettstein et al., 1963) increased purity in fasted ribosome preparations to $A_{260/280}$ and $A_{260/230}$ ratios of 1.8 and 1.5 respectively. Mammalian ribosomes were then subjected to further purification by DEAE cellulose chromatography according to Furano (1966) as seen in Figure 5. The run-off peak contained ferritin and the second peak, the purified ribosomes as determined by their UV absorption spectra (Figure 2). The resulting ribosomes were therefore pure by spectral criteria, having $A_{260/280}$ and $A_{260/235}$ ratios of 2.0 and 1.7 respectively. The UV spectra resemble those of RNA because of the low levels of tyrosine and tryptophane in the ribosomal proteins. DEAE cellulose purified ribosomes were monodisperse on sucrose gradient centrifugation (Figure 3), although some aggregation was suggested by the asymmetry on the heavy side of the peak. In contrast to mammalian ribosomes, the ribosomes of peas and E. coli were purified to $A_{260/280}$ ratios of 2.0 by differential centrifugation and were not usually subjected to DEAE cellulose chromatography (Figure 2).

Nuclear Ribosomes: A ribonucleoprotein fraction was isolated from beef and rat liver nuclei according to Allfrey's (1963) procedure

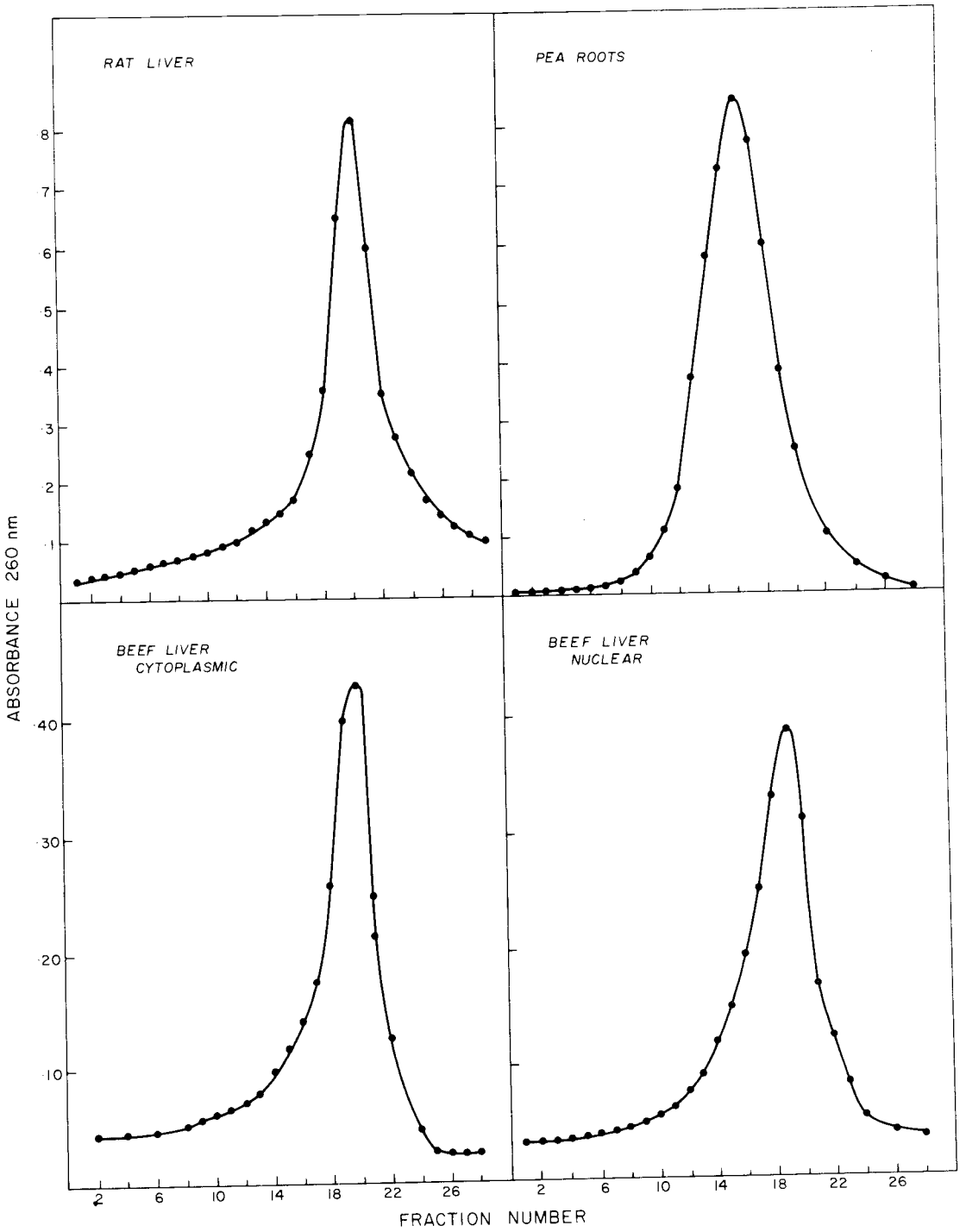

Figure 3. Sucrose gradient centrifugation of ribosomes isolated from pea roots, rat liver, beef liver and beef liver nuclei. The ribosomes (10 to 20 A_{260} units) were suspended in 0.4 ml of buffer and layered over 4.6 ml of 10 to 35% sucrose gradients. The material was centrifuged in a Spinco SW 50 rotor for 60 minutes at 210,000 x g and at 0.5°C. The tubes were punctured at the bottom and 0.2 ml fractions collected.

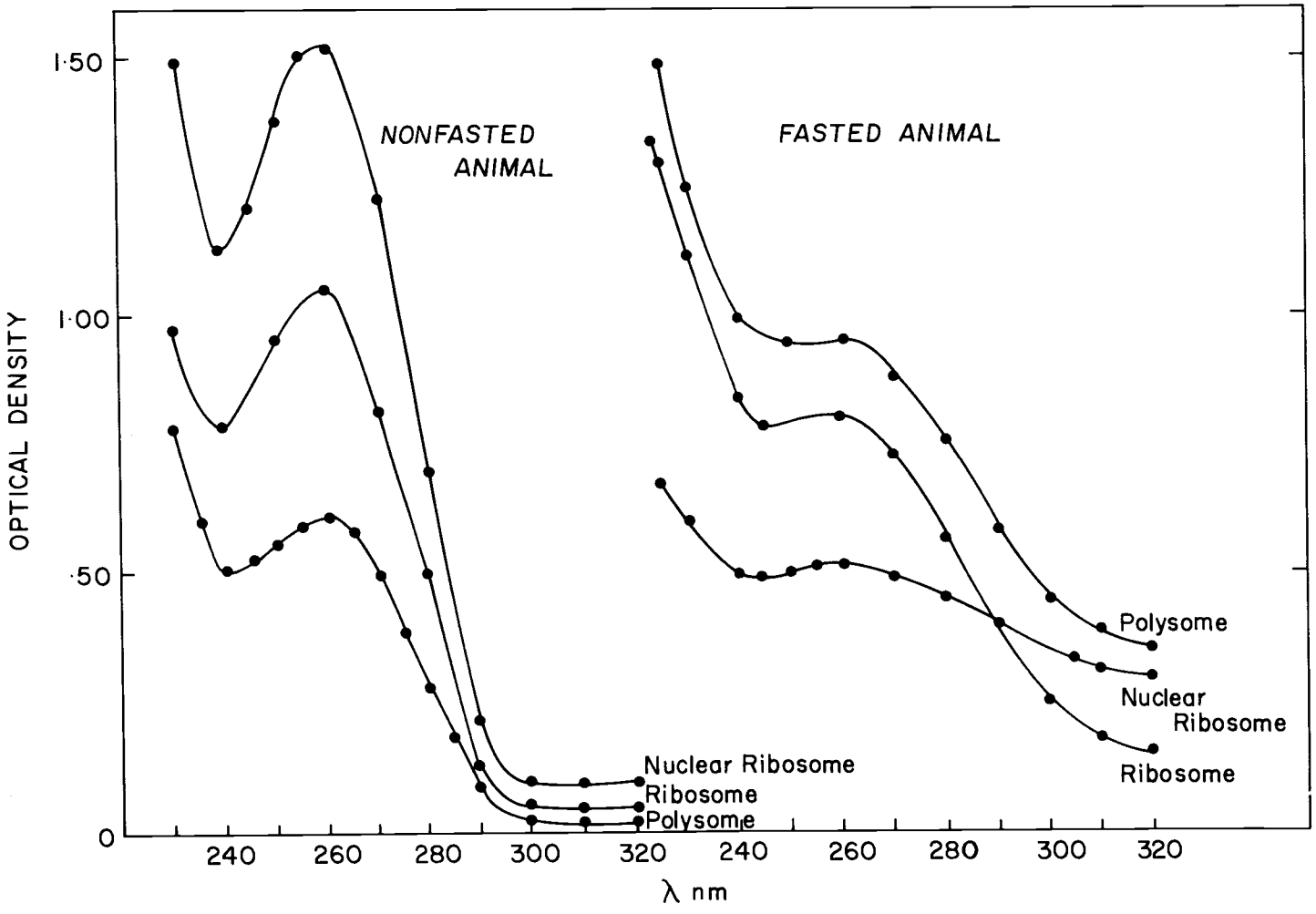

Figure 4. The ultraviolet absorption spectra for crude polysomes, ribosomes and nuclear ribosomes isolated from the livers of fasted and nonfasted rats. Curves were plotted from a Cary continuous recording spectrophotometer.

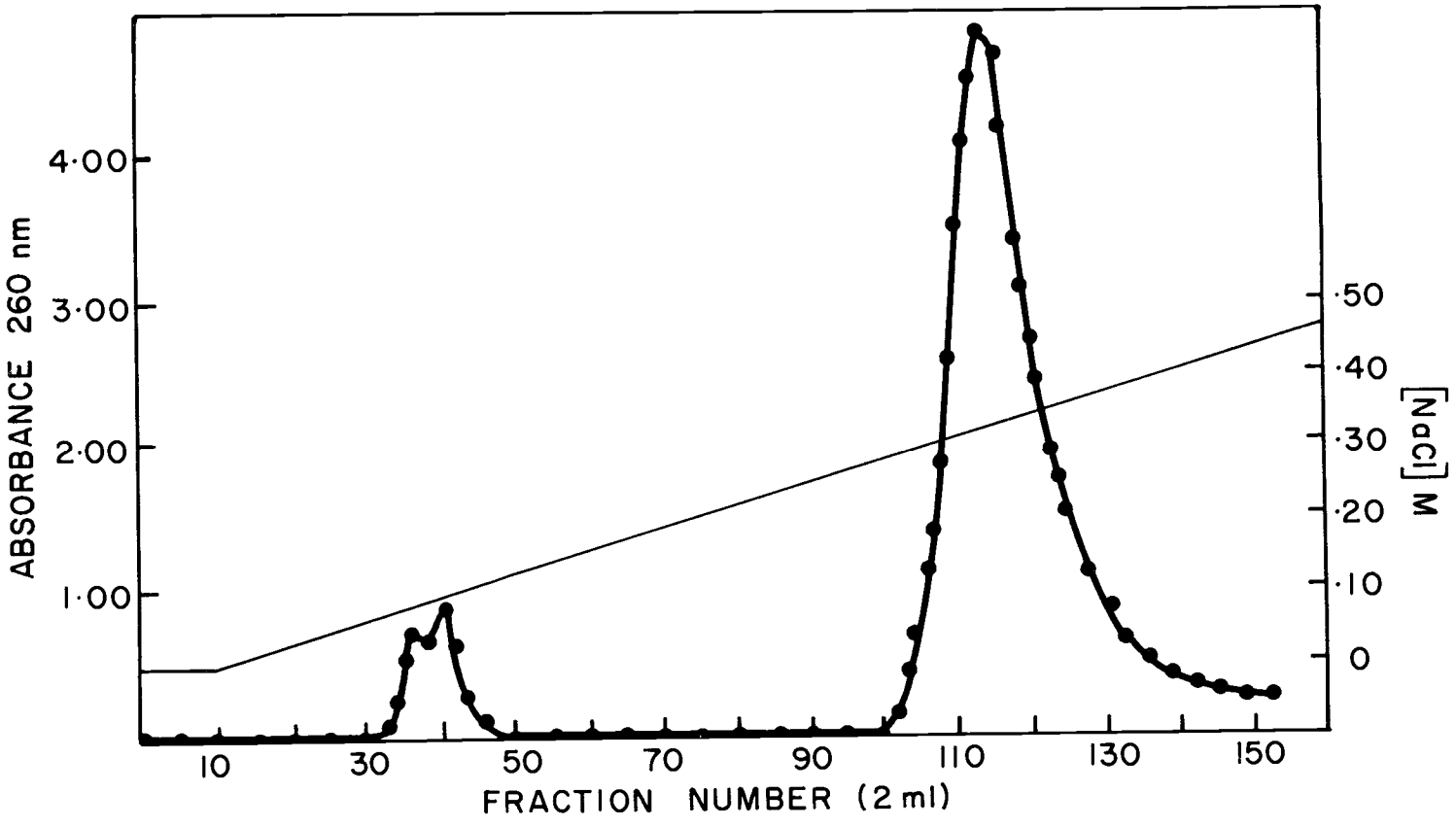

Figure 5. DEAE cellulose chromatography of beef liver ribosomes. Ribosomes (200 mg) were adsorbed to 2.5 x 10 cm column and eluted with a linear gradient of NaCl in 1 mM $MgCl_2$ and 20 mM Tris-HCl, pH 7.6. Fractions (2 ml) were collected at the rate of 30 ml/hour.

and designated as "nuclear ribosomes." This fraction has not been well characterized to date and may contain other nuclear ribonucleoprotein particles such as the "informosome" (Samarina et al., 1968) or ribosomal precursors. According to Samarina et al. the nuclear ribosomes were extracted at pH 7 while the "informosomes" (messenger ribonucleoprotein complex) were released at pH 8. When extracted at pH 7.4 as recommended by Pogo et al. (1962) the particle was monodisperse on sucrose gradient centrifugation (Figure 3). Due to the lack of characterization no attempt was made to purify the fraction except to note that by spectral criteria it was contaminated with ferritin. The acid-extractable proteins from particles isolated at pH 8 (beef liver nuclei) were fractionated on CM-cellulose and represent a preliminary characterization (Figure 12).

Extraction of Histones and Ribosomal Proteins

Satake's et al. (1960) method for extracting histones by titrating to pH 0.5 with 6 N H_2SO_4 has been adopted, but no differences were detected using other methods (Setterfield et al., 1960). Ribosomal protein can be extracted with either acid or high salt (LiCl-urea), however, when mammalian or plant ribosomes were extracted with LiCl-urea the resolution was poor. The proteins were separated from the salt or acid immediately after extraction and prior to adsorbing the proteins to the cellulose column (Figure 6). Protein

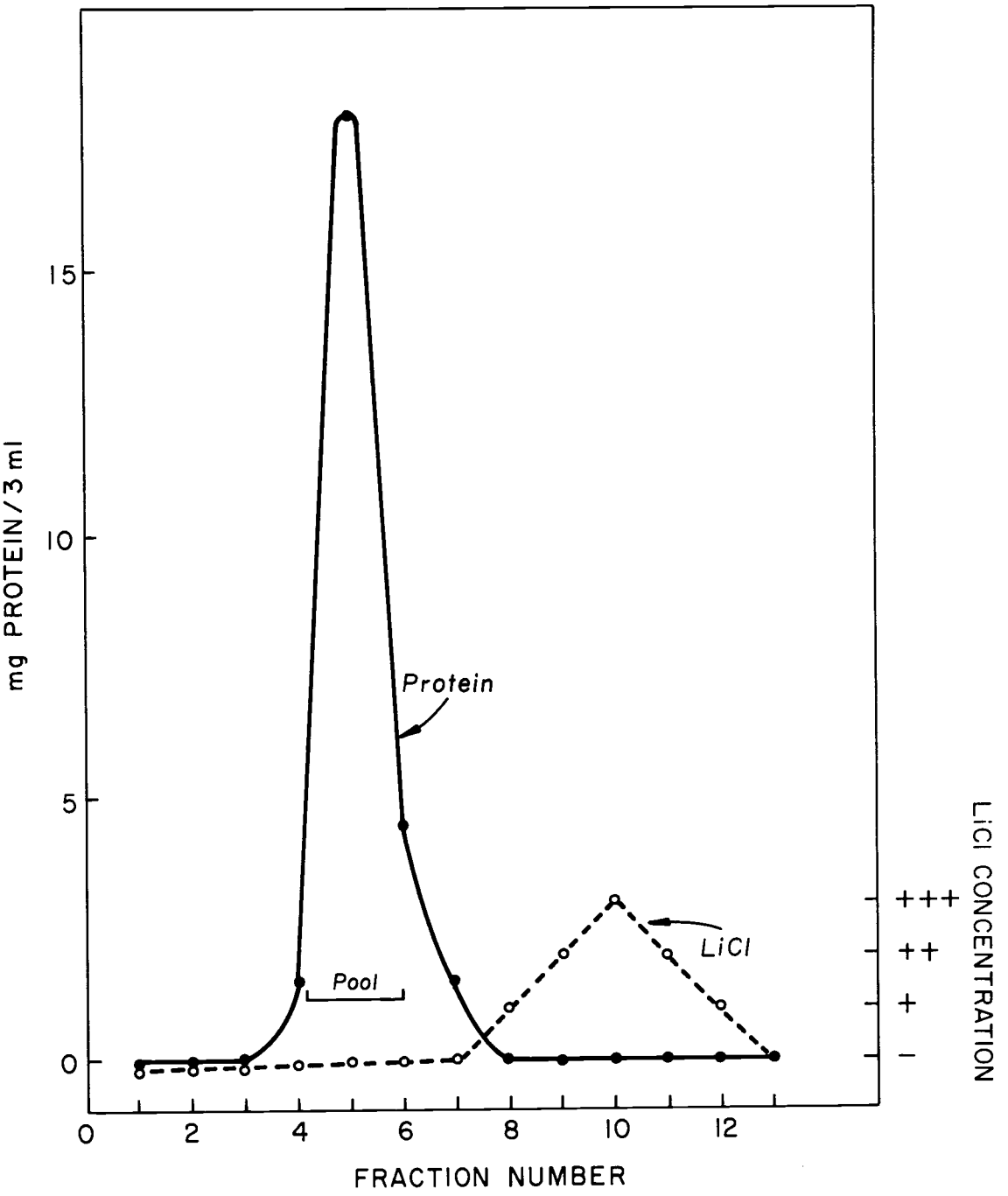

Figure 6. Gel filtration of histones on Bio-Gel P-2. Six ml of histones were applied to a 1.2 x 20 cm column and eluted with 0.01 M NaOAc, pH 5.6 in 6 M urea. Fractions (3 ml) were collected at a rate of 20 ml/hour.

degradation (measured both chromatographically and electrophoretically) was observed when storage in 6 M urea at -70°C. exceeded one week.

Carboxymethyl Cellulose Chromatography

Escherichia coli: Ribosomal protein from E. coli Q 13 was extracted with LiCl-urea and fractionated by means of carboxymethyl cellulose chromatography (Figure 7). On a 200 ml gradient 21 peaks were obtained, each of which displayed one to three components upon electrophoresis. The chromatographic profile of 70S ribosomal proteins agrees with the data of Otaka et al. (1967) if their 50S and 30S patterns are superimposed and demonstrates that the technique has been handled properly. Protein was bound irreversibly to the cellulose under Otaka et al. conditions of adsorption in 0.005 M NaOAc buffer, but were elutable if adsorbed in 0.01 M NaOAc.

Pea Roots: Histones and ribosomal proteins were extracted with acid from 48 hour germinating pea roots and fractionated on separate columns under similar conditions (Figure 8). On a 200 ml gradient, the histones fractionated into six major peaks which appeared similar to the fractions obtained from IRC-50 chromatography (see Luck et al., 1958). There was very little protein in the run-off, but a peak (R-O) would appear to correspond to the run-off peak of IRC-50. The fraction corresponding to peak III, IV contained a shoulder which

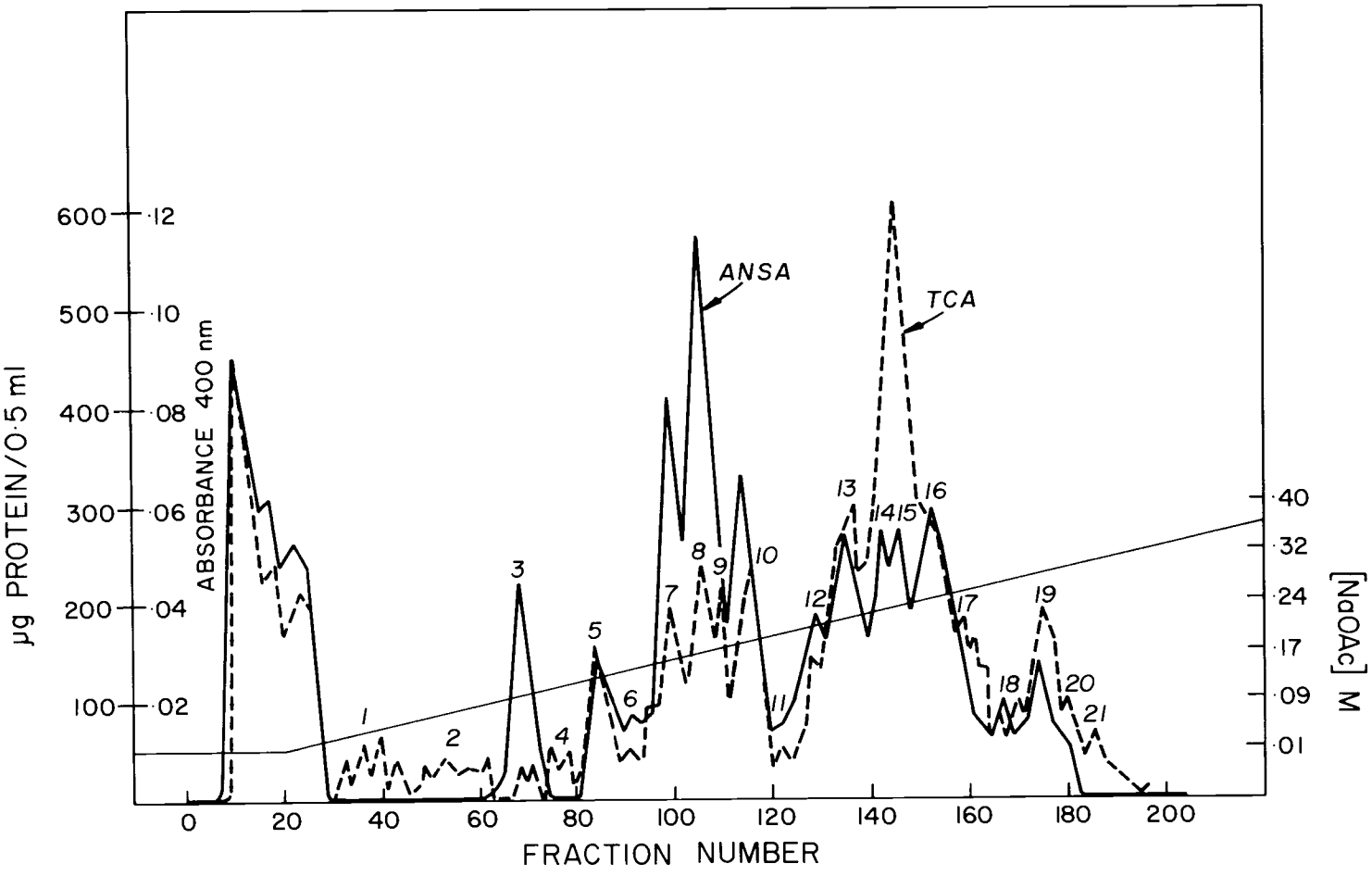

Figure 7. Carboxymethyl cellulose chromatography of ribosomal protein isolated from Escherichia coli. The protein (40 mg) was adsorbed to a 1.2 x 15 cm column and eluted with an increasing gradient (200 ml) of NaOAc, pH 5.6 in 6 M urea. Fractions were collected at a rate of 6 ml/hour at 10°C. The protein concentration was estimated by TCA (---) and ANSA (——) as described in the text. Sodium acetate concentration was estimated from the conductivity (thin line ——).

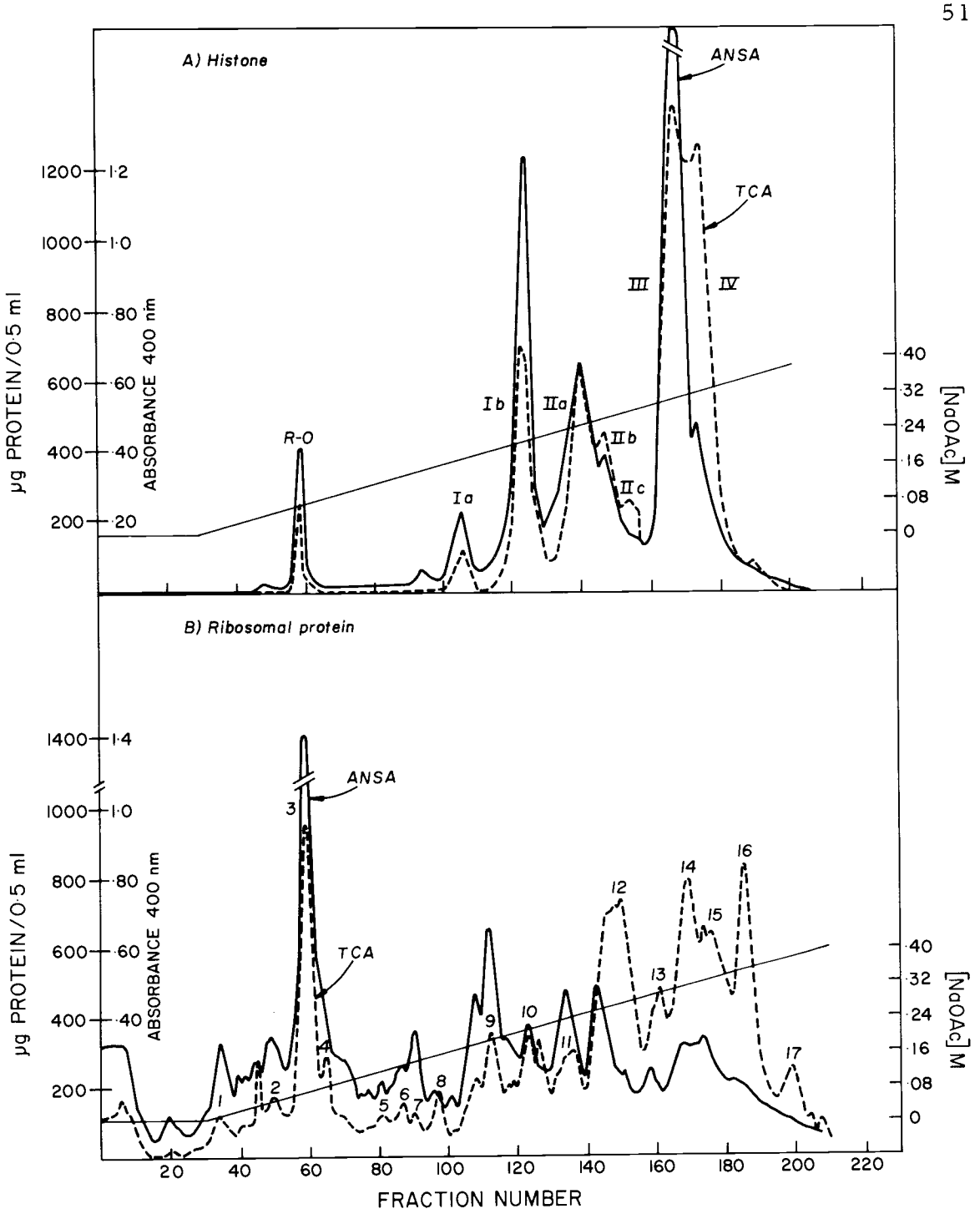

Figure 8. Carboxymethyl cellulose chromatography of A) histone and B) ribosomal protein isolated from 48 hour germinating pea roots. Protein concentration was estimated by TCA (---) and ANSA (—) as described in Figure 7. The elution conditions were similar to Figure 7. Forty-five mg of histone and 80 mg of ribosomal protein was added to the two columns. The thin line (—) represents the molarity of sodium acetate.

represents a partial separation of fraction III from fraction IV. The ribosomal protein pattern contained 17 peaks, with the major fraction (3) corresponding to the R-O peak of the histone profile. There appeared to be two ribosomal proteins (1 and 2) less basic than this fraction, in addition to some protein material in the run-off. The ribosomal proteins were spread throughout the elution gradient in contrast to the histones which elute from 0.2 to 0.4 M NaOAc. A comparison of the histones with the ribosomal proteins showed that for each histone fraction, including the arginine-rich peak, the ribosomal proteins (9 through 16) contained corresponding fractions.

Beef Liver: Histones and ribosomal proteins were prepared from beef liver and fractionated on separate columns under similar conditions (Figure 9). The elution gradient consisted of 120 ml of 0.01 to 0.16 M and 240 ml of 0.16 to 0.4 M. Therefore, the concentration of fractions eluting between 0.01 to 0.16 M are two-fold greater than the later fractions. Under these conditions the histone pattern resembled the IRC-50 profiles for calf thymus. The histone fractions were poorly resolved on a 200 ml gradient as seen in Figure 11, and fractions III and IV eluted as a single peak under both conditions. The first peak (R-O) on the histone profile (similar to the peas) corresponded to the major ribosomal protein peak (2). The second major ribosomal peak (6) was represented in the histone profile, but was never prominent. The ribosomal protein pattern

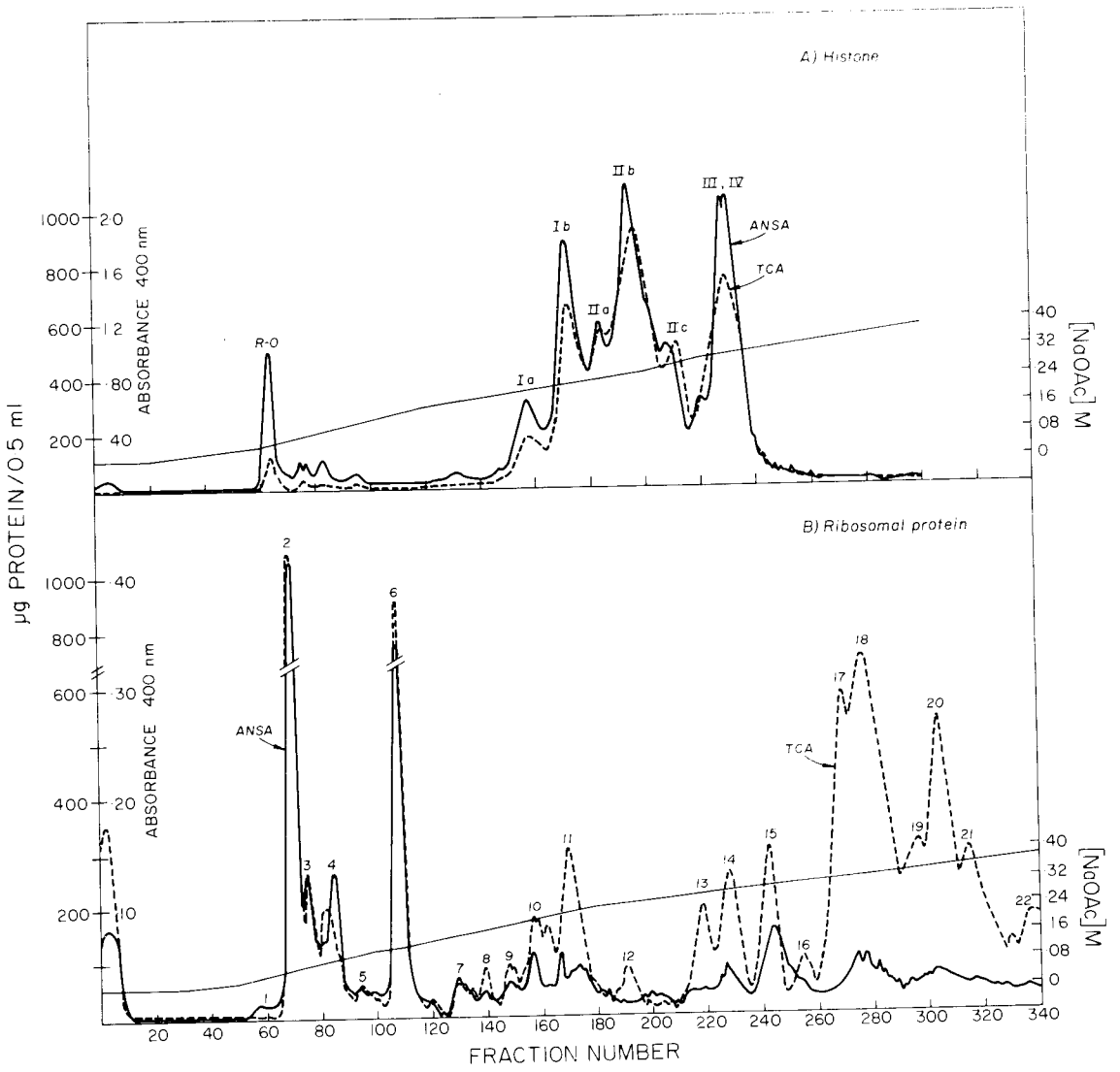

Figure 9. Carboxymethyl cellulose chromatography of A) histone and B) ribosomal protein isolated from beef liver. Protein concentration was estimated by TCA (---) and ANSA (—) as described in Figure 7. Elution occurred with 120 ml of 0.01 to 0.16 M NaOAc followed by 240 ml of 0.16 to 0.4 M NaOAc. One hundred mg of histone and 90 mg of ribosomal protein was applied to the two columns. The thin line (—) represents the molarity of sodium acetate.

contained 22 peaks that were distributed throughout the gradient. There was less correspondence between histones and ribosomal proteins than was found for peas. In addition, there was a group of six ribosomal proteins (17 to 22) that appeared to be more basic than the corresponding histones. Although these peaks were reproducible between runs, they may represent aggregation rather than an increased basicity. This region is difficult to understand because it was not present in either the pea root or rat liver profiles. Furthermore, the single attempt to fractionate the proteins from nuclear ribosomes did not possess these peaks. Aggregation in 6 M urea would appear unlikely, but cannot be ruled out.

Rat Liver: The profiles of histones and ribosomal proteins (Figure 10) isolated from rat liver were similar to the corresponding beef liver profiles, but the resolution of individual histone fractions was intermediate between beef liver and the pea roots. The intermediate position was partly apparent in that histone fractions Ib and IIc were absent and partly real and in that fractions III and IV began to resolve on the 200 ml gradient. The histone profiles contained fractions Ia, IIa, IIb, III and IV. The R-O peaks observed in beef and pea histone profiles were present, but inconspicuous. Heterogeneity among the five fractions did not increase when the slope of the gradient was lowered as in Figure 13 (200 ml gradient 0.2 to 0.4 M). The fractions were clearly resolved including peaks III and

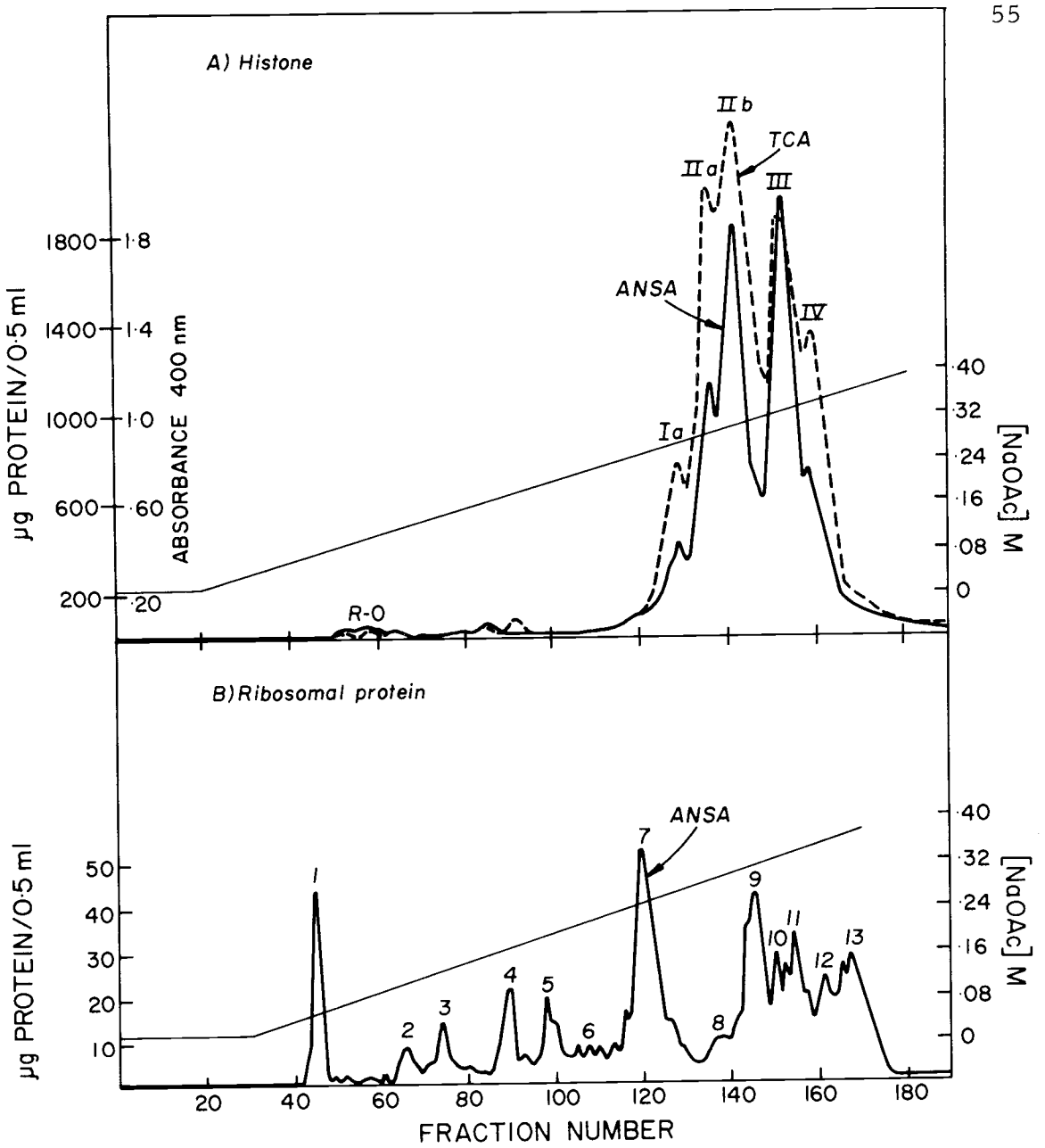

Figure 10. Carboxymethyl cellulose chromatography of A) histone and B) ribosomal protein isolated from rat liver. Protein concentration was estimated by TCA (---) and ANSA (——) as described in Figure 7. The elution conditions were the same as for Figure 7. Twenty-four mg of histone and five mg of ribosomal protein was added to the two columns. The thin line (——) represents the molarity of sodium acetate.

IV. Under these conditions the proteins normally eluting between 0.01 and 0.2 M NaOAc were eluted as a single polydisperse peak. The profile of the rat liver ribosomal proteins contained 13 peaks spread throughout the gradient. The low number of fractions obtained were accounted for by the low level of protein applied to the column. For this reason the TCA analysis was not made.

Chromatin Pre-treatment: In order to assess the degree of contamination of chromatin the total acid soluble nuclear proteins were compared with histones extracted from exhaustively washed chromatin (Figure 11). The total nuclear proteins were substantially more heterogeneous than the saline-citrate treated histones. Most significantly, saline-citrate selectively extracted out both the R-O peaks and a portion of the lysine-rich histones, resulting in a lower ratio of peak heights between the lysine-rich and arginine-rich histone regions. Saline-citrate qualitatively extracted *in toto* several of the early eluting peaks as well as two peaks from the lysine-rich region. Although the profile of the total acid soluble nuclear proteins did not equal the superimposed profiles of histones and ribosomal protein, the partial saline extraction of all but the arginine-rich histones indicated that these proteins were not tightly bound to chromatin. The second most prominent ribosomal protein was conspicuously reduced in the total nuclear protein profile (see Figure 9, peak 6).

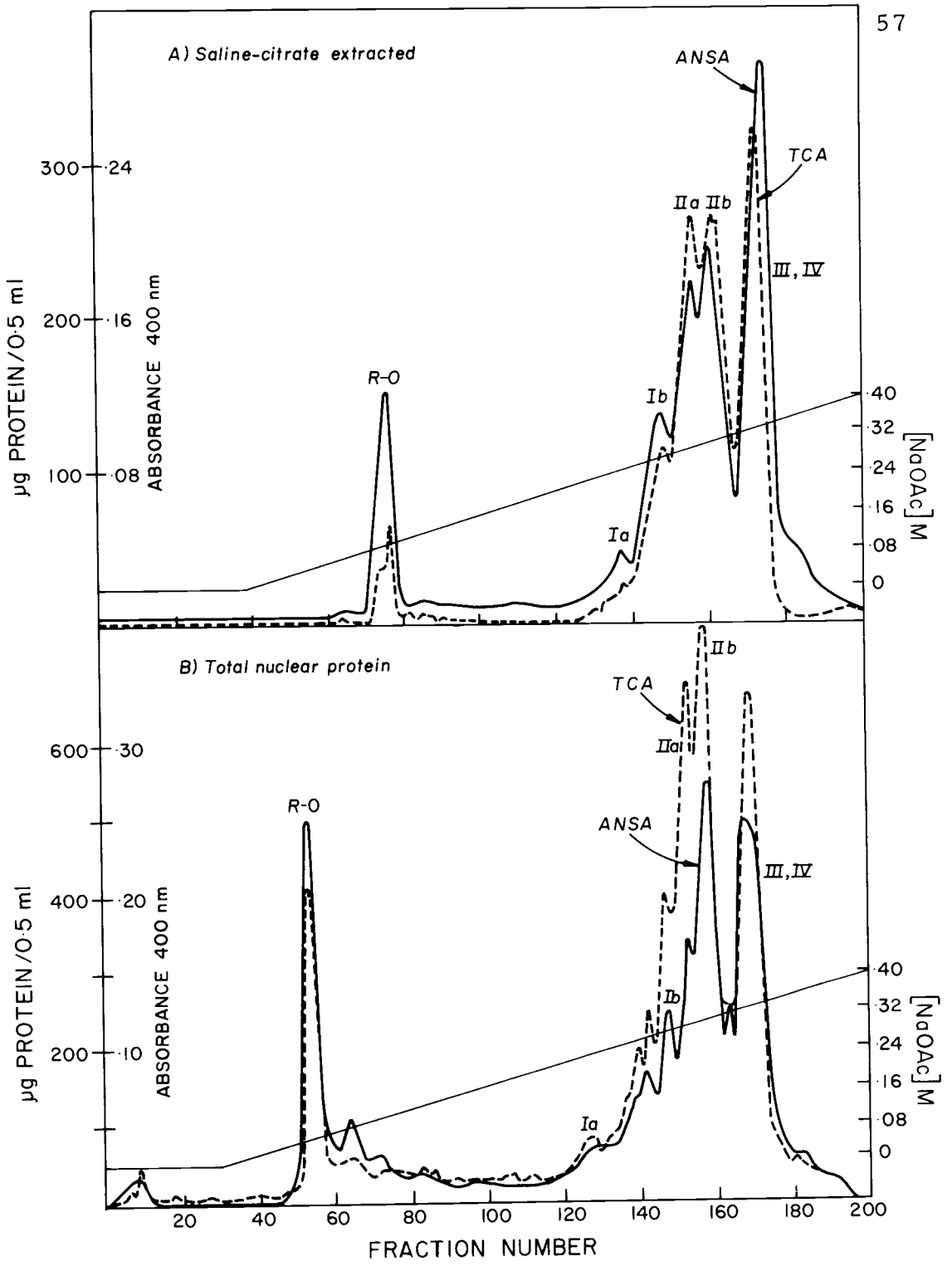

Figure 11. Carboxymethyl cellulose chromatography of A) histone from saline-citrate extracted beef liver chromatin and B) total nuclear acid soluble protein. Protein concentration was estimated by TCA (---) and ANSA (——) as described in Figure 7. The elution conditions were the same as for Figure 7. Twenty mg of protein was added to each column. The thin line (——) represents the molarity of sodium acetate.

Nuclear Ribosomes: Nuclear ribosomes were present in much lower concentration than cytoplasmic ribosomes and have not been well characterized. Reproducible preparations were obtained if the nuclei were extracted at pH's lower than 7.4. Extraction at higher pH's produced increasing heterogeneity that may represent contamination from nucleoprotein particles associated with messenger RNA. In the single opportunity for fractionating the basic proteins from such particles, the extraction was made at pH 8. The profile displayed in Figure 12 has been difficult to interpret because it would include any basic proteins from these ribonucleoprotein particles. The proteins eluting within the gradient portion resembled a histone profile without the arginine-rich peak. The proteins included within the run-off and extending slightly into the gradient represent the major portion of the total protein. The excessive run-off was originally interpreted as degradation resulting from the dissociation of the ribosomes at pH 8. However, if the nuclear extract contained other ribonucleoprotein particles this peak may represent native proteins. The second major fraction (6) from cytoplasmic ribosomal proteins was absent as were those cytoplasmic ribosomal proteins (17 to 22) that elute after the arginine-rich histones (see Figure 9).

Subfractionation by Gel Electrophoresis

Polyacrylamide gel electrophoresis was used to fractionate and

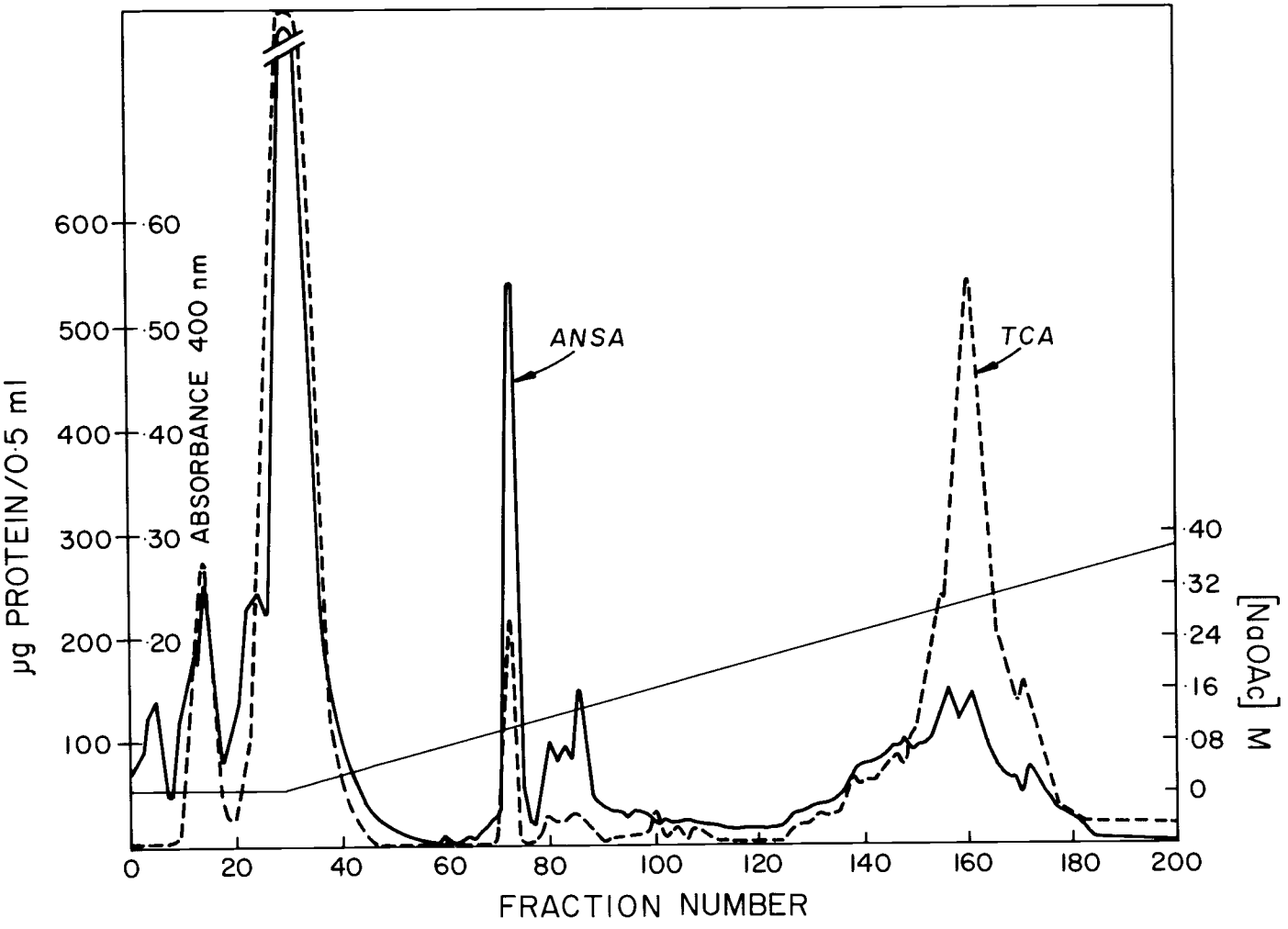

Figure 12. Carboxymethyl cellulose chromatography of ribosomal protein isolated from beef liver nuclear ribosomes. Protein concentration was estimated by TCA (---) and ANSA (——) as described in Figure 7. The elution conditions were the same as for Figure 7. Sixteen mg of protein was applied to the column. The thin line (——) represents the molarity of sodium acetate.

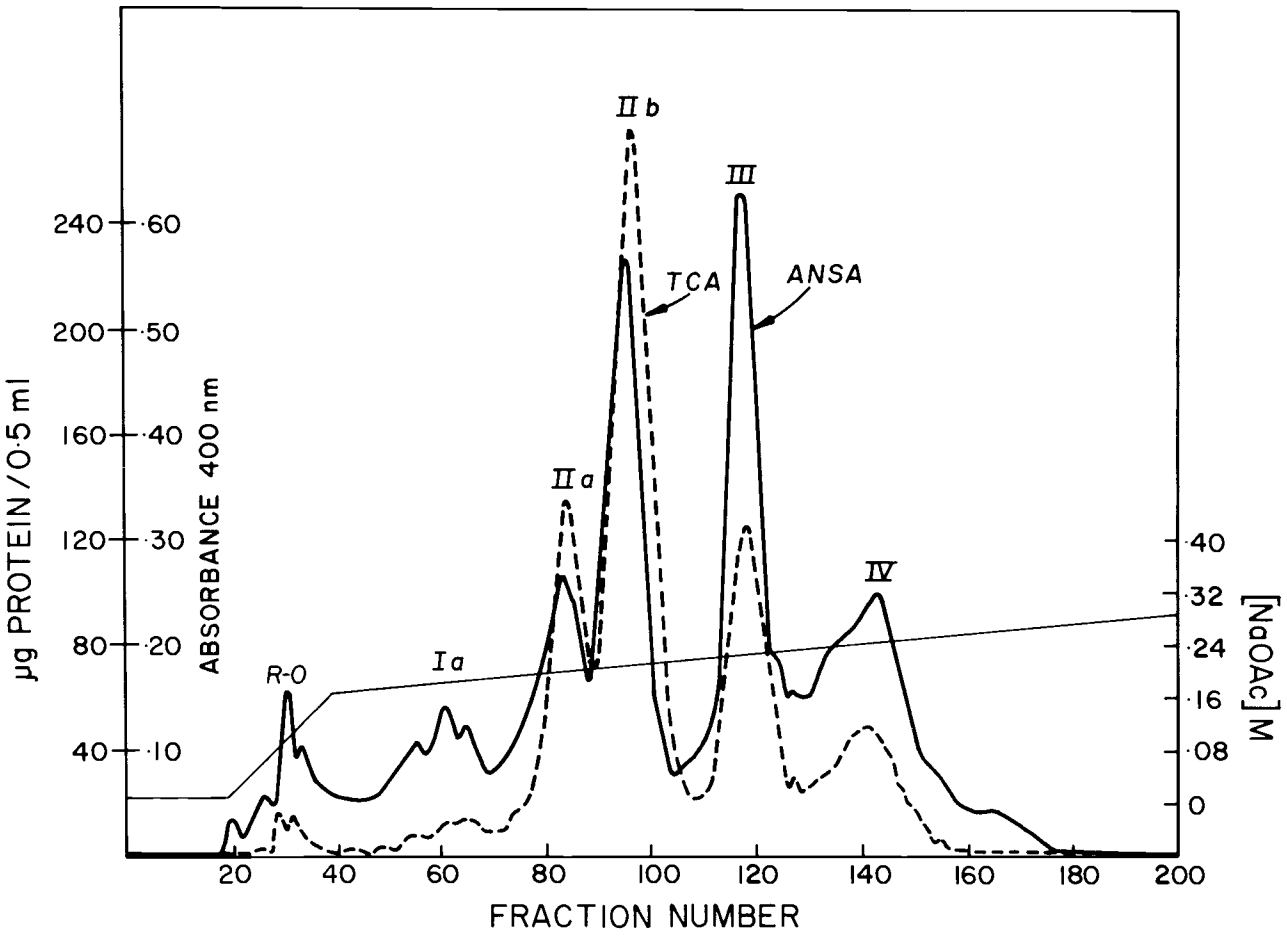

Figure 13. Carboxymethyl cellulose chromatography of rat liver histone. Protein concentration was estimated by TCA (---) and ANSA (——) as described in Figure 7. The elution conditions were similar to Figure 7, except the 200 ml gradient began at 0.16 M NaOAc instead of 0.01 M NaOAc. Ten mg of histone was applied to the column. The thin line (——) represents the molarity of sodium acetate.

subfractionate basic proteins. Several methods were compared during this investigation. The most satisfactory was Reisfeld's et al. (1962) procedure, modified by Leboy et al. (1964) to include 8 M urea. The The technique of Fambrough and Bonner (1966), similar to Leboy, but lacking the stacking gel, was more convenient, but was satisfactory only with highly concentrated samples (100 μg/50μl).

The histone electrophoretograms were stained and scanned at 525 nm with the linear transport (Figure 14). The beef and rat liver scans were similar to each other with the four major bands grouped so as to resemble their chromatographic profiles. The bands of the electrophoretogram were in the same order as their elution pattern from CM-cellulose. Pea histone also resolved well, but the electrophoretogram diverged more from the chromatographic profile. Three bands from peak IV had lower R_f values than would be predicted from their chromatographic elution position.

Scans of the ribosomal electrophoretograms are displayed in Figure 15. The electrophoretogram from E. coli closely resembled the chromatographic elution profile as well as previous reports (Low and Wool, 1967; Sells and Davis, 1968). The rat liver electrophoretograms were similar to Low and Wool with the scan from beef showing some similarity to the rat pattern. The conditions of Setterfield et al. (1960) were too divergent to make a comparison with the electrophoretogram obtained from peas. In general, more electrophoretic

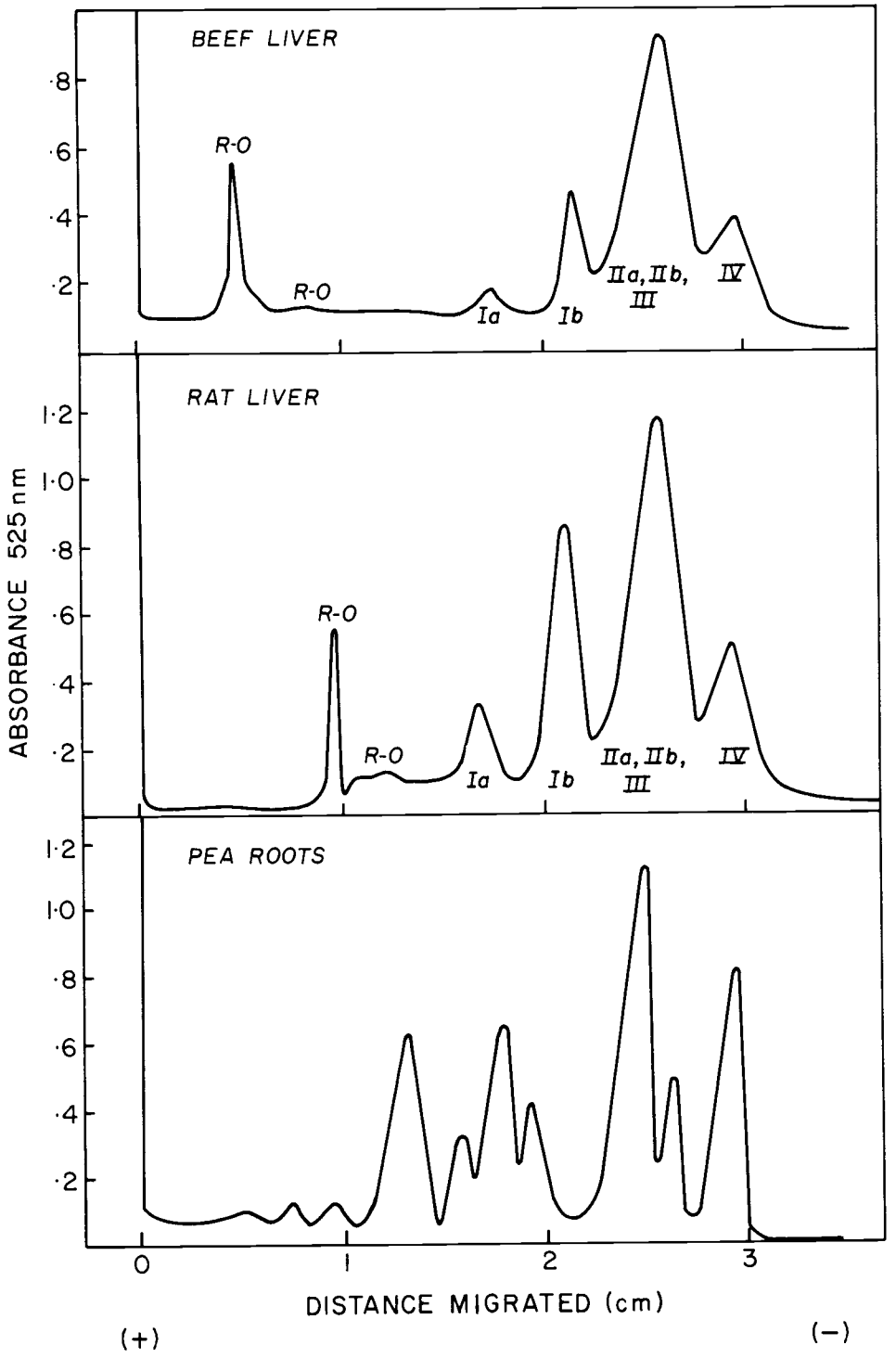

Figure 14. Scans from histone electrophoretograms. Histones extracted from beef liver, rat liver and pea roots were electrophoresed at 3 ma/tube at pH 4.3, stained with 0.5% Amido Schwarz and scanned at 525 nm. The abscissa represents distance of migration from the origin to the cathode. The numbers are for convenience and do not imply homology. Samples of 100 μg were added to each gel.

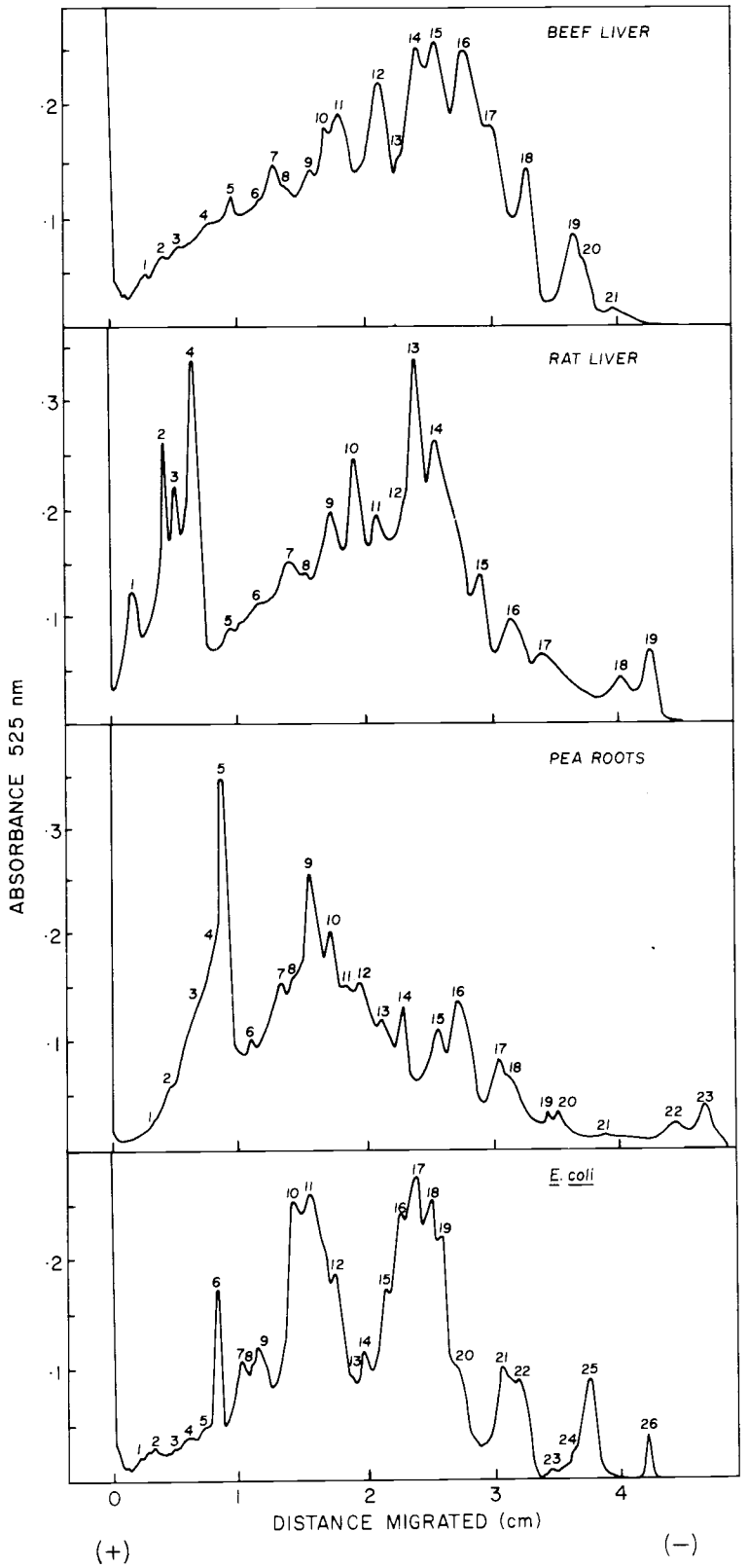

Figure 15. Scans from ribosomal protein electrophoretograms. Ribosomal proteins extracted from beef liver, rat liver, pea roots and E. coli were electrophoresed as described in Figure 14. Samples of 150 μg were added to each gel.

bands were observed than were chromatographic peaks because the major chromatographic peaks (beef 2 and pea root 3) each contained four to six bands.

Subfractionation of the chromatographic fractions from pea root histones showed that chromatographic peaks Ia and Ib were homogeneous (Figure 16). Peaks IIa, IIb and IIc contained four bands, two major and two minor, with the major bands corresponding to IIa and IIb. Peak III, IV contained six bands. Of these, three had R_f's less than peak Ia and correspond to fraction IV and three had R_f's greater than Ia and correspond to fraction III. Altogether 12 bands were observed.

Subfractionation of the chromatographic fractions from beef liver histones have not revealed any homogeneous fractions (Figure 17). Peak R-O contained six bands, peak Ia contained a major (probably Ia) and a minor (possibly Ib) band. The complex of peaks Ib, IIa, IIb, IIc appeared to have four bands which overlap in the four peaks. However, the concentration of the major band would have obscured any minor components. Peak III, IV contained two bands. Excluding the R-O peak, seven bands were observed.

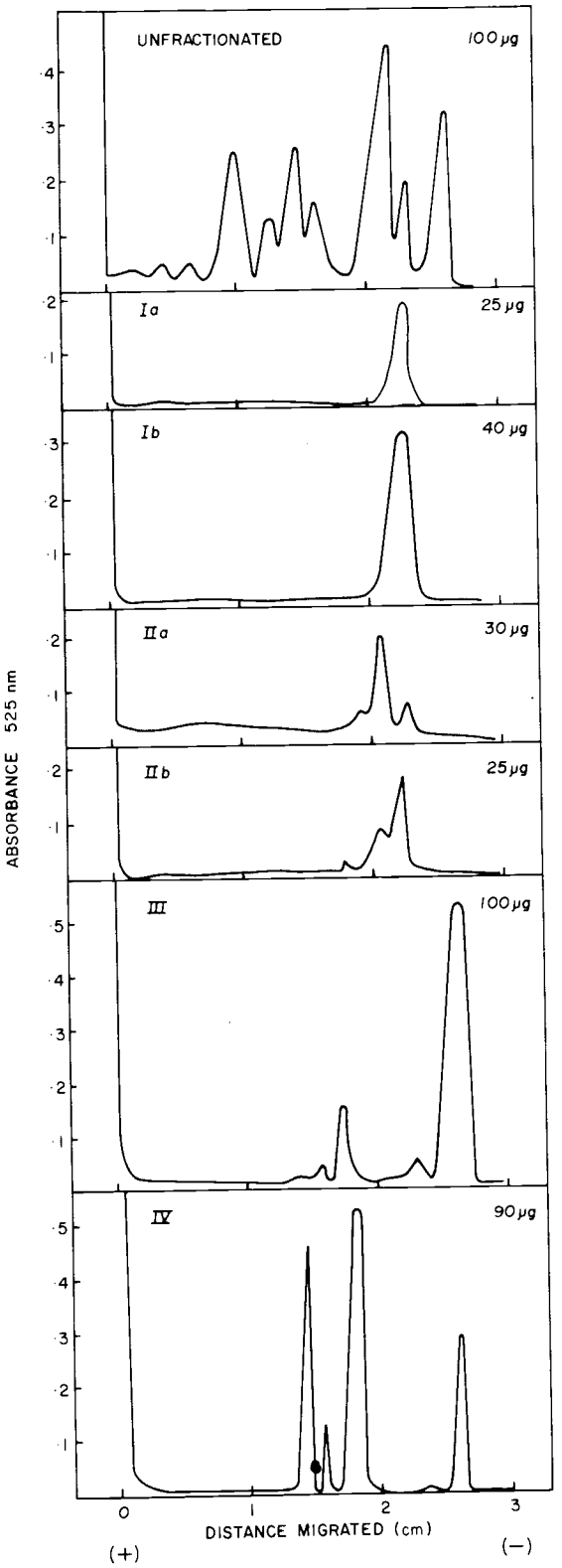

Figure 16. Scans from electrophoretograms of pea root histone chromatographic fractions. CM-cellulose chromatographic fractions were dialyzed against 0.01 M NaOAc in 6 M urea for one hour and electrophoresed as described in Figure 14. The amount of protein added is listed for each gel.

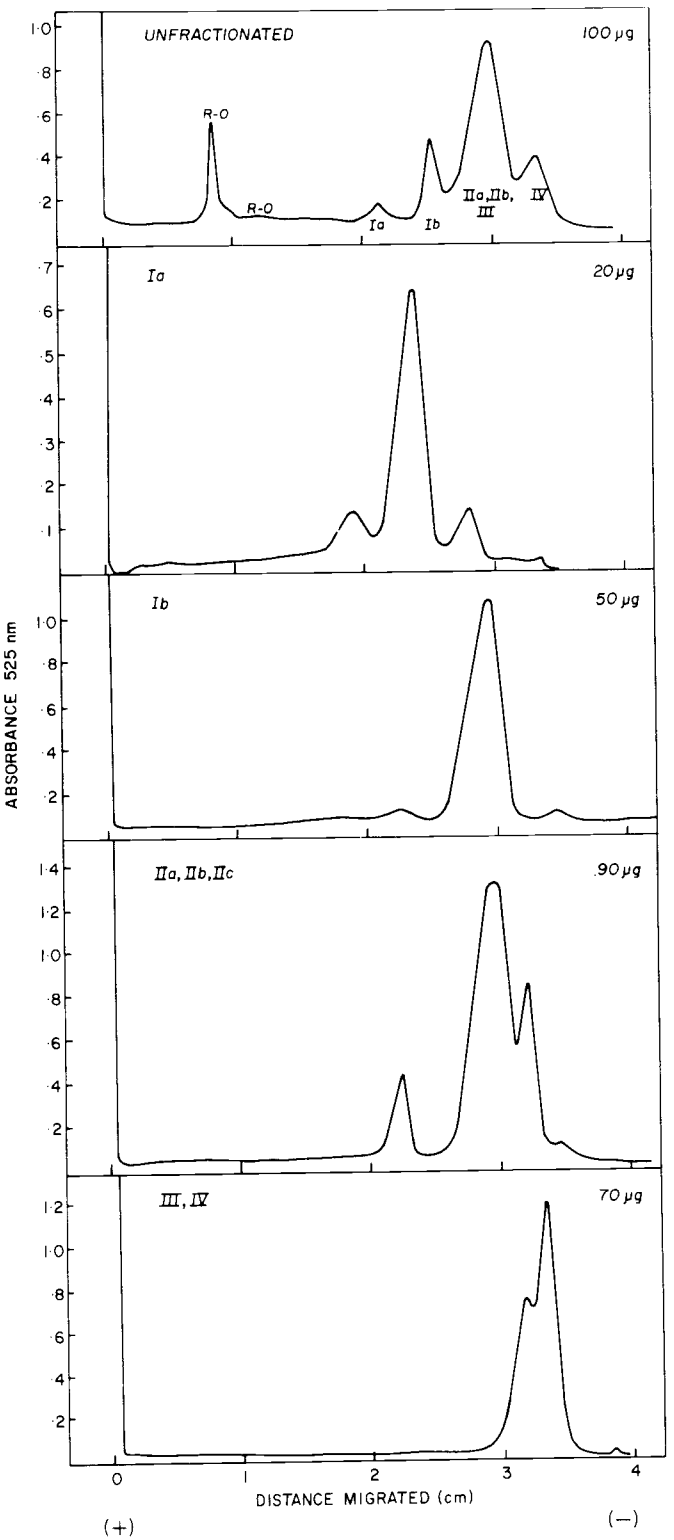

Figure 17. Scans from electrophoretograms of beef liver histone chromatographic fractions. CM-cellulose chromatographic fractions were dialyzed against 0.01 M NaOAc in 6 M urea for one hour and electrophoresed as described in Figure 14. The amount of protein is listed for each gel.

DISCUSSION

Carboxymethyl cellulose chromatography, originally designed to fractionate ribosomal protein from bacteria (Otaka et al., 1968) has, in this study, been successfully adapted to both mammalian and higher plant histones and ribosomal proteins. It has several advantages over IRC-50 chromatography: 1) little, if any, protein is irreversibly adsorbed to the exchanger, 2) all fractions (both lysine-rich and arginine-rich histones) elute with a single uninterrupted gradient, 3) both histones and ribosomal protein can be eluted under similar conditions, 4) the eluting buffer and cellulose are more easily obtained and purified and 5) the arginine-rich peak can be resolved into fraction III and fraction IV (except in beef).

Under strict adherence to the procedure the chromatograms were highly reproducible. The concentration of the buffer used to adsorb the proteins to the cellulose was critical; at 5 mM NaOAc the proteins were bound irreversibly and could not be eluted, whereas at 50 mM the proteins were not adsorbed to the cellulose. Provided the cellulose was pre-equilibrated at high buffer concentration (0.75 M) during column preparation all of the basic proteins could be eluted with a 0.01 to 0.4 M NaOAc gradient. Changes in pH were the major cause of irreproducibility and were eliminated by using freshly deionized urea.

Carboxymethyl cellulose chromatography differs from conventional IRC-50 chromatography in that the concentration of the denaturing agent (urea) remains constant with the proteins eluting with increased salt concentration, whereas IRC-50 chromatography employs an increasing gradient of denaturing agent (GuCl). This may account for any differences in the observed elution profiles.

IRC-50 chromatography produces five peaks, labelled Ia, Ib, IIa, IIb and III, IV and the profiles obtained with pea roots and calf thymus histone under similar conditions are alike (Luck et al., 1958; Fambrough and Bonner, 1966). When pea root histone was chromatographed on CM-cellulose the fractions were well resolved and the degree of separation was comparable to the separation achieved with IRC-50. However, when beef liver histones were chromatographed on CM-cellulose under the same conditions as peas, the fractions eluted within a smaller range of salt concentration and were not well separated. For this reason we conclude that pea root and beef liver histones are not homologous. If liver and thymus histone are assumed to be equal this conclusion is at variance with the report of Fambrough and Bonner (1966).

When rat liver histones were chromatographed on CM-cellulose, the profile resembled that obtained with beef liver. If the slope of the eluting gradient was reduced (either by increasing the volume or beginning elution at 0.16 M NaOAc) the fractions were well separated

(Figures 9 and 13) and comparable to peas. In addition, we found when using shallow gradients that the arginine-rich histones could be fractionated into separate peaks III and IV, whereas this is not possible with IRC-50.

Beef liver and pea root histones were fractionated into six peaks corresponding in appearance to the fractions Ia, Ib, IIa, IIb, IIc and III, IV of IRC-50 chromatography (Luck et al., 1958). This does not mean, however, that these fractions are identical to the IRC-50 fractions and further characterization will be necessary to establish this point. Rat liver histone lacked both peaks Ib and IIc, but peak III was resolved from peak IV on a 400 ml gradient.

In contrast to the histones, which have been extensively characterized following IRC-50 chromatography, the ribosomal proteins were not bound to IRC-50 and therefore chromatographic data and subsequent characterization is lacking (Berlowitz and Birnstiel, 1967; Neelin and Vidali, 1968). Ribosomal proteins from mammals and higher plants have been partially characterized from electrophoretic data (Setterfield et al., 1960; Hamilton and Ruth, 1967). We observed that CM-cellulose fractionates ribosomal protein into 13 peaks (rat liver), 22 peaks (beef liver) and 17 peaks (pea root). Although the number of fractions is less than that observed in electrophoretic gels, a partial characterization of their physical and chemical properties is possible now that the fractions can be obtained

in large quantities. In addition, further separation is possible and is likely to increase the number of fractions. The short 200 ml gradients were adequate for resolving the histones (7 to 12 proteins), but would need to be extended to adequately fractionate the ribosomal proteins into homogeneous peaks necessary for further characterization. A short gradient might be adequate if proteins from each ribosomal subunit were to be examined separately. To date only Hamilton and Ruth (1967) have achieved sufficient yields of large subunits to characterize the proteins.

When assayed turbidimetrically (TCA) some of the histones and ribosomal proteins exhibited identical chromatographic mobility. True identity (identical primary structure) was not probable however, in that: 1) relative peak heights and shapes did not often correspond and 2) the possible ribosomal homologs were more numerous than their histone counterparts (Figures 8 to 10). The preliminary conclusion, substantiated by the electrophoretic data, is that chromatographic identity is not a sufficiently sensitive criterion. When the histones and ribosomal protein chromatograms were compared for the 0.01 M to 0.2 M NaOAc region there was, however, considerable identity (Figure 9).

The second line of evidence for similarity but nonidentity of histones and ribosomal proteins came from the comparison between the TCA and ANSA assays. ANSA has been used to: 1) estimate

protein concentration (Shepherd and Noland, 1965) and 2) probe for conformational changes and differences in primary structure (Stryer, 1968). When used to analyse column effluents, we found that ANSA often provided superior peak resolution to TCA (larger difference between peak and trough height). The two assays showed good agreement when applied to histone fractions (using peak height and shape as criteria). However, when applied to ribosomal protein they diverge substantially and significantly. For the region 0.01 to 0.2 M NaOAc the ratio of TCA to ANSA was about one, whereas the ratio of TCA to ANSA was two or three to one for the 0.2 to 0.4 M region (Figures 7 to 10). This shift suggests a lack of identity between histones and ribosomal protein fractions, even though their chromatographic mobilities were similar. This shift in ratio occurred for the three sources investigated (E. coli, peas and beef liver) and presumbly would also occur in rat liver (not investigated). The only other shift observed with ANSA occurred in the arginine-rich region of pea histones where peak IV was reduced by one half as compared to TCA. A third comparison using Amido Schwarz staining of electrophoretic patterns as criteria confirmed the TCA analysis. Although the TCA assays indicated chromatographic identity between some histones and ribosomal fractions, the relative ratio of TCA/ANSA showed that these components were in fact dissimilar. The comparison of TCA to ANSA reiterates the conclusion

that chromatographic identity cannot be used as a criteria for true identity and must be accompanied by peptide mapping of tryptic digests to have meaning.

A third line of evidence for similarity, but nonidentity of the histones and ribosomal proteins was provided by the experiment to assay chromatin purity (Figure 11). The chromatogram from total nuclear acid soluble protein in which prior extraction with saline-citrate was omitted gave: 1) increased heterogeneity of fractions in the lysine-rich region and 2) peak height ratios of lysine-rich to arginine-rich histones greater than one as compared to the exhaustively saline-citrate extracted chromatin. Saline-citrate extraction both qualitatively and quantitatively altered the chromatographic profile so that the only fractions to increase in relative proportion following treatment were the arginine-rich histones (peak III, IV).

Identities between histones and ribosomal protein have been reported using electrophoretic mobility as the primary criterion (Lindsay, 1966; Beeson and Triplett, 1967; Shepherd and Noland, 1968). However, the work presented here reveals that electrophoretic mobility or any other single criterion is not sufficient to prove identity and must be used in conjunction with other techniques (multiple levels of discrimination). No standards of purity were established in the above citations and cross contamination of their

fractions is evident.

The chromatographic identity established in this study confirmed the observations, based on electrophoretic identity, which showed that histones and ribosomal proteins do in fact have similar charge properties (Lindsay, 1966; Beeson and Triplett, 1967; Shepherd and Noland, 1968).

This data is at variance with the observations of Berlowitz and Birnstiel (1967) and Neelin and Vidali (1968) who reported that contrary to histones the ribosomal proteins did not bind to IRC-50 and were by this criteria less basic. However, both Berlowitz and Neelin used ethanol to precipitate the proteins from the extracting acid and experienced difficulty in resuspending the denatured proteins. Neelin and Vidali, in particular, reported that less than 50% of the ribosomal protein precipitate could be redissolved. Perhaps this accounts for the observed lack of binding and could be reconciled with our data on this basis, remembering that in our experiments the acid extractable proteins were exchanged directly into 0.01 M NaOAc in 6 M urea on Bio-Gel P-2.

Chromatographic or electrophoretic heterogeneity may be explained in at least three ways: 1) cross contamination of cytoplasmic ribosomes by nuclear particles during isolation, 2) contamination of chromatin by nuclear ribonucleoprotein particles including nucleolar proteins and 3) aggregation. Knowing this, our first

objective was to prepare organelles by procedures that lower cross contamination. Nuclei were prepared in isotonic sucrose in the presence of detergent. Centrifugation in 2 M (hypertonic) sucrose with the resulting precipitation of intranuclear material was thereby eliminated (Maggio et al., 1963). Ribosomes were also prepared under gentle homogenization conditions in order to reduce cross contamination by particles released from broken nuclei. A substantial effort was made to achieve purity of ribosomal preparations. Ribosomes were thereby purified to $A_{260/280}$ and $A_{260/235}$ ratios of 2.0 and 1.7 respectively; substantially better than previous reports of 1.75 and 1.5 by Low and Wool (1967). Ribosomes prepared by DEAE cellulose chromatography were free from ferritin.

The second source of heterogeneity, from intranuclear ribonucleoprotein particles, was considered during the preparation of chromatin. Chromatin prepared from intact nuclei must exclude "nuclear ribosomes" and "messenger ribonucleoprotein particles." Nucleoli are a source of "nuclear ribosomes" or ribosomal precursors and hence the major source of contamination in conventionally prepared chromatin. They retain their integrity in aqueous solution and pellet at 1200 × g. Nucleoli could not be dispersed with 0.5% deoxycholate because of its solubilizing effect on chromatin. Saline-citrate extraction was the most effective method for washing chromatin and reducing heterogeneity as determined by comparing

the saline-citrate treated chromatogram with the total nuclear protein (Figure 11). Nevertheless, saline-citrate extracted chromatin may still be substantially contaminated with both nuclear ribonucleoprotein particles and proteins which form the fibrillar structure of the nucleolus. It may well be that some of the basic proteins (e.g. the lysine-rich histones) observed here are nucleolar in origin.

The third source of heterogeneity among chromatographic fractions is due to aggregation and is more easily recognized. Aggregation may either be due to: 1) nonspecific (noncovalent) aggregation and 2) oxidative dimerization of SH groups (covalent). Nonspecific aggregation can be reduced by using urea and guanidine hydrochloride at high molarities, although in 6 M urea some of the beef liver ribosomal proteins (17 to 22) appeared to aggregate (Figure 9). Cysteinyl dimerization can be reduced and reversed by including sulfhydryl compounds in all of the extraction media (Fambrough and Bonner, 1968). An attempt was made to include sulfhydryl compounds (DTT) throughout the preparation of materials, except during the actual protein fractionation on CM-cellulose. Dimerization could thereby occur following adsorption to the cellulose and prior to electrophoresis on polyacrylamide gels. Peak III of the arginine-rich histones contains cysteine and is susceptible to dimerization (Fambrough and Bonner, 1968). The presence of six electrophoretic

bands in peaks III, IV of peas as compared to two bands from beef and rat liver made these fractions suspect. The observation on peas, however, was the reverse of Fambrough and Bonner's (1968) report of extensive dimerization of oxidized calf thymus histones as compared to pea root histones. The number of histones present in any source remains an open question. Fambrough, Fujimura and Bonner (1968) discussed six fractions from pea roots, while clearly showing data for seven. They believed the "six" fractions to be homologous from calf thymus to peas (Fambrough and Bonner, 1968) in contrast to the lack of homology that we report for beef liver and peas. This study reports seven fractions from beef and twelve from peas with the major difference occurring in peaks III and IV. Beef possessed two bands while peas showed six bands in peaks III, IV. If, in peas, peaks III, IV were the result of dimerization the total number of components would be close to eight and in good agreement with Bonner's results. Regardless of the final outcome as to the number of fractions, there appears to be only a small number of distinct proteins which would make histones unlikely participants in any mechanisms of gene action based on a one-gene-one-repressor protein concept.

The total number of ribosomal proteins for any species has not been determined. Nineteen are known for the 30S subunit of E. coli with as many as 40 postulated for the 50S (Traut et al., 1967). It is anticipated that refined fractionation procedures will reveal at

least this number for mammalian and higher plant ribosomes. To date, less than 30 proteins from 80S ribosomes have been observed by electrophoresis (Low and Wool, 1967; Setterfield et al., 1960). We observed more than 25 ribosomal proteins on electrophoretic gels, although not all were detected by the scan. The chromatographic fractionation of proteins from 80S ribosomes produced 17 peaks (peas) and 22 peaks (beef) although longer gradients (greater than 400 ml) appear likely to revise these numbers upwards as has proved to be the case for E. coli.

In summary, carboxymethyl cellulose has been an extremely valuable technique for studying milligram quantities of histones and ribosomal proteins. The method shows: 1) minimal nonspecific adsorption, 2) elution of all fractions with an uninterrupted gradient, 3) elution of histones and ribosomal protein under similar conditions, 4) resolution of the arginine-rich fractions and 5) ease in preparation. Although the limit of resolution has not been attained, the chromatographic profiles are comparable to IRC-50 chromatography and superior to preparative gel electrophoresis. Whereas certain fractions of histones and ribosomal protein were chromatographically identical, structural identity was not likely.

BIBLIOGRAPHY

Allfrey, V. G. 1963. Nuclear ribosomes, m-RNA, protein synthesis. Experimental Cell Research, sup., 9:183-212.

Allfrey, V. G., R. Faulkner and A. E. Mirsky. 1964. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proceedings of the National Academy of Sciences 51:786-793.

Allfrey, V. G., V. C. Littau and A. E. Mirsky. 1963. On the role of histones in regulating RNA synthesis in the cell nucleus. Proceedings of the National Academy of Sciences 49:414-421.

Arnstein, H. R. V. 1963. The structure and function of ribosomes in protein biosynthesis. Chemical Society Annual Reports LX:512-528.

Beeson, J. L. and E. L. Triplett. 1967. Localization and characterization of rat and chicken histones. Experimental Cell Research 48:61-70.

Benjamin, W. and A. Gellhorn. 1968. Acidic proteins of mammalian nuclei: Isolation and characterization. Proceedings of the National Academy of Sciences 59:262-268.

Berlowitz, L. and M. Birnstiel. 1967. Histones in the wild-type and the anucleolate mutant of Xenopus laevis. Science 156:78-80.

Bonner, J. and R. C. Huang. 1963. Properties of chromosomal nucleohistone. Journal of Molecular Biology 6:169-174.

Bonner, J., R. C. Huang and R. V. Gilden. 1963. Chromosomally directed protein synthesis. Proceedings of the National Academy of Sciences 50:893-900.

Bonner, J. and J. Widholm. 1967. Molecular complementarity between nuclear DNA and organ-specific chromosomal RNA. Proceedings of the National Academy of Sciences 57:1379-1385.

Brown, D. D. and J. B. Gurdon. 1964. Absence of ribosomal RNA synthesis in the anucleolate mutant of Xenopus laevis. Proceedings of the National Academy of Sciences 51:139.

Busch, H. 1965. Histones and other nuclear proteins. New York, Academic Press. 266 p.

Chargaff, E. 1950. Chemical specificity of nucleic acids and mechanism of their enzymatic degradation. Experientia 16:201-209.

Chersi, A., M. Dzionara, D. Donner and H. G. Wittman. 1968. Ribosomal proteins. IV. Isolation, amino acid compositions, peptide maps and molecular weights of yeast ribosomal proteins. Molecular and General Genetics 101:82-88.

Crampton, C. F., S. Moore and W. H. Stein. 1955. Chromatographic fractionation of calf thymus histones. Journal of Biological Chemistry 215:787-801.

Cruft, H. J., C. M. Mauritzen and E. Stedman. 1957. The nature and physicochemical properties of histones. Philosophical Transactions of the Royal Society of London, ser. B, 241:93-145.

Daly, M. M. and A. E. Mirsky. 1954. Histones with high lysine content. Journal of General Physiology 38:405-413.

Davis, J. R. and H. Busch. 1959. Chromatographic analysis of radioactive cationic nuclear proteins of tissues of tumor-bearing rats. Cancer Research 19:1157-1166.

Davison, P. F. 1957. Chromatography of histones. Biochemical Journal 66:708-712.

DeLange, R. J., E. L. Smith, D. M. Fambrough and J. Bonner. 1968. Amino acid sequence of histone. IV. Presence of ϵ-N-Acetyllysine. Proceedings of the National Academy of Sciences 61:1145-1146.

Duesberg, P. H. and R. R. Rueckert. 1965. Preparative zone electrophoresis of proteins on polyacrylamide gels in 8 M urea. Analytical Biochemistry 11:342-361.

Fambrough, D. M. and J. Bonner. 1966. On the similarity of plant and animal histone. Biochemistry 5:2563-2569.

_____ 1968. Sequence homology and role of cysteine in plant and animal arginine-rich histones. Journal of Biological Chemistry 243:4434-4439.

Fambrough, D. M., F. Fujimura and J. Bonner. 1968. Quantitative distribution of histone components in the pea plant. Biochemistry 7:575-584.

Flaks, J., P. S. Leboy, E. A. Birge and C. G. Kurland. 1966. Mutations and genetics concerned with the ribosome. Cold Spring Harbor Symposium on Quantitative Biology 31:623-631.

Furano, A. V. 1966. Chromatography of Escherichia coli ribosomes on diethylaminoethyl cellulose. Journal of Biological Chemistry 241:2237-2244.

Gurley, L. R., J. L. Irvin and D. J. Holbrook. 1964. Inhibition of DNA polymerase by histones. Biochemical and Biophysical Research Communications 14:527-532.

Hamilton, M. G. and M. E. Ruth. 1967. Characterization of some of the proteins of the large subunit of rat liver ribosomes. Biochemistry 6:2585-2590.

Huang, R. C. and J. Bonner. 1962. Histone, a suppressor of RNA synthesis. Proceedings of the National Academy of Sciences 48:1216-1222.

_____ 1965. Histone-bound RNA a component of native nucleohistone. Proceedings of the National Academy of Sciences 54:960-967.

Huang, R. C., J. Bonner and K. Murray. 1964. Physical and biological properties of soluble nucleohistones. Journal of Molecular Biology 8:54-64.

Hymer, W. C. and E. L. Kuff. 1964. Isolation of nuclei from mammalian tissues through the use of Triton X-100. Journal of Histochemistry and Cytochemistry 12:359-363.

Izawa, M., V. G. Allfrey and A. E. Mirsky. 1963. The relationship between RNA synthesis and loop structure in lampbrush chromosomes. Proceedings of the National Academy of Sciences 49:544-551.

Johns, E. W. 1964. Studies on histones. 7. Preparative methods for histone fractions from calf thymus. Biochemical Journal 92:55-59.

Johns, E. W. and J. A. V. Butler. 1962. Further fractionation of histones from calf thymus. Biochemical Journal 82:15-18.

_____ 1964. Specificity of the interactions between histones and deoxyribonucleic acid. Nature 204:853-855.

Kleinsmith, L. J., V. G. Allfrey and A. E. Mirsky. 1966a. Phosphoprotein metabolism in isolated lymphocyte nuclei. Proceedings of the National Academy of Sciences 55:1182-1189.

_____ 1966b. Phosphorylation of nuclear proteins early in the course of gene activation in lymphocytes. Science 154: 780-781.

Kornguth, S. E. and L. G. Tomasi. 1968. Changes in the distribution of a histone in dorsal root ganglion neurons during development. Journal of Cell Biology 38:515-522.

Kossel, A. 1884. Ueber einen peptonartigen Bestandtheil des Zelkerns. Zeitschrift für Physiologische Chemie 8:511-515.

Kossel, A. and E. G. Schenk. 1928. Untersuchunger über die basischen Eiweiß stoffe, ein Beitrag zu ihrer Entwicklungsgeschichte. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 173:278-308.

Kruh, J. and D. Labie. 1968. Action d'histones de diverse origines sur la biosynthèse acellulaire de l'hémoglobin et sur les acides ribonucléiques de réticulocytes. Biochimica et Biophysica Acta 161:518-531.

Leboy, P., E. Cox and J. Flaks. 1964. The chromosomal site specifying a ribosomal protein in Escherichia coli. Proceedings of the National Academy of Sciences 52:1367-1374.

Levene, P. A. and L. W. Bass. 1931. Nucleic acids. New York, Chemical Catalog Company. 337 p.

Lilienfeld, L. 1893. Zur Chemie der Leucocyten. Zeitschrift für Physiologische Chemie 18:472-486.

Lindsay, D. T. 1966. Electrophoretically identical histones from ribosomes and chromosomes of chicken liver. Archives of Biochemistry and Biophysics 113:687-694.

Low, R. G. and I. G. Wool. 1967. Mammalian ribosomal protein: Analysis by electrophoresis on polyacrylamide gel. Science 155:330-332.

Luck, J. M., P. S. Rasmussen, K. Satake and A. N. Tsvetikov. 1958. Further studies on the fractionation of calf thymus histone. Journal of Biological Chemistry 233:1407-1414.

Maggio, R., P. Siekevitz and G. Palade. 1963. Studies on isolated nuclei. I. Isolation and chemical characterization of a nuclear fraction from guinea pig liver. Journal of Cell Biology 18:267-291.

Maio, J. and C. Schildkraut. 1967. Isolated mammalian metaphase chromosomes. I. General characteristics of nucleic acids and proteins. Journal of Molecular Biology 24:29-39.

Mauritzen, C. M., W. C. Starbuck, I. S. Saroja, C. W. Taylor and H. Busch. 1967. The fractionation of arginine-rich histones from fetal calf thymus by exclusion chromatography. Journal of Biological Chemistry 242:2240-2245.

Mauritzen, C. M. and E. Stedman. 1959. Cell specificity of β-histones in the domestic fowl. Proceedings of the Royal Society of London, ser. B, 150:299-311.

Miescher, F. 1897. Die histochemischen und physiologischen Arbeiten. Leipzig, Vogel. 229 p.

Mirsky, A. E., C. J. Burdick, E. H. Davidson and V. C. Littau. 1968. The role of lysine-rich histone in the maintenance of chromatin structure in metaphase chromosomes. Proceedings of the National Academy of Sciences 61:592-597.

Neelin, J. M. and G. Vidali. 1968. Ribosomal protein from goose reticulocytes are not histones. Canadian Journal of Biochemistry 46:1507-1514.

Otaka, E., T. Itoh and S. Osawa. 1967. Protein components in the 40s ribonucleoprotein particles in Escherichia coli. Science 157:1452-1454.

_____ 1968. Ribosomal proteins of bacterial cells: Strain- and species-specificity. Journal of Molecular Biology 33:93-107.

Phillips, D. M. P. 1963. The presence of acetyl groups in histones. Biochemical Journal 87:258-263.

Pogo, A. O., B. G. T. Pogo, V. C. Littau, V. G. Allfrey, A. E. Mirsky and M. G. Hamilton. 1962. The purification and properties of ribosomes from the thymus nucleus. Biochimica et Biophysica Acta 55:849-864.

Pogo, B. G. T., V. G. Allfrey and A. E. Mirsky. 1966. RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. Proceedings of the National Academy of Sciences 55:805-812.

Poulik, M. D. and W. Smithies. 1958. Comparison and combination of the starch gel and filter paper electrophoretic methods applied to human sera: Two-dimensional electrophoresis. Biochemical Journal 68:636-643.

Rasmussen, P. S., K. Murray and J. M. Luck. 1962. On the complexity of calf thymus histone. Biochemistry 1:79-89.

Reisfeld, R. A., V. J. Lewis and D. E. Williams. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281-283.

Sadowski, P. D. and J. A. Howden. 1968. Isolation of two distinct classes of polysomes from a nuclear fraction of rat liver. Journal of Cell Biology 37:163-181.

Samarina, O. P., E. M. Lukanidin, J. Molnar and G. P. Georgiev. 1968. Structural organization of nuclear complexes containing DNA-like RNA. Journal of Molecular Biology 33:251-263.

Satake, K., P. S. Rasmussen, J. M. Luck. 1960. Arginine peptides obtained from histone fractions after partial hydrolysis with Streptomyces griseus proteinase. Journal of Biological Chemistry 235:2801-2809.

Schmidt, J. and B. R. Reid. 1968. Fractionation of yeast ribosomal proteins. Biochemical and Biophysical Research Communications 31:654-659.

Sells, B. H. and F. C. Davis, Jr. 1968. Ribosome biogenesis: Nonrandon addition of structural proteins to 50S subunits. Science 159:1240-1242.

Setterfield, G., J. M. Neelin, E. M. Neelin and S. T. Bayley. 1960. Studies on basic proteins from ribosomes of buds of pea seedlings. Journal of Molecular Biology 2:416-424.

Shepherd, G. R. and L. R. Gurley. 1966. High-resolution disc electrophoresis of histones. I. An improved method. Analytical Biochemistry 14:356-363.

Shepherd, G. R. and B. J. Noland. 1965. A fluorescence assay suitable for histone solutions. Analytical Biochemistry 11:443-448.

_____ 1968. The intracellular distribution of basic proteins in the Chinese hamster ovary cell. Experimental Cell Research 49:238-250.

Spitnik-Elson, P. 1965. Preparation of ribosomal protein from Escherichia coli with lithium chloride and urea. Biochemical and Biophysical Research Communications 18:557-562.

Stedman, E. and E. Stedman. 1943. Probable function of histone as a regulator of mitosis. Nature 152:556-557.

_____ 1951. The basic proteins of cell nuclei. Philosophical Transactions of the Royal Society of London, ser. B, 235:565-596.

Stryer, L. 1968. Fluorescence spectroscopy of proteins. Science 162:526-533.

Takanami, M. 1960. A stable ribonucleoprotein for amino acid incorporation. Biochimica et Biophysica Acta 39:318-326.

Tanaka, K., H. Teraoka, M. Tamaki, E. Otaka and S. Osawa. 1968. Erythromycin-resistant mutant of Escherichia coli with altered ribosomal protein component. Science 162:576-578.

Tissières, A., J. D. Watson, D. Schlessinger and B. R. Hollingworth. 1959. Ribonucleoprotein particles from Escherichia coli. Journal of Molecular Biology 1:221-233.

Traub, P., K. Hosokawa, G. R. Craven and M. Nomura. 1967. Structure and function of E. coli ribosomes. IV. Isolation and characterization of funcationally active ribosomal proteins. Proceedings of the National Academy of Sciences 58:2430-2436.

Traub, P. and M. Nomura. 1968. Structure and function of E. coli ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins. Proceedings of the National Academy of Sciences 59:777-784.

Traut, R. R., P. B. Moore, H. Delius, H. Noller and A. Tissieres. 1967. Ribosomal proteins of Escherichia coli. I. Demonstration of different primary structures. Proceedings of the National Academy of Sciences 57:1294-1301.

Waller, J. P. 1964. Fractionation of the ribosomal protein from Escherichia coli. Journal of Molecular Biology 10:319-336.

Weiss, S. B. 1960. Enzymatic incorporation of ribonucleoside triphosphate into the interpolynucleotide linkages of ribonucleic acid. Proceedings of the National Academy of Sciences 46: 1020-1030.

Wettstein, R. O., T. Staehelin and H. Noll. 1963. Ribosomal aggregate engaged in protein synthesis: Characterization of the ergosome. Nature 197:430-435.

Wilson, S. H. and M. B. Hoagland. 1965. Studies on the physiology of rat liver polyribosomes: Quantitative and intracellular distribution of ribosomes. Proceedings of the National Academy of Sciences 54:600-607.