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NON-HISTONE NUCLEAR PROTEINS : PATTERNS OF SYNTHESIS AND PHOSPHORYLATION DURING THE CELL CYCLE, REDISTRIBUTION AT TIMES OF GENE ACTIVATION, AND PROPERTIES OF SUBSETS INTERACTING WITH HnRNA

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
by

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ABSTRACT

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CHAPTER ONE : INTRODUCTION

The interphase nucleus contains large numbers of non-histone proteins, the majority of which are associated with chromatin. The amount and number of these proteins varies according to cell type. Actively proliferating cells with high transcriptional activity contain considerably more non-histone nuclear proteins than do quiescent cells. In every cell type, the nuclear proteins are heterogeneous ; recent estimates based on two-dimensional gel electrophoresis place the total number of different HeLa cell chromosomal proteins at over 470 (Peterson and McConkey, 1976). Many of these proteins exhibit enzymatic activities associated with nuclear functions (such as DNA synthesis and repair, RNA synthesis, post-synthetic modifications of histones and nucleic acid, nuclear energy metabolism, chromosome movement, etc...). However, most of the nuclear proteins do not appear to be enzymes, and are therefore difficult to analyze or classify. In general, two groups of proteins may be distinguished: proteins that are structural elements of the nucleus, and proteins that bind to DNA and appear to influence the rate and specificity of RNA synthesis. (Even these two broad categories cannot account for a number of proteins that are transiently associated with chromatin, such as steriod receptors and considerable overlap between these categories undoubtedly exists.)

In this thesis, two different aspects of the non-histone nuclear proteins are examined. In the first part, the non-histone nuclear proteins are treated as a group, and aspects

of their metabolism are examined in synchronized HeLa cells, and in lymphocytes stimulated to proliferate by mitogens. These experiments lend support to the already substantial evidence that many of the nuclear proteins are intimately involved in the control of cell proliferation (Stein and Baserga, 1972; review). The second part describes the chemistry of nuclear RNA-binding proteins. These proteins are likely to be structural elements of chromatin active in transcription.

Isolation of Non-histone Nuclear Proteins

The non-histone chromosomal proteins are defined functionally: they are the proteins that remain associated with chromatin or nuclei after soluble proteins and histones have been extracted. The characteristics and yields of these proteins are critically dependent on the isolation methods employed, and many of the discrepancies in the literature may be attributed to the use of different isolation techniques by different laboratories.

The mandatory first step in the preparation of non-histone nuclear proteins is the isolation of morphologically intact nuclei. The methods generally used for the isolation of mammalian cell nuclei fall into two categories. The first uses aqueous homogenizing media and differential centrifugation (e.g., Dounce, 1955; Allfrey, 1959; Wang, 1967; Busch, 1967; Hogeboom et al., 1957; Chauveau et al., 1956). The second is the nonaqueous technique (Behrens, 1932; Allfrey, et al., 1952; Allfrey, 1959; Kirsch et al., 1970) which uses nonaqueous solvent mixtures of suitable specific gravity for homogenization of lyophilized tissue and separation of the nuclei from

cytoplasm according to differences in density. The aqueous procedures are most commonly used because of short preparation times, ease of manipulation, and absence of protein denaturation. The nonaqueous procedures provide useful checks for the preservation of nuclear components, since many proteins solubilized during the isolation of nuclei in aqueous media are retained in nuclear fractions prepared in nonaqueous solvents. (Georgiev, 1967; Allfrey, 1959). Kirsch et al. (1970) introduced the use of glycerol and 3-chloro-1,2-propanediol in the nonaqueous isolation of nuclei. Many nuclear enzyme activities, including RNA polymerase activity, are preserved when nuclei are prepared by this method. Methods employing ionic detergents (Penman, et al., 1969), pH extremes (Dounce, 1950), or organic solvents in aqueous media (Wray, 1975) often result in the extraction of many nuclear proteins found in nuclei purified by alternative procedures, and are therefore not recommended.

Several methods have been developed for fractionation of chromatin from isolated nuclei into histones and non-histone proteins. The development of methods for fractionation of nuclear proteins has been impeded since many of these proteins are insoluble in non-denaturing solvents and tend to aggregate with each other when dissociated from DNA. In fractionating nuclear proteins, one is forced to choose between mild procedures where separation and recoveries are poor, or methods where high recoveries and clean separations are obtained, but highly denaturing solvents are employed.

Most frequently, nuclear proteins have been fractionated

by their differential solubility in salt and dilute mineral acids. (Elgin, 1975; MacGillivray, et al., 1975; Wilson and Spelsberg, 1975). After removal of soluble components with isotonic saline, the nuclei are extracted in acid, usually 0.25 M HCl or 0.25 N H_2SO_4 . Treatment of chromatin with acid selectively solubilizes most of the histones. The remaining non-histone proteins are recovered as acid-insoluble precipitates which may be subsequently solubilized in SDS or phenol. The phenol procedure, originally introduced by Shelton and Allfrey (1970), for isolation of nuclear phosphoproteins, is a variation of the procedure of Viñuela et al. (1967) for the solubilization of phage proteins. This procedure is particularly suited for studies of nuclear phosphoproteins since contaminating nucleic acids are completely separated from the proteins. Nuclear residual proteins may also be solubilized in SDS after acid extraction (Elgin, 1975). The proteins in the SDS-soluble fraction are similar, but not identical to the proteins in the phenol-soluble fraction (Kleinsmith and Kish, 1975). SDS solubilization of residual proteins is convenient when proteins are to be analyzed by SDS-polyacrylamide gel electrophoresis. Nucleic acids are present in this fraction, but much of the DNA may be removed by centrifugation (Elgin, 1975) or by hydrolysis with DNase I (Wilson and Spelsberg, 1975).

Much recent effort has been devoted to developing procedures for fractionation of nuclear proteins solubilized in salt and urea. It is hoped that the high ionic strength utilized in the salt extraction techniques are not as deleterious to some protein properties as exposure to acid and organic solvents.

The histones are separated from the non-histone proteins and nucleic acids by chromatography either on cation exchangers such as SP-Sephadex (Elgin, 1975) or Bio-Rex 70 (Levy, et al., 1973) or chromatography on hydroxylapatite (MacGillivray, 1975). In other procedures, DNA is separated from chromosomal proteins after salt dissociation by prolonged high speed centrifugation (Hnilica, 1973) or by gel filtration (van den Broek et al., 1973).

Chromatography of proteins extracted in dilute salt (0.35 M NaCl to 0.6 M NaCl) on DNA-affinity columns is finding increasing use as a mild preparative procedure for nuclear proteins with DNA binding activity (Allfrey, et al., 1975). The method does not permit isolation of all the nuclear proteins; however, a limited set of nuclear proteins with biological activities may be obtained. Affinity columns using nucleohistone, or chromatin, will undoubtedly be developed for the fractionation of proteins that bind to chromatin by interacting with histones or other chromosomal proteins but do not bind directly to DNA.

Nuclear Fractionation

An alternative approach to fractionating nuclear proteins involves the initial separation of subnuclear structures. Nuclear fractionation has lagged considerably behind cell fractionation, partly because of the lack of good enzymatic markers (or other biochemical markers) and morphological criteria for subnuclear structures, and partly because of the fragility of subnuclear components compared with the relative stability of the intact nucleus.

The first nuclear component to be effectively purified

was the nucleolus, which was recognized as early as the 18th century because of its distinctive morphology (Busch, 1967). Most procedures for nucleolar isolation (and nuclear fractionation in general) are variations of the original technique of Maggio et al. (1963). Nuclei are disrupted by sonication in isotonic or hypotonic buffers, and nucleoli purified by differential centrifugation through barriers of 0.88 M sucrose. Modifications in this methodology, largely introduced in the laboratory of Harris Busch, have considerably improved the quality of nucleolar preparations. Although nucleoli can be obtained with well preserved morphologies, most of these preparations appear contaminated by extranucleolar DNA, when the DNA is analyzed by CsCl density separations of the GC -rich ribosomal DNA from chromosomal DNA. Only in a few organisms, such as Physarum polycephalum can nucleolar preparations with greater than 60% rDNA be obtained (Bradbury, et al. 1973). Useful techniques for isolation of nucleoli in which rRNA and its precursors are preserved were developed by Penman and his associates (Weinberg et al., 1967). These techniques involve lysis of nuclei in high salt, and subsequent DNase treatment. Unfortunately, these nucleoli do not have well preserved morphologies (Busch, 1967).

Chromatin is most satisfactorily isolated from post nucleolar supernatants of nuclear lysates or sonicated nuclei, by centrifugation through concentrated sucrose solutions (Bhorjee and Pederson, 1973; Tata and Baker, 1974). Chromatin isolated directly from whole tissues (Bonner et al., 1968) is subject to contamination with cytoplasmic proteins

(Johns and Forrester, 1969). Until recently, there were no good criteria for the preservation of chromatin structures during isolation procedures. Usually a preparation was considered satisfactory when the protein to DNA ratio did not exceed 4 : 1, the RNA to DNA ratio was low, gross contamination by ribosomal RNA eliminated, and the histone to DNA ratio about 1 : 1 (Bonner et al., 1968). The observation that intact nucleohistone structures give rise to DNA digestion products that are multiples of a unit length (due to the nucleosome structure of chromatin) provides an independent measure for histone rearrangments during chromatin isolation. It has already been observed that sonication of nuclei may disrupt the native nucleohistone structure (Nöll, 1975). Hopefully, chromatin digestion patterns will be routinely used to monitor chromatin preparations in the future.

Chromatin fractionation has often been attempted, but has usually met with limited success (Nasser and McCarthy, 1975). Although it was early recognized that chromatin can exist in the form of either euchromatin or heterochromatin, and that these fractions have different morphologies and template activities (Frenster, et al., 1963; Littau et al., 1964; Yasmineh and Yunis, 1969), effective separation of these components has not been achieved. Most workers have followed the procedures of Frenster et al. (1963) and relied on sonication followed by differential centrifugation to prepare initial fractions. (Frenster et al., 1963; Yasmineh and Yunis, 1969; Chalkley and Jensen, 1968; Duerksen and McCarthy, 1971). The last few years has seen the application of nonionic

compounds in equilibrium bouyant density separations of unfixed chromatin fractions (Rickwood, et al., 1973; Hossainy et al., 1973; Chan and Scheffler, 1974), as well as other fractionation procedures involving thermal elution from hydroxylapatite (McConaughy and McCarthy, 1972) differential precipitation of chromatin (Bonner et al., 1975) and separations of chromatin by ion-exchange chromatography (Reeck et al., 1970). Chromatin subfractions prepared in these procedures differ in template activity, presence of nascent chains of RNA, and hybridization characteristics of the DNA. However, there have been no demonstrations of complete separations of a specific gene (such as the globin gene from erythrocyte chromatin). A major problem appears to be the reliance on random shearing to obtain fragments sufficiently small (1 to 3 x 10^6 daltons, DNA) to be handled with the available techniques. Enzymatic procedures, especially involving the use of restriction enzymes to prepare chromatin fragments will probably be required in order to obtain satisfactory chromatin fragments under mild conditions.

Recent results, especially from the laboratory of Günter Blobel, have led to purification of nuclear pore complexes, and the recognition that the nuclear envelope consists of three structures : the double membrane system, pore complexes, and a peripheral lamina (Aaronson and Blobel, 1974). The nuclear pores may be isolated after solubilization of the outer nuclear membrane with detergents, and extensive nuclease digestion to remove chromatin and RNA.

Attention has also been directed towards the separation of nuclear ribonucleoprotein complexes from chromatin. The

various isolation procedures for these structures are considered in detail in chapter three.

Non-histone Nuclear Proteins and Transcriptional Control

The non-histone nuclear proteins have been intensively studied in recent years because of indications that these proteins interact with DNA to influence the rate and specificity of RNA synthesis. In vivo evidence that changes in transcriptional activity of cells are preceded by changes in the non-histone nuclear proteins, have been recently substantiate by in vitro evidence that proteins in this fraction can influence the transcription of chromatin by bacterial polymerases and permit selective transcription of specific messenger RNA sequences.

When interpreting the evidence for the involvement of non-histone proteins in the regulation of transcription, two cautionary points should be considered. Proteins that directly interact with DNA sequences and influence transcription in a manner analogous to the transcriptional regulators known in prokaryotes, such as lac repressor, are likely to be present in only a few hundred copies per eukaryotic genome, and therefore have probably escaped detection by most of the techniqu employed by workers in this field. Secondly, there has been no direct demonstration that an isolated protein can bind to specific DNA sequences and thereby permit selective synthesis of a particular messenger RNA. Nonetheless, activation of genomes during development, cell transformation, and cell response to a wide variety of stimuli, are accompanied by major morphological and functional changes in the nucleus, and corresponding changes in the distribution and amount of nuclear

proteins. Many of these changes in chromatin preceding gene activation may be attributed to reorganization of nuclear structures to allow for transcription, rather than primary control events, yet it seems inconceivable that these events are not closely regulated, and that they in turn do not have regulatory influences on RNA transcription.

Much of this evidence has been recently reviewed (Allfrey, 1974; Stein et al., 1974; Spelsberg et al., 1972; MacGillivray et al., 1972; Stein and Baserga, 1972; Stein and Kleinsmith, 1975; Smith and DeLange, 1975; Elgin and Weintraub, 1975) and the current state of the field is summarized briefly below:

1) There are strong correlations between the non-histone protein content of chromatin and the RNA-synthetic capacity of the tissue (Dingman and Sporn, 1964; Bonner et al., 1968; Gershey and Kleinsmith, 1969; Ruiz-Carrillo et al., 1974). Tissues that are low in transcriptional activity such as mature avian erythrocytes (Vidali et al., 1973; Ruiz-Carrillo et al., 1974) or calf thymus (Allfrey et al., 1952) have low levels of non-histone nuclear proteins, while tissues active in transcription, such as rat liver, have relatively high levels of non-histone nuclear proteins (Teng et al., 1970).

2) The non-histone nuclear proteins are preferentially localized in the euchromatic RNA synthesizing regions of the chromatin (Frenster et al., 1963; Frenster, 1965; Dolbeare and Koenig, 1970; Marushige and Bonner, 1971; Reeck et al., 1972; Warnecke et al., 1973; Bonner et al., 1975), although some of them are present in higher amounts in heterochromatin (Comings and Harris, 1975; Bonner et al., 1975).

3) The distribution of non-histone proteins varies in different somatic tissues of the same organism (Teng et al., 1971; Wang, 1971; Richter and Sekeris, 1972; Bekhor et al., 1974; Wilson and Spelsberg, 1973; MacGillivray and Rickwood, 1974; Tashiro, et al., 1974). While many of these proteins are generally present in a variety of nuclear types (Elgin and Bonner, 1970), the best evidence, based on immunological tests for specificity, supports the view that real differences exist between the nuclear complements of non-histone proteins in different cell types of the same animal (Chytil and Spelsberg, 1971; Wakabayashi et al., 1974; Zardi et al., 1973).

4) The nature and amount of the non-histone nuclear proteins changes during embryogenesis and early development (Marushige and Ozaki, 1967; Hill et al., 1971; Seale and Aaronson, 1973; Cognetti et al., 1972; Teng, 1974; Elgin and Hood, 1973).

5) The complement of nuclear acidic proteins is dramatically altered during differentiation of particular cell types (Vidali et al., 1973; Ruiz-Carrillo, et al., 1974; LeStourgeon and Rusch, 1971; LeStourgeon and Rusch, 1973; Spelsberg et. al., 1973; Hemminki and Bolund, 1975; Platz et al., 1975). For example, maturation of avian erythrocytes is accompanied by progressive loss of nuclear proteins, and a simplification of the non-histone nuclear protein complement (Vidali et al., 1973). In contrast, differentiation of the slime mold Physarum polycephalum is accompanied by specific increases and decreases of proteins in the nucleus (LeStourgeon and Rusch, 1971, 1974).

6) Increased synthesis of nuclear acidic proteins is observed early after stimulation of salivary glands to proliferate by isoproterenol (Stein and Baserga, 1971) and in lymphocytes

stimulated by phytohemagglutinin (Levy et al., 1973). When resting WI-38 fibroblasts are stimulated to proliferate by changing the medium, the incorporation of amino acids into the nuclear non-histone proteins is promptly increased (Rovera and Baserga, 1971).

7) The activated puffing regions of Dipteran chromosomes accumulate specific non-histone proteins after treatment with ecdysone or other stimuli (Helmsing and Berendes, 1971; Gorovsky and Woodard, 1967; Berendes, 1968; Holt, 1971). Recent immunoflourescent evidence points to site specific localization of specific nuclear proteins in Drosophila polytene chromosomes (Elgin, 1976).

8) Changes in the metabolism of nuclear acidic proteins occur in target tissues during stimulation by hormones (Shelton and Allfrey, 1970; Teng and Hamilton, 1970; Swanek et al., 1970; Buck and Schauder, 1970; Enea and Allfrey, 1973; Helmsing and Berendes, 1971; Jungmann and Schweppe, 1972; Cohen and Hamilton, 1975) and by drugs (Stein and Baserga, 1971; Ruddon and Rainey, 1970).

9) Changes in the synthesis and distribution of nuclear non-histone proteins occur in early stages of carcinogenesis (Boffa et al., 1970; Gronow and Thackrah, 1974a and b), and differences exist between normal and tumour cells in this regard (Arnold et al., 1973; Chae et al., 1974; Chiu et al., 1974; Baserga, 1974; Kadohama and Turkington, 1973; Orrick et al., 1973; Stein et al., 1974; Yeoman et al., 1974; Reeck and Morris, 1974; Lea et al., 1975; Wilson et al., 1975; Ballal and Busch, 1973; Yeoman et al., 1973; Fujitani and

Holoubek, 1975; Biessmann and Rajewsky, 1975; Bresnick, 1970; Chiu et al., 1974; Augenlicht, et al. 1975). "Transformation" of WI 38 fibroblasts by SV-40 vrius results in an immunologically detectable alteration in the non-histone proteins (Zardi et al., 1973).

10) Some of the nuclear non-histone proteins interact with DNA in a highly selective manner. The specificity of the DNA-binding has been shown by centrifugational methods (Teng, et al., 1970; Patel and Thomas, 1973), affinity chromatography of nuclear proteins on DNA columns (Kleinsmith et al., 1970; Kleinsmith, 1973; van den Broek et al., 1973; Allfrey et al., 1973, 1975, 1976), and filter binding assays (Johnson et al., 1975; Sevall et al., 1975; Patel and Thomas, 1975).

11) Activation of chicken erythrocyte nuclei in heterokaryons with HeLa cells is associated with progressive enlargement of the erythrocyte nucleus and accumulation of HeLa cell specific nuclear proteins (Ringertz, et al., 1971; Carlsson et al., 1973; Appels et al., 1974, 1975; Goto and Ringertz, 1974). This is not simply due to random leakage through the nuclear envelope because a variety of marker proteins (such as lactate, isocitrate and malate dehydrogenases) failed to accumulate in the chick nucleus (Appels et al., 1975).

12) Nuclear non-histone fractions include components of polypeptide nature that alter the rate of transcription and influence the nature of the transcript in reconstituted chromatin fractions (Paul and Gilmour, 1968; Gilmour and Paul, 1969, 1970; Teng and Hamilton, 1969; Teng et al., 1970, 1971; Kamiyama and Wang, 1971; Kostraba and Wang, 1972; Stein et al., 1972;

Kamiyama et al., 1972; Shea and Kleinsmith, 1973; Rickwood et al., 1972; Spelsberg et al., 1971; Kostraba et al., 1975). It has generally been observed that the source of the histones does not modify the nature of the RNA synthesized in reconstituted chromatin (Gilmour and Paul, 1970; Stein et al., 1970; Spelsberg et al., 1971), while varying the origin of the non-histone protein in such chromatin leads to altered patterns of transcription. Several laboratories have demonstrated that the in vitro transcription of globin genes requires the presence of non-histone proteins from erythroid cell chromatin (Axel et al., 1973; Barrett et al., 1974; Steggles et al., 1971; Gilmour et al., 1975; Chiu et al., 1975; Young et al., 1974) or from hemopoietic tissues such as embryonic liver cells (Paul et al., 1974). Similar evidence for transcriptional control of histone genes by chromosomal proteins from S-phase HeLa cells has recently been reported (Stein et al., 1975).

These experiments are subject to a number of criticisms: 1) RNA polymerase of bacterial orgin is employed and these enzymes have been repeatedly shown to transcribe chromatin in an aberrant manner (Reeder, 1973; Wilson, et al., 1975a and b). 2) Chromatin is usually reconstituted from high salt-urea extracts of nuclei by stepwise dialysis. It has not been demonstrated that in the particular preparations used for transcription experiments that either the nucleohistone structure or specific enzyme marker activities are restored. 3) While the experiments demonstrate increases in the level of synthesis of sequences hybridizable to cDNA probes, it is not clear how much these increases reflect general increases in transcriptio

A more satisfactory experiment would be the demonstration of an increase in the rate of synthesis of one set of sequences and the simultaneous decrease in the rate of synthesis of another set of sequences. One recent report describes a chromosomal protein fraction which represses RNA initiation *in vitro* (Kostraba and Wang, 1975). 4) The chromatin transcripts are usually small, and the level of transcription of a specific sequences may therefore be more a reflection of the accessibility of that region of the chromatin than a measure of the selective activation of transcription of a particular genetic locus. Still, these experiments provide convincing evidence that proteinsfrom the non-histone fraction are obligatorily required for specific *in vitro* transcription of chromatin.

Phosphorylation of Non-histone Nuclear Proteins

Many of the non-histone nuclear proteins are phosphoproteins (Teng *et al.*, 1970; LeStourgeon and Rusch, 1973; Jungmann and Schweppe, 1972; Kleinsmith *et al.*, 1966; Benjamin and Gelhorn, 1968; Langan, 1967; Johnson and Allfrey, 1972; Platz and Hnilica, 1973; Rickwood *et al.*, 1973; Olson *et al.*, 1974; Prestayko *et al.*, 1974; Davidson *et al.*, 1951). The fact that some nuclear proteins are phosphorylated *in vivo* was first reported by J. N. Davidson and coworkers (1951) and by Johnson and Albert in 1953. After a long lapse of interest, the ATP - dependence of the reaction, the identification of phosphoserine and phosphothreonine in the modified proteins, and the nuclear localization of the substrates were firmly established (Kleinsmith *et al.*, 1966; Langan, 1967; Kleinsmith

and Allfrey, 1969).

A large number of correlations have been noted between the phosphorylation of nuclear proteins and gene activation. For example : Nuclear protein phosphorylation is an early event in embryonic development (Platz et al., 1973). Phosphorylation is increased at very early stages of gene activation when lymphocytes are stimulated by phytohemagglutinin (Kleinsmith et al., 1966). The phosphorylation of certain nuclear proteins is selectively augmented in target tissues responding to hormones such as cortisol and corticosterone (Allfrey et al., 1971; Bottoms and Jungmann, 1973), estradiol (Turkington and Riddle, 1969), testosterone (Ahmed, 1971; Ahmed and Ishida, 1971; Schauder et al., 1974), aldosterone (Liew et al., 1973) and gonadotropins (Jungmann and Schweppe, 1972). Cyclic AMP selectively enhances the phosphorylation of some, but not all nuclear phosphoproteins (Johnson and Allfrey, 1972; Kish and Kleinsmith, 1974; Kleinsmith, 1975). Phosphorylation of non-histone nuclear proteins varies during liver regeneration (Ballal et al., 1975). Protein kinase activity is enriched in the transcriptionally active fraction of estrogen stimulated oviduct chromatin (Keller et al., 1975) and protein phosphorylation is most pronounced in the RNA synthesizing "puffs" of Dipteran chromosomes (Benjamin and Goodman, 1969).

Phosphorylation of nuclear proteins has been observed to alter the properties of these proteins in vitro. Certain nuclear phosphproteins bind preferentially to DNA of the species of origin (Teng et al., 1971; Kleinsmith et al., 1970;

Kleinsmith, 1973). Enzymatic phosphorylation of the proteins in isolated chromatin fractions increase the template activity of the chromatin in RNA synthesis (Kamiyama et al. , 1971; Martelo and Hirsch, 1974), while dephosphorylation of liver nuclear phosphoproteins destroys their stimulatory effects on transcription from free DNA (Shea and Kleinsmith, 1973). Enzymatic dephosphorylation of the nuclear non-histone proteins diminishes their capacity to stimulate the synthesis of histone messenger RNAs in reconstituted chromatin (Kleinsmith et al., 1975).

These observations, taken together, lend support to earlier proposals that the post-synthetic modification of nuclear proteins by phosphorylation/dephosphorylation reactions may determine whether or not they can function (Allfrey et al., 1971, 1973). The choice of mechanisms is broad since the nuclear phosphoproteins are known to interact both with DNA (Teng et al., Kleinsmith et al., 1970; Kleinsmith, 1973) and with histones (Wang and Johns, 1968; Spelsberg and Hnilica, 1969). In any case, the importance of phosphorylation mechanisms in the nucleus is suggested by the great number of proteins modified in this way, and by the multiplicity of nuclear protein kinases (Kish and Kleinsmith, 1974; Desjardins et al., 1972; Takeda et al. , 1971; Ruddon and Anderson, 1972; Kemp et al., 1975; Rikans and Ruddon, 1973; Wilson and Ahmed, 1975; Walsh and Ashby, 1973). Additional controls are provided by the dependancy of some of the nuclear protein kinases on cyclic AMP (Johnson et al., 1975; Johnson and Allfrey, 1972: Kish and Kleinsmith, 1974) and on cyclic GMP (Johnson and Haddon,

1975), resulting in differential phosphorylation of their substrates.

CHAPTER TWO : NUCLEAR ACIDIC PROTEINS DURING THE CELL CYCLE

The experiments described in this chapter deal with changes in the metabolic activities of nuclear phosphoproteins during the replication cycle of HeLa cells and in lymphocytes stimulated to proliferate by mitogens. The question arises whether the nuclear phosphoproteins, which are major structural and functional elements of chromatin and nuclei, are altered at specific times during synchronous growth, and the induction of growth. Changes in the amount and phosphorylation of these nuclear proteins may represent important biochemical events in the replication of cells and coordination of transcriptional activity.

A number of observations have been made of synthesis and phosphorylation of nuclear acidic proteins in cultured cells. (Stein and Baserga, 1972; review). The synthesis and accumulation of acidic nuclear proteins proceeds throughout the cell cycle of continously dividing population of HeLa S-3 cells (Stein and Baserga, 1970). In synchronously dividing cell populations, there is an increased rate of synthesis and accumulation of these proteins which precedes the onset of DNA syntheis (Stein and Borun, 1972). The amount of nuclear protein synthesized transported, and retained in the acidic chromosomal protein fraction is greater immediately after mitosis and later in G_1 than in the S or G_2 phases of the cell cycle (Borun and Stein, 1972). Many of the newly synthesized non-histone proteins of rapidly dividing cells are metabolically stable (Seale, 1975; Tsanov *et al.*, 1974), whereas others "turn over" at rates which

depend on cell cycle position and conditions of growth. Synthesis of nuclear acidic proteins has been observed early in the induction of proliferation of WI-38 fibroblasts (Rovera and Baserga, 1971) as well as of lymphocytes (Levy et al., 1973). Preliminary studies of phosphorylation of HeLa nuclear proteins during the cell cycle indicate that the rate of phosphorylation is maximal in the early S phase and decreases in the late S and G_2 phases (Allfrey et al., 1973). The suppression of nuclear acidic protein phosphorylation during M has been reported by Platz et al. (1973).

The present chapter emphasizes several aspects of the metabolism of nuclear proteins in cultured cells. The non-histone nuclear proteins accumulate in the nucleus of cells in the early pre-replicative phase of the cell cycle both in synchronously dividing cells and in lymphocytes stimulated to proliferate by the mitogen, concanavalin A. Although the amount of these proteins in the nucleus increases multifold, there are no major qualitative differences in the molecular weight distribution of accumulated proteins as judged by uni-directional SDS-polyacrylamide gel electrophoresis. Many of these proteins are phosphoproteins, and in synchronously dividing cells, rates of phosphate uptake into most major phosphoprotein species are increased during the early G_1 and early S phases and are minimal during the late G_2 to M period. Similarly, accumulation of protein in nuclei of stimulated lymphocytes is accompanied by an increase in the rate of phosphorylation of the nuclear proteins. The effects of a variety of inhibitors of protein synthesis on the synthesis of

non-histone nuclear proteins as compared with proteins derived from the cytoplasm are also described. Clear distinctions can be made between histones, non-histone nuclear proteins and cytoplasmic proteins on the basis of differential sensitivities to these drugs. The behavior of the non-histone nuclear proteins in these cells is consistent with the model that the abundant proteins in this class are involved in the maintenance and replication of nuclear structures. Increases in the amount and phosphorylation of non-histone nuclear proteins correlate well with the induction of transcription in these, as well as numerous other systems (see Chapter One for review).

MATERIALS AND METHODS

Culture and Synchronization of HeLa S-3 Cells - HeLa S-3 cells were maintained in suspension culture at 2 to 6 x 10^5 cells per ml by daily dilution with fresh Joklik-modified minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum supplemented with 2.5 units per ml of penicillin G, 2.5 µg per ml of streptomycin, and 20 units per ml of mycostatin, or in Joklik-modified minimal essential medium supplemented with 5% fetal calf serum. Cells were gifts of Dr. Lawrence A. Caliguiri and Dr. Warren Jelinek of the Rockefeller University.

Synchronization was obtained by the double thymidine block method (Bootsma et al., 1964; Puck, 1964). Cells were exposed to 2 mM thymidine for 14 hours, to normal medium for the

following 9 hours, and to 2 mM thymidine for an additional 14 hours. The cells were harvested by centrifugation at 1,500 x g for 4 minutes and resuspended in one-fifth the original volume of thymidine-free medium. The time of release from the thymidine block is taken as starting from this resuspension. The cells were then centrifuged and resuspended in the original volume of thymidine-free medium. The cell cycle was monitored by measurements of cell concentration, mitotic index, and [^{3}H]-thymidine incorporation rate. The cell number was measured at different times in the cell cycle using a Coulter counter. For determination of the mitotic index, the cells were fixed in 3 : 1 ethanol-acetic acid and stained with 1% crystal violet in water. The number of cells showing condensed chromosomes was scored in at least 500 cells at each time point. Rates of thymidine incorporation into DNA were determined by incubating 1 ml aliquots of the cell suspension in the presence of 5 μCi [^{3}H]-thymidine of specific activity 20 Ci/ m mole (New England Nuclear, Inc., Boston, Mass.) for 30 minutes at 37 ^{o}C. Three ml of cold medium were added at the end of the incubation period and the cells were collected by centrifugation at 2,000 x g for 3 minutes. The cells were resuspended in 3 ml of 5% TCA and transferred to a Millipore filter (pore size 0.45 μ). The incubation tube was rinsed twice with 3 ml aliquots of 5% TCA and the washings passed through the filter. The filter was finally washed with 10 ml of 70% ethanol (v/v) and dried at 60 ^{o}C for 30 minutes. The dried filters were submerged in 20 ml Bray's scintillation fluid (Bray, 1960) and radioactivity monitored using a Packard Tri-

Carb model 3375 scintillation spectrometer.

Culture of Equine Lymphocytes - Lymphocytes were prepared under sterile conditions from peripheral blood of adult male horses by centrifugation through a layer of Ficoll-Isopaque. One liter of heparinized blood was allowed to settle at 37 ^{o}C for 1 hour. Heparin (Connaught, Toronto, Canada) was used at 10,000 units per liter. The upper plasma layer was then decanted and 35 ml aliquots were layered over 15 ml of a sterile solution consisting of 10 % Ficoll (Pharmacia, Uppsala Sweden; Ficoll was extensively dialyzed against deionized water prior to use) and 6.4% Isopaque (sodium metrizoate; Nyegaard and Sons, Oslo) adjusted to specific gravity 1.078 to 1.080 with water. This preparation was centrifuged at 850 x g for 20 minutes at room temperature, and the discrete band of lymphocytes suspended in the Ficoll-Isopaque layer was collected and washed to remove contaminating platelets. The lymphocyte suspension was diluted with 4 volumes of sterile isotonic 0.9% NaCl solution, and centrifuged for 10 minutes at 400 x g. The pellet was resuspended in sterile isotonic saline and centrifuged at 100 x g for 10 minutes. Resuspension of the lymphocyte pellets in sterile isotonic saline and centrifugation at 100 x g was repeated twice, after which the purified lymphocytes were cultured in Eagle's minimal essential medium with Earle's salts (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum, penicillin (2.5 units per ml), streptomycin (2.5 µg per ml), and mycostatin (20 units per ml) at a concentration of 2.5 x 10^{6} lymphocytes per ml. Cells prepared as described

were 98-99 % small lymphocytes, the remaining white cells consisting primarily of monocytes. Platelet contamination was small. Platelets could be eliminated by repeated washings which also reduced the yield of lymphocytes. After 20 hours in culture at 37 ^{o}C, more than 95% of the lymphocytes were viable as measured by exclusion of trypan blue dye. Lymphocytes were cultured for 20 hours prior to use in order to minimize traumatic effects of the isolation procedure. The lymphocyte culture was stirred slowly during this time. Lymphocytes were incubated with Con A and radioisotopes as described below. Lymphocytes were not stirred during the period of Con A treatment.

Culture of Human Lymphocytes - Human lymphocytes were prepared from fresh whole blood by isopycnic banding in Lymphoprep (Nyegaard and Sons, Oslo). Sixty ml of blood from male donors were drawn into sterile heparinized tubes (Vacutainer, BD Scientific Company, N. Y.), diluted 1 : 1 with sterile saline containing heparin (10,000 units/liter, Connaught laboratories). The donors had fasted 20 hours prior to the drawing of blood. Eight ml aliquots of the diluted blood were layered over 4 ml of Lymphoprep in 16 x 125 mm sterile culture tubes. The gradients were spun at 1,500 rpm for 40 minutes in a GLC 2 desk top centrifuge (Sorvall) at room temperature. The discrete band of lymphocytes was washed 3 times in MEM without serum by pelleting at 400 x g for 10 minutes and resuspending the cells in fresh medium. Aliquots of 2.5 x 10^{6} cells were then cultured in 1 ml aliquots in MEM supplemented with 10% fetal calf serum, in sterile 13 x 75 mm tubes for determination

of the dose response curve to Con A. In the turnover experiments, cells were cultured at 2.5 to 4 x 10^6 cells per ml in 75 cm^2 "T" flasks (Falcon plastics, New York). The lymphocytes were maintained in culture at least 24 hours prior to use, to minimize the traumatic effects of the isolation procedure.

Phosphorylation of HeLa Cell Nuclear Proteins - At different times after removal of the thymidine block, 4 to 8 x 10^7 cells were harvested by centrifugation at 1,500 x g for 4 minutes and gently resuspended in 20 ml of culture medium containing 2 mCi of carrier-free [^{32}P]-orthophosphate (New England Nuclear, Inc., Boston, Mass.). After 15 minutes incubation at 37 ^{o}C, the cells were centrifuged as before and the cell pellet was frozen by immersing the tube in acetone at -70 ^{o}C. The cells were stored at -80 ^{o}C. Nuclei were isolated within the next 24 hours.

Estimates of turnover in the nuclear phosphoprotein fraction were based on measurements of isotope retention in the nuclear proteins of cells which had been prelabeled with [^{32}P]-orthophosphate and [^{14}C]-leucine. In studies of ^{32}P and ^{14}C turnover in synchronously-dividing cells, 1 liter of cell suspension containing 4 to 6 x 10^5 cells per ml was taken after the first thymidine block and exposed to 25 mCi of [^{32}P]-orthophosphate and 0.50 mCi of L-[U-^{14}C]-leucine of specific activity 316 mCi per m mole (Schwarz-Mann, Inc., Orangeburg, N. Y.) for 9 hours in the thymidine-free medium, and resuspended in 1 liter of thymidine-free medium for the cold chase experiments. At the indicated times after removal of the second thymidine block, 100 ml aliquots of the suspension

were centrifuged at 1,500 x g for 4 minutes. The cell pellets were frozen and stored at -80 °C prior to the isolation of nuclei. For turnover studies in non-synchronized cultures, 1 liter of cells were incubated in the presence of 25 mCi of [^{32}P]-orthophosphate and 170 μCi of [^{14}C]-leucine for 23 hours. The cells were washed in isotope-free medium and resuspended in 1 liter fresh medium for cold chase experiments.

Phosphorylation of Lymphocyte Nuclear Proteins - Equine lymphocytes were exposed to [^{32}P]-orthophosphate for various times at 37 °C under the following conditions: Cells subjected to experimental manipulations were harvested by centrifugation and resuspended in 10 ml of phosphate free Eagle's MEM with Earle's salts and 10% fetal calf serum at a concentration of 1 to 5 x 10^7 cells per ml. Con A (25 μg per ml) was included during phosphorylation in those samples in which the mitogen was being tested. The reaction was begun by adding 2 mCi of carrier-free NaH_2[$^{32}PO_4$] to each 10 ml sample. In order to stop the phosphorylation reaction, cells were centrifuged for 5 minutes at 1,500 x g at room temperature and quick-frozen with acetone at -70 °C. Nuclear proteins were then extracted and analyzed as described.

Turnover Measurements of Lymphocyte Nuclear Proteins - Equine lymphocytes (2.5 x 10^6 per ml) were cultured as described in the presence of 0.60 μCi L-[U-^{14}C]-leucine (270 mCi per m mole, New England Nuclear). After 24 hours in culture, the cells were centrifuged at 100 x g for 5 minutes and resuspended in 20 volumes of unlabeled Eagle's MEM with Earle's salts and 10% fetal calf serum in the presence or absence of Con A

(25 μg/ml). Protein turnover was calculated from the time at which the cells were exposed to Con A, about 20 minutes after removal of the cells from the radioactive medium. At various times after removal from the radioactive medium, lymphocytes were collected by centrifugation at 1000 x g for 5 minutes, and quick-frozen with acetone at -70 °C. Nuclear proteins were then extracted and analyzed as described.

Turnover measurements in human lymphocytes followed essentially the same procedure as for equine lymphocytes. Cells were pre-labeled for 24 hours in the presence of 10 μCi per ml [^{3}H]-leucine (50 Ci/m mole, New England Nuclear) in leucine-free MEM supplemented with 2.5 units/ml penicillin, 2.5 μg/ml streptomycin, and 10% fetal calf serum. The cells were washed three times in complete MEM supplemented with 10% fetal calf serum and aliquots of 7.5 x 10^6 cells were placed in 3 ml cultures in 13 x 75 mm tubes. Con A dissolved in MEM (100 μg/ml) was added to the cultures to a final concentration of 10 μg per ml. At various times after addition of the drug, Con A-treated and control cultures were collected by centrifugation at 400 x g for 5 minutes and quick-frozen in ethanol at -70 °C. Three parallel cultures were taken for each time point. The nuclear proteins were extracted and analyzed as described.

Measurements of the Synthesis of Lymphocyte Nuclear Proteins - Aliquots of 10^7 equine lymphocytes were incubated for various times at 37 °C at 2.5 x 10^6 cells per ml in Eagle's MEM with Earle's salts and 10% fetal calf serum in the presence or absence of Con A (25 μg/ml) and cycloheximide (Sigma, St. Louis

Mo., 10 μg/ ml) and in the presence of 150 μCi of L-[4,5 - ^{3}H (n)]-leucine (30-50 Ci per m mole; New England Nuclear, Boston, Mass.) At various times after addition of Con A, cycloheximide, and labeled leucine, cells were collected by centrifugation at 1000 x g for 5 minutes and quick-frozen with acetone at -70 °C. Nuclear proteins were then extracted and analyzed as described.

Effects of Inhibitors of Protein Synthesis on HeLa Cell Nuclear Protein Synthesis - HeLa S-3 cells were harvested by centrifugation at 1000 x g for 5 minutes, washed once with Earle's balanced saline, and resuspended at a concentration of 2 x 10^{6} cells per ml in Earle's solution. The cells were incubated for various times at 37 °C in the presence of isotopic amino acids and in the presence and absence of protein synthesis inhibitors. Unless otherwise indicated, the cell suspensions contained 10 μCi/ml of one of the following tritiated precursors : L-[4,5-^{3}H]-leucine, specific activity 50 Ci/m mole; L-[methyl-^{3}H]-methionine, specific activity 11.0 Ci/m mole; L-[^{3}H (U)]-amino acid mixture or 2.5 μCi/ml of L-[^{14}C (U)]-amino acid mixture. The inhibitors tested were L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK; Sigma Chemical Co., St. Louis, Mo.), pactamycin, puromycin, and cycloheximide (Sigma). The time course of amino acid incorporation was determined on replicate cell suspensions, each containing 2 x 10^{7} cells, incubated for periods ranging from 5 to 20 minutes. The incubations were stopped by rapid chilling of the cell suspensions on an ice bath, and centrifugation at 2000 x g for 2 minutes. The cell pellets were immediately

frozen in a bath of dry ice-acetone.

TPCK dissolved in ethanol at a final concentration of 10 mg/ml was added to cell suspensions to obtain a final concentration of 25 μg per ml. An equal volume of ethanol was added to control cultures. In some experiments, TPCK was added to control cultures at the end of the incubation period as a control to ensure that the effects we observed were not the result of nonspecific proteolysis during the preparation of protein samples in cells that were not exposed to the drug. Cycloheximide and puromycin were stored in neutralized 10 mM stock solutions in water. Pactamycin was stored in 0.4 mM stock solutions in 50% ethanol. Serial dilutions of the stock solutions were made and 50 μl aliquots of each were added to cell suspensions to obtain the final concentrations listed in Table X.

Thymidine Incorporation by Cultured Lymphocytes - The dose response curve of cultured lymphocytes ot Con A was measured as the incorporation of [^{3}H]-thymidine into DNA 24 to 36 hours after exposure of cultures to the mitogen. Twenty-four hours after isolating the cells, Con A was added to a final concentration of 2.5, 5, 10, 15, 25, 30, 50 or 100 μg per ml to one ml cultures containing 2.5 x 10^6 cells. Twenty-four hours after addition of the drug, 5 μCi of [^{3}H]-thymidine (20 Ci/ m mole, New England Nuclear) was added to each culture and the cells were incubated for an additional 12 hours. The cells were collected by centrifugation at 1000 x g for 5 minutes, washed three times in Earle's balanced salt solution and lysed by adding 2 ml of 10% TCA. The cell lysate was collected on

Whatman GF/A glass fiber filters, washed with 10 ml TCA and 10 ml ethanol. The filters were dried and immersed in 0.5ml 4M NH_4OH-Protosol (1: 12 v/v) for 3 hours at 25 ^oC. Fifteen ml of Liquiflour in toluene (New England Nuclear) were added and the samples counted.

Isolation of HeLa Cell Nuclei and Chromatin - HeLa cell nuclei and chromatin (from mitotic cells) were isolated by a modification of the method of Hancock (1969). Four to 8 x 10^7 cells were suspended in 5 ml of 80 mM NaCl - 20 mM EDTA containing 1% (v/v) Triton X-100, pH 7.2, and homogenized with 20 strokes in a Dounce-type glass homogenizer using a pestle of 0.0025 - 0.0075 mm clearance (Kontes, Inc. Vineland, N. J.). The homogenate was centrifuged at 2,000 x g for 5 minutes to pellet nuclei and intact cells, and the pellet resuspended and re-homogenized as before. After centrifugation at 2,000 x g for 5 minutes, the pellet was again homogenized and centrifuged to sediment intact nuclei. At this point the nuclei were free of cytoplasmic or whole cell contamination as judged by phase contrast microscopy. The protein to DNA ratio of the final nuclear pellet was about 2.8 to 1. In the experiments testing the effects of inhibitors of protein synthesis on the incorporation of label into nuclear proteins, the nuclear fraction was further purified by sedimentation throught 2.2 M sucrose - 5 mM $MgCl_2$ at 25,000 rpm for 60 minutes using an SW 41 rotor (Beckman Instruments).

Preparation of Lymphocyte Nuclei - Nuclei were prepared from equine and human lymphocytes essentially as described by Levy et al. (1973). Pellets of 5 x 10^7 lymphocytes suspended

in 5 ml of ice cold 0.32 M sucrose containing 3 mM $CaCl_2$, 0.5% Triton X-100, and 0.01 M Tris-HCl, pH 8.0, were homogenized by 20 strokes of a Dounce-type homogenizer and centrifuged at 900 x g for 10 minutes. The homogenization and centrifugation were repeated once, and the nuclear pellet was suspended and washed once more with the sucrose-$CaCl_2$ - Triton buffer. Nuclei prepared as described were free of cytoplasmic or whole cell contamination as judged by phase-contrast microscopy.

For some experiments nuclei from equine lymphocytes were prepared in nonaqueous media according to the method of Kirsch et al. (1970). Lymphocytes, quick frozen with chilled Freon -12 at -156 ^{o}C and lyophilized to complete dryness, were suspended in 100% glycerol at 2 ^{o}C (8 ml of glycerol per 10^7 cells) and sonicated for 30 seconds as maximum intensity with the medium tip of a Bronwell sonifier. The sonicated suspension was layered over an equal volume of 85% glycerol-15% 3-chloro-1,2-propanediol and centrifuged at 120,000 x g for 40 minutes. The pellet thus obtained, washed once with 100% glycerol, contained nuclei free of cytoplasmic or whole cell contamination. Nuclei prepared by nonaqueous means retain many soluble components lost during homogenization in aqueous media (Georgiev, 1967).

Isolation of Non-histone Nuclear Proteins - The nuclear phosphoprotein fractions from HeLa cells and lymphocytes were prepared from isolated nuclei by the method of Shelton and Allfrey (1970) as modified by Teng et al. (1971). The nuclei were extracted twice with 0.14 M NaCl and twice with 0.25 N HCl. The residue was washed once with 1 : 1 chloroform-methanol

containing 0.2 N HCl and once with 2 : 1 chloroform - methanol containing 0.2 N HCl. The residue was then resuspended in 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol. The suspension was mixed gently with an equal volume of phenol (saturated with buffer) and allowed to stand for 14 hours at 2 ^{o}C. The mixture was homogenized briefly and centrifuged at 12,000 x g for 10 minutes. The aqueous phase was collected, and re-extracted with an equal volume of phenol, and centrifuged as before. The combined phenol extracts containing the nuclear phosphoproteins were dialyzed against a series of urea-containing buffers as described by Teng et al. (1971) to restore the proteins to the aqueous phase.

Alternatively, salt-extracted nuclear pellets were extracted with 2 ml of 0.2 M H_2SO_4 to obtain the acid-extractable nuclear proteins. (This fraction was composed mainly of histones). The acid-extracted nuclear pellets were resuspended in 2 ml 1% SDS, 1% 2-mercaptoethanol, resuspended with brief sonication (2 minutes of 15-second pulses at 40 watts with a Branson sonifier) and allowed to dissolve overnight. Aliquots of this fraction, the nuclear residual protein fraction, were counted and analyzed by SDS-polyacrylamide gel electrophoresis as described.

Electrophoretic Analyses of Nuclear Proteins - The phenol-soluble non-histone nuclear proteins from HeLa cells were separated by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS prepared as described by Teng et al. (1971). The fractions to be analyzed were dialyzed against 0.01 M

sodium phosphate buffer, pH 7.4, containing 0.1% SDS and 0.14 M 2-mercaptoethanol. Electrophoresis was carried out at 6 ma per tube until a bromophenol blue marker reached the bottom of the tube. About 10 hours were usually required.

In other experiments, the non-histone nuclear protein fractions were analyzed on discontinuous polyacrylamide gels containing 0.1% SDS prepared according to the method of Laemmli (1970) as modified by LeStourgeon and Rusch (1973). Samples were dialyzed against 0.375 M Tris-HCl buffer, pH 6.8, containing 3% SDS, 10% (v/v) glycerol, and 5% (v/v) 2-mercaptoethanol. Protein samples were overlaid on stacking gels (6 mm x 1 cm; 3% acrylamide) containing 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The stacking gels were polymerized over 8.75% acrylamide gels (6 mm x 9.5 cm) containing 0.375 M Tris-HCl, 0.1% SDS, pH 8.0. Electrophoresis was carried out at 1 ma per tube until the bromophenol blue tracking dye had migrated 1 mm into the separating gel, at which time the current was adjusted to 2 ma per tube until the marker dye had reached the bottom of the gel (about 6 hours). The electrode buffer was 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.4.

The gels were stained with 0.1% fast green in 7% acetic-acid-35% methanol for 12 hours and then destained in 7% acetic acid- 35% methanol. Densitometric analysis at 615 nm of the stained gels was carried out in a Gilford spectrophotometer equipped with a model 2410 S linear transport device. Estimates of the molecular weights of individual protein bands were based on mobility versus molecular weight plots for proteins of known molecular weight, using as standards,

horse heart cytochrome c (12,000 daltons), myoglobin (17,800 daltons), alcohol dehydrogenase (36,000 daltons), ovalbumin (45,000 daltons), bovine serum albumin (68,000 daltons) and gamma globulin (160,000 daltons), all measured under identical electrophoretic conditions.

For measurement of isotope distribution in different protein bands the gels were swollen by immersion in 7% acetic acid, frozen by contact with solid CO_2, and sliced transversly into 1 mm slices. When ^{32}P, ^{3}H or ^{14}C were the only labels, the gel slices were solubilized by incubation with 200 µl of 50% H_2O_2 at 60 ^{o}C for 12 hours. Scintillation liquid, 15 ml (Omniflour (New England Nuclear) 4 g per liter of 1 : 1 methyl cellosolve - toluene), was added and the radioactivity measured by scintillation spectrometry. In double-labeling experiments, using ^{3}H and ^{14}C as labels, the slices were dried on filter-paper discs and combusted in a Packard model 305 Sample Oxidizer. The radioactivity of the combustion products was measured in the recommended scintillation mixtures for ^{14}C and ^{3}H.

The nuclear acid-soluble proteins were analyzed for histone composition by electrophoresis in polyacrylamide gels containing 4 M urea and 0.9 N acetic acid as described by Panyim and Chalkley (1969). The gels were stained with 0.1% amido black in 7% acetic acid - 35 % methanol for 12 hours and then destained in 7% acetic acid - 35 % methanol. Densitometric analyses and measurement of radioactivity in the gels were carried out as described above.

Determination of Protein Radioactivity - The total radioactivity

in extracts containing the non-histone proteins was measured by precipitating the proteins in 10% TCA. After filtration on Whatman glass-fiber filters (GF/A) , the precipitates were washed with 10% TCA, dried and immersed in 0.5 ml of 4M NH_4OH - Protosol (1 : 12 v/v) for 3 hours at 25 ^oC. Fifteen ml of Liquiflour (New England Nuclear) in toluene were added and the samples counted. The total radioactivity of the acid-soluble nuclear proteins was determined by drying aliquots of the extract on glass fiber filter discs and counting as described above for TCA-precipitable proteins.

Isolation of HeLa Cell Polysomes - Suspensions of HeLa S-3 cells were incubated in the presence or absence of 25 µg/ml TPCK for 5 minutes and then pulse-labeled for 2 minutes with 10 µCi/ml of [^{3}H]-leucine. The control cells and TPCK-treated cells were chilled on ice, harvested by centrifugation at 1,000 x g for 2 minutes, and washed with 10 volumes of ice-cold Earle's balanced salt solution. The cells were lysed in 0.14 M NaCl - 10 mM Tris-HCl - 1 mM $MgCl_2$ by addition of Nonidet NP-40 to a final concentration of 0.5%. The lysates were vortexed vigorously for 15 seconds and centrifuged for 5 minutes at 2,000 x g to pellet nuclei and cell debris. The supernatant containing the polysomes was applied to linear 15 - 40 % sucrose gradients in 0.14 M NaCl, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5 and centrifuged for 90 minutes at 35,000 rpm in a SW 41 rotor. In some experiments, a 70% cushion of sucrose was placed beneath the gradient. The gradients were collected by displacement in an ISCO gradient collection apparatus and absorbancy monitored at 254 nm with an LKB Uvicord

II spectrophotometer. Fractions of 0.4 ml were collected for determination of TCA precipitable radioactivity.

Pulse-labeling of ATP pools with [^{32}P]-orthophosphate - HeLa cells and equine lymphocytes were gently resuspended in 5 ml of culture medium containing 2 mCi of carrier-free [^{32}P]-orthophosphate. After 15 minutes incubation at 37 ^{o}C, the cells were centrifuged as before, and the cell pellet was frozen by immersing the tube in acetone at -70 ^{o}C. The frozen cells were then homogenized in 4 ml of ice cold 0.6 N $HClO_4$, centrifuged and re-extracted with 4 ml 0.2 N $HClO_4$. The extracts were combined and titrated to pH 7.0 with 5 N KOH. After 12 hours at 4 ^{o}C, the $KClO_4$ precipitate was removed by centrifugation and the supernatant was filtered through Whatman No. 3 paper. Aliquots of the clear supernatant were subjected to chromatography on Dowex-1 (formate) columns (0.6 x 5.0 cm) as described by Hurlbert (1957). After application of the sample, the columns were washed with distilled water and with 1.0 M ammonium formate. The ATP fraction was eluted with 2 M ammonium formate, 0.75 M formic acid, and the ATP further purified by chromatography of Whatman No. 3 paper in ethanol - 1 M ammonium acetate, pH 7.3 - water, 66.5 : 30 : 3.5 (v/v/v). Spots corresponding in R_f to authentic ATP standards were cut out and the ATP eluted with water and analyzed by measuring absorbance at 259 nm and ^{32}P radioactivity.

Chemical Analyses - Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard for nuclear acidic protein determinations, and calf thymus

histones as a standard for histone analyses. DNA was determined by the diphenylamine reaction as described by Burton (1956), using highly polymerized calf-thymus DNA as a standard.

The amino acid compositions of the phosphoprotein fractions were determined by ion-exchange chromatography after the method of Spackman et al. (1958) using a Beckman amino acid analyzer 120B modified for a 10-fold increase in sensitivity by insertion of a Honeywell expanded range card. For determination of alkali-labile phosphorous, the proteins were dialyzed exhaustively against distilled water and hydrolysed in 1.0 N NaOH at 100 ^{o}C for 5 minutes. Inorganic phosphate released into the supernatant was analyzed as the phosphomolybdate complex after acidification with 0.1 ml of 0.1 M silicotungstic acid in 0.1 M H_2SO_4. To the clear supernatant was added 1.5 ml of 5% ammonium molybdate in 4 N H_2SO_4. The phosphomolybdate complex was extracted in 2.5 ml of 1 : 1 isobutyl alcohol-benzene, reduced with $SnCl_2$ and measured at 660 nm (Teng et al., 1971; Kleinsmith et al., 1966).

RESULTS

Composition of HeLa Nuclei (or Chromatin) at Different Stages in the Cell Cycle - Nuclear protein and DNA contents have been examined in synchronously growing HeLa S-3 cells at different times after release from a double thymidine block. The degree of synchrony obtained by this procedure is illustrated

in Figure 1, which plots three parameters of growth : cell number, mitotic index, and rate of [^{3}H]-thymidine incorporation into DNA. The S phase, as measured by the rate of DNA labeling begins immediately after release of the cells from the thymidine block and lasts for 5 to 6 hours. Maximal rates of DNA synthesis occur at 3 hours. The peak of mitosis occurs by 8 hours. New cells are first evident at about 7½ hours; by 12 hours the population has almost doubled. The timing of these events was highly reproducible, and was determined for each of the isotope labeling experiments described in this chapter. The degree of synchronization, as calculated by the method of Engleberg (1961) is 63% for the cells shown in Figure 1. On the basis of this data, we consider the G_2 period of the cell cycle to extend from 5½ to 7½ hours, while the G_1 phase of the following cycle is taken as the period from 8½ to 12 hours.

In order to monitor changes in nuclear proteins during the cell cycle, the number of cells in the population were counted and analyzed for DNA content and protein distribution in different nuclear extracts. The results are summarized in Table I. A comparison of the figures for DNA content of the original cell suspension (column 3) and the DNA recovered in the nuclear (or chromatin) fraction (column 4) shows that recovery is very high at all stages in the cell cycle with an average recovery of 86.1 ± 1.7 %. This minimizes risks of artifact due to the selection of a small or variable fraction of the nuclei for analysis.

There are significant variations in the protein to DNA

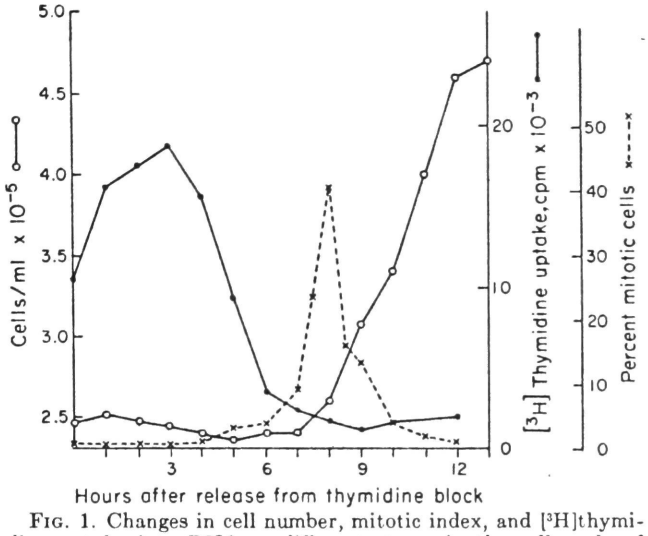

FIG. 1. Changes in cell number, mitotic index, and [^{3}H]thymidine uptake into DNA at different stages in the cell cycle of synchronously growing HeLa S-3 cells. The cells were synchronized by the double thymidine block method and aliquots of the suspension were analyzed as described under "Materials and Methods." The growth parameters are plotted against time after release from the thymidine block.

TABLE I

Composition of nuclear (or chromatin) fractions at different stages in cell cycle of synchronously dividing HeLa S-3 cells

Time after release from thymidine block	Cell concentration	DNA content of cells	Composition of nuclei (or chromatin)				Distribution of protein in nuclear extracts						
			DNA	Protein	Protein to DNA ratio	DNA recovery	0.14 M NaCl-soluble	0.25 N HCl-soluble	$CHCl_3$—CH_3OH—HCl-soluble	Phenol-soluble			Recovery[b]
										Protein	Protein to DNA ratio	Fraction[a]	
hrs	*cells/100 ml*	*μg/100 ml*	*μg/100 ml*			%	*μg/100 ml*			*μg/100 ml*		%	%
0	4.88×10^7	1157	970	2125	2.19	83.8	1020	990	100	330	0.340	15.5	114.8
1.5	5.02×10^7	1210	1000	3125	3.13	80.6	1210	1260	150	420	0.420	13.4	97.3
3.0	4.92×10^7	1230	1050	3375	3.21	85.4	1260	1310	100	460	0.438	13.0	92.7
4.5	4.84×10^7	1370	1070	3500	3.27	78.1	1260	1370	n.d.[c]	470	0.431	13.4	88.6
6.0	4.52×10^7	1620	1330	3750	2.82	82.1	1540	1410	150	440	0.330	11.7	94.4
7.5	4.86×10^7	1950	1810	3875	2.14	92.8	1760	1350	150	520	0.287	13.4	97.5
9.0	5.60×10^7	2130	1870	4250	2.27	87.7	1920	1800	150	540	0.289	12.7	104.7
10.5	6.80×10^7	1940	1900	4750	2.50	97.9	1900	1900	n.d.	510	0.263	10.7	90.5
12.0	8.00×10^7	2200	1830	4875	2.66	83.1	1900	2100	n.d.	550	0.284	11.2	92.7
13.5	9.40×10^7	2050	1850	5000	2.70	90.2	1650	2050	150	550	0.281	11.0	87.4

[a] Fraction of the total nuclear protein in the phenol-soluble fraction.

[b] Recovery = sum of proteins recovered in each fraction/total nuclear proteins.

[c] Not detectable.

ratio of the nuclei during synchronous growth, ranging from 2.19 to 3.27 to 1 (Table I; column 6). This ratio drops in the late S phase and increases during the G_1 period of the following cycle. The changes are largely due to varying proportions of the non-histone nuclear proteins because the histone to DNA ratios are not appreciably altered during the cell cycle but remain constant at about 1.1 to 1. This is consistent with observations that histone and DNA synthesis proceed concomitantly throughout the S phase of HeLa cells (Robbins and Borun, 1972; Hancock, 1969; Spalding et al., 1966).

In the fractionation procedure employed to separate nuclear proteins, about 40% of the total nuclear protein is removed when the nuclei are washed twice with 0.14 M NaCl (Table I, column 8). The acid-soluble proteins, mainly histones, comprise another 40% of the total nuclear proteins. Little loss of protein occurs in the chloroform-methanol-HCl washes; the bulk of the extracted materials comprises lipids and phospholipids.

The residual proteins, which are then extracted in phenol, comprise about 13% of the total protein in the isolated nuclei. This fraction includes many of the nuclear phosphoproteins. The amount of the phenol-soluble protein per nucleus varies during the cell cycle, as judged by the phosphoprotein to DNA ratio at different times after release from the thymidine block. This ratio falls from 0.42 to 1 in the early S phase to 0.28 to 1 in G_2 to M (Table I, column 12). It follows that a substantial increase in the acidic protein complement of the

nucleus must occur in the prereplicative phase of the cycle.

It should be noted that the high recoveries of protein obtained (approximately 96%) minimize the possibilities of artifact due to differential extraction of proteins depending upon stages in the cell cycle. As a further check, the recovery of phenol-soluble proteins from metaphase chromosomes isolated from cells blocked in mitosis (by exposure to vinblastine sulfate for 16 hours) and from nuclei obtained from an unsynchronized cell population were compared. No indications of differential extractability were obtained.

The distribution of alkali-labile phosphorous in the different nuclear subfractions is shown in Table II. It can be seen that the phenol-soluble proteins, representing only 13 to 14% of the total nuclear protein, contain about one-third of the total phosphorous and also account for about one-third of the total [^{32}P]-phosphate incorporation in a long term (23 hr) labeling period. The proteins extractable in 0.14 M NaCl contain only 7% of the total ^{32}P incorporated. The HCl-soluble protein fraction, mainly histones, contains about half of the total counts. It should be noted that the proteins of the phenol-soluble fraction have both the highest specific ^{32}P activity and the highest phosphorous content of the nuclear fractions analyzed.

Characterization of the Nuclear Phosphoprotein Fraction - The nuclear phosphoprotein fraction comprises a heterogeneous mixture of proteins differing in molecular weight, amino acid composition and degree of phosphorylation. The molecular size heterogeneity is indicated by differences in electrophoretic

TABLE II

Phosphorus content of nuclei and nuclear subfractions in logarithmically growing HeLa S-3 cells[a]

Nuclear fraction	Protein content		Phosphorus content			^{32}P radioactivity			
	μg	% total	μg	% total	μg P/μg protein	cpm	% total	cpm/μg P	cpm/mg protein
Intact nuclei	38,100	100.0	21.65	100.0	0.06	101,000	100.0	4,670	2,650
NaCl extract	10,400	36.7	4.03	18.6	0.04	7,270	7.2	1,800	700
HCl extract	16,200	42.5	7.02	32.4	0.04	48,170	47.7	6,860	2,971
$CHCl_3$—CH_3OH[b] extract	1,500	3.9	3.38	15.6		5,650	5.6	1,670	
Phenol extract	5,400	14.2	6.50	30.0	0.12	33,200	32.9	5,110	6,130

[a] One liter of cell suspension (4×10^8 cells) containing 25 mCi of [^{32}P]orthophosphate was incubated for 23 hours. An aliquot containing 4×10^7 cells was mixed with 4×10^8 unlabeled cells for isolation of nuclei and extraction of the nuclear proteins as described under "Materials and Methods." The protein content, alkali-labile phosphorus content, and ^{32}P activity were determined for each fraction.

[b] The phosphorus content per μg of protein and the specific ^{32}P activity per mg of protein are not given for the chloroform-methanol extract because of the high contamination by radioactive phospholipids in this fraction.

mobility in SDS-polyacrylamide gels. A complex banding pattern which is obtained which shows the presence of multiple polypeptide chains ranging in molecular weight from 18,000 to 170,000 daltons (Figure 2). At least 21 major bands are resolved under these electrophoretic condtions. These in turn include many different protein species of similar or identical molecular weights which may be resolved by two-dimensional electrophoresis (Peterson and McConkey, 1975).

A comparison of the banding patterns of the phenol-soluble nuclear proteins at different stages in the cell cycle is presented in Figure 2. The results show that although there is close to a two fold variation in the total amount of the proteins in this fraction, there are no major qualitative differences in the banding patterns. Some minor differences are detectable by densitometry, in agreement with the findings of Bhorjee and Pederson (1972), but , on the whole, it is the uniformity of the protein pattern rather than its variability, which attracts attention. This result is in contrast with the findings that the nuclear phosphoprotein complement changes appreciably in cells during the course of differentiation (LeStourgeon and Rusch, 1971, 1973; Vidali et al., 1973; Conner and Patel, 1972; Hill et al., 1975).

The amino acid analyses presented in Table II confirm the impression of constant proportionality of the phenol-soluble proteins throughout the HeLa cell cycle, although one would only expect to detect gross differences by this technique. The average amino acid composition of the nuclear phenol-soluble protein fraction indicates a clear predominance of the acidic

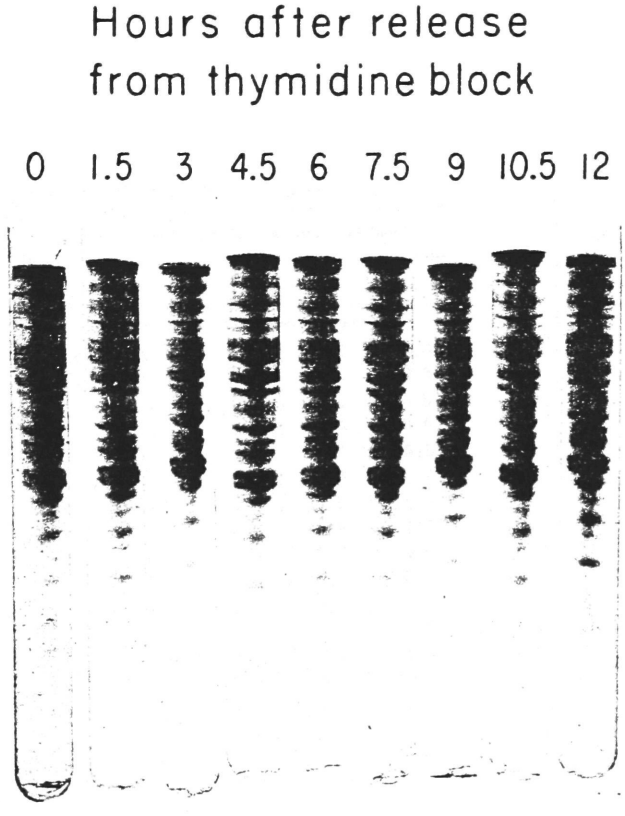

FIG. 2. Electrophoretic patterns in SDS-polyacrylamide gels of HeLa nuclear phenol-soluble proteins prepared at different stages of the cell cycle. The proteins were extracted from nuclei isolated at the indicated times after release from the thymidine block. All gels in this and in succeeding figures contained 125 μg of total protein. Note the uniformity in protein-banding patterns throughout the cycle.

TABLE III

Amino acid composition of nuclear phosphoprotein fraction at different stages of HeLa S-3 cell cycle

Amino acid	Hrs after release of cells from thymidine block				
	0	2	5	9	12
	moles/100 moles total amino acids[a]				
Lysine	6.3	6.8	6.4	6.6	6.3
Histidine	2.2	2.4	2.5	2.4	2.3
Arginine	6.0	5.7	5.8	6.4	5.9
Aspartic acid	9.7	9.5	9.7	9.8	9.6
Threonine	5.4	5.6	5.1	4.7	5.5
Serine	7.3	7.3	7.3	7.2	6.7
Glutamic acid	12.5	12.5	12.7	11.9	11.5
Proline	4.6	4.6	5.1	4.6	4.9
Glycine	9.0	9.5	9.3	9.2	9.0
Alanine	7.2	7.0	7.2	7.3	7.0
Valine	6.2	6.3	6.4	6.2	5.9
Methionine	1.1	1.5	1.7	2.0	1.5
Isoleucine	5.8	4.8	4.7	4.9	5.8
Leucine	9.7	9.4	8.9	9.3	10.4
Tyrosine	2.9	3.3	3.1	3.4	3.6
Phenylalanine	3.8	3.9	3.7	4.0	3.9

[a] Values not corrected for hydrolytic losses.

amino acids, aspartic and glutamic acid (21 mole %) over the basic amino acids lysine, arginine, and histidine (15 mole %). This is in accord with findings in other cell types (Teng et al., 1971).

Nuclear Protein Phosphorylation During Cell Cycle - Rates of [^{32}P]-phosphate incorporation into the nuclear phosphoprotein fraction vary at different stages in the cell cycle. Comparisons were made by selecting aliquots of the cell suspension at different times after release from the thymidine block and pulse-labeling for 15 minutes in the presence of [^{32}P]-orthophosphate. The cells were frozen immediately and the nuclear proteins were fractionated as described under "Materials and Methods." The specific activity of the phosphoprotein fraction was determined and plotted against time as shown in Figure 3.

Two peaks of phosphate incorporation are evident. The first occurs early in the S phase (between 1½ and 3 hours) and the second peak occurs early in G_1 (at about 10 hours). The rate of phosphate uptake appears to be somewhat greater in S than in G_1 ; an uptake ratio of about 1.2 : 1.0 was observed consistently. The phosphorylation of the phenol-soluble nuclear acidic protein is markedly reduced in the late S and G_2 phases of the cell cycle and remains low in the M period. Thus, the rate of [^{32}P]-phosphate incorporation into nuclear phosphoproteins is high a periods of intense RNA synthesis and low when transcription is suppressed (Pfeiffer and Tolmach, 1968; Farber et al., 1972; Johnson and Holland, 1965). It is interesting to note that analogous cell cycle

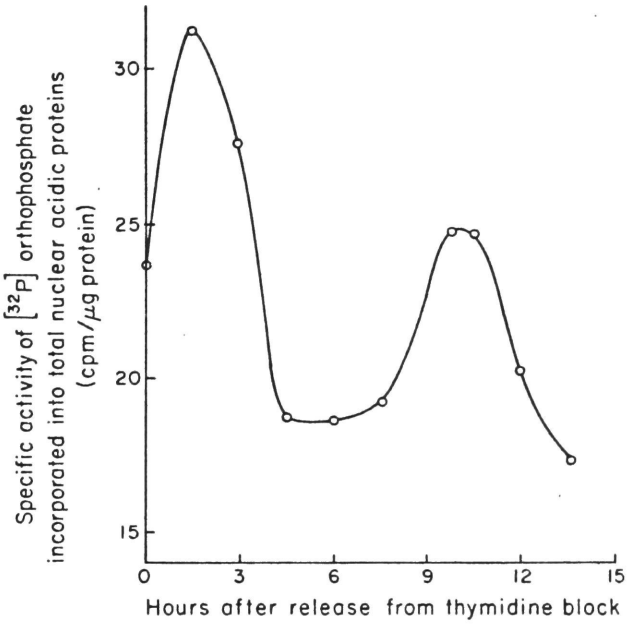

FIG. 3. Changes in rate of nuclear phenol-soluble protein phosphorylation at different times in the cell cycle. Aliquots of the HeLa cell suspension were taken at the indicated times and incubated for 15 min with [^{32}P]orthophosphate. The nuclear phosphoprotein fraction was isolated and its [^{32}P] activity measured. The specific activity is plotted against time after release from the thymidine block. This is maximal in S and in G1, but minimal in the G2 to M period.

fluctuations in the rate of [^{32}P]-phosphate incorporation into protein phosphate in tubulin have been reported (Piras and Piras, 1975).

The possibility that estimates of the rate of nuclear protein phosphorylation might be in error due to injurious effects of the thymidine double block was tested by comparing [^{32}P]-phosphate uptake in control (unsynchronized) cell cultures and in cultures exposed to 2 mM thymidine for 2 or 4 hours. As can be seen from the data in Table IV, the phosphate uptake into the nuclear proteins of control and thymidine-treated cells is virtually identical. Moreover, no differences in gel electrophoretic patterns could be discerned (data not shown). Other control experiments have established that cytoplasmic protein fractions do not contribute significantly to the radioactivity of the nuclear fraction we have analyzed. This possibility was tested by preparing the nuclear phosphoproteins from unlabeled cells which were homogenized in the presence of a [^{32}P]-labeled post-nuclear supernatant fraction from homogenates of cells incubated in the usual way with 2 mCi of [^{32}P]-orthophosphate. Less than 4.8% contamination was observed (Table IV).

The distribution of [^{32}P]-phosphate in different size classes of nuclear phosphoproteins after pulse-labeling for 15 minutes was determined by radioassay of the multiple bands separated by SDS-polyacrylamide gel electrophoresis (Figure 4). The staining pattern shown at the bottom of the figure is aligned with the corresponding densitometer tracing in the top panel. The other panels compare the distribution and specific

TABLE IV

Tests for contamination of nuclear phosphoproteins by radioactive cytoplasmic proteins, and for possible effects of 2 mM thymidine on protein phosphorylation by HeLa cells

Conditions of experiment	Specific activity of phenol-soluble proteins
	cpm/mg protein
Nonradioactive nuclei + ^{32}P-labeled cytoplasm[a]	992
Cells incubated without thymidine[b]	20,590
Cells exposed to 2 mM thymidine for 2 hrs	19,930
Cells exposed to 2 mM thymidine for 4 hrs	20,050

[a] Nuclei from 4×10^7 nonradioactive cells were mixed with the postnuclear supernatant fraction obtained from 4×10^7 cells which had been labeled for 15 min in the presence of 2 mCi of [^{32}P]orthophosphate. The nuclei were then reisolated and the phenol-soluble proteins were prepared and counted.

[b] Cells (4×10^7) were pulse labeled for 15 min in the presence of 2 mCi of [^{32}P]orthophosphate in the presence or absence of 2 mM thymidine as described under "Materials and Methods."

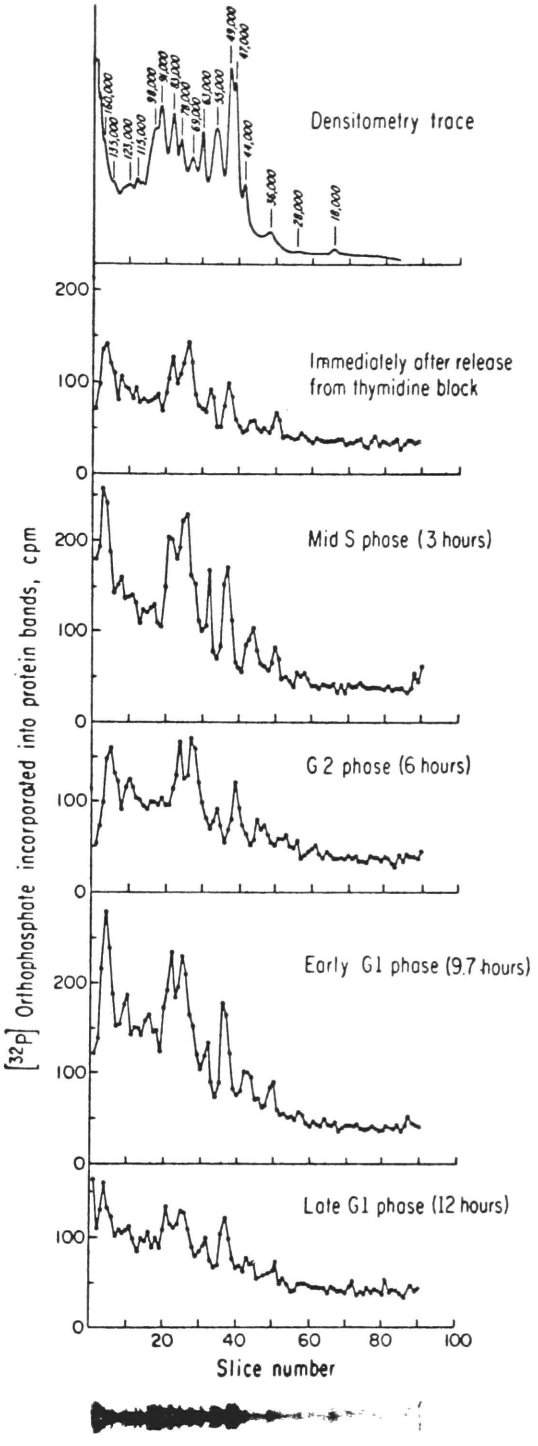

FIG. 4. Distribution of [^{32}P]phosphate in the nuclear phenol-soluble proteins of HeLa S-3 cells at different stages of the cell cycle. Aliquots of the cell suspension were pulse-labeled at the indicated times by exposure to [^{32}P]orthophosphate for 15 min. The nuclear phosphoproteins were extracted in phenol and separated by electrophoresis in 0.1% SDS-10% polyacrylamide gels. The protein-banding pattern is shown at the bottom of the figure and the corresponding densitometer tracing is shown in the top panel. Molecular weights are indicated above the major protein peaks. Intermediate panels show the patterns of [^{32}P]phosphate distribution in the nuclear proteins at different times after release from the thymidine block. Note the heterogeneity of the labeling pattern, the increase in rate of ^{32}P uptake in mid S and in early G1, and the relatively low activity in the G2 period.

activities of the nuclear phosphoproteins at the indicated stages in the cell cycle.

It is evident that there is a marked heterogeneity in [^{32}P]-phosphate incoroporation into the proteins at different regions of the gel. In some cases, the peaks of [^{32}P]-activity do not coincide exactly with the position of the major protein bands. This strongly suggests that minor bands, not visible because of their low concentration, may be contributing disproportionately to the [^{32}P]-uptake measurements.

The [^{32}P] activity of the individual protein bands follows quite closely the cell cycle-dependent changes described for the total phenol-soluble protein fraction. Labeling is greatest in the S and G_1 phases and is depressed in the G_2 to M phase. Some minor differences in the rate of labeling of different bands can be observed, but, on the whole, the pulse labeling experiments indicate a parallel response of many diverse nuclear proteins to events occurring at different stages of the cell cycle.

The fluctuations observed in the rate of [^{32}P] incorporation into the non-histone proteins at different stages in synchronous cell growth does not appear to depend on fluctuations in the specific activity of the cellular ATP pools. The incorporation of [^{32}P]-orthophosphate into ATP pools was measured in cells in different stages of the cycle. As the results in Table V show, the specific activity of the cellular ATP pools remain relatively uniform in the course of synchronous growth. The minor variations listed in Table V do not seem significant and are not likely to account for the large differences in

TABLE V

[^{32}P]Phosphate incorporation into ATP pools of HeLa cells at different times of cell cycle

Time after release from thymidine block	Specific activity of ATP[a]
hrs	*cpm/pmole*
0	9.7
2	10.8
5	10.5
9	11.3

[a] Cells (4×10^7) were incubated for 15 min in the presence of 1 mCi of [^{32}P]orthophosphate, and the ATP was isolated and analyzed as described under "Materials and Methods."

the specific activity of the phosphoprotein fraction at different stages. The fluctuations in [^{32}P]-phosphate incorporation into the nuclear proteins are more likely to represent alterations in protein kinase activities in nuclei at different stages of the cycle. It is known that the phosphorylation of some acidic nuclear proteins is cAMP dependent (Johnson and Allfrey, 1972; Kish and Kleinsmith, 1972). Cyclic AMP levels are known to fluctuate throughout the cell cycle of synchronously growing HeLa cells (Zeilig et al., 1972) with minimal concentrations in the late G_2 to M period. This suggests that the major changes in nuclear protein phosphorylation in the HeLa cell cycle reflect the changing activities of cAMP-dependent protein kinases.

Histone Phosphorylation in Cell Cycle - The rate of [^{32}P]-orthophosphate incorporation into the histones of synchronized HeLa cells varies throughout the cell cycle as shown in Figure 5. Panel A shows the rate of total histone phosphorylation while Panel B shows the rate of incorporation of phosphate into histone H-1 purified by electrophoresis. The peak of total histone phosphorylation is observed at 3 hours, which coincides with the peak of DNA synthesis. Correlations between histone phosphorylation and DNA synthesis have been noted before (Ord and Stocken, 1968; Balhorn et al., 1972; Oliver et al., 1972; Gurley et al., 1973). The phosphorylation of histone fraction H-1 is known to dependent on cell cycle position and to be active in S phase (Balhorn et al., 1972;

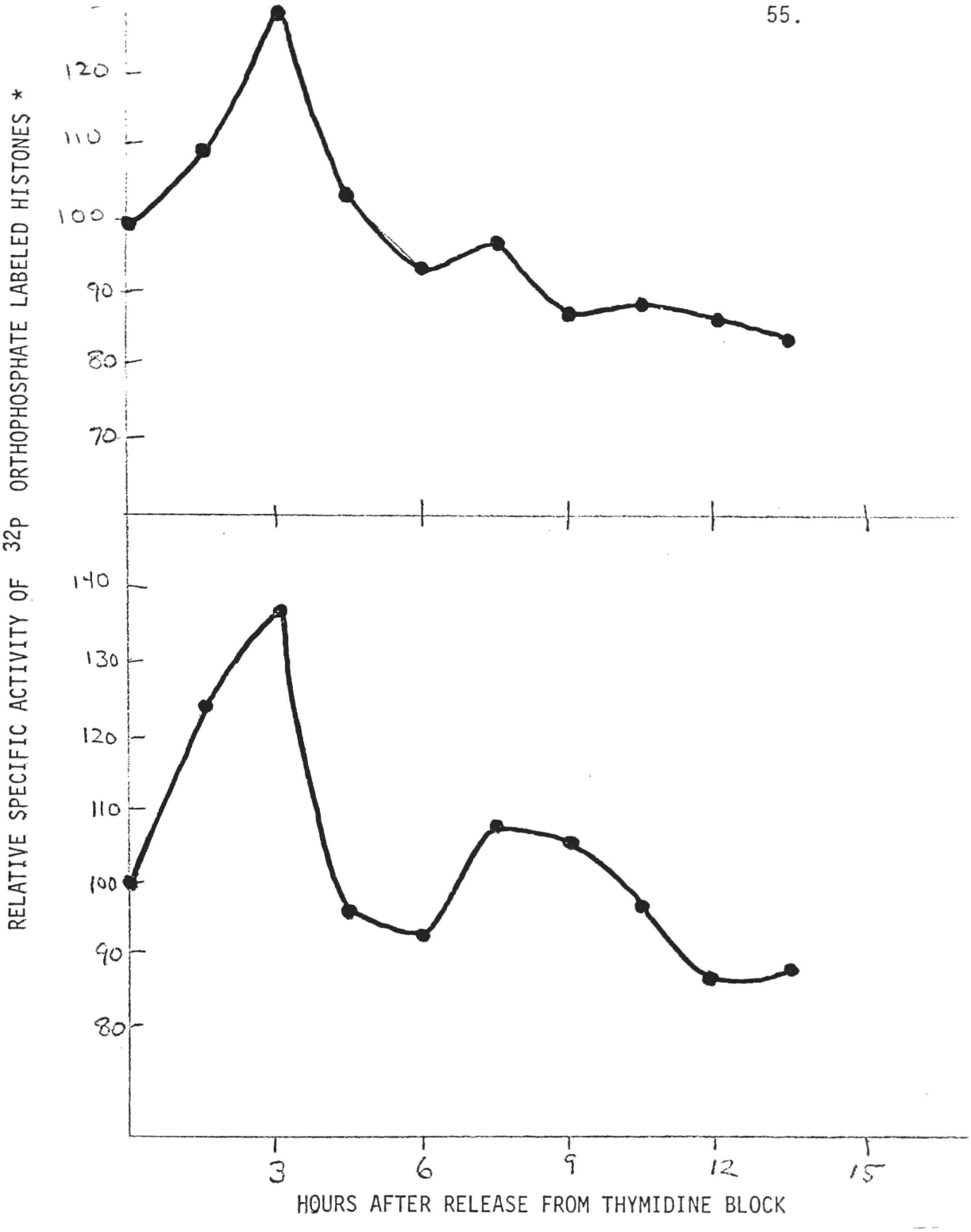

FIGURE 5 - Altered rates of histone phosphorylation at different stages of the cell cycle. Aliquots of the HeLa cell suspension were withdrawn at the indicated times after release from the thymidine block and pulse-labeled for 15 minutes in the presence of [^{32}P] orthophosphate. The specific activities of the histone bands purified in polyacrylamide gels by the method of Panyim and Chalkley (1969) were determined and plotted against time. Panel A, total histones; Panel B, histone H-1.

*Expressed as percent activity immediately after release from double thymidine block.

Oliver et al., 1972; Gurley et al., 1973). More recently, it has been recognized that H-1 is also phosphorylated in M (Marks et al., 1973; Bradbury et al..1973; Gurley et al., 1974;). The data in Figure 5 confirms the biphasic nature of H-1 phosphorylation.

Some differences exist between the timing of histone phosphorylation and phosphate uptake into the non-histone nuclear proteins. The peak of histone phosphorylation occurs somewhat later in the cell cycle than the corresponding peak for the nuclear phenol-soluble proteins (Figure 3). Moreover, no major peak in total histone phosphorylation apppears in G_1 as it does for the more acidic protein fraction. These differences argue strongly against the view that the variable rates of phosphorylation of the nuclear acidic proteins simply reflect differences in the specific activities of nuclear ATP pools at different phases of the cycle.

Turnover of Phosphoprotein-Phosphate at Different Stages of Cell Cycle - The retention by nuclear proteins of previously incorporated phosphate groups was compared at different times after release of cells from the thymidine block. In these experiments, the acidic nuclear proteins were prelabeled in a 23 hour incubation in the presence of $[^{32}P]$-orthophosphate and $[^{14}C]$-leucine, as described under "Materials and Methods." After washing to remove the radioactive precursors (and thymidine), the cells were incubated in a nonradioactive medium. Aliquots of the suspension were withdrawn at different times during the "cold chase" for preparation and analysis of the nuclear phenol-solubel phosphoprôtein fraction.

The results summarized in Figure 6 compare the retention of [^{32}P]-phosphate and [^{14}C]-leucine in the total phenol-soluble protein fraction. After a brief initial period in which specific activities increase slightly, the radioactivity is lost following an exponential decay curve. The rate of ^{32}P loss greatly exceeds that of ^{14}C label; protein phosphate activity declines with an average half-life of 6.7 hours, while the ^{14}C activity of the proteins falls to 50% of the original activity in about 25 hours. These divergent results indicate that the phosphoryl groups in the proteins are subject to removal without a corresponding degredation of the polypeptide chain. The loss of [^{32}P]-ortho-phosphate from the proteins has also been confirmed by analysis of the alkali labile phosphate activity in the protein samples.

The rate of ^{32}P turnover varies in different nuclear phosphoproteins. The distribution of [^{32}P]-phosphate in different size class of proteins separated by SDS-polyacrylamide gel electrophoresis is shown in Figure 7. The staining pattern (shown at the bottom of the figure) and the densitometer tracing (in the top panel) show the positions and molecular weights of the major protein bands. (These are fully comparable with the results presented in Figure 4 for the ^{32}P pulse-labeling experiments.) The other panels compare the distribution and specific activities of the nuclear phosphoproteins at the indicated stages of the cell cycle.

Each band appears to have a distinctive half-life for its ^{32}P labeled phosphoryl groups. For example, a band appearing at molecular weight 28,000 decays at the slowest rate, with a

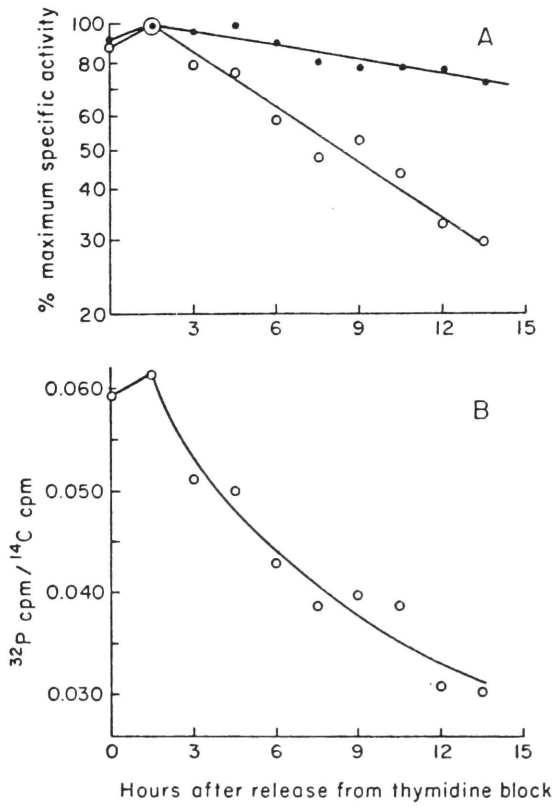

FIG. 6. Relative rates of turnover of [^{32}P]phosphate and [^{14}C]-leucine in nuclear phosphoproteins of synchronously growing HeLa S-3 cells. The acidic nuclear proteins were prelabeled for 23 hours in the presence of [^{14}C]leucine and [^{32}P]orthophosphate as described under "Materials and Methods." After washing, the cells were incubated under cold chase conditions, and aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear phosphoprotein fraction. *A*, the per cent of the maximal specific activity (counts per min per mg of protein) is plotted against time for [^{14}C]leucine (●——●) and for [^{32}P]phosphate (○——○). *B*, the ratio of ^{32}P activity to ^{14}C activity is plotted against time. The decreasing ratio indicates that phosphate groups in the protein are subject to replacement without a corresponding degradation of the polypeptide chain.

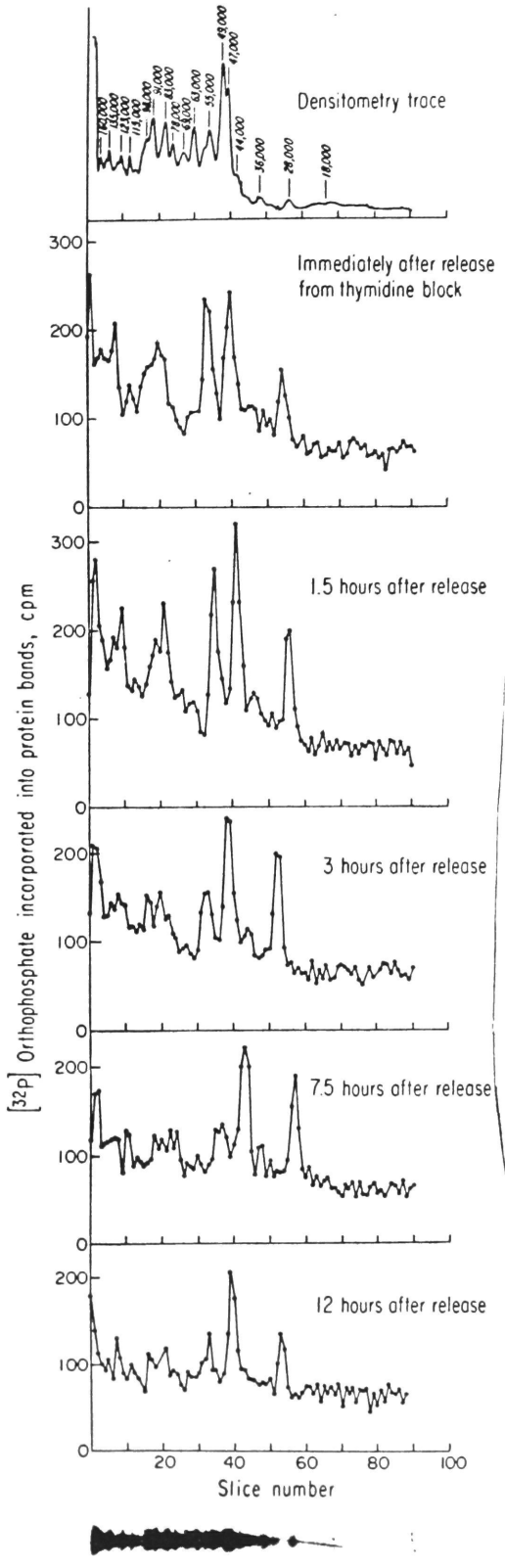

FIG. 7. Differential rates of [^{32}P]phosphate turnover in HeLa nuclear phosphoproteins during synchronous growth. The cell suspension was prelabeled with [^{32}P]orthophosphate for 23 hours as described under "Materials and Methods." After washing, the cells were incubated under cold chase conditions, and aliquots were withdrawn at the indicated times for preparation and electrophoretic analysis of the nuclear phenol-soluble phosphoprotein fraction. The proteins were separated by electrophoresis in 0.1% SDS-10% polyacrylamide gels. The protein-banding pattern is shown at the bottom of the figure and its densitometer tracing is shown in the top panel. Molecular weights are indicated above the major protein peaks. The intermediate panels show the patterns of ^{32}P distribution in the nuclear proteins at different times after release from the thymidine block. Note the rapid loss of ^{32}P activity from the band at molecular weight 55,000, and the relatively slow decreases in ^{32}P activity of bands at molecular weights 28,000 and 47,000.

half-life of about 12 hours. Another band of molecular weight 47,000 loses its [^{32}P]-phosphate at an intermediate rate, with a half-life of about 9 hours. Many of the bands in the higher molecular weight regions have half-lives of the order of 6 hours. Such differences in [^{32}P]-phosphate metabolism are not readily evident in the short term pulse-labeling experiments. It follows that the retention of [^{32}P]-phosphate is a more sensitive index of differential phosphoryl group turnover in different nuclear acidic proteins than is the uptake of [^{32}P]-phosphate in short term incubations.

To rule out the possibility that such different rates of ^{32}P turnover in different nuclear phosphoproteins might be an artifact due to prolonged exposure of the cells to thymidine (possibly permitting selective protein degradation during growth arrest), similar experiments were carried out in non-synchronously growing HeLa S-3 cultures. The results summarized in Figure 8 compare the retention of [^{32}P]-phosphate and [^{14}C]-leucine in the protein subfractions soluble in 0.14 M NaCl, 0.25 N HCl, and phenol. As in the synchronously-growing cell cultures, the rate of ^{32}P loss from the phenol-soluble proteins exceeds the rate of decline of ^{14}C specific activity. the ratio of ^{32}P to ^{14}C activities consequently decreases rapidly with time during the cold chase (Figure 8). The kinetics of ^{32}P turnover appear to be characteristic for the different nuclear subfractions.

In agreement with the reults obtained in synchronously-growing cells, the rate of ^{32}P turnover varies for different proteins in the phenol-soluble fraction. The distribution of

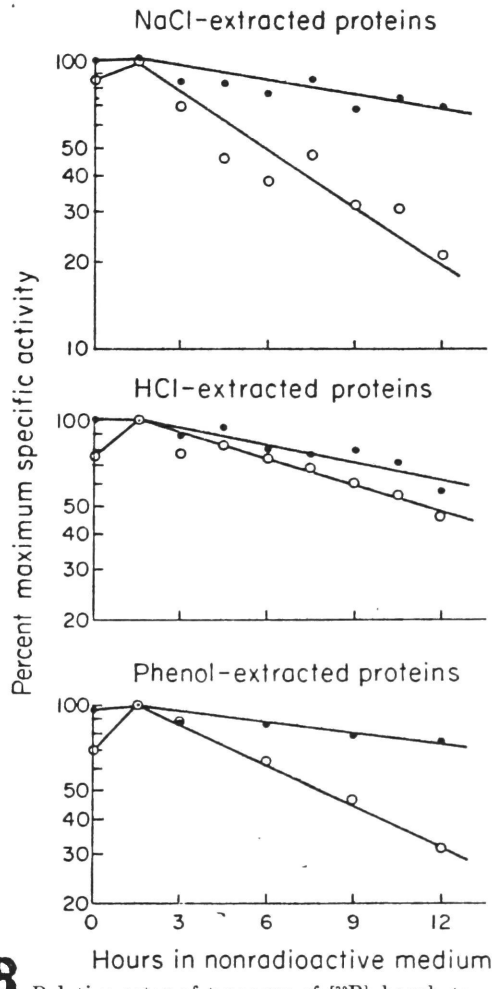

FIG. 8. Relative rates of turnover of [^{32}P]phosphate and [^{14}C]-leucine in protein fractions of logarithmically growing HeLa S-3 cells. The proteins were prelabeled for 23 hours in continuously growing but asynchronous cultures. After washing, the cells were incubated under cold chase conditions, and aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear protein fractions. The per cent of the maximal specific activity (counts per min per mg of protein) is plotted against time for [^{14}C]leucine (●——●) and for [^{32}P]phosphate (○——○) for proteins soluble in 0.14 M NaCl (*top panel*), 0.25 N HCl (*center panel*), and phenol (*lower panel*).

[^{32}P]-phosphate in different protein bands separated by SDS-polyacrylamide gel electrophoresis is shown in Figure 9 for 3 and 9 after commencement of the cold chase. Three major phosphorylated species of molecular weights 28,000 47,000 and 55,000 daltons are evident. The rapid ^{32}P turnover in the protein of molecular weight 55,000 is in agreement with results obtained in the synchronously-growing cell populations (Figure 7). Figure 9 also shows the distribution of the ^{14}C labeled proteins in the electrophoretic pattern. It is clear that in some cases, the peaks of ^{32}P activity do not coinicide exactly with the positions of the major [^{14}C]-leucine-labeled bands.

Phosphate Content of Isolated Nuclear Phosphoproteins - The average phosphorous content of the total nuclear phenol-soluble protein fraction was determined at various times after release from the thymidine block. There are no major differences in alkali-labile phosphorous content of the proteins prepared at different stages of the cycle (Table VII). The steady-state level determined by direct analysis represents a balance between the turnover and replacement of phosphate groups on "old" protein molecules, as well as an incorporation of phosphate into newly-synthesized acidic proteins of the nucleus. It is clear from the ^{32}P incorporation data that rates of phosphorylation vary in different proteins and change at different periods of the cell cycle. The studies of isotope retention in both synchronous and non-synchronous cell cultures show that individual nuclear proteins exchange their phosphate groups at differing rates. The complexity and diversity of

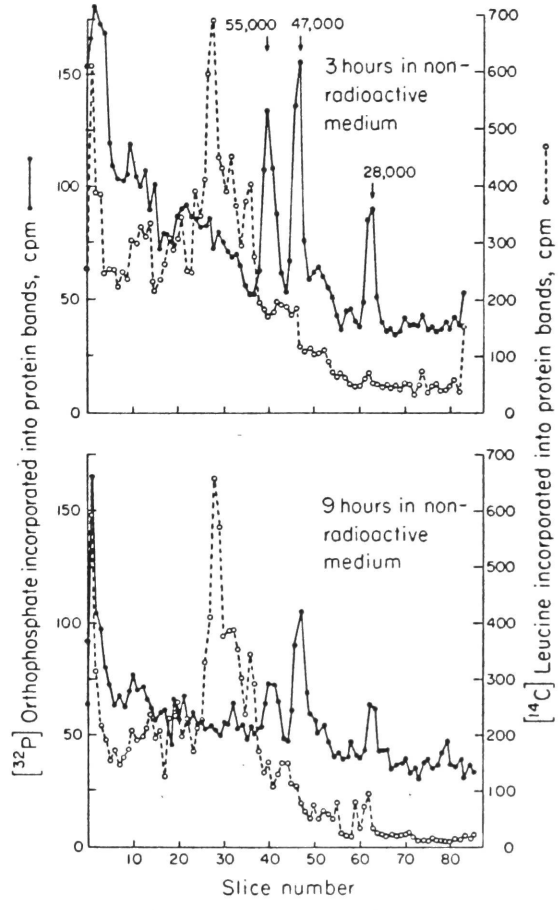

FIG. 9. Differential rates of [^{32}P]phosphate turnover in HeLa nuclear phosphoproteins in unsynchronized, logarithmically growing cell populations. The cells were prelabeled with [^{32}P]-phosphate and [^{14}C]leucine for 23 hours. After washing, the cells were incubated under cold chase conditions and aliquots of the suspension were withdrawn at the indicated times for preparation and electrophoretic analysis of the nuclear phenol-soluble protein fraction. The proteins were separated by electrophoresis in 0.1% SDS-10% polyacrylamide gels. The distribution of ^{32}P (●——●) and ^{14}C (O- - -O) activity is shown after 3 hours and after 9 hours in the nonradioactive medium. Note the rapid rate of loss of ^{32}P from the protein band at molecular weight 55,000 and the relatively slower loss from bands at molecular weights 28,000 and 47,000.

TABLE VI

Phosphorus content of nuclear phosphoprotein fraction at different stages of cell cycle of synchronized HeLa cells

Time after release from thymidine block	Phosphorus content of protein[a]
hrs	%
0	0.099 ± 0.003
1.5	0.144 ± 0.024
3.0	0.115 ± 0.018
4.5	0.091 ± 0.014
6.0	0.100 ± 0.008
7.5	0.083 ± 0.001
9.0	0.104 ± 0.007
10.5	0.079 ± 0.004
12.0	0.100 ± 0.009

[a] Phosphorus determined as alkali-labile phosphate as described under "Materials and Methods." Data presented as average of three values ±S.E.

these structural modifications are not evident in the over-all phosphate level of the phenol-soluble fraction.

Based on an average phophorous content of about 0.1% by weight, it can be estimated that an average protein of molecular weight 120,000 containing 73 seryl residues (Table III) might contain only about 4 of these in the phosphorylated form. (Since not all proteins in the phenol extract are equally phosphorylated, some will be more and others less phosphorylated than this average figure indicates.)

The G_0 to G_1 Transition : Effects of Con A Stimulation on the Nuclear Proteins of Lymphocytes - The preceding experiments have detailed changes in nuclear phosphoproteins during the cell cycle of HeLa cells. It was noted that early in the pre-replicative stage of the cell cycle the amount and rate of phosphorylation of nuclear proteins was increased. Are analogous increases observed when quiescent cells are stimulated to proliferate?

As a model system, we have examined the effects of the mitogen, Concanavalin A, on human and equine lymphocytes. Mitogenic plant lectins such as Con A induce cultured peripheral lymphocytes to undergo progressive enlargement, DNA synthesis, and mitosis, resulting in transformation of the normally dormant cells to a rapidly proliferating blast-like state (Powell and Leon, 1970; Nowell, 1960; Moorhead et al. (1960); Robbins, 1964). As revealed by several experiments performed with the mitogen phytohemagglutinin, early events in this transformation process include a modification of chromatin proteins. PHA stimulates histone acetylation (Pogo et al., 1966),

nuclear protein phosphorylation (Kleinsmith, et al., 1966) and RNA synthesis within minutes of addition to cultured human lymphocytes. This early elevation of RNA synthesis by PHA may include increased synthesis of ribosomal RNA (Cooper, 1969). A stimulation of RNA polymerase I activity has also been observed within 15 minutes of PHA addition (Pogo, 1972). RNA polymerase II activity (Pogo, 1972) and the synthesis of messenger RNA (Rosenfeld et al., 1972) are elevated within 2 hours of PHA stimulation. Synthesis of the non-histone nuclear proteins is stimulated within an hour of PHA addition to rat lymphocytes (Levy et al., 1973). In contrast to these early events, DNA synthesis is only slightly elevated 24 hours after PHA addition (Haddon et al., 1972). It should be noted than an important difference between studies of lymphocytes and HeLa cell populations is that the lymphocytes do not represent a homogeneous population of cells. The response of lymphocytes to mitogens may in fact be a multicellular event involving the interaction of two or more possibly different cell types (Beyer and Bowers, 1975).

Stimulation of Phosphorylation of Equine Lymphocyte Nuclear Acidic Proteins by Con A - The ability of Con A to stimulate the rate of phosphorylation of phenol-soluble non-histone proteins is shown in Figure 10. In this experiment, the rate of phosphorylation was stimulated about 4-fold, 2 hours after addition of Con A. The rate of phenol-soluble non-histone protein phosphorylation increased to about 10-fold that of control levels at 8 hours after addition of Con A. Con A reproducibly stimulated phosphorylation of the nuclear non-

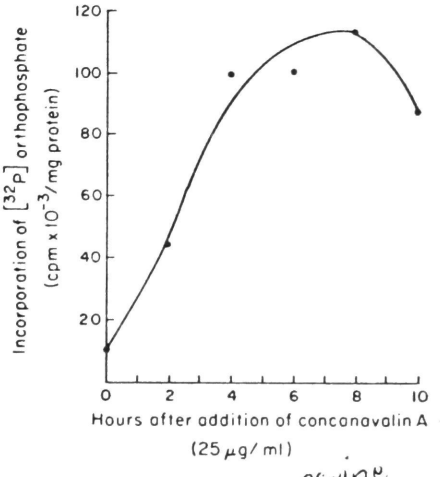

FIG. 10. Stimulation of phosphorylation of lymphocyte phenol-soluble nuclear protein by Con A. Aliquots of cells cultured in the presence or absence of Con A for the indicated times were pulsed for 15 min with [^{32}P]phosphate as described in the text. Chromatin proteins were extracted and analyzed for radioactivity as described. Note that Con A stimulates protein phosphorylation more than 4-fold in 2 hours.

histone proteins although variability of the degree of stimulation (from 2 to 12 fold) was encountered with different preparations of horse lymphocytes. The source of this variability appears to be a variable degree of "background" stimulation induced at some point prior to ^{32}P uptake measurements. Although it is likely that some lymphocytes are stimulated naturally in the horse, it is also possible that cell contacts during handling procedures could activate a small percentage of lymphocytes.

The Con A-induced phosphorylation of phenol-soluble non-histone nuclear proteins is expressed in Figure 10 as an increase in specific activity (counts per minute per mg of protein). This change in specific activity of phosphate incorporation is a complex event since the amount of these proteins associated with the chromatin also increases upon treatment of the lymphocytes with Con A. This change is shown in Figure 11. The amount of lymphocyte phenol-soluble nuclear acidic protein progressively increases from about 200 μg per 10^7 cells in control cultures to about 800 μg per 10^7 cells in cultures incubated with Con A for 6 hours. A comparison of Figures 10 and 11 (which depict data from the same experiment) shows that Con A induces simultaneous increases in the rate at which the nuclear acidic proteins are phosphorylated and the levels with which they are associated with chromatin. The rate of phosphorylation increases rapidly within 4 hours after Con A treatment and is maximal at 8 hours, whereas the amount of nuclear protein increases progressively and becomes constant about 6 hours after exposure of the

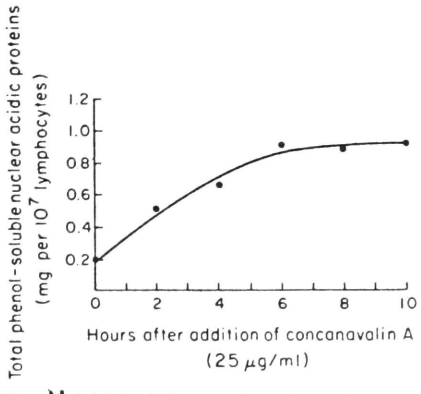

FIG. 11 (*right*). Effect of Con A on the amount of phenol-soluble non-histone protein associated with lymphocyte chromatin. At the indicated times after addition of Con A, cells were harvested, chromatin proteins were isolated, and protein amounts were measured as described in the text. Note that Con A increases the amount of protein in the phenol-soluble fraction about 4-fold in 6 hours.

lymphocytes to Con A.

Figure 12 is a photograph of the electrophoretic sodium dodecylsulfate disc gel patterns of phenol-soluble non-histone nuclear proteins recovered from equal populations of lymphocytes exposed to Con A for different times. Con A - induced increases in the amount of acidic protein associated with chromatin may be seen as an increase in the amount of stainable material applied to the gels. There is some specificity in the Con A-induced increases of phenol-soluble proteins associated with chromatin, and this specificity can be seen from the densitometry tracings of the stained gels shown in Figure 13. The top panels in Figure 13 illustrate the distribution of [^{32}P]-phosphate in the SDS gels of the phenol-soluble non-histone proteins. Con A stimulates a general increase in phosphate incorporation into the nuclear acidic protein although disproportionate increases in the phosphorylation of proteins in the molecular weight range of 90,000 130,000 and 52,000 daltons are evident. Comparison of the gel patterns of protein phosphate labeling and protein distribution (Figure 12) shows that the peaks of increase in ^{32}P labeling of individual proteins do not necessarily coincide with peaks of increase in protein content. For instance, the large relative increases in phosphorylation of protein fractions of molecular weight 28,000 and 52,000 are not accompanied by relative increases in the amounts of these proteins. Changes in the rate of phosphorylation of nuclear proteins cannot be attributed to changes in the specific activity of the ATP pools which in this system, as in HeLa

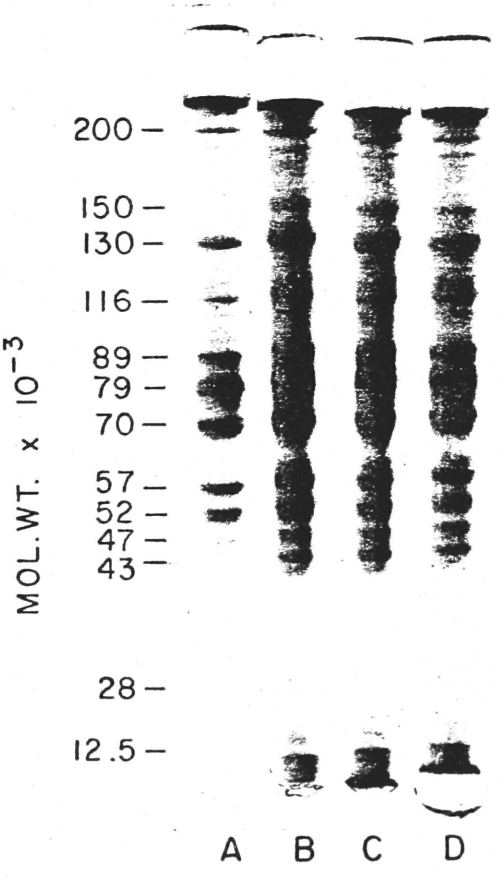

FIG 12. Effects of Con A on sodium dodecyl sulfate disc gel electrophoretic patterns of phenol-soluble non-histone nuclear proteins. At various times after addition of Con A, cells were harvested, and nuclei and chromatin proteins were extracted. Electrophoresis was performed on gels (9.5 cm × 6 mm) of 10% polyacrylamide with a 1-cm stacking gel of 3% polyacrylamide as described. Gels were stained with fast green. Each gel contains protein obtained from 2.5×10^6 lymphocyte nuclei. *A*, no Con A; *B*, 2 hours after addition of Con A; *C*, 4 hours after addition of Con A; *D*, 6 hours after addition of Con A. Note that Con A preferentially increases the intensity of bands at molecular weights 43,000 and 150,000.

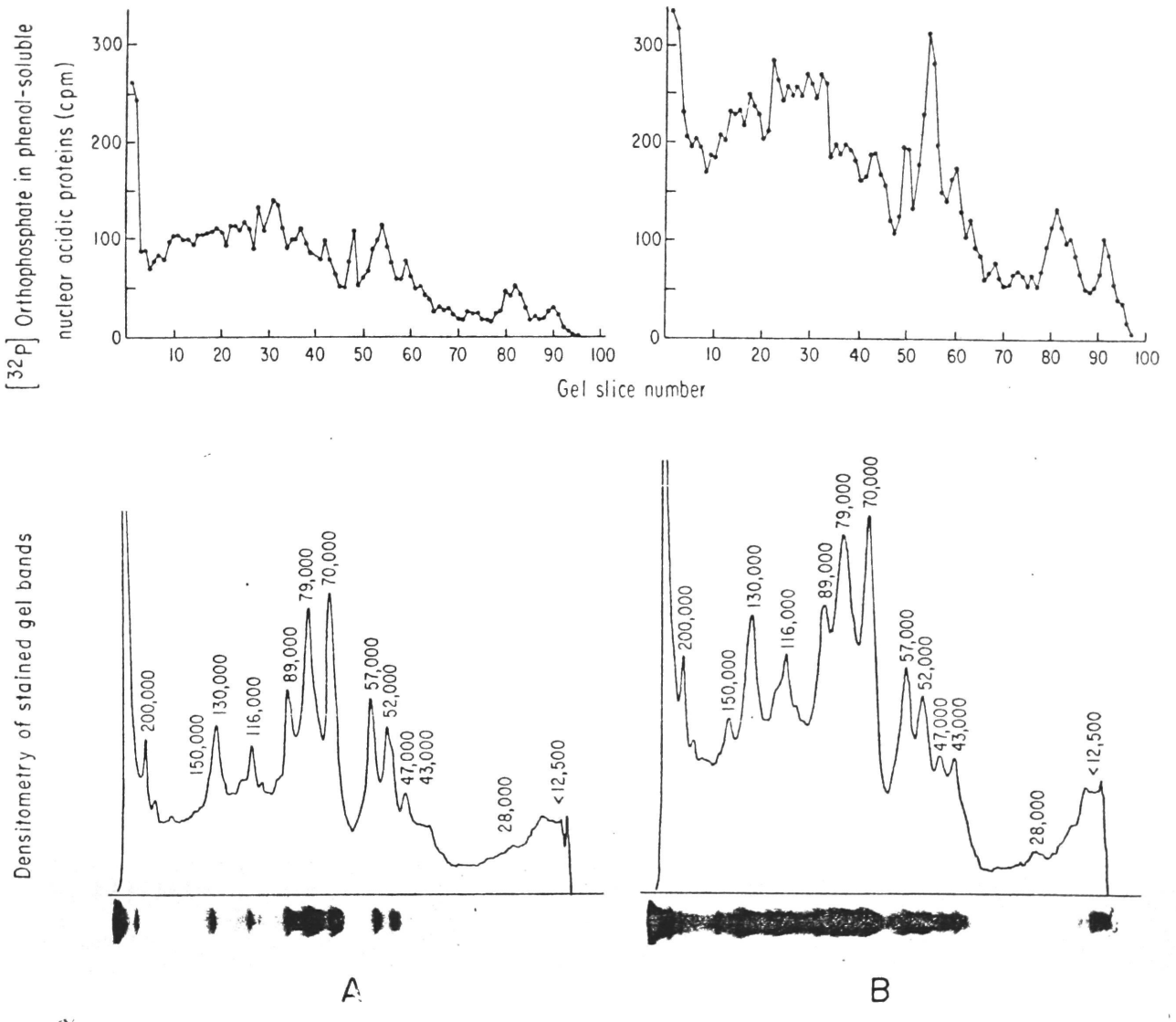

FIG. 13. Effects of Con A on electrophoretic distribution and phosphorylation of phenol-soluble non-histone nuclear proteins. After culturing in the presence or absence of Con A, lymphocytes were pulsed for 30 min with [^{32}P]phosphate. Nuclear proteins were isolated, and sodium dodecyl sulfate disc gel electrophoresis was performed as described in the text. Each gel received nuclear protein from 2.5×10^6 lymphocyte nuclei. After recording the densitometric pattern of protein staining, each gel was sliced transversely and assayed for ^{32}P radioactivity as described. *A*, [^{32}P]phosphate distribution (*top*) and densitometric pattern (*bottom*) of a single electrophoretic gel of nuclear proteins from resting lymphocytes (no Con A). *B*, [^{32}P]phosphate distribution (*top*) and densitometric pattern (*bottom*) of a single electrophoretic gel of nuclear proteins from lymphocytes incubated 2 hours in the presence of Con A. Panels have been aligned for vertical comparison. Note the preferential stimulation of phosphorylation of a protein fraction of molecular weight 52,000 in the presence of Con A.

cells, remain constant.

Distribution of Protein in Equine Lymphocyte Nuclear Protein Fractions - Table VII summarizes the ratios of protein to total nuclear DNA for different nuclear fractions from lymphocytes incubated with or without Con A. Nuclei described in Table VII were isolated in medium containing Triton X-100 as described. It can be seen that the protein to DNA ratio for whole lymphocyte nuclei increases about 1.7-fold after 6 hours of incubation of the cells with Con A. This increase in total nuclear protein in response to Con A is reflected in increases in protein to DNA ratios for all of the nuclear acidic protein extracts. Con A increased the amounts of the 0.14 M NaCl-extractable protein about 1.3-fold in 6 hours. Amounts of protein in this fraction were quite low and difficult to measure (probably because the nuclear isolation procedure removed the more loosely-bound proteins). The HCl-extractable protein fraction increased only slightly in response to Con A. This fraction contains the histones as well as other acid-extractable components. The ratios of acid-extractable protein to DNA shown in Table VII (0.72 to 1.34) are comparable to values obtained from cell types other than lymphocytes. In most cell nuclei, histones are present at a ratio of about 1 : 1 with the DNA (Georgiev, 1967). The chloroform : methanol - extractable nuclear proteins increased about 2-fold in response to Con A. The largest protein increase induced by Con A was localized in the phenol-soluble fraction. Protein in this fraction increased as much as 3-fold in response to Con A treatment of lymphocytes. It can be seen from Table VII that close to all the nuclear

TABLE VII

Effects of Con A on protein content of nuclear protein fractions

Con A[b]	Incubation time	Protein to DNA ratios in nuclear fractions[a]					Protein recovery[d]
		Whole nuclei[c]	0.14 M NaCl extract	0.25 N HCl extract	$CHCl_3$-CH_3OH extract	Phenol extract	
	hrs	*mg/mg*					%
−	0	2.25	0.10	1.15	0.70	0.18	95
+	0	2.14	0.08	1.04	0.74	0.20	96
−	3	2.28	0.13	1.31	0.90	0.15	109
+	3	3.85	0.15	1.03	1.93	0.78	101
−	6	1.94	0.09	0.72	0.96	0.32	108
+	6	3.79	0.12	1.34	1.70	1.02	110

[a] DNA values used were those for whole lymphocyte nuclei.

[b] Con A was used at 25 μg per ml of culture medium; lymphocytes at 2.5×10^6 cells per ml as described in the text.

[c] Nuclei were prepared after homogenization of cells in the presence of Triton X-100 as described in the text.

[d] (Milligrams of protein in extracted fractions)/(milligrams of protein in whole nuclei) × 100.

proteins are removed from the DNA by the various extraction procedures. A similar high recovery of protein was obtained with HeLa cells.

Nuclei from lymphocytes incubated with or without Con A were also isolated by nonaqueous procedures in order to preserve the soluble nuclear components which are lost upon homogenization in aqueous media. Table VIII describes the distribution of proteins in the nonaqueous lymphocyte nuclei. As in Triton X-100 treated nuclei, the 0.14 M NaCl-soluble proteins constitute only a small percentage (11%) of the total nuclear protein. This fraction undergoes a 3-fold increase in protein in response to Con A. The HCl-soluble fraction does not exhibit a protein increase in response to Con A. These proteins are mostly histones, as evident from the protein to DNA ratio of 1.2 : 1. The phenol-soluble fraction is somewhat lower in protein content in nonaqueous nuclei than in Triton X-100-extracted nuclei. These proteins increase about 4-fold in response to Con A. Lymphocyte chromatin is low in the amount of phenol-soluble protein compared to HeLa cell chromatin. Resting lymphocytes contain phenol-soluble nuclear protein at a ratio of protein to DNA of 0.1. The ratio of these proteins in HeLa cells varies from 0.26 to 0.44 to 1.0 during the cell cycle. Nuclei from resting lymphocytes isolated in either nonaqueous or aqueous media are similar in total protein content. This would suggest that the bulk of the lymphocyte nuclear proteins are not readily solubilized during the aqueous isolation procedures. It is most interesting that even in nuclei isolated by rigorous

TABLE VIII

Effects of Con A on content of nuclear protein fractions from lymphocyte nuclei isolated in nonaqueous media

Nuclear protein fractions	Protein to DNA[a] ratios	
	− Con A[b]	+ Con A
	mg/mg	
Whole nuclei[c]	2.07	3.22
0.14 M NaCl extract	0.11	0.29
0.25 N HCl extract	1.19	1.20
$CHCl_3$–CH_3OH extract	0.45	1.05
Phenol extract	0.11	0.45
Recovery (%)	90	93

[a] DNA values used were those for whole lymphocyte nuclei.

[b] Lymphocyte cultures (2.5×10^6 cells per ml) were incubated for 3 hours in the presence or absence of Con A (25 μg per ml) as described in the text.

[c] Nuclei were isolated from quick-frozen, lyophilized cells in media consisting of glycerol and 3-chloro-1,2-propanediol as described in the text.

nonaqueous procedures, there is an increase in total protein in nuclei from Con A-treated cells. Thus it appears that early after stimulation by Con A, lymphocytes accumulate non-histone nuclear proteins. This process may be analogous to the increase in nuclear protein observed in late G_1 and early S in HeLa cells.

Effects of Con A on Synthesis of Nuclear Proteins - Cycloheximide was employed in experiments to determine whether Con A-induced increases in nuclear protein content could be due to the observed ability of mitogens to stimulate nuclear protein synthesis (Levy et al., 1973). Table IX describes the effects of cyclohexmide on nuclear acidic protein synthesis in lymphocytes incubated for 6 hours in the presence or absence of Con A. In 6 hours, Con A stimulates the synthesis of the phenol-soluble proteins about 1.3-fold while increasing the amount of these proteins in the nucleus about 2-fold. Cycloheximide inhibits phenol-soluble protein synthesis by 94% but has virtually no effect on the Con A-induced increase in nuclear protein content. Thus, the Con A-induced increase in nuclear protein associated with chromatin is not entirely due to a stimulation of the synthesis of these proteins. While Con A does stimulate non-histone nuclear protein synthesis, the amount of protein synthesized is evidently not large enough to account for the increase in the level of the phenol-soluble chromatin proteins. The increases in the amount of protein in the phenol-soluble fraction, independent of protein synthesis, suggests that Con A induces an intracellular rearrangement of presynthesized proteins. The additional proteins may be of cytoplasmic origin.

TABLE IX

Effects of cycloheximide on Con A-induced increases in synthesis and total amount of phenol-soluble nuclear protein

Time of incubation[a]	Additions		[^{3}H]Leucine incorporation	Phenol-soluble protein level
	Con A[b]	CHX[c]		
hrs			*cpm/10^7 cells*	*mg/10^7 cells*
0	−	−	104	0.25
0	−	+	82	0.25
6	−	−	17,000	0.37
6	−	+	549	0.23
6	+	−	22,800	0.62
6	+	+	1,460	0.70

[a] All lymphocyte aliquots received [^{3}H]leucine. Zero time points were centrifuged for 5 min at 0° in the presence of [^{3}H]-leucine before freezing.

[b] 25 μg per ml.

[c] CHX = cycloheximide, 10 μg per ml.

Turnover of Phenol-Soluble Non-histone Nuclear Proteins - Equine lymphocytes were prelabeled in a 24-hour incubation with [^{14}C]-leucine as described under "Materials and Methods." After washing to remove the radioactive precursors, the cells were incubated in nonradioactive medium in the presence of absence of Con A. Figure 14 illustrates the effects of Con A on the retention of ^{14}C by the phenol-soluble chromatin proteins. In the absence of Con A, non-histone proteins turn over quite slowly; virtually all the radioactivity is retained in the fraction after 6 hours. Under these cold chase conditions, Con A actually stimulates an increase in total ^{14}C label in the phenol-soluble non-histone nuclear proteins (Figure 14). This presumably reflects an influx of ^{14}C labeled protein from the cytoplasm. Simultaneously, Con A stimulates an increase of about 2.5-fold in the amount of phenol-soluble protein while slightly inhibiting the loss of ^{14}C label from the entrire nuclear fraction.

Effects of Con A on Nuclear Residual Proteins from Human Lymphocytes - In order to test the generality of a Con A-induced increase in nuclear residual proteins in lymphocytes, we examined the effects of Con A on human lymphocytes. Human lymphocytes respond to lower doses of Con A than do equine lymphocytes, and the maximum stimulation of thymidine incorporation into DNA is observed using 10 μg/ml Con A (Figure 15).

Resting human lymphocytes contain somewhat more residual nuclear protein than do resting equine lymphocytes, the SDS-soluble protein to DNA ratio of the human lymphocyte

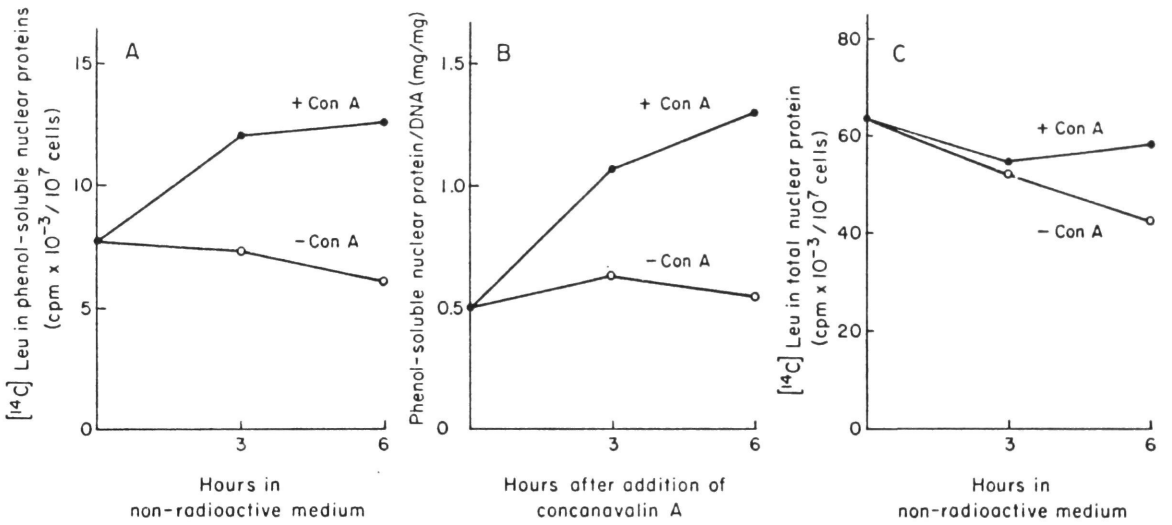

FIG. 14. Effects of Con A on turnover of lymphocyte nuclear proteins. Lymphocyte proteins were prelabeled in a 24-hour incubation with [^{14}C]leucine. After washing to remove radioactive precursors, cells were resuspended in nonradioactive medium in the presence or absence of Con A, and radioactivity in nuclear proteins was monitored during the cold chase period as described in the text. *A*, effect of Con A on amount of label in total phenol-soluble non-histone protein; *B*, effect of Con A on amount of total phenol-soluble non-histone protein; *C*, effect of Con A on retention of label by total proteins of lymphocyte nuclei. Note that Con A increases the total amount of ^{14}C label in the phenol-soluble nuclear proteins under cold chase conditions.

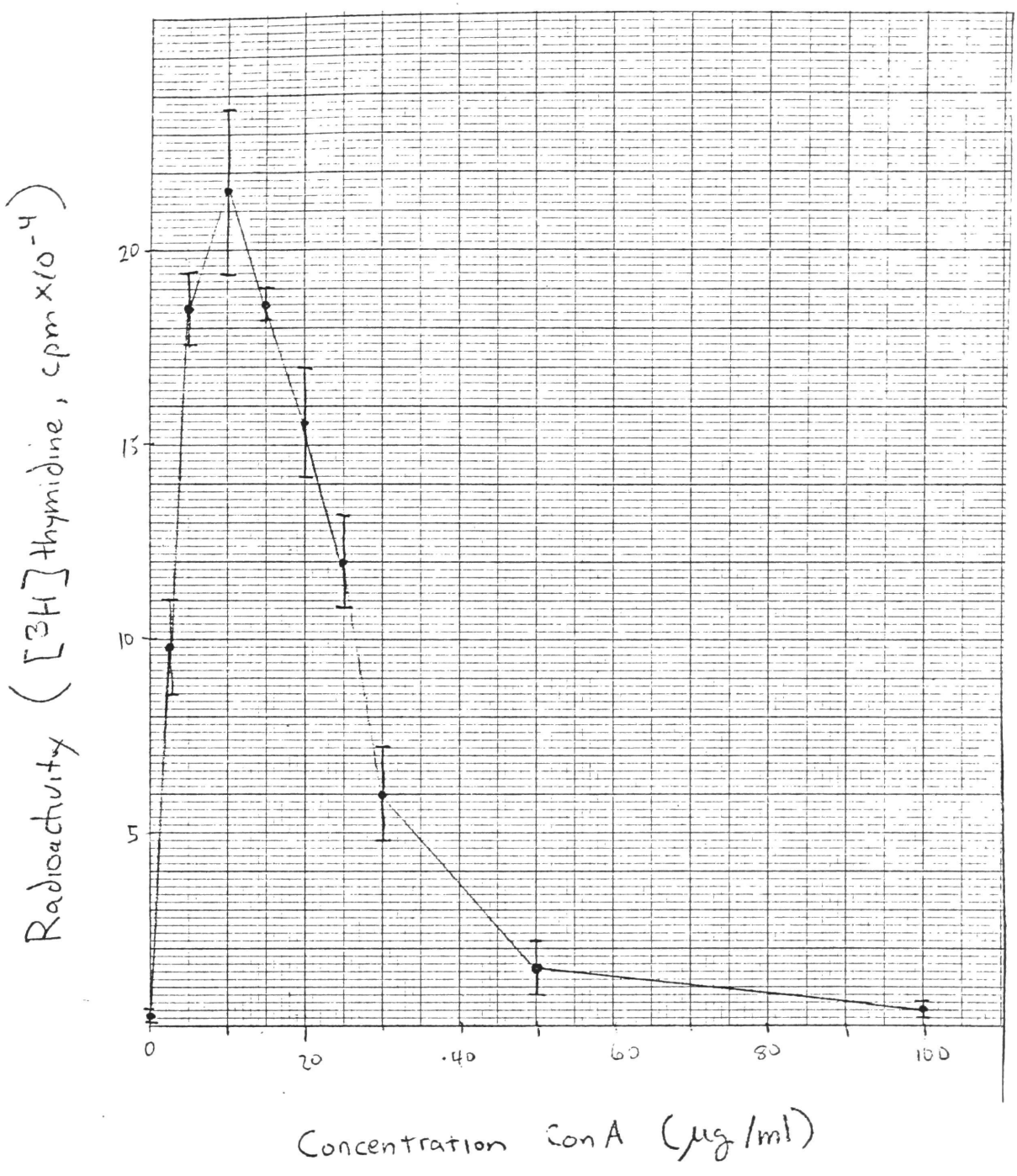

FIGURE 15 - Effects of various concentrations of Con A on thymidine incorporation into DNA by cultured human lymphocytes. 2.5 x 10^6 cells were preincubated for 24 hours in 1 ml MEM supplemented with 10% fetal calf serum, exposed to various concentration of Con A for 12 hours, and labeled for an additional 12 hours with 0.5 µCi [^{3}H]thymidine in the presence of the drug. The cells were harvested and radioactivity incorporated into DNA determined as described in MATERIALS AND METHODS. Each point is the average of 3 parallel cultures, and is plotted as the average ± S.E.M.

nucleus being about 0.33 to 1.00. After exposure to Con A for 4 hours, the amount of residual protein increased somewhat, the protein to DNA ratio increasing almost 2-fold to 0.58 to 1.00. Hemminki (1975) has observed increases of about this magnitude in the amount of non-histone protein associated with chromatin in human lymphocytes stimulated by leucoagglutinin. The increase in the amount of protein associated with chromatin in human lymphocytes is considerably less than the effect observed in equine lymphocytes, possibly because a smaller fraction of the human lymphocytes have been transformed, or possibly because there is increased cell death in the cultures of human lymphocytes.

Figure 16 shows the results of a typical experiment in which the turnover of prelabeled nuclear proteins is measured in Con A-stimulated and control human lymphocytes. It may be seen that Con A decreases the rate of turnover of the nuclear proteins, although an increase in the total ^{14}C label in the residual nuclear protein fraction, such as was observed in the experiments with phenol-soluble proteins of equine lymphocytes is not evident.

It appears that human lymphocytes behave similarly to equine lymphocytes, and accumulate nuclear proteins soon after stimulation by mitogens. The magnitude of the effect in the two systems differs considerably. The mechanism for the accumulation of nuclear proteins is unknown. The present data suggests that the increase is not due to new protein synthesis alone, and that the increase is due either to intracellular rearrangements of proteins, or accumulation due

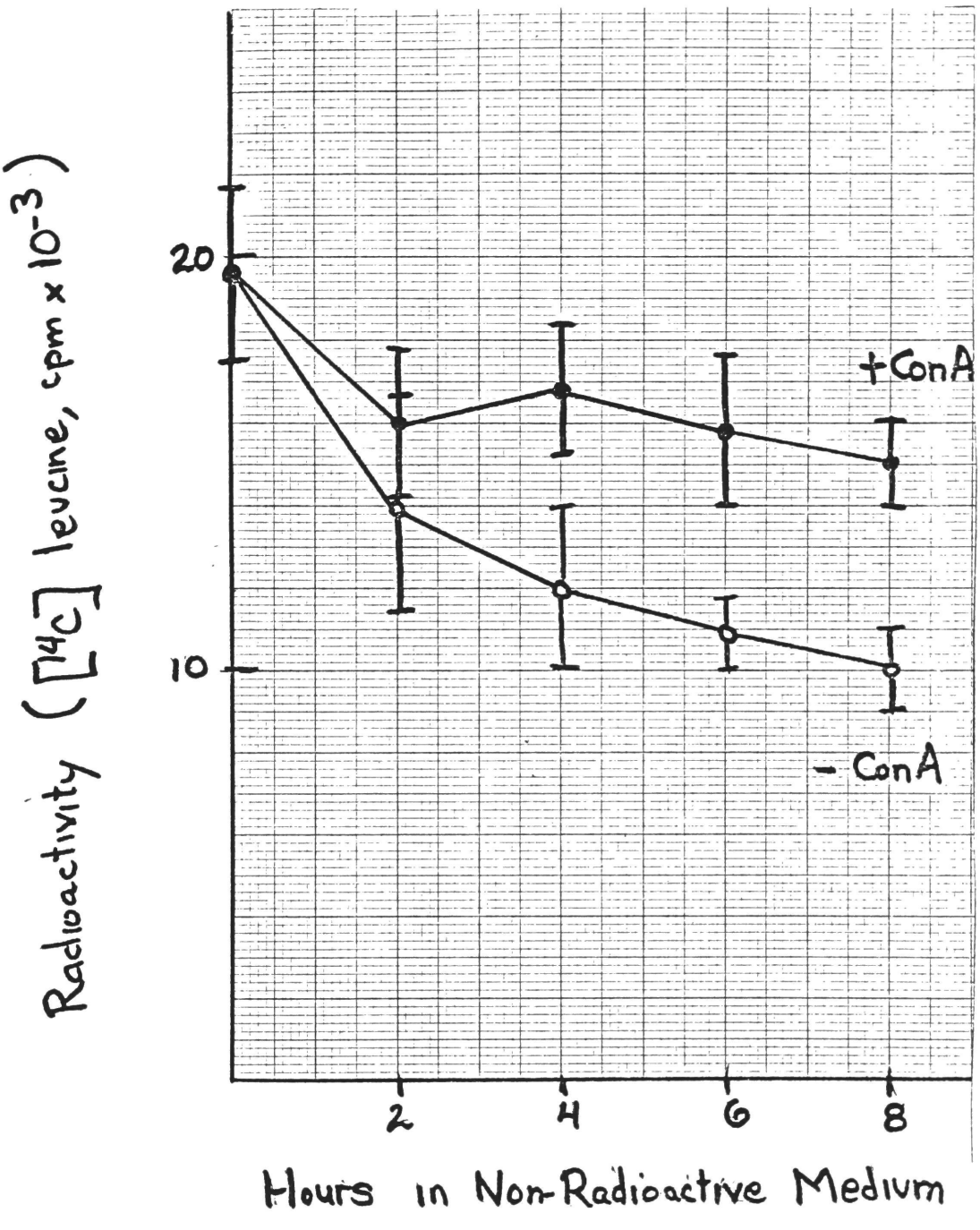

FIGURE 16 - Effects of Con A on turnover of human lymphocyte, nuclear residual proteins. Lymphocyte proteins were prelabeled in a 24 hour incubation with [^{14}C]leucine. After washing to remove the radioactive precursors, cells were cultured in 3 ml cultures containing 2.5 x10^6 cells per ml in nonradioactive medium in the presence or absence of 10µg/ml Con A. The radioactivity in the SDS-soluble nuclear proteins was monitored during the cold chase period as described in the text. Each point is the average of 3 parallel cultures, and is plotted as the average ± S.E.M. ●—● +Con A; O—O -ConA.

to changes in the rate of turnover. Ruddon (1974) has independantly observed increases in nuclear protein after mitogen stimulation of lymphocytes, and has suggested that lymphocytes accumulate nuclear proteins by decreasing the turnover rate of these proteins.

Differential Effects of Inhibitors of Protein Synthesis on the Synthesis of Nuclear and Cytoplasmic Proteins - In both HeLa cells and lymphocytes, specific non-histone nuclear proteins accumulate in the nucleus prior to DNA synthesis. The accumulation of these proteins is probably a complex event, involving changes in the turnover rates of proteins, transport of proteins from the cytoplasm and synthesis of new proteins. In order to learn whether the synthesis of non-histone nuclear proteins in HeLa cells is coordinated at the level of translation, we have tested the effects of a variety of inhibitors of protein synthesis on the synthesis of nuclear and cytoplasmic proteins.

Table X illustrates the effects of the antibiotic protein synthesis inhibitors pactamycin, puromycin and cycloheximide on the synthesis of total cellular proteins and acid-soluble and residual nuclear proteins.

Pactamycin, an inhibitor of protein chain initiation at low concentrations, and elongation at higher concentrations (Pestka, 1970) causes a marked inhibition of the incorporation of label into each of the protein fractions. However, the nuclear residual proteins seem to show some resistance to the drug, relative to the other protein fractions. For example, at a drug concentration of 0.5 μM, [^{3}H]-leucine incorporation

TABLE X : Effect of antibiotic protein synthesis inhibitors on the incorporation of [^{3}H]-leucine into nuclear and cytoplasmic proteins. 2 x 10^6 cells were harvested and incubated in 10 ml Earle's balanced saline in the presence of 2 μCi/ml (pactamycin) or 10 μCi/ml (puromycin and cycloheximide) L-[4,5-^{3}H]-leucine in the presence or absence of antibiotic at the indicated concentrations. The cells were harvested after 20 minutes by centrifugation and fractionated as described under "Materials and Methods." Data is expressed as the average of 3 parallel-culture experiments.

Drug Conc.	Cytoplasmic Protein cpm	%	Nuclear Protein Acid-Soluble cpm	%	Residual cpm	%
I. Pactamycin						
0	69,400 ± 2,400	100	26,300 ± 100	100	7,000 ± 800	100
0.01 μM	57,100 ± 1,200	82	20,600 ± 800	78	7,400 ± 400	105
0.05 μM	28,900 ± 200	42	10,600 ± 300	40	4,200 ± 200	59
0.1 μM	16,300 ± 200	24	5,200 ± 600	20	2,200 ± 700	31
0.5 μM	5,900 ± 300	8	1,300 ± 600	5	1,000 ± 300	15
1.0 μM	4,700 ± 100	7	900 ± 200	3	900 ± 50	13
II, Puromycin						
0	238,600 ± 4,000	100	77,400 ± 1,145	100	36,000 ± 600	100
0.5 μM	243,900 ± 5,200	102	78,600 ± 800	102	35,300 ± 900	98
2.5 μM	199,900 ± 2,200	84	67,500 ± 1,200	87	24,000 ± 1,600	67
5.0 μM	160,900 ± 3,000	67	58,300 ± 1,000	75	17,800 ± 400	50
25 μM	66,000 ± 800	28	18,600 ± 300	24	4,900 ± 100	14
50 μM	28,000 ± 500	12	5,300 ± 200	7	1,400 ± 300	4
III. Cycloheximide						
0	238,900 ± 7,000	100	76,600 ± 900	100	37,000 ± 1,300	100
0.50 μM	155,700 ± 12,000	65	58,000 ± 1,400	76	23,400 ± 400	62
2.5 μM	87,000 ± 2,500	36	28,000 ± 400	36	10,000 ± 300	27
5.0 μM	60,800 ± 400	25	17,830 ± 200	23	6,630 ± 800	18
25 μM	31,800 ± 200	13	8,800 ± 100	11	2,800 ± 200	8
50 μM	27,200 ± 600	11	6,400 ± 700	8	1,800 ± 200	5

into nuclear residual proteins was 14.5 ± 0.5 % of the control values, as compared with 8.2 ± 0.3 % for total cellular proteins and only 4.7 ± 0.1% for histones. Puromycin and cycloheximide, both of which interfer with elongation (Pestka, 1970) also caused marked inhibition of the synthesis of the various protein fractions. In contrast to the effect of pactamycin, the nuclear residual proteins appear to be more sensitive than cytoplasmic proteins to elongation inhibitions by puromycin or cycloheximide over a wide range of inhibitor concentrations. For example, at 25 μM puromycin, [^{3}H]-leucine uptake into the non-histone nuclear proteins is 13.6 ± 0.3% of the control values; the corresponding figure for cytoplasmic proteins is 27.9 ± 0.3%. At 50 μM cycloheximide, [^{3}H]-leucine uptake into the nuclear non-histone proteins is only 4.9 ± 0.5% of the uptake observed in control cells, whereas cytoplasmic protein radioactivity is 11.4 ± 0.6% of the corresponding control value.

Dramatic evidence for differential sensitivity of nuclear proteins to protein synthesis inhibitors was obtained with the chloromethylketone derivative, TPCK (L-1-tosylamido-2 phenylethyl chloromethyl ketone). TPCK was originally introduced as a reagent for the selective inhibition of chymotrypic activity (Shaw, 1970). It has since been shown to inhibit the post-translational cleavage of long polypeptide chains synthesized on polycistronic viral messenger RNAs (Summers *et al.*, 1972; Pfefferkorn and Boyle, 1972). More recently, it has been found that TPCK acts as a potent and specific inhibitor of the initiation step in the biosynthesis of proteins

(Pong et al., 1975).

Figure 17 compares the effects of 25 µg/ml TPCK on the incorporation of [^{3}H]-leucine into total cellular proteins (mainly of cytoplasmic origin) and into two nuclear protein fractions : the acid-soluble proteins (mainly histones) and the residual non-histone protein fraction. Leucine uptake into the cytoplasmic proteins ceases after about 5 minutes in TPCK-treated cells, but proceeds actively in control cells (figure 17A). Similar kinetics of inhibition of cellular protein synthesis by TPCK have been reported by Taber et al. (1973) and Pong et al. (1975). Lower concentrations of TPCK have progresssively diminishing effect on [^{3}H]-leucine uptake (Taber et al. 1973).

Figure 17 B shows the effects of 25 µg/ml TPCK on the incorporation of [^{3}H]-leucine into the acid-soluble nuclear proteins. It is clear that TPCK inhibits the synthesis of histones, even at early times. In contrast, the uptake of [^{3}H]-leucine into the nuclear non-histone proteins shows a striking resistance to TPCK (figure 17C). No significant inhibition is observed until 15 minutes after addition of the drug, after which time the incorporation begins to level off. After 20 minutes there was a 20 to 30% inhibition of [^{3}H]leucine incorporation. The effects were highly reproducible in replicate experiments using [^{3}H]-leucine and identical kinetics were observed in parallel experiments in which L-[methyl - ^{3}H]-methionine was the precursor.

This differential sensitivity of TPCK makes it possible to selectively label this fraction of nuclear proteins by

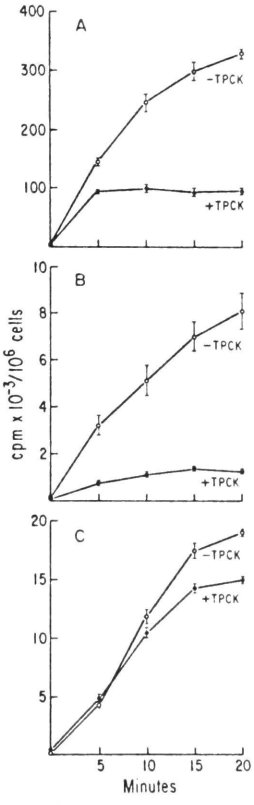

FIG. 17 Comparative kinetics of amino-acid incorporation into cytoplasmic and nuclear proteins of control and TPCK-treated HeLa S-3 cells. [^{3}H]Leucine uptake is plotted versus time for (A) total cellular proteins (mainly cytoplasmic); (B) acid-soluble nuclear proteins (mainly histones); and (C) nuclear residual (non-histone) proteins, in control (O——O) and TPCK-treated (●——●) cells. Points represent the average of three determinations. Error bars show the standard deviation from the mean. The failure of TPCK to inhibit amino-acid uptake into nuclear non-histone proteins at early times is evident under these conditions, and has been confirmed in HeLa S-3 cells growing in Joklik-modified Eagle's minimal essential medium.

exposing cells to TPCK for 5 minutes prior to addition of the radioactive amino acids. The nuclear non-histone proteins continue to incorporate amino acids after uptake into the histone and cytoplasmic proteins has effectively ceased.

Does TPCK Affect the Size or Rate of Synthesis of New Synthesized Proteins ? - In viral systems involving polycistronic messenger RNAs such as poliovirus, TPCK prevents cleavage of newly synthesized polypeptide chains and high molecular weight precursors of the viral proteins accumulate (Summers et al., 1972; Korant, 1972). We have tested for corresponding effects of TPCK on the processing of nuclear and cytoplasmic proteins of HeLa cells by comparing the molecular weight distributions of newly synthesized proteins from TPCK-treated cells (incubated with [^{3}H]-leucine) with those of control cells (incubated with [^{14}C]-leucine). After 20 minutes incubation, both cultures were combined prior to extraction of protein fractions, which were then separated by electrophoresis in SDS-polyacrylamide gels. The ^{3}H to ^{14}C ratio of each band in the gel serves as an indication of the relative rates of synthesis and accumulation of proteins of the indicated molecular weight in the presence and absence of TPCK. The distribution of ^{3}H and ^{14}C activities and the corresponding isotope ratios are plotted in Figure 18. In the case of the total cellular proteins (mainly cytoplasmic in origin) the ratio increases from 3 to 1 for proteins of molecular weight less than 90,000 to 6 to 1 for proteins of higher molecular weight. Similar results have been reported by Taber et al., who interpreted

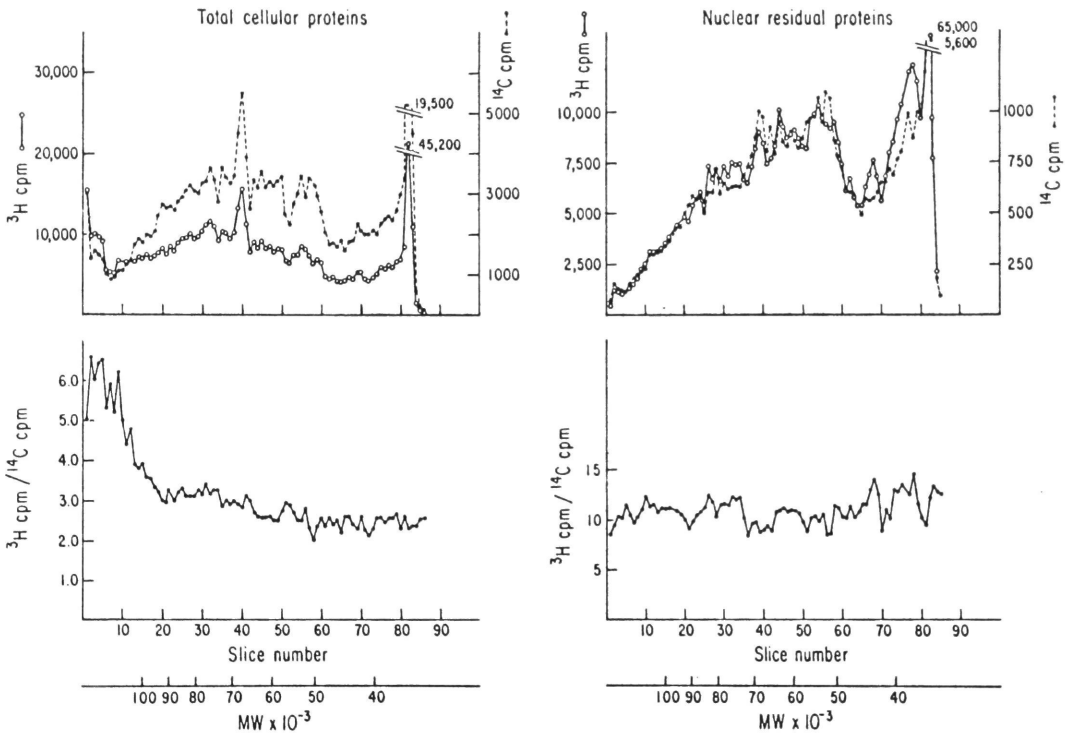

FIG. 18 Size distribution of newly synthesized cytoplasmic and nuclear proteins of control and TPCK-treated HeLa S-3 cells. Control cells (●- - - - -●) were incubated for 20 min with 2.5 μCi/ml of [^{14}C]leucine; cells, exposed to 25 μg/ml of TPCK (○——○), received 10 μCi/ml of [^{3}H]leucine for 20 min. The cell suspensions were combined prior to extraction and electrophoretic analysis on $NaDodSO_4$-polyacrylamide gels of the total cellular proteins (left-hand panels) and of the nuclear non-histone proteins (right-hand panels). The distributions of ^{3}H and ^{14}C activities (upper panels) and the corresponding ^{3}H:^{14}C ratios (lower panels) are plotted as a function of protein mobility (molecular weight). Note that TPCK alters [^{3}H]leucine incorporation to favor labeling of cytoplasmic proteins of molecular weight greater than 90,000, but TPCK does not correspondingly shift the pattern of labeling of nuclear non-histone proteins.

this to be the result of inhibition of proteolysis and Pong et al. who argued on the basis of a Dintzis-type experiment that the accumulation of radioactivity in high molecular weight proteins resulted from disproportionate labeling of higher molecular weight proteins which continue to elongate on long messenger RNAs after smaller mRNAs have run off the ribosomes.

In contrast to the results obtained with cytoplasmic proteins, the nuclear residual proteins do not show such a preferential labeling of the high molecular weight fractions of TPCK-treated cells, i.e., the ratio of [^{3}H]-leucine uptake into the non-histone nuclear proteins of TPCK-treated cells to [^{14}C]-leucine uptake into the nuclear proteins of control cells remains relatively constant throughout the entire gel (Figure 18). This result, taken together with the evidence that the synthesis of the nuclear non-histone proteins is resistant to TPCK (Figure 17), indicates that the entire molecular weight range of nuclear non-histone proteins continues to incorporate radioactive amino acids in the presence of TPCK regardless of the final chain length.

An important distinction exists between the histones and the non-histone nuclear proteins in this regard. When the labeling patterns of individual histones are compared in control and TPCK treated cells, the results (Figure 19) show a suppression of synthesis of all histone classes.

TPCK Effects on Labeling of Polysome Associated Proteins - An attempt was made to isolate polysomes involved in the synthesis of nuclear non-histone proteins based on

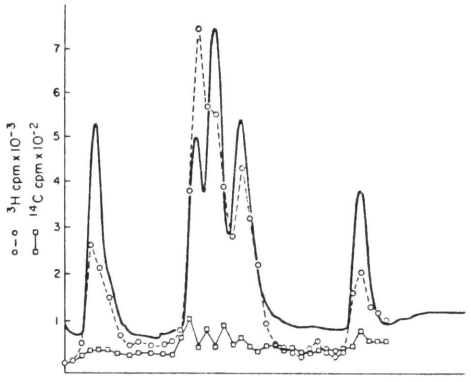

FIG. 19. Inhibition of histone synthesis by TPCK. HeLa S-3 cells were continuously labeled with [^{3}H]leucine (control) or with [^{14}C]leucine (TPCK-treated cells) as indicated in the legend for Fig. 2. The cultures were combined prior to extraction and electrophoretic separation of the nuclear histones. The direction of migration is from right to left. The densitometric tracing of the histone banding pattern is shown by the heavy solid line. The major histone classes migrate in the sequence: H4 (f2a1); H2a (f2a2); H2b (f2b); H3 (f3); H1 (f1). [^{3}H]leucine uptake (O- - - -O) and [^{14}C]leucine uptake (□——□) are plotted as a function of histone mobility.

their expected resistance to TPCK inhibition. HeLa cells were incubated in the presence of TPCK for 5 minutes and then pulse-labeled for 2 minutes with [^{3}H]-leucine. Under these conditions, the polysomes synthesizing nuclear non-histone proteins should be labeled preferentially. Figure 20 shows the distribution of radioactivity in the free cytoplasmic polysomes of control and TPCK-treated cells. In both cases, the drug causes an extensive disaggregation of large polysomes into monosomes and a corresponding loss of radioactive nascent chains from the polysome region of the gradient.

In control cells, very large polysomes could be collected at the interface between the sucrose gradient and at 70% sucrose cushion; no such fraction was recovered from the TPCK-treated cells. Any pelleted polysomes were also sensitive to TPCK inhibition (Figure 20). We examined polysomes remaining with the nuclear fraction, extracting them in a mixture of Tween 80 and sodium deoxycholate (Penman, 1969). The polysomes from the detergent-treated nuclei showed the same sensitivity to TPCK as did the majority of the cellular polysomes. Thus, attempts to isolate a special class of TPCK-resistant polysomes have so far proven negative; this is probably because such polysomes exist in very low concentrations.

Inhibitors of polypeptide chain initiation, TPCK in particular, can be used to discriminate between the synthesis of nuclear non-histone proteins and cytoplasmic proteins. Why this discrimination takes place is not yet clear,

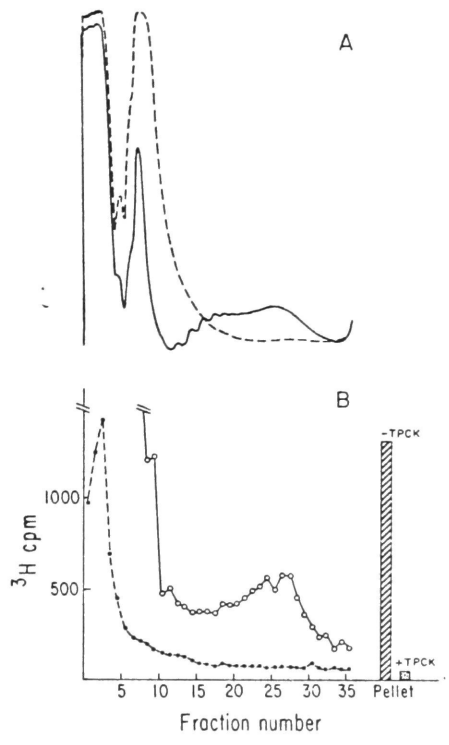

FIG. 20 Effects of TPCK on size and [^{3}H]leucine activity of HeLa polysomes. The polysomes of control cells and of cells exposed to TPCK for 5 min before pulse-labeling for 2 min with [^{3}H]leucine were analyzed on 15–40% sucrose density gradients. (A) The distribution of polysomes along the gradient in control (solid line) and TPCK-treated cells (dashed line). The direction of sedimentation is from left to right; (B) The distribution of radioactivity in polysome-associated proteins of control (O——O) and TPCK-treated cells (●- - - - - -●). The histogram shows that TPCK also inhibits [^{3}H]leucine incorporation into the pellet recovered from the bottom of the gradient.

although several possibilities deserve mention: 1) Nuclear non-histone proteins may be synthesized on polycistronic messenger RNAs and large precursor protein(s) may be subsequently cleaved. (Thus, protection against initiation inhibitors is a consequence of the size of the message). 2) Initiation factors required for the synthesis of nuclear proteins may not be effected by TPCK, whereas initiation factors for histones and cytoplasmic protein synthesis may be inactivated by this reagent. 3) The sites of synthesis of the nuclear non-histone proteins may not be as accessible to TPCK as most of the cytoplasmic polysomes are.(In view of the small size of TPCK and the greater sensitivity of nuclear protein synthesis to puromycin and cycloheximide, this seems unlikely.) 4) In a competition between messenger RNAs for factors required for protein chain initiation (or ribosomes), the messengers for nuclear non-histone proteins may be more efficient than mRNAs for cytoplasmic proteins.

The use of TPCK to selectively block amino acid incorporation into histones and cytoplasmic proteins offers a new range of possibilities for the study of the synthesis, processing and translocation of chromosomal and DNA-binding proteins.

CHAPTER THREE : SUBSETS OF NON-HISTONE NUCLEAR PROTEINS ASSOCIATED WITH RADIPLY LABELED HIGH MOLECULAR WEIGHT RNA

In the previous chapter, the nuclear proteins were separated into three solubility classes: 0.14 M NaCl-soluble, acid-soluble, and residual. Many of the residual proteins, which may be solubilized in either SDS or phenol, are tightly associated with chromatin in the nucleus. Proteins in this fraction, particularly proteins with DNA-binding capacity, have been intensively studied as possible gene regulators in eukaryotes (Allfrey, 1974). The experiments described in this chapter illustrate that in addition to DNA-binding proteins, the residual non-histone nuclear proteins contain a number of RNA-binding proteins.

Although the existence of nuclear RNA-binding proteins has been known for some time (see Georgiev and Samarina, 1971 for review), the precise number of these proteins and their chemistry has not been clearly defined. Nearly 10% of the non-histone nuclear proteins of rat liver nuclei are isolatable in the form of a ribonucleoprotein particle that sediments at about 40S [1] ("Informosomes") . This chapter presents a detailed analysis of the physical properties of the ribonucleo-

1 Estimates of the sedimentation coefficient of the nuclear ribonucleoprotein particles range from 30S to 50S in the literature. The best data, based on analytical ultracentrifugatio studies, is from Faiferman et al. who obtained values of about 40S for the size of these complexes. In this chapter we will refer to ribonucleoprotein particles of this size as 40S RNP.

protein particle, and its associated proteins. This system offers several advantages in the investigation of nuclear proteins at a molecular level. The RNA-binding proteins of the cell nucleus are a limited subset of proteins, so that they may be characterized on an individual basis. The proteins may be isolated under non-denaturing conditions, so that some aspects of their in vivo functions may be duplicated in vitro. Since these proteins are found in large amounts in the nuclei of animal cells, they may be obtained in sufficient quantities to begin an investigation of their chemistry. Finally, the association of these proteins with rapidly labeled high molecular weight RNA of DNA-like base composition (HnRNA) suggests possible physiological roles for these proteins in the synthesis, processing and transport of HnRNA.

Cytological studies provided the first evidence that HnRNA might be associated with proteins in the nucleus (Gall, 1956; Callan and Lloyd, 1960; Swift, 1963; Stevens and Swift, 1966; Monneron and Bernhard, 1969). These early observations have been extended by detailed electron microscopic observations of nuclei differentially stained to detect RNA and protein components by Bernhard and his colleagues. (Puvion and Bernhard, 1975; Monneron and Bernhard, 1969). The recent visualization of presumptive HnRNA transcripts as ribonucleoprotein fibrils in spreads of HeLa cell and amphibian chromatin provides further evidence for the hypothesis that specific proteins may associate with nascent HnRNA chains in the interphase nucleus. (Miller and Bakken, 1972; Hamkalo et al.,

1973).

Biochemical investigation of nuclear RNA -protein complexes were initiated by Georgiev and his colleagues in the Soviet Union (Georgiev and Samarina, 1971). They reported that most of the nuclear RNA of DNA-like base composition may be extracted from the nuclei of animal cells in the form of specific ribonucleoprotein complexes (RNP). When a ribonuclease inhibitor is present these complexes are obtained as large heterodisperse structures, with sedimentation values as high as 200S. After mild RNase treatment (or after extraction in the absence of RNase inhibitor), all the RNP sediments at about 40S. Since virtually all the RNA may be recovered in these 40S particles after ribonuclease treatment, it was proposed that these particles represent a monomeric degradation product of a larger structure containing HnRNA and protein. Recently, it has been observed that the poly A region of the HnRNA is complexed with a distinct set of proteins. Upon degradation of ribonucleoprotein complexes, the poly A is released in a structure that sediments at about 15S (Billings et al., 1974; Samarina et al., 1974). This fraction is enriched in a protein of about 78,000 daltons. A similar, perhaps identical, protein has been found in association with poly A from messenger RNA released from cytoplasmic polysomes by dissociation with puromycin or EDTA (Blobel, 1970; Schwartz and Darnell, 1976).

Controversy has arisen about the nature of the proteins found in the nuclear 40S RNP particle. Georgiev and coworkers initially reported that a single protein of molecular weight

40,000 daltons was present in the complex. Their data was based largely on the observation that protein from rat liver 40S particles, and similar RNP preparations from other animal tissues, migrates as a single band in acid-urea gels using the buffer system of Reisfeld (Samarina et al., 1968) However, other investigators have observed heterogeneous banding patterns from similar preparations on SDS-polyacrylamide gels (Niessing and Sekeris, 1971; Albrecht and Van Zyl, 1973; Faiferman et al., 1971; Martin et al., 1973). Pederson (1974) has prepared RNP fractions from HeLa cells, and other tissue culture cell lines, by sonication of the nuclei, and has observed heterogeneous, cell-specific proteins. He therefore proposed that HnRNA molecules that differ in nucleotide sequence are complexed with different sets of proteins.

This chapter presents evidence that nuclear 40S particles contain a limited subset of basic proteins, with distinctive chemical properties. Homologous structures appear to be present in ribonucleoprotein isolated from the nuclei of a variety of tissues, including HnRNP isolatable from HeLa cells by the methods of Pederson (1974).

MATERIALS AND METHODS

Preparation of Rat Liver Nuclei - Nuclei were prepared from 100 - 115 gm (wet weight) portions of rat liver. Both male and female rats of the Charles River or Sprague-Dawley strains were used in this study. The animals were maintained on standard laboratory feed, and allowed to eat ad libitum before

sacrifice. Animals were sacrificed by cervical dislocation and decapitated. The livers were quickly excised, and placed in ice-cold 0.32 M sucrose, 3 mM $MgCl_2$. All subsequent operations were carried out at 0 - 4 ^{o}C. The rinsed livers were drained of excess fluid, freed from remaining connective tissue, weighed and minced with scissors to approximately 3 mm^3 pieces. The minced tissue was homogenized in 550 ml 0.32 M sucrose, 3 mM $MgCl_2$ with 20 strokes of a motor-driven glass-Teflon homogenizer (180 ml capacity, 170 mm grinding length, 75 mm pestle head, and a clearance of 0.15 to 0.22 mm; Arthur H. Thomas, Inc., Philadelphia, Pennsylvania), rotating at 1560 rpm. The homogenate was filtered through 4 layers of cheesecloth, transferred to 180 ml centrifuge tubes, and a crude nuclear fraction was pelleted at 1,100 x g for 15 minutes. The supernatant was decanted by aspiration and the nuclear pellets were resuspended in 2.2 M sucrose, 5 mM $MgCl_2$ to a final volume of 360 ml. Resuspension of the crude nuclear pellets was facilitated by homogenizing the nuclear suspension in heavy sucrose by hand with two or three strokes of a Teflon pestle in a loose homogenizer. (A suitable homogenizer can be constructed using a 100 ml graduated cylinder, i.d. 290 mm, and a Teflon pestle from a 55 ml capacity Thomas homogenizer.) The nuclear suspensions were layered over 10 ml 2.2 M sucrose, 5 mM $MgCl_2$ in 1" x 3½" cellulose nitrate tubes (Beckman Instruments), the interphase was mixed with a spatula, and the nuclei were sedimented at 25,000 rpm for 60 minutes in a SW 27 rotor (Beckman Instruments). The purified nuclear pellets contained 40 to 60% of the DNA

in the original liver homogenate. Typically, 10^{10} nuclei were prepared from 100 gm (wet weight) of liver. In some preparations rat liver RNA and protein were labeled *in vivo* with the following precursors : orotic acid [5 -^{3}H] (17.8 Ci/ mM, New England Nuclear, Boston, Mass.) L-[4,5-^{3}H] leucine (50 Ci/ mM, New England Nuclear), [^{32}P]-orthophosphate, carrier-free (New England Nuclear). Isotopes were administered by interperitoneal injections using the schedules indicated in the legends to the figures.

Preparation of Duck Liver Nuclei - Nuclei were prepared from the livers of 5 month old Pekin ducks, essentially as described for rat liver nuclei. One animal is sufficient for a single preparation of nuclei. The animals were exsanguinated by bleeding from the jugular vein, decapitated, and the livers removed. Care must be taken to avoid rupture of the gall baldder when removing the liver. The connective tissue was removed, and the livers weighed, rinsed in 0.32 M sucrose, 3 mM $MgCl_2$, and minced using a Rival meat grinder (Rival, Inc,, New York). The ground tissue (100 gm) was homogenized in 550 ml 0.32 M sucrose, 3 mM $MgCl_2$ with 20 strokes of a motor-driven glass-Teflon homogenizer as described above. The homogenate was filtered through 4 layers of cheesecloth and crude nuclear pellets were prepared by centrifugation at 1,100 x g for 15 minutes. The crude nuclear pellets were resuspended in 500 ml 0.32 M sucrose, 3 mM $MgCl_2$ and homogenized with an additional 20 strokes. After the second homogenization, nearly all the nuclei appeared free of gross cytoplasmic contamination. Crude nuclear pellets were

prepared as before, and repurified by centrifugation through heavy sucrose as described above.

Preparation of Rat and Duck Liver Ribonucleoprotein Particles - Ribonucleoprotein particles were prepared from nuclei by one of four procedures: extraction with hypotonic buffers; sonication; hypotonic lysis; and hypertonic lysis.

The extraction procedure is essentially that of Samarina et al. (1968). Nuclear pellets from the sucrose gradients, containing a total of 10^{10} nuclei were resuspended in 60 ml 0.1 M NaCl, 0.01 M triethanolamine-HCl, pH 7.0, 1 mM $MgCl_2$. (Dispersion of the nuclei with a spatula avoids lysis which may occur if nuclei are aspirated into a pipette or dispersed by strong agitation of the suspensions.) The nuclei were pelleted by low-speed centrifugation (800 x g, 5 minutes) and the washing procedure repeated two more times. The nuclear pellets were washed one time in 60 ml 0.1 M NaCl, 0.01 M triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$, and resuspended in 4.5 ml of the pH 8.0 buffer. The nuclear suspensions were gently stirred for 30 minutes. The nuclei were pelleted by low-speed centrifugation, and resuspended in pH 8.0 buffer, as before, for a total of four extractions. We have found it convenient to stir the nuclear suspensions during the extraction in 30 ml glass centrifuge tubes mounted over a magnetic stirrer. The suspensions should be stirred at a speed just sufficient to prevent settling of the nuclei. If violent agitation is avoided during the nuclear extraction, and the nuclei pelleted at low speed, the nuclei will remain intact throughout the extraction procedure, and no clumping

of nuclei resulting from the extrusion of a nucleohistone gel will be observed. Lysis of the nuclei during the extraction results in drastic reductions in the yields of ribonucleoprotein particles and in contamination of the preparations with high molecular weight nuclear acidic proteins. When preparing ribonucleoprotein particles from smaller quantities of nuclei, the volumes of extraction and washing buffers were reduced proportionately. The nuclear extracts were combined, clarified by centrifugation at 2,000 x g for 10 minutes (to remove chromatin and nuclei) and applied to sucrose gradients as described below.

Ribonucleoprotein particles were prepared by sonication as follows : 5 to 10 x 10^8 nuclei, washed as described above were resuspended in 4.5 ml 0.1 M NaCl, 0.01 M triethanol-amine-HCl, pH 8.0, 1 mM $MgCl_2$, and disrupted with approximately five 20-second bursts at 40 watts output of a Branson sonifier using a microtip probe. The nuclei were cooled on an ice-bath during the sonication. Sonication was continued until no intact nuclei were observed by phase contrast microscopy. Since the sonication procedure is effected by the geometry of the sonication vessel, the volume of the nuclear suspension, and the concentration of nuclei, it is necessary to continuously monitor nuclear fragmentation during the sonication procedure by phase-contrast microscopy. Extensive sonication results in disruption of the nucleoli, and in contamination of the ribonucleoprotein preparations with nucleolar proteins; too little sonication reduces the yields. The sonicate was layered over 35 ml 0.88 M sucrose in 0.1 M NaCl, 0.01 M

triethanolamine-HCl, pH 8.0, and spun for 15 minutes at 27,000 rpm in a SW 27 rotor to pellet nucleoli and most of the chromatin. If nuclei are sonicated in extremely hypotonic buffers, such as described by Pederson (1974), the chromatin in solubilized, and will not pellet through 0.88 M sucrose at low speed.

RNP-particles were prepared by hypotonic lysis as follows: 5 to 10 x 10^8 nuclei, washed as described, were resuspended in 10 ml 10 mM tris-HCl, 10 mM EDTA, pH 8.0, and allowed to swell for 10 minutes at 0 ^{o}C. The swollen nuclei were pelleted by low-speed centrifugation (800 x g for 5 minutes) and gently resuspended in 4.0 ml 10 mM tris-HCl, pH 8.0. The nuclear suspension was transfered to a 7 ml Dounce homogenizer (Kontes Glass, Inc.), and allowed to swell for an additional 10 minutes at 0 ^{o}C. The swollen nuclei were lysed by homogenizing the suspension with 3 strokes of the loosely fitting pestle. When the nuclei lyse, the suspension becomes an extremely viscous nucleohistone gel. Nuclei in these hypotonic buffers are extremely fragile, and will lyse prematurely during resuspension unless care is taken to handle them gently. High yields of particles can only be obtained if the nuclei are lysed uniformly. The viscosity of the nuclear lysate was reduced by DNase-treatment as follows : 0.5 ml 20 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, containing 200 µg electrophoretically-purified DNase I (Worthington; treated with iodoacetate to inactivate ribonuclease, Zimmerman and Sandeen, 1966), was added to the nuclear lysate, mixed by homogenization using a few strokes of the loosely

fitting pestle, and allowed to incubate for 10 minutes at 0 ^{o}C. A low-speed supernatant fraction was prepared as described for the sonicated nuclei, by centrifugation over cushions of 0.88 M sucrose, and the ribonucleoprotein particles were further purified by centrifugation on sucrose gradients, as described below.

Hypertonic lysis of nuclei to obtain ribonucleoprotein particles results in reduced and variable yields, because of the salt-lability of these RNP complexes. Five to 10 x 10^{8} nuclei, washed as described, were resuspended in 2.0 ml of 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$. The suspensions were rapidly stirred on a vortex mixer and KCl added dropwise for a 1 M stock solution until lysis of the nuclei was observed. The nuclei usually lyse when the KCl molarity is 0.35 M or more. Care must be taken to mantain the KCl molarity below 0.5 M. The lysate was immediately diluted with 1 volume of 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$. The viscosity of the suspension was reduced by adding 200 μg of DNase, and incubating for 10 minutes at 0 ^{o}C. The DNase can be rapidly mixed with the nuclear lysate by brief homogenization with the loosely fitting pestle in a Dounce homogenizer. We have not been successful when divalent cations (Ca^{++} or Mg^{++}) are used in place of monovalent cations (K^{+} or Na^{+}) for the nuclear lysis. A low-speed supernatant fraction was prepared by centrifugation of the lysate over cushions of 0.88 M sucrose, as described. Ribonucleoprotein particles were further purified by centrifugation in sucrose gradients as described below.

Ribonucleoprotein particles were purified from nuclear

extracts and low-speed supernatant fractions of nuclear lysates by centrifugation on linear 15 - 30% sucrose gradients in 100 mM NaCl, 10 mM triethanolamine-HCl, pH 8.0 (or 10 mM Tris-HCl, pH 8.0), 1 mM $MgCl_2$. Three ml of nuclear extract were applied to 39 ml gradients and spun at 23,000 rpm for 15 hours using an SW 27 rotor. The gradients were collected by displacement with 60% sucrose using an Isco gradient collection apparatus, and the absorbancy was monitored at 254 nm with an LKB Uvicord II spectrophotometer. Under these conditions, the ribonucleoprotein particles appeared as a discrete peak of optical density sedimenting in the center of the sucrose gradients (at about 40S). Fractions containing the particles were pooled on the basis of the optical density profiles. When radioactivity was present in the samples, fractions of 0.2 to 0.4 ml were collected, and aliquots monitored for TCA-precipitable radioactivity as described below. Typically, 50 absorbancy units of ribonucleoprotein particles can be obtained from extracts of 10^{10} nuclei.

Isotopic Labeling and Fractionation of HeLa Cells - HeLa cells were maintained in suspension culture at 2 - 4 x 10^5 cells per ml by daily dilution with Joklik-modified MEM supplemented with 5% fetal calf serum. Cells were labeled in the presence of low doses of actinomycin D (to inhibit ribosomal RNA synthesis) as follows : 2 x 10^8 cells were harvested by centrifugation at 1.500 x g for 10 minutes. When cells were to be labeled with [^{32}P]-orthophosphate, the cells were washed 3 times in 200 ml Joklik - modified MEM containing 0.1 mM PO_4. The cells

were resuspended in 100 ml Joklik-modified MEM containing 0.1 mM PO_4, and dialyzed fetal calf serum was added to a final concentration of 5%. Actinomycin D was added to a final concentration of 0.05 μg/ml and the cells were incubated with stirring for 30 minutes at 37 °C. After this preincubation period, 20 mCi [^{32}P]-orthophosphate was added and the cell suspension was stirred for an additional 2 hours. At the end of the incubation period, cells were harvested by centrifugation at 1.500 x g for 10 minutes and fractionated as described below. When [^{3}H]-uridine was the label, cells were resuspended in 100 ml Joklik-modified MEM, 5% fetal calf serum without washing. Cells were preincubated with actinomycin D, labeled for 2 hours with 2.0 mCi [^{3}H]-uridine, and harvested by centrifugation at 1,500 x g.

The cells labeled with either [^{3}H]-uridine or [^{32}P]-orthophosphate were washed 3 times with 70 ml phosphate buffered saline, then resuspended in 30 ml 1.5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.2, 10 mM NaCl, and allowed to swell for 10 minutes. The cells were disrupted with 20 strokes of a Dounce homogenizer using a tightly fitting pestle (Kontes, Inc.). Nuclei were pelleted by centrifugation at 1,000 x g for 10 minutes. The crude nuclear pellets were washed three times in 30 ml 1.5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.2, 10 mM NaCl, and fractionated as described below. In some experiments, the nuclear pellets were purified by resedimentation through cushions of 2.2 M sucrose, 5 mM $MgCl_2$, and then fractionated as described below.

Polysomes were isolated from a post-mitochondrial supernatant of the cytoplasmic fraction. The post-nuclear supernatant was centrifuged at 10,000 x g for 15 minutes and aliquots of 2 ml were applied to linear 15-40% sucrose gradients in 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, and centrifuged at 25,000 rpm for 150 minutes in an SW 27 rotor. The gradients containing the polysomes were collected by upward displacement, as before, and the regions from each tube containing 3-8 mers were pooled.

The nuclear fractionation procedure was essentially that of Pederson (1974). Nuclei from 2 x 10^8 cells were resuspended in 5 ml 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 10 mM NaCl, and disrupted by brief sonication. A crude nucleolar fraction was prepared by centrifuging the sonicate through 0.88 M sucrose in sonication buffer. Nucleoli, unbroken nuclei, heterochromatin, and cell debris pellet under these conditions. The material remaining on top of the sucrose was removed and fractionated by centrifugation through a discontinuous sucrose gradient (Kish and Pederson, 1975) composed of layers containing 25.0 ml 60% sucrose, 2.0 ml 45% sucrose, and 8.0 ml 10% sucrose, all in 10 mM Tris-HCl, pH 7.2, 10mM NaCl, 1.5 mM $MgCl_2$. Centrifugation was for 90 minutes at 26,000 rpm in a Beckman SW 27 rotor. HnRNP particles are recovered as an opalescent band spanning the 45% sucrose layer. Chromatin was recoverable as a pellet in the bottom of the tube. The RNP fraction recovered in this manner was analyzed directly by SDS-polyacrylamide gel electrophoresis, or partially digested with ribonuclease, and recentrifuged on sucrose gradients as described below.

Ribonuclease Digestion Experiments - [^{3}H]-uridine or [^{32}P]-phosphate labeled RNP isolated from HeLa cells were partially digested with pancreatic ribonuclease, and the digestion products were analyzed by sucrose gradient centrifugation. RNP recovered from step gradients was dialysed against 0.1 M NaCl, 10 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, to remove the sucrose. Aliquots were incubated for 30 minutes at 0^o C with 0, 0.01 μg/ml, 0.1 μg/ml, and 1.0 μg/ml pancreatic ribonuclease, and the reaction mixtures were subsequently layered over linear sucrose gradients and centrifuged in an SW 27 rotor for 17 hours at 15,000 rpm. Under these conditions, undigested RNP sediments as a heterogeneous peak with a modal distribution around 76S (Pederson, 1974). The gradients were fractionated by displacement and the amount of acid-insoluble radioactivity in each fraction determined as described below.

Recentrifugation and Isopycnic Banding of Unfixed RNP Particles in Meterizamide - Regions of sucrose gradients containing rat liver 40S ribonucleoprotein particles were pooled and the particles were precipitated by addition of solid ammonium sulfate to 70% saturation (5.1 g/10 ml). The particles were stirred for 2 hours and the suspension cleared by centrifugation for 40 minutes at 10,000 x g. The RNP particles were recovered as a white precipitate which readily dissolved in hypotonic or isotonic buffers. Quantitative precipitation of both RNA and protein is achieved by this method. (Alternatively, the particles may be collected by high-speed centrifugation (150,000 x g for 6 hours). However, the pellets resulting from high-speed centrifugation do not redissolve

readily, resulting in poor recovery of ribonucleoprotein particles upon recentrifugation.) The redissolved particles were centrifuged on linear 15-30% sucrose gradients, as before. Occasionally, the particles will aggregate and resediment as an apparent dimer; usually however, the particles will resediment as a discrete peak of optical density or radioactivity as 40S particles.

RNP particles from sucrose gradients were applied to gradients of Metrizamide (Nyegaard and Sons, Oslo, Norway) for isopycnic banding. The material from the sucrose gradients was dialyzed overnight against 10 mM NaCl, 50 mM PO_4, 1 mM $MgCl_2$, pH 7.0. In tests of the salt stability of the particles, 0.6 M NaCl replaced 10 mM NaCl in this buffer. Eight ml of the dialyzed particles were applied to discontinuous gradients of Metrizamide, composed of 5 ml of 60% (w/v) Metrizamide(R.I. = 1.4310) and 10 ml of 40% (w/v) Metrizamide (R.I. = 1.3981) in phosphate buffer containing high or low NaCl concentrations. The gradients were centrifuged for 60 hours at 40,000 rpm in a Ti 42 fixed-angle rotor. The gradients were fractionated by displacement from the bottom of the tubes, and aliquots were monitored for TCA-precipitable radioactivity, as described below. Because of the high absorbance of Metrizamide in the ultraviolet region, it was not possible to record the distribution of UV-absorbing material throughout the gradients. The density of every third fraction was determined from the refractive index using the equation, $\rho = 3.350$ (R.I.) - 3.462, after appropriate corrections for the contribution of the salt in buffers.

Isopycnic Banding of Fixed RNP and RNA in CsCl or $CsSO_4$ Density Gradients - Samples of 40S particles from sucrose gradients, and metrizamide gradients were fixed with glutaraldehyde by dialysis overnight against 50 mM PO_4, pH 7.0, 1 mM $MgCl_2$, 1% glutaraldehyde. The fixed samples were applied to gradients of CsCl or Cs_2SO_4 as follows: Preformed Cs_2SO_4 gradients were prepared from 2.2 ml 1.65 gm/cm^3 Cs_2SO_4 (in 10 mM NaCl, 50 mM PO_4, pH 7.0, 1 mM $MgCl_2$, 0.1% Brij 35, R.I. = 1.3850) and 2.2 ml 1.25 gm/cm^3 Cs_2SO_4 (R.I. = 1.3550). A slight turbidity in the stock solutions was removed by Millipore (0.45μ) filtration. One ml of sample was layered over the gradients, and the gradients were centrifuged for 60 hours at 40,000 rpm in a SW 65 swinging-bucket rotor. The gradients were collected into 12-drop fractions and the density of every 5th fraction determined from the refractive index using the equation ρ = 13.75 (R.I.) - 17.38. The fractions were monitored for TCA-precipitable radioactivity as described below. For CsCl gradient centrifugation, 1.0 ml samples were layered over 4.4 ml 50% (w/w) CsCl, in 10 mM NaCl, 50 mM PO_4, pH 7.0, 1 mM $MgCl_2$, 0.1% Brij 35, and the gradients centrifuged at 40,000 rpm for 60 hours in an SW 65 rotor. The density of every fifth fraction was determined from the refractive index using the equation ρ = 10.8601 (R.I.) - 13.4974.

Spectral Measurements of RNP and RNA - Circular dichroism measurements were performed in the laboratory of Dr. Gerald M. Fasman, Brandeis University. Samples of 40S particles for circular dichroism measurements were collected from sucrose gradients, and dialyzed overnight against 10 mM

Tris-HCl, pH 7.2, 1 mM $MgCl_2$ and salt as indicated in Table XI. RNA was prepared from samples of 40S RNP by phenol extraction. Samples of 40S particles were made 1% in Sarkosyl, and 1 volume of phenol plus 1 volume of chloroform containing 1% isoamyl alcohol (v/v) were added. The mixture was homogenized in a Dounce homogenizer, and the aqueous layer was recovered by centrifugation at 7,000 x g for 15 minutes. The interphase layer and the phenol layer were washed with one volume of 10 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, and the aqueous extracts were combined. The aqueous extracts were extracted twice with chloroform-isoamyl alcohol, and the RNA was precipitated by addition of five volumes of ethanol. The RNA was allowed to precipitate for 4 hours at -20 ^{o}C, and was recovered by centrifugation at 10,000 x g for 15 minutes. The pellets were redissolved in 10 mM Tris-HCl, pH 7.2, 1 mM $MgCl_2$, reprecipitated two additional times, and dissolved in an appropriate buffer for circular dichroism measurements.

Circular dichroism measurements were performed at 23 ^{o}C on a Cary 60 spectropolarimeter with 6001 CD attachment. The path length for all samples was 1 cm in fused quartz cells (Optical Cell Company). The concentration range for circular dichroism measurements aws 6 -10 x 10^{-5} M RNA-nucleotide residues. The concentration was determined from ultraviolet absorption spectra. The half-bandwidth was set for 1.5 nm, the time constant was usually 3 seconds. Mean residual ellipticity (θ_λ) is reported in degree cm^2 per decimole of nucleotide residues in the sample.

Ultraviolet absorption spectra were recorded on a Pye-Unicam model SP 1800 recording ultraviolet spectrophotometer. The path-length for all samples was 1 cm; spectra were recorded against an appropriate blank as indicated in the legend to figure 37.

In vitro labeling of RNP proteins by reductive alkylation - The reaction of Rice and Means (1971) for introduction of [^{14}C]-methyl groups into protein amino groups by reductive alkylation was modified for use with proteins from 40 S ribonucleoprotein particles. 40 S particles from six sucrose gradients were collected by precipitation with ammonium sulfate, dissolved in 3 ml of 0.1 M NaCl, 10 mM triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$ and dialyzed overnight against 50 mM Na borate, pH 9.0, 1 mM $MgCl_2$. The final concentration of particles was adjusted to 10 A_{254} units/ ml, and aliquots of 3 ml each were used in the reaction. To the solution cooled on ice were added 150 µl 10% Na deoxycholate and 100 µl 5 M NaCl to disrupt the ribonucleoprotein complexes. Immediately, 100 µl of 0.04 M [^{14}C]-formaldehyde (Amersham, 19 mCi/m mole; approximately 50 µCi at this dilution per reaction) was added. This was followed in 30 seconds by four 20 µl sequential additions of sodium borohydride (5 mg/ml). To ensure complete reduction of the formaldehyde, an additional 100 µl of sodium borohydride solution was added after 1 minute. The reaction may also be performed when the particles are

disrupted in 1 M NaCl or allowed to remain intact: however, under these conditions, radioactivity is not introduced uniformly into the proteins (figure 45). The low molecular weight reaction products were removed by dialysis and gel filtration. Because sodium borohydride will react with Sephadex, it is necessary to remove most of the reducing agent before gel filtration. Typically , the reaction mixture was dialyzed against 6 M urea, 10 mM Tris-HCl, pH 8.0, containing 50 μl 2-mercaptoethanol per liter, for about 4 hours, and applied to DEAE-Sephadex columns as described below.

In vitro labeling of RNP proteins by protein kinase - Intact ribonucleoprotein particles were phosphorylated in vitro with protein kinase as follows: 40 S particles from sucrose gradients were dialyzed overnight against 0.1 M Na acetate pH 6.3, 10^{-5} M papavarine, 1.0 mM EGTA. Aliquots of 250 μl were made 10 mM in $MgCl_2$ immediately before the reaction. The reactions were conducted in the presence or absence of 10^{-6} M cAMP (from a 10^{-5} M stock solution of cAMP in water; equivalent volumes of water were added to reactions carried out in the absence of cAMP.), and in the presence or absence of 2 μg of partially purified cAMP-dependent protein kinase from calf thymus (gift of Dr. E. M. Johnson). The reactions were started by adding $[^{32}P]$-γ-ATP (New England Nuclear) to a final concentration of 10^{-6} M (4.50 x 10^6 cpm/pmole at the time of the reaction depicted in figure 46, and terminated after 1 or 15 minute incubations at 30 ^{o}C,

by addition of 20 µl of 10% SDS. The reaction mixtures were dialyzed against sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis, as described below.

Chromatographic separation of RNP proteins - The basic proteins of the RNP complex were separated from the acidic proteins and RNA by chromotography on DEAE-Sephadex, after disruption of the complex with urea. Samples in 10 mM KCl, 10 mM Tris-HCl, pH 8.0, 6 M urea containing 50 µl per liter 2-mercaptoethanol, were applied to 1.7 cm x 26 cm columns of DEAE-Sephadex equilibrated in the same buffer, and eluted by gravity. Approximately forty 1 ml fractions were collected and the columns eluted with 1 M KCl in the same buffer. Twenty microliter aliquots of each fraction were monitored for radioactivity. The DEAE-Sephadex was equilibrated as follows: 50 grams of ion-exchanger was allowed to swell overnight in 2 liters of 10 mM Tris-HCl, pH 8.0, 10 mM KCl. The supernatant liquid was discarded and the exchanger stirred into another 2 liter volume of buffer, and allowed to settle in 2 liters of buffer containing 1 M KCl. This buffer was replaced repeatedly with buffer containing 10 mM KCl, until the pH of the supernatant was the same as that of the starting buffer. 6M urea was added to the buffer, and the buffer replaced 3 more times. The equilibrated DEAE-Sephadex in 6 M urea, 10 mM Tris-HCl, pH 8.0, 10 mM KCl, was stored at 4 ^{o}C with chloroform added as a preservative. All buffer solutions were degassed under vacuum and filtered

through 0.45 micron-Millipore filters before use. (It is necessary to readjust the pH of the buffer after degassing and filtration.)

Proteins in the DEAE run-off peak were further fractionated on columns of phosphocellulose (0.7 cm x 25 cm) equilibrated with a buffer of 50 mM methylammonium phosphate, pH 6.8, 6 M urea, containing 50 µl 2-mercaptoethanol per liter. The samples were applied directly to the phosphocellulose columns in 10 mM Tris buffer after adjusting the pH of the buffer to 6.8, washed with 2 column volumes of starting buffer, and eluted with 400 ml linear gradients ranging in NaCl concentration from 0 to 300 mM in methylammonium phosphate buffer. The columns were eluted at a flow rate of 25 ml/hour and 5 minute fractions were collected. Aliquots of 250 µl were monitored for radioactivity. Alternatively, columns were equilibrated in 10 mM Tris-HCl, pH 7.1, 10 mM KCl, 6 M urea, 1 mM dethiothreitol, and eluted with linear 400 ml gradients ranging in KCl concentration from 0 to 150 mM.

Phosphocellulose was equilibrated as follows: 100 gm ion exchanger was stirred into 2000 ml of 0.1 N NaOH, and allowed to settle in a 2 liter graduated cylinder for 60 minutes. The supernatant containing fines was removed by aspiration, and the exchanger washed with 2 liter batches of water, by settling, until the pH was near neutral. Fines are removed during each of these settling steps. The settled exchanger was mixed with 2 liters of 0.1 N HCl and allowed to settle, and the process repeated 2 times. The

settled exchanger was then washed with 2 liter batches of water until the pH was near 6.0. The supernatant was removed and the exchanger allowed to settle in 2 liter batches of 50 mM NaH_2PO_4 buffer adjusted to pH 6.8 with methylamine and containing 50 µl per liter 2-mercaptoethanol. This process was repeated until the pH of the supernatant was 6.80 ± 0.02, at which time the exchanger was equilibrated with 3 batches of buffer containing 6 M urea. The equilibrated buffer was stored at 4 °C with chloroform added as a preservative. In order to minimize the nonspecific adsorption of protein, fresh columns were packed for each chromatography step. When fresh resin is used, more than 95% of the applied radioactivity may be recovered. Columns were washed with 500 ml of fresh starting buffer before the application of the sample. All solutions were degassed and filtered through 0.45 micron Millipore filters before use in either the chromotography or equilibration of the resin. It is necessary to readjust the pH of the buffers after filtration.

In some experiments, fractions from the phosphocellulose columns were rechromatographed on phosphocellulose columns equilibrated at pH 6.8 or 6.5; or on carboxymethyl cellulose (CM) columns (0.7 cm x 30 cm) equilibrated with a buffer in 6 M urea containing 10 mM methylamine adjusted to pH 5.6 with acetic acid, and 50 µl per liter 2-mercaptoethanol. The CM columns were eluted with 400 ml gradients of sodium acetate in the methylamine acetate buffer, the final acetate

concentration being 300 mM, pH 5.6. Samples were dialyzed against appropriate buffers before rechromatography on phosphocellulose or on CM cellulose. The CM cellulose resin was equilibrated in essentially the same manner as the phosphocellulose resin, with the appropriate buffers. During the preparative pre-equilibration steps the concentrations of NaOH and HCl were 0.5 M.

Electron Microscopy - Aliquots of rat liver 40 S particles were visualized in the electron microscope by negative staining with uranyl acetate or phosphotungstic acid. Samples from sucrose gradients were dialyzed against 50 mM PO_4 buffer, pH 7.0, 1 mM $MgCl_2$ to remove sucrose, and fixed by dialysis against phosphate buffer containing 1% glutaraldehyde. The excess glutaraldehyde was removed by dialysis against the original buffer. Samples were applied to carbon-coated formvar grids, stained with 1% uranyl acetate or 1% phosphotungstic acid, (neutralized aqueous solutions) and examined in a Siemens Elmiskop model 102.

Polyacrylamide gel electrophoresis - Protein samples were analysed by polyacrylamide gel electrophoresis in the presence of SDS using the discontinuous buffer systems of Ornstein and Davis (Laemmli, 1970). Gels were cast as linear gradients of 8.75 - 14% acrylamide (in 0.375 M Tris-HCl, pH 8.8) in 1 mm x 150 mm x 150 mm slabs. Stacking gels of 3% acrylamide, 10-15 mm in length, were polymerized on top of the separating gels (in 0.1% SDS, 0.125 M Tris-HCl, pH 6.8). Protein samples were dialysed against sample buffer, 3% SDS, 5% 2-mercaptoethanol, 50 % glycerol, 0.375 M Tris-HCl, pH 6.8, and aliquots of no

more than 50 µl were applied to each gel slot. Bromophenol blue was added to each sample as tracking dye. Electrophoresis was for 18 to 20 hours at 12.5 ma. The running buffer was 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.4. The gels were stained with 0.1% Coomassie brilliant blue R-250 (Sigma) in 50% ethanol, 7.5% acetic acid, for 4 to 6 hours, and destained in 7.5% acetic acid. The gels were dried on Whatman 3 MM paper and exposed to medical X-ray film (Kodak, Blue Shield) when autoradiographs were desired. Protein in these gels was quantitated by microdensitometry of diapositive photographic negatives of the gels, using a Joyce-Loebl densitometer.

Alternatively, electrophoresis was performed in 6 mm x 9.5 cm columns of 8.75 % acrylamide overlayered with 6 mm x 1 cm 3% stacking gels using the discontinuous buffer systems described above. Electrophoresis was at 2 ma / tube until the bromophenol blue tracking dye had reached the bottom of the tube. Samples of up to 250 µl may be applied to these gels without affecting the resolution. Gels were stained in 0.1% Fast green in 35% methanol, 7.5% acetic acid, and destained by diffusion in 35% methanol, 7.5% acetic acid. Protein was quantitated by direct densitometric analysis of the stained gels at 615 nm using a Gilford spectrophotometer equipped with a model 2410 S linear transport device.

Proteins were also analyzed by electrophoresis at pH 4.5

in polyacrylamide gels containing 6 M urea, using the buffer system of Reisfeld (1962). The separating gel was 7.5% acrylamide in 6 M urea, 0.06 N NaOH adjusted to pH 4.3 with acetic acid. Stacking gels were 3% acrylamide in 6 M urea, 0.06 N NaOH adjusted to pH 6.8 with acetic acid. Gels were polymerized by adding 0.0033 volume 10 % ammonium persulfate and 0.0005 volume of TEMED. The running buffer was 0.07 M β-alanine adjusted to pH 4.5 with acetic acid. Electrophoresis was performed in 0.6 x 9 cm tubes at 4 ma/ tube with the anode at the top. Samples were applied in 8 M urea, 1% 2-mercaptoethanol, 0.06 N NaOH adjusted to pH 6.8 with acetic acid. Pyronin Y was added as tracking dye, and electrophoresis was stopped when the dye reached the bottom of the tube.

Chemical analyses - Proteins from the phosphocellulose columns were concentrated for amino acid analyses by lyophilization after exhaustive dialysis against 0.1% SDS. The SDS serves as carrier during the lyophilization, so that small quantities of protein (less than 100 micrograms) could be recovered with 80-100% yields from volumes as large as 10 ml.

The lyophilized protein-SDS samples were redisolved in 1 ml of water, dialyzed exhaustively against water, and made 6 N in HCl for acid hydrolysis. The samples were degassed, sealed under vacuum, and hydrolysed for 18 hours at 110 ^{o}C. The hydrolysates were lyophilized, and analyzed on either a Beckman 120 B analyzer modified for a 10-fold increase in

sensitivity with a Honeywell scale expander, or on a Durrum amine acid analyzer.

Protein was determined by the method of Lowry et al. (1951)

Determination of Radiactivity - Unless otherwise specified, radioactivity measurements of samples from sucrose gradients and isopycnic gradients, were of TCA-insoluble material precipitated on to Millipore filters (0.45 micron pore size) and washed with 10 ml TCA (10%), and 10 ml absolute ethanol. The filters were counted in 15 ml Liquifluor-toluene (New England Nuclear) in a Beckman model LS 330 liquid scintillation spectrometer. Eluted fractions from phosphocellulose and carboxymethylcellulose columns were monitored directly for radioactivity without TCA precipitation. 250 µl aliquots were diluted with 750 µl water and dissolved in 15 ml Formula 950A (New England Nuclear) 33% Triton X-100, a toluene based, scintillation liquid. Samples were counted in a Beckman LS 330 scintillation spectrometer.

RESULTS

Isolation of 40S ribonucleoprotein particles from rat liver - As initially reported by Samarina et al. (1967), isolated rat liver nuclei washed in slightly hypotonic buffers at pH 8.0 release ribonucleoprotein particles which may be banded in a sucrose gradient. Figure 21 plots the distribution of optical density at 254 nm, orotic acid incorporated into RNA during a 2 hour period, ^{32}P incorporated into

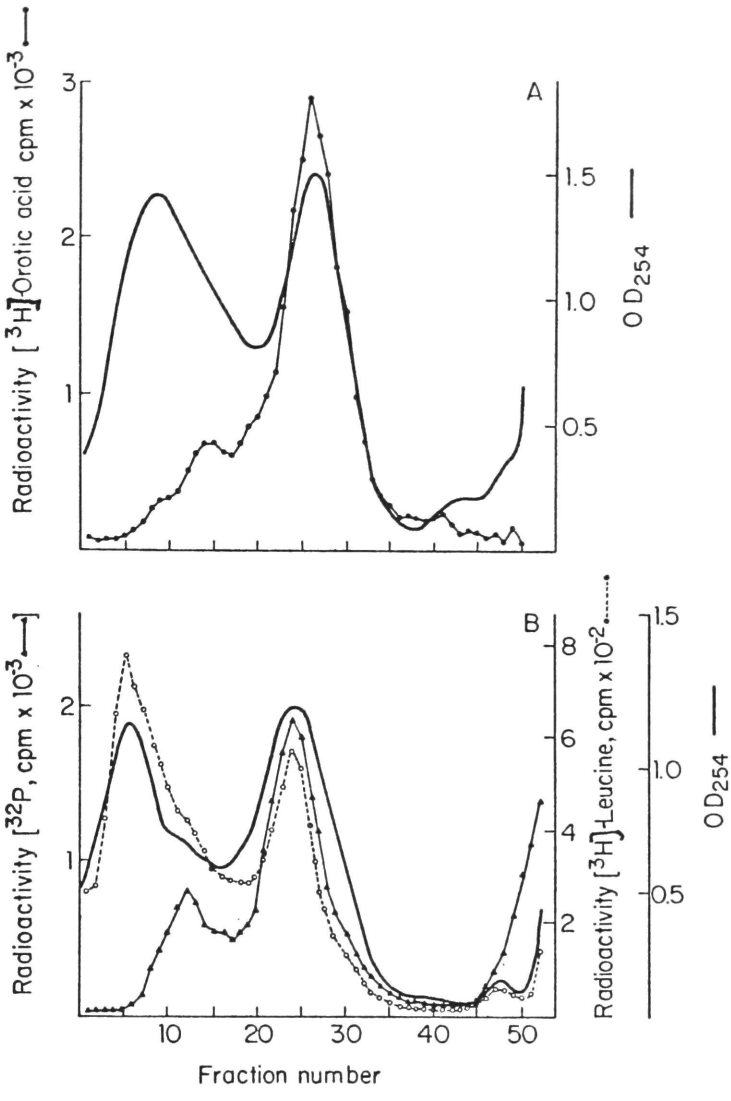

Figure 21. Fractionation of rat liver nuclear extracts containing 40 S ribonucleoprotein particles on sucrose gradients. Rats received interperitoneal injections of 200 μCi orotic acid -[5 -^{3}H] 2 hours before sacrifice, or 1 mCi of [^{32}P]-orthophosphate 1 hour before sacrifice. Protein was labeled over a 20 hour period in rats receiving three successive interperitoneal injections of [^{3}H - 4,5]-leucine at 6 hour intervals. Nuclear extracts were prepared from isolated rat liver nuclei, as described in "Materials and Methods." (pH 8.0 extraction) Aliquots of nuclear extracts were applied to 39 ml linear 15 - 30 % sucrose gradients and centrifuged for 15 hours at 23,000 rpm in a SW 27 rotor. The gradients were fractionated by displacement with 60% sucrose and the optical density at 254 nm and TCA-precipitable radioactivity were monitored across the gradients. The direction of sedimentation is from left to right. The 40S ribonucleoprotein particles sediment in the center of the gradient and appear as a prominent peak of radioactivity or optical density. ●—● [^{3}H]orotic acid, radioactivity; ▲—▲, ^{32}P radioactivity; O---O [^{3}H]-leucine, radioactivity; —— optical density, 254 nm.

nucleic acid during a 1 hour period, and [^{3}H]-leucine incorporated into protein during a 20 hour period, in nuclear extracts fractionated on 15-30% linear sucrose gradients. The majority of the orotic acid incorporated into TCA-insoluble material in the nuclear extracts sedimented as a discrete peak at about 40S. The distribution of radioactivity coincides with the predominant peak of optical density in the gradient. The optical density at 254 nm may be regarded as a measure of the steady state distribution of ribonucleoprotein, whereas radiactivity incorporated during short labeling periods may be regarded as a measure of the distribution of newly synthesized sequences found in RNP. A small shoulder of radioactivity appears in the 15-20 S region of the sucrose gradient, corresponding to a shoulder of optical density. This region of the sucrose gradient is known to contain RNA enriched in poly A sequences, associated with protein (Quinlan *et al.*, 1974; Lukanidin *et al.*, 1974). The small amount of orotic acid incorporated into TCA-precipitable material sedimenting in this region may be associated with these structures. Phosphate incorporated into nucleic acid during a short labeling period distributes on the sucrose gradients similarly to the orotic acid, indicating that there is no major contamination of the ribonucleoprotein particles with radioactive DNA or chromatin. The shoulder in the 15-20 S region of the sucrose gradient is slightly exaggerated relative to the main peak of radioactivity, when ^{32}P is used as a label instead of [^{3}H]-orotic acid, consistent with the presence of ^{32}P-labeled poly A

sequences in this region of the gradients. The majority of the [^{3}H]-leucine incorporated into TCA-precipitable material, and extractable from isolated nuclei, does not enter the sucrose gradients, but remains at the top of the gradient, along with a prominent peak of UV-absorbing material. This material is probably protein that has been solubilized during the extraction procedure. Approximately 40% of the leucine incorporated in a long term label enters the gradients, and cosediments with the peak of ^{32}P-radioactivity, indicating the presence of protein in the material sedimenting at 40S. The proteins associated with rat liver ribonucleoprotein particles label poorly with leucine and long labeling periods are required before a significant amount of protein cosedimenting with RNA is labeled. The slow labeling of these proteins suggests that they are metabolically stable. In contrast, most RNA in the nucleus of animal cells turns over extremely rapidly (Darnell, 1968) and may be labeled to high specific activities in short periods.

Approximately 30-50% of the TCA-insoluble radioactivity in the nuclear fraction labeled with orotic acid for 2 hours may be extracted in hypotonic buffers. In good preparations, the predominant peak of optical density displayed in the sucrose gradients corresponds to 40% of the labeled protein in the nuclear extract and over 80% of the orotic acid labeled RNA. Electron microscopy shows the presence of particulate material in this zone of the gradient (Figure 22). Thus, much of the rapidly labeled

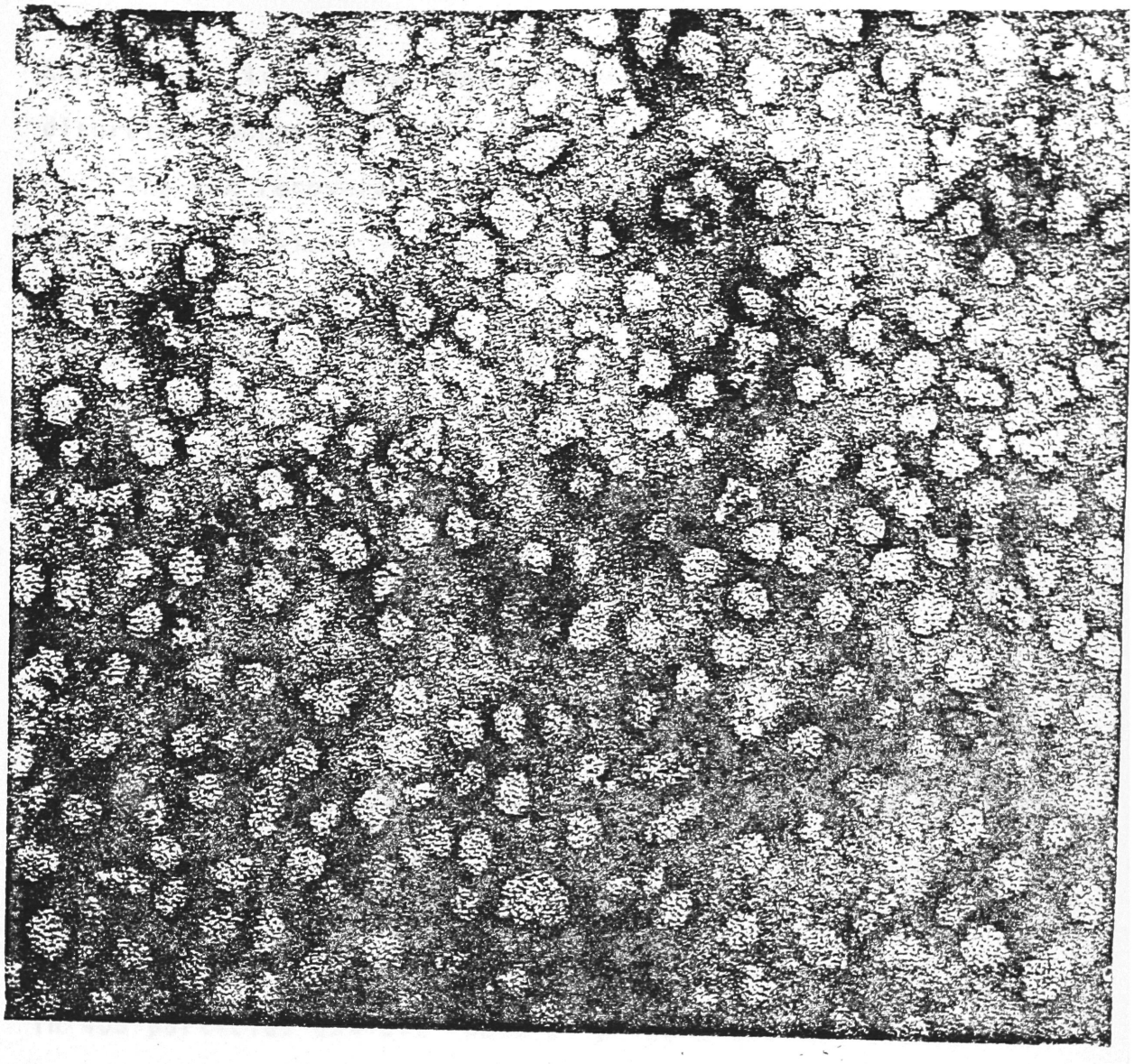

Figure 22. Rat liver nuclear ribonucleoprotein particles released by extraction of isolated nuclei with hypotonic buffer (pH 8.0). Uranyl acetate negative staining, X 240,000. (Courtesy of Dr. H. P. Hoffman).

RNA of rat liver nuclei may be recovered in the form of discrete ribonucleoprotein particles.

The RNA recovered in the 40S ribonucleoprotein particles is almost certainly derived from HnRNA molecules. The base composition of this RNA is DNA-like (Samarina et al., 1965, 1968; Moulé and Chauveau, 1968) and includes sequences that hybridize to the moderately-repetitive DNA as well as sequences not present in the cytoplasm (Martin and McCarthy, 1972). Other sequences in the 40S ribonucleoportein particles will hybridize to cDNA copies of total cytoplasmic messenger RNA (Martin, 1976). In FL cells infected with adenovirus, viral-specific sequences are isolatable as 40S ribonucleoprotein particles (Lukanidin, et al., 1972). In tissue culture cells, the RNA sequences recoverable in this kind of structure are rapidly labeled, even in the presence of low doses of actinomycin D (Pederson, 1974). Similarly, in mouse Taper hepatoma cells, RNA sequences recovered in 40S particles exhibit "natural pulse-chase kinetics", as does HnRNA in these cells. (Martin and McCarthy, 1972; Martin et al., 1973). Since the RNA in these particles is only 4-8S (Samarina et al., 1968; Pederson, 1974), it must be concluded that the 40S complexes derive from much larger nuclear ribonucleoprotein complexes.

The isolated ribonucleoprotein particles appear to be rather uniform structures 200 to 300 Å in diameter, when visualized in negative contrast in the electron microscope (figure 22). Smaller subcomponents of 50 to 70A in diameter are also visible. Similar electronmicrographs have

been published by Albrecht and Van Zyle (1973), Monneron and Moulé (1971), and Lukanidin et al.(1972). Some size heterogeneity in these preparations is observed, perhaps due to breakdown of the particles or to aggregation of subpopulations of RNP structures. It should be emphasized however, that the predominant species in these preparations is a discrete, morphologically-distinct macromolecular complex. No major contamination of the preparations by chromatin, membranous material or ribosomes is observed.

Protein composition of the 40S Ribonucleoprotein particle from rat liver nuclei - When rat liver nuclear ribonucleoprotein particles are examined on SDS-polyacrylamide gels a heterogeneous banding pattern is observed (figures 23,24,26). On SDS gels containing a gradient of acrylamide from 8.75 - 14% more than 35 protein bands were reproducibly resolved. The proteins range in molecular weight from 30,000 to over 200,000 daltons (figure 23). Less resolution was obtained on 8.75% disc gels (figure 26); however, the banding patterns are similar in the two gel systems, and the mobility of a given protein band on the two gel systems can be easily determined from an examination of the banding patterns.

Extremely reproducible banding patterns are obtained in replicate preparations of 40S particles prepared as described. (Figure 23). During the course of developing these isolation procedures, it was noted that lysis of the nuclei during the extraction procedure, or poor preparations of nuclei resulted in preparations with high amounts of

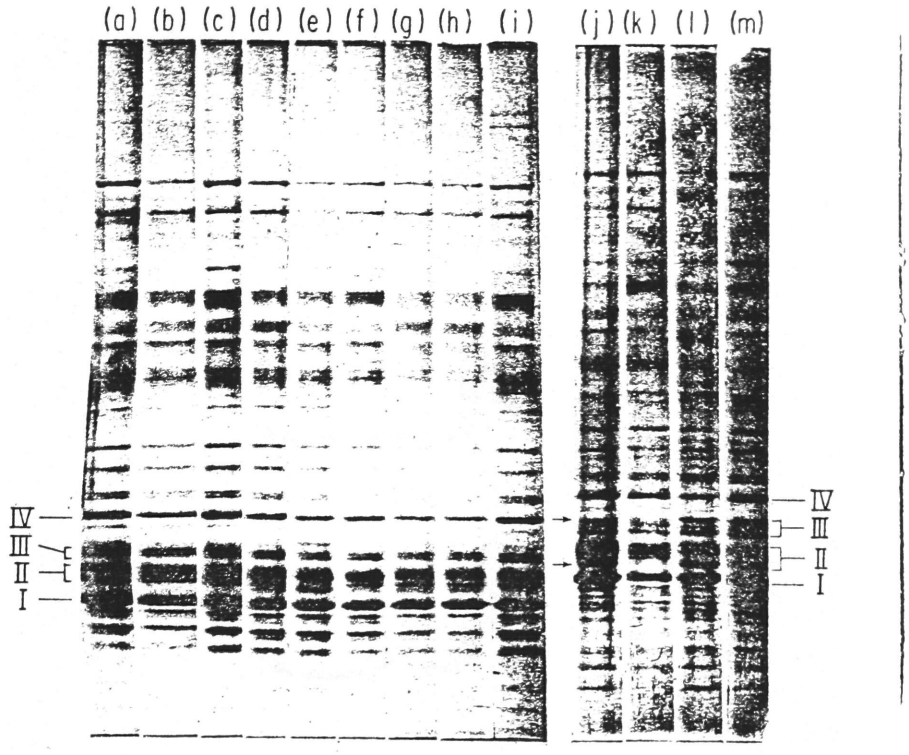

Figure 23. Comparison of various preparations of rat liver 40S ribonucleoprotein particles by SDS-polyacrylamide gel electrophoresis of the proteins. Gels were gradients of 8.75-14% acrylamide prepared and run as described in Materials and Methods. The proteins are fractionated according to molecular weight, proteins of high mobility (bottom of gel) have lower molecular weights than proteins of lower mobility. The Roman numerals indicate the positions of the prominent proteins present in the 40S particles. Note that these proteins are present in each of the preparations in the same relative amounts. Arrows indicate two proteins present in variable quantities in each of the preparations. Other variable components are present, especially among the proteins with mobilities greaterthan protein I. Panels a - i were taken from one gel and panels j-m from another. Panel a, 1/29/76; Panel b, 1/30/76; Panel c, 2/4/76, Panel d, date unknown, Panel e, 1/4/76; Panel f, 1/5/76; Panel g, 1/5/76 (sonication); Panel h, date unknown; Panel i, 30S particles after reductive alkylation, date unknown; Panel j, 12/16/75; Panel k, 1/4/76; Panel l, 1/2/76; Panel m, date unknown.

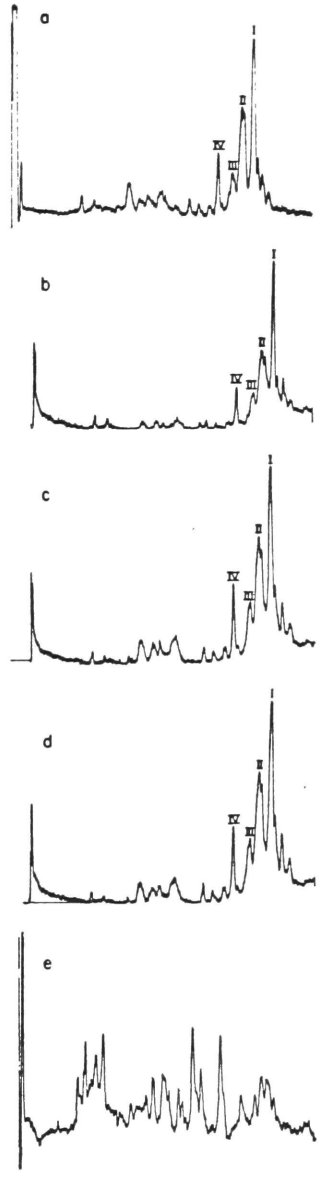

Figure 24. Densitometry tracings showing proteins from rat liver 40S ribonucleoprotein particles fractionated by SDS-polyacrylamide gradient gel electrophoresis. The proteins indicated by the Roman numerals represent 70% of the protein mass of the 40S particles. Note that these proteins are present in the same relative amounts in each of the densitometer scans. Panel e shows the distribution of proteins sedimenting at 15-20S in the sucrose gradients and fractionated under identical electrophoretic conditions. Note that different proteins are present in the 40S and 15-20S regions of the gradients. Panels b, c, and d are scans of panels b, c, and d of the gel shown in figure 23. Panels a and e are scans taken from a different gel.

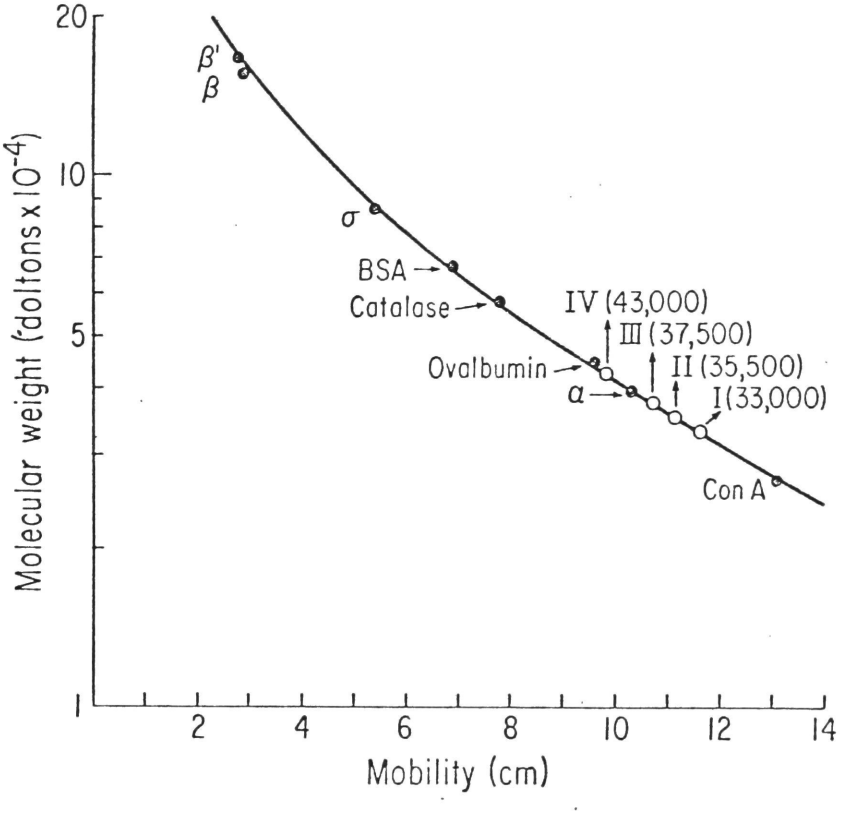

Figure 25 . Estimation of the molecular weights of the major RNP proteins by SDS-polyacrylamide gradient gel electrophoresis. The relative mobilities of the major RNP proteins and proteins of known molecular weight are plotted as the migration distance and the log of the molecular weight. The calibration curve deviates from linearity because of the gradient of polyacrylamide. Standards were E.coli RNA polymerase, β subunit, 165,000 daltons, β' subunit, 155,000 daltons, σ subunit, 87,000 daltons, α subunit, 39,000 daltons; bovine serum albumin, 68,000 daltons; catalase, 57,000 daltons; ovalbumin, 45,000 daltons; and concanavalin A, 27,000 daltons.

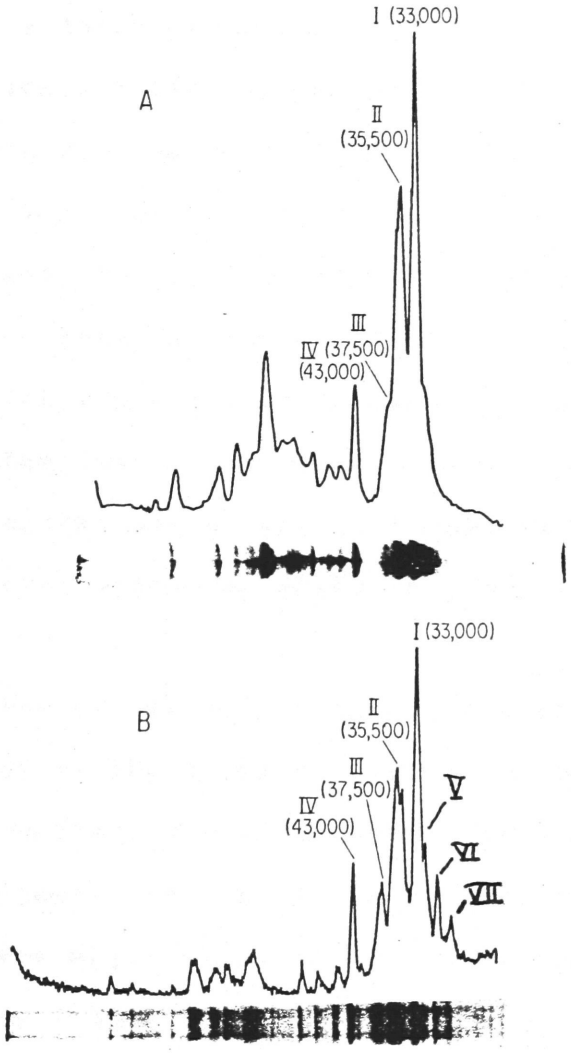

Figure 26. Comparison of RNP proteins separated on SDS-polyacrylamide gels of 8.75% (panel A) and 8.75-14.0 % gradients (panel B) of polyacrylamide. Considerably better resolution was obtained on gels containing a gradient of acrylamide. The 8.75% gels were stained with Fast Green while the gradient gels were stained with Coomassie Brilliant Blue. (See Materials and Methods for details on the preparation and running of the gels.) Above the gel photographs are densitometry scans of the gels. Note that although the gels have been stained with different stains and densitometric tracings prepared on different instruments, the prominent protein bands are readily identifiable in both systems.

proteins with molecular weights greater than 40,000 daltons. (Compare panel A of figure 26, an early preparation, with panel B, figure 26, a later preparation.) Since variable components such as these higher molecular weight proteins are prob bly contaminents, we have chosen to regard only those proteins that are reproducibly isolatable in the ribonucleoprotein particles as required for the structure. Thus the proteins indicated by the arrows in figure 23, which appear in variable quantities in each of the preparations, are regarded as contaminants. Likewise, the variable proteins of low molecular weight are also regarded as contaminents (proteins migrating ahead of band I in figure 23).

Microdensitometry of the gel patterns (figures 24, 25) shows that nearly 70% of the protein in these preparations is represented by proteins ranging in molecular weight from 33,000 to 43,000 daltons. For convenience in referring to these proteins, the major bands have been assigned Roman numerals I-VII. Band I appears to be a single protein species. Band II is partially resolved into three species referred to as IIA, IIB, IIC being the component of highest mobility. Band III is partially resolved into two bands, IIIA and IIIB. Migrating just ahead of the prominent band in region IV and partially obscured by it are two bands, referred to as proteins IVA and IVB. The prominent peak in region IV is therefore referred to as band IVC. The three major proteins with mobilities greated than I, are referred to as V, VI, and VII (figure 26).

The proteins in the 33,000 to 43,000 dalton group appear in constant relative proportions in microdensitometer scans of the polyacrylamide gels (figure 24). Approximately the same relative peak heights are observed in densitometry tracings made directly from gels stained with fast green, and in tracings made from photographic negatives of gels stained with Coomassie brilliant blue R-250, suggesting that these tracings permit reliable estimates of the relative amount of protein in the gels (figure 26).

The proteins that are isolated as 40S particles are a unique subset of the proteins in the nuclear extracts. Different proteins are present in the 15-20 S region of the sucrose gradients, and in the total extract. (figure 24, panel e, and figure 38 panels a,b,c.)

Protein composition of rat liver ribonucleoprotein particles prepared by alternative procedures - The protein compositions of ribonucleoprotein particles prepared from nuclei by four separate procedures (extraction, sonication, hyper- and hypotonic lysis) were compared. Figure 27 compares the the optical density profiles of sucrose gradients of the ribonucleoprotein particle -containing nuclear extracts prepared by each of these methods. The yield in particles is proportional to the amount of UV-absorbing material in the 40S region of the gradients (indicated by the bars). Approximately equal recoveries of particles were obtained by the sonication and extraction procedures. Hypotonic lysis of nuclei resulted in somewhat decreased yields of particles. The large amount of UV-absorbing material on

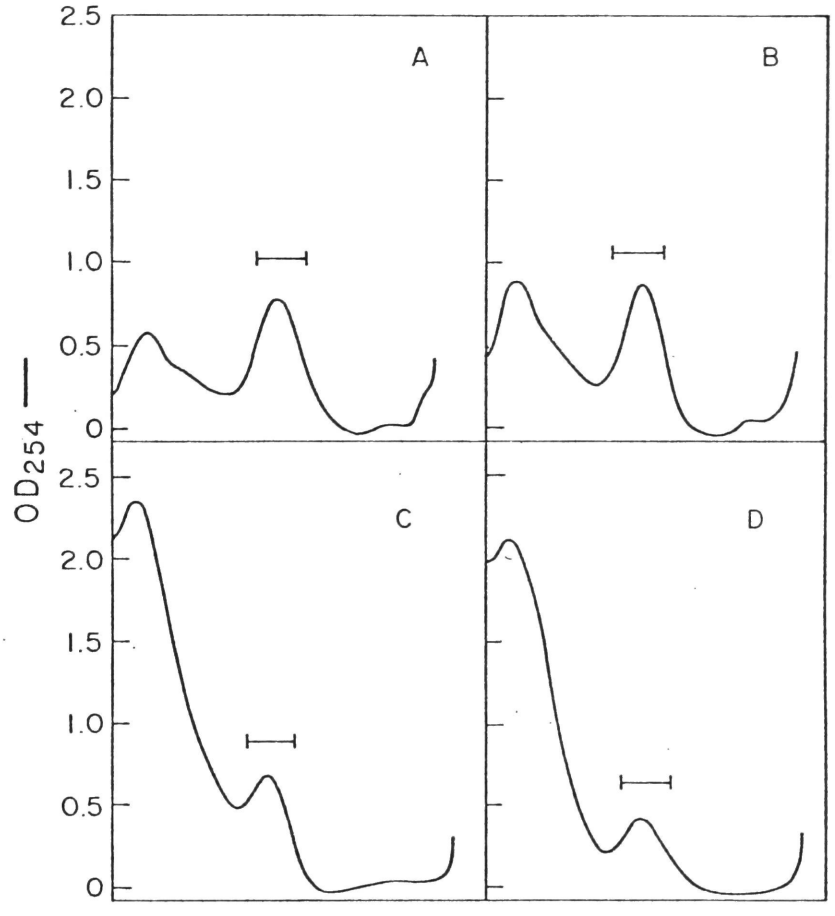

Figure 27. Fractionation of nuclear extracts and lysates containing 40S ribonucleoprotein particles on 15-30% linear sucrose gradients. Panel A, nuclei extracted with 0.1M NaCl, 0.01 M triethanolamine-HCl, pH 8.0, 0.001 M $MgCl_2$; Panel B, low speed supernatant fraction from sonicated nuclei; Panel C, low speed supernatant fraction from nuclei lysed in hypotonic buffers; Panel D, low speed supernatant fraction from nuclei lysed in hypertonic buffer. Procedures for preparing nuclear extracts and low speed supernatant fractions from isolated rat liver nuclei are described in "Materials and Methods". Centrifugation was for 15 hours at 23,000 rpm in an SW 27 rotor. The gradients were fractionated by displacement with 60% sucrose, and the optical density at 254 nm monitored. The direction of sedimentation is from left to right, bars indicate the regions of the sucrose gradients containing the 40S particles. The large peaks of optical density at the top of the gradients in panels C and D is due to DNA solubilized by DNase during the preparation of the nuclear lysates.

the top of the sucrose gradient in panels C and D is nucleic acid solublized during the DNase treatment of the nuclear lysate. Low and variable yields were obtained with hypertonic lysis of the nuclei, presumably because of the salt lability of the ribonucleoprotein complexes (see below). In the experiment shown in panel D of figure 27, relatively high yields were obtained.

Figure 28 compares the SDS-polyacrylamide gel banding patterns of proteins in the ribonucleoprotein preparations shown in figure 27, and in another experiment. The banding patterns in each of these preparations is remarkably constant, and no variability greater than that obtained in replicate preparations is observed when particles are prepared by extraction, sonication, or hypotonic lysis of the nuclei. Particles prepared by hypertonic lysis of the nuclei are somewhat depleted in protein, although all the protein species found in other preparations are observed (figure 28, slot d). The ability to prepare indistinguishable ribonucleoprotein particles by four independent methods, strongly suggests that these particles represent a well-defined nuclear structure, with a fixed protein composition, and are not an artifactual aggregate generated during the isolation procedure.

Stability of ribonucleoprotein particles to recentrifugation and isopycnic banding without fixation - In order to determine whether all the proteins components sedimenting in the 40S region of the sucrose gradients are associated with the ribonucleoprotein particle, or another structure of similar

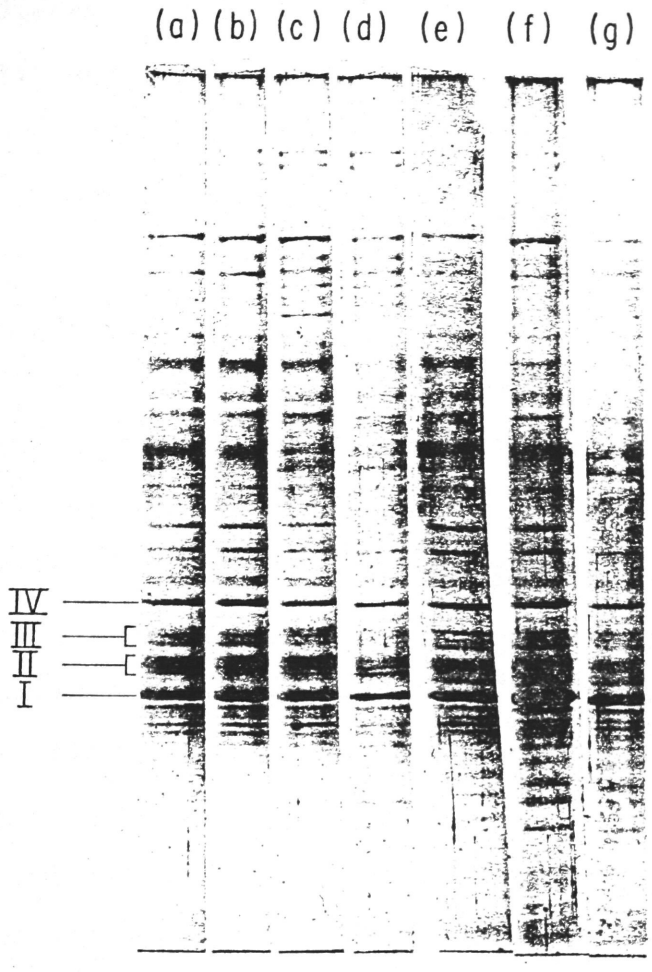

Figure 28. Comparison of proteins co-isolating with 40S ribonucleoprotein particles prepared by different procedures, by SDS-polyacrylamide gradient gel electrophoresis. Panel a, 40S particles prepared by extraction of nuclei, Panel b, 40s particles prepared by sonication of nuclei, Panel c, 40S particles prepared by lysis of nuclei in hypotonic buffers, Panel d, 40S particles prepared by lysis of nuclei in hypertonic buffers. The fractions shown in panels a through d are from the particles prepared on the sucrose gradients shown in Figure 27. Panels e to g show 40S particles prepared in another experiment. Panel e, 40S particles prepared by extraction of nuclei, Panel f, 40S particles prepared by sonication of nuclei, Panel g, 40S particles prepared by lysis of nuclei in hypotonic buffers. Note that the same proteins are present in the 40S particles prepared by each of the different procedures. Conditions for electrophoresis and preparation of the 40S particles are given in "Materials and Methods."

size, we have banded ribonucleoprotein particles obtained from sucrose gradients on gradients of Metrizamide, a tri-iodinated benzamido derivative of glucose (2-(3-acetoamido-5-N-methyl-acetamido-2,4,6-triodobenzamido)-2-deoxy-D-glucose, Nyegaard and Sons, Oslo), and examined the proteins which remain associated with RNA after isopycnic centrifugation by SDS-polyacrylamide gel electrophoresis.

Metrizamide is an inert, non-ionic compound of low polarity that forms solutions of high density (greater than 1.400 gm/cm^3) and low viscosity (58 cP at 1.38 gm/cm^3, 5 ^{o}C). Stable gradients of metrizamide are generated during centrifugation. (Birnie et al., 1973; Rickwood, et al., 1974). This compound has been successfully used to band isopycnically DNA, RNA, protein and chromatin (Rickwood et al., 1974). The buoyant densities of nucleic acids are extremely low in metrizamide and correspond to those of fully hydrated DNA and RNA. The bouyant densities of proteins are higher than those of nucleic acids, but also correspond to those of fully hydrated molecules (Birnie et al., 1973)

Figure 29A shows that isopycnic banding of ^{32}P- and [^{3}H]-leucine-labeled ribonucleoprotein particles from sucrose gradients on gradients of Metrizamide in a buffer of low ionic strength. Most of the ^{3}H and ^{32}P radioactivity bands together at a density of 1.29 gm/cm^3 as a sharp peak. Approximately 20% of the ^{3}H leucine is lost from the ribonucleoprotein complex and recovered as a small peak banding at p= 1.38 gm/cm^3. When the same material is applied to gradients of Metrizamide containing 0.6 M NaCl two discrete

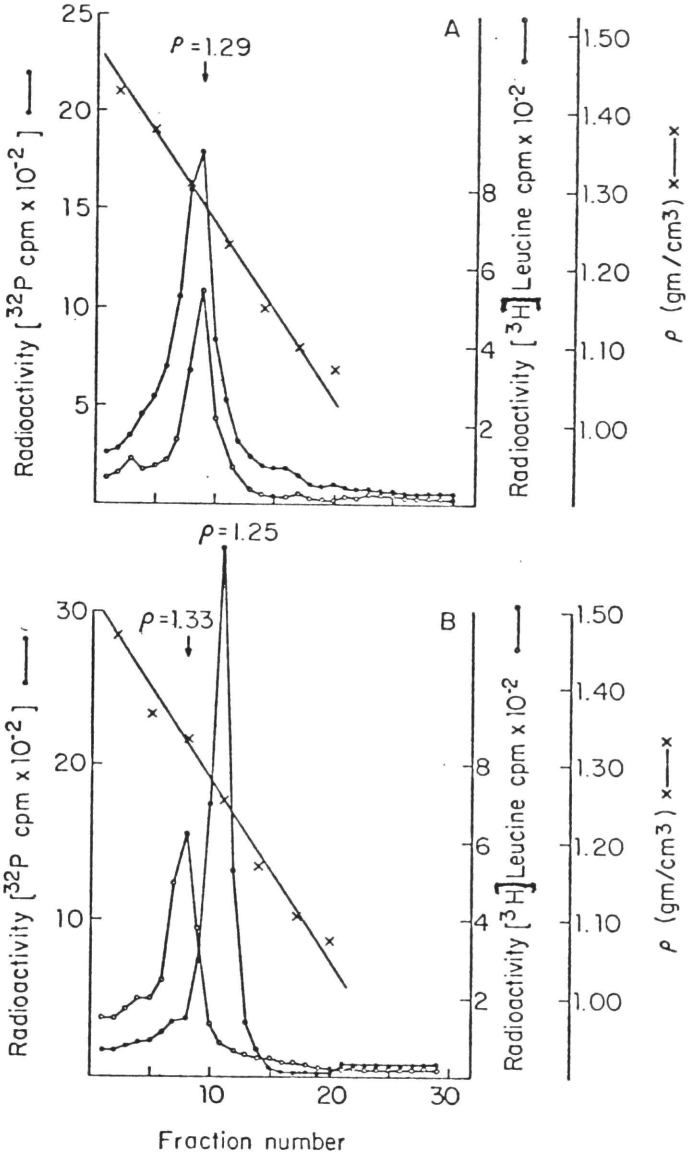

Figure 29. Isopycnic banding of unfixed 40S ribonucleoprotein particles from rat liver in gradients of Metrizamide. Panel A, gradients in 50mM PO_4 , pH 7.0, 1mM $MgCl_2$, 10mM NaCl; Panel B, gradients in 50mM PO_4 , pH 7.0, 1mM $MgCl_2$, 0.6 M NaCl. 40S ribonucleoprotein particles labeled with [^{32}P]orthophosphate and [^{3}H]leucine were isolated by sucrose gradient centrifugation of nuclear extracts (Figure 21 , Panel B), dialyzed overnight against phosphate buffer of high or low ionic strength, and applied to 15 ml gradients of Metrizamide. Gradients were centrifuged for 60 hours at 40,000 rpm in a Ti 60 rotor. Fractions were monitored for TCA-precipitable radioactivity, and the density of every third fraction determined from the refractive index. ●——●, ^{32}P radioactivity; O——O, [^{3}H]leucine radioactivity; x----x, density (gm/cm3)

peaks are observed (figure 29B). Virtually all the ^{32}P-labeled RNA bands at a density of 1.25 gm/cm^3, whereas the ^{3}H-labeled protein bands at a density of 1.33 gm/cm^3. The ability to band ribonucleoprotein particles isopycnically is further evidence for the existence of these structures. Under these conditions, the structures are labile to high salt, and complete separation of the protein and RNA components may be achieved in gradients centrifuged in the presence of 0.6 M NaCl. The intact ribonucleoprotein particle bands at a density intermediate to the separated RNA and protein components.

The alterations in bouyant densities of the ribonucleoprotein particles after banding in Metrizamide in high or low salt were confirmed by isopycnic banding of the corresponding fractions in gradients of CsCl or Cs_2SO_4, after fixation with glutaraldehyde. Figure 30 compares the equilibrium bouyant densities in Cs_2SO_4 of [^{3}H]-orotic acid-labeled ribonucleoprotein particles from sucrose gradients (panel a), and of the same material after isopycnic banding in Metrizamide at low (panel b) and high (panel c) ionic strength. Particles from sucrose gradients band in Cs_2SO_4 at a density of 1.43 gm/cm^3. No appreciable shift in the equilibrium bouyant density was observed after the particles were rebanded in Metrizamide at low ionic strength, indicating that the complex had been preserved during the Metrizamide gradient centrifugation. After exposure to 0.6 M KCl and centrifugation in Metrizamide the labeled RNA banded in Cs_2SO_4 with a heterogeneous distribution. Most

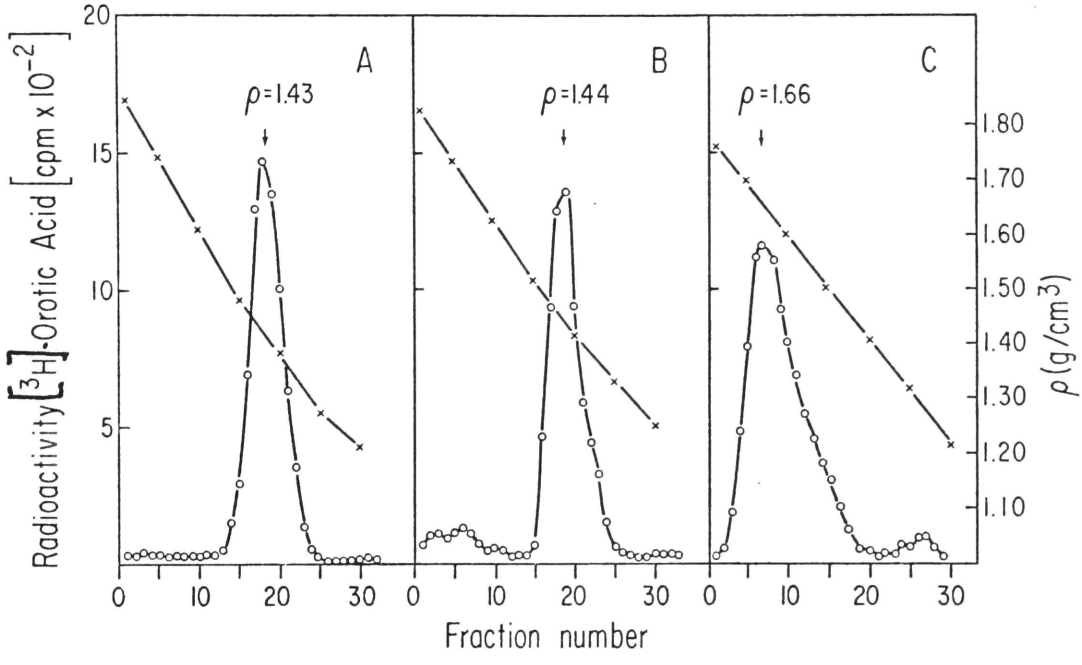

Figure 30. Isopycnic banding of rat liver 40S RNP after centrifugation in sucrose gradients, and in Metrizamide gradients of high or low ionic strength, in Cs_2SO_4. Particles from sucrose and metrizamide gradients were dialyzed against 50 mM PO_4 , 1 mM $MgCl_2$, and fixed with 1% glutaraldehyde. Aliquots of 1 ml were applied to preformed Cs_2SO_4 gradients and centrifuged as described in "Materials and Methods." A) Particles from sucrose gradients, B) Particles from Metrizamide gradients of low ionic strength, C) Particles from Metrizamide gradients of high ionic strength. Equal amounts of radioactivity were applied to each gradient. Approximately 60% of the radioactivity was recoverable as TCA-precipitable radioactivity after centrifugation. O⟶O radioactivity derived from [^{3}H]orotic acid, x⟶x density (gm/cm^3)

of the label shifted towards the density of free RNA and banded at about 1.66 gm/cm^3. This shift in bouyant density of the fixed nucleic acid in Cs_2SO_4 confirms the density shifts observed in Metrizamide, and indicates that virtually complete dissociation of the RNA and protein is obtained. ^{32}P-labeled ribonucleoprotein particles banded in CsCl behave similarly (figure 31). After sucrose gradient centrifugation, and rebanding in Metrizamide at low ionic strength the ribonucleoprotein particles banded as sharp peaks at about 1.38 - 1.39 gm/cm^3 in CsCl. (figure 31, panels A and B). These values agree well with previously published values for the equilibrium bouyant density of ribonucleoprotein in CsCl (Lukanidan et al., 1972). After centrifugation in Metrizamide at high ionic strength most of the radioactivity applied to the CsCl gradients was not recoverable as a peak of isopycnically banded material (figure 31, panel C). A small peak of radioactivity was observed at 1.44 gm/cm^3 in the experiment shown in figure 31 C.

Figure 32 demonstrates that the ribonucleoprotein particles are stable to resedimentation in sucrose gradients (panel A), and that even after isopycnic banding in Metrizamide at low ionic strength, the majority of the radioactivity will resediment as a discrete peak at 40S (figure 32 C). Exposure of the particles to 0.6 M KCl causes disaggregation of the 40S particles, and all the ^{32}P-radioactivity applied to the sucrose gradient is recovered in the 4 to 10 S region. These sedimentation properties emphasize the lability

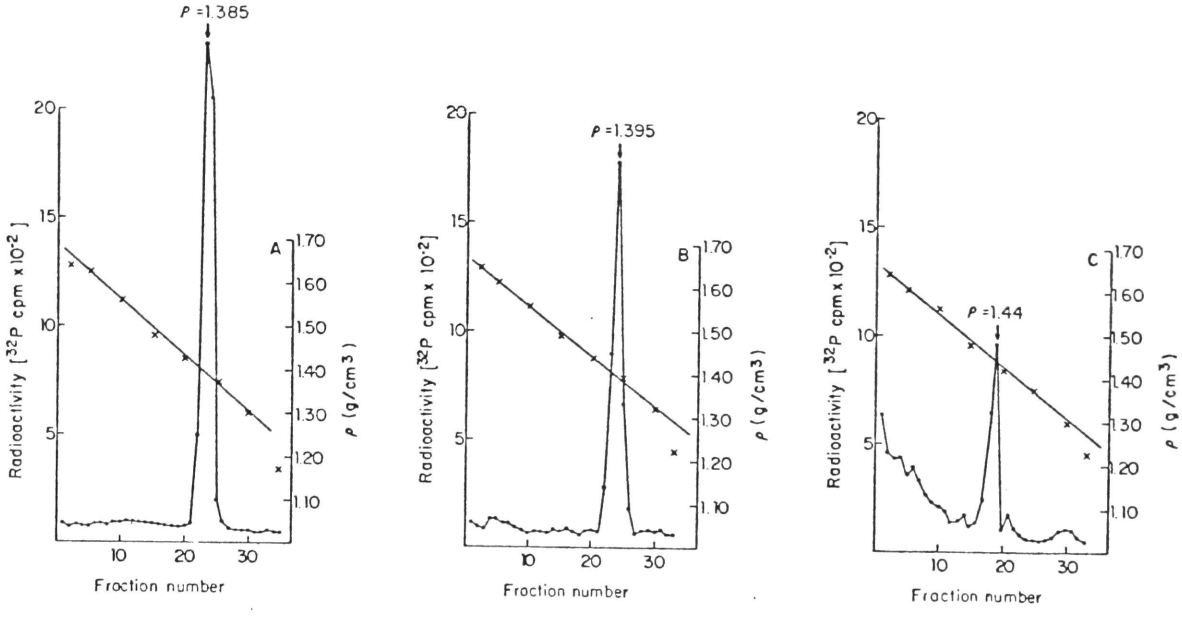

Figure 31. Isopycnic banding of rat liver 40 S RNP in CsCl after centrifugation in sucrose gradients and in Metrizamide gradients of high or low ionic strength. Fractions were fixed as described in the legend to figure 30, and aliquots of 1 ml were applied to CsCl gradients and centrifuged as described in "Materials and Methods." A. Particles from sucrose gradients (recovery of radioactivity, 63%) B. Particles from Metrizamide gradients of low ionic strength (recovery, 45%) C. Particles from Metrizamide gradients of high ionic strength (recovery 31%). ●——● ^{32}P radioactivity, x----x density (gm/cm^3)

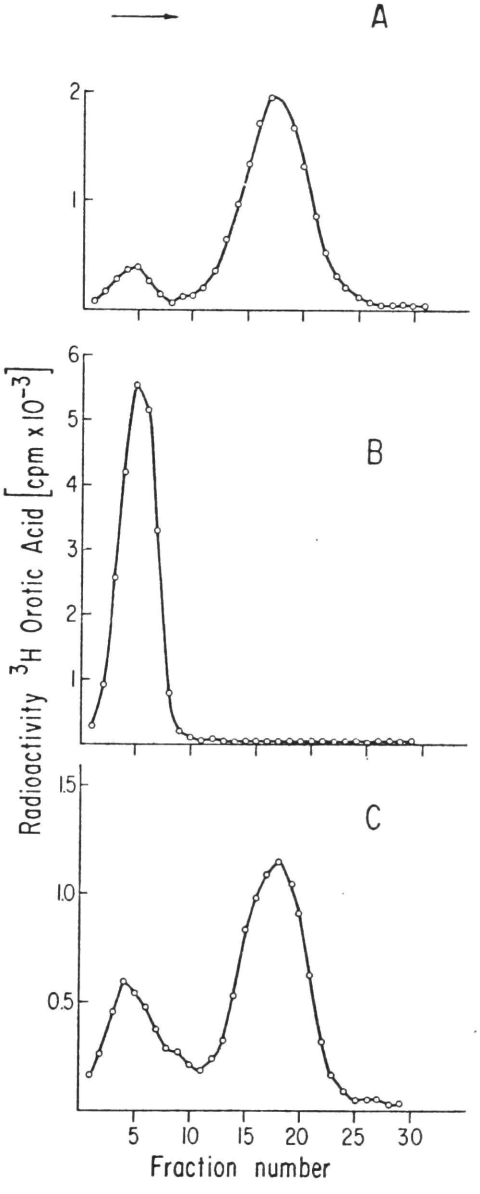

Figure 32. Resedimentation of rat liver 40S RNP. A) 40S particles from sucrose gradients, precipitated with ammonium sulfate and applied to another sucrose gradient in 0.1 M NaCl, 0.01 M triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$; B) Same as A, but buffer in sample and gradients contained 0.6 M NaCl; C) RNP particles after isopycnic banding in Metrizamide gradients of low ionic strength, dialyzed against 0.1 M NaCl, 0.01 M triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$ and applied to sucrose gradient. Linear 15-30% sucrose gradients were centrifuged for 15 hours at 23,000 rpm (SW 27 rotor), fractionated by displacement with 60% sucrose and the total radioactivity in each fraction determined. The direction of sedimentation is from left to right.

of the particles to high salt, and indicate that the RNA recovered from the particles is of low molecular weight. Similar observations on the sedimentation characteristics of 40 S particles in high salt have been reported by Samarina et al., (1967).

Figure 33 compares densitometric tracings of the electrophoretic banding patterns in SDS-polyacrylamide gels of the proteins associated with ribonucleoprotein particles before (panel A) and after (panel B) centrifugation in Metrizamide. The majority of the proteins of the complex are retained after isopycnic banding. Note that the proteins in region I to IV of the gel are retained in the same proportions after the isopycnic centrifugation. There is some loss of proteins of higher molecular weight, consistent with the loss of ^{3}H leucine during the centrifugation. Since it is possible to resediment the 40 S particle after isopycnic banding in Metrizamide (figure 32 C), this data suggests that the high molecular weight proteins dissociated at low ionic strength from the ribonucleoprotein complex are not required to maintain the structure of the 40 S particle.

The stability of the ribonucleoprotein particles to resedimentation and to isopycnic banding in Metrizamide, suggested that these two steps might be effecively used in a purification scheme for ribonucleoprotein particles. The results of this kind of purification are illustrated in figure 34 which compares the SDS-polyacrylamide gel profiles of the proteins associated with a single preparation of

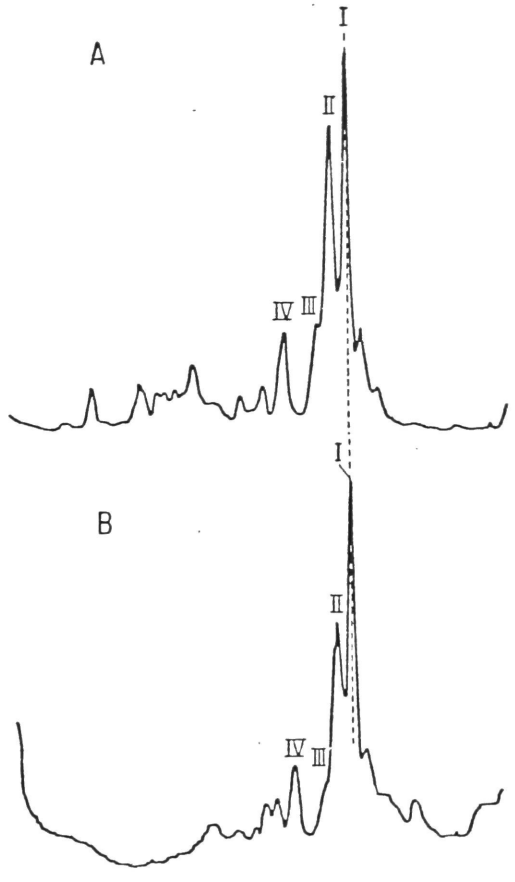

Figure 33. Comparison of proteins associated with rat liver 40S ribonucleoprotein particles before and after isopycnic banding in Metrizamide by SDS-polyacrylamide gel electrophoresis. A. RNP from sucrose gradients, B. The same preparation after isopycnic banding in Metrizamide gradients of low ionic strength. 8.75% SDS-polyacrylamide gels were run as described in "Materials and Methods" stained with Fast Green and analysed by densitometry. Recentrifugation in Metrizamide result in the loss of many proteins of high molecular weight. Proteins I - IV are retained in the same relative proportions.

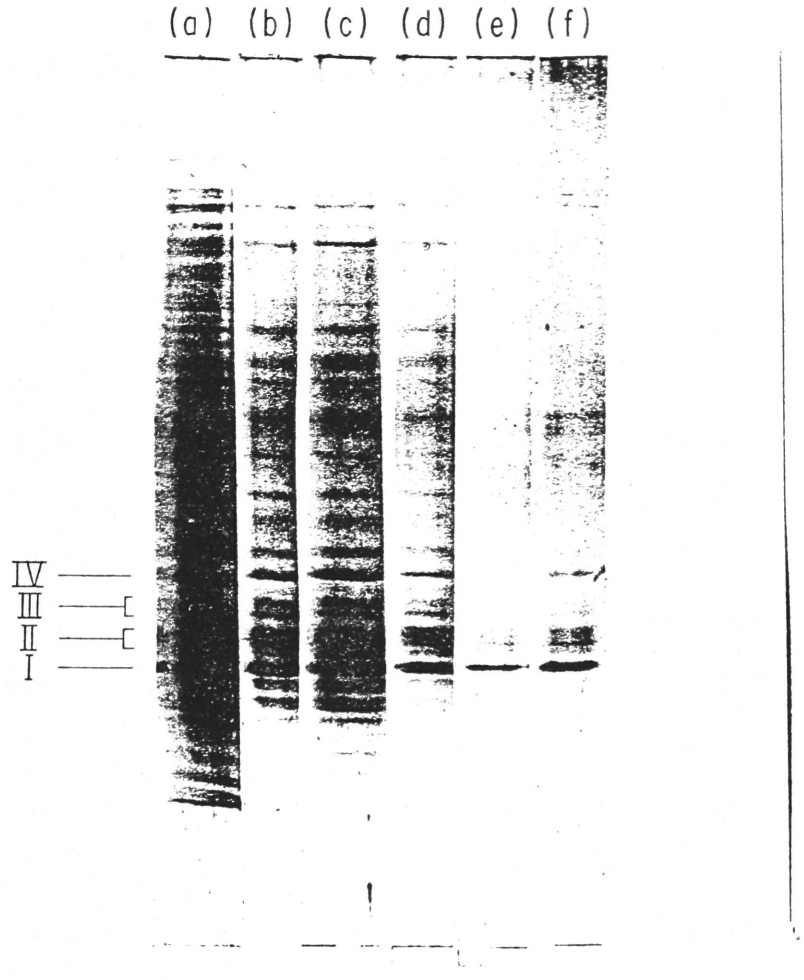

Figure 34. Effects of successive recentrifugation and isopycnic banding on proteins associated with 40S RNP. The figure depicts analysis of the same preparation of ribonucleoprotein at different stages of purification by SDS-polyacrylamide gradeint gel electrophoresis. a) Nuclear extract b) 40S particles after first sucrose gradient centrifugation c) 40S particles after precipitation with ammonium sulfate d) 40S particles after recentrifugation on sucrose gradients e) 40S particles from the second sucrose gradients banded isopycnically in Metrizamide f) 40S particles treated with 0.01 µg/ml pancreatic ribonuclease for 30 minutes at 4 °C, recentrifuged on sucrose gradients and banded isopycnically in Metrizamide. Conditions for centrifugation and electrophoresis are given in "Materials and Methods."

ribonucleoprotein particles at various stages of purification. Particles obtained from sucrose gradients as previously described (panel b) were precipitated with ammonium sulfate (panel c), recentrifuged (panel d) and banded isopycnically in Metrizamide (panel e). Another preparation was treated identically but digested with 0.01 μg RNase for 30 minutes at 0 °C before the second sucrose gradient centrifugation step. This material is shown after isopycnic banding in Metrizamide in panel f. It can be seen that each step in this scheme results in a simplification of the banding patterns of the proteins associated with ribonucleoprotein. After the Metrizamide gradient step, virtually only the proteins of molecular weight 33,000 - 43,000 daltons remain associated with RNA. The rat liver ribonucleoprotein particles are partially resistant to pancreatic ribonuclease and treatment of the particle with this enzyme does not result in the loss of additional proteins from the ribonucleoprotein particles purified through the Metrizamide gradient step. It is difficult to determine whether the proteins lost during this rigorous isolation procedure are components present *in vitro* or contaminants. It seems clear, however, that the proteins required to maintain a core structure that sediments at 40S are limited in number, and may involve only those proteins of molecular weight 33,000 to 43,000 daltons. As noted previously, these proteins are the predominant proteins in less-rigorously prepared ribonucleoprotein particles.

Is there a salt-resistant protein core in the rat liver 40S particle? - A number of investigators have noted that when ribonucleoprotein complexes are exposed to various concentrations of salt some proteins are selectively solubilized (Pederson, 1974; Gallinaro-Martringe, 1975) This as been interpreted as evidence for ribonucleoprotein structures of high and low resistance stability in salt (Kumar and Pederson, 1975).

Figure 35 illustrates an experiment in which selective solubilization of proteins I and IIA from rat liver ribonucleoprotein particles was observed. Ribonucleoprotein particles were dialyzed against various concentrations of NaCl, in 10 mM triethanolamine-HCl buffer, pH 8.0, and soluble and insoluble proteins were fractionated by high speed centrifugation. The pellets and supernatant fractions were assayed for protein content by SDS-polyacrylamide gel electrophoresis. In this particular preparation, there was noticeable contamination of the 40S particles with proteins of high molecular weight. Most of the high molecular weight proteins pelleted with the particles under these conditions of increasing ionic strength. The solubilization of proteins I and IIA appears to be a progressive phenomenon: no clear-cut transitions are observed, and RNA as well as protein was released into the supernatant. This data combined with the previous data concerning the complete dissociation of rat liver ribonucleoprotein particles in Metrizamide gradients in 0.6 M KCl suggest that there is no

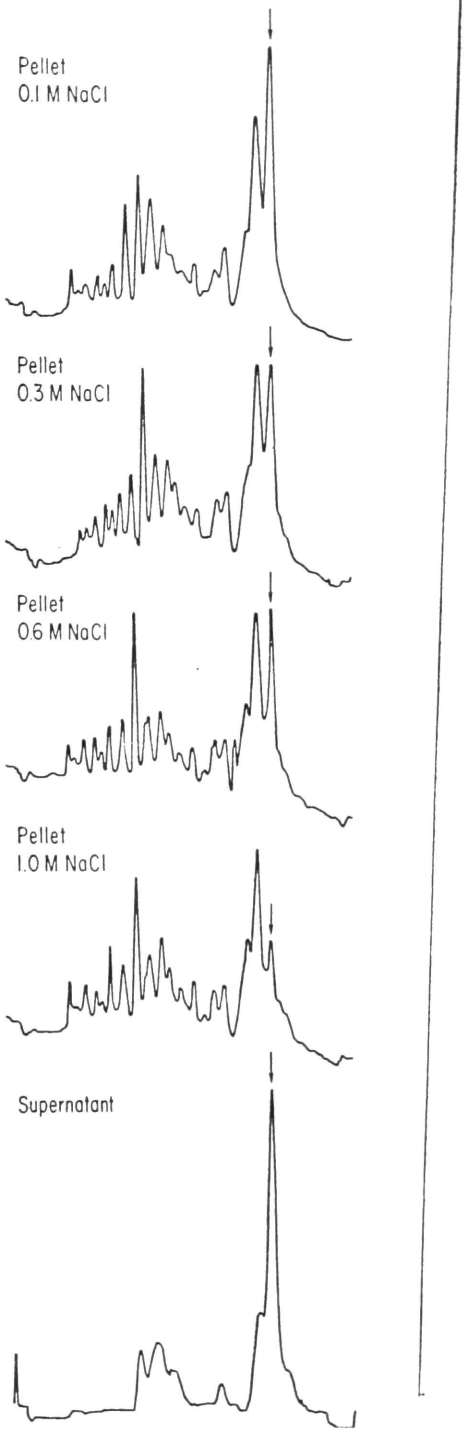

Figure 35. Differential solubilization of proteins from 40S ribonucleoprotein particles in buffers of high ionic strength. Particles were isolated by velocity centrifugation on sucrose gradients as described in "Materials and Methods." Fractions were dialyzed against 0.1 M, 0.3 M, 0.6 M or 1.0 M NaCl all with 10 mM triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$ at 4 °C. The samples were then centrifuged at 368,000 x g for 6 hours and the pelleted particles and solubilized proteins were analyzed by electrophoresis on 8.75% SDS-polyacrylamide gels. Proteins I (indicated by arrow), IIA and some proteins of higher molecular weight are selectively solubilized under these conditions.

salt resistant core particle. However, a differential solubility of the ribonucleoprotein particle proteins appears evident, and selective separations of proteins I and IIA from the other proteins in the rat liver 40S particle may be reproducibly obtained.

Further evidence concerning the lability of ribonucleoprotein particles to high salt was obtained from circular dichroism studies of RNP in buffers of various ionic strength, and of phenol-extracted RNA. (Table XI). Purified RNA and ribonucleotide homopolymers have extremely high positive ellipticity at about 260 nm. RNA prepared from 40S particles by phenol extraction has an ellipticity of 18,000 to 14,000 deg cm^2 per decimole nucleotide residues, depending on the buffer. In native 40S particles, by contrast, there is an almost complete suppression of the ellipticity of the RNA and ellipticities of only 5,000 to 6,000 deg cm^2 per decimole nucleotide residues are observed. This suppression of ellipticity is a direct spectral measurement of the interactions between RNA and protein in the particles. Analogous shifts in the circular dichroism spectrum of poly A were noted by Durand et al. (1975) when peptides are bound to this ribohomopolymer. Similarly, when DNA is eomplexed with histones, its circular dichroism spectrum is altered (Adler et al., 1974).

When nuclear RNA particles are exposed to increasing concentrations of salt, there is a progressive increase in the ellipticity of the RNA, indicating a progressive dissociation of the RNA and protein. (Table XI). There

TABLE XI

CIRCULAR DICHROISM PROPERTIES OF RAT LIVER RNP AND RNA

SAMPLE	BUFFER*	Conc.x 10^{-5}M	λ(nm)	θ_{λ} †
40S RNA	0.1 M NaCl	13.40	266	18,079
40S RNA	4.0 Gd-HCl¶	7.05	268	14,017
40S RNP	0.1 M NaCl	5.65	265	5,945
40S RNP	0.3 M NaCl	8.39	267	7,912
40S RNP	0.6 M NaCl	7.93	266	9,935
40S RNP	1.0 M NaCl	6.79	267	12,969
40S RNP	1.5 M NaCl	7.29	266	14,164
40S RNP	2.0 M NaF	9.36	269	9,181
40S RNP	4.0 Gd-HCl¶	8.06	269	10,765
40S RNP	50 mM EDTA	9.78	268	5,033
40S RNP	0.25% SDS	7.94	277	13,997

* 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2 and salt as indicated

† deg cm^2/decimole

¶ guanidine-HCl

is a good correlation between the change in ellipticity of the RNA and extent of solubilization of proteins I and IIA. As noted previously, no transitions such as might be expected if a salt-resistant core particle were present are observed. At 1.5 M NaCl, the ellipticity of RNP is nearly the same as that of free RNA, indicating nearly complete dissociation of the proteins and RNA in buffers containing salt at this concentration. Other dissociating agents such as 4M guanidine hydrochloride, and 0.25% SDS are effective in completely dissociating the RNA and protein, as measured by circular dichroism. From centrifugation studies, the 40S particles are known to be resistant to EDTA (Faiferman et al., 1970). Even in the presence of 50 mM EDTA, the ellipticity of the RNP is indistinguishable from that of the native particles.

The circular dichroism data provides direct spectral evidence of the association of RNA and protein in rat liver 40S particles. These complexes appear labile to high salt, and increasing the salt concentration of buffers above 0.6 M NaCl promotes shifts in the sedimentation coefficient, equilibrium bouyant density, and circular dichroism spectra of the particles. No evidence for salt-resistant ribonucleoprotein particles was obtained in these studies.

The major structural proteins of rat liver ribonucleoprotein particles are basic - In order to further characterize the proteins in the ribonucleoprotein complexes, we have begun an investigation of their chemistry. About 70% of the proteins that coisolate on sucrose gradients with RNA,

behave chromatographically as basic proteins. Figure 36 shows the elution profile of ribonucleoprotein particles disrupted in urea and applied to a column of DEAE Sephadex. In order to follow the proteins better during chromatography, the proteins were labeled by reductive methylation of lysine residues *in vitro* following the method of Rice and Means (1970). Most of the protein applied to the column elutes as a run-off peak at about fraction 10. The small peak of radioactivity eluting at fraction 20 is unreacted [^{14}C]-formaldehyde, which is separated because of the gel filtration properties of DEAE-Sephadex. Approximately 30% of the applied radioactivity eluted from the column with 1 M KCl.

The DEAE-Sephadex chromatography separates RNA from the basic proteins as well as separating the acidic and basic protein components. Evidence for the removal of RNA from the DEAE run-off fraction is shown in figure 37, which compares the UV absorption spectra of ribonucleoprotein particles and of proteins eluting from the DEAE columns. Ribonucleoprotein exhibits a characteristic spectrum with an OD maximum at 258 nm and a shoulder at 280 nm. The proteins in the DEAE run off peak have a OD maximum at 276 nm and a shoulder at 283 nm. There is no spectral evidence for RNA contaminating the DEAE run-off fraction. Experiments in which RNA was labeled *in vivo* confirm that the RNA is completely dissociated from the basic proteins by chromatography on DEAE-Sephadex in the presence of urea.

Figure 38 compares the SDS-polyacrylamide gel banding patterns of proteins in the ribonucleoprotein particles,

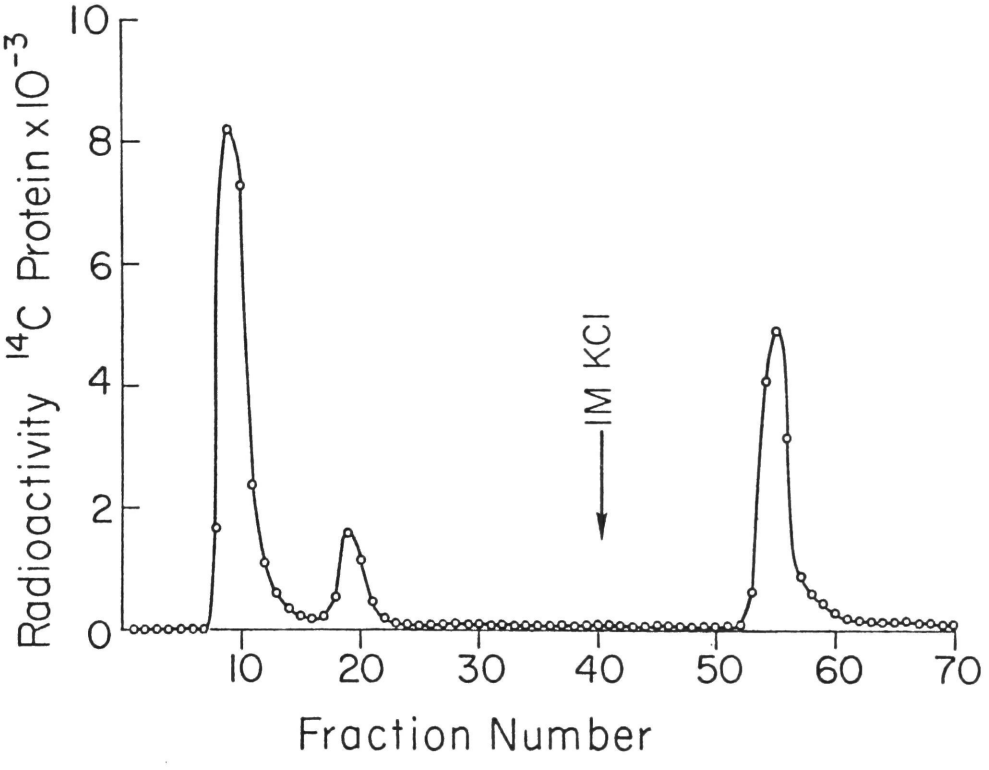

Figure 36. Chromatography of ^{14}C-labeled 40S ribonucleoprotein particle proteins on DEAE-Sephadex. 40S particles were isolated from sucrose gradients, labeled *in vitro* by reductive alkylation of lysine residues with [^{14}C]-formaldehyde and sodium borohydride, and applied to 1.7 x 25 cm columns of DEAE Sephadex equilibrated with 6 M urea, 10 mM Tris-HCl, pH 8.0, 10 mM KCl, 50 µl/ℓ 2-mercaptoethanol, as described in "Materials and Methods." Columns were eluted with starting buffer, and fractions of 1 ml collected. After 40 fractions, the columns were eluted with 1 M KCl in starting buffer. Radioactivity was monitored in 20 µl aliquots of the fractions as described in "Materials and Methods."

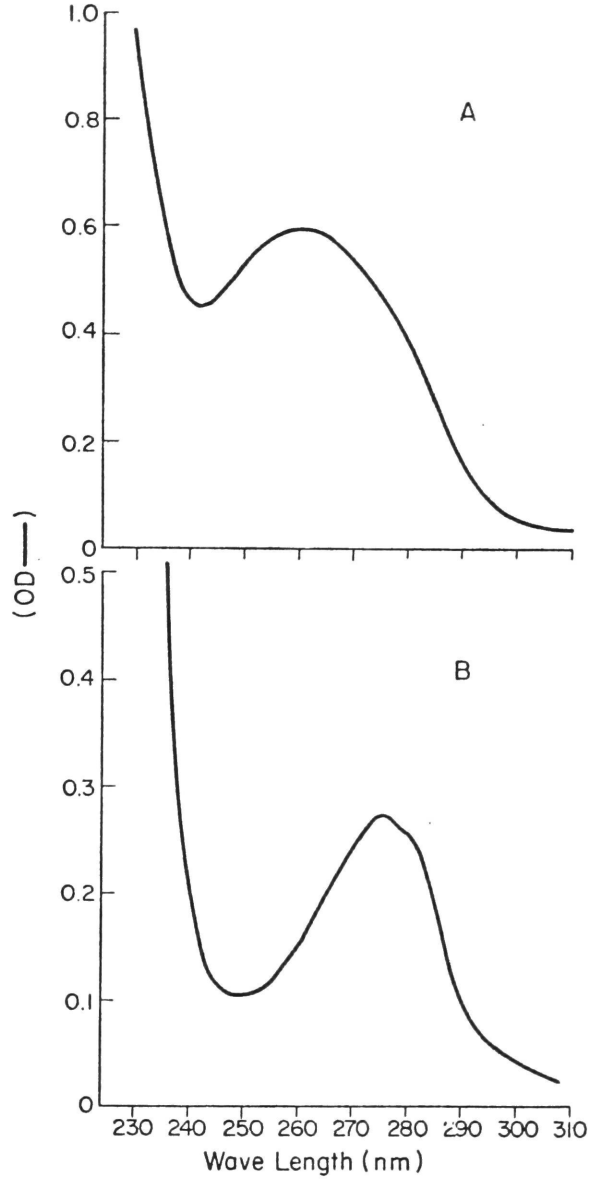

Figure 37. Ultraviolet absorption spectra of 40S ribonucleoprotein particles and basic proteins purified by chromatography on DEAE-Sephadex. Panel A. 40 S ribonucleoprotein particles in 0.1 M NaCl, 10 mM triethanolamine-HCl, pH 8.0, 1 mM $MgCl_2$. Panel B. Proteins from DEAE-Sephadex "run-off" fraction in 6 M urea, 10 mM Tris-HCl, pH 8.0, 10 mM KCl. Spectra were recorded against appropriate blanks in a 1 cm quartz cells using a Pye-Unicam model SP 1800 recording spectrophotometer.

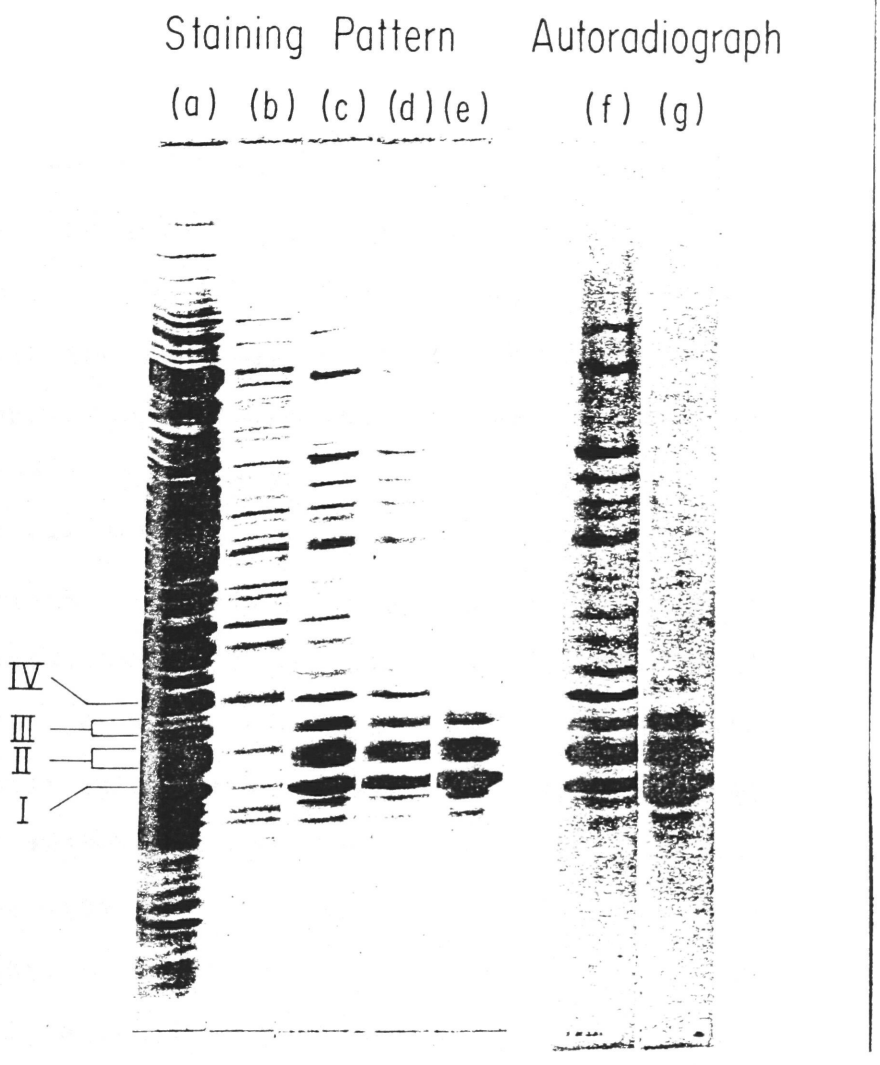

Figure 38. Comparison by SDS-polyacrylamide gradient gel electrophoresis of ribonucleoprotein fractions and basic proteins isolated by chromatography on DEAE-Sephadex. a) Nuclear extract; b) 15-20S region of sucrose gradients; c) 40S ribonucleoprotein particles; d) 40S ribonucleoprotein particles after labeling with [^{14}C] formaldehyde by reductive alkylation of lysine residues; e) Basic proteins selected by chromatography on DEAE-Sephadex; f) Autoradiograph of slot d; g) autoradiograph of slot e. Note that no high molecular weight products are generated by the alkylation reaction, and that all the proteins are labeled. The conditions for centrifugation, alkylation and chromatography are given in "Materials and Methods."

and the basic proteins selected by chromatography on DEAE. An autoradiograph of the same gel is included to illustrate the efficacy of the labeling procedure. Comparison of panels c, d, and e shows that the proteins in fractions I-VII, with the exception of IVC are basic proteins. All the higher molecular weight proteins and IVC are retained on the DEAE column. This evidence of chemical similarity, together with the previous evidence for copurification after isopycnic centrifugation, provides evidence that these prominent, basic proteins are the major structural elements of the 40S ribonucleoprotein particle.

Comparison of panels c to g shows that under the labeling conditions used, all the proteins are labeled, and that there is no cross-linking of the proteins generating high molecular weight aggregates. Comparisons of densitometric tracings of autoradiographs and stained gels, as well as visual comparisons of the autoradiographs and gels, demonstrates that the labeling of the proteins is to a first approximation proportional to the concentration of protein in the sample.

In light of this chromatographic evidence of the charge similarity of many of the major proteins in the 40S ribonucleoprotein particle, it is possible to reinterpret the original observation of Samarina _et al_. that a single protein component is observed when ribonucleoprotein particles are electrophoresed in polyacrylamide gels containing urea, using the buffer system of Reisfeld. Figure 39 illustrates a gel run under these conditions. The single band that results is a mixture of the DEAE run-off

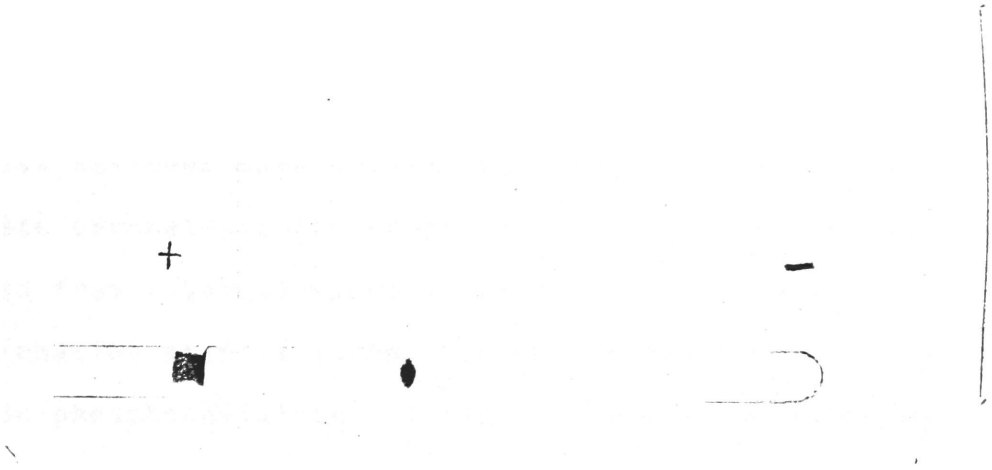

Figure 39. Fractionation of 40S ribonucleoprotein particle proteins on polyacrylamide gels containing 6M urea, pH 4.3. The basic proteins from the 40S ribonucleoprotein particles migrate toward the cathode at this pH. Under these conditions, the proteins in the DEAE run-off fraction are not resolved electrophoreticly and co-migrate as an apparently homogenous band. Conditions for the preparation of sample and electrophoresis are given in "Materials and Methods."

peak proteins, and may be further fractionated according to molecular size simply by rerunning on SDS-polyacrylamide gels.

Fractionation of the basic proteins from rat liver 40S particles - Progress has been made towards our goal of obtaining a complete chromatographic resolution of the basic proteins derived from ribonucleoprotein particles. The primary fractionation step is chromatography of the DEAE - run-off peak on phosphocellulose. A typical elution profile and SDS-polyacrylamide gel analysis of the resulting fractions is shown in figures 40 and 41. The elution order of the proteins is indicated by the Roman numeral on the chromatogram. Fraction IA is a protein that comigrates with fraction I on SDS gels (Figure 42 panels A,B,D) but is resolved by charge on the phosphocellulose column. As can be seen from the gel patterns depicted in figure 41, fractions I and IA can be obtained in a relatively pure state after this first chromatographic separation. Fraction IIBC contains the proteins IIB and IIC, which are not resolved although they are well separated from the other proteins in the basic fraction. In the later half of the chromatogram the resolution of the components is less good. Fraction IIA is almost 50% cross contaminated by fraction I, and fraction III contains IIIA, IIIB, IIA, I and another protein in the II region of the gel designated IID. The fraction containing IVA and IVB is likewise heavily contaminated by IIIA, IIIB, IIID, and I.

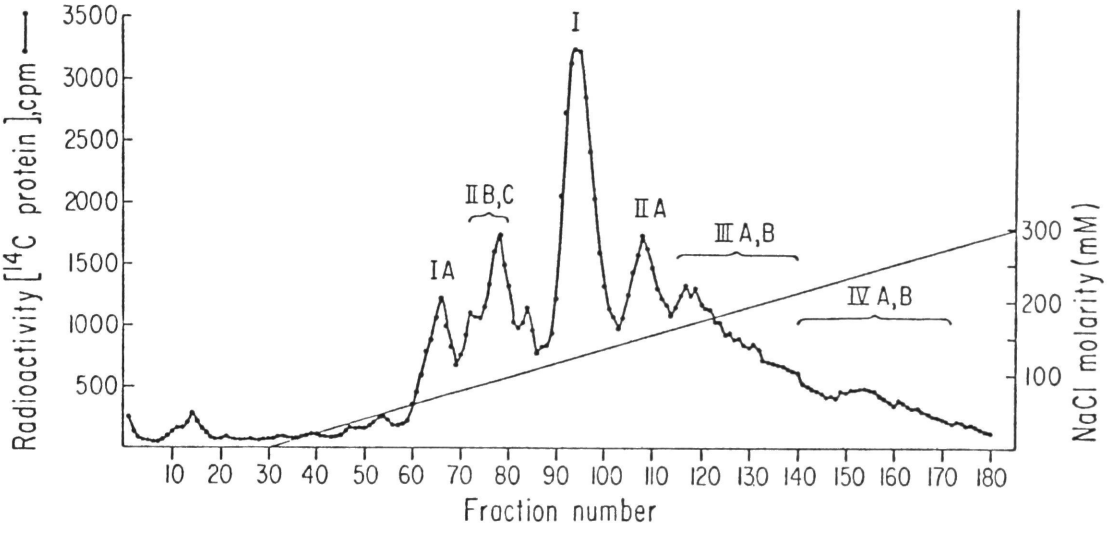

Figure 40. Fractionation of the basic proteins from rat liver 40S ribonucleoprotein particles by chromatography on phosphocellulose. The proteins from the DEAE-Sephadex run-off fraction, labeled with ^{14}C *in vitro* by reductive alkylation with [^{14}C] formaldehyde and sodium borohydride, were applied to 0.7 cm x 26 cm columns of phosphocellulose equilibrated with 50 mM methylammonium phosphate, pH 6.8 buffer containing 50 µl per liter 2 -mercaptoethanol. Proteins were eluted with a 400 ml gradient from 0-300 mM NaCl in methylammonium phosphate buffer. Fractions of approximately 2.5 ml were collected and aliquots of 250 µl monitored for radioactivity. Greater than 90% recovery of radioactivity was obtained. The elution order of the proteins is indicated by the Roman numerals which refer to proteins fractionated by molecular weight on SDS-polyacrylamide gels. Analysis of the fractions by SDS-polyacrylamide gel electrophoresis is shown in figure 41. Details concerning the preparation of phosphocellulose columns and ribonucleoprotein particles are given in "Materials and Methods."

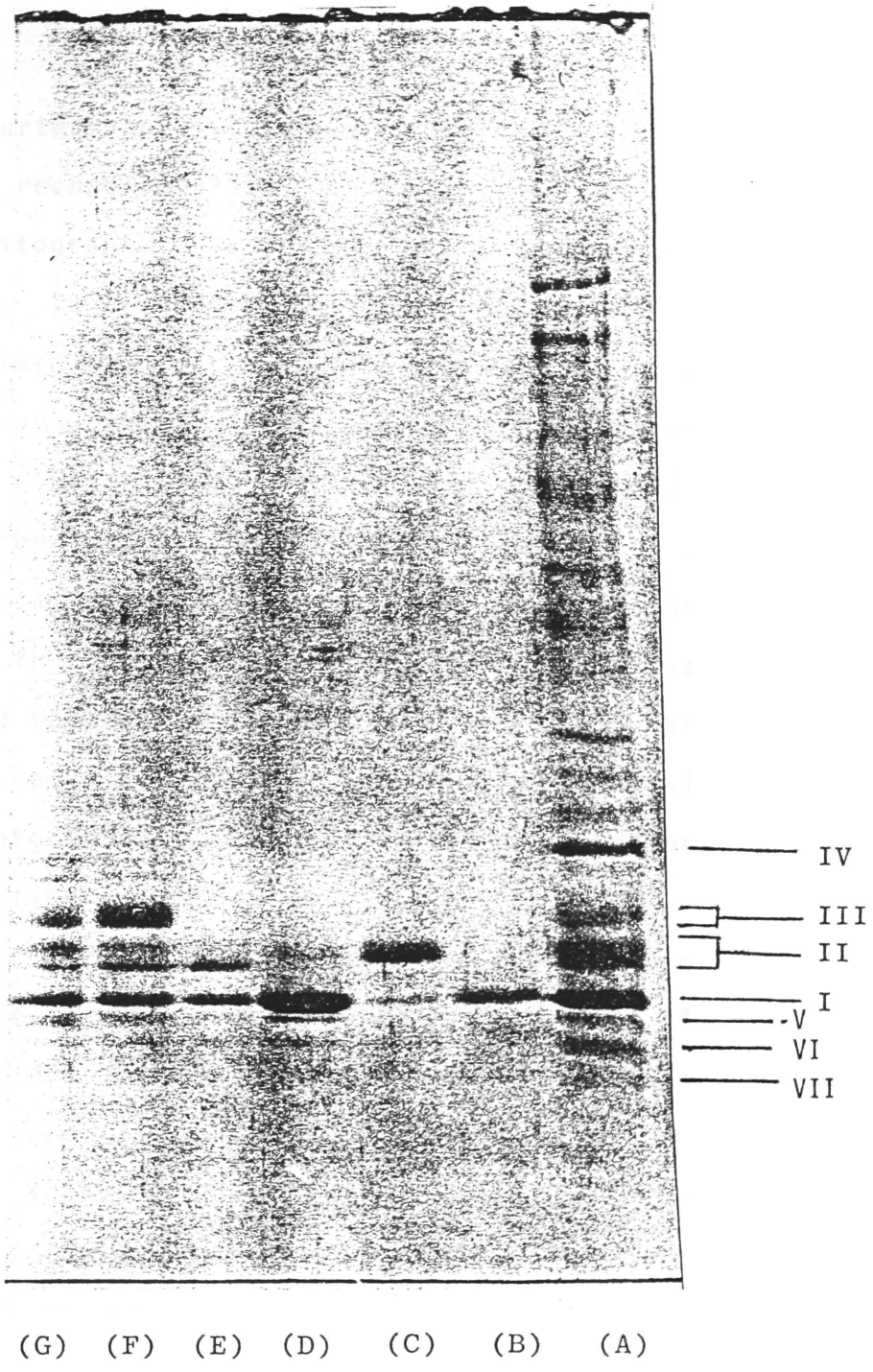

Figure 41. Analysis by SDS-polyacrylamide gel electrophoresis of pooled fractions from the phosphocellulose column depicted in figure 40. A) Total 40S ribonucleoprotein particles; B) Fractions 63-68 (Protein IA, trace VII); C) Fractions 76-80 (Proteins IIB, IIC, trace I); D) Fractions 92-98 (Protein I, traces V, VI); E) Fractions 106-111 (Proteins IIA and I); F) Fractions 116-135 (Proteins IIIA, IIIB, IID, IIA, I, V); G) Fractions 136-160 (Proteins IVA, IVB, IIIB, IID, IIA, I, V). Conditions for chromatography and electrophoresis are given in the legend to figure 40, and in "Materials and Methods."

Some further purification of the proteins can be achieved by rechromatography on phosphocellulose at pH 6.8 or by chromatography on carboxymethyl cellulose at pH 5.6 (Figure 42). In this way fractions I, IIA, and IIBC can be obtained with approximately 80% purity. Fraction IIIA, B is not well resolved on CM cellulose and remains extensively contaminated by proteins I, IIA, and IID.

The chromatographic separations, in conjunction with SDS-polyacrylamide gels, place a lower limit on the number of proteins in the DEAE run-off fraction, since separations are achieved both according to charge and molecular weight. On the basis of a bidimensional analysis of this kind, it is estimated that 12-15 distinct proteins are present in this fraction.

Amino acid compositions have been obtained for the partially purified fractions shown in figure 43. (Table XII). The amino acid compositions of each of the fractions are practically identical, reinforcing the idea of chemical similarities among the proteins in this group. Fractions IIB, C and IIIA,B, contain the unusual amino acid N^G,N^G-dimethyl-arginine. This amino acid probably arises as the result of postsynthetic modifications of the arginine residues *in vivo* and may have a significant regulatory role. Dimethyl-arginine elutes between ammonia and arginine on the amino acid analyzer column run under standard conditions (figure 44). This amino acid has been further identified by Dr. Lidia C. Boffa in our laboratory, by chromatography with known standards in a variety of systems. The amino acid compositions of the

Figure 42. Rechromatography of protein fractions separated by chromatography on phosphocellulose. From top to bottom: Total DEAE Sephadex fraction on phosphocellulose pH 6.8; rechromatography of protein I (fraction 73-82) on phosphocellulose pH 6.8; rechromatography of protein IIA (fractions 84-92) on phosphocellulose pH 6.8; rechromatography of proteins IIB and IIC (fractions 55 - 70) on phosphocellulose pH 6.5; rechromatography of proteins IIIA and IIIB (fractions 95-105) on carboxymethylcellulose pH 5.6. Chromatography conditions are given in "Materials and Methods."

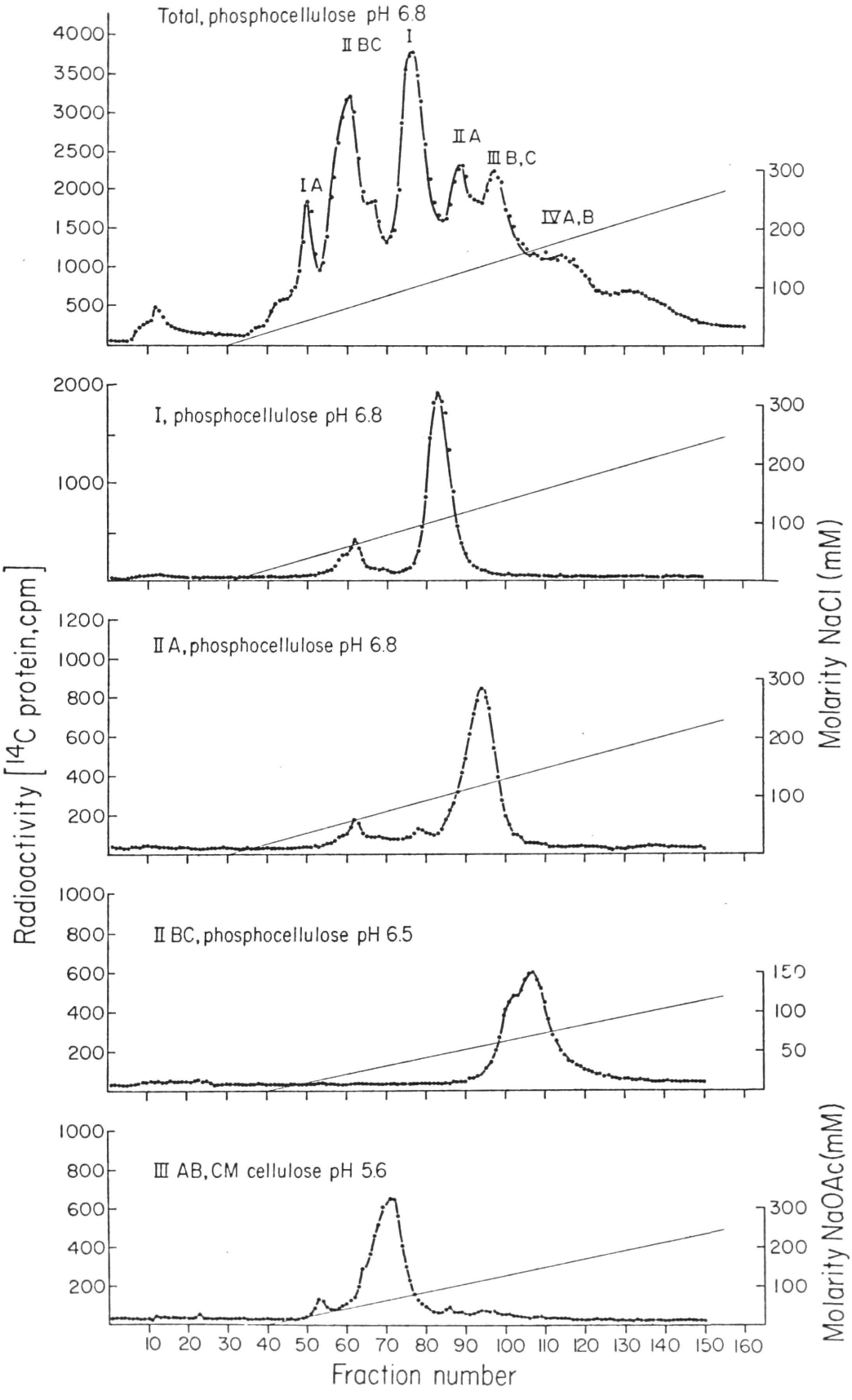
Total, phosphocellulose pH 6.8
IIBC
I
IIA
IIIB,C
IA
IVA,B
I, phosphocellulose pH 6.8
IIA, phosphocellulose pH 6.8
IIBC, phosphocellulose pH 6.5
IIIAB, CM cellulose pH 5.6
Radioactivity [14C protein, cpm]
Molarity NaCl (mM)
Molarity NaOAc(mM)
Fraction number

Table XII

AMINO ACID COMPOSITIONS OF RAT LIVER RNP PROTEINS #

moles / 100 moles total amino acids *

Amino Acid	I	IIBC	IIA	III
Aspartic Acid	10.74	10.39	10.83	9.83
Threonine	3.23	3.70	3.89	3.59
Serine	7.15	7.62	9.16	9.12
Glutamic Acid	10.53	11.18	12.03	11.74
Proline	3 .65	2.82	3.29	3.85
Glycine	25.54	27.72	25.95	24.83
Alanine	3.63	3.25	4.04	4.04
Half Cystine	0.63	0.57	0.57	0.67
Valine	4.16	4.79	4.33	4.41
Methionine	---	---	---	---
Isoleucine	2.37	2.12	2.37	2.06
Leucine	2.58	2.69	3.32	2.86
Tyrosine	5.37	4.85	4.38	3.39
Phenylalanine	5.58	5.39	5.37	4.45
Lysine	5.34	6.24	6.16	5.66
Histidine	2.21	2.03	2.07	1.77
N^G, N^G dimethyl-Arginine	---	1.70	---	1.18
Arginine	7.29	5.75	7.62	6.53

*Values not corrected for hydrolytic losses

Analysis of the protein fractions by SDS-polyacrylamide gel electrophoresis is shown in figure 43.

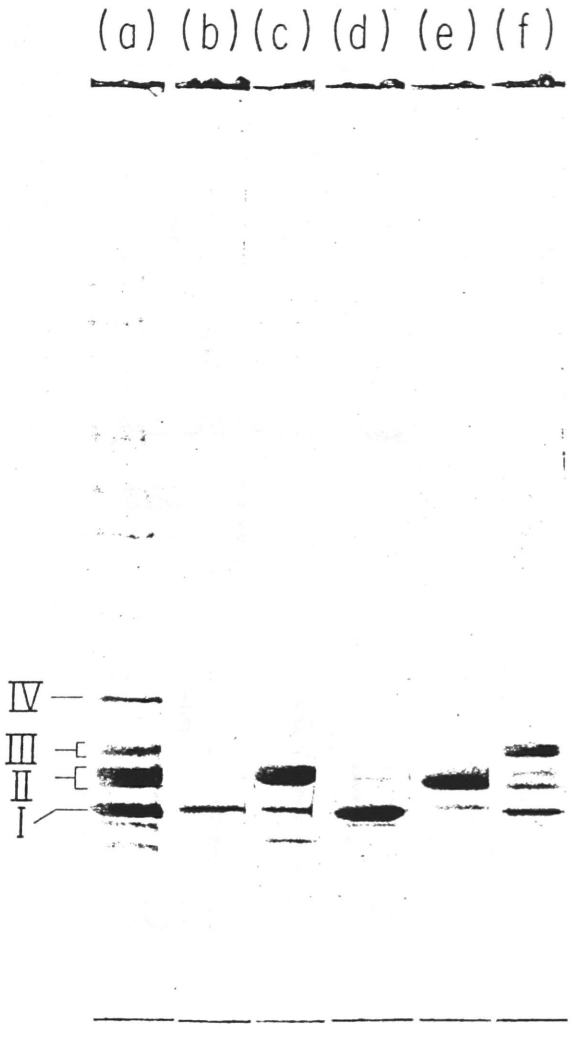

Figure 43. Analysis of protein fractions analysed for amino acid content in Table XII by SDS-polyacrylamide gradient gel electrophoresis. a) Total 40S ribonucleoprotein particles b) Fraction IA, c) Fraction IIB, C d) Fraction I e) Fraction IIA f) Fraction IIIA,B. These fractions were obtained from two separate experiments. Fractions IIB,C and III A,B were rechromatographed as described in Figure 42. These fractions illustrate typical purifications resulting from rechromatography. The other fractions were obtained directly after a single chromatography step on phosphocellulose, pH 6.8. Conditions for chromatography and electrophoresis are given in "Materials and Methods."

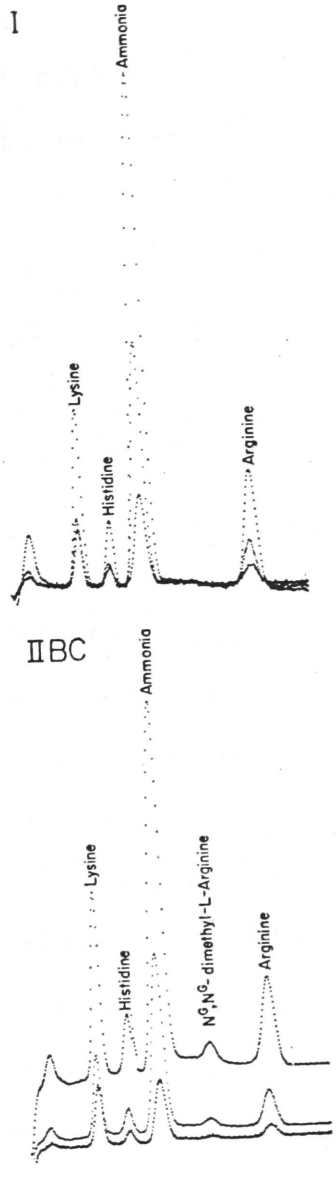

Figure 44. Separation of N^G, N^G -dimethyl-L-arginine from arginine on the amino acid analyzer. Top panel: elution profile of the basic amino acids from acid hydrolysates of protein fraction I. Bottom panel: elution profile of the basic amino acids from acid hydrolysates of protein fraction IIA, IIB. N^G, N^G-dimethyl-L-arginine is well resolved from arginine and elutes as a small peak after ammonia. The amino acid was identified by co-chromatography with known standards. Analyses were performed on a Beckman 120B amino acid analyser, modified for a 10-fold increase in sensitivity with a Honeywell scale expander.

partially purified RNP proteins agrees well with published compositions for the total proteins in this fraction, and are distinct from the compositions of nuclear phosphoproteins or histone proteins. (Table XIII).

Protection of lysine residues against alkylation in intact 40S particles - During the development of techniques for labeling the proteins of the 40S ribonucleoprotein particle in vitro , it was noted that the specific activity of the proteins increased dramatically when the alkylation reaction was run under conditions that resulted in the disruption of the ribonucleoprotein particles. Figure 45 illustrates an experiment in which the labeling of the proteins in intact particles and in 1 M KCl and 0.2% sodium deoxycholate disrupted particles were compared after chromatography on phosphocellulose. Disruption of the particles with high salt resulted in a disproportionate uptake of label into proteins I and IIA. These are the same proteins that appear to be solubilized in high salt (figure 35). The highest specific activities were obtained when the particles were disrupted with the detergent sodium deoxycholate. As illustrated in figure 45, proteins labeled under these conditions have nearly 4-fold greater specific activities than do proteins in control samples. Under these labeling conditions, the proteins appear to be uniformly labeled as judged by autoradiography of protein bands separated by SDS-polyacrylamide gel electrophoresis (figure 38).

The increase in the specific activity of the proteins following disruption of the particles suggests that in

TABLE XIII

AMINO ACID COMPOSITIONS OF NUCLEAR PROTEIN FRACTIONS

	Nuclear RNP					Nuclear Phosphoproteins			Histones		
Amino Acid	Mouse Ascites[a]	Duck Liver[a]	1[b]	Rat Liver 2[c]	3[d]	Rat Liver[e]	HeLa[f]	Calf Thymus[g]	H_2b[h]	H_3[h]	H_{2a2}[h]
Lysine	6.27	5.44	6.5	6.85	7.4	6.04	6.3	9.4	14.0	8.0	11.4
Histidine	1.99	1.98	2.6	1.77	2.4	2.52	2.3	1.9	2.1	1.3	3.0
Arginine	6.01	5.83	7.6	5.18	6.2	5.89	5.9	8.5	8.3	13.0	10.3
Aspartic Acid	10.80	9.46	10.6	12.00	10.4	10.28	9.6	10.5	5.1	4.1	5.6
Threonine	3.28	3.69	3.9	3.83	4.6	3.81	5.5	3.8	6.0	7.4	4.3
Serine	7.62	8.96	7.6	6.29	6.1	4.38	6.6	10.3	9.0	4.2	3.5
Glutamic Acid	10.53	13.07	10.6	11.30	11.9	12.16	11.5	14.9	8.5	11.3	9.6
Proline	3.67	3.90	5.7	5.21	4.6	6.49	4.9	6.2	7.7	3.4	3.9
Glycine	22.41	17.90	17.1	16.68	14.6	15.28	9.0	6.8	6.1	5.6	10.8
Alanine	3.96	7.16	5.5	5.53	5.5	6.73	7.0	6.2	10.0	14.4	12.0
Cysteine	-	-	0.68	0.27	0.19	-	-	0.6	-	1.5	-
Valine	4.75	4.35	4.1	5.33	5.7	6.10	5.9	4.5	6.6	4.6	6.6
Methionine	1.75	1.62	-	2.20	2.0	1.92	1.5	1.9	1.6	1.3	0.4
Isoleucine	2.54	2.62	2.4	3.04	3.3	4.13	5.8	3.0	4.5	5.3	4.1
Leucine	3.30	4.19	4.8	4.89	5.2	6.79	10.4	6.2	5.5	9.6	11.0
Tyrosine	5.64	3.73	4.5	4.65	4.0	3.26	3.6	2.4	3.2	1.9	2.6
Phenylalanine	5.42	4.09	4.2	4.83	5.1	3.96	3.9	2.8	1.6	2.8	0.9
X		2.01	1.4								

a. Martin et al., 1973

b. Krischevskaya and Georgiev, 1969

c. Ishikawa, et al., 1970

d. Sarasin, 1969

e. Teng et al., 1971

f. Karn et al., 1974

g. Kleinsmith, 1968

h. Sommer and Chalkley, 1974

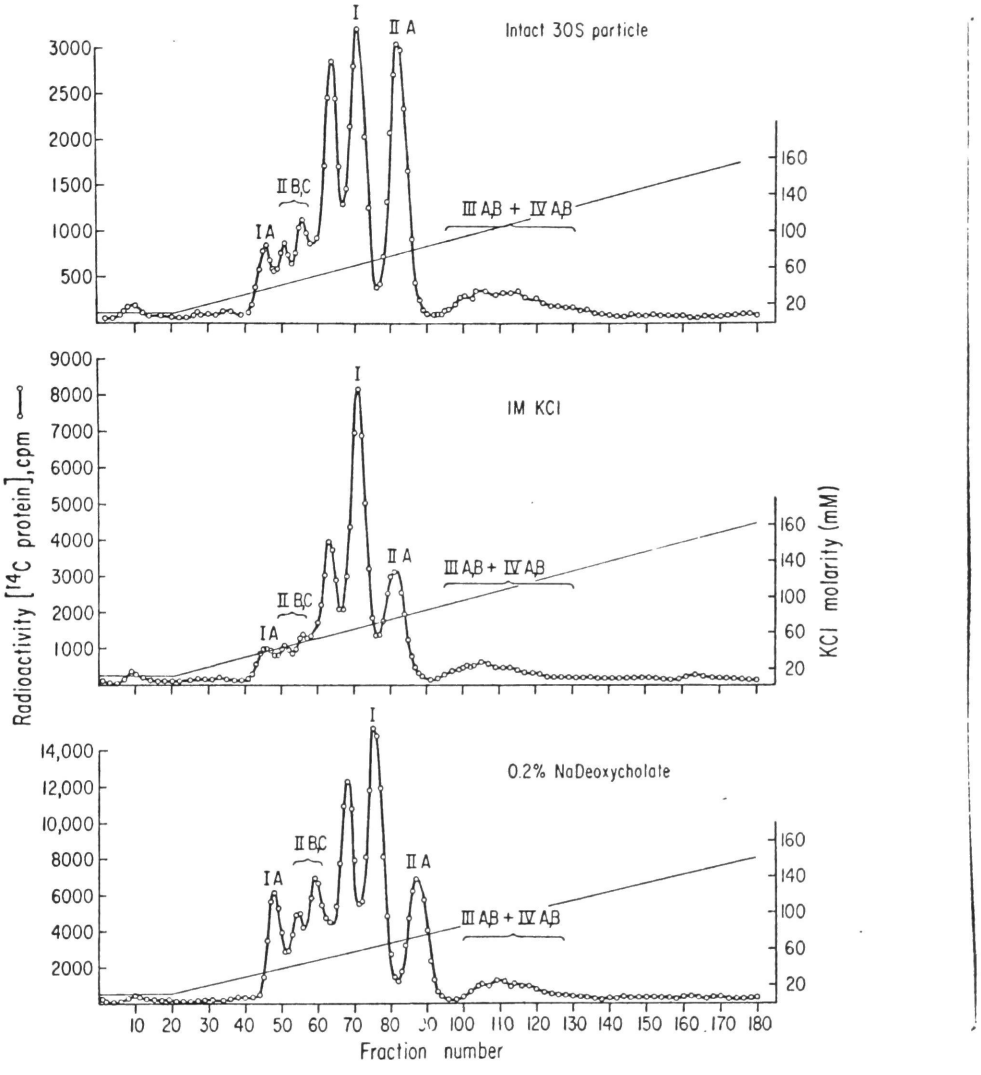

Figure 45. Accessibility of protein lysine residues to modification in vitro in intact, and in partially-disrupted 40S ribonucleoprotein particles. Ribonucleoprotein particles were isolated on sucrose gradients and equal aliquots were labeled with ^{14}C formaldehyde by reductive alkylation of lysine residues. The reaction products were analysed by chromatography on phosphocellulose after chromatography on DEAE-Sephadex. Top panel : labeling of proteins in particles in intact 40 S particles; Middle panel : labeling of proteins in particles disrupted with 1M KCl; Bottom panel: labeling of proteins in particles disrupted with 0.2% sodium deoxycholate. The specific activity of the proteins increases almost 4-fold when the particles are disrupted with detergent (note changing radioactivity scales). Phosphocellulose columns were equilibrated with 10 mM Tris-HCl, pH 7.2, 10 mM KCl, 1 mM DTT, and eluted with a 400 ml linear gradient of KCl from 10 mM KCl to 150 mM KCl. The protein eluting between fractions IIB, IIC and I is an unusual component which appeared in this preparation.

in the intact particle the lysine residues are stereochemically protected against alkylation. Since lysine residues do not usually intercalate with ribonucleotide homopolymers (Durand et al., 1974) it seems likely that the protection of the lysine residues is a measure of the interactions of the proteins with each other. It would be of interest to examine the accessibility of other amino acid residues in the structure by similar modification reactions (nitrosylation of tyrosine residues, etc.).

In vitro phosphorylation of ribonucleoprotein particles - It was noted that some of the proteins in the ribonucleoprotein complex are subject to postsynthetic modification of arginine residues resulting in the formation of dimethyl-arginine. Are these proteins phosphoproteins as well? We have examined the ability of protein kinases to phosphorylate 40S ribonucleoprotein particles in vitro. Recent in vivo evidence suggests that the ribonucleoproteins may be phosphorylated (Prestayko et al., 1975).

Crude 40S particles isolated from sucrose gradients have an associated protein kinase activity (Blanchard et al., 1975). When 40S particles are incubated in an appropriate buffer in the presence of [^{32}P-γ]ATP, they will autophosphorylate. Figure 46A presents an analysis of the reaction products from this kind of experiment, separated by SDS-polyacrylamide gel electrophoresis. The reaction was allowed to procede for 1 or 15 minutes in the presence or absence of cAMP. A great many of the proteins in the crude ribonucleoprotein particle preparation are substrates for the kinase reaction;

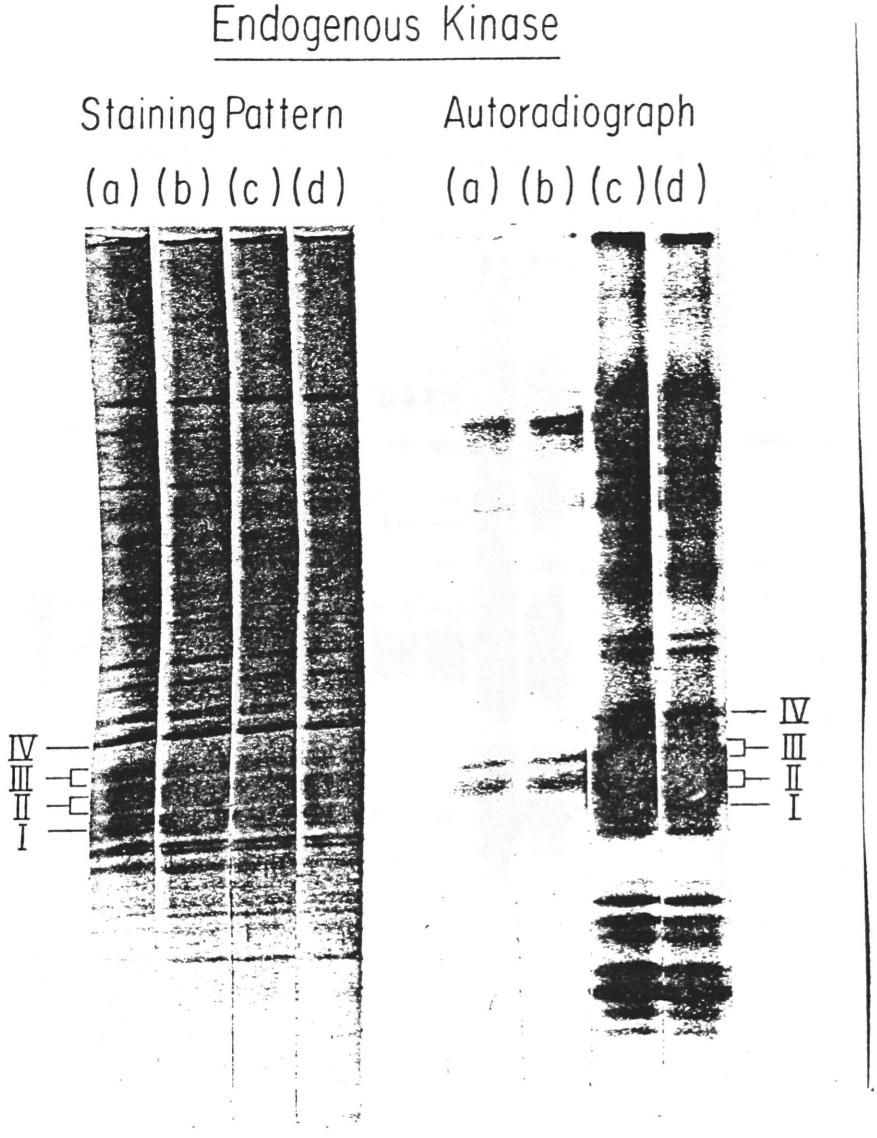

Figure 46. Phosphorylation of 40S ribonucleoprotein particles in vitro by protein kinases.

Figure 46 A. Autophosphorylation of 40S ribonucleoprotein particles in vitro by protein kinase activity present in the crude 40S particle preparations. 40S particles from sucrose gradients were incubated in the presence or absence of 10^{-6} M cAMP, in 0.1 M NaOAc, pH 6.3, 10^{-5} M papavarine, 0.1 mM EGTA, 10 mM $MgCl_2$, 10^{-6} M [^{32}P -γ]- ATP, for 1 or 15 minutes at 30 ^{o}C. The reactions were terminated by the addition of SDS and the reaction products analyzed by autoradiography of SDS-polyacrylamide gradient gels. Pictured at left is a photograph of the stained gel. The corresponding autoradiograph is shown to the right. a) -cAMP, 1 min; b) +cAMP, 1 min; c) -cAMP, 15 min; d) +cAMP, 15 min.

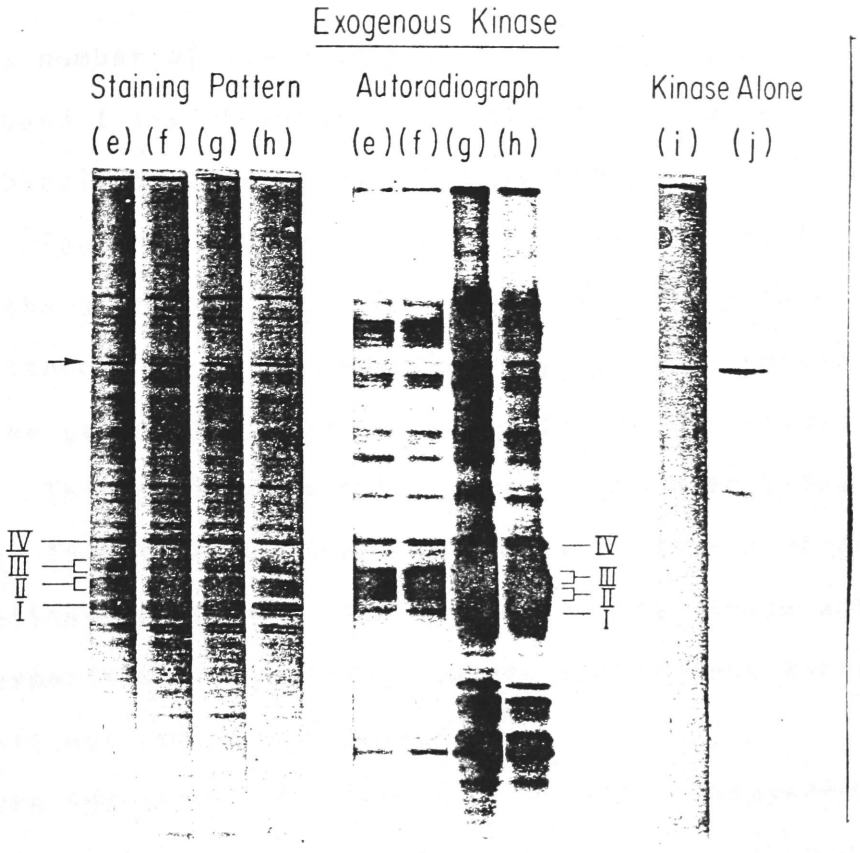

Figure 46 B. Phosphorylation of 40S ribonucleoprotein particles in vitro by calf thymus cAMP dependent protein kinase. 40S particles, isolated from sucrose gradients, were incubated in the reaction mixture described in the legend to Figure 46A, in the presence or absence of 2 μg of partially-purified protein kinase from calf thymus. Arrow points to the position of the protein kinase on the SDS-polyacrylamide gels: e) -cAMP, 1 min; f) +cAMP, 1 min; g) -cAMP, 15 min; h) +cAMP, 15 min; i) kinase alone, staining pattern; j) autoradiograph of slot i.

however, considerable variations in the final specific activities of the reaction products are evident. For example, a number of low molecular weight proteins migrating ahead of band I are phosphorylated to a high degree, although they are barely detectable by staining (Figure 46A). Of the basic ribonucleoprotein bands I-VII, bands IIB, IIC and IIIA are the best substrates for the modification reaction. Band I, although it is the most heavily-stained protein band on the gel is the poorest substrate for the kinase reaction. This protein is only nominally phosphorylated, even after 15 minute incubations. There is little effect of cAMP on either the rate or specificity of the kinase activity in this experiment, suggesting that the endogeneous kinase activity is not cyclic AMP-dependent.

Figure 46B shows the effects of adding an exogeneous protein kinase to the reaction mixture. Again, incubations were for 1 or 15 minutes in the presence or absence of cAMP. The exogeneous kinase markedly increased the rate of the phosphorylation reaction. (Compare panels e and f which are one minute incubations in the presence of the added kinase with panels a and b, which are one minute incubations in the absence of added kinase.) There was little apparent change in the substrate specificity when the exogeneous kinase is added to the reaction mixtures. Most of the proteins continue to be phosphorylated to the same relative extent by the exogeneous kinase as they were by the endogenous kinase activity. A notable exception is band IVC which appears to

be a particularly good substrate for the exogeneous, but not for the endogeneous kinase. It is interesting to note that even in the presence of the exogeneous kinase, band I is a poor substrate for the kinase reaction. Since the serine and threonine content of each of the basic ribonucleoproteins is almost constant (Table XII), this suggests that band I is protected against the phosphorylation reaction by the stereochemistry of the particle structure. (An alternative explanation is that the sequences in which the serine and threonine residues appear in protein I are not recognized by the enzyme. However, in view of the high content of these residues in protein I (10.4 mole percent), this seems less likely.)

Proteins IIB, IIC and IIIA seem to contain "hot spots" for multiple modifications. These proteins are the best substrates for the kinase reactions, in addition to being the proteins that show methylation of arginine residues. The significance of these modification reactions in not yet known, but it seems likely that the organization of the ribonucleoprotein particle may be regulated *in vivo* by chemical alterations of these proteins.

Protein composition of ribonucleoprotein particles from duck liver - Immunological data from Georgiev's laboratory (Lukanidan, *et al.*, 1976) suggest that antibodies prepared against rat liver 40S ribonucleoprotein particles will cross-react with other nuclear ribonucleoproteins from different species, but not with ribonucleoproteins derived from ribosomes, or with ribosomal proteins. This data plus

electrophoretic evidence that the proteins from 40S ribonucleoprotein particles from a variety of species will comigrate on acid-urea gels (Samarina et al., 1968) suggests that these proteins are similar in a wide variety of species. Martin et al.(1973) reached similar conclusions on the basis of comparisons of the electrophoretic mobility of rat liver, duck liver, chicken liver, monkey liver, and mouse Taper hepatoma ribonucleoproteins on SDS-gels. Since the resolving capacity of the gel systems used by Samarina et al. and Martin et al. is not as good as the resolution of the gels used in this study, we have reexamined the relative mobilities of ribonucleoproteins from 40S particles isolated from duck liver and rat liver. (In figure 9 of Martin et al., 1973, rat liver proteins IIA, IIB, and IIC comigrate. IIIA and IIIB are barely resolved from the II group. The higher molecular weight components are not detectable, probably because of the small amount of total protein applied to the gel. Band IVC is not evident in this photograph, however, in their figure 11, which shows an SDS-polyacrylamide gel of mouse Taper hepatoma proteins, a protein that appears to have the same mobility as the rat liver band IVC is evident.)

Figure 47 compares the banding patterns of duck liver ribonucleoprotein particle proteins prepared by sonication and extraction of the nuclei to rat liver ribonucleoproteins from particles isolated by extraction of nuclei. In these crude preparations, both the rat liver and duck liver ribonucleoprotein particles display heterogeneous banding

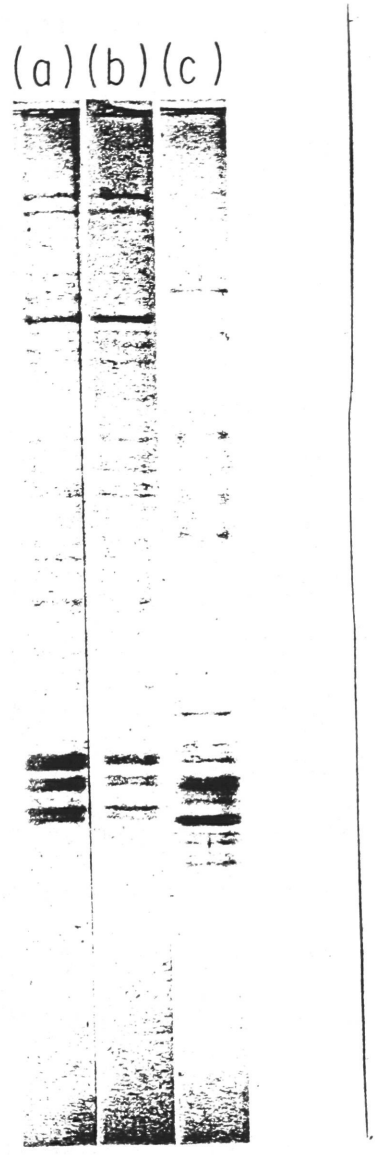

Figure 47. Comparison of 40S ribonucleoprotein particles isolated from duck and rat liver by SDS-polyacrylamide gel electrophoresis. a) Duck liver 40S particles prepared by sonication of nuclei; b) Duck liver 40S particles prepared by extraction of nuclei; c) Rat liver 40S particles prepared by extraction of nuclei. Six prominent proteins are present in the duck liver ribonucleoprotein particles that migrate with approximately the same mobilities as the basic proteins from rat liver ribonucleoprotein particles. Conditions for electrophoresis and isolation of RNP particles are given in "Materials and Methods."

patterns. Six major proteins are present in the duck liver preparations, and are likely to be homologous proteins. This impression is reinforced by the similarity of the total amino acid composition of duck liver ribonucleoprotein particles and rat liver ribonucleoprotein particles . (Data of Martin et al., 1973, reproduced in Table XIII). Further chemical analysis of the proteins from these two species will be required before the relatedness of these proteins can be firmly established. A number of the higher molecular weight proteins in the two preparations also comigrate, and these may also be homologous proteins. It is interesting that there is no homologue to protein IVC in the duck liver preparations, suggesting that this acidic protein may not be generally present in ribonucleoprotein particles. In general, comparison of the ribonucleoproteins isolatable from duck and rat liver reinforces the impression that the 40S ribonucleoprotein particle is composed of a limited number of basic proteins.

Protein structure of ribonucleoprotein from HeLa cells - Pederson (1974) has prepared ribonucleoprotein particles from tissue culture cells by sonication of the nuclei in hypotonic buffers, followed by removal of most of the chromatin by centrifugation through sucrose gradients. Since the endogenous ribonuclease activity in the nuclei of most tissue culture cells appears to be less than that in the nuclei of many animal tissues, the ribonucleoprotein particles in these preparations sedimented as large heterogeneous structures of up to 200S with a modal distribution about 76S.

Pederson's preparations contained more high molecular weight proteins than are typically observed in preparations of 40S particles from animal tissues, and Pederson has observed tissue-specificity in these proteins of higher molecular weight (Pederson, 1974).

The ribonucleoprotein complexes prepared as described by Pederson share a number of characteristics with the rat liver 40S particles described here: 1) the bouyant density of the fixed ribonucleoprotein complexes is 1.43 gm/cm^3 in CsCl; 2) the particles are composed of RNA associated with a group of proteins in the range of 40,000 daltons that have isoelectric points from 7.6 to 8.3 and another group of proteins of higher molecular weights with isoelectric points from 4.9 to 6.5; 3) upon ribonuclease digestion, the primary digestion product of Pederson's large particles is a ribonucleoprotein complex that sediments at about 40S; 4) some proteins, including one with a molecular weight of about 40,000 daltons, are selectively solubilized by 0.5 M NaCl.

In order to make a direct comparison of ribonucleoprotein complexes prepared by Pederson's procedures and 40S ribonucleoprotein particles, we have fractionated HeLa cells according to Pederson (1974). Figure 48 presents a flow diagram of the fractionation scheme. Cells were labeled in the presence of low doses of actinomycin D (to inhibit rRNA synthesis) with [^{32}P]-orthophosphate, or [^{3}H]-uridine. The cells were disrupted by Dounce homogenization in hypotonic buffer. Crude nuclei were recovered by low speed

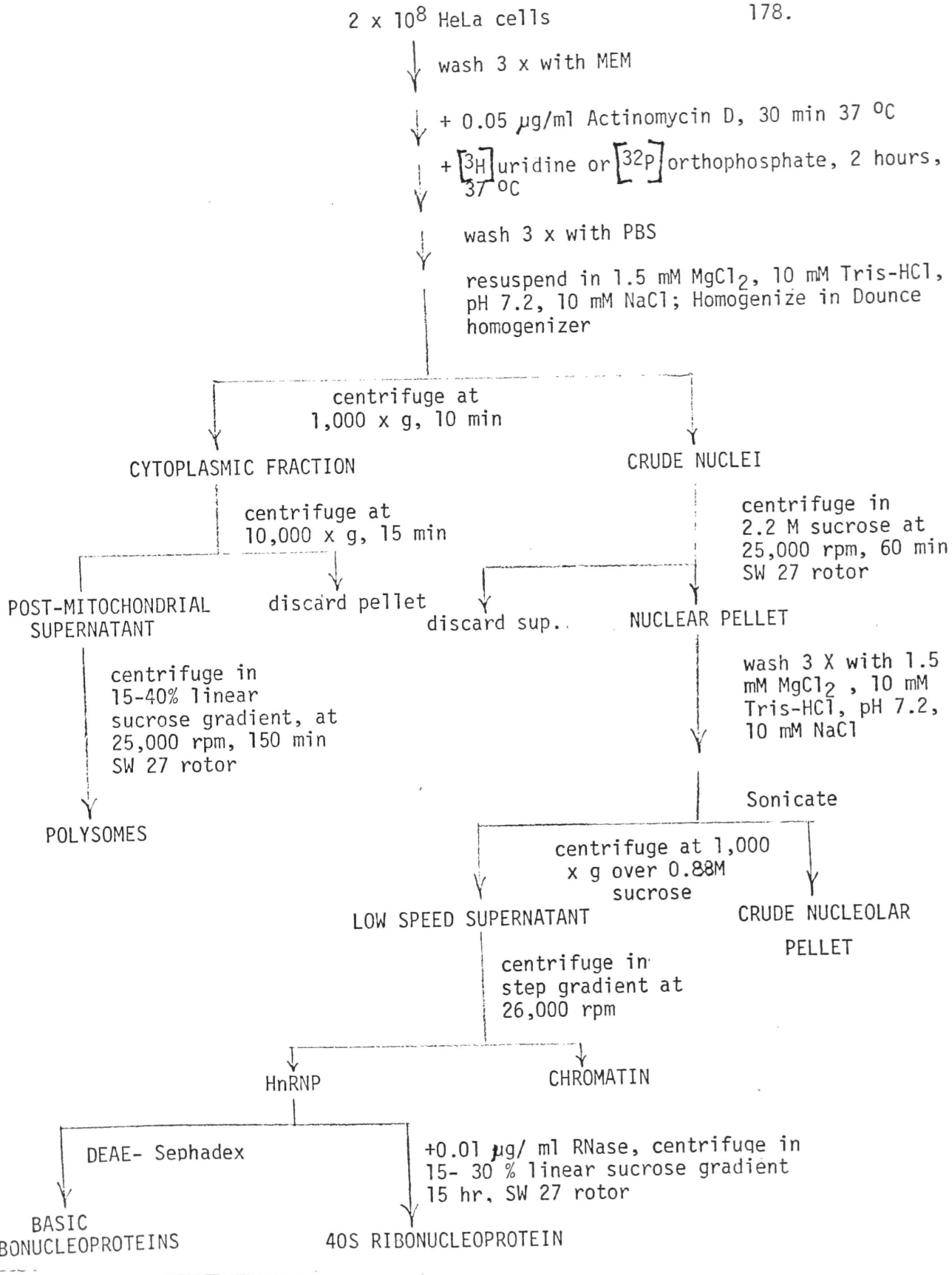

Figure 48. Flow diagram for the fractionation of HeLa cells. Fractionation procedure is described in detail in "Materials and Methods."

centrifugation, and further purified by sedimentation through 2.2 M sucrose. Polysomes were isolated from the post-nuclear supernatant. The nuclei were disrupted by brief sonication in hypotonic buffer, and crude nucleolar pellets were obtained by low-speed centrifugation through 0.88 M sucrose. Chromatin and RNP particles were separated on discontinuous sucrose gradients in hypotonic buffer. (Kish and Pederson, 1975). Under these conditions, most of the chromatin is pelleted and HnRNP particles are recoverable as a narrow opalescent band in the sucrose gradients. The HnRNP fraction was further fractionated by either partial nuclease digestion to generate 40S particles, or by chromatography on DEAE-Sephadex to separate the basic and acid protein components of the complex. Each of these fractions was analyzed by SDS-polyacrylamide gel electrophoresis.

Figure 49 compares the banding patterns of the various HeLa cell fractions separated by SDS-polyacrylamide gel electrophoresis. Panel a is total HeLa cell nuclear protein; panel b, a postnucleolar supernatant fraction; panel c, a chromatin fraction; panel d, a nucleolar fraction, and panels e,k, and n show HnRNP fractions. Each of these fractions contains numerous proteins. Few differences in the electrophoretic banding patterns of the prominent protein species are evident when total HeLa nuclear protein, chromatin, and nucleolar fractions are compared, suggesting that these fractions are not well separated by the isolation procedure. The proteins associated with HnRNP appear to be a subset of

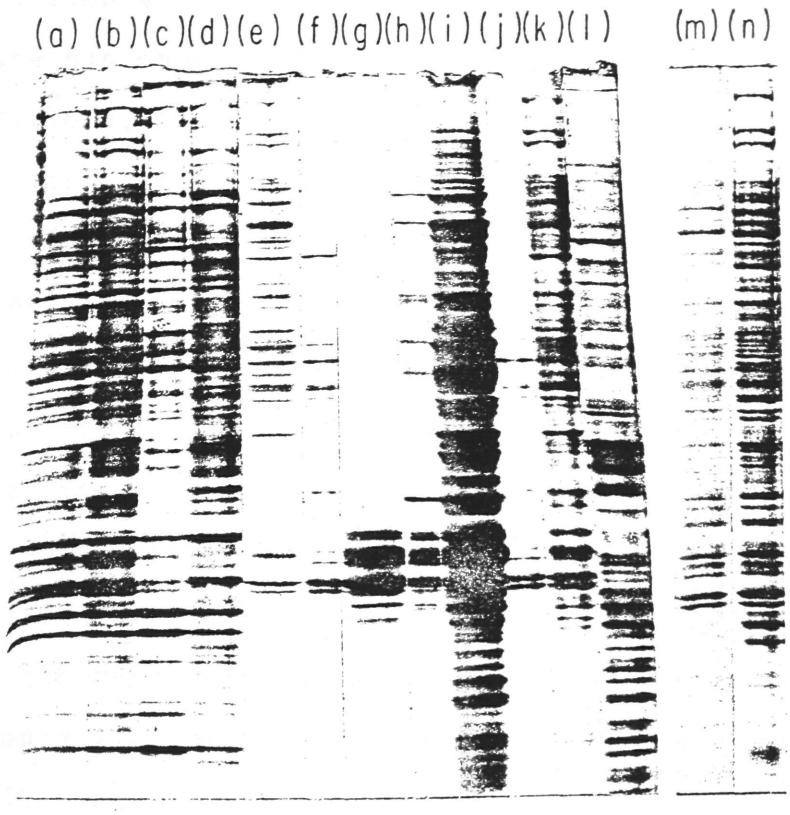

Figure 49. SDS-polyacrylamide gradient gel electrophoresis of nuclear fractions from HeLa cells and rat liver. The method of fractionation of the HeLa cells is shown in the flow diagram in figure 48, and described in the text and "Materials and Methods."

a) Total HeLa cell nuclear protein
b) Low speed supernatant fraction from sonicated HeLa cell nuclei.
c) HeLa cell chromatin fraction
d) HeLa cell nucleolar fraction
e) HeLa cell HnRNP fraction
f) HeLa cell HnRNP fraction after chromatography on DEAE-Sephadex to separate the basic proteins
g) Rat liver basic proteins from 40S particles selected on DEAE-Sephadex
h) Rat liver 40S ribonucleoprotein particles
i) Total rat liver nuclear extract
j) HeLa cell HnRNP fraction after DEAE-Sephadex chromatography
k) HeLa cell HnRNP fraction
l) HeLa cell polysomes
m) HeLa cell 40S RNP generated from HnRNP by nuclease digestion
n) HeLa cell HnRNP before nuclease digestion.

the total nuclear proteins; however many of the proteins in this fraction are present in other nuclear fractions. (Most of the histones migrate off the gel when run under these conditions, histone H-1 migrates with an anonomously high molecular weight in SDS gels and appears in these gels as the two prominent proteins in the chromatin and total nuclear fractions with molecular weights in the range of 30,000 daltons, Figure 49 a,b,c) Panel 1 shows HeLa cell polysomal proteins. These proteins are not prominent in any of the nuclear fractions.

Comparison of the HnRNP protein from HeLa cells with RNP preparations from rat liver (panel h, with e, k and n) shows that the HeLa cell preparations contain considerably more high molecular weight components than do the crude rat liver 40S particles. The complexity of the HeLa cell fraction is comparable to that of the rat liver total nuclear extracts. Migrating in the range of the rat liver proteins I- IV are a number of HeLa cell RNP proteins. When HeLa cell RNP proteins are fractionated into basic and acidic components by chromatography on DEAE-Sephadex (panels f and j) these protein bands are found in the basic fraction. Panel g is an analysis of the DEAE run-off proteins from rat liver 40S particles, included for comparison. In addition to the proteins of about 30,000 to 40,000 daltons, the basic protein fraction of HeLa cell RNP contains a group of proteins clustered at about 60,000 daltons. Similar proteins occasionally contaminate the rat liver 40S preparations, and these proteins may be derived from another nuclear structure. Proteins of

this molecular weight occur as components of nuclear pores (Aaronson and Blobel, 1975) and it would be interesting to make a direct comparison of the proteins isolated from nuclear pores with proteins of the HnRNP fraction of HeLa cells.

When HnRNP from HeLa cells are sedimented on sucrose gradients, the material distributes heterogeneously, with a modal distribution of about 76S, as reported by Pederson (1974). Mild nuclease digestion generates ribonucleoprotein particles of about 40S (Figure 50; Pederson, 1974; Lukanidan et al., 1972). When the proteins associated with HnRNP before and after the nuclease digestion are compared by SDS-gel electrophoresis, it is observed that many of the high molecular weight components are lost, whereas the basic proteins are retained. This suggests that at least some of the high molecular weight proteins present in the HnRNP preparations are not required to maintain a ribonucleoprotein structure sedimenting at 40S.

When ^{32}P is used as a label instead of [^{3}H]uridine, digestion of the ribonucleoprotein particles appears to be a more complex phenomenon than the simple generation of a monomeric subunit. Figure 51 shows the results of mild ribonuclease digestion of ^{32}P-labeled HeLa cell HnRNP on the sedimentation of the HnRNP complexes. When 0.01 μg/ml pancreatic ribonuclease was used, multiple peaks of radioactivity were observed (Figure 51, panel A). These peaks resediment as discrete bands (Figure 52). As the digestion conditions are made more severe, (Figure 51, panel B), two peaks of radioactivity become

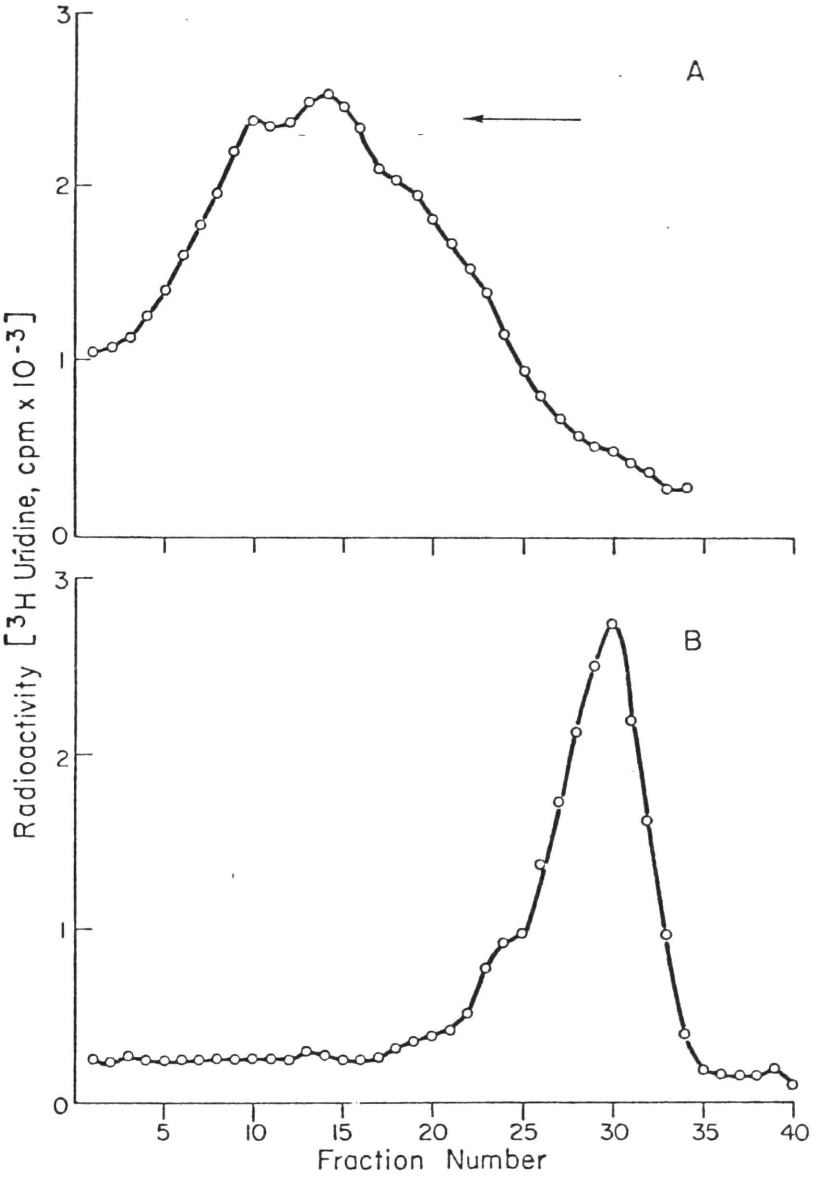

Figure 50. Effect of ribonuclease on the sedimentation of HnRNP particles. Particles were isolated and incubated for 30 minutes at 4 °C in the presence or absence of 0.01 µg/ml pancreatic ribonuclease. Samples were applied to 15- 30% linear sucrose gradients in hypotonic buffer and centrifuged at 15,000 rpm for 17 hours in an SW27 rotor. Panel A, particle incubated in absence of RNase; Panel B, particles incubated in the presence of RNase. Gradients were fractionated and monitored for TCA precipitable radioactivity. Note that the direction of sedimentation is from right to left.

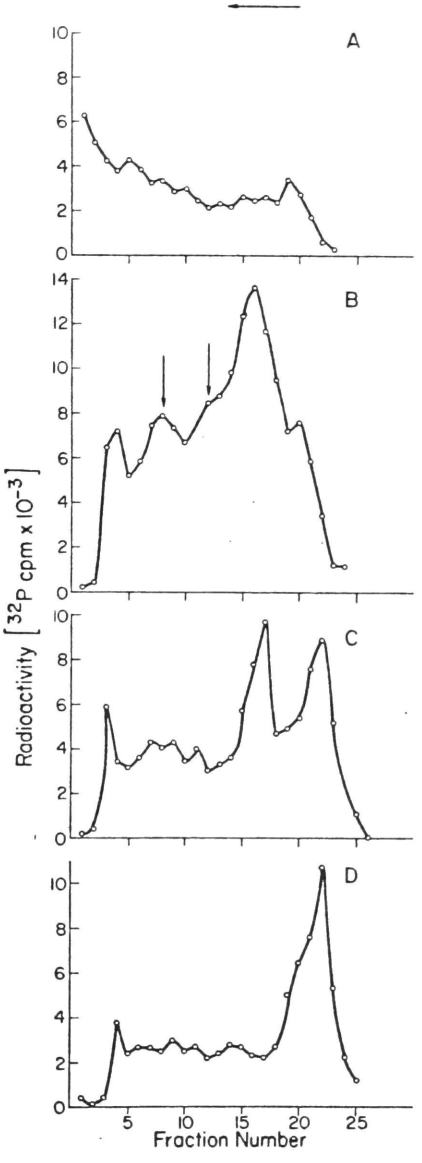

Figure 51. Effects of various concentrations of ribonuclease on the sedimentation of [^{32}P]-labeled HnRNP from HeLa cells. Particles were isolated and incubated for 30 minutes at 4 °C in the presence of pancreatic ribonuclease of the concentrations indicated below. Samples were applied to 15 - 30% linear sucrose gradients in hyptonic buffer an centrifuged at 23,000 rpm for 15 hours in an SW 27 rotor. Under these conditions, most of the undigested HnRNP is pelleted. The arrows in panel B indicate fractions resedimented as shown in Figure 52. Gradients were fractionated and monitored for TCA-precipitable radioactivity. The direction of sedimentation is from right to left. A) no ribonuclease B) 0.01 µg/ml C) 0.1 µg/ml D) 1.0 µg/ml

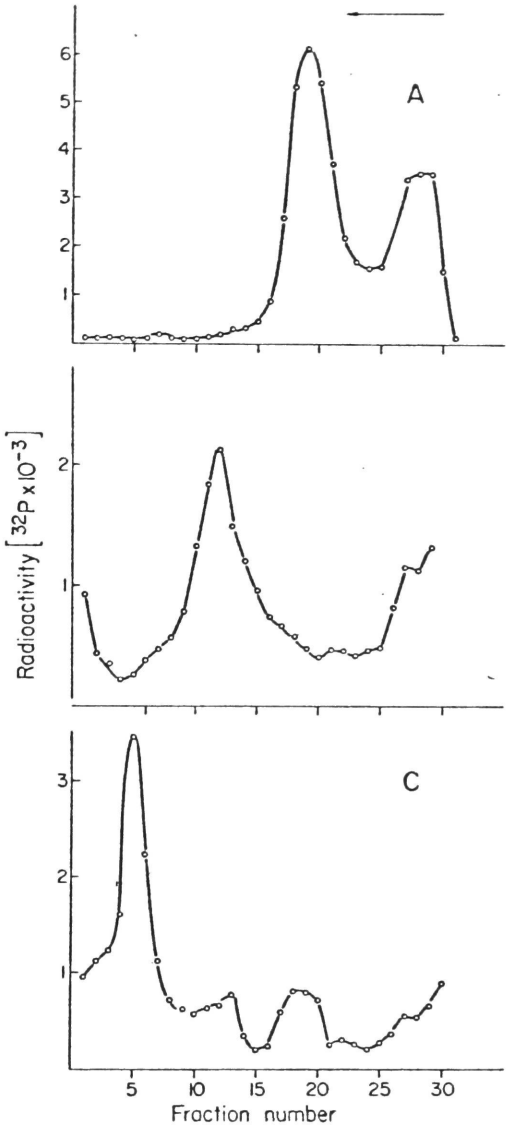

Figure 52. Resedimentation of HnRNP digestion products. The fractions indicated by the arrows in figure 51 were resedimented in 15-30% sucrose gradients centrifuged under the same conditions described in the legend to figure 51. The direction of sedimentation is from right to left. Gradient fractions were monitored for total radioactivity. A. fraction 14; B. fraction 10; C. fraction 7.

evident over a high background of radioactivity. One peak corresponds to the 40S ribonucleoprotein particles, and is associated with a discrete peak of optical density (data not shown). The slower sedimenting peak probably corresponds to poly A and its associated proteins (Quinlan et al., 1974). Under severe digestion conditions (1.0 µg/ml ribonuclease, Figure 51 panel C), the 40S ribonucleoprotein particle is destroyed, whereas the poly A-containing peak remains. Even under these harsh digestion conditions, the high ^{32}P-background persists. This data suggests that the HnRNP prepared by Pederson's methods is not simply a multimeric structure composed of 40S particles and poly A. The high background radioactivity detected with ^{32}P label (but not with ^{3}H label) raises suspicions that the Pederson RNP fraction is contaminated by material labeled with ^{32}P that is not RNA.

This data suggests that large HnRNP aggregates prepared according to Pederson's techniques may contain structures that give rise to 40S particles that are similar, but not necessarily identical to the 40S RNP particles we have described. The complexity of these large structures, and the decreased heterogenity of the protein components in their derivative 40S particles, suggests that the species specificities reported by Pederson may involve nuclear structures distinct from our 40 S HnRNPs.

SUMMARY AND CONCLUSIONS

The evidence presented in this chapter suggests that the 40S ribonucleoprotein complexes isolated from the nuclei of animal tissues and tissue culture cells are composed of HnRNA and include a limited number of basic proteins with molecular weights ranging from 30,000 to 40,000 daltons.

Most of the conclusions in this chapter are based on observations on 40S ribonucleoprotein complexes isolated from rat liver nuclei. Comparisons with similar preparations from HeLa cells and duck liver nuclei, as well as examination of published data concerning similar preparations from other tissues, suggests that homologous structures exist in all eukaryotes.

Rat liver is a good source of ribonucleoprotein since optically-measurable quantities can be obtained with relative ease and in high purity. Ribonucleoprotein sedimenting at 40S is the major component of rat liver nuclear extracts, and appears as a relatively homogeneous particle population with distinctive morphology when viewed in negative contrast in the electron microscope.

The proteins in the 40S particle from rat liver, isolated by velocity sedimentation on sucrose gradients, are a heterogeneous group of proteins ranging in molecular weight from 30,000 to over 200,000 daltons. A group of proteins with molecular weights ranging from 32,000 to 43,000 daltons accounts for approximately 70% of the protein mass of the particles. The proteins are always found in the same relative proportions,

whereas variability is encountered from preparation to preparation in the relative amounts of the other protein components. This variability is particularly evident in the proteins with molecular weights greater than 43,000 daltons. The same proteins are present when 40S particles are isolated from nuclei by extraction with hypotonic buffers at elevated pH, by sonication, by hypotonic lysis of the nuclei, or by hypertonic lysis of the nuclei, suggesting that the 40S ribonucleoprotein particle is not an artifactual association of protein and RNA generated by the isolation procedure.

When the 40S particles are repurified by a rigorous procedure involving precipitation of the complex with ammonium sulfate, resedimentation, and isopycnic banding without fixation in the non-ionic density medium Metrizamide, approximately 20% of the protein mass is lost. The proteins in the 33,000 to 43,000 dalton group are retained throughout this isolation procedure. In contrast, many of the proteins of higher molecular weight are selectively lost. This data supports the view that the proteins of 33,000 to 43,000 daltons are required for the maintenance of the structure of the 40S particle.

The proteins in the 33,000 to 43,000 dalton group may also be distinguished from the proteins of higher molecular weight on the basis of charge. Most of the proteins that remain associated with 40S particles after isopycnic centrifugation are basic proteins that may be purified as a group by chromatography on DEAE-Sephadex. The proteins of higher

molecular weight, have charge characteristics similar to the remainder of the non-histone proteins, and are retained on the DEAE-Sephadex columns. The basic proteins account for 70% of the protein mass of the 40S ribonucleoprotein particles isolated by velocity centrifugation.

The basic proteins were further fractionated by chromatography on phosphocellulose. A combined analysis of the proteins by chromatography and SDS-polyacrylamide gel electrophoresis indicates that there are 12 to 15 basic proteins in the rat liver RNP preparations. Two of these proteins can be isolated to near homogenity by chromatography on phosphocellulose. Two other fractions, each a mixture of two closely related proteins, were also obtained. All the partially purified fractions have extremely similar amino acid compositions, indicating a high degree of chemical relatedness in the basic proteins from nuclear ribonucleoprotein complexes.

The proteins of the 40S ribonucleoprotein particle are subject to post-synthetic modifications. We have observed both methylation of the arginine residues of some of the basic proteins *in vivo*, and phosphorylation of serine residues of these and other proteins *in vitro*. These observations of chemical modifications in the proteins in ribonucleoprotein complexes suggest mechanisms by which the structure of these complexes may be selectively altered *in vivo*.

Physical studies of the 40S particle provide direct evidence that the proteins that copurify with RNA, interact

with RNA and with each other to form a specific structure. The ribonucleoprotein particles may be progressively disaggregated by increasing the ionic strength of the medium in which the particles are suspended. Disruption of the particles in high salt results in solubilization of specific proteins, alterations in the circular dichroism spectrum and changes in the sedimentation velocity and equilibrium bouyant density of the complex, as well as increased accessibility of lysine residues to chemical modification *in vitro*.

The protein compositions of ribonucleoprotein particles isolated from rat liver, duck liver and HeLa cells (prepared as large heterogeneous structures by the method of Pederson, 1974) were compared. Basic proteins of similar molecular weights were present in each of these preparations, suggesting that each of these structures contain homologous proteins. The ribonucleoprotein complexes isolated from HeLa cells contain considerably more acidic, high molecular weight proteins than do the 40S particles from rat liver; however, some of these proteins are lost when 40S particles are prepared from the HeLa cell ribonucleoprotein fraction by partial nuclease digestion of the RNA.

With the present techniques, it is difficult to distinguish between protein actually associated with HnRNP and simple contaminants. Preparations of large HnRNP structures, such as Pederson's, are particularly difficult to purify since they are heterogeneous in size and therefore cannot be effectively purified by sedimentation velocity alone. In

the absence of additional purification steps, involving charge or density separations, these preparations must be evaluated with caution. From a re-examination of Pederson's procedures it seems that his preparations contain structures that can give rise to 40S particles that are similar both in protein composition and physical properties to the 40S particles isolatable from animal tissues. At least for these structures, the evidence for tissue-specificity of the associated proteins is not convincing, and similar proteins appear present in every 40S particle prepared to date. The proteins in the 40S particles are primarily, a set of basic proteins ranging in molecular weight from 33,000 to 43,000 daltons. The proteins appear to have distinctive solubility properties and amino acid compositions.

It is difficult to evaluate the significance of the higher molecular weight proteins present in many preparations of RNP. These proteins may be loosely associated with 40S particles in vivo, proteins associated with other structures that copurify with HnRNP, legitimate RNA-binding proteins of yet unknown specificities, or simple contaminants. More data will be required before the role of these proteins in HnRNP structures can be determined.

It has been suggested by Georgiev and coworkers (Georgiev and Samarina, 1971) and by Pederson (1974) that HnRNP is a multimeric structure composed of subunits of about 40S and poly A. Our preliminary data, while consistent with this model, also suggests that there may be additional structures generated during nuclease digestion (these could be multimers

of the 40S particle). Considerably more data will be required before the model can be regarded as having been demonstrated.

We have emphasized the role of basic proteins in the structural organization of newly-synthesized RNA in the nucleus of animal cells. It seems likely that further chemical analyses of these proteins will provide a wealth of data concerning the role of these proteins in the structural organization of nuclear RNP. When purified protein fractions become available, immunological localizations of these proteins would become feasible. It would be of particular interest to learn whether these proteins are associated with HnRNA chains on chromatin, such as those visualized in spreads of interphase chromatin prepared by the techniques of Miller and his associates (Miller and Bakken, 1972). It would also be of interest to examine the mode of synthesis of these proteins, their interactions with each other, and other aspects of their physiology.

While much work is needed to clarify all the phenomena described here, the picture emerging from this data and previously published data from the laboratories of Georgiev, Martin, Pederson, and others, strongly suggests that a limited number of basic proteins, with distinctive chemical properties form the core of a repeating subunit structure on chains of HnRNA. It seems likely that these structures represent the native state of HnRNA in the nucleus of animal cells.

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