STUDIES OF RNA IN THE AVIAN NERVOUS SYSTEM DURING DEVELOPMENT

by

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IN MEMORY OF MY FATHER

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Abstract

Myelination in the chick nervous system commences after the fourteenth day of embryonic development. To explore certain biochemical aspects of this process, a study was undertaken of the transcription of mitochondrial and messenger-like RNA in the nervous system of pre-myelinating 14-day embryos and of myelinating 17-day embryos and 3-day chicks.

Using differential centrifugation and mild "osmotic shock" treatment of crude mitochondrial fractions, mitochondrial RNA could be isolated from the brain and nerve tissues of 17-day embryos and 3-day chicks in a relatively pure and undegraded form. The purified mitochondrial fraction contained three major RNA components. Two of these (having molecular weights of 0.72 and 0.45 million daltons respectively) probably originated from mitochondrial ribosomes. The third mitochondrial RNA component had an electrophoretic mobility on polyacrylamide gels similar to that of transfer RNA. Base analysis of the presumed mitochondrial ribosomal RNAs showed that these species had significantly lower G+C contents than did their cytoplasmic counterparts. The labelling of mitochondrial rRNA and tRNA could be demonstrated both in vitro and in organ culture. Mitochondrial RNA labelling could be selectively

inhibited by low doses of ethidium bromide. To estimate the contribution of mitochondrial RNA labelling to that of total, rapidly-labelled RNA of the sciatic nerve, the specific radioactivities of total nerve RNA and of mitochondrial RNA were compared in 17-day embryos and 3-day chicks. Less than 1% of the total radioactivity in RNA was associated with the purified mitochondrial fractions at both developmental stages. Therefore, the low-molecular-weight, high-specific-activity RNA species previously isolated by Hu and Mezei (1971) from the myelinating nerve presumably contained only a small proportion of mitochondrial RNA. These results, however, do not exclude the possibility that mitochondrial RNA synthesis plays an important, but as yet unrecognized, role in myelinogenesis.

To characterize some of the messenger-like or hnRNAs in the developing nervous system, brain and nerve tissues of embryonic and post-hatch chicks were incubated with radioactive precursors *in vitro*. Radioactive RNA was isolated, purified and fractionated on oligo(dT)-cellulose columns into bound, poly(A)+ and unbound, poly(A)$^-$ fractions. Base analysis of these fractions indicated that the adenylate content of the bound RNA fractions was higher than that of the unbound fractions. After a 2 h incubation time with [^{3}H]-adenosine *in vitro*, the nuclear and cytoplasmic subfractions of brains and sciatic nerves contained

about 5-20% of poly(A)+ RNAs. The relative proportion of poly(A)+ RNAs in the tissues decreased during development. Polyacrylamide gel electropherograms of preparations labelled *in vitro* showed that the bound fractions contained heterodisperse RNA peaking mainly between the 28S and 18S marker rRNAs. The poly(A)+ RNA fractions of the rapidly-myelinating nerve tissues of 17-day embryos and post-hatch chicks contained a relatively larger proportion of smaller-than-18S molecules than those of the pre-myelinating 14-day embryos.

To further characterize the types of non-ribosomal RNA molecules in the developing sciatic nerve, the inhibitor 5-fluoroorotic acid was used in organ cultures to selectively suppress the labelling of rRNA in this tissue. 5-Fluoroorotic acid inhibited the labelling of mature rRNA in the sciatic nerve of 14-day, 17-day embryos and 3-day chicks without affecting appreciably the radioactive electrophoretic pattern of the heterodisperse RNA species. Incorporation of [5-^{3}H]uridine and [5-^{3}H] orotic acid in the 5-fluoroorotic acid-treated embryonic nerves was mostly into RNA species larger than 18S, whereas in the treated 3-day chicks, most of the radioactivity appeared in heterodisperse RNA migrating between the 18S and tRNA components. Ribosomal RNA of purified polyribosomes was also found to be unlabelled in the presence of

the drug. The heterodisperse 5-fluoroorotic acid-resistant RNA of the embryonic polyribosomes contained relatively more large-molecular-weight RNA species (>18S) than that of the 3-day chicks. These results are consistent with the hypothesis that the embryonic nerve tissue contains a larger proportion of high-molecular-weight RNA molecules than does nerve tissue of young chicks or adults.

List of Abbreviations

BHK	Baby hamster kidney
CNS	Central nervous system
cpm	counts per minute
Cyto c oxid	Cytochrome C oxidase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
5-Foro	5-fluoroorotic acid
LDH	Lactate dehydrogenase
nm	nanometer (10^{-9} meter)
oligo(dC)	Oligo (deoxycytidylic acid)
oligo(dT)	Oligo (Deoxythymidylic acid)
PNS	Peripheral nervous system
poly(A)	poly (adenylic acid)
poly(U)	poly (uridylic acid)
RNA	Ribonucleic acid
rRNA	ribosomal RNA
mRNA	messenger RNA
hnRNA	heterogeneous nuclear RNA
tRNA	transfer RNA
RNase	ribonuclease
SDS	Sodium dodecyl culfate
TCA	Trichloroacetic acid

List of Abbreviations (cont.)

TEMED	N,N, N',N' - Tetramethylethylenediamine
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
U.V.	Ultraviolet

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Section I Introduction

The Present Investigation

Electron microscopic, x-ray diffraction and chemical studies of developing central and peripheral nerves of animals of different species have shown that an association must exist between a Schwann or glial cell and an axon prior to the onset of myelin formation (for review see Davison & Peters, 1970). At different stages of development, varying in time with species studied, the Schwann or glial cells multiply, invade the nerve bundles, and wrap themselves around the axons to form the myelin sheath (Peters & Muir, 1959). Therefore, myelinogenesis can be regarded as a special kind of differentiation.

A great number of enzymes and other chemical constituents are synthesized in the nervous system just prior to or during the active phase of myelination. For example, it has been shown that both axons and Schwann cells are actively synthesizing macromolecules such as RNA and proteins in the myelinating nerve (Koenig & Koelle, 1961; Peterson et al., 1968; Edstrom et al., 1969). The chemical signals for the initiation of myelination are not known, but one may assume that myelinogenesis, like other processes of cell differentiation, is controlled at the level of transcription, processing of transcripts, translation, or a combination of these. To explore whether

transcription plays an important role in the control of myelination it is important to characterize the particular transcription products in the developing peripheral nerve.

In the sciatic nerve of chick, myelin is laid down most rapidly three to four days before and after hatching (Geren, 1954). Therefore, as a first approach, the pattern of RNA synthesis was studied in organ culture of the sciatic nerve and brain from 14-day and 17-day embryos, and 3-day chicks. During these previous studies (Hu & Mezei, 1971) it was established that the sedimentation and electrophoretic properties of the rapidly-labelled RNA species were different in pre- and post-hatch chicks. In younger animals, the rapidly-labelled RNA was distributed mainly in the high-molecular-weight region of the sucrose gradient. However, as the animals became older more low-molecular-weight RNA appeared in the less dense region of the sucrose gradient. Studies on the pattern of methylation of RNA in the peripheral nerve of the chick during development (Mezei & Hu, 1972) indicated that the rapidly-labelled high-molecular-weight RNA in the embryonic chicks might be a mixture of ribosomal RNA precursors and heterogeneous nuclear DNA-like RNA. In contrast, in the three-day chicks during a short (0.5 hr.) pulse with both radioactive uridine and methionine, methylation was almost entirely limited to the tRNA species. Furthermore, the incorporation of uridine in the 3-day animals revealed the presence of a

heterogeneous population of rapidly-labelled, non-methylated RNA species, most of which migrated between the smaller ribosomal RNA and tRNA components of the bulk RNA on gel electrophoresis.

In the present investigation several hypotheses were considered concerning the nature of rapidly-labelled low-molecular-weight RNA of the peripheral nerve. First, this RNA could have been transcribed in the mitochondria of the peripheral nerve, since this RNA fraction was poorly methylated and its synthesis could be specifically reduced by inhibitors of mitochondrial RNA synthesis, such as ethidium bromide and acriflavine (Mezei et al., 1971). It is also known that the number of mitochondria per cell increases during nerve development (Friede & Samorajsky, 1968). Furthermore, Nass (1969) has suggested that mitochondria might play a special role in cell differentiation in conjunction with nuclear genetic control mechanisms. Therefore, in the present investigation a more detailed characterization of the properties and labelling of mitochondrial RNA was carried out in the developing sciatic nerve and brain of chicken.

For these studies the method of Hernandez et al. (1971) was adopted, which involves a series of differential steps in sucrose media followed by osmotic shock treatment of crude subcellular fractions from brain and nerve. This technique was successful in yielding mitochondrial RNA

preparations in reasonable yield and purity, and exhibiting properties distinctly different from cytoplasmic RNA. However, a very low percentage of the total radioactivity in RNA was incorporated in the purified mitochondrial fractions from both pre- and post-hatch animals. Therefore, the low-molecular-weight, high-specific-activity species previously demonstrated in myelinating nerve (Hu & Mezei, 1971) contained only a small portion of mitochondrial RNA.

The second hypothesis concerning the nature of the low-molecular-weight, rapidly-labelled and poorly-methylated RNA of the 17-day old embryos and 3-day chicks proposed that these polynucleptides could represent some of the mRNA fraction of the myelinating nerve, because the methylation, sedimentation and electrophoretic properties of these molecules were similar to those of mRNA of most eucaryotic cells (Hiatt, 1962; Peterson, 1970; Penman et al., 1968; Nemer et al., 1974; 1975).

There is increasing evidence that a number of mRNAs and hnRNA from eucaryotic cells contain adenylate-rich sequences (Edmonds & Caramella, 1969; Lim & Canellakis, 1970; Edmonds et al., 1971; Nakazato & Edmonds, 1972). The presence of these residues can be used to advantage in the selective isolation of mRNA free of other RNA species, since polyadenylic sequences bind preferentially to a variety of materials such as oligo(dT)-cellulose, poly(U)-containing filter paper, and nitrocellulose (Millipore)

filters (Lee et al., 1971). Moreover, a number of investigations have recently shown that a high proportion of RNA synthesized by brain or brain nuclei contains molecules that are associated with a polyadenylic acid sequence (DeLarco & Guroff, 1972; Banks & Johnson, 1973; Hemminki, 1974; Lim et al., 1974; Mahony & Brown, 1975). Therefore, in the present investigation, the isolation and characterization of the poly(A)-containing RNA of the developing brain and nerve tissue of chicks was attempted using the technique of oligo(dT)-cellulose column fractionation. From total RNA of the chick peripheral and central nervous system, this procedure yielded an RNA fraction which was retained on oligo-(dT)-cellulose columns and had properties similar to those described by others for a variety of tissues and organisms.

If some of the low-molecular-weight rapidly-labelled RNA represents the mRNA fraction of the developing sciatic nerve, it could contain a mixture of poly(A)-containing and poly(A)-deficient molecules, since it has been shown that not all mRNA molecules possess poly(A) sequences (Nemer et al, 1974; Milcarek et al., 1974). Recently Wilkinson et al., (1971), Wilkinson and Pitot (1973), and Hadjiolova et al., (1973) showed that 5-fluoroorotic acid (5-Foro) may be useful for the specific inhibition of rRNA synthesis, resulting in the selective incorporation of a labelled precursor into cytoplasmic RNA having several properties of mRNA. In the

present investigation, therefore, the effect of this inhibitor on the pattern of RNA synthesis of the developing sciatic nerve was investigated in order to further characterize total mRNA during nerve development.

The data reported in this thesis show that 5-Foro inhibited labelling of mature rRNA of the sciatic nerve of developing chicks without affecting appreciably the radioactive electrophoretic pattern of the heterodisperse RNA species . The radioelectropherograms of the 5-Foro-resistant RNA species from the three developmental stages were almost identical to those observed previously in this laboratory after a relatively short "pulse" (30 to 60 min.) by the radioactive precursor alone (Mezei & Hu, 1972). These results are consistent with the conclusion that the embryonic nerve tissue contains a larger proportion of high-molecular-weight mRNA molecules than that of the young chicks or adults.

Literature Review

A. Myelinogenesis as a differentiation model

1. The nervous system and myelin

The nervous system is composed of an aggregation of units, each of which comprises the nerve cell proper (which, with all its processes (dendrites and axons) is termed a neuron) plus satellite cells such as neuroglia and astrocytes. The neurons are specialized for reception of stimuli and transmission and conduction of nerve impulse.

In the peripheral (PNS) and central (CNS) nervous systems, the axons are of two types, myelinated and unmyelinated, and the present literature review will deal mainly with the myelinated ones. A typical myelinated neuron is shown in Figure 1.

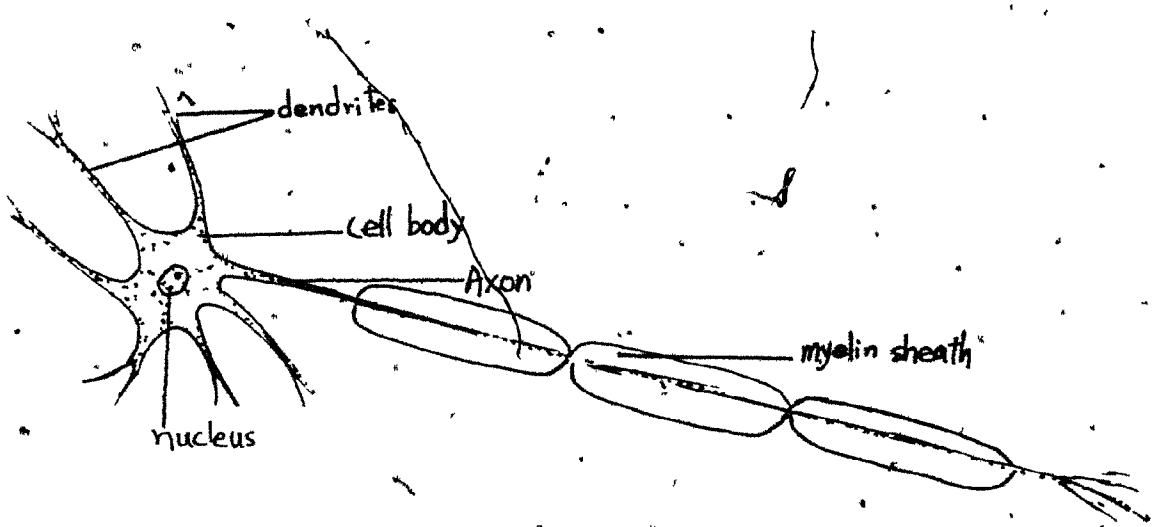


Fig. 1. Diagram of a Neuron in PNS

Early in the seventeenth century Leeuwenhoek demonstrated by light micorscopy, a sheath around the axon (Rossiter, 1962). Since then a great deal of data has accumulated on the structure of myelinated nerve fibers and their associated cells.

A historical review of the structure of the PNS is given by Bunge (1968). Work on the detailed structure of myelin began with polarized light microscopy. This technique was to give the first indication of the radial arrangement of myelin lipid components (Schmitt & Bear, 1939). A number of investigators (Schmitt & Bear, 1939; Schmitt et al., 1941) obtained x-ray diffraction patterns from peripheral nerve preparations which led to the suggestion that the myelin sheath is constructed from layers, or lamellae. Each myelin lamella contains two bimolecular lipid layers, about 55Å thick, alternating with protein layers about 30Å thick. This work was confirmed later by electron microscopic studies (Sjostrand, 1950; Fernandez-Moran, 1950).

2. Myelinogenesis

(a) Morphological evidence.

It is generally accepted that the Schwann cells originate from the embryological neural crest cells which migrate along the peripheral nerve and cover the axons. There is a very close correlation between the axons and the Schwann cells in their segmental distribution (Harrison, 1924). The Schwann cells increase in number by mitosis after

their initial migration into the developing peripheral nerve, and form a complete layer surrounding the entire bundle of the young nerve fibers. The Schwann cells next start to invade the central core of the axons. These invading Schwann cells, which are undergoing mitosis, form partitions between the axons until the latter are separated into very small bundles (Peters & Muir, 1959).

The formation of the peripheral myelin sheath was first studied with the electron microscope by Geren (1954). In the sciatic nerve of developing chicken, Geren noted the spiral nature of the earliest myelin lamellae and was able to correlate this with the spiralling of the infolded Schwann surface membrane with its external mesaxon. Based on these morphological observations she suggested that the myelin sheath consists of a greatly-extended Schwann cell membrane wrapped in a spiral manner around the enclosed axons. Geren's postulation was soon supported by Robertson (1955), who showed that the structure and the formation of the myelinated sheath in vertebrate nerve tissue is consistent with Geren's model of myelin formation.

(b) Current concept of myelination

During development, an axon destined to become myelinated lies in a furrow indenting the Schwann cell along its long axis. The furrow containing the axon then deepens, and its free edges extend towards each other until they come into close approximation. This leads to the

complete envelopment of the axon by the Schwann cell, and where the edges of the furrow meet, the outer surface of their bounding plasma membranes come into apposition to form the mesaxon, as it was termed by Gasser (1952). Once the mesaxon is formed, it soon proceeds to elongate. The elongation of the mesaxon takes place in a basically spiral fashion, and this leads to the production of a loosely-coiled mesaxon. Once a number of turns have been completed, the cytoplasm is lost between them. Therefore, the apposition of the cytoplasmic surface of the consecutive turns of the mesaxon leads to the formation of compact myelin (Davison & Peters, 1970). Figure 2 shows the process of myelination.

(c) Relationship between cell types

It was shown by Speidel (1964) that myelin formation in the PNS is the result of an interaction between the axon and the Schwann cells. Neither the axon nor the Schwann cell alone was able to form myelin (Peterson & Murray, 1955). Friede and Samorajski (1967) studied the relationship between the number of myelin lamellae and axon circumference, and showed that there is a constant linear relationship between these two. The critical diameter for the nerve fiber which is to be myelinated was about 1.1μ for fresh nerve. Therefore, they proposed that this relationship in adult nerve fiber may indicate the mechanism by which the axon controls deposition of myelin during

development. In 1964 Speidel emphasized that there is a special affinity between myelin-emergent fibers and Schwann cells, and that this affinity is greater than the one which exists between Schwann cells and non-myelinated emergent fibers. Thus he considered Schwann cells to move preferentially towards myelin-emergent fibers. However, the chemical basis of this affinity is unknown. Speidel also speculated that the axon does not participate metabolically in the process of myelin formation, which is carried out entirely by the Schwann cell.

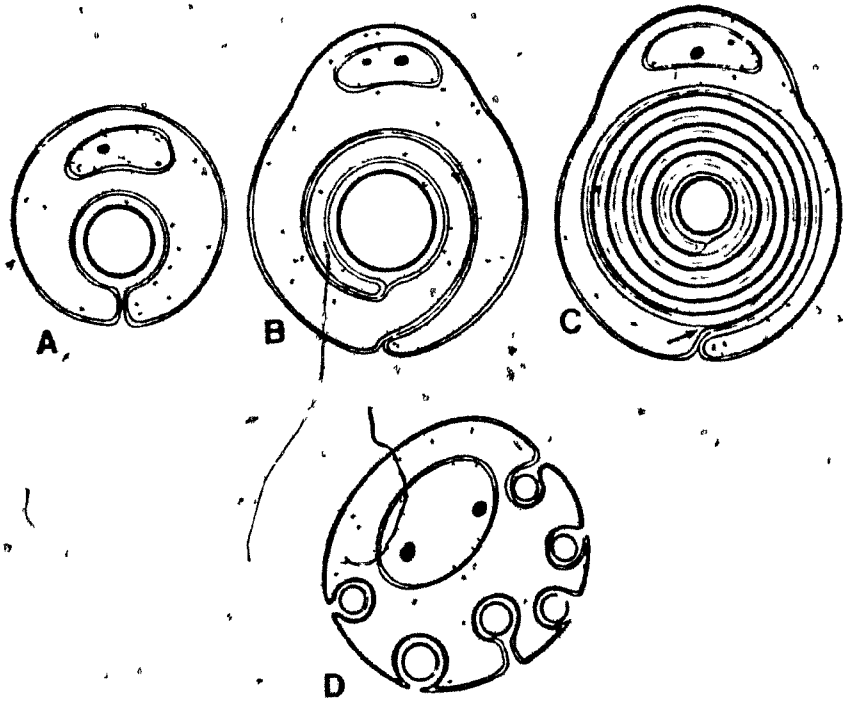


Fig. 2 - Successive stages in the development of the myelin sheath from the plasma membrane of a Schwann cell (A, B, C). Relation of a Schwann cell to several unmyelinated fibers (D). (See Barr, 1972; p. 39)

(d) Biochemical evidence

Myelinogenesis occurs at two levels. The first is concerned with the biosynthesis of the chemical constituents, the second with their assembly. During myelination the great increase in the enfolded surface of the Schwann cells suggests the incorporation of lipid and proteins into the myelin membrane in large amounts (Geren, 1954). In brain, during early development, many substances are incorporated as the result of two processes, growth and myelination (Rossiter, 1962). Wells & Dittmer (1967) have studied changes in lipids associated solely with myelination of the rat brain during development. They found a general increase in the content of 21 different classes of lipids during rat brain development. More specifically, the lipids considered to be associated solely with myelination (such as cerebrosides, sphingomyelins, triphosphoinositides, phosphatidic acid, galactosyl diglyceride, and ethanolamine plasmalogens) increased maximally during the different periods of myelination (Davison & Peters, 1970). Myelin isolated from sciatic nerves of chicks was found to undergo marked changes in lipid composition during development, particularly in the phospholipid and cerebroside content (Oulton & Mezei, 1973). Yates and Wherrett (1974) described lipid changes occurring in post-natal rabbit sciatic nerve during development. Recently the lipid composition of chick brain and sciatic nerve during development was determined

by Shaikh and Palmer (1976).

The activities of many enzymes in lipid metabolism in rat brain are increased during the period of rapid myelination (Salway et al., 1967; Davison & Peters, 1970).

There are three major types of proteins present in myelin: the proteolipid protein; the basic protein(s) and Wolfgram, acidic protein. Proteolipid proteins (Folch & Lees, 1951), named because they are soluble in organic solvents, comprise approximately 50% and 23% of the total proteins in CNS and PNS myelin respectively (Wolfgram & Kotorii, 1968). The basic protein is characterized by a high content of basic amino acid residues and represents 30% of the total protein of CNS myelin and 21% of the total protein of PNS myelin (Eng et al., 1968). There is only one basic protein present in CNS myelin in most species (Shooter & Einstein, 1971), whereas PNS myelin contains two basic proteins. Of the myelin proteins, the basic protein has unique immunological properties. When injected into experimental animals, it causes a condition called experimental allergic encephalomyelitis (EAE), characterized by hind-leg paralysis and pathological changes in the brain. One of the basic proteins of PNS myelin can produce a similar disease, experimental allergic neuritis (EAN), which specifically affects peripheral nervous tissue. The Wolfgram protein (soluble in acidic chloroform-methanol) accounts for 17% of the total protein of CNS myelin and

approximately 50% of the protein of PNS myelin.

Besides the characteristic myelin proteins, Levine and Moore (1965) have described a brain-specific, acidic protein (S-100), which has been identified in glial cells and axonal plasma membrane of most species (Davison & Peters, 1970).

Proteins, particularly those unique to the nervous system, have been studied during development (Greaney, 1961; Mokrasch & Manner, 1963; Einstein et al., 1970). It has been found that the composition of brain proteins changes during development (Prensky & Moser, 1967). There are changes in certain enzyme proteins and in the chemical composition of the proteolipid proteins (Davison & Peters, 1970). Einstein et al. (1970) demonstrated that both the basic proteins and the proteolipid proteins of mouse brain myelin increase significantly during development. Furthermore, it was shown that the encephalitogenic protein accumulated in the CNS with increasing age (Einstein & Csejter, 1966; Eng et al., 1968). Levine and Moore (1965) found that the acidic protein (S-100) increased sharply during the period of rapid myelination in the brain. Recently, Wiggins et al. (1975) studied myelin proteins in rat sciatic nerve during development. They showed that there was less change in the protein composition of sciatic nerve myelin during development than in that of the CNS. Shashoua and Wolf (1971) reported that at the onset of myelination there

was a marked increase in the synthesis of basic myelin proteins and proteolipid proteins, and suggested that such an increase of proteins and enzymes should be preceded by an abrupt synthesis of the mRNA molecules coding for these proteins (Shashoua, 1974). The results of experiments with mutant mice also support this hypothesis. Three recessive mutations in this species cause grossly defective myelination in the CNS (Sidman et al., 1964; Meier & MacPike, 1970). Mandel et al. (1972) observed in these mutant a decrease in the rate of synthesis of several enzymes concerned with biosynthesis of myelin lipids and a striking decrease in myelin proteolipid proteins and basic proteins. Therefore, they suggested that these results indicated that the biogenesis of myelin is a coordinated phenomenon during which a series of enzymes is transcribed and translated in parallel as a function of age. The regulation of this transcription can be envisaged either by an induction of a myelin enzyme operon, or by sequential transcription, in which the synthesis of one enzyme or its products regulated the transcription of enzymes involved in latter stages of myelin biosynthesis.

(e) Involvement of RNA in growth and differentiation

. Experiments have been performed to investigate the relationship between RNA and embryonic development. Brown and co-workers (Brown & Littna, 1966; Brown & Gurdon, 1966) demonstrated the synthesis of different types of RNA

during development of the toad embryo. In sea urchin and amphibian embryos, interference with RNA synthesis alters the pattern of differentiation. In the amphibian embryo, low doses of actinomycin-D inhibit the development of the nervous tissue (Tiedmann, 1966). Experiments on the specific hybridization of a particular RNA from one developmental stage to homologous DNA, and the competition of this binding by RNAs from various other developmental stages, demonstrate that there are changing populations of m-RNA during sea urchin development (Whitely et al., 1966).

RNA changes in the nervous system during development have been studied in several laboratories (Dellweg et al., 1968; Guroff et al., 1968; Yamagami & Mori, 1970; Mandel & Jacob, 1971; Shashoua, 1974). The experimental results indicate changes in the brain RNA content and rate of synthesis as a function of age. Gene transcription and gene expression during development has been reviewed by Denis (1974) and Paul (1974), who suggest that the direction of protein synthesis upon which development and differentiation depend is accomplished by recent transcription as well as by stored m-RNA.

B. RNA metabolism in eucaryotic cells

A general model for the steps in gene expression has been suggested by Jacob and Monod (1961). The model states that genetic information in DNA nucleotide sequences is transcribed into complementary RNA sequences and that these

are translated into polypeptide chains which form the final products of the gene. Control of gene expression might occur at the level of either transcription or translation, or at the level of processing of RNA and protein. It is becoming increasingly clear that in both procaryotes and eucaryotes transcriptional regulation is one of the important control mechanisms. Thus an understanding of the events in transcription might provide some indications of how cell differentiation is controlled (Denis, 1974).

1. Differences between eucaryotes and procaryotes

In procaryotes, the ribosomes interact with mRNA immediately after the start of RNA synthesis. As a result a complex structure is formed containing DNA, RNA polymerase, newly-formed mRNA and ribosomes with their growing polypeptide chains (Miller, et al., 1970). Therefore, in procaryotes transcription and translation are coupled and there are no intermediate steps.

However, in the larger and structurally-more-complex eucaryotic cell, transcription is uncoupled from translation. Eucaryotes have a nucleus separated from the cytoplasm by a nuclear membrane. This nuclear membrane, separates the processes of transcription and translation. The extra step of mRNA transport is essential in eucaryotes (Georgiev, 1972). Because of the extensive physical compartmentalization in higher organisms, the nucleus,

nucleolus, chloroplast, and mitochondria all appear to be unique transcriptional sites with their own genetic information and their own transcriptional machinery (Miller & Beatly, 1969; Miller, et al., 1970). Furthermore, the DNA of eucaryotes is covered by proteins (MacGillivary & Rickwood, 1974), such as histones and acidic proteins, which are probably involved in gene regulation.

These complications make the study of transcription in eucaryotes difficult. Inhibitors which selectively inhibit different transcriptional systems have become fundamental tools in the investigation of these processes in eucaryotes, and knowledge of transcription in eucaryotes has been derived mainly from the use of inhibitors (Penman et al., 1970a; Perry et al., 1970), such as actinomycin-D, fluorinated pyrimidines, ethidium bromide and cordycepin.

2. Eucaryotic RNA polymerases

In procaryotes a single species of RNA polymerase is responsible for all DNA-directed RNA synthesis. However, in eucaryotes each RNA synthetic system has its own RNA polymerase. This enzyme activity is not only found in the nucleus but also in the nucleolus, mitochondria and chloroplasts. These different polymerases, which show different sensitivities to the drug α-amanitin, have been designated RNA polymerase I, II, III and IV (Blatti et al., 1970). Enzyme I is found in the nucleolus and is mainly responsible for ribosomal RNA synthesis. Enzymes II and

III are primarily located in the nucleoplasm. Polymerase IV found in yeast might be a unique yeast mitochondrial RNA polymerase; it is composed of only one polypeptide component and is not inhibited by α-amanitin. However, it is sensitive to rifampicin. All the above enzymes have the basic properties of a DNA-dependent RNA polymerase, and Mn^{+2} or Mg^{+2} ions are required for their activities, although at different optimal concentrations (Chambron, 1974). The differential inhibition of the RNA polymerases in eucaryotic cells by specific drugs simplifies the study of transcription in these organisms.

3. Types of Eucaryotic RNA

(a) Ribosomal RNA

(i) Properties: Ribosomes, the major components of the protein synthetic machinery in all organisms, are composed of two subunits which differ in size (60S and 40S for eucaryotes). Each subunit contains an RNA species of high molecular weight, methylated, stable, and having a high G + C content. In addition to the high-molecular-weight rRNA species ribosomes also contain low molecular weight molecules such as "7S" and 5S" RNAs (Dalgarno & Shine, 1973).

(ii) Synthesis: The transcription of rRNA is well-described in eucaryotes (Burdon, 1971). The genes for rRNA are clustered in the nucleolus (Perry, 1964). The following experiments suggested that nucleolar RNA is an

obligatory precursor of the rRNA of the cytoplasm. (1) Irradiation of nucleoli with an ultraviolet microbeam resulted in the cessation of cytoplasmic rRNA accumulation. (2) Kinetic studies of radioactive precursor incorporation into RNA showed that the radioactive precursor was incorporated into the nucleolar RNA very rapidly, whereas radioactivity only appeared in cytoplasmic rRNA after a lag period. (3) When HeLa and L cells were treated with low doses of actinomycin-D, which blocked nucleolar RNA synthesis, cytoplasmic rRNA accumulation was subsequently also reduced. Since actinomycin-D binds to DNA and interfers with RNA synthesis, it is widely used to study RNA catabolism in a variety of cells, inhibiting specifically any further supply of newly-synthesized RNA.

Actinomycin-D was first reported by Waksman and Woodruff (1940) and was isolated from Actinomycetes. It is a peptide-containing antibiotic and a potent antitumor substance but its clinical application is limited by its toxicity. The general action of actinomycin-D on nucleic acids has been reviewed by Reich and Goldberg (1964). At low doses it inhibits selectively the DNA-directed synthesis of RNA, but not of DNA, and markedly affects the rate of incorporation of guanine and cytosine residues into the growing RNA chain (Hyman & Davidson, 1970). A model based on x-ray analysis for the structure of the actinomycin-DNA complex has been proposed. According to this model,

actinomycin-D is hydrogen-bonded to the guanine residue of helical DNA (Reich & Goldberg, 1964). Hyman and Davidson (1970) suggested that the G-C base pair is essential for the formation of actinomycin-D-DNA complex. They also concluded that the effect of actinomycin-D on RNA synthesis is mainly on chain elongation, although the compound may also have a minor effect on initiation and termination. However, the results of Wells and Larson (1970) demonstrated that the presence of deoxyguanylic acid is not essential for the actinomycin-D-DNA complex formation, but rather a particular nucleotide sequence seems to be necessary for the complex formation. At the present there is still a great deal of uncertainty about the mode of actinomycin-D-DNA complex formation.

In eucaryotes, the processing of high-molecular-weight 45 S rRNA precursor into mature 28 S and 18 S rRNA is particularly sensitive to low concentrations of this drug, whereas normal or near-normal amounts of 4 S, 5 S and heterogeneous nuclear RNA are transcribed (Perry, 1963; Perry et al., 1964; Perry & Kelly, 1968). This differential sensitivity is of fundamental importance in the study of all the other minor forms of RNA in eurcaryotic systems.

(iii) Post-transcriptional modifrcation: With the development by Loening (1967) of polyacrylamide gel electrophoresis, a method for the separation of molecules

as large as rRNA, it has become possible to study in detail the events of nuclear RNA processing. Weinberg et al. (1967) obtained information concerning nucleolar RNA processing using gel electrophoresis. Penman and his coworkers (Greenberg & Penman, 1966; Weinberg & Penman, 1970) demonstrated that the rRNAs are synthesized in the nucleolus as a single transcriptional unit, a 45 S, rRNA precursor, which is subsequently cleaved specifically into 41 S, 36 S, 32 S and 20 S rRNA species; with the 32 S species ultimately yielding 28 S rRNA, and the 20 S species yielding 18 S rRNA (Burdon, 1971). Recently Wellauer and Dawid (1973) investigated the secondary structure of rRNA and its precursors by electron microscopy. The secondary-structure maps and the measurement of various rRNA molecules strongly suggested the same processing pathway that was deduced previously by kinetic experiments. Therefore, the process of rRNA maturation can be outlined as shown in Fig. 3.

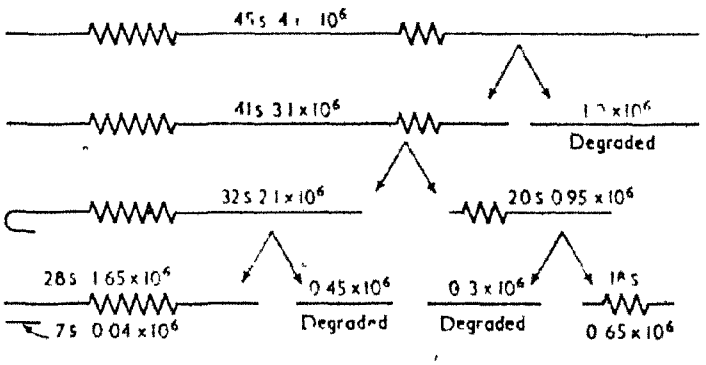


Fig. 3. Schemes for the processing of nucleolar RNA. (Weinberg & Penman, 1970).

Greenberg and Penman (1966) studied the incorporation of methyl groups into rRNA by incubating HeLa cells in the presence of [methyl-^{14}C]methionine, and found that the methylated RNAs were located largely in the 45 S RNA fraction. That the nucleolar 45 S RNA serves as a precursor of cytoplasmic 28 S and 18 S RNA was demonstrated by incubating HeLa cells previously labelled with [methyl-^{14}C] methionine for various times in the presence of a large excess of unlabelled methionine. Concomitant with loss in radioactivity in the 45 S material, increased labelling occurred in the 32 S RNA of the nucleolus and 18 S RNA of the cytoplasm. This observation suggests that cleavage of 45 S RNA occurs in the nucleolus with a rapid exit of 18 S RNA to the cytoplasm and a delay in the release of the 32 S cleavage product. Loss of radioactivity from the 32 S component was closely correlated with the appearance of radioactive 28 S. These results also suggest that most of the methyl groups found in rRNA are incorporated into molecules at the level of 45 S RNA in the nucleolus. Similar results have been demonstrated by Zimmerman and Holler (1967). However, Salim and Maden (1973) reported that in HeLa cells additional methylations occurred at the level of 18 S rRNA. Approximately 80-90% of the methyl groups on the 18 S and 28 S RNA respectively, consist of $O^{2'}$-methylribose units present as nucleotide residues (Attardi & Amaldi, 1970).

(b) Heterogeneous nuclear RNA

(i) Properties: When mammalian and avian cells are briefly (<5 min.) incubated with radioactive RNA precursors and the labelled RNA examined by zonal sedimentation, a high proportion of the radioactivity is found to sediment as material larger than 45 S rRNA precursor. The distribution of this high-molecular-weight RNA is heterogeneous (Houssais & Attardi, 1966; Soeiro et al., 1966). Using cell fractionation techniques (Scherrer et al., 1966), this RNA, which has a DNA-like (low mol.% G+C) base composition, was found to be localized outside the nucleolus. Georgiev and coworkers (for review see Georgiev, 1972) have described an RNA fraction (designated D-RNA) having similar properties, and isolated by a hot phenol extraction technique. This RNA is also extractable by relatively high pH buffer (approx. 8.5) and is metabolically unstable (Brawerman, 1963). In 1966, Darnell's group called this material "heterogeneous nuclear RNA" (hnRNA) (Warner et al., 1966), and demonstrated that it was not a precursor of rRNA.

(ii) Synthesis and degradation of hnRNA: Georgiev (1972) suggested that hn RNA might be synthesized in the form of a long polycistronic chain which contains the nucleotide sequences for several proteins. Recently, Daneholt (1975) and Newlon et al. (1975) reported that in Drosophila the hnRNA primary transcription product might

even be much larger than one can observe, since the processing time is very fast.

In HeLa cells the average half-life of hn RNA has been calculated to be 3 min. (Soeiro et al., 1968), whereas, Penman et al. (1968), Daneholt and Svedhem (1971) have found that the average half-life of hnRNA is not more than 25 minutes. These experiments, along with the results of short pulse labelling, indicate that hnRNA is rapidly synthesized. Kinetic labelling experiments have shown that not more than 10-20% of hnRNA migrates to the cytoplasm after processing (Scherrer et al., 1966; Attardi et al., 1966; Soeiro et al., 1968; Penman et al., 1968), suggesting that degradation of hnRNA occurs in the nucleus with the majority of the products returning to an acid-soluble pool.

(iii) Post-transcriptional modification and possible mRNA precursor role: After the discovery of hn RNA, a rapdily-labelled RNA with similar properties was also found in the cytoplasm when HeLa cells were treated with a low dose of actinomycin-D. This cytoplasmic RNA species was found to be associated with polysomes, and had a sedimentation value between 6-25 S and a DNA-like base composition (Penman et al., 1963). The template activity of this RNA in a cell-free system was also demonstrated (DiGirolamo et al., 1964). Scherrer et al., (1966) showed by base analysis, autoradiography and pulse

labelling experiemnts that the rapidly-labelled high-molecular-weight RNA was a polymer of 15,000 nucleotides with the properties of messenger RNA. Because the chain length of the cytoplasmic messenger RNA is roughly equivalent to the size of a monocistronic message for a given protein, it was suggested that cytoplasmic mRNA is formed from hnRNA by specific cleavage, by a complex mechanism which selects the specific sequences to be translated (Georgiev, 1972).

This suggestion has been supported by RNA-DNA hybridization experiments. Competitive hybridization between hnRNA and cytoplasmic mRNA showed that the latter competed to a small extent with total hnRNA, whereas total hnRNA inhibited completely the hybridization of cytoplasmic mRNA to DNA (Shearer & McCarthy, 1966; Soeiro & Darnell, 1970). Imaizumi et al. (1973) reported direct evidence that hnRNA from duck erythroblasts does indeed contain the same nucleotide sequences as the globin mRNA. Lewin (1974) recently suggested that the repetitive sequence content of hnRNA is usually greater than that of mRNA. He speculated that if hn RNA molecules act as mRNA precursors, then at least some repetitive sequences in hnRNA act as elements that control mRNA processing, since in HeLa cells and sea urchin oocytes the mRNA is largely derived from the non-repetitive sequence of these hnRNA. Other evidence also suggests the existence of precursor

mRNAs in the nucleus. It has been shown that cells transformed by the small DNA tumor virus SV40 contain the virus DNA as part of the cell genome (Lindberg & Darnell, 1970). In such transformed cells, hnRNA molecules considerably larger than virus-specific polysomal mRNA are found to hybridize to virus DNA. Recent work has also established that both hnRNA and mRNA contain a fairly discrete poly(A) segment as part of the polynucleotide chain (Lim & Canellakis, 1970; Lee et al., 1971; Darnell et al., 1971b; Nakazato & Edmonds, 1972). Furthermore methylated ribonucleosides form the common sequence $m^7G^{5'}ppp^{5'}YmpZp$ (in which 7-methyl guanosine is joined by a 5'-5' pyrophosphate linkage to an $O^{2'}$-methylnucleoside residue Ym) which has been detected at the 5' terminus of both hn RNA and mRNA (Perry & Kelly, 1974; Perry et al., 1975).

It has been suggested that hnRNA is in the form of a ribonucleoprotein particle with the RNA distributed on the surface. The RNA in this form may be easily attacked by processing enzymes, such as endo- or exonucleases. The hn RNA which does not leave the nucleus will be destroyed rapidly, whereas those hnRNA molecules destined to become cytoplasmic mRNA are transferred to the membrane and then into the cytoplasm to join the pre-existing pool. Some of the cytoplasmic mRNAs may then be activated and attached to ribosomes to form polysomes; others may be inactivated and remain stored, or may decay at a constant rate (Georgiev, 1972).

The above observations and hypotheses suggest that hnRNA is a precursor of cytoplasmic mRNA. However, it should be emphasized that conclusive experiments demonstrating the function of hnRNA are still lacking.

(c) Messenger RNA

(i) Properties: MRNA has been defined as "a polynucleotide which determines the sequence of amino acids in polypeptide chains." The mRNA is a carrier of the information of the genome (Singer & Leder, 1966). Cytoplasmic RNA is classifed as mRNA if it possesses the following properties: if it is rapidly synthesized and degraded (with exceptions of those stable mRNA molecules with a life time of several days, such as the mRNA for hemoglobin in the reticulocyte and for silk fibroin in the silk worm); if it represents a very small amount of the total cellular RNA; if it has a DNA-like base composition; and if it has a low content of methylated constituents. It is found in association with ribosomes during protein synthesis, and has the ability to direct protein synthesis in a cell-free system. Messenger RNA can also exist in the form of ribonucleoprotein particles ("informosomes"), as stored mRNA which is not translated immediately (Neyfakh, 1971; Glisin & Savic, 1971).

The study of mRNA has been greatly facilitated by the use of inhibitors of rRNA synthesis, such as actinomycin-D (Perry, 1962; 1963; Penman et al., 1963) and 5-Fluoroorotic

acid (Cihak & Pitot, 1970; Wilkinson et al., 1971). The fluorinated pyrimidines have been used with bacteria as well as mammalian cells for both clinical and scientific investigations. Incorporation of fluorouracil into nucleic acids has been studied with the aid of [2-^{14}C] fluorouracil. Fluorouridylic acid was the only radioactive nucleotide identified in the RNA hydrolysates (Heidelberger, 1965). This demonstrated the incorporation of fluorouracil into internucleotide linkage in the total RNA of mouse tissue. It was shown the 5-Fluorouracil causes a delayed release of rRNA into the cytoplasm (Willen & Stenram, 1967). Since 5-Fluorouracil is not an efficient precursor of hepatic RNA (Wagner & Heidelberger, 1962), Wilkinson et al. (1971) investigated mechanism of action of 5-Fluoroorotic acid (5-Foro). This analogue, which undergoes metabolic conversion similar to that of orotic acid in rat liver (Hurlbert & Potter, 1952;1954), is converted enzymatically to fluoruridine nucleotides via the following pathway: F-orotate→F-OMP→F-UMP→F-UDP→F-UTP→F-RNA (Heidelberger, 1965). Experiments with 5-Foro revealed that the drug is not only selectively incorporated into messenger-like RNA, but that it probably also affects the specific cleavage of the large ribosomal RNA precursor (Cihak & Pitot, 1970). This observation has been confirmed by Wilkinson et al. (1971). The latter workers demonstrated that 5-Foro is incorporated into nuclear and

cytoplasmic ribonucleoprotein particles containing messenger-like RNA, while the labelling of 28 S and 18 S RNA is inhibited. Since 5-fluoroorotic acid apparently does not affect the synthesis of 45 S RNA, these authors concluded that 5-fluoroorotic acid inhibited rRNA processing. Hadjiolova et al. (1973) studied the pattern of labelling of RNA in mouse liver cells treated with this drug, and found that the labelling of 28 S and 18 S rRNA was inhibited. In contrast, the labelling of 45 S, 32 S and 21 S precursor rRNA was even greater in treated mice, due to accumulation of these rRNA components. Therefore, they also concluded that this drug blocks rRNA maturation. The early steps of rRNA maturation leading to the formation of 32 S and 21 S precursor rRNA were not affected by the drug, while the last steps occurring in nuclei, namely conversion of 32 S to 28 S rRNA and of 21 S to 18 S rRNA, were almost completely blocked. In contrast, Hadjiolova et al. (1973) demonstrated that the labelling of hnRNA, messenger-like RNA in the 6 S to 20 S zone, and tRNA remained unaltered.

Thus the site of the inhibitory action of 5-fluoroorotic acid is restricted to a definite stage in the maturation of rRNA. At present it is difficult to envisage a mechanism for the observed selective action of 5-fluoroorotic acid. It is possible that the incorporation of this compound into RNA chains prevents modification of the molecules

which is critical for the removal of the extra pieces of 32 S and 21 S precursor by specific endo- or exonucleases.

(ii) Synthesis and degradation: The current concept of mRNA synthesis in eucaryotes is that mRNA is derived from hnRNA. The experimental evidence which supports this idea and those common factors which they both possess have been discussed in the previous section. However, one feature in which these two species of RNA differ is stability. MRNA is much more stable than hnRNA, although there are indications that embryonic mRNAs have much shorter half-lives than those of adult organisms (Nemer et al., 1974; 1975).

The initial approach to the study of mRNA stability was to treat cells or organisms with a dose of actinomycin-D sufficient to arrest all RNA synthesis, and to measure the quantity of mRNA remaining at various times after administration of the drug. In HeLa cells, high doses of actinomycin-D caused a gradual disappearance of polysomes, with a decay curve suggesting a half-life of 3 - 4 hours (Penman et al., 1963). Measurements of the decay of uridine-labelled mRNA in HeLa cells after a chase with unlabelled uridine gave a half-life of 2-3 days (Murphy & Attardi, 1973). Singer and Penman (1973) also studied the decay of poly(A)-containing RNA in these cells by a technique which did not involve the use of a chase with cold uridine, and found two classes of mRNA, with

half-lives of 7 hr. and 24 hr. Kinetic measuremnts of labelling of poly(A)-containing RNA in exponentially growing mouse L-cells indicated a mean life time of 14.4 hr. for the mRNA (Greenberg, 1972). The explanation for the difference between earlier and more recent results might be that one cannot equate poly(A)-containing RNA with total mRNA and that the actinomycin-D used in the early experiments might have caused abnormal decay of polyribosomes.

Further evidence of the relative stability of eucaryotic mRNA comes from the observations that eucaryotic protein synthesis is not immediately affected by inhibitors of transcription (Singer & Penman, 1973), whereas bacterial protein synthesis ceases within several minutes after inhibition of RNA transcription (Levinthal et al., 1962).

(iii) Post-transcriptional modification; poly(A) addition and Methylation ("Capping"): Recent experiments have demonstrated that a high proportion of mRNA of mammalian cells is covalently linked to a sequence of polyadenylic acid (Poly(A)), 150-200 nucleotides long, located at the 3' terminus of the RNA molecules (Edmonds & Caramela, 1969; Lim & Canellakis, 1970; Edmonds et al., 1971; Nakazato & Edmonds, 1972). The finding that both hnRNA and mRNA have poly(A) sequences (Darnell et al., 1971b; Lee et al., 1971) provides further evidence that at least

some mRNA molecules may arise via specific nuclear processing of hnRNA.

The poly(A) sequence was originally detected in labelled mRNA by its resistance to pancreatic and T_1 RNase (Kates, 1970), by its ability to bind to oligo (dT)-cellulose at high ionic strength, and by the specific temperature or pH requirement for its extraction (Hadjivassiliou & Brawerman, 1967). It has been postulated that poly(A) sequences are transcribed on a DNA template, since their synthesis is sensitive to actinomycin-D (Edmonds et al., 1971). However, since poly(A) addition is not inhibited to the same extent as hnRNA synthesis, Darnell et al. (1971a) have suggested that the synthesis of poly (A) is probably not DNA-dependent, but is a post-transcriptional event following the synthesis of hnRNA by DNA-dependent RNA polymerase. It was shown that the poly(A) segment is synthesized by stepwise addition of adenylate residues to the 3' end of the complete hnRNA and mRNA. Poly(A) chain elongation has been observed both in the nucleus and in the cytoplasm of mouse sarcoma and chinese hamster cells (Brawerman & Diez, 1975). Murthy et al. (1975) have shown that there are slight differences in the size of poly(A) segments associated with different mRNAs. Globin mRNA contains a poly(A) sequence of specific size; which, however, decreases as a function of in vivo incubation time (Gorski et al., 1975).

The process of poly(A) addition is sensitive to the inhibitor cordycepin. Cordycepin (3'-deoxyadenosine) is an analogue of adenosine, and probably inhibits the normal 5'-3'-phosphodiester bond formation in polyribonucleotides. Penman et al. (1970[b]) demonstrated that this drug selectively suppresses the labelling of mRNA, while hnRNA is unaffected. Penman and his coworkers concluded that in mammalian cells cordycepin has several major effects on RNA synthesis. First, it inhibits mRNA and mitochondrial RNA formation. Second, it causes premature termination of RNA synthesis in the nucleus, thereby producing shorter chains (Penman et al., 1970[b]; Zylber et al., 1971). However, hn RNA synthesis per se is insensitive to this drug (Penman et al., 1970[b]). These differential effects of the drug seem to be inconsistent with the idea of precursor-product relationship of hnRNA and mRNA. However, it is possible that cordycepin might interfere with post-transcriptional addition of poly(A) onto the preformed hnRNA chains and cause the production of incomplete hnRNA. These incomplete molecules then cannot pass to the cytoplasm.

Enzymes responsible for poly(A) addition have been detected in a variety of cells (Edmonds & Caramela, 1969; Mans & Walter, 1971; Haff & Keller, 1973). It has been postulated that poly(A) addition might be essential for mRNA processing (Weinberg, 1973; Jelinek et al., 1973).

However, it has been clearly shown that there are at least two classes of mRNAs without the poly(A) segment, namely the histone mRNA and a special mRNA in sea urchin embryos (Adesnik & Darnell, 1972; Nermer et al., 1974; 1975). Furthermore, when polysomal RNA preparations labelled in the presence of rRNA inhibitors are fractionated by the poly(A) selection technique, only 60 to 85% of the radioactive RNA behaves like poly(A)+mRNA (Lee et al., 1971; Lindberg & Persson, 1972). The experiments of Milcarek et al. (1974) also suggested that 30% of the total polysomal mRNA lacks the poly(A) segment but behaves as mRNA in other respects. The latter authors also demonstrated a close parallel between the kinetics of appearance and decay of poly(A)+ and poly(A)$^-$ mRNA in HeLa cells. Therefore, besides histone and sea urchin special mRNAs, other mRNAs lacking poly(A) are also present in eucaryotic cells. Furthermore, mRNA that has been stripped of its poly(A) segment is still an effective template for protein synthesis (Williamson et al., 1974; Bard et al., 1974; Soreq, 1974). Therefore, it is concluded that there are mRNAs which function even without poly(A) segment. This suggests that the poly(A) segment might not be responsible for the stability of mRNA and that the addition of a poly(A) segment is not necessarily the primary requirement for mRNA processing. As yet there is no clear role for the poly(A) segment of eucaryotic mRNA.

Methylated nucleotides have been observed in hn RNA and mRNA from mammalian cells (Perry & Kelly, 1974; Desrosiers et al., 1974). The sites of methylation in each case are at or near the 5'-termini of the mRNA and hnRNA (Wei & Moss, 1975; Furuichi et al., 1975; Perry et al., 1975). On the average there are about 6.7 methyl groups for each 3000 nucleotides of mRNA and 4 methyl groups for each 10,000 nucleotides of hn RNA (Perry et al., 1975). The 5'-terminal structure has been identified as $m^7G^{5'}ppp^{5'}YmZp$---, in which 7-methyl guanosine is joined in a 5'-5' pyrophosphate linkage to an $o^{2'}$-methylated nucleoside residue, Ym (Wei & Moss, 1975; Furuichi et al., 1975; Perry et al., 1975). The biological role of this methylated 5'-terminal structure in animal cells remains to be determined, but its appearance in a wide variety of mRNAs and hn RNAs is indicative of an important function. Methylated residues in cellular hnRNA may be recognized as cleavage sites by processing enzymes responsible for mRNA formation (Rottman et al., 1974). It has been clearly shown recently that the formation of the methylated terminal structure ("Capping" reaction) is essential for translation of reovirus mRNA and globin mRNA (Muthukrishnan et al., 1975). These findings suggest that this methylated 5' termini of mRNA may play a key role in regulation of protein synthesis in eucaryotic cells.

(d) Transfer RNA

Transfer RNA, a low-molecular-weight, relatively stable species of RNA, plays a vital role in protein synthesis, accepting amino acids for transfer to growing polypeptide chains on ribosomes. The properties and metabolism of tRNA have been reviewed extensively by Letham (1973) and Nishimura (1974).

(d) Mitochondrial RNA

(i) Properties: Mitochondria from animal tissues contain ribosomes (having a sedimentation coefficient of 55-60 S) which can be dissociated into 43 S and 32 S subunits. Two RNA species can be isolated from these two subunits. The 43 S particles contain 18 S RNA, while the 32 S particles contain 14 S (Borst & Grivell, 1971). The values determined by gel electrophoresis are slightly different from the above values, which are determined by sedimentation density gradients. The values obtained by gel electrophoresis are between 17 - 21 S_E for the large species of mitochondrial RNA and 12 - 13 S_E for the small mitochondrial RNA (Bartoov et al., 1970; Swanson & Dawid, 1970). This discrepancy appears to be due to the low G+C content of mitochondrial RNA coupled with differences in the ionic conditions and temperature of RNA fractionation in the different experiments. These parameters are known to affect the secondary structure of the RNA (Borst & Grivell, 1971). The

molecular weights of these two RNA species have been accurately determined by electron microscopy under denaturing conditions (Robberson et al., 1971) and by band centrifugation (Dawid & Chase, 1972). For HeLa cells and Xenopus cells the values are 0.53 - 0.56 x 10^6 daltons for the larger species of mitochondrial RNA and 0.30 - 0.36 x 10^6 daltons for the smaller species.

Mitochondria from a variety of sources contain distinctive low-molecular-weight (4 S) RNA that is believed to correspond, at least largely to mitochondrion-specific transfer RNA. Such 4 S RNA differs from its cytoplasmic counterpart in nucleotide composition and in degree of methylation (for review see Stewart, 1973).

In addition to the transfer-and ribosomal-like RNA, a heterogeneous fraction of mitochondrial DNA-specific RNA has been observed (Attardi & Attardi, 1969). Its properties have been difficult to characterize because of contamination by heterogeneous RNA of nuclear origin associated with cytoplasmic membrane-bound polyribosomes in the mitochondrial fraction. Abelson and Penman (1972) demonstrated a mitochondrial-specific heterogeneous RNA by selective inhibition of nuclear RNA synthesis. It has been shown to contain a stretch of poly(A) approximately 55 nucleotides in length (Perlman et al., 1973). Hirsch and Penman (1973) reported that the properties of the poly(A)-containing, heterogeneous mitochondrial RNA of

HeLa cells, had a poly (A) segment at the 3' end which was also complementary to mitochondrial DNA.

(ii) Synthesis: The occurrence of mitochondrial RNA polymerase has been reported in Neurospora and yeast mitochondria (Küntzel & Schäfer, 1971; Tsai et al., 1971). The enzyme consists of a single polypeptide chain with a molecular weight of 64,000. It is sensitive to rifampicin and resistant to α-amanitin, preferring native mitochondiral DNA as a transcriptional template. Saccone et al. (1969) reported incorporation of all four labelled ribonucleotide triphosphates into rat liver mitochondrial RNA. This RNA synthesis was inhibited by actinomycin-D, indicating that DNA is involved in the process. The RNA synthesized in vitro hybridized with liver mitochondrial DNA, but not with liver nuclear DNA, and more specifically, with only the heavy strand of liver mitochondrial DNA (Borst & Aaij, 1969). Attardi and his co-workers (Attardi et al., 1970) extensively studied mitochondrial RNA synthesis in HeLa cells. They found in long term labelling studies that the mitochondrial RNA hybridized almost exclusively with the heavy strand of mitochondrial DNA. This result was similar to those obtained in liver (Borst & Aaij, 1969) and amphibian egg mitochondria (Dawid, 1972). However, after pulse labelling, both heavy and light strands of DNA were transcribed almost equally indicating that symmetrical transcription

occurred in vivo (Attardi et al., 1970; Borst, 1972). These results may indicate that mitochondria transcribe both strands of their DNA, but soon after synthesis most of the light-strand transcriptional products are degraded or exported from this intracellular compartment.

RNA synthesis in mitochondria of most cells is sensitive to rifampicin (Tsai et al., 1971; Gamble & McCluer, 1970). Preferential inhibition of mitochondrial RNA synthesis by ethidium bromide has also been observed with mitochondria from different sources (Penman et al., 1970a). Therefore, these compounds are the most widely used inhibitors in studies of mitochondrial RNA synthesis.

Ethidium bromide, a trypanocidal drug (2,7 diamino-9-phenyl-10-ethyl phenanthridinium bromide) has been found to inhibit nucleic acid synthesis in vivo in a variety of organisms (Tomchick & Mandel, 1964). In cell free systems, ethidium bromide inhibits DNA polymerase and DNA-dependent RNA polymerase activity. It is postulated that the action of the drug is through the formation of a complex between ethidium bromide and DNA. Waring (1964) characterized the ethidium-DNA complex and obtained data to support the idea that the primary binding of ethidium bromide to DNA involves an intercalation process. In E. coli. ethidium bromide is a very effective inhibitor of the RNA synthesis reaction catalyzed by DNA-depended RNA polymerase (Waring, 1964). However, the effect of ethidium bromide can be overcome by increasing the DNA concentration. These results demonstrated that the inhibition is related in a competitive

fashion to the amount of DNA present, and indicates that ethidum bromide inhibits RNA synthesis by binding to the DNA template, resulting in a complex which is not suitable for the action of RNA polymerase.

Richardson (1973) demonstrated in E. coli. that the rate of RNA chain initiation was affected by low doses of ethidium bromide. This sensitivity of RNA chain initiation to the drug might be caused by the binding of ethidium bromide to the initiation site. There was a variation in sensitivity to the drug using different DNA templates, suggesting differences in initiation sites in various templates.

At relatively low doses of ethidium bromide, mitochondrial RNA synthesis is also inhibited. In their studies on the synthesis of RNA in isolated yeast mitochondria, South and Mahler (1968) reported that ethidium bromide inhibited the incorporation of radioactive precursors into RNA. With HeLa cells growing in culture as little as 0.2 µg. of ethidium bromide/ml. inhibits completely the synthesis of 21 S and 12 S RNA species in mitochondria. In contrast, this low dose causes no inhibition of the incorporation of radioactive precursor in RNA originating in the nucleus (Zylber et al., 1969).

It was shown by Radloff et al. (1967) that the closed circular mitochondrial DNA could be separated easily from the linear nuclear DNA in a cesium chloride density gradient when treated with ethidium bromide.

It therefore seemed possible that ethidium bromide might selectively inhibit transcription of the circular mitochondrial DNA by altering the tertiary structure of such molecules, e.g. by removing or introducing superhelical twists. The transcription of linear DNA might be less sensitive since the drug does not produce large changes in the tertiary structure of such molecules (Radloff et al., 1967; Bauer & Vinograd, 1968). Therefore, ethidium bromide became a useful tool in the study of mitochondrial RNA synthesis.

(iii) Post-transcriptional modification, including ribosomal RNA processing and methylation: Pulse-labelling, pulse chase experiments and comparison of base composition of mitochondrial rRNAs and their precursors strongly support the idea that mitochondrial ribosomal RNAs in Neurospora originate from a single common polynucleotide precursor molecule (32 S in size) transcribed from the mitochondrial DNA (Kuriyama and Luck, 1973). Competitive hybridization experiments have shown that the 32 S mitochondrial rRNA precursor molecules contain nucleotide sequences in excess of the content represented by the mature rRNAs. These extra sequences are equivalent to approximately 20% of the total mass of the precursor molecule.

One controversial point is the degree of methylation of mitochondrial RNA. Dubin and Montenecourt (1970)

and Dubin and Friend (1971) reported that ribosomal RNA of hamster cell mitochondria is very poorly methylated, and virtually no methylation was found by Vesco and Penman (1969). However, Attardi et al. (1970) found significant methylation (one methyl group/100 nucleotides) of mitochondrial ribosomal RNA in HeLa cells. Dubin (1974) examined the state of methylation of mitochondrial rRNA from BHK (Baby Hamster Kidney) cells. The level of methylation in mitochondria was determined by [^{14}C] methionine, [^{3}H] methionine and [^{32}P] Pi multiple isotope labelling techniques. He estimated that there were 2.2 and 4.0 methyl groups per molecule of 17 S and 13 S RNA, respectively. From base analyses, Dubin (1974) concluded that most or all of the 17 S rRNA molecules contained one ribose methylated residue per molecule and one less well-characterized methylated residue; and that most or all of the 13 S molecules contained one methylated cytosine residue, one N^6-dimethyladenine residue and one presumed thymine residue per molecule. The important conclusion of Dubin's (1974) work is that hamster cell mitochondrial rRNA is significantly methylated, albeit less so than any cytoplasmic ribosomal RNA so far studied. However, all these experiments are subject to the criticism that incorporation of the methyl label from methionine occurred into the heterocyclic purine rings (via the

"1-c pool") rather than exclusively into the exocyclic methyl groups. This objection has been met by including formate or purine nucleosides in the growth medium (Lambowitz & Luck, 1975).

The function of RNA methylation is unknown, but it is thought to play a role in RNA processing and ribosome assembly (Maden, 1971). The low level of methylation of ribosomal RNA in mitochondria suggests that at most only a few methyl groups per molecule are required for this process.

(iv) Mitochondrial RNA and development

Studies have been carried out to relate changes in mitochondrial RNA synthesis, or in the types of RNA synthesized, to changes in developmental patterns in the cell (for review see Stewart, 1973; Cantatore et al., 1974). Hormones which affect differentiation, such as thyroid hormone and ACTH, have been found to stimulate mitochondrial RNA synthesis (Schimmelpfennig et al., 1970). It has been found that human leukemic leukocytes contain circular dimer forms of mitochondrial DNA (Clayton & Vinograd, 1969), suggesting the possibility that mutation in mitochondrial DNA might cause abnormal mitochondrial RNA synthesis, which in turn may be associated with occurrence of tumors. Cytoplasmic respiratory-deficient mutants of yeast and Neurospora have been described by Kellerman et al. (1971), who showed that mitochondria

of a petite mutant of yeast lacked high-molecular-weight RNA. In another mutant (the poky mutant of Neurospora) the rate of synthesis of the small subunit of mitochondrial ribosomes was much less than that of the large subunit during the log phase of growth (Rifkin & Luck, 1971).

Although the area of organelle development and its relationship to the development of the whole organism is only beginning to be explored, these mutants might provide a useful means to examine questions concerning the nature of transcripts formed in the organelle, and the control mechanisms exerted on trascription both within and outside the mitochondria. Therefore, information from these mutants should provide important insight into the involvement of mitochondrial RNA in developmental processes.

4. RNA in nerve tissue

Within the past decade a number of investigations have suggested involvement of RNA in the process of acquisition and storage of new patterns of behavior. The synthesis, distribution and possible function of these macromolecules have been reviewed by a number of investigators (Hyden, 1967; Glassman, 1969; Mandel & Jacob, 1971; Horn et al., 1973; Shashoua, 1974).

(a) Ribosomal RNA and transfer RNA

Brain cells contain all the known types of RNA.

The most abundant class of RNA molecules are the ribosomal RNAs. Synthesis of RNA and the bulk RNA components have been demonstrated in rat brain (Jacob et al., 1966; Mahler et al., 1966; Dawson, 1967; Dutton & Mahler, 1968; Dutton et al., 1969), in chicken brain (Rose et al., 1970; Hu & Mezei, 1971; Mezei & Hu, 1972; Judes & Jacob, 1973), and in goldfish brain (Shashoua, 1973).

The pattern of transformation of larger RNA macromolecules into rRNA and mRNA has not been extensively studied in brain tissue. Gel electrophoretic analysis of the products in rat brain nuclear RNA showed the presence of 45 S and 31 S peaks (Tencheva & Hadjiolov, 1969). Additional RNA peaks corresponding to 24 S, 22 S, 14 S, 9 S and 6 S were also detected. The 28 S and 18 S rRNA were present in a ratio of 2:1 (Dawson, 1967; Shashoua, 1973). These results were similar to those reported for HeLa cells (Scherrer et al., 1963; Penman, 1966; Weinberg et al., 1967).

Saborio and Aleman (1972) studied [^{14}C]-uridine and [methyl-^{3}H] methionine incorporation into nuclear RNA of rat brain. They found two types of RNA, methylated and non-methylated; the methylated species seemed to correspond to rRNA precursors. Mezei & Hu (1972) found similar results for the rapidly-labelled high-molecular-weight RNA species in chicken sciatic nerve. Judes and Jacob (1973) reported the incorporation of methyl groups

into 29 S, 18 S and 4 S RNAs in chicken embryonic cerebral hemispheres in vitro. They indicated that ribosomal RNAs were methylated at the level of the early precursor molecules (45 S) of rRNA. There were more methyl groups incorporated into 18 S than into 29 S rRNA. It was, therefore, concluded that in the developing chicken cerebral hemispheres, the molecular events leading to RNA methylation were similar to those in mammalian cells (Greenberg & Penman, 1966; Zimmerman & Holler, 1967).

Studies of transfer RNA in brain tissue have been reviewed by Mandel and Jacob (1971). Recent studies of tRNA in brain and its role in protein synthesis have been carried out in several laboratories; for details see Murthy et al. (1974) and Borkowski and Brzuskiewicz-Zaruowska (1975).

(b) Heterogeneous nuclear and mRNA

Methods have been developed to obtain mRNA from brain tissue (Brawerman & Hadjivassiliou, 1967; Murthy, 1968; Zomzely et al., 1970; 1971). The kinetics of the synthesis of thesemacromolecules have been investigated in several laboratories (Dutton et al., 1969; Murthy, 1968; Zomzely et al., 1971). Murthy (1972) studied the time course of in vivo incorporation of [^{14}C]uridine and methyl groups of [methyl-^{3}H]methionine into the rRNA and mRNA of free and membrane bound

ribosome components of rat cortex. He found that the rate of incorporation of [^{14}C]uridine relative to [^{3}H] methionine was higher in mRNA and discussed the possible role of the cytoplasmic RNAs in regulating cerebral protein synthesis.

Zomzely et al. (1970; 1971) reported that RNA could be dissociated from purified cerebral polyribosomes of rat brain in the presence of EDTA. The base composition of the RNA resembled that of rat DNA. This fraction of RNA readily hybridized with rat DNA and also exhibited high template activity in a cell-free-protein synthesis system. Poly(A)-containing RNAs were isolated from polysome preparations of rat brain. The poly(A)+ RNAs were found to stimulate amino acid incorporation into polypeptide chains in an in vitro ribosomal system, indicating that they possessed messenger activity (Murthy et al., 1975). Several investigators (Zomzely et al., 1971; Lernerd & Herschman, 1972; Zomzely-Neurath et al., 1973; Amaldi et al., 1973; Murthy & Roux, 1974) reported that the synthesis of protein and of soluble brain-specific proteins (S-100 and 14-3-2) on purified rat brain polysomes in a homologous cell-free system. Since these brain-specific soluble proteins have been implicated in neural activity (Zomzely-Neurath et al., 1973) it was assumed that polysomes engaged in the synthesis of these classes of proteins may play an important role in the unique function of the brain.

Hybridization experiments showed that RNA from brain hybridized with homologous DNA to a greater extent than did liver RNA. These results indicated the presence of a wider variety of mRNAs in brain than in peripheral organs (Singh, 1965; Bondy & Roberts, 1968; Hahn & Laird, 1971; Grouse et al., 1973). Wintzerith et al. (1975) also demonstrated that pulse labelled cerebral hnRNA hybridized with homologous DNA to a greater extent than did cytoplasmic RNA. This suggested that nuclei contain hybridizable components that are not transferred into the cytoplasm. The hnRNA found in brain sediments between 80 and 300 S (Stevenin et al., 1970). These molecules are very susceptible to the action of ribonuclease and the presence of ribonuclease inhibitors is indispensable if they are to be obtained in an undegraded form. The relationship between this hnRNA and mRNA in the nervous system has not been completely elucidated, but it seems probable that the hn RNA and mRNA are part of the same giant-sized molecules synthesized in the nucleus (Mandel & Jackob, 1971). The products of some cistrons would be transported into the cytoplasm, while others would remain in the nucleus. Their role and fate in the nucleus is unknown. The half-life of hnRNA in rat brain has been estimated to be 20 - 30 minutes (Singh, 1965), while the half-life of mRNA in brain was determined to be 3 hours (Appel, 1967).

Dutton et al. (1969) and Vesco and Giuditta (1967) also demonstrated that nuclear heterodisperse and cytoplasmic heterodisperse RNAs are present in brain and have high turnover rates.

(c) RNA in axons

Although most of the RNA in the nervous system is synthesized in the cell body it has been demonstrated that nerve fibers, and axons contain RNA (Edström et al., 1969; Koening, 1965; Hu & Mezei, 1971; Lasek, 1970). Edström et al. (1969) demonstrated that the myelinated Mauthner fiber isolated from spinal cord after incubation with radioactive material, showed RNA synthesis. A considerable part of the material sedimented at 4 S, but part of the nucleic acid was recovered at higher sedimentation values up to 30 S. Newly-synthesized RNA was extracted from the isolated myelin sheath as well as from the axon. Isolated myelinated mauthner fibres were also incubated in vitro with RNA precursors. In this case incorporation occurred exclusively into material sedimenting at 4 S or lower. Similar results have been reported in squid giant axon by Lasek (1970) and Lasek et al. (1973).

It is still not clear whether axonal RNA is synthesized in situ or transported from the nerve cell body. Peterson and coworkers (1968;1970) reported the formation of RNA and its transport down the sciatic and optic nerves

of the chick embryo following an injection of labelled orotic acid into the spinal cord and the posterior chamber of the eye. Autoradiographic studies demonstrated that labelled granules were present in the axoplasm of both the sciatic nerve and the optic nerve, as well as in the Schwann cell sheath of the axon. More recent studies indicated that RNA is also transported in the chicken optic nerve axon (Bondy, 1971), goldfish optic nerve (Ingoglia et al., 1973) and in the nervous system of the rat (Ochs, 1972).

(d) The RNA of neurons, glial cells and Schwann cells

The RNA content of specific neruons and glial cells can be determined with the aid of microtechniques to isolate these cell types (Egyházi, 1966). RNA metabolism in these cell types has been studied extensively (Egyhazi, 1966; Egyházi & Hydén, 1966; Volpe & Giuditta, 1967). Differences in RNA metabolism were observed in neurons and glial cells (Daneholt & Brattgard, 1966; Volpe & Giuditta, 1967; Yanagihara, 1974; Soga & Takahashi, 1975). Little is known of the RNA metabolism in isolated Schwann cells due to the difficulty in isolating these cells.

(e) RNA in subcellular fractions of nervous system

The occurrence of RNA including poly(A)-containing RNA has been demonstrated in brain subcellular particles such as mitochondria, synaptosomes, microsomes

and polysomes (Shashoua, 1973; Lim et al., 1974; De Larco et al., 1975; Cupello & Hydén, 1975; Murthy et al., 1975). Cupello and Hydén (1975) showed that RNA species with electrophoretic characteristics similar to those reported for liver mitochondrial RNA were found in brain mitochondria. However, properties of their mitochondrial RNA preparation indicated the presence of large amounts of cytoplasmic rRNA. Recently, fractionation of mitochondrial tRNA from calf brain was reported by Borkowski and Brzuszkiewicz-Zarnowska (1975). Nevertheless, extensive studies on the characterization and synthesis of mitochondrial RNA have not been carried out in the nervous system.

Section II

Characterization of Mitochondrial RNA from Brain and Peripheral Nerve of Developing Chick

A. Introduction

In recent years it has been recognized that mitochondria are not only subcellular organelles which serve to provide membrane surfaces upon which energy conversion can occur, but that they are also autonomous bodies containing genetic informational molecules, DNA (Luck & Reich, 1964) and RNA (Borst, 1972), plus the components necessary to protein synthesis (Gorden & Deanin, 1968). The existence of an abundance of mitochondria in a number of specialized sensory nerve terminals is well-established (Merrillees, 1960). It has also been demonstrated that the content of mitochondria per cell increases during nerve development (Friede & Samorajsky, 1968). Although the details are still obscure, these results suggest that mitochondria may play an important role in the nervous system.

Recent studies have indicated that the sciatic nerve of chicks contains a class of relatively low-molecular-weight RNA components which sediment or migrate between the 18S and 4 S components of the bulk RNA (Hu & Mezei, 1971; Mezei & Hu, 1972; Muirhead & Mezei, 1973). The RNA species become increasingly labelled by radioactive precursors at a developmental stage at which rapid myelination of peripheral nerve commences. The

sedimentation and electrophoretic properties of the low-molecular-weight, rapidly-labelled RNA species of the sciatic nerve are quite similar to those which have been described for cytoplasmic RNA of other fully-differentiated eucaryotic tissues (Hiatt, 1962; Peterson, 1970). Furthermore, labelling of these RNAs is inhibited by relatively high doses of ethidium bromide (5-10 µg/ml of incubation medium) or acriflavine (5 µg/ml of incubation medium) (Mezei et al., 1971), compounds which are believed to inhibit mitochondrial RNA synthesis selectively (Zylber et al., 1969). Since the concentration of mitochondria increases during myelinogenesis (Friede & Samorajsky, 1968), it seemed possible that part of the rapidly-labelled RNA of the myelinating nerve might be transcriptional products of mitochondrial DNA. To examine this hypothesis, the synthesis of mitochondrial RNA in the sciatic nerve was investigated. Since the amount of peripheral nerve available for these studies was limited, brain tissue was used for preparation of bulk material in preliminary experiments.

The first experimental approach was to isolate purified mitochondria which could be identified and characterized by morphological (electronmicroscopy) and biochemical means. It was expected, for example, that in such preparations the activities of characteristic marker enzymes (cytochrome c oxidase) would be maximized, whereas those of enzymes such as lactate dehydrogenase, which are located mainly in other subcellular compartments,

would be minimized (Salway, et al., 1967; Waksman et al., 1968).

The second criterion for the investigation of mitochondrial RNA synthesis was the isolation of these molecules in a pure, undegraded form, and in reasonable and reproducible yields. Although mitochondrial RNAs have been isolated and characterized from a wide variety of organisms (Borst, 1972), few such studies had been carried out on nervous tissue (Borkowski, 1971; Cupello & Hyden, 1975). On the basis of other investigations (for review see Borst, 1972), it was expected that mitochondrial rRNA from the chick nervous system would show a different molecular weight distribution and base composition than its cytoplasmic counterpart and that its synthesis would be sensitive to well-known inhibitors of mitochondrial transcription, such as ethidium bromide.

Having established methods for isolation, purification and characterization of mitochondrial RNA of nervous tissue, it would be possible to compare the pattern of labelling by specific precursors and the specific activities of such preparations to those previously obtained in this laboratory (Hu & Mezei, 1971; Mezei et al., 1971) for total RNA of the chick sciatic nerve. If the specific activities and the pattern of labelling of purified mitochondrial RNA were comparable

to those previously demonstrated for the low-molecular-weight, rapidly-labelled RNA of the myelinating nerve, one could then postulate that these molecules may have originated from mitochondria of Schwann cells or axons.

Several methods are available for the purification of mitochondria from brain tissue (Gray & Whittaker, 1962; Whittaker et al., 1964; Gorden & Deanin, 1968). Differential and sucrose density gradient centrifugations are widely used as preliminary enrichment and purification procedures. In sucrose gradients, mitochondria band at a densityof 1.15-1.19 gm/cm^3; while the density at which synaptosomes band is 1.13-1.17 gm/cm^3. Therefore, mitochondria purified by this method are in most cases heavily-contaminated with synaptosomes (Whittaker, 1968; Cotman, et al., 1971) As an alternative approach ficoll-sucrose gradients were employed by Cotman et al. (1971) to further resolve the crude mitochondrial fraction of brain homogenates. Ficoll, a synthetic high-molecular weight polysaccharide contains no ionized groups, but is extremely soluble in aqueous solution due to a high content of hydroxyl groups. Its advantage in density gradient centrifugation is that there is a slow increase in osmotic pressure with increasing concentration, whereas in sucrose gradients the relationship between the increase of osmotic pressure and sucrose concentration is almost linear (Pharmacia, 1968). Furthermore, ficoll-

gradients are less viscous than sucrose gradients of the same densities (Brown, 1968). Therefore, in the present investigation preliminary experiments employed the ficoll-gradient method of Cotman et al. (1971).

The major method of isolation and purification of mitochondria in this dissertation was that of Haldar et al. (1967). These authors subjected crude mitochondrial fractions to mild osmotic shock treatment. In 1964, Whittaker et al. demonstrated that when synaptosomes were suspended in a medium hypoosmotic to plasma, 80% of the synaptosomes were ruptured. Haldar et al. (1967), reasoned that this method would be suitable for the purification of brain mitochondria heavily-contaminated with synaptosomes and other organelles, since mitochondria could tolerate osmotic pressure change better than other organelles. These workers showed that after brief osmotic shock treatment of crude mitochondrial fractions, other cell organelles had been ruptured and could be removed by centrifugation; leaving the mitochondria in a relatively intact state. Haldar et al. (1967) and Hernandez et al. (1971) claimed that the morphology and biological activity of the mitochondria after this brief osmotic shock treatment were normal.

The purity of the mitochondria in the present investigation was checked by electron microscopy, assay of marker enzyme activities, and determination of yields

of mitochondrial protein and RNA. Cytochrome c oxidase was selected as a suitable marker because this enzyme is an integral part of the mitochondrial respiratory chain; is firmly membrane-bound, being associated with the inner membrane of the organelle. (Schnaitman et al., 1967). It was shown in mouse brain that the subcellular distribution of cytochrome c oxidase and lactate dehydrogenase was different: approximately 81% of cytochrome c oxidase activity was found in the mitochondrial fraction, whereas most of the LDH activity appeared in the soluble cytoplasmic fraction (Waksman et al., 1968).

Hernandez et al. (1971) reported that the yield of mitochondrial protein was 1 mg per gram of wet tissue. At present, considerable variation exists in the reported values for the yield of mitochondrial RNA from a variety of animal tissues. Kroon (1971) plotted the RNA content of mitochondrial fractions as a function the glucose-6-phosphatase activity in these preparations and calculated the absolute content of mitochondrial RNA by extrapolating to zero concentration of this microsomal enzyme. By this approach, the "real" RNA content of normal liver mitochondria was on the order of 1 µg RNA/mg protein. Bartoov et al. (1970) reported a value of 3 µg RNA/mg protein for purified rat liver mitochondria, while a value of 6.6 µg RNA/mg of mitochondrial protein was found by O'Brien and Kalf (1967).

In the present dissertation the apparent size of mitochondrial RNAs from chick nervous tissues was estimated by gel electrophoresis. A number of recent reports have described discrete high-molecular-weight RNA species in mitochondria. These rRNA species have been shown to sediment like bacterial rRNA and more slowly than homologous cytoplasmic rRNA (Kuntzel & Noll, 1967; Rifkin et al., 1967; Zylber et al, 1969; Vesco & Penman, 1969; Attardi & Attardi, 1969). Montenecourt et al. (1970) studied mitochondrial rRNA in cultured hamster and mouse cells and found two species of mitochondrial RNA with molecular weights of 0.72×10^6 and 0.42×10^6 dalton respectively. Rabbitts and Work (1971) reported the existence of 18 S and 12 S RNAs in chick liver mitochondria. Molecular weight determinations of these two species were carried out by gel electrophoresis. The values obtained were 0.70×10^6 and 0.33×10^6 dalton for the large and small species, respectively. It should be emphasized that due to the unusual secondary structure and low G+C content of mitochondrial RNAs, molecular weight determination of these RNAs are very sensitive to changes in temperature and ionic strength of the experimental milieu (Grivell et al., 1971). For example, Groot et al. (1970) demonstrated that between 2° and 28°C, the relative mobility of the small mitochondrial RNA component gradually decreased, and hence, the calculated apparent molecular

weight of mitochondrial RNA is 40% higher at 28° than at 2°.

In the present investigation an attempt was made to analyse the base composition of mitochondrial RNA to further characterize these species in the chick nervous system. Conventional methods of base analysis were not suitable for this purpose because of the low content of mitochondrial RNA and the poor incorporation of ^{32}p into brain mitochondrial RNA *in vitro*. Therefore, a method was sought which would require only a small amount of RNA (µg levels) yet give reliable and reproducible results. Randerath *et al.* (1972) have described a procedure in which the base composition of ribopolynucleotides can be determined by a tritium labelling technique. The main advantage of this method are its good resolution, great sensitivity, simplicity and speed. Therefore, this method was adapted to analyse the base ratio of mitochondrial RNA from chicken brain.

Mitochondrial RNA from rat liver and mouse L-cells (Bartoov *et al.*, 1970) and HeLa cells (Vesco & Penman, 1969) has characteristically low G+C content. Bartoov *et al.* (1970) have suggested that the extremely low G+C content in mouse cell mitochondrial RNA might be due to contamination of this species with heterodisperse cytoplasmic RNA. Results similar to those of Bartoov *et al.* (1970) have been reported by Montenecourt and co-workers

(Montenecourt et al., 1970; Dubin & Montenecourt, 1970) who compared the base composition of mouse L-cell and BHK cell mitochondrial rRNAs and tRNA with their cytoplasmic counterparts. It was found that the G+C content of the mitochondrial rRNA and tRNA were 47.7% and 46.2%, respectively, whereas that of the cytoplasmic 28 S, 18 S rRNAs and tRNA was 64.5, 56.9 and 60.1%, respectively. The situation appears to be the same in Neurospora and yeast (Rifkin et al., 1967; Fauman et al., 1969). On the average the difference in the percentage of G+C content between mitochondrial RNAs (rRNAs and tRNA) and the analogous cytoplasmic RNA species is around 15-25% in various higher organisms. On the basis of the above results it is generally accepted that mitochondrial RNA has a relatively low G+C content when compared to its cytoplasmic counterparts.

The effects of two inhibitors of mitochondrial RNA synthesis, ethidium bromide and cordycepin, were also studied in the present investigation. It is now generally accepted that ethidium bromide inhibits selectively mitochondrial RNA synthesis (for review see Borst, 1972). It has been shown by DeVries and Kroon (1970) that ethidium bromide inhibits mitochondrial cytochrome c oxidase formation in regenerating rat liver, presumably because transcription of mitochondrial RNA is inhibited. Zylber et al. (1971) reported that cordycepin inhibits mitochondrial

RNA synthesis, and that this drug apparently has no other direct effect on mitochondria. The mechanism of cordycepin inhibition of mitochondrial RNA synthesis is quite different from that of ethidium bromide, with cordycepin probably acting at the level of the RNA polymerase.

B. Materials and Methods

Materials

Ethidium bromide (2,7-diamino-9-phenyl-10-ethyl-phenanthridium bromide) was purchased from Calbiochem. (Los Angeles, Calif.). [5-^{3}H]uridine (25.6 ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.) and [^{3}H]KBH_4 (9.3 ci/mmol) from Amersham-Searle Co., (Arlington Heights, Ill., U.S.A.) respectively. Acrylamide and bis-acrylamide were purchased from Canalco Industries Corp; Rockville, Maryland. Nucleosides were the products of Sigma Chemical Company, St. Louis, Missouri. Sodium periodate was obtained from the British Drug Houses LTD, England and was of analytical reagent grade. Omnifluor (98% ppo, 2% Bis-MSB), medical x-fay film (Kx-Fuji) and cellulose sheets on plastic backing (No. 6064) were products of New England Nuclear Corp; Boston, Mass.; Fuji Co., Japan; and Eastman-Kodak, Rochester, N.Y., respectively. Organic solvents and other chemicals were of analytical reagent grade from J.T. Baker Chemical Co., Phillipsburg, N.J.

Methods

1. Preparation of the tissues and incubation procedure

Fertile eggs (White Leghorn strain) were obtained from Lone Pine Farm LTD. (Berwick, N.S.) and incubated in a rotating incubator (Humidair Comp.) at 37°C. To dissect nerve tissue, the dorsal and leg skin of the embyro or of the hatched chick was excised, and the sciatic nerve was exposed by severing the superficial muscles of the upper thigh. The nerve was severed proximally near the spinal cord just distal to the spinal ganglia, and severed distally just below the knee joint. Adhering material was removed prior to incubation. The brain and nerve tissue were kept in aseptically-prepared oxygenated incubation medium (1.2×10^{-1} *M* NaCl, 2.1×10^{-3} *M* $CaCl_2$, 5.6×10^{-3} *M* KCl, 8.1×10^{-4} *M* $MgSO_4$, 1.0×10^{-3} *M* NaH_2PO_4, 1.2×10^{-2} *M* glucose, and 2.4×10^{-2} $NaHCO_3$ final pH 7.8) at 0-4°C until 0.2 g (nerve) and 10 g (brain) of tissue was accumulated. The nerves were then incubated for the times indicated in 2-3 ml of incubation medium containing 50 µCi/ml of [5-^{3}H]uridine (25.6 mCi/µmole, New England Nuclear Corp., Boston, Mass.) in shaking water bath at 37°C and under a constant stream of oxygen. After incubation the tissue was washed in 10 volumes of incubation medium and drained on a piece of filter paper. To test the possibility of bacterial contamination, aliquots of the incubation mixture were plated on blood agar. The low level of colonies obtained eliminated any substantial possibility of contamination (Shirley, 1974).

2. Preparation and Purification of Mitochondria

(a) "Osmotic Shock" treatment procedure: Brains from freshly-killed chicks or sciatic nerves after incubation were rinsed three times with 10 volumes of homogenizing medium containing 0.32 M sucrose, 0.5 mM EDTA (pH 7.4) and homogenized at 0-4°C in 10 volumes of the above homogenizing medium in a Potter Elvehjem teflon tissue grinder (clearance 0.3-0.4 mm; Kontes Glass Co., Vineland, N.J. U.S.A.) at a speed of 2500 r.p.m. for 1 minute. All the following steps were carried out at 0-4°C Prior to subcellular fractionation, nerve homogenates were mixed with 10 volumes of fresh chicken brain homogenate of the same developmental stage to serve as a source of carrier mitochondria. Mitochondria were prepared and purified from the homogenates according to the following procedure (Hernandez et al., 1971): After homogenization the mixture was centrifuged at 700xg for 15 min. in a Sorvall RC2B centrifuge at 4°C. The supernatant was saved and the pellet was washed once with homogenizing medium (1/3 of the original volume) and the suspension was centrifuged as before. The first and second supernatants were combined and centrifuged at 12,000 xg for 15 min. and the supernatant was discarded. The pellet from this step (12,000 x g) constituted the crude mitochondrial fraction.

The crude mitochondrial fraction was suspended in

homogenizing medium (1/2 of the original volume of the homogenate) and subjected to centrifugation for 15 min. at 8000 x g; the supernatant was discarded and the pellet saved. The pellet was resuspended in homogenizing medium (1/3 of the original homogenate volume) and the mixture was recentrifuged at 9000 x g for 15 min. The supernatant and the top fluffy material were discarded. The pellet was resuspended in homogenizing medium (1/3 of the original homogenate volume) and subjected to low speed centrifugation (700 x g) for 10 min. to eliminate any remaining contamination by nuclei or cell debris. The supernatant from this step was subjected to centrifugation at 9000 x g. The pellet from this step was then subjected to a mild osmotic shock treatment, as follows: distilled water at 4° was added to the pellet in the ratio of 1 ml/g of tissue and the mixture was shaken gently for 5 min. at 4°C. After this step the osmotic pressure of the mixture was raised by adding albumin (10 mg/ml) in the ratio of 0.2 ml/ml of added distilled water. The above mixture was mixed well and centrifuged at 9000 x g for 10 min. The resulting pellet was washed twice by resuspension in 15 ml of the homogenizing medium, followed by centrifugation at 9000 x g. The final washed pellet consisted of purified mitochondria. The overall procedure is shown in Fig. 4.

(b) Buoyant density sedimentation in ficoll gradients: In preliminary experiments an attempt was made to purify

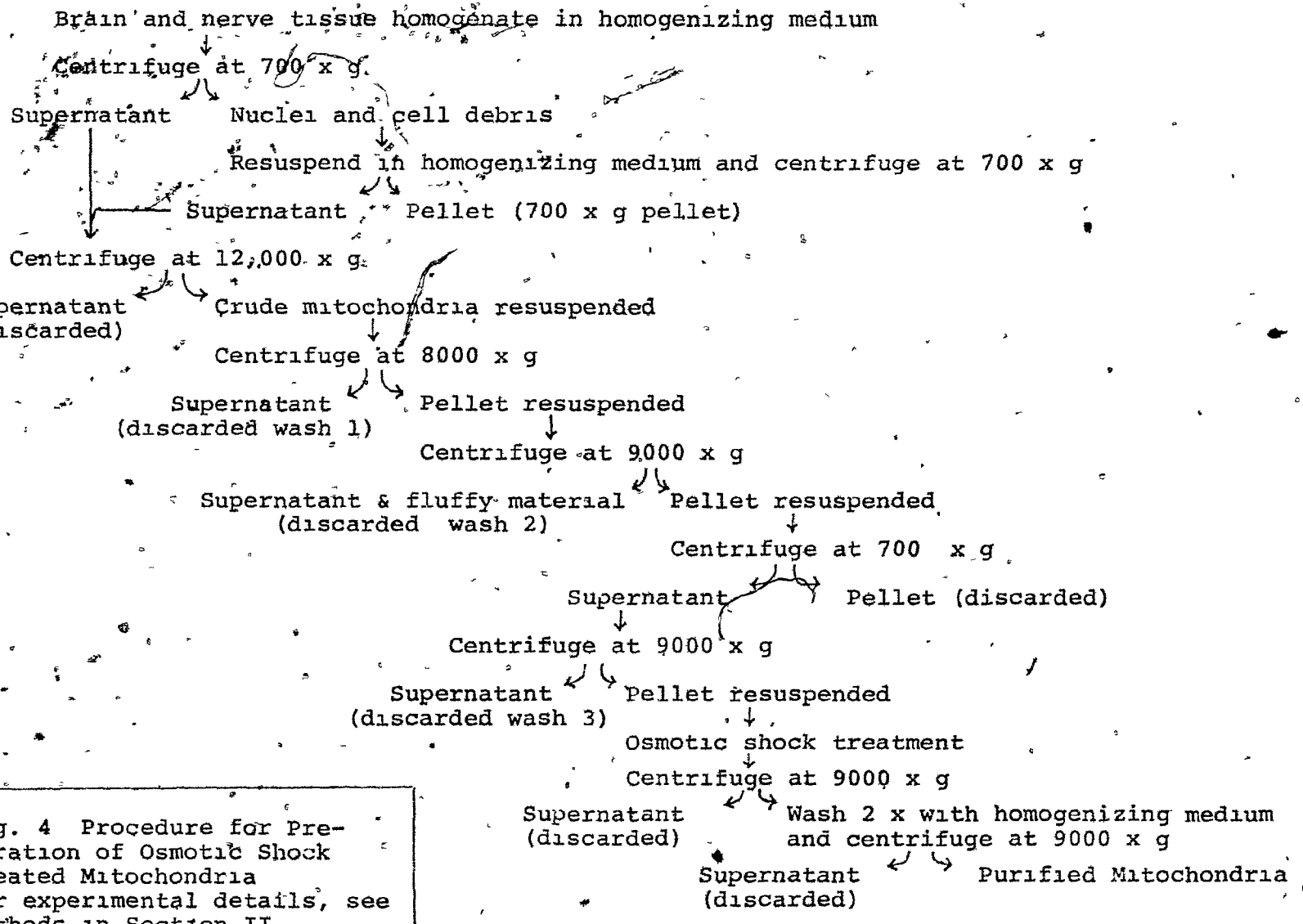


Fig. 4 Procedure for Preparation of Osmotic Shock Treated Mitochondria
For experimental details, see Methods in Section II

brain mitochondria using ficoll-sucrose gradients to further fractionate crude mitochondrial preparations (Cotman et al., 1971). The crude mitochondrial fraction was suspended in 3 ml. homogenizing medium and layered on a discontinuous ficoll gradient composed of 3 ml of each of 10%, 20%, 30% ficoll in 0.32 M sucrose. The gradient was prepared at room temperature (22°C) and was allowed to stand for one hour at 4°. The crude mitochondrial preparation was layered on the gradient, which was then spun at 100,000 x g for 70 min. in a Beckman $L_2$65B ultracentrifuge in a Ti41 rotor at 4°C. This procedure gave three fractions, designated M1, M2, and M3, respectively, in order of increasing density. Fraction M1, the top-most layer, probably consisted of myelin. Mitochondria were expected to band at the highest density position (fraction M3).

3. Extraction of RNA

Purified mitochondria were suspended in 5 volumes of a solution containing 0.01 *M* Tris(pH 7.2), $1x10^{-2}$ *M* $Cacl_2$, $1x10^{-2}$ *M* sucrose, 0.3% SDS and 0.04% purified bentonite (Brownhill et. al., 1959) and an equal volume of a solution containing $2x10^{-2}$ *M* sodium acetate (pH 5.0) $2.8x10^{-1}$ *M* LiCl, $2x10^{-3}$ *M* $MgCl_2$, 1% of (w/v) SDS and 0.01% bentonite. After three cycles of freezing and thawing to disrupt the membranes, 2 volumes of water-saturated phenol, preheated to 60°C were added. The mixture was heated in a water bath at 60°C for 5 min. and shaken vigorously after which it was centrifuged at 8000 x g for 5 min. in a Sorvall RC_2 B

centrifuge at 4° C. The aqueous phase was removed and the interphase was re-extracted with 1/4 of the original volume of the Tris buffer and acetate buffer, respectively. Centrifugation was repeated as in the above step and the resulting aqueous phase was added to the previous one. The combined aqueous supernatants were re-extracted with an equal volume of phenol at 4° C for 2 min. The RNA was precipitated from the aqueous phase by addition of 2 volumes of 95% ethanol and the suspension was allowed to stand overnight at -20° C. The RNA precipitate was collected by centrifugation and dissolved in 2-3 ml of a dialyzing solution containing 2×10^{-2} *M* sodium acetate (pH 5.0), 1×10^{-1} *M* NaCl, and 0.03% (w/v) bentonite. The RNA solution was then dialyzed against 800 ml of the above solution for 6-8 h at 4° C. The dialyzing solution was changed three times during this period. After dialysis, the RNA was precipitated by addition of 2 volumes of cold 95% ethanol and allowed to stand overnight at -20° C. The precipitate was dissolved in 0.2 ml of the dialyzing solution, and centrifuged at 800 x g for 5 min at 4° C to remove the bentonite particles. The RNA preparations were monitored for purity by determining the ratio of the ultraviolet absorbance at 260-280 nm. The ratio in all cases was greater than 1.90. For purposes of comparison, total brain RNA was extracted by the same procedure.

4. Gel Preparation

Polyacrylamide gel electrophoresis was performed essentially as described by Bishop et. al. (1967). The gels were prepared from an aqueous stock solution containing 15% acrylamide, recrystallized from acetone, and 0.75% bis-acrylamide, recrystallized from 95% ethanol. To prepare 2.6% (w/v) polyacrylamide gels, 5 ml 3E Buffer (0.12*M* Tris base, 0.06*M* Sodium acetate, 0.03*M* Sodium EDTA titrated with glacial acetic acid to pH 7.6) were mixed with 2.6 ml of acrylamide stock solution and 7.1 ml of distilled water. The mixture was degassed for 15 seconds, then 0.025 ml of TEMED (N,N,N',N' - Tetramethyl ethylene diamine) and 0.25 ml of 10% ammonium persulfate were added in succession, with thorough mixing after each addition. Cylindrical gels (0.7 cm dia. and 7 cm length) were cast in plexiglass tubes. Dialysis tubing was wrapped around the bottom of each tube to prevent the solution from escaping during polymerization, and to prevent the gels from sliding out of the tubes during electrophoresis. The gels were allowed to set for 30 min. E Buffer (1/3 dilution of 3 E Buffer) containing 0.2% SDS was then layered on the gels and they were left standing for another 30 min.

Gels were pre-run for one hour in a Canalco electrophoresis apparatus (Canalco Industries Corp; Rockville, Maryland) with a Canalco power pack at 5.0 milliamperes (MA)

per gel. The electrophoresis buffer was E buffer containing 0.2% SDS.

5. Fractionation

For resolution by polyacrylamide gel electrophoresis 20-60 µg of RNA in 20% sucrose was layered on top of each gel, and electrophoresis was continued for 2 h at 5m A/gel. The gels were removed from their plastic tubes and scanned at 260 nm in order to obtain a profile of ultraviolet-absorbing components. The gels were then frozen on dry ice and sliced. The slices (1mm) were laid on fiber glass discs and digested with 10% NH_4OH in a closed chamber overnight at room temperature. The discs were then dried, placed in 10 ml of a scintillation fluid (containing 4 gm omnifluor per liter of toulene). Counting efficiency for tritium was 36% (determined by an internal standardization method). Samples were counted for 10 min. in a Philips liquid scintillation analyzer.

6. Assay of Activities of Marker Enzymes

Marker enzyme studies were performed on all the fractions collected during the mitochondrial purification procedures. The activities of L-lactate:NAD+oxidoreductase (Lactate dehydrogenase, E.C.1·1·1·27) and ferrocytochrome C: oxygen oxidoreductase (cytochrome c oxidase, E·C·1·9·3·1) were determined using a Cary model 16 K spectrophotometer (Cary Instruments, Monrovia, Calif.).

(a) Lactate dehydrogenase: Lactate dehydrogenase, a soluble enzyme (Salway et al., 1967) catalyses the reaction: Pyruvate + NADH + H^+ = Lactate + NAD^+. The

activity of this enzyme was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADH. Reaction mixtures contained (in a final volume of 3 ml) 10 µmoles Na pyruvate, 0.2 µmoles NADH (freshly prepared) 100 µmoles KH_2PO_4-K_2HPO_4 buffer; pH 7.4. The reaction was started by addition of 0.05 ml of sample (Kornberg, 1955). One unit of enzyme activity was defined as that amount of lactate dehydrogenase which changed the absorbance of NADH at 340 nm by 0.001 per min. at 22° C. Specific activity was expressed as units per mg protein, which was determined by the method of Lowry et al. (1951).

(b) Cytochrome C Oxidase: Cytochrome c oxidase is an enzyme of the mitochondrial transport system (Waksman et al., 1968). It oxidizes reduced cytochrome c and is itself oxidized by oxygen. Cytochrome oxidase was assayed by the method of Cooperstein & Lazarow (1967) which measures the decrease in absorbance at 550 nm due to the oxidation of reduced cytochrome c. Incubation mixtures contained 3.0 ml of a reduced cytochrome c solution, and the reaction was started by addition of 0.05 ml of sample. The reduced cytochrome c solution was prepared by mixing 30 ml of a solution of cytochrome c ($1.7 \times 10^{-5} M$ in $0.03M$ phosphate buffer, pH 7.4) and 100 µl of a freshly-prepared solution of sodium hydrosulfite ($1.2M$). Air was bubbled through the solution for 15 min. to remove excess

hydrosulfite. One unit of cytochrome c oxidase was defined as that amount of enzyme which results in a decrease in the absorbance at 550 nm of 0.001 per min at 22° C. Specific activity was expressed as units per mg. protein.

7. Base Composition of RNA

Determination of base compositions of different RNAs was carried out essentially according to the method of Randerath et al. (1972).

(a) Preparation of tritium-labelled potassium borohydride solution: tritium-labelled potassium borohydride (Spec. Act. 9.3 ci/m mole) was dissolved in standardized CO_2-free 0.1NKOH and unlabelled KBH_4 (in 0.1N KOH) was added to give final specific activity of 2-3 ci/m moles at 0.1 *M* total potassium borohydride concentration. The solution was stored in small portions (approx. 50 µl each) at -70° C.

(b) Enzymatic digestion of RNA: to each µg RNA was added 30 n moles Bicine [N, N-Bis(2-hydroxy-ethyl) glycine] Na buffer (pH 8), 10 n moles $MgCl_2$, 0.20 mg venom phosphodiesterase, 0.20 µg ribonuclease A, 0.15 µg alkaline phosphatase and water to obtain a final concentration of 1 µg RNA/5 µl incubation mixture. Incubation was at 37° for 16 hours. The enzyme digest was then diluted with distilled water to obtain a final concentration of 1 µg RNA/ 30 µl.

(c) Sodium periodate oxidation and potassium borohydride reduction: Five µl of $NaIO_4$ (12.2 mg/50ml) solution was added to each µg of the digested RNA, and the solution was allowed to stand in the dark for 100 min. at 22° C. After the oxidation reaction, the mixture was placed in a well-ventilated hood, 0.5 µl of tritiated potassium borohydride solution (2-3 ci/ µmole) was added and the mixture was allowed to react in the dark for 100 min. After the reduction step 25 µl of 1 N acetic acid was added to stop the reaction and release excess tritium gas. The mixture was allowed to stand for another 30 min. then evaporated to dryness and the dry residue dissolved in 5 µl 0.1 N Formic acid. It was found that the above procedure was most conveniently carried out with 6 µg of RNA.

(d) Thin layer chromatography: 12-18 µl (3 µl per portion) of RNA digests (in formic acid) were applied to 20 x 20 cm cellulose sheets with a microsyringe and resolved by two dimensional thin layer chromotography. The solvents were: (I) (first dimension) Acetonitrile/Ethyl acetate/n-butanol/isopropanol/6N NH_4OH/(4:3:1:2:2.7) and (II) (second dimension) t-amylalcohol/Methyl ethyl ketone/ acetonitrile/ethyl acetate/water/Formic acid/(4:2:1.5:2:2:0.18) (v/v). Development in the first dimension was to 17 cm from the origin. After drying in a stream of cool air, then in a stream of warm air (50-60° C) for 2-3 min., the chromatogram

was developed in the second dimension with solvent II to 5 cm on a Whatman No. 1 Wick attached to the top (the original right hand side) of the sheet by stapling. After the second dimension, sheets were dried for further manipulations.

(e) Auto-radiographic detection, extraction of compounds and liquid scintillation counting: medical x-ray film (Kx Fuji) was exposed directly on the cellulose sheets in the dark for 7 days. Compounds were located by superimposing the exposed x-ray film on the chromatogram. Areas on the chromatogram corresponding to the compounds were cut out and eluted in 5 ml 2N NH_4OH for 2 hr. with constant shaking, washed 3 times with an additional 1 ml NH_4OH, and made up to a final volume of 10.0 ml. Aliquots (0.2 ml) were counted in 6 ml "Aquasol" (New England Nuclear) in a Philips liquid scintillation counter. The entire procedure is summarized in the following scheme (Fig. 5).

8. Electron microscopy

Electron microscopy was carried out by Mr. Bora Merdsoy (Biology Department, Dalhousie University).

C. Results

1. Purification of mitochondria by buoyant density in ficoll-gradients.

Electron microscopy, RNA fractionation studies and

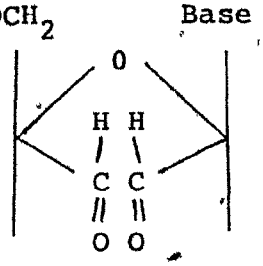
RNA

↓ Digestion to nucleosides: Ribonuclease A; Snake venom phosphodiesterase; Alkaline phosphatase; pH 8.0 37°C 16 hours

Adenosine, Cytidine, Uridine, Guanosine

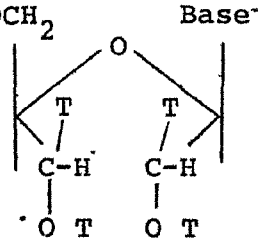
↓ Oxidation: $NaIO_4$ pH 6 22°C 100 min.

Nucleoside Dialdehyde =



↓ Tritium incorporation: KB^3H_4 pH 7-8 22°C 100 min.

Tritium-labelled trialcohols =



↓ TLC of aliquot and Liquid Scintillation

counts/min. of 4 nucleoside trialcohols

Fig. 5. Base composition analysis of ribopolynucleotide by enzymatic digestion and stoichiometric tritium incorporation.

assays of marker enzymes indicated that this procedure did not give satisfactory results. For example, a significant increase in cytochrome c oxidase activity was not observed in the M_3 fraction, although LDH activity was somewhat decreased in this fraction (Table I). Electronmicrographs of material from the M_3 layer showed that this fraction was contaminated by other subcellular particles (Fig. 6). Gel electrophoresis of RNA extracted from the M_2 and M_3 bands indicated the presence of 28 S, 18 S and 4 S RNA components. An extra peak corresponding to a species of 0.45×10^6 daltons was also observed by both ultraviolet absorbance scanning and radioactive labelling techniques, as shown in Fig. 7 and Fig. 8. Therefore, in subsequent experiments, the purification procedure of Hernandez et al. (1971) was adapted for the investigation of the physico-chemical properties of mitochondrial RNA.

2. Purification of mitochondria by mild "osmotic shock" treatment.

Electron microscopy: the purity of the mitochondrial preparation was checked by several procedures. Fig. 9 A and B shows the electron micrographs of crude mitochondrial fraction and purfied mitochondria obtained by mild osmotic shock treatment of mixed brain and nerve tissues from 3-day chicks. Whereas electron microscopy of crude mitochondria indicate contamination of these fractions by

other cytoplasmic membranous elements, osmotic shock treatment results in a fairly pure preparation, although some unidentified membrane fragments are also noticeable.

3. Marker enzyme specific activities and yield of RNA in the subcellular fractions.

The data in Table II indicate a definite enrichment of cytochrome c oxidase specific activity in the purified mitochondrial fraction. In contrast, while the cytochrome c oxidase specific activity became increasingly greater during purification, lactate dehydrogenase specific activity decreased, especially in those fractions of high cytochrome c oxidase activity. The recovery of total cytochrome c oxidase activity in "osmotic shock" treated mitochondria was 29%, relative to that present in the initial homogenate whereas the recovery of LDH was only 5%. RNA and protein contents of the mitochondrial fractions were progressively reduced during the purification procedure. These results, therefore, indicate an effective separation of mitochondria from other subcellular organelles. The behaviour of LDH activity in different subcellular fractions of chicken brain was unusual, since the specific activity and % recovery or this enzyme was greatly increased after the removal of nuclear and mitochondrial fractions. This might indicate that there were endogenous inhibitors of LDH activity in total brain homogenate which were removed in the nuclear and in crude

mitochondrial fractions. Nevertheless, the specific activity of LDH was still decreased in those fractions which showed increased cytochrome c oxidase activity.

Table 1

Comparison of Specific Activities of Marker Enzymes in Subcellular Fractions From Discontinuous Ficoll-Gradients

Fractions	Units of Cytochrome c oxidase/mg protein	Units of Lactate dehydrogenase/mg protein
Total	11.0	7.7
Crude Mitochondria	13.7	5.5
M_1	4.3	6.3
M_2	9.5	5.1
M_3	5.3	3.7

Enzyme activities and units were determined and defined as described in Methods.

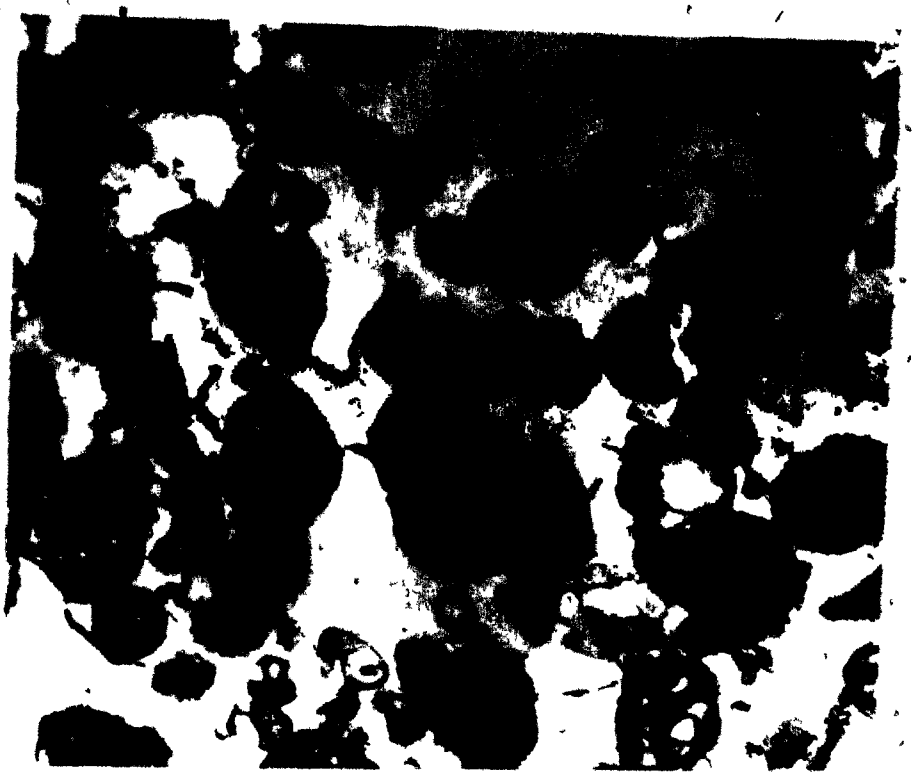


Fig. 6. Electronmicrograph of M_3 layer of Ficoll gradient (magnification x 12,500).

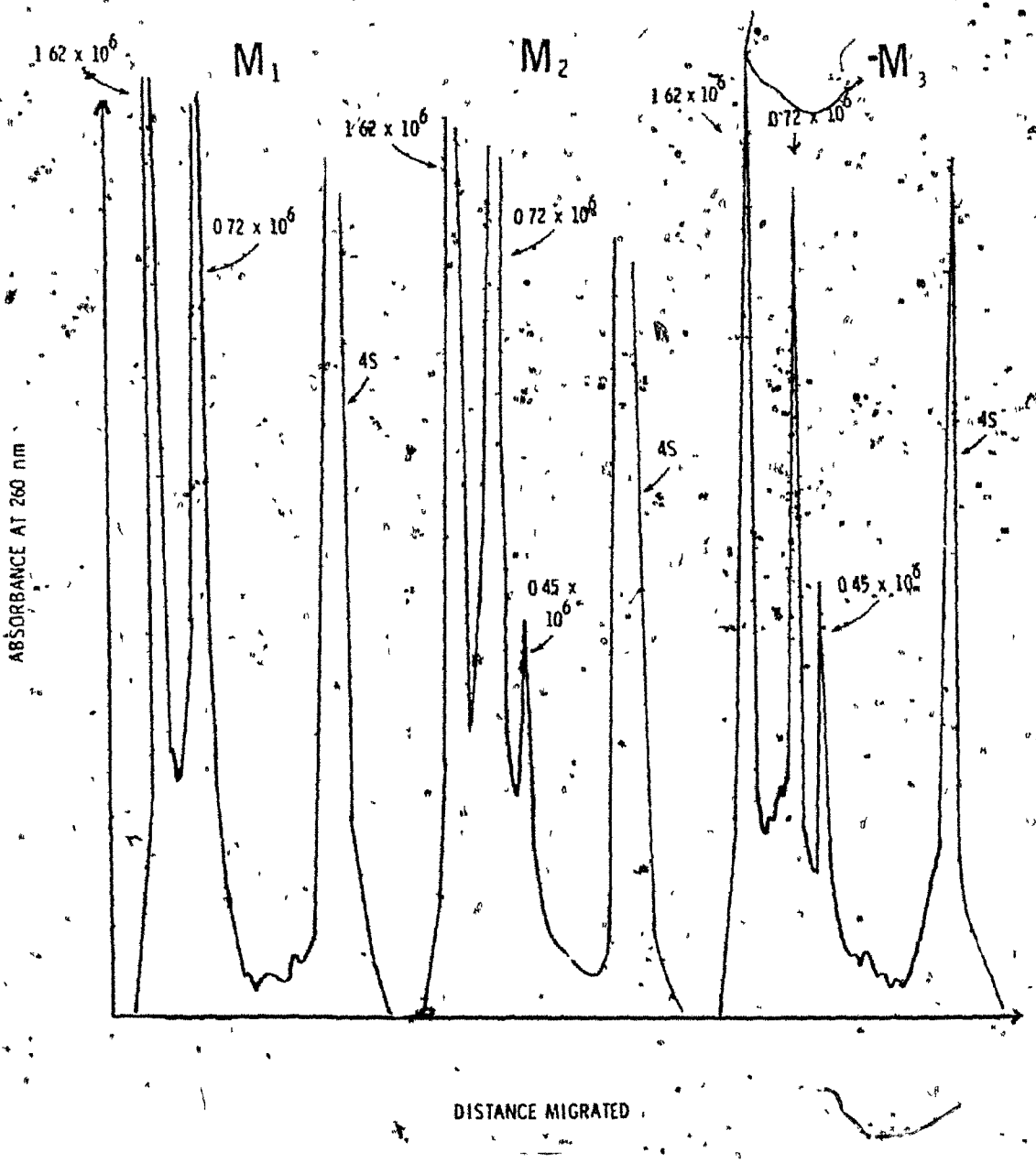


Fig. 7. Spectrophotometric tracings of RNA from submitochondrial fractions M_1 M_2 and M_3 fractionated on 2.6% polyacrylamide gels for 2 h as described in Method.

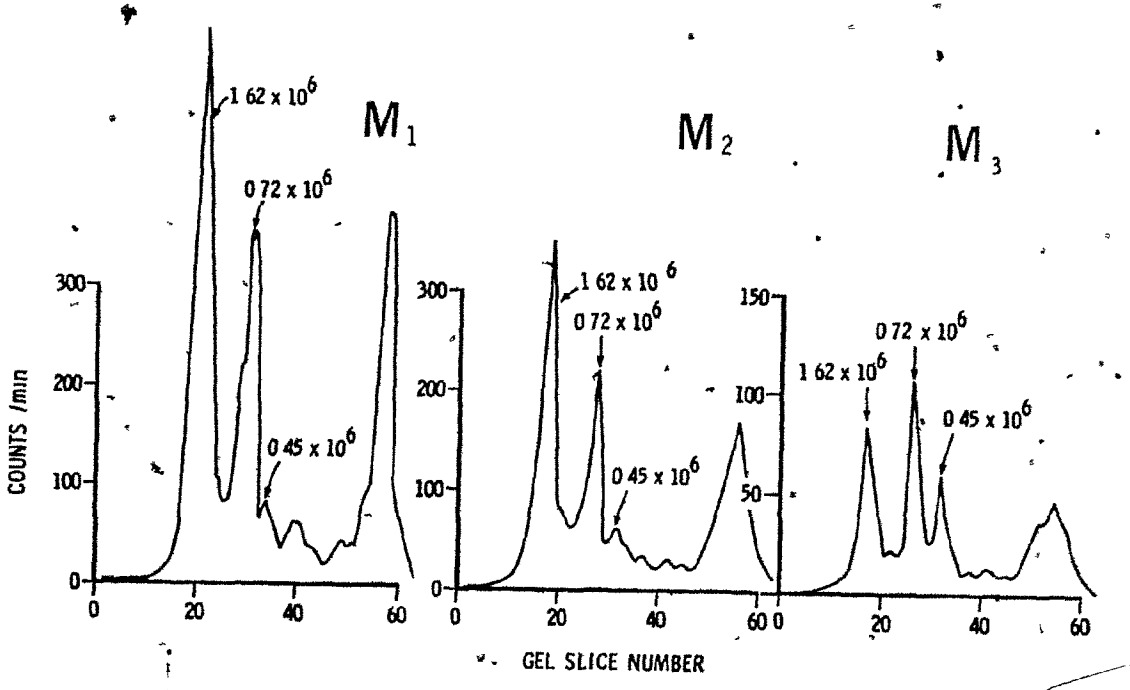


Fig: 8. Electrophoretic patterns of RNA extracted from the M_1, M_2 and M_3 fractions of 16-day embryonic brains labelled for 48 hr. in vivo by injecting 50 µci [5-^{3}H]uridine into the air space. Conditions of RNA extraction and fractionation are described in Methods.

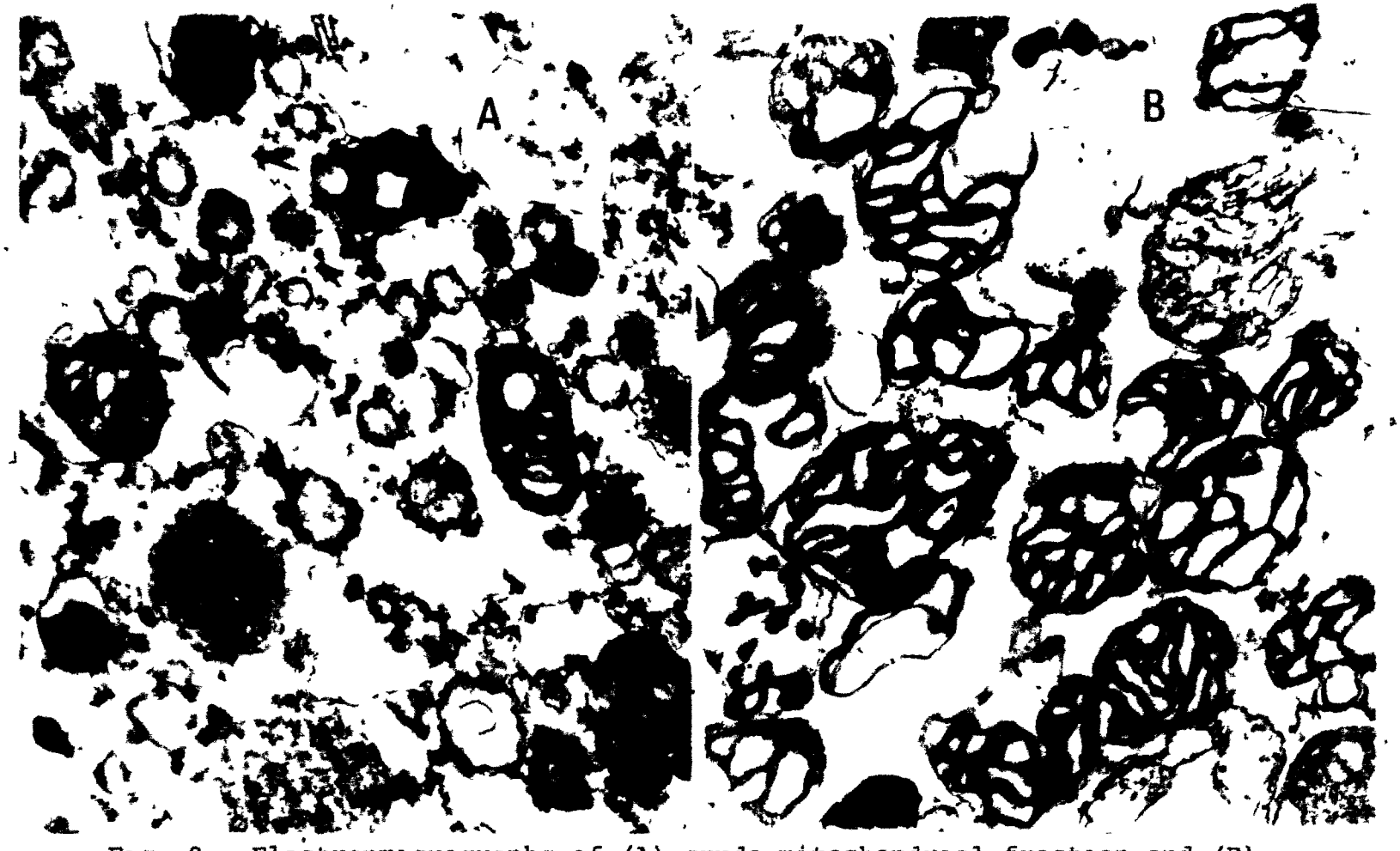


Fig. 9. Electronmicrographs of (A) crude mitochondrial fraction and (B) purified mitochondrial fraction after mild osmotic shock treatment. (Magnification x 12,500)

Table II

Comparison of Marker Enzyme Activities, Protein and RNA Contents of Subcellular Fractions

Fractions	Units of Cyto. c Oxidase/mg Protein	Cyto. c Oxid Recovery % of Total	Units of LDH/mg Protein	LDH Recovery % of Total	mg Protein/g Wet Weight of Tissue	µg RNA/g Wet Weight of Tissue
Total homogenate	12.5	100	8.0	100	17.9	1300
Supernatant after 700 x g	9.3	95.0	7.5	123	---	---
Supernatant after 12,000 x g	3.7	30.7	12.9	200.7	---	---
Pellet of 12,000 x g (crude mitochondria)	14.7	64.5	8.1	60	4.3	22
Combined washes 1,2,3 of crude mitochondria	1.8	27.6	7.3	57.7	---	---
Suspension of "Osmotic shock mitochondria"	57.0	28.8	6.0	4.7	0.50	3

Enzyme specific activities and units and protein content were determined and defined as described in Materials Methods in Section II. RNA content of the purified RNA extracts was determined from their absorbance ratios at 260 and 280 nm according to Warburg and Christian (1942).

4. Characterization of RNA by polyacrylamide gel electrophoresis.

The ratio of absorbance at 260 to that of 280 nm for the RNA was consistently 1.9 or higher, an indication that the preparations were relatively free of protein. Fig. 10 shows the ultraviolet scans of the gel electropherograms of RNA from total homogenate and the mitochondrial fractions. The total RNA contains the expected 28 S and 18 S rRNA and 4 S tRNA components. Although crude mitochondrial RNA is heavily contaminated by the two cytoplasmic rRNA species, the 28 S peak is greatly reduced in the osmotic shock treated preprartion (Fig. 10). Furthermore, RNA species with molecular weights of 0.45 x 10^6 dalton and 0.72 x 10^6 dalton are significantly enriched in purified mitochondria (Fig. 10 c). The molar ratio of these two RNA species was found to be 1.12±0.02 (mean ± S.D.) by measurement of the peak area by weight of the 0.72 x 10^6 and 0.45 x 10^6 dalton species (the area of a blank gel was subtracted for these measurements).

5. Determination of molecular weight by polyacrylamide gel electrophoresis.

Molecular weightsof mitochondrial RNA components were estimated graphically assuming an inverse linear relation between mobility and the logarithm of molecular weight, as described by Bishop et al. (1967) and Loening

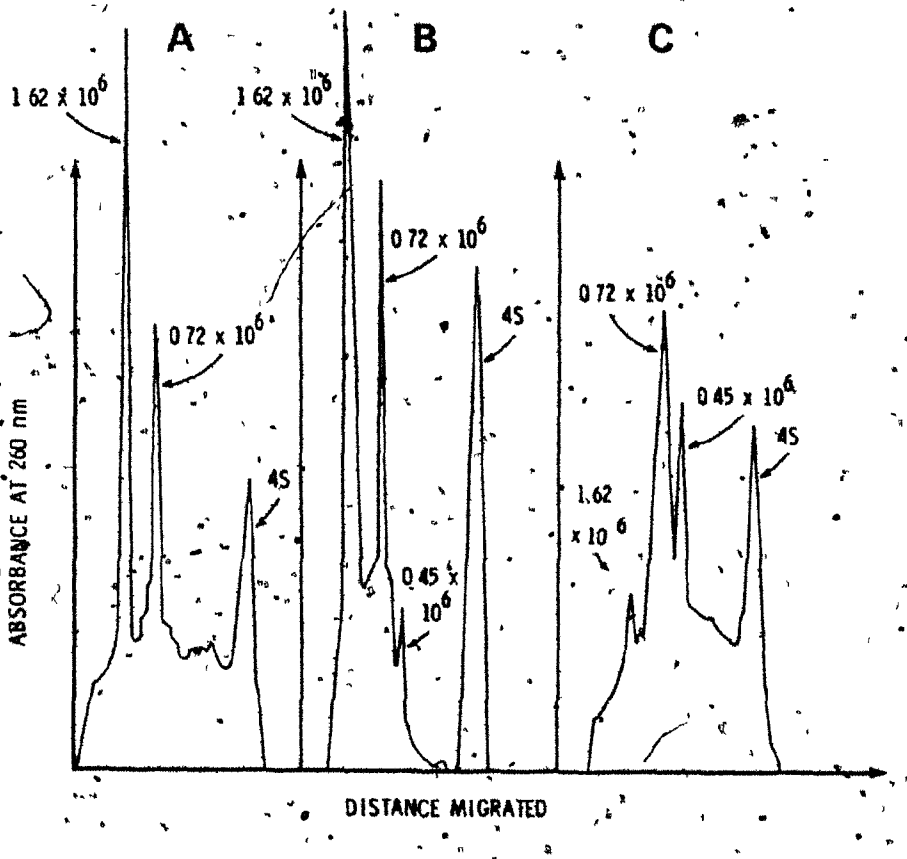


Fig. 10. Spectrophotometric tracings of RNA from mixed nerve and brain tissues of chicks; (A) total homogenate; (B) crude mitochondria; (C) purified mitochondria. Tissues were fractionated, and the RNA was extracted and fractionated an 2.6% (w/v) polyacrylamide gels, as described in Methods.

(1967). RNA from E. coli was purified according to Hayashi and Spiegelman (1961) and was used as an internal standard. On the basis of a consensus in the literature the 23 S and 16 S rRNA components of E. coli were assigned values of 1.10×10^6 and 0.50×10^6 daltons respectively.

Fig. 11 shows that the molecular weights of the major mitochondrial RNA components were 0.72×10^6 and 0.45×10^6 dalton respectively.

6. Conversion of standard nucleosides to nucleoside trialcohols.

In order to explore the quantitative aspects of the Method of Randerath for polynucleotide base composition determination, 30 nmoles of each of the four major ribonucleosides (adenosine, uridine, guanosine and cytidine) were subjected to sodium periodate and tritiated borohydride reactions as stated in Methods. The specific activities of the nucleoside trialcohols recovered from the final dry residues were:

A' = 0.60 µci/n mol.
U' = 0.60 µci/n mol.
C' = 0.58 µci/n mol.
G' = 0.57 µci/n mol.

The results indicated a similar extent of conversion of the four nucleosides to nucleoside trialcohols.

The relative positions of the four nucleoside trialcohols after thin layer chromatography and x-ray film detection are shown in Fig. 12.

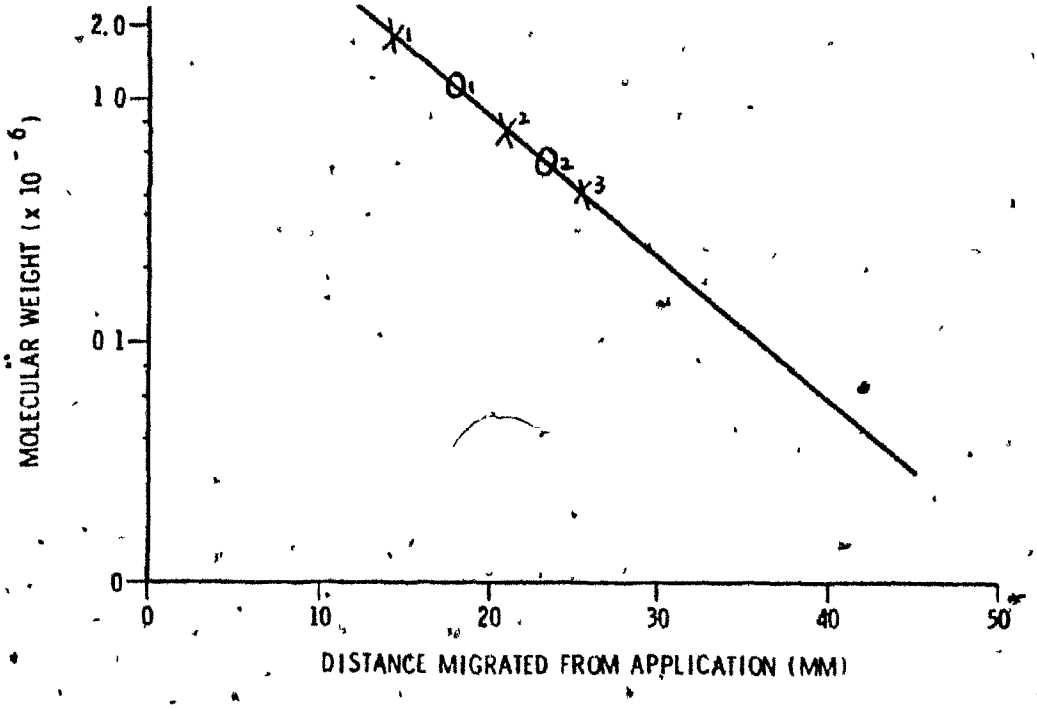


Fig. 11. Molecular weight electrophoretic mobility relation in 2.6% polyacrylamide gel for RNA species from mitochondria. The two points marked "O_1, O_2" are the 23 S and 16 S rRNAs of E. coli. The two points marked "X_1, X_2" are the 28 S and 18 S rRNAs of chicken brain. The two peaks of chicken brain mitochondrial RNA were found to correspond to the points of X_2 and X_3 for 0.72×10^6 and 0.45×10^6 dalton RNA species respectively.

Fig. 12. Two dimensional TLC separation of a mixture of standard nucleoside trialcohols. Details of methodology are described in Materials and Methods of Section II.

7. Base composition of mitochondrial RNA and brain total RNA

The results of tritium exchange and thin layer chromatography of RNA digests are shown in Fig. 13 and Table III. Fig. 13 indicates that the relative positions of nucleoside trialcohols from the mitochondrial RNA digest were comparable to those of the standard nucleoside trialcohols. Table III indicates that the G + C content of mitochondrial RNA was lower than those of total brain RNA. The base composition of total brain RNA was similar to the base composition of chicken fibroblast RNA reported by Sholtissek (1965). The base ratio of wheat germ RNA determined in the present investigation was very close to the base ratio determined by another method (Lane, 1965). These experiments, therefore, indicate that the tritium exchange method of Randerath et al. (1972) yields results comparable to those reported by other methods and is a reproducible and satisfactory procedure for the analysis of very small amounts of nonradioactive RNA specimens. Base compositions of rat liver ribosomal RNAs and rat liver mitochondrial RNA were also included for comparison. The G+C content of rat liver mitochondrial RNA is slightly lower than that of chicken brain mitochondrial RNA reported here.

8. Pattern of labelling of brain mitochondrial RNA. In vivo experiment.

To investigate the incorporation of label into mitochondrial RNA in vivo, [5-^{3}H]uridine (50 µci/embryo)

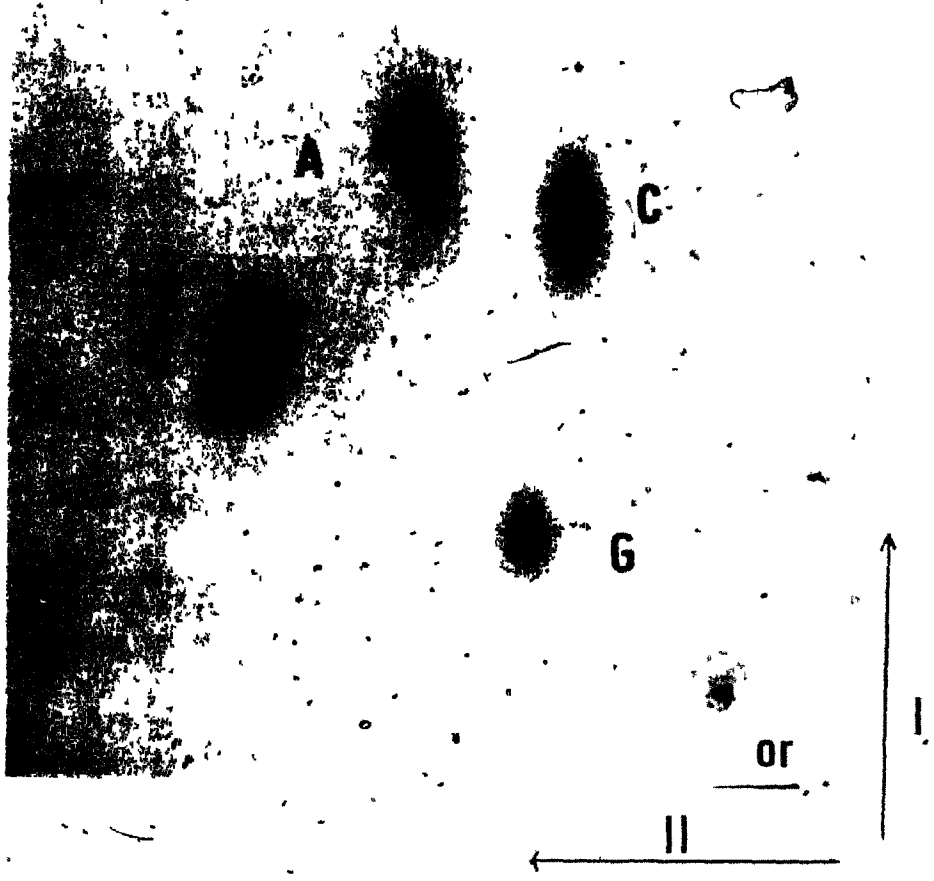


Fig. 13. Two dimensional TLC separation of a mixture of mitochondrial RNA digest. Details of methodology are described in Materials and Methods of Section II.

Table III

*Base Ratios of Mitochondrial RNA and Brain Total RNA

RNA Samples	Bases C	G	A	U	G+C/A+U
*Mito.-rRNA	30.2±0.8	20.1 ± 1.1	28.0 ± 0.5	21.6±0.7	1.01 ± 0.01
total brain RNA	31.1	32.9	17.7	18.2	1.78
wheat germ rRNA	25.6	31.0	22.7	20.7	1.30
Reported					
wheat germ rRNA (Lane, 1965)	25.8	31.3	24.1	18.8	1.33
chicken fibroblast RNA (Sholtissek, 1965)	31.6	33.3	18.2	16.9	1.85
Rat Liver 18 S RNA	29.8	32.4	19.8	18.0	1.64
28 S RNA	32.2	33.0	17.8	17.0	1.87
Mito. rRNA (Bartoov, 1970)	24.9	21.6	30.5	22.9	0.87
BHK 18 S RNA	25.8	31.1	21.2	21.9	1.32
28 S RNA	28.9	35.5	17.8	18.2	1.39
Mito. rRNA (Dubin & Montenecourt, 1970)	23.5	27.4	24.5	24.3	1.04

Mitochondrial RNA and brain RNA were extracted and treated with enzymes, sodium periodate and tritiated potassium borohydride as described in Methods of section II. The base composition of mitochondrial RNA represents the mean ±S.D. of three separate expriments. The data of brain RNA represent the mean of two determinations. Only one analysis was carried out for wheat germ RNA.

*Mitochondrial rRNA was precipitated (as verified by gel analysis) from total mitochondrial RNA in the presence of 1 M NaCl. RNA precipitated by this treatment was assumed to be mitochondrial rRNA.
Wheat germ rRNA was a gift from Dr. M. Gray.

was injected into the air space of 15-day chick embryos. After 48-hr. incubation in ovo, RNA was extracted from total brain tissues and from the purified mitochondrial fraction. The electropherogram of Fig. 14 A shows that the radioactivity profile coincides with the u.v. absorbance pattern (obtained in a different experiment) as shown in Fig. 10 A. Three major radioactive peaks, corresponding to the 28 S, 18 S and 4 S components of the bulk RNA can be observed. In contrast, in the purified mitochondrial preparation, the relative amount of radioactivity in the 28 S rRNA component is reduced, in comparison to the significantly labelled 0.72×10^6 and 0.45×10^6 dalton RNA (Fig. 14 B). In this experiment, a very sharp peak was found around fraction number 52. The nature of this species is not known at the present.

9. Pattern of labelling of nerve mitochondrial RNA. Organ culture experiments.

Since brain mitochondrial RNA was shown to be significantly labelled in ovo, the capacity of nerve tissue to synthesize these species was investigated in organ culture. Fig. 15 A to F shows the pattern of radioactivity on gel-electropherograms of RNA from total homogenates, crude and purified mitochondria of 17-day embryonic and 3-day chick sciatic nerves. The time of incubation with [5-^{3}H]uridine was 2.0 hr. Fig. 15 A, B, D and E demonstrated components having electrophoretic mobilities of

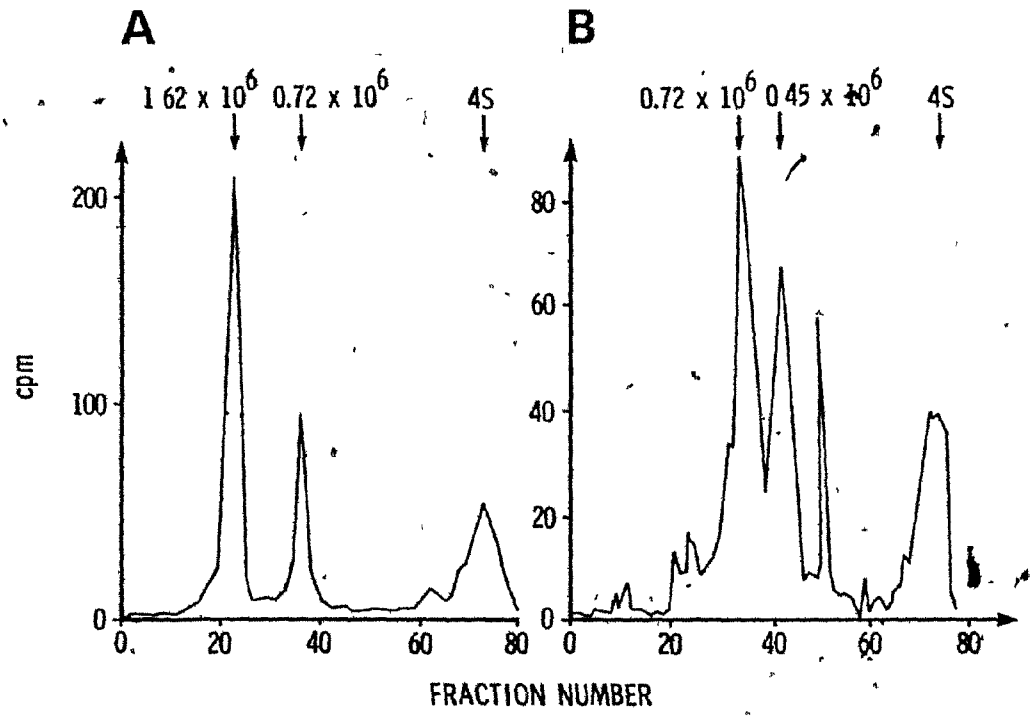


Fig. 14. Electrophoretic patterns of RNA from total brain (A; 72 µg RNA applied) and purified mitochondria of 17-day embryonic brains (B; 50 µg RNA applied). Chick embryos (15-day old) were labelled with 50 µCi [5-^{3}H]-uridine/embryo for 48 h in ovo. Conditions of the experiment, RNA extraction and fractionation are described in Methods and text. Arrows represent the position of 28 S, 18 S and 4 S RNA peaks of the bulk RNA determined by scanning of gels at 260 nm. Samples were counted for 10 min.

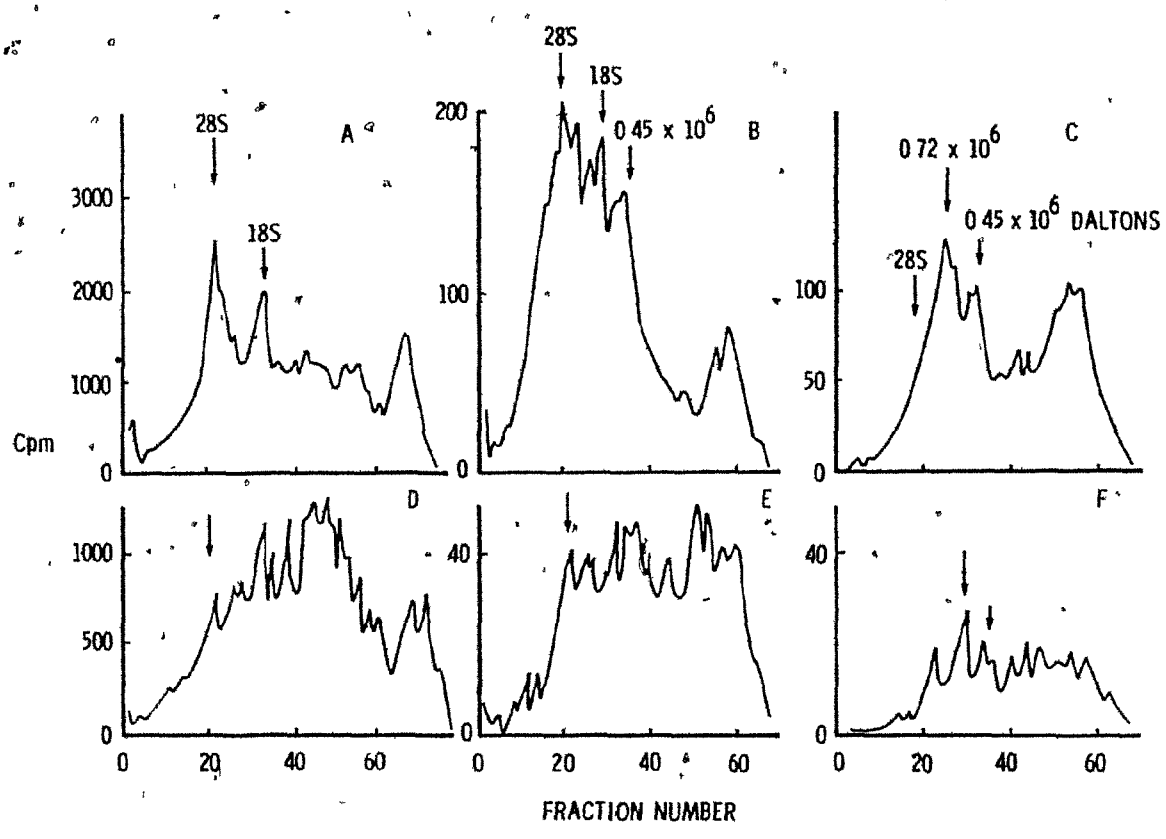


Fig. 15. Electrophoretic patterns of nerve RNA from total homogenates of 17-day embryos (A, 22 µg RNA applied) and 3-day chicks (D, 21 µg RNA applied; crude mitochondria of 17-day embryos (B, 127 µg RNA applied) and 3-day chicks (E, 126 µg RNA applied); purified mitochondria of 17-day embryos (C, 64 µg RNA applied) and 3-day chicks (F, 36 µg RNA applied). Sciatic nerves from 90, 17-day embryos and 60, 3-day chicks were incubated with 100 µCi [5-^{3}H]-uridine/ml of incubation medium for 2 h. Conditions of RNA labelling in organ culture, extraction and fractionation are described in Methods. Samples were counted for 10 min.

28 S, 18 S and 4 S RNA become significantly labelled in both total RNA and crude mitochondrial fractions from the 2 developmental stages. The 0.72 and 0.45 x 10^6 dalton species in crude and purified mitochondrial fractions were also labelled. (Figs. 15 B, C, E and F). Furthermore, Figure 15 C and F shows that the proportion of radioactivity in the 28 S region of the electropherograms was reduced in purified mitochondrial RNA, particularly from the 17-day embryonic nerve. The molar ratio of radioactive 0.72 to 0.45 x 10^6 dalton species was 1.07 0.06 by measurement of the radioactive counts in the peaks of 0.72 x 10^6 and 0.45 x 10^6 dalton species, a result comparable to that obtained from the ultraviolet scan of these preparations (compare Fig. 10 C). These results indicate that mitochondrial RNA became labelled in organ culture after a 2.0 hr. incubation period.

10. Effect of ethidium bromide

Since ethidium bromide is believed to selectively inhibit mitochondrial RNA synthesis (Zylber et al., 1969), the effect of different doses of this drug on the labelling of purified mitochondrial preparations was investigated. In some experiments, for comparison, the effect of the drug on RNA synthesis in the "700 x g" pellet fraction (which was greatly enriched in nuclei and whole cells) was also examined.

Preliminary experiments with high doses of ethidium

bromide (5 µg/ml of incubation mixture) indicated an overall inhibition of incorporation of [5-^{3}H]uridine into the crude nuclear and purified mitochondrial fractions. However, when 0.5 µg of ethidium bromide/ml of incubation medium was used, the pattern of radioactivity in RNA from ethidium bromide-treated tissue showed a selective inhibition of labelling of the 0.72 and 0.45 x 10^6 dalton species and the tRNA in the purified mitochondrial fraction (Fig. 16 A and B). In contrast, labelling of RNA species in the "700 x g pellet," and especially of high-molecular-weight material, was not selectively inhibited. Similar results were obtained with 3-day chick sciatic nerve (Fig. 17).

11. Effect of cordycepin.

Cordycepin (5 µg/ml of incubation medium) also inhibited the incorporation of [5-^{3}H]uridine into the 0.72 and 0.45 x 10^6 dalton species of purified mitochondria, although the inhibition was not as definite as that obtained with ethidium bromide (Fig. 18 A). The "700 x g pellet" fraction showed marked inhibition between the region of 28 S and 4 S, whereas the labelling of fractions heavier than 28 S were relatively unaffected.

12. Comparison of specific radioactivities of RNA in subcellular fractions of sciatic nerve.

To estimate the contribution of mitochondrial RNA

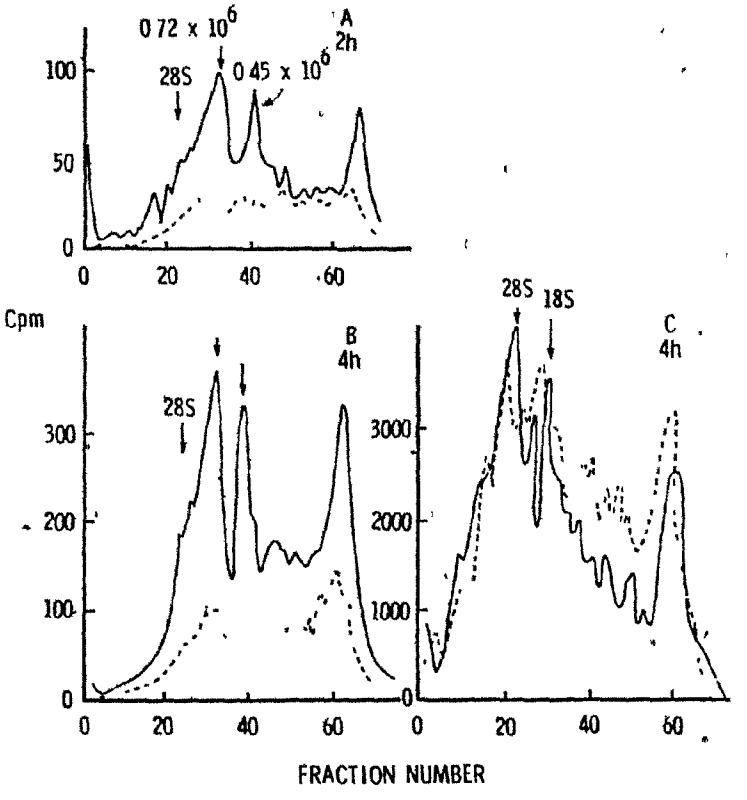


Fig. 16. Effect of ethidium bromide (0.5 µg/ml of incubation medium) on the labelling of RNA from purified mitochondria (A and B) and "700 x g pellet" (C) of 17-day embryonic nerves. The time of incubation was 2 h (A) and 4 h (B and C). Solid line represents radioactivity in control and dotted line represents readioactivity in ethidium bromide treated preparations respectively. Conditions of RNA labelling in organ culture, extraction and fractionation are described in Methods. The amounts of RNA applied on gels for the control samples in A, B and C were 33, 45 and 24 µg, and those of ethidium bromide treated preparations 40, 54 and 24 µg respectively. Samples were counted for 10 min.

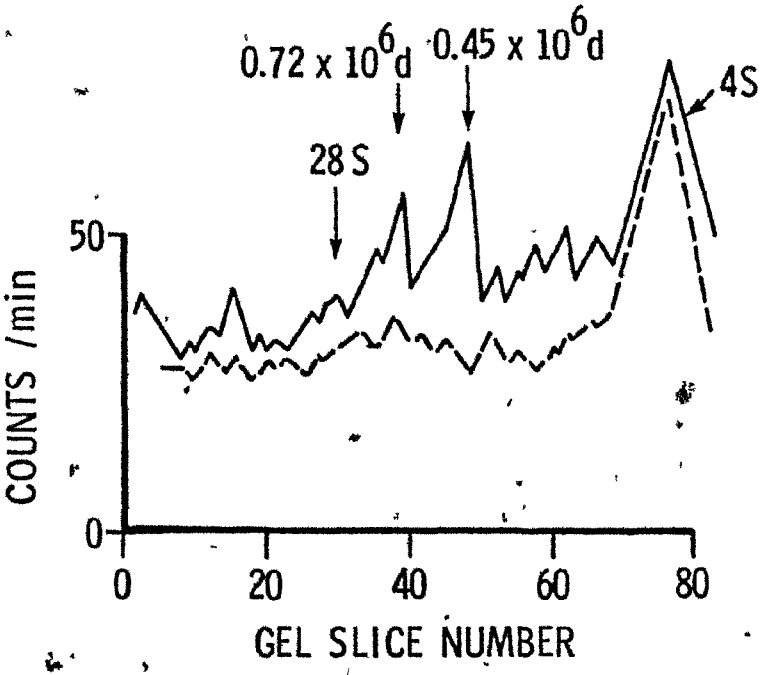


Fig. 17. Effect of ethidium bromide on the labelling of RNA from purified mitochondria of 3-day chick nerve. The time of incubation was 4 hr. For further details see legend of Fig. 16. Samples were counted for 10 min.

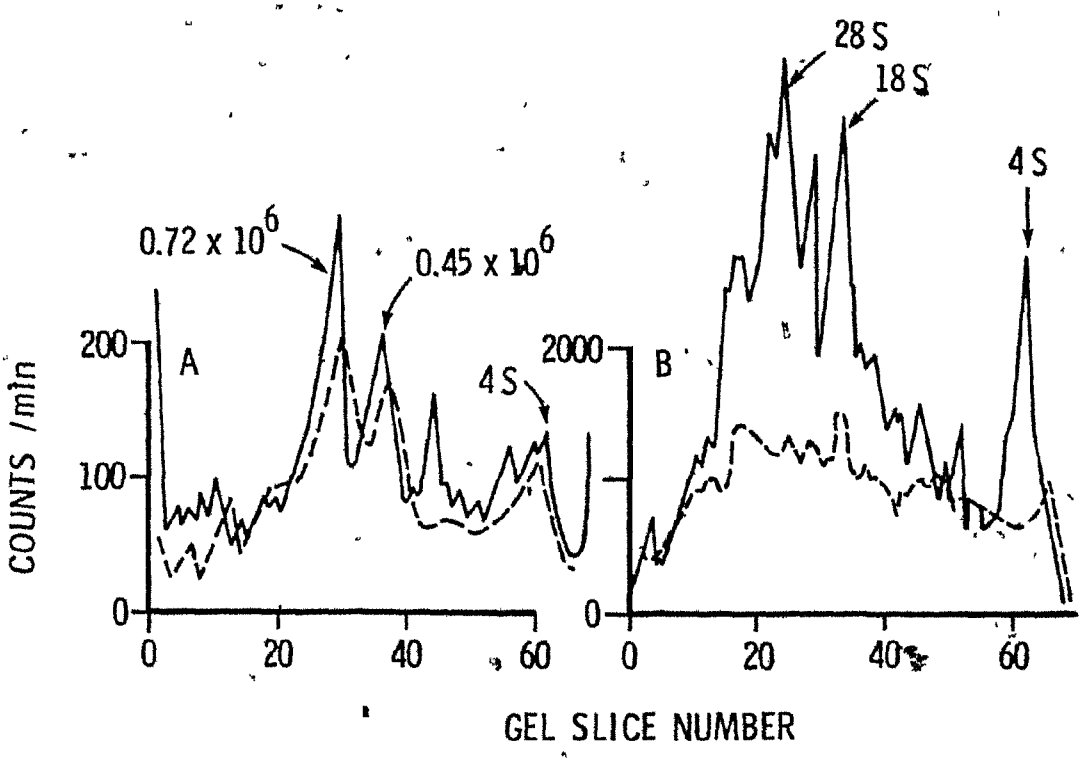


Fig. 18. Effect of cordycepin (5 µg/ml of incubation medium) on the labelling of RNA from purified mitochondria (A) and 700 x g pellet (B) of 17-day embryonic nerve. The time of incubation was 4 hr. The protocol for RNA labelling in organ culture, extraction and fractionation are described in Methods. The amount of RNA applied on gels for the control samples in A and B were 40 and 20 µg and those of cordycepin treated preparations 49 and 25 µg respectively.

Table IV

Specific Radioactivities of RNA and % Recoveries of Radioactivity and Cytochrome c Oxidase Activity from Total Homogenate Crude Mitochondria and Purified Mitochondria of Sciatic Nerve at two Developmental Stages

Fractions	Specific Radioactivity: Counts/min:/g. tissue		% Recovery of Radio-activity in fractions		% Recovery of Cyto. c Oxidase Activity in fractions (from Table II)	% Rec. of Rad., % Rec. of Cyto. c Oxidase Act.
	17-day em.	3-day chick	17-day em.	3-day chick	17-day Embryo	17-day Embryo
total homogenate	3.1×10^{7}	1.5×10^{7}	100	100	100	1
Crude mitochondria	1.9×10^{4}	0.66×10^{4}	6×10^{-4}	4×10^{-4}	64.5	0.92×10^{-5}
Purified mitochondria	0.8×10^{4}	0.33×10^{4}	3×10^{-4}	2×10^{-4}	28.8	1.0×10^{-5}

RNA was extracted and purified from the subcellular fractions as described in Materials and Methods. Specific radioactivities were expressed as counts/min/g wet weight of nerve tissue, since in each case cold brain RNA was added to the preparations at the beginning of the fractionation procedure to facilitate precipitation of nerve mitochondrial RNA. Data for % recovery of radioactivity and % recovery of cyto. c oxidase activity were obtained from different experiments.

labelling to that of total, rapidly-labelled RNA of the sciatic nerve, a comparison was made of the specific radioactivities of total nerve RNA and crude and purified mitochondrial RNA from 17-day embryos and 3-day chicks after a 2 h incubation of the tissues with [5-^{3}H]uridine in organ culture. Table IV shows that the specific activities of the crude and purified mitochondrial RNA preparations were much lower than that of the total nerve RNA. Less than one per cent of the total radioactivity in RNA was incorporated into the purified mitochondrial RNA fractions from both developmental stages. The recovery of cytochrome c oxidase activity in the mitochondrial fractions was much higher than that of the radioactivity in mitochondrial RNA (Table IV) indicating that the low incorporation of radioactivity into mitochondrial RNA was not due to preferential loss of mitochondria during the isolation and purification procedure.

D. Discussion.

Purification of mitochondria from brain homogenates is complicated because a variety of subcellular structures in this tissue band in the same density range during isopycnic centrifugation in sucrose gradients (Whittaker, 1968; Cotman et al., 1971). Therefore, specific procedures are needed to isolate these subcellular organelles from nervous tissue. In the present investigation the procedure of Hernandez et al. (1971) was adopted. This

osmotic shock treatment of crude mitochondrial fractions from sciatic nerve and brain tissue of chicks was successful in removing a considerable amount of membranous contaminants (Fig. 9 & Table II).

There are further problems which must be overcome in the isolation of RNA from mitochondria, particularly from the peripheral nerve. These include: (1) the low content of RNA in mitochondria; and (2) the presence of ribonuclease in this organelle (Rahman, 1966); (3)contamination by cytoplasmic ribosomes; and (4) the relatively small amount of peripheral nerve tissue available for these studies. The first and fourth problems can be solved by labelling the tissues with radioactive RNA precursors, such as [5-^{3}H]uridine, and isolating the labelled nerve RNA in the presence of a carrier tissue such as brain. However, it is difficult to label nerve and brain RNA *in vivo* due to the low permeability and incorporation of radioactive precursors in these tissues, particularly *in ovo* (Judes *et al.*, 1973). This difficulty can be partially overcome by incubating isolated nervous tissue in organ culture for relatively short periods of time (Hu & Mezei, 1971, Judes *et al.*, 1973). However, using these *in vitro* techniques one must consider the consequences of the use of artificial conditions and the great sensitivity of nerve cells to tissue damage, such as Wallerian degeneration, activation of degradative enzymes, etc. (Wolman,

1968; Porcellati, 1972). Both the in vivo and in vitro appraoches have been used in the present investigation, with partial success.

In order to solve the second problem, the RNase inhibitors bentonite and SDS were included in the extraction medium. The third problem was partially overcome by osmotic shock treatment and several washings of the mitochondrial fraction with sucrose-EDTA solutions.

Several lines of evidence presented here enable one to conclude that the purity of osmotic shock-treated mitochondria of chick nervous tissue is comparable to that of highly-purified mitochondrial preparations from other tissues. First, the morphology of these preparations indicated that most of the particles had the appearance of typical mitochondria (Fig. 9), and being similar to those obtained by Hernandez et al. (1971) for osmotically-shocked mitochondria of rat brain. Second, the removal of a considerable amount of cytoplasmic contaminants was indicated by the decreased specific activity of lactate dehydrogenase and the increased specific acitivity of cytochrome c oxidase in the osmotic shock-treated preparations (Table II). The amount of protein and RNA was also reduced by this treatment, indicating removal of impurities. The pattern of distribution of the marker enzyme activities in the subcellular fractions of chick nervous tissue was very similar to that obtained by

Waksman (1968) for mouse brain. Third, the RNA content of purified mitochondria from chick brains was about .6 µg/mg protein; a yield which is within the range reported by others for highly-purified mitochondria from other organisms (O'Brien & Kalf, 1967; Bartoov et al., 1970; Kroon, 1971). However, the yield of mitochondrial protein per gram of wet weight of brain tissue was only about one third of that reported by Hernandez et al. (1971) for rat cortex (which contains mainly cell bodies). This discrepancy may be due to the fact that whole brain tissue, including white matter containing mainly nerve fibers, was used as the starting material in the present investigation.

The following properties of RNA extracted from the osmotic shock-treated subcellular fractions also provide evidence that these species are indeed mitochondrial components. First, ultraviolet electrophoretic patterns of purified mitochondrial RNA indicated that the 28 S cytoplasmic component was greatly reduced and that two RNA species with apparent molecular weights of 0.72×10^6 and 0.45×10^6 dalton, together with a component the size of tRNA, constituted the major species in purified mitochondria (Fig. 10). The 0.72 and 0.45×10^6 dalton species were extracted in an equimolar ratio, as expected if these molecules arise from a mitochondrial ribosome containing one heavy and one light rRNA species. These

data are comparable to published values for the molecular weights of mitochondrial rRNA of BHK cells and mouse cells (Dubin & Montenecourt, 1970). Second, the G+C content of the mitochondrial RNA species was significantly lower than that of total chick brain RNA. Third, the synthesis of these RNA species was sensitive to low doses of specific inhibitors of RNA synthesis, such as ethidium bromide and cordycepin.

Working with chicken liver mitochondria, Rabbitts and Work (1971) recently described the presence of 55 S ribosomes, which contained two rRNA species of lower molecular weights than those obtained in the present investigation. Gel electrophoresis of chick liver mitochondrial RNA was carried out at 4°C, whereas the experiments reported in the present investigation were performed at room temperature. Although the effects of temperature and ionic strength on the electrophoretic mobility of brain mitochondrial RNA were not explored in the present investigation, the differences in the experimental conditions might account for the differences bewteen the molecular weights reported here and those obtained by Rabbitts and Work (1971). It is unlikely that there are variations in the sizes of mitochondrial RNA species in different tissues from the same animal. Experiments in which mitochondrial RNA is extracted from the liver and brain of the same animal and compared under identical conditions

might provide an answer to this problem.

Reports on the size of mitochondrial RNA from nervous tissue are relatively scarce. Recently, Cupello and Hyden (1975) reported the presence of two RNA species in rat brain mitochondria, with electrophoretic characteristics similar to those reported for liver mitochondrial RNA by Aaji and Borst (1970) and Groot et al. (1970). However, their mitochondrial preparations were heavily contaminated with 28 S and 18 S rRNA.

One must also consider the possiblity that the 0.72 and 0.45 x 10^6 dalton mitochondrial RNA species observed in the present investigation are degradation products of either a larger mitochondrial RNA molecule or of the 28 S and 18 S cytoplasmic rRNAs. However, this seems unlikely in view of the presence of the RNase inhibitors bentonite and SDS in the RNA extraction medium.

The G+C content of a variety of mitochondrial rRNAs is relatively low (for review see Grivell & Borst, 1971; Borst, 1972). Although Borkowski (1971) reported that mitochondrial RNAs from calf brain contained high concentrations of guanylic and cytidylic nucleotides, the relatively low G+C content of chicken brain mitochondrial RNA was similar to (though still somewhat higher than) that reported for other organisms (Sholtissek, 1965; Bartoov et al., 1970; Dubin & Montenecourt, 1970; Table III). In the present investigation mitochondrial RNA was

precipitated from 1 M NaCl and, presumably, represented mitochondrial rRNAs. The difference between the G+C content in mitochondrial rRNA and the homologous total RNAs in the present investigation is around 15%, which falls within the range of 15-25% differences reported by others for different organisms. The slightly higher G+C content in the chick brain mitochondrial RNA might be a reflection of true differences in the base compositions of mitochondrial RNA from various organisms. This suggestion is supported by the great variation in base composition of mitochondrial DNA among different species (Borst & Kroon, 1969). For example , the G+C contents of mitochondrial DNA from sea urchin and domestic chicken were found to be 43% and 50% respectively. On the basis of these latter results it seems reasonable to expect that the base composition of mitochondrial RNA may also be different in different species. However, contamination by the homologous cytoplasmic RNA, which might have caused an apparent increase of the G+C content in the mitochondrial RNA preparation, cannot be completely excluded at present.

There are only a limited number of investigations dealing with the in vivo labelling of mitochondrial RNA in animal tissues; most of these studies have employed mammalian cell lines and used relatively low doses of actinomycin-D to suppress selectively cytoplasmic rRNA synthesis (for review see Gravell & Borst, 1971). The

electrophoretic profile of brain mitochondrial RNA labelled *in vivo* in the present investigation was similar to that from the sciatic nerve incubated with the precursor for 4 hr. in organ culture (Fig. 14, 16). This, therefore, supports the hypothesis that the properties of mitochondrial RNA are similar in the PNS and CNS. The pattern of labelling of RNA from purified mitochondria showed a relatively low percentage of total radioactivity in the 28 S cytoplasmic RNA species, indicating that the purification procedure yielded reasonably pure mitochondrial species (Fig. 14 B, 15 C and 16 A,B). Furthermore, the larger mitochondrial RNA component contained about twice the radioactivity of the smaller component. These results which are similar to those reported by Grivell and Borst (1971) are expected if the two mitochondrial RNA species are synthesized at an equal rate and are components of a mitochondrial ribosome. An extra radioactive component with slightly higher electrophoretic mobility than that of the 0.45×10^6 dalton species was also apparent in one of the electropherograms of the mitochondrial RNAs labelled *in vivo*. At the present it is not clear whether this radioactive material represents an artifact or is a true radioactive mitochondrial RNA species, since only one *in vivo* experiment was carried out and the presence of this radioactive component was not as prominent on electropherograms of the *in vitro*

labelled preparations. In a study of RNA synthesis in chicken liver mitochondria *in vivo*, Rabbitts and Work (1971) found that a subcellular particle carrying nascent polypeptide chains sedimented with an S value of 55 on sucrose gradients, suggesting that mitochondrial ribosomes are capable of carrying out protein synthesis. The *in vivo* labelled RNA was extracted from these 55 S mitochondrial ribonucleoprotein particles and characterized by gel electrophoresis. The sizes of the two mitochondrial ribosomal RNAs were similar to those reported in the present investigation. Comparable results have been obtained with BHK cells, in which 55 S mitochondrial ribosomes containing 18 S and 12 S RNA have been identified (Rabbitts & Work, 1971).

It is now generally accepted that ethidium bromide inhibits selectively mitochondrial RNA synthesis (for review see Borst, 1972), although high concentrations of this mutagenic dye inhibit nuclear RNA synthesis as well (Meyer *et al.*, 1972). This latter observation might explain the inhibition of the rapidly-labelled RNA of the sciatic nerve of 17-day embryos and 3-day chicks observed in this laboratory using 5 µg ethidium bromide or acriflavine per ml of incubation medium (Mezei *et al.*, 1971). When a low dose of ethidium bromide was used (0.5 µg/ml of incubation medium) the

drug selectively inhibited the labelling of the 0.72 x 10^6 and 0.45 x 10^6 dalton RNAs, and the tRNA component of the sciatic nerve mitochondrial fraction (Fig. 16 A and B). However, some heterodisperse, ethidium bromide-resistant species were still apparent in the osmotic shock-treated preparations, perhaps indicating cytoplasmic RNA contamination.

According to Zylber et al. (1971), cordycepin is also a selective inhibitor of mitochondrial transcription. The pattern of inhibition obtained in the present investigation with 5 µg/ml cordycepin is quite similar to that reported by Zylber et al. (1971) for HeLa cells. Zylber et al. (1971) also claimed that the half-life of mitochondrial rRNA in cordycepin-treated HeLa cells was approximately 3 hr. It is rather surprising to find such a short half-life for rRNA species, and it is possible that this result was due to the toxic side effects of the rather high dose of the drug, with consequent accelerated degradation of mitochondrial RNA. In the present investigation, the turnover rate of mitochondrial RNA was not investigated because of the low levels of radioactivity incorporated into the mitochondrial RNA species both in vivo and in organ culture.

The conclusions of the present dissertation regarding the identity and purity of the mitochondrial RNA preparations of the chick nervous system could be further

substantiated by the following experiments: (a) hybridization of labelled RNA with homologous DNA from mitochondria and nucleus; (b) extraction of RNA from purified mitochondrial ribosomes and ribosomal subunits; (c) examination of additional marker enzymes known to be specifically associated with cytoplasmic microsomes, such as Glucose-6-phosphatase (Shirley, 1974); (d) mixing experiments with homologous, radioactive ribosomes of the cytoplasm to exclude a specific association of radioactive degradation products with the osmotically-shocked purified mitochondrial preparations.

To examine the quantitative contribution of mitochondrial transcription in the synthesis of rapidly-labelled RNA of the myelinating embryonic and post-hatch nerves, the specific radioactivities of total nerve RNA and purified mitochondrial RNA were compared after a relatively short incubation of these tissues with [5-^{3}H]uridine. Less than 1% of the total radioactivity in RNA was incorporated into purified mitochondrial fractions at both developmental stages. Therefore, the low-molecular-weight, high-specific-activity species previously isolated by Hu and Mezei (1971) from the myelinating nerve presumably contained only a small proportion of mitochondrial RNA. On the basis of the present investigation, (particularly those in section III and IV), it is now postulated that most of the above species repre-

sent mRNA of the peripheral nerve.

Studies by Gurdon and Brown (1966), DeTerra (1967) provided evidence that the cytoplasm exerts a powerful influence over both DNA and RNA synthesis in nuclei. For frog eggs, Weber and Boell (1962) claimed that the region of highest mitochondrial activity becomes the center of differentiation. According to Nass (1969) nuclei and mitochondria interact strongly in early embryonic cells. Since mitochondria are necessary for the large amounts of energy required at the stage of rapid myelination, it is possible that mitochondrial transcription is especially important in the myelinating nerve. It is clear, for example, that although the combined mitochondrial translational products account for only 5-15% of the mitochondrial membrane, they are indispensible for the assembly of a functional mitochondrion (for review see Schatz & Mason, 1974). Furthermore, it has been shown that the concentration of mitochondria in Schwann cell cytoplasm increases during development (Friede & Samorajski, 1968). Although the results of the present investigation indicate that RNA synthesis of nerve mitochondria does not contribute significantly in quantitative terms to the overall labelling of low-molecular-weight total RNA species, it is still possible that mitochondrial translational products could specifically interact with nuclear gene

products to give special signals for starting cytodifferentiation process(es). It is also possible that the onset of myelination might be controlled by an interaction of mitochondrial and nuclear genes in the developing nervous system.

Section III

Isolation and Characterization of Polyadneylate-Containing RNA of Brain and Nerve Tissue of Developing Chicks

A. Introduction

Results of experiments reported from this laboratory (Hu & Mezei, 1971; Mezei & Hu, 1972) have indicated that part of the rapidly-labelled RNA of the sciatic nerve has properties similar to those described for mRNA from a variety of eucaryotic tissues (Hiatt, 1962; Peterson, 1970; Milcarek et al., 1974; Nemer et al., 1975). These properties are especially evident in the heterodisperse RNA of the rapidly-myelinating sciatic nerve of 3-day chicks. This RNA has a relatively short half-life, is poorly methylated and sediments or migrates between the 18 S and 4 S components of bulk RNA.

Two hypotheses have been advanced to explain the origin of these RNA species. The first hypothesis considered that these species originated in the mitochondrial compartment of the peripheral nerve. However, the results described in Section II of this thesis showed that only a very small proportion of radioactive uridine incorporated into RNA was found in mitochondrial RNA after a relatively short (2 hr.) incubation period. The second hypothesis assumed that most of the rapidly-labelled, low-molecular-weight RNA (smaller than 18 S and larger than 4 S)

represented the mRNA fraction of the sciatic nerve. In subsequent experiments, therefore, an attempt was made to further characterize these species of RNA in nerve tissues from different developmental stages.

There is increasing evidence that a number of eucaryotic mRNAs and large heterogeneous nuclear RNAs contained adenylate-rich sequences about 200 nucleotides long at the 3'-OH terminus (Kates, 1970; Lim & Canellakis, 1970; Lee et al., 1970; Edmonds et al., 1971; Darnell et al., 1971 a, b). Moreover, a number of investigations (Delarco & Guroff, 1972; Banks & Johnson, 1975; Hemminki, 1974; Lim et al., 1974; Murthy et al., 1975; Berthold et al., 1975) have recently shown that a high proportion of RNA synthesized in brains of rodents and chicks contains molecules associated with polyadenylate sequences.

The occurrence of a poly(A) segment in most eucaryotic mRNA provides a criterion for its identification, as well as a method for its isolation. The poly(A) segments can be adsorbed on Millipore filters at high ionic strength (Lee et al., 1971) or annealed to poly(U) immobilized on glass fiber filter (Sheldon et al., 1972). It was first reported by Gilham (1964) that the poly(A) content of a polynucleotide can be measured by a technique dependent on the hybridization of poly(A) sequences to oligo (dT)-cellulose. Biologically-active rabbit globin mRNA has been

purified by this procedure and was shown to direct synthesis of globin in a cell-free extract (Aviv & Ledar, 1972).

Oligo(dT)-cellulose is highly effective in selectively binding molecules containing a poly(A) segment, without retaining RNA molecules known to lack them, i.e. tRNA, 5 S RNA and ribosomal RNAs (Nakazato & Edmonds, 1972). In studying the behaviour of ribosomal RNA on oligo(dT)-cellulose columns, Faust et. al. (1973) found that the bulk of the rRNA (98.6%) was not absorbed when RNA was applied in buffers of high ionic strength. This unbound fraction was found to contain no poly(A)+ RNA species. However, poly(A)+RNA in which the poly(A) segment is shorter than 20 nucleotides does not bind to oligo(dT)-cellulose (Hendler et. al., 1975). The binding efficiency of oligo(dT)-cellulose was studied by Milcarek et. al. (1974), who showed that after unlabelled cytoplasmic RNA had been passed twice over oligo(dT)-cellulose less than 4% of the poly(A)+ RNA was present in material which did not bind to oligo(dT)-cellulose. This result indicated that most of the poly(A)+ RNA in bulk cytoplasmic RNA is bound to oligo(dT)-cellulose. DeLarco and Guroff(1973) have shown that brain "messenger RNA" binds to oligo(dT)-cellulose, but not to oligo(dC)-cellulose. Neither of these materials bound brain or E. coli ribosomal RNA. Therefore, it is believed that oligo-(dT)-cellulose column chromatography is a reliable technique for

isolating and characterizing poly(A)+ mRNAs and hnRNAs from myelinating CNS and PNS of chicks.

DeLarco and Guroff (1972) found that a high proportion (up to 40%) of the total radioactivity in RNA newly-synthesized by rat brain either *in vivo* (60 min. labelling) or *in vitro* (20 min. labelling) was bound to oligo(dT)-cellulose. With 10-day-old rats, a higher proportion of total brain RNA was bound to oligo(dT)-cellulose than in the case of the adult animals. Furthermore, the specific activity of the bound RNA was always higher than that of the unbound RNA. On the basis of this experimental evidence, these workers concluded that both *in vivo* and *in vitro* brain produces RNA which binds to oligo(dT)-cellulose. In both whole animals and tissue slices a somewhat greater proportion of the RNA synthesized by young animals appeared to have poly(A) sequences than did RNA synthesized by adults. Thus, not only is the young animal making more RNA but a greater proportion of this RNA appears to be messenger RNA. In a recent report, DeLarco *et al.* (1975) demonstrated more convincingly that the amount of poly(A)+ RNA in these species was greater in the brain tissue of 10-day-old rats than in adult brain. The average poly(A) sequence in the brain RNA of 10-day-old rat was shown to be somewhat longer than in adult brain RNA. The proportion of total rat brain RNA binding to oligo(dT)-cellulose was

5.9 - 7.2% for adult animals but 7.9 - 9.1% in the case of the 10-day-old rat. In these *in vitro* experiments, RNA from the nuclear fraction was bound to oligo-(dT)-cellulose to a relatively greater extent than was total RNA or RNA from the cytoplasmic fraction. The bound RNA fraction was a better template for *in vitro* protein synthesis experiments than the unbound fractions. These experiments are consistent with the hypothesis that the bound RNA fraction contains some of the mRNA species of the rat brain. Lim *et al.* (1974) reported the isolation from rat brain of poly(A)-containing RNA which in *Xenopus* oocytes directed the synthesis of myelin-specific encephalitogenic protein. Murthy *et al.* (1975) also isolated from rat brain, poly (A)+ RNA which directed protein synthesis. Hemminki (1974) showed that 60% of the cytoplasmic RNA from 9 to 13-day-old chick brain was bound to oligo(dT)-cellulose, whereas only 30% of the nuclear RNA fraction was retained by this material. Berthold *et al.* (1975) demonstrated that in rat brain incubated with ^{32}p for 2.5 hr., 30% and 20% of poly(A)+ RNA was present in the nuclear and cytoplasmic RNA fractions respectively. The mean molecular size of nuclear poly(A)+ RNA was always larger than the cytoplasmic poly(A)+ RNA.

DeLarco *et al.* (1975) examined (as a function of time of labelling of the subcellular RNAs) the proportion

of the labelled RNA which was bound to oligo(dT)-cellulose. For the nuclear fraction the amount of bound radioactive RNA was 16.7% at 1 hr., increasing to 18.4% at 8 hr., and decreasing to 12% at 24 hr. and to 4.8% at 144 hr. For the polysomal fraction the amount of bound RNA was 10.0% at 1 hr. and decreased to 5.8% at 24 hr. and to 3.1% at 144 hr. These experiments suggest that this pattern was due to the relatively higher turnover rate of the poly(A)+ RNA compared with stable RNA lacking poly(A), the labelling of the latter increasing steadily during long incubation periods. Therefore, the proportion of radioactive poly(A)+ RNA binding to oligo(dT)-cellulose depends on the proportion of labelled non-poly(A)-RNA present in the RNA preparations. The above investigations then indicate that the nervous systems from different species contain and synthesize poly(A)+ RNA species which represent some of the mRNA of these tissues. However, extensive studies of poly(A)+ RNA in developing chicken brain and sciatic nerve have not been carried out to date.

The experimental approach for this problem was to isolate total "rapidly-labelled" RNA with constant efficiency and high yield from the tissues in question.

It has been known for some time that RNA fractions enriched in components with a DNA-like base composition can be obtained from animal cells by differential phenol

extraction (Georgiev & Mantieva, 1962). Treatment of rat liver with aqueous phenol at low temperature (4° C) preferentially releases rRNA into the aqueous phase, while re-extraction at elevated temperature (60° C) yields RNA components with a nucleotide composition similar to that of DNA. A similar separation has been obtained by sequential phenol extractions with neutral and alkaline Tris-HCl buffer (Brawerman, 1963; Hadjivassilou & Brawerman, 1965). The RNA obtained at alkaline pH is observed to be particularly rich in adenylic acid, with a DNA-like base composition and high template activity (Hadjivassilou & Brawerman, 1967; Brawerman et al., 1972; Lee et al., 1971). This selective extraction of poly(A) rich RNA by high pH buffer takes advantage of the failure of poly(A)-containing RNA molecules to enter the aqueous phase at pH 7.6. The behaviour of these RNA molecules in Tris-buffer is highly dependent on the ionic strength of the water-phenol mixture. Phenol extraction in the presence of sodium acetate (pH 5.2) at 60° C also leads to the appearance of the poly(A)-containing molecules in the aqueous phase (Edmonds & Caramela, 1969; Mendecki et al., 1972). Therefore, the "hot phenol" (60° C) extraction procedure followed by re-extraction of the phenol-water interphase with pH 9 Tris-buffer was adapted in the investigation reported here to obtain total

RNA (poly(A)$^+$ and poly (A)$^-$) from brain and nerve tissues of chicks.

RNA fractions containing poly(A) sequences (poly(A)$^+$ RNA) could then be isolated and identified by their capacity to bind to oligo(dT)-cellulose at high ionic strength. It was expected that such preparations would have base compositions similar to that of homologous DNA, and contain a high proportion of adenylate residues. Furthermore, on the basis of previous investigations on brain tissue of various species, it was expected that nervous tissues from embryos will contain a relatively higher proportion of poly(A)$^+$RNA molecules than nerve tissue from post-hatch animals.

With the methods available for extraction and purification of these poly(A)$^+$ species, it became possible to compare the polyacrylamide gel electrophoretic patterns of this RNA with those obtained previously for total rapidly-labelled RNA of the corresponding developmental stage. If a sizable portion of poly(A)$^+$RNA could be isolated from embryonic and post-hatch tissues and if it was found that the average molecular size of the poly(A)$^+$ RNA decreased during nerve development one could conclude that at least part of the rapidly-labelled RNA observed previously in the peripheral nerve was poly(A)$^+$, probably mRNA or hnRNA.

B. Materials and Methods

Materials

[2-^{3}H]-Adenosine (19.3; 10.8 ci/mmoles) was purchased from New England Nuclear Company (Boston, Mass., U.S.A.); Oligo(dT)-cellulpse was obtained from Collaborative Research Inc. (Waltham, Mass., U.S.A.), other reagents were as described in Section II Materials.

Methods

1. Preparation of tissues

Brain and nerve from various developmental stages was dissected and stored in oxygenated incubation medium at 0-4° C, until enough material was accumulated for incubation. The incubation medium contained 48 ml of an aseptically-prepared solution of 1.2×10^{-4} *M* NaCl, 2.1×10^{-3} *M* $CaCl_2$, 5.6×10^{-3} *M* KCl, 8.1×10^{-6} *M* $MgSO_4$, 1×10^{-3} *M* NaH_2PO_4, 1.2×10^{-2} *M* glucose and 2.4×10^{-2} *M* $NaHCO_3$, (Kreb's-Ringer-bicarbonate-glucose medium pH 7.8), 1 ml of a mixed solution of MEM amino acids (50 x) solution and MEM vitamins (100 x) solution (2 : 1 v/v) (Grand Island Biological Company, Grand Island, N.Y.) and 0.5 ml freshly-prepared glutamine solution (29.2 mg/ml). The brain was then sliced into 0.4 mm slices with McIlwain tissue slicer (Mickle Laboratory Engineering Co., Gomshall Surrey, England). The sliced brain or dissected nerve was pre-incubated for 15 min. at a concentration of 1 g tissue/5 ml of incubation medium, then [2- ^{3}H]-adenosine (100 µci/ml)

was added and incubation was continued for 2 hr. under a constant stream of 95% O_2 : 5% CO_2 mixture.

2. RNA extraction

(a) Total RNA extraction: Total RNA was extracted as described in "Methods", Section II; except that the interphase was re-extracted with 5 volumes of 0.01 *M* Tris-buffer (pH 9).

(b) Differential temperature extraction of nuclear and cytoplasmic RNA: The extraction procedure of Hu and Mezei (1971), was followed, with some modifications. After incubation, the tissues were rinsed three times with unlabelled incubation medium, and homogenized in a Duall tissue grinder (Kontes laboratories) for 1 min. at 2500 rpm at 4° C with 5 volumes of a solution containing 0.01 *M* Tris (pH 7.2), 0.01 *M* $CaCl_2$, 0.01 *M* Sucrose, 0.3% SDS and 0.04% of purified bentonite (Brownhill et al., 1959). After homogenization, 5 volumes of a solution containing 2×10^{-2} *M* sodium acetate (pH 5.0), 2.8×10^{-1} *M* LiCl, 2×10^{-3} *M* $MgCl_2$, 1% (w/v) SDS and 0.01% bentonite was added. The mixture was vigorously shaken with 2 volumes of water-saturated phenol, at 4° C for 5 min. and then centrifuged at 8000 x g for 5 min., after which the aqueous layer was removed. Subsequently the phenol-water interphase was re-extracted at 37° C with a solution containing 0.01M Tris (pH 9.0), 0.01 *M* $CaCl_2$,

0.01M Sucrose, 0.3% (w/v) SDS, and 0 04% (w/v) purified bentonite. After vigorous shaking for 5 min. the mixture was centrifuged at 8000 x g for 5 min. The aqueous layer was removed and combined with the aqueous layer obtained from the first "cold-phenol" extraction. After one more "cold-phenol" extraction of the combined aqueous phase, RNA was precipitated and purified as described previously in "Methods," Section II. The RNA from the above extracts was designated the "cold phenol RNA fraction" and was assumed to represent the cytoplasmic RNAs of the tissues.

To obtain the nuclear fraction of the cells, the phenol-water interphase remaining after the "cold phenol" extractions was re-extracted at 65° C with 5 volumes of a preheated (65° C) 1:1 mixture of the solutions used in the initial "cold phenol" extraction procedure . After shaking for 5 min. at 65° C the mixture was rapidly cooled, centrifuged, and the aqueous layer was re-extracted with an equal volume of cold phenol for 2 min., then RNA was isolated and purified from the aqueous extract as described above. The RNA from this extract was designated "the hot phenol RNA fraction" and was assumed to contain the nuclear RNA of the tissues.

3. Oligo(dT)-cellulose column chromatography

Poly(A)+RNA from total "Hot phenol" and "Cold phenol"

fractions was obtained by fractionating these RNA preparations on oligo(dT)-cellulose columns according to the procedures of Edmonds and Caramela (1969), with some modifications. Oligo(dT)-cellulose (70 mg.) was suspended in binding buffer containing 0.01M Tris (pH 7.6), 0.4 M Nacl, 0.001M EDTA, and loaded in a 1 ml. syringe. The column was washed 5 times with 1 ml. binding buffer and placed at 4° C for 30 min. One ml. of RNA sample dissolved in the binding buffer and containing 40-400 µg RNA was applied on the column. The fraction which passed through the column was collected and re-applied on the column. This fraction was then collected together with 3 x 1 ml. of binding buffer used to further wash the column. The collected fractions obtained with binding buffer at 4° C were designated the poly(A)$^-$ RNA samples. The column was then washed 4 more times with 1 ml. binding buffer and afterwards brought to room temperature for 30 min. After this step, the column was placed into a 37° C room for 15 min. and the poly(A)$^+$ RNA fraction was recovered with 4 x 1 ml. eluting buffer containing 0.01 M Tris (pH 7.6), 0.001M EDTA. The column was then washed with a further 4 x 1 ml. of eluting buffer. Aliquots (0.2 ml.) of the unbound (poly(A)$^-$) and bound (poly(A)$^+$) RNA fractions and of the final column washings were counted in 6.0 ml. Aquasol (New England

Nuclear Corp., Boston, U.S.A.) in a liquid scintillation counter. It was found that about 90 ±10% of the total RNA counts applied on the column were recovered in the unbound and bound RNA fractions, whereas the column-wash fractions contained negligible amounts of radioactivity.

RNA in the bound and unbound fractions was precipitated with 2 volumes of absolute ethanol as described previously, after addition to the 4 ml fraction of 0.5 ml bentonite-treated 20% sodium acetate solution and 150 µg of unlabelled brain RNA as carrier. The re-precipitated RNA samples were characterized by 2.6% polyacrylamide gel electrophoresis, as described previously in "Methods," Section II.

4. Base analysis

RNA from the bound and unbound fractions was reprecipitated as described above, but without carrier RNA. After centrifugation the RNA was dissolved in distilled water and subjected to enzymatic digestion and base analysis as described in "Methods," Section II.

C. Results

1. RNA extractions

RNA was extracted from brain and nerve tissue by several different methods including: (1) the "hot-phenol" method used previously (Hu & Mezei, 1971); (2) the thermal fractionation method combined with pH 9 buffer

(0.01 *M* Tris) extraction described in "Methods" of this section; and (3) the chloroform-phenol extraction procedure reported by Perry et al.(1972) (Table VI).

Table V indicates that similar and reasonably-reproducible yields were obtained using the first two extraction procedures for brain and embryonic nerve tissue. However, methods which utilized an extra pH 9 buffer extraction step generally increased yields. Therefore, the "hot-phenol" procedure combined with an extra pH 9 buffer extraction step was used for the extraction of total RNA in subsequent experiments. Since the chloroform-phenol procedure caused losses of bulk RNA (Table VI) this method was not utilized. Table V also indicates that the yield of RNA decreased in both brain and nerve tissues during development.

2. Calibration of oligo(dT)-cellulose column

To calibrate the oligo(dT)-cellulose columns a [^{3}H]-poly(A) stock solution was prepared in binding buffer (Miles Lab., Inc., Elkhart, Ind.). Increasing amounts of the stock solution containing 6.0×10^4 dpm/ml, 0.42 µg poly(A)/ml, were passed through the columns as described above. Fig. 19 shows a linear relationship between the amount of [^{3}H]-poly(A) applied and [^{3}H]-counts recovered in the bound-fraction. Almost 95% of the applied counts were found to be bound on the oligo(dT)-cellulose and no saturation of the column could be achieved even with 1.0 ml

Table V

Content of RNA in Brain and Nerve Tissues of Different Stages of Development

Tissue	Age of Animal	RNA content µg/g wet weight of tissue	
		Extraction procedure according to (Hu & Mezei, 1971)	Extraction procedure described in "Methods" of this section
Brain	14-day embryo	1150±50	1305±50
	17-day embryo	858±57	1116±123
	3-day chick	532±80	808±150
Nerve	14-day embryo	1049±152	1200±138
	17-day embryo	611±34	1115±170
	3-day chick	520±20	725±68

RNA was extracted from tissues as indicated in the reference and "Methods," Section III. The yield of RNA was determined from measurement of absorbance of the purified RNA preparations at 260 nm and 280 nm according to Warburg and Christian (1942). Results represent the mean ±S.D. of three separate experiments.

Table VI

Yield of Nerve RNA from 3-day Chick Extracted by Different Methods.

Methods	Polysomal fraction		Combined pellets after centrifugation of nerve homogenate at 700xg & 12000xg	
	Sp.Act. cpm/µg RNA	RNAµg/g tissue	Sp.Act. cpm/µg RNA	RNAµg/g tissue
Extraction procedure according to Hu & Mezei (1971)	1251	70	2400	140
pH 9 buffer only (10mM Tris)	998	42	2000	140
Chloroform-phenol (Perry et al., 1972)	1050	38	1675	80

3-day chick nerve was dissected and incubated with [^{3}H]-uridine for 2 hr. The polysomal fraction was obtained as described in "Methods," section IV.

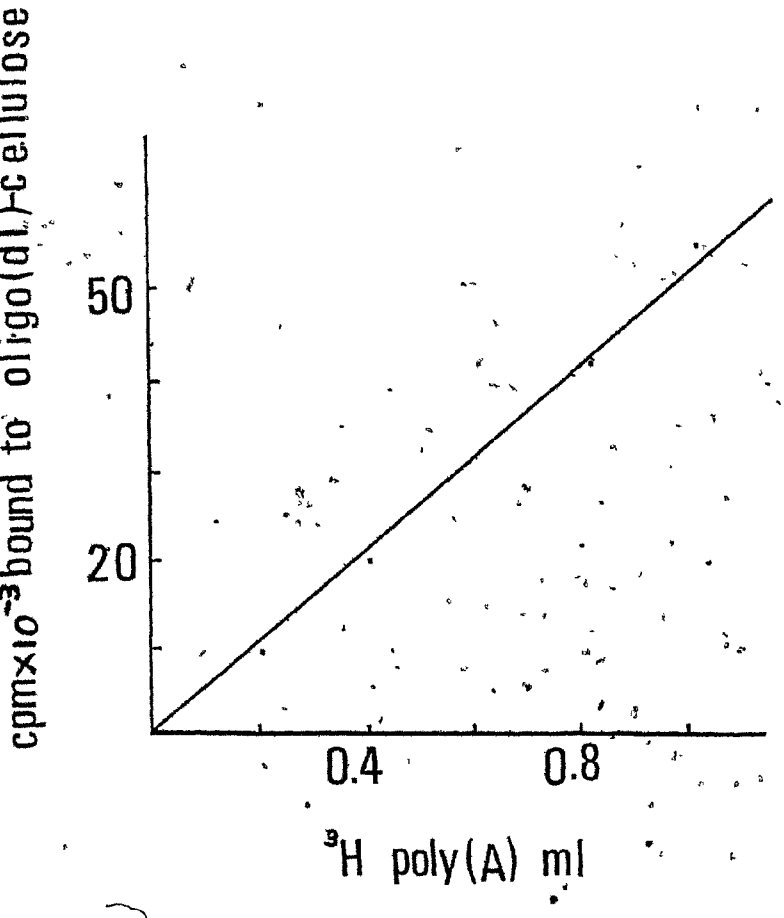


Fig. 19. Relationship between amount of [^{3}H]-poly(A) applied and amount bound on oligo(dT)-cellulose columns. Experimental details are described in "Methods" of Section III.

of [^{3}H]-poly(A), since the amount of [^{3}H]-poly(A) added was still far below the capacity of the column.

To evaluate the reproducibility of the fractionation procedure, different amounts of [^{3}H]-adenosine-labelled brain RNA ("cold phenol" fraction) from 14-day embryos were applied on the oligo(dT)-cellulose column. Fig. 20 shows that the % bound RNA was independent of the amount of RNA applied to the column. The same brain RNA was found not to bind to cellulose alone. This result indicates a high degree of reproducibility for the technique. Furthermore, Fig. 20 demonstrates that 70 mg of oligo(dT)-cellulose was not saturated even with 360 µg of the brain RNA (according to information supplied by manufacturer [Collaborative Res. Inc.] 100 mg of oligo(dT)-cellulose binds approximately 4.7 O.D. units of poly(A)).

3. Behaviour of purified wheat germ ribosomal RNA on oligo(dT)-cellulose column

To test further the specificity of oligo(dT)-cellulose chromatography, purified wheat germ ribosomal RNA was applied on this material. Table VII indicates that all the RNA applied on the column was recovered in the unbound fraction, very small amounts of u.v. absorbing material were found in the bound fraction, and this extra u.v. absorbing material was also found in the unbound fraction. This material probably represents background u.v.-absorbing impurities eluted from the oligo(dT)-cellulose itself.

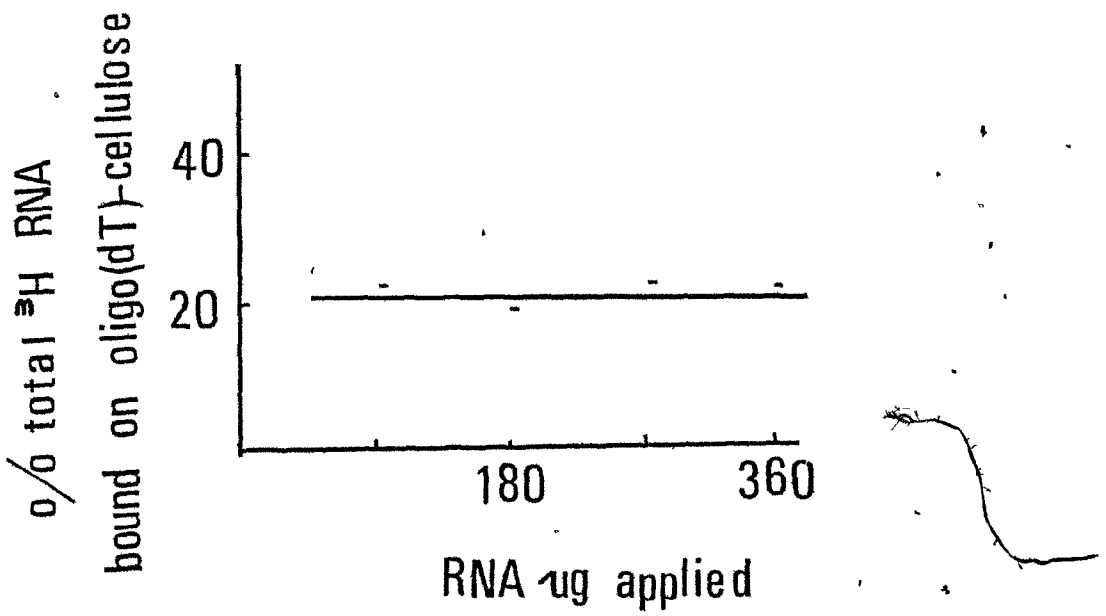


Fig. 20. Relationship between amount of [^{3}H]-RNA applied and % of total RNA bound on oligo(dT)-cellulose columns. 14-day embryonic brains (1 g.) were sliced and incubated in incubation medium (1 g/5 ml) with 100 µci/ml [^{3}H] adenosine (19.3 ci/mmole) for 2 hr. RNA was fractionated into "cold" and "hot" phenol fractions as described in Methods. "Cold" phenol-extracted RNA was applied to the column according to the procedure described in "Methods," Section III.

Table VII

Recovery of Ribosomal RNA after Oligo(dT)-Cellulose Column Chromatography

Fractions	Total absorbance units recovered
Unbound	10.2
Bound	0.1

One ml of wheat germ ribosomal RNA solution (10 absorbance units/ml) in high ionic strength buffer was fractionated on an oligo(dT)-cellulose column as described in "Methods" of Section III. Wheat germ ribosomal RNA was a gift from Dr. M. Gray.

4. Proportion of total and thermally-fractionated RNA retained on oligo(dT)-cellulose columns

Table VIII shows the results of experiments to determine the proportion of poly(A)+RNA in the total rapidly-labelled RNA from brain and nerve tissues of animals of different developmental stages. The results indicate that the proportion of poly(A)$^+$ RNA in brain and nerve decreases as the animals become older.

Table IX shows the results of experiments to determine the proportion of poly(A)+RNA in thermally-fractionated brain and nerve RNA at different developmental stages. The results indicate that in both the "hot" and "cold phenol" fractions the proportion of poly(A)+RNA decreases during development. The hot-phenol fractions always contained a higher proportion of poly(A)+RNA than the cold-phenol fractions.

5. Base analysis

To demonstrate that the RNA fractions retained on oligo(dT)-cellulose columns were indeed enriched in adenylic acid, total RNA from 14-day embryonic brain was fractionated as described in "Methods," and the base compositions of RNAs from the unbound and bound fractions obtained after this procedure were determined according to the procedure described in "Methods," Section II. Table X shows that the RNA fraction retained on the column had a

Table VIII

Percent of Total RNA Bound on Oligo(dT)-Cellulose Columns

Tissue	Age of Animals	% of Binding
Brain	14-day embryo	21.3 - 27.3
	17-day embryo	12.8 - 16.1
	3-day chick	7.1 - 7.8
Nerve	14-day embryo	16.2
	17-day embryo	4.4 - 8.3
	3-day chick	2.2 - 4.6

RNA from brain and nerve tissue was labelled with [2-^{3}H] adenosine (10.8 ci/mmole) for 2 hr., and then was extracted and fractionated on oligo(dT)-cellulose columns as described in the "Methods" of this section. Each value indicates the average of two determinations in one separate experiment.

Table IX

Percent of Cold-Phenol Extracted RNA and Hot-Phenol Extracted RNA Bound on Oligo(dT)-Cellulose Column

Tissue	Age of Animal	% of Binding	
		"Cold-phenol" RNA	"Hot-phenol" RNA
Brain	14-day embryo	10.5-22.6	27 -39
	17-day embryo	5.2- 6.2	24.5-30.5
	3-day chick	4.8- 6.0	7.3-11.8
Nerve	14-day embryo	6.4	---
	17-day embryo	5.1	8.5
	3-day chick	3.8	3.9

RNA from brain and nerve tissue was incubated with [2-^{3}H] adenosine (19.3 ci/mmole, or 10.8 ci/mole) for 2 hr. and then was extracted and fractionated on oligo(dT)-columns as described in the "Methods" of this section. Each value is the average of two determinations in one separate experiment.

Table X

Base Analysis of Oligo(dT)-Cellulose Bound and Unbound RNAs

Base / Fractions	C	G	A	U	G+C/A+U	A/C
Unbound	28.7	33.5	21.8	16.0	1.65	0.76
Bound	20.2	29.7	32.2	17.9	1.00	1.60
Reported ovalalbumin mRNA (Woo et al., 1975)	20.7	21.0	33.3	25.7	0.72	1.56
Poly(A) rich 10-12 S RNA (Hadjivassiliou & Brawerman, 1967)	22.4	23.0	32.2	22.4	0.83	1.44

Total RNA of 14-day embryonic brain was extracted and fractionated by oligo(dT)-cellulose column chromatography as described in "Methods." The unbound and bound fractions were reprecipitated, dissolved in distilled water, and their base composition determined as described in "Methods" of Section II.

relatively high A content compared to that of the unbound fraction. Moreover, the G+C/A+U ratio for the bound fraction was 1.0, similar to the base ratio for DNA (Greenberg & Perry, 1972). The base composition of the unbound fraction was close to that of transfer RNA (Dubin & Montencourt, 1970), as well as to total brain RNA (Table V), probably due to the high proportion of tRNA present in the unbound fractions (see Fig. 21A). The mole % of A in the bound fraction was similar to that reported in other systems (Table X).

6. Gel fractionation of poly(A)+ and poly(A)$^-$ RNA from 14-day embryonic, 17-day embryonic and 3-day chick brain

Fig. 21 shows radioelectropherograms of total brain RNA from three developmental stages. The three major radioactive peaks on the gel patterns of the unbound fractions (A.B.C.) correspond to the 28S, 18S and 4S components of the bulk RNA. The presence of some high-molecular-weight RNA, probably representing rRNA precursors or aggregates is also apparent in these fractions from younger animals (A.B.). However, the resolution of RNA on the 2.6% gels is not sufficient to clearly define the nature of the heavy RNA species. In the bound fractions (Fig. 21 D.E.) of 14-day and 17-day embryonic brain, the RNA is mainly distributed in the high-molecular-region of the gels, whereas in the

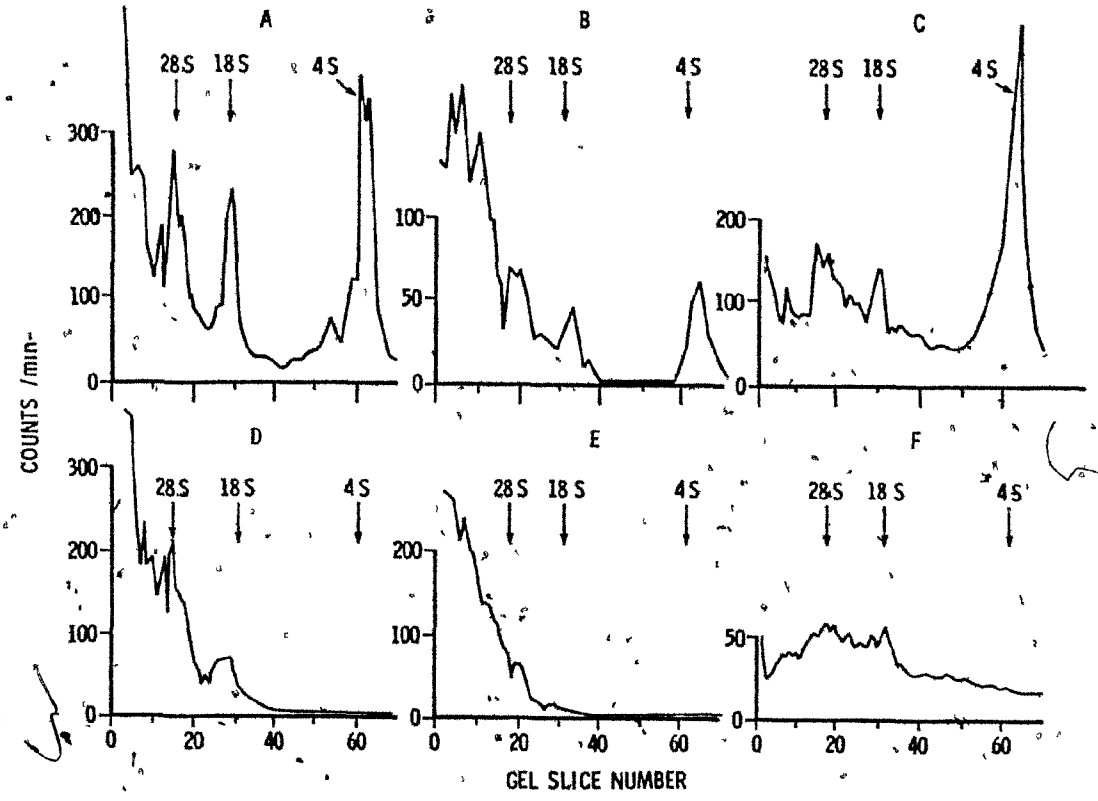


Fig. 21. Electrophoretic patterns of total RNA fractions of 14-day embryonic (A,D), 17-day (B,E) and 3-day chick (C,F) brain, fractionated by oligo(dT)-cellulose chromatography. Brain tissue (1 g) was sliced and incubated in incubation medium (1 g/5 ml) with [^{3}H]adenosine (100 µci/ml) for 2.0 hr. as described in "Methods." Total RNA was extracted as described in "Methods." The RNA was then separated by oligo(dT)-cellulose into poly(A)$^-$ (unbound, A, B,C) and poly(A)$^+$ (bound, D,E,F) RNA fractions and characterized by 2.6% polyacrylamide gel electrophoresis as described in "Methods," Section II. The arrows indicate the position and sedimentation values of the three major peaks of bulk RNA, obtained by scanning the gel at 260 nm.

case of 3-day chick brain (Fig. 21 F.) a large proportion of the total radioactivity is distributed in the region between the 18S and 28S markers. No peak corresponding to the 4S RNA species can be seen in these gel patterns; however, radioactive peaks migrating in the position of the 28S and 18S rRNA species are apparent in some cases. These results indicate that in 14-day and 17-day embryonic brain the poly(A)+ RNA contains a larger proportion of high-molecular-weight RNA species than does that of 3-day chick brain.

For comparison, total RNA from 14-day, 17-day embryonic and 3-day chick brain was further fractionated into "cytoplasmic" and and "nuclear" fractions using the "cold-phenol" and "hot-phenol" extraction procedure described in "Methods" of this section. Fig. 22 and Fig. 23 show the radioelectropherograms of RNAs extracted by the "cold-phenol" (A.B.) and "hot-phenol" (C.D.) procedures from 14-day and 17-day embryonic brain. As expected, the unbound fraction of the "cold-phenol" extracts contains three major cytoplasmic RNAs, the 28S, 18S and 4S components (Fig. 22A, 23A). The bound fraction of the "cold-phenol" extract contains mostly heterodisperse RNA species, peaking around the 18S - 28S region (Fig. 22B, 23B). No definite 4S component is apparent in these preparations, indicating a reasonably good resolution by the

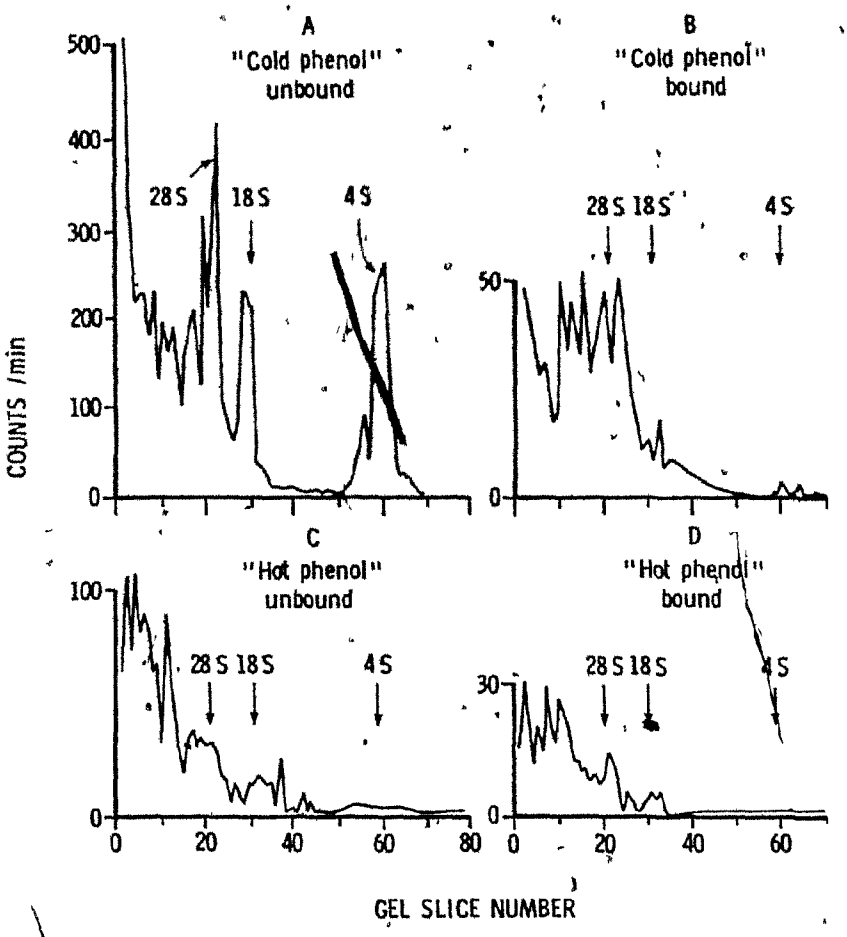


Fig. 22. Electrophoretic patterns of RNA fractions from 14-day embryonic brains, fractionated by oligo(dT)-cellulose chromatography. Brain tissue (1 g) was incubated with [^{3}H] adenosine for 2 hr. as described in the legend of Fig. 21, and fractionated by cold-phenol extraction into "cytoplasmic" (A.B.) and by hot-phenol extraction into "nuclear" (C.D.) RNA, as described in "Methods". The RNA from these extracts was then separated into poly(A)+ and poly(A)$^-$ RNAs, as described in the legend of Fig. 21. The gel patterns of these poly(A)$^-$ (A.C.) and poly(A)+ (B.D.) RNA fractions were obtained as described in the legend of Fig. 21. Samples were counted for 10 min.

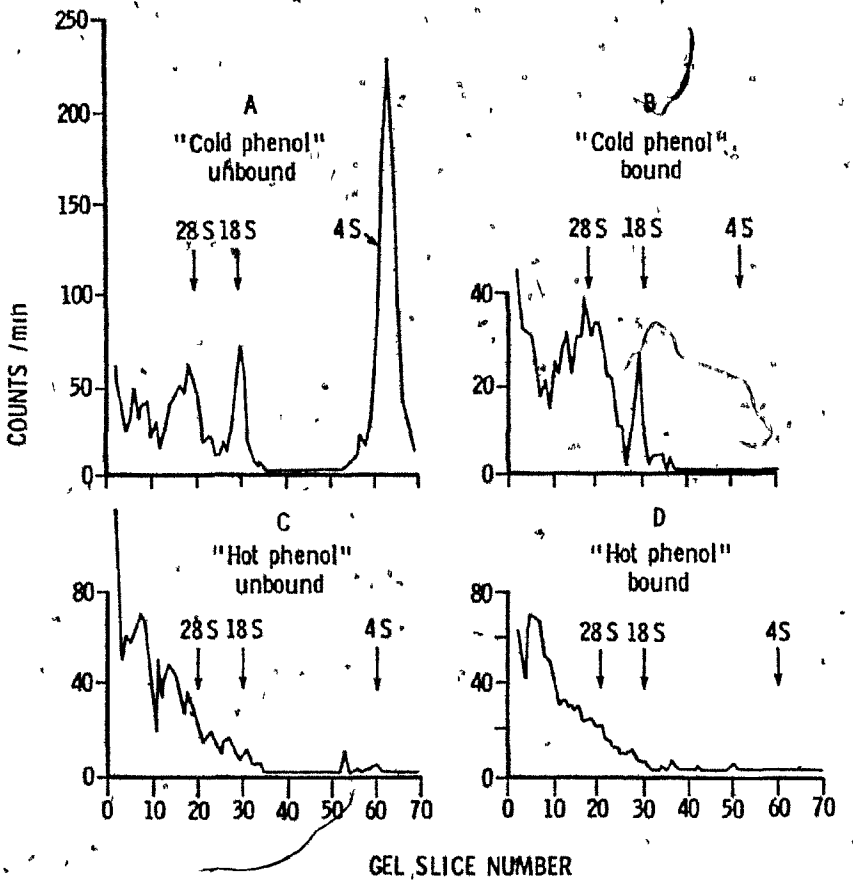


Fig. 23. Electrophoretic patterns of RNA fractions from 17-day embryonic brains. Brain tissue was incubated as described in legend of Fig. 22; "cold" and "hot phenol" RNA was extracted, fractionated by oligo(dT)-cellulose chromatography and characterized by polyacrylamide gel electrophoresis, as described in Fig. 22.

oligo(dT) fractionation procedure. In contrast, the unbound RNA in the "hot-phenol" fraction is mainly distributed in the high molecular weight region >18S (Fig. 22C, 23C). This shift towards the appearance of very high-molecular-weight, heterodisperse species is also evident for the radio-electropherogram of the bound, "hot-phenol" fraction (Fig. 22D, 23D). These patterns are expected, if the "hot-phenol" RNA fractions contain a mixture of rRNA precursors and high-molecular-weight hnRHAs.

Unfortunately, not enough labelled material could be obtained from 3-day chick brain after "hot" and "cold-phenol" extraction and oligo(dT)-cellulose fractionation for meaningful polyacrylamide gel analysis.

7. Gel fractionation of poly(A)+ and poly(A)$^-$ RNA from 14-day, 17-day embryonic and 3-day chick sciatic nerve.

Fig. 24(A.B.C.) indicates that the unbound fractions from total RNA of 14-day, 17-day embryonic nerve and 3-day chick nerve contains three major radioactive RNA species, representing the 28S, 18S and 4S RNA of nerve tissue. These results are similar to those obtained for brain RNA. In contrast, the bound fractions, (Fig. 24 D.E.F.) show the presence of a high proportion of heterodisperse RNA species with a mean molecular weight distribution $\geq$ 28S in younger animals. The bound fraction of 3-day chick nerve RNA is mainly distributed around the 28S and 18S region (Fig. 24F)

with a relatively high percentage of the radioactivity present in molecules ≤18S. No radioactive 4S peak can be detected in the oligo(dT) bound fractions, however, contamination by the 28S and 18S rRNA species cannot be excluded.

To compare the size distribution of the bound nerve RNA from these three developmental stages, the proportion of the total radioactivity in each of the polyacrylamide gel fractions was plotted against fraction number. Fig. 25 indicates that 91% of the total counts in the bound fraction of 14-day embryonic nerve was distributed in the region between fractions 1- 30 (≥18S). For 17-day embryonic nerve 73% of the label was in the same area, whereas for 3-day chick nerve the proportion was 62%. Most of the experiments with post-hatch animals gave a similar result. However, one experiment (results not shown) showed a different pattern indicating an apparent shift in the poly(A)+ RNA distribution to a high molecular weight, peaking around 28S, and with 70% of the total radioactivity in the first 30 fractions. This difference might be due to aggregation of poly(A)+RNA (caused perhaps by the higher RNA concentration used in this particular experiment) or to some other factors, such as the different batch of bentonite (an RNase inhibitor) used in the extraction procedure, biological variations or partial

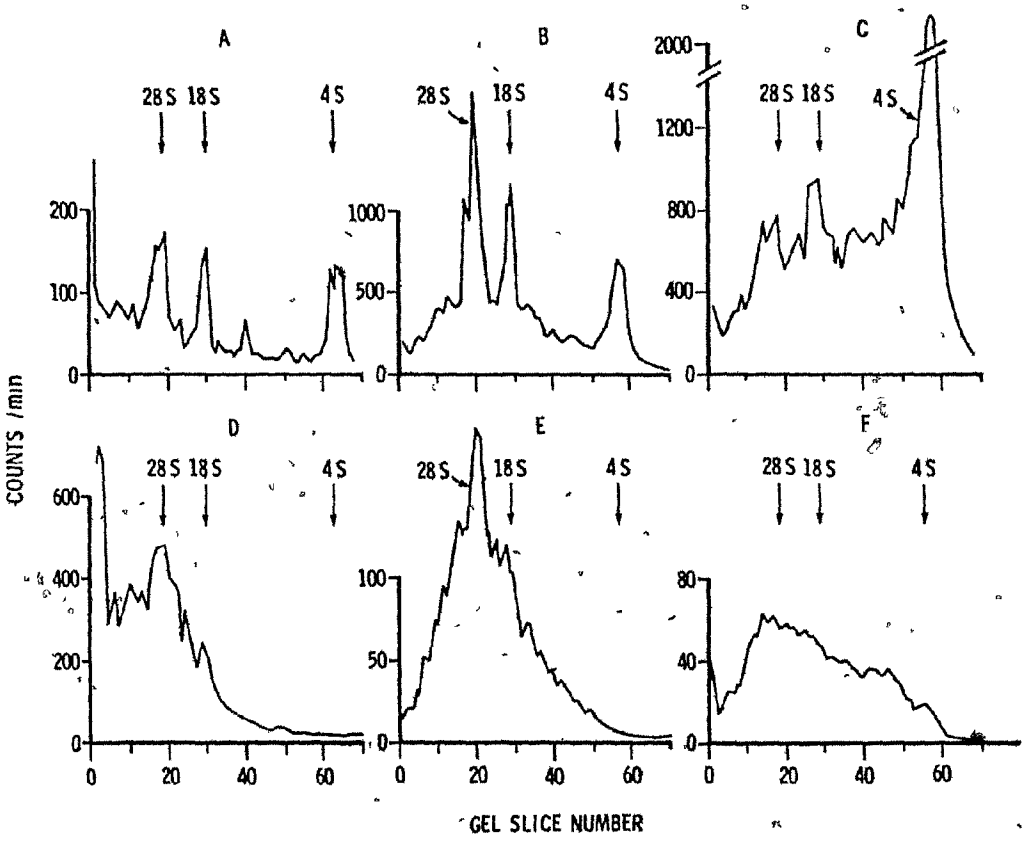


Fig. 24. Electrophoretic patterns of total RNA fractions from 14-day, 17-day embryonic nerve and 3-day chick nerve. Nerve tissue was incubated, total RNA was extracted, fractionated by oligo(dT)-column chromatography and characterized by polyacrylamide gel electrophoresis, as described in the legend of Fig. 21.

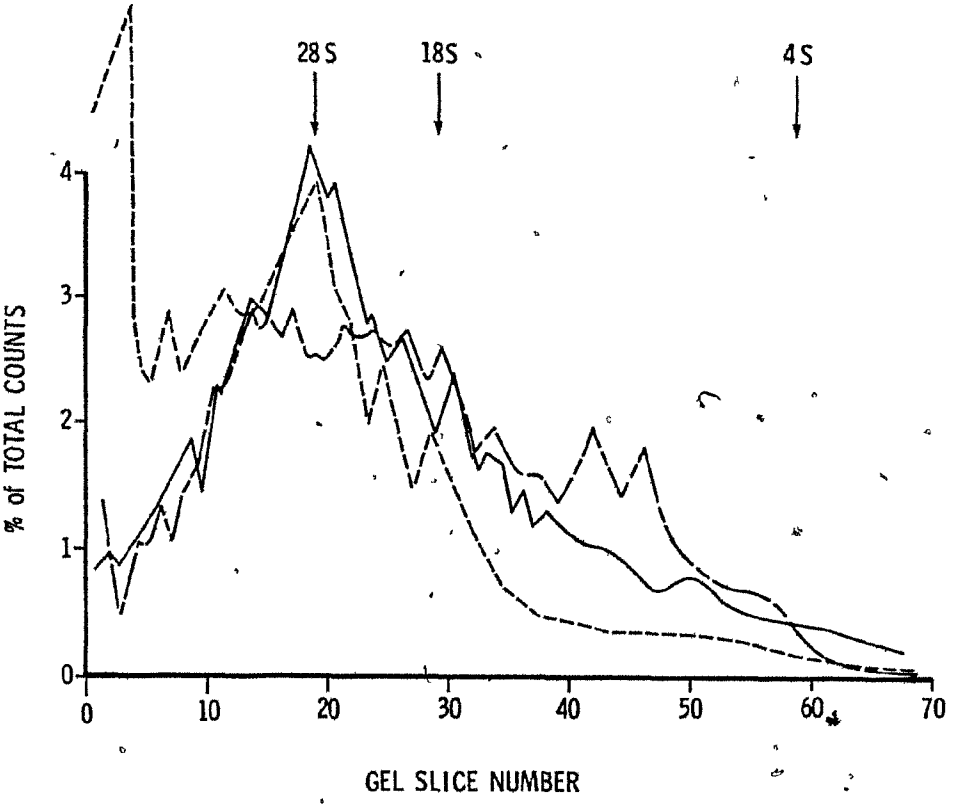


Fig. 25. Percentage distribution of total counts of oligo (dT)-cellulose bound RNA. RNA from 14-day embryonic (----), 17-day embryonic (——) and 3-day chick (- --) nerve were fractionated on polyacrylamide gel. The percentage distribution of total counts were calculated from the gel patterns of the bound fractions of Fig. 24. The arrows indicate the positions of 28S, 18S and 4S RNA markers determined from the U.V. scans of these gels.

degradation of RNA preparations. However, the general trend of these results indicates that the average size distribution of the poly(A)+ RNA diminishes during nerve development.

For comparative purposes, an attempt was made to fractionate total nerve RNA from these three developmental stages into "cytoplasmic" and "nuclear" fractions. However, due to the small amounts of nerve tissue available from 14-day embryos, not enough radioactive RNA could be recovered for polyacrylamide gel fractionation.

Fig. 26 shows the radioelectropherograms of RNA from 17-day embryonic nerve extracted by the "cold-phenol" (A.B.) and the "hot-phenol" (C.D.) procedure. The unbound fraction of the cold-phenol extract contains three major cytoplasmic RNAs, corresponding to the 28S, 18S and 4S RNA components of the bulk RNA (Fig. 26A). The bound fraction of the cold-phenol extract contains mostly heterodisperse RNA species, peaking around the 28S region (Fig. 26B). No definite 18S or 4S components are apparent in this preparation. The unbound material in the hot-phenol fraction is mainly distributed in the region between 18S and 28S, with some heavier material also present (Fig. 26C). The bound fraction in the hot-phenol extract is distributed in the 28S region; no 4S peak can be seen (Fig. 26D).

Total RNA from 3-day chick nerve was also fractionated

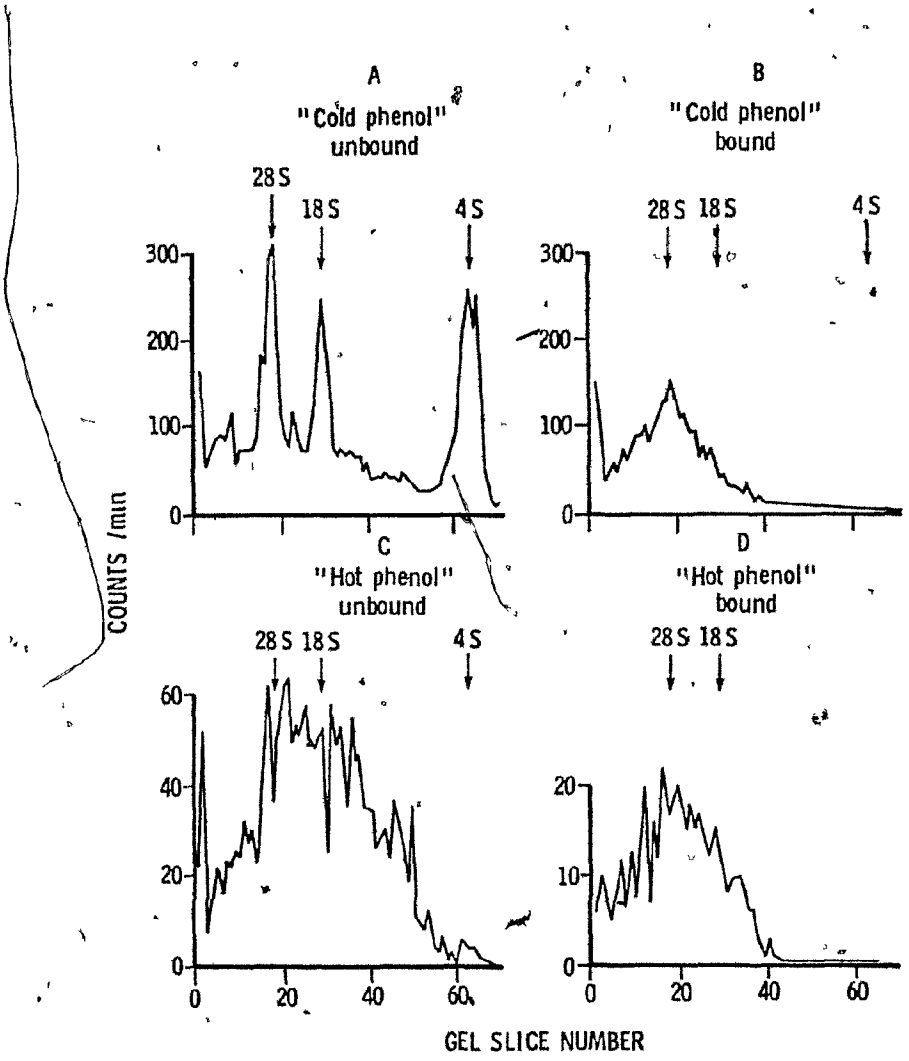


Fig. 26. Electrohporetic patterns of RNA fractions of 17-day embryonic nerve. Nerve tissue was incubated as described in the legend of Fig. 22. RNA was extracted from the cold-phenol and hot-phenol fractions then these materials were subfractionated by oligo(dT)-cellulose column chromatography and characterized by polyacrylamide gel electrophoresis as described in the legend of Fig. 22.

into "nuclear" and "cytoplasmic" fractionsby "hot" and "cold-phenol". extraction procedures. The gel patterns of the unbound and bound fraction of "cold-phenol" extracted RNA were similar to those of the total RNA (results not shown); however, not enough radioactivity was recovered from the bound fraction of the "hot-phenol" extract to obtain meaningful polyacrylamide gel patterns for these preparations.

D. Discussion

There are several variables which must be considered in studies on poly(A)+ RNA. The first is the efficiency of RNA extraction. Preferential isolation or loss of certain RNA species could greatly affect the results, especially if the amount of binding is expressed as a % of the total RNA applied to oligo(dT)-cellulose columns. Therefore, it is important to ascertain that RNA is extracted from tissues of animals of different developmental stages with constant efficiency and without preferential loss of certain species of RNA. In the present investigation different procedures were tested, and it was found that the hot-phenol-SDS procedure combined with an alkaline Tris buffer extraction step gave RNA preparations with a consistent yield (Table V). The amount of RNA obtained from 17-day embryos and 3-day chicks was somewhat higher using this method . When total RNA content

of developing chick brain and sciatic nerve was determined by a TCA precipitation method (Shaikh & Palmer, 1976) the results indicated that the content of RNA at different developmental stages decreased by approximately 50% as the age of the animals increased (going from 13-day embryo to 4-day chicks). In the present study yields of RNA from 14-day embryo to 3-day chick nervous tissues were lower than those reported by Shaikh & Palmer (1976) for comparable stages. However, the relative decrease in the RNA content during development was similar. This indicates that although the extraction and purification of undegraded RNA may result in losses, these losses are similar for each developmental stage. The second variable is the binding specificity and efficiency of the oligo(dT)-cellulose. Although it is generally accepted that this material binds only those RNA molecules possessing poly(A) sequences at least 20 nucleotides long (Hendler et al., 1975), the methodology and results using this technique vary a great deal from one laboratory to another, depending on the procedures of RNA extraction, time of incubation with radioactive precursors or other factors (Hemminki, 1974; DeLarco et al., 1975; Berthold et al., 1975). In the present investigation satisfactory results were obtained by the following criteria: (a) purified wheat germ rRNA was not bound on the oligo(dT)-cellulose column

(Table VII). (b) The percentage binding of total brain RNA was found to be independent of the amount of RNA applied (Fig. 20); whereas the same brain RNA was found not to bind to ordinary cellulose. (c) When radioactive poly(A) was applied to the column, 95% of the radioactive material was recovered in the bound fraction and there was a linear relationship between the radioactivity in the oligo(dT)-cellulose bound fraction and the amount of radioactive poly(A) applied (Fig. 19). (d) Base analysis of the oligo(dT)-cellulose-bound RNA demonstrated that these species had high A and low G+C contents, properties similar to those of poly(A)+ RNA from different tissues or species (Table X). (e) Preliminary experiments from this laboratory indicated that over 80% of the oligo(dT)-cellulose bound fraction, when reapplied on a second oligo(dT)-cellulose column was recovered in the bound fraction (Murphy, C., 1975; personal communication).

The third variable, important in the characterization of poly(A)+ RNA molecules by gel electrophoresis, is the possibility of aggregation of these molecules during the isolation or fractionation procedure. In the present investigation this problem was partially overcome by using hot-phenol extractions followed by rapid cooling. Preliminary experiments using incubation at 37°C with

dimethylsulfoxide (DMSO) or heat treatment for 4 min. at 60°C in the presence of SDS followed by rapid cooling of 14-day embryonic brain or 17-day embryonic nerve RNA preparations before gel-electrophoresis indicated no extensive aggregation of these samples (personal communication from C. Murphy). Therefore the electropherograms of oligo(dT)-cellulose bound and unbound fractions of RNA preparation reported in the present investigation are likely to contain no significant aggregation artifacts, although a developmental stage-dependent aggregation of the poly(A)+ RNA molecules cannot be excluded at present. Future experiments using disaggregating conditions such as DMSO, heat treatment and formamide gels should resolve this problem (Mach et al., 1973; Nemer et al., 1975; Ouellette et al., 1975).

In the present investigation, certain developmental stage-dependent changes were found for the poly(A)+ RNA of the CNS and PNS of chicks. For easy comparison, the results are summarized in Table XI. For example, the percentage of newly-synthesized poly(A)+ RNA from both brain and nerve tissues (either total or thermally-fractionated RNAs) became smaller with increasing age of the animals (Table XI). These results are similar to those obtained for rat brain (DeLarco & Guroff, 1972; DeLarco et al., 1975).

Table XI

Summary of Results of Oligo(dT)-Cellulose Bound RNA Fractions From Different Developmental Stages and Different RNA Fractions

Tissue	Age of Animal	% Binding			% of Oligo(dT)-Cellulose Bound Material Heavier than 18S RNA on Gel Electrophoresis (Total RNA)
		Total RNA	Hot-Phenol RNA	Cold-Phenol RNA	
Brain	14-day emb.	21.3-27.3	27 -39	10.5-22.6	93
	17-day emb.	12.8-16.1	24.5-30.5	5.2- 6.2	98
	3-day ch.	7.1- 7.8	7.3- 7.8	4.8- 6.0	57
Nerve	14-day emb.	16.2	---	6.4	91
	17-day emb.	4.4-8.3	8.5	5.1	73
	3-day ch.	2.2-4.6	3.9	3.8	62

Data are combined from Tables VIII, IX, Fig. 21, and Fig. 25.

The decrease in relative proportion of poly(A)+ RNA in the post-hatch animals might be due to several factors. First, the requirement for mRNA synthesis and translation might be greater in the 14-day and 17-day embryonic nervous system, since at these stages of nerve development myelination has just commenced. In the nervous system of the 3-day old chick myelination is still active; however, enzymes and other constituents necessary for this process are already available, and the synthesis of new mRNA species might not be las extensive as in the younger animals. Second, nucleases cleaving the poly(A) segment from mRNA may be more active in the post-hatch than in the embryonic chick. Cleavage of poly(A) segments from mRNA in the older chick may have been so extensive that its resulting mRNA species no longer bind to oligo(dT)-cellulose, behaving instead like poly(A)$^-$ RNA. With respect to this latter hypothesis, the report of Sheiness and Darnell (1973) is especially noteworthy. These investigators claimed that the poly(A) segment in HeLa cell cytoplasmic RNA becomes shorter with time and that the mRNA may eventually lose most or all of its poly(A) segment. Thus failure to detect poly(A) on mRNA does not constitute proof that it was never there. Sheiness and Darnell speculated that this shortening of poly(A) segments in the cytoplasm might play an important

physiological role in the utilization of mRNA during protein synthesis. However, other experiments indicate that this age dependent shortening of poly(A) segments does not hinder mRNA from actively participating in protein synthesis in vitro (Sheiness & Darnell, 1973).

The third reason for the differential proportions of the poly(A)-containing RNA in the pre- and post-hatch nervous system of chick might be changes during nerve development in the pool sizes of precursors engaged either in mRNA or in bulk RNA synthesis. The experiments in this thesis, and those reported by others for brain tissue cannot distinguish among the above-mentioned possiblities.

Preliminary experiments indicated that the hot-phenol fractions, presumably representing the nuclear component of brain and nerve tissues from 14-day and 17-day old embryos, contained a higher percentage of poly(A)+ RNA than the cold phenol (presumably cytoplasmic) fractions (Table XI). These results are similar to those reported by DeLarco et al. (1975) and Berthold et al. (1975), who demonstrated that in rat brain the percentage of bound nuclear RNA was always higher than in the case of the cytoplasmic fraction.

In contrast, Hemminki (1974) showed that after 10 hr. of labelling with [^{3}H]uridine in vivo 60% of the

total radioactivity in cytoplasmic brain RNA of 13-day old chick embryos was bound on oligo(dT)-cellulose columns, whereas in the nuclear fraction only 30% of the total radioactivity was bound. Mahony and Brown (1975) reported that in rabbit brain there was 7% poly(A)-containing RNA in the nuclear fraction, and 13% in the polysomal RNA fraction, after 1 hr. of in vivo incubation. These results are hard to reconcile with those of other investigators (DeLarco et al., 1975) and with the results of the present investigation. It is unlikely that there is such a high proportion of cytoplasmic poly(A)+RNA in any tissue, especially after the relatively long radioactive labelling period used by Hemminki (1974). It is probable that the chloroform-phenol extraction procedure used by him led to preferential loss of rRNA and isolation of poly(A)+ RNA-rich fractions from embryonic chick brain (see results in Table VI). The greater proportion of poly(A)+RNA in the cytoplasmic fraction than in the nuclear fraction in Hemminki's work might be explained by the relatively long-term in vivo labelling conditions used in his experiment. During the 10 hr. incubation period sufficient time would have elapsed for part of the hnRNA to be processed and transported into the cytoplasm, whereas the remainder of the hnRNA could have entered into the

rapidly-turning-over nuclear pool. The experimental design in the present investigation was different. It is plausible that during the 2 hr. *in vitro* incubation period used here a greater proportion of the radioactivity was incorporated into the nuclear fraction as hnRNA, allowing relatively little time for the processing of the presumed mRNA precursors into the cytoplasmic components of nerve cells. The discrepancy between the results of Hemminki (1974) and those reported by DeLarco *et al.* (1975) and those of the present investigation can be further explained by the age difference in the experimental animals used in the experiments. Hemminki used 9-13 day old chick embryos, whereas the present investigation utilized older animals. Since in the total RNA preparation the labelling of the poly(A)+ RNA decreases with increasing developmental stage (Table XI) possibly the capacity of 13-day old or younger embryos for poly(A)+ RNA synthesis is even greater than that reported in the present investigation for the 14-day animals. Furthermore the processing of hnRNA might be more rapid and efficient in the very young embryonic brain than in that of the adult. Preliminary experiments by Lim (personal communication) indicate that this is true for the developing rat brain.

To investigate the utility and reproducibility of

the thermal-phenol RNA fractionation technique, the electrophoretic properties of "cold" and "hot phenol" fractions from brain and nerve RNA have been investigated. Those fractions presumably representing the cytoplasmic and nuclear RNA species were then further fractionated into poly(A)+ and poly(A)$^-$ molecules by oligo(dT)-cellulose chromatography (Fig. 22, 23, 26). As expected, the poly(A)$^-$, cold-phenol-extracted RNA fraction contained mainly 28S, 18S and 4 S labelled RNA species. The cytoplasmic "cold phenol" extracts also contained some RNA species of size greater than 28S. The nature of these high-molecular-weight molecules in the "cytoplasmic fraction" is undetermined at present. They might represent nuclear RNA contamination, since there may be no clear-cut separation of nuclear from cytoplasmic RNAs by the thermal fractionation method of Georgiev and Mantiva (1962). Or the apparently high-molecular-weight RNA species in the "cold phenol" RNA fraction might be aggregated lower-molecular-weight RNA. Finally, this high-molecular-weight RNA might represent the so-called "giant cytoplasmic RNA" recently found in other developing systems such as the sea urchin embryo Giudice et al., 1974). Nonetheless, except for these high-molecular weight RNA species, the results obtained by the cold phenol fractionation technique in the present

investigation agree with those originally reported by Georgiev and Mantieva (1962) who claimed that cold phenol extracts of eucaryotic cells contain mainly rRNA and tRNA species. The bound fraction of the cold phenol extract contains mainly high-molecular-weight, heterodisperse RNA, peaking in the 28S region of the elctropherogram (Fig. 22B, 23B, 26B). However, contamination by 28S and 18S rRNA species cannot be excluded at the present. Gel electrophoresis did not show the presence of prominently-labelled tRNA species in this fraction, as expected if the bound RNA of cold phenol extracts contains mostly poly(A)+ RNA molecules. The electrophoretic pattern of rat brain bound microsomal RNA described by Lim et al.(1974) is similar to those obtained in the present investigation for the bound RNA of cold phenol extracts of chicken brain and nerve. The majority of the poly(A)+ RNA of rat brain was also centered around the 28S region of the polyacrylamide gels. However, the electropherogram shown by Lim et al. (1974) did not indicate the presence of very high-molecular weight RNA on the top of the gel; probably because they treated their RNA with DMSO before application of these samples on the gels. In contrast, the gel patterns obtained in the present investigation always showed some very high-molecular-weight RNA, of apparent size >28S. Although the RNA species in the first 10 gel-fractions might have

been true high-molecular-weight molecules, the possibility of aggregation of the RNA cannot be excluded at present, the RNA preparation was not treated with DMSO before polyacrylamide gel fractionation, and it is therefore possible that some of the chick brain and nerve RNA fractions contained aggregates. However, the probability of aggregation was reduced in the hot phenol fractions, where RNA was extracted at elevated temperature followed by rapid cooling (see "Methods" of this section). Extraction at 60°C followed by rapid cooling is sufficient to prevent aggregation of most RNAs (Gross & Goldwasser, 1969).

The radioelectropherograms of bound and unbound RNA fractions of the hot phenol extract showed no definite rRNA or tRNA peaks (Fig. 22C, 23C, 26C), as expected if the hot-phenol extracts represent the nuclear fraction of the cells. It is possible that the species which did not bind to oligo(dT)-cellulose contained a mixture of poly(A)$^-$ hnRNA and nuclear rRNA precursors. The large size of these molecules, indicated by their polyacrylamide-gel patterns, is consistent with this hypothesis. A large percentage of the radioactivity of the bound RNA of the hot-phenol extract was distributed in gel fractions heavier than 28S. This pattern is similar to the profile of poly(A)+ RNA from rat brain nuclei reported by Lim et al. (1974) and Berthold et al. (1975), and might represent the poly(A)+ hnRNA of the chick nervous system.

A further developmental stage-dependent change was noticeable in the properties of poly(A)+ RNA fractions from brain and nerve tissues when the eletrophoretic properties of these preparations were investigated. The bound total RNA of 14-day and 17-day embryonic brains was heterodisperse, with an average molecular size larger than 28S (Fig. 21D.E, Table XI). These results are similar to the combined patterns of nuclear and polysomal poly(A)+ RNA of rat brain (Lim et al., 1974) and are consistent with those obtained for the "cold" and "hot phenol" bound (presumably poly(A)+) fractions of 14-day and 17-day embryonic brain RNA (compare Fig. 21 D,E with Fig. 22, 23). In contrast, the total bound RNA from brain tissue of 3-day old chick was distributed on polyacrylamide gels in the regions 18S - 28S and <18S (Table XI). These results indicate that the size of the presumptive poly(A)+ RNA molecules becomes smaller during later development (17-day embryo to 3-day chick).

The above developmental changes were also apparent for the presumptive poly(A)+ nerve RNA (Fig. 24; Table XI). The gel patterns of these fractions from 14-day embryos, 17-day embryos and 3-day chicks indicate that the average size distribution of the bound molecules decreased gradually as the animals became older. In the 14-day embryonic nerve 91% of the total oligo(dT)-cellulose

bound radioactive RNA was distributed in gel fractions 1 to 30 (≥18S), whereas the proportion in the same gel fractions of the RNA from 17-day old embryonic and 3-day chick nerve was 73% and 62% respectively (Fig. 25, Table XI).

The change in size of the poly(A)+ RNA is comparable with those reported previously for the sedimentation and electrophoretic properties of rapidly-labelled total nerve RNA (Hu & Mezei, 1971; Mezei & Hu, 1972). These results then indicate that some of the rapidly-labelled species represents the mRNA fraction of the sciatic nerve. Furthermore, it is interesting to note that the poly(A)+ RNA of nerve tissue from the three developmental stages always contained labelled material peaking in the region slightly greater than 28S during gel electrophoresis (Fig. 25). These results are very similar to those concerning polysomal poly(A)+ RNA reported by Lim et al. (1974) and DeLarco et al. (1975). Based on these results one might speculate that there are also common mRNA species in nervous tissues of chicks or other species regardless of their developmental stages (Zomzey et al., 1971; Davison & Peters, 1970).

However, it should be emphasized that some of the results presented in this section are from one experiment only, and therefore, more extensive investigations are

needed to clearly elucidate the nature and characteristics of oligo(dT)-cellulose bound RNA of the chick nervous system. Such experiments would involve determination of the location and size of poly(A) segment on these molecules (Edmonds et al., 1971; Lee et al., 1971), the study of the template activity of the poly(A)+ RNA species in protein synthesis (Aviv & Leder, 1972), and mixing experiments in which labelled nerve or brain tissue from one developmental stage would be extracted and analysed in the presence of large amounts of unlabelled tissue from another developmental stage. Such additional experiments would provide additional assurance that the relative proportion and the electrophoretic properties of poly(A)+ RNA remain unchanged and characteristic for a particular developmental stage.

Section IV

Effect of 5-Fluoroorotic Acid on the Synthesis of RNA in Chick Peripheral Nerve During Development

A. Introduction

Studies described in Section II and III and previously-published reports from this laboratory demonstrated that the sedimentation and electrophoretic properties of the rapdily-labelled RNA of the chick sciatic nerve changes during development (Hu & Mezei, 1971; Mezei & Hu, 1972). A noticeable change in the physico-chemical properties of this RNA occurred in the 17-day embryonic nerve, during the onset of rapid myelination of peripheral nerve. It was considered possible that myelinogenesis is accompanied by the switching "on or off" of certain groups of genes in neurons and/or satellite cells and that some of the fractions of the rapidly-labelled RNA represent different populations of mRNA of these cells. This hypothesis was supported by the results of experiments described in Section III, which indicated that a significant portion of the rapidly-labelled RNA of the chick CNS and PNS contained poly(A) sequences. Furthermore, poly(A)+ RNA fractions labelled <u>in vitro</u> in the rapidly-myelinating nerve tissues of 17-day embryos and post-hatch chicks contained a relatively larger proportion of molecules smaller than 18S than did the poly(A)+ RNA of the pre-myelinating 14-day embryos.

It has been shown recently that certain mammalian (HeLa) and embryonic sea urchin cells contain mRNAs lacking poly(A) sequences (Milcarek et al., 1974; Nemer et al., 1975; Dubroff & Nemer, 1976). Milcarek et al. (1974) demonstrated that about 30% of HeLa cell mRNA lacks poly(A) when labelled in the presence of different rRNA inhibitors, such as 5-Fluorouridine and actinomycin-D. They found that 5-Fluorouridine had little effect on the amount of [^{3}H]adenosine incorporation into poly(A)+ mRNA and appeared to be less toxic to cells than was actinomycin-D. Nemer and his coworkers (Nemer, 1975; Nemer et al., 1975) demonstrated that in sea urchin embryos the poly(A)+ and poly(A)$^-$ mRNA had similar metabolic properties, and the ratio of poly(A)+ to poly(A)$^-$ mRNA changed during development. On the basis of these results it was considered important to study some of the physicochemical properties (such as the behaviour on polyacrylamide gel electrophoresis) of total mRNA in the developing [illegible]ic nerve.

There are serious technical problems in examining the properties of mRNA, expecially poly(A)$^-$ mRNA. Presumptive mRNA must be studied in the presence of a much larger amount of ribosomal RNA, and steps must be taken to inhibit selectively the labelling of the ribosomal components. Recently a number of investigators reported that 5-Fluoroorotic

acid (5-Foro) inhibited maturation of rRNA precursors in eucaryotes and caused selective incorporation of labelled precursors into cytoplasmic messenger-like RNA (Wilkinson et al., 1971; Wilkinson & Pitot, 1973; Hadjiolova et al., 1973). Since the base analogue seemed to be a useful tool to distinguish between messenger-like RNA and rRNA, the effect of the drug on the labelling of nerve RNA during development was investigated. If 5-Foro is a selective inhibitor of rRNA synthesis in this tissue one would expect the electrophoretic properties of the radioactive 5-Foro resistant RNA species of the 14-day, 17-day embryonic and 3-day chick nerve to be comparable to those observed previously in this laboratory after a relatively short "pulse" (30 to 60 min.) by the radioactive precursor alone (Mezei & Hu, 1972).

B. Materials and Methods

Materials. [5-^{3}H] Uridine (spec. act. 25.6 ci/mM), [2-^{14}C]5-fluoroorotic acid (spec. act. 8.3 mCi/mM), [5-^{3}H] orotic acid (spec. act. 12.2 ci/mM) were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.). Actinomycin D and 5-fluoroorotic acid were generous gifts of Merck, Sharp and Dohme of Canada, Ltd. (Montreal, Canada) and Hoffmann-La Roche Inc. (Nutley, N.J. 07110) respectively.

Methods.

1. Preparation of tissue, isolation of total nerve RNA and gel electrophoresis.

Most of the techniques used here have been described in detail in previous sections and publications. Sciatic nerves from pre- and post-hatch chicks (White Leghorn strain) were dissected and incubated as described in Section II. RNA was extracted from the nerve tissue, purified and analysed by electrophoresis on 2.6% or 2.4% (w/v) polyacrylamide gels as described by Mezei and Hu (1972) and in "Methods", Section II. The absorbance patterns of the gels at 260 nm are not included on the figures of this section; however, they were similar for all of the RNA preparations (for ref. see Mezei and Hu (1972)). Three major absorbance peaks were obtained each time, corresponding to the 28S, 18S rRNA and 4S (tRNA) components of the bulk RNA.

2. Preparation of total sciatic nerve polyribosomes

Total sciatic nerve polyribosomes were prepared according to Means et al. (1971) with some modifications. After incubation, nerve tissue (1g wet weight) was homogenized in 2.5 volumes of a homogenizing medium containing 0.025 M KCl, 0.01 M $MgCl_2$, 0.25 M sucrose and 0.035 M Tris-HCl pH 7.6, for 2 min. at 2500 r.p.m. at 4°C in a Potter Elvehjem teflon tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.). All subsequent steps were carried out at 4°C. The nerve homogenate was then mixed with 6 volumes of fresh chicken brain homogenate of the same

developmental stage as the nerve tissue to serve as a source of carrier polyribosomes. The combined homogenates were centrifuged at 1080 x g for 10 min. The supernatant was further centrifuged at 14,000 x g for 20 min. The resulting post-mitochondrial supernatant was mixed gently with 5% (w/v) deoxycholate solution in homogenizing medium to obtain a final concentration of 1% (w/v) deoxycholate in the solution. After 10 min. standing with occasional shaking, 6 ml of the deoxycholate-treated post-mitochondrial supernatant was layered over 3 ml of 2 M sucrose in the homogenizing medium. The gradients were centrifuged in a SWTi41 rotor at 110,000 x g for 3.5 h in a Beckman L2-65B ultracentrifuge. RNA was extracted from the polyribosome pellet by the hot phenol-sodium dodecyl sulfate extraction as described in Section II, except that the phenol-water interphase was reextracted with a pH 9 Tris buffer (0.01 M).

C. Results

1. Effect of actinomycin D

To compare the effects of a known inhibitor of transcription with those of 5-Foro the pattern of RNA labelling in 3-day chick sciatic nerve was investigated after incubation of the tissues with low dose of actinomycin D (0,1 µg/ml of incubation medium). Fig. 27A and B shows that the drug suppressed somewhat the incorporation

of [5-^{3}H] uridine into the two rRNA peaks after a 3.0 h incubation period, without affecting the pattern of labelling of the heterogeneous species migrating between the 18S and 4S components. The pattern of labelling in the presence of actinomycin D was similar to that obtained previously for "pulse-labelled" (0.5 - 1.0 h) RNA of 3-day chick sciatic nerve (Hu & Mezei, 1971).

2. Effect of increasing doses of 5-Foro

Since most investigators have used 5-Foro *in vivo* a dose-response relationship had to be determined for the sciatic nerve *in vitro*. Fig. 28 shows that when the same amount of nerve tissue (0.6 g wet weight) of 3-day chicks was incubated for 3.0 h with [5-^{3}H] orotic acid in the absence or in the presence of increasing amounts of unlabelled 5-Foro, the base analogue almost completely inhibited the incorporation of orotic acid into the 28S and 18S rRNA peaks. This inhibition was evident even at the lowest dose of drug used (0.5 μmole/ml of incubation medium). The pattern of labelling of the heterogeneous species migrating between the 18S and 4S components was relatively unaffected by the drug. The overall patterns of radioactivity of the 5-Foro treated preparations were similar to that obtained with a low dose of actinomycin D (compare Figs. 27A, B and 28A, B, C and D). However, increasing doses of 5-Foro caused a progressive decrease

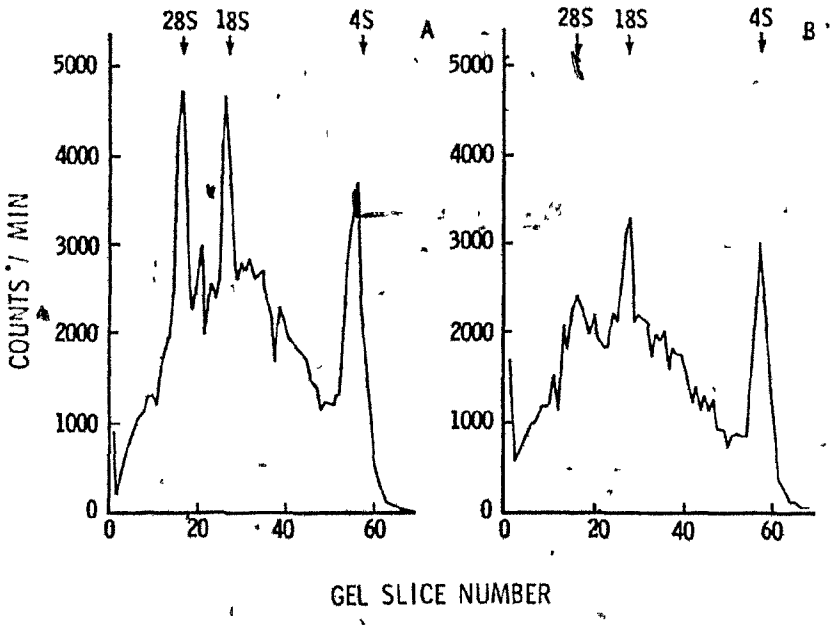
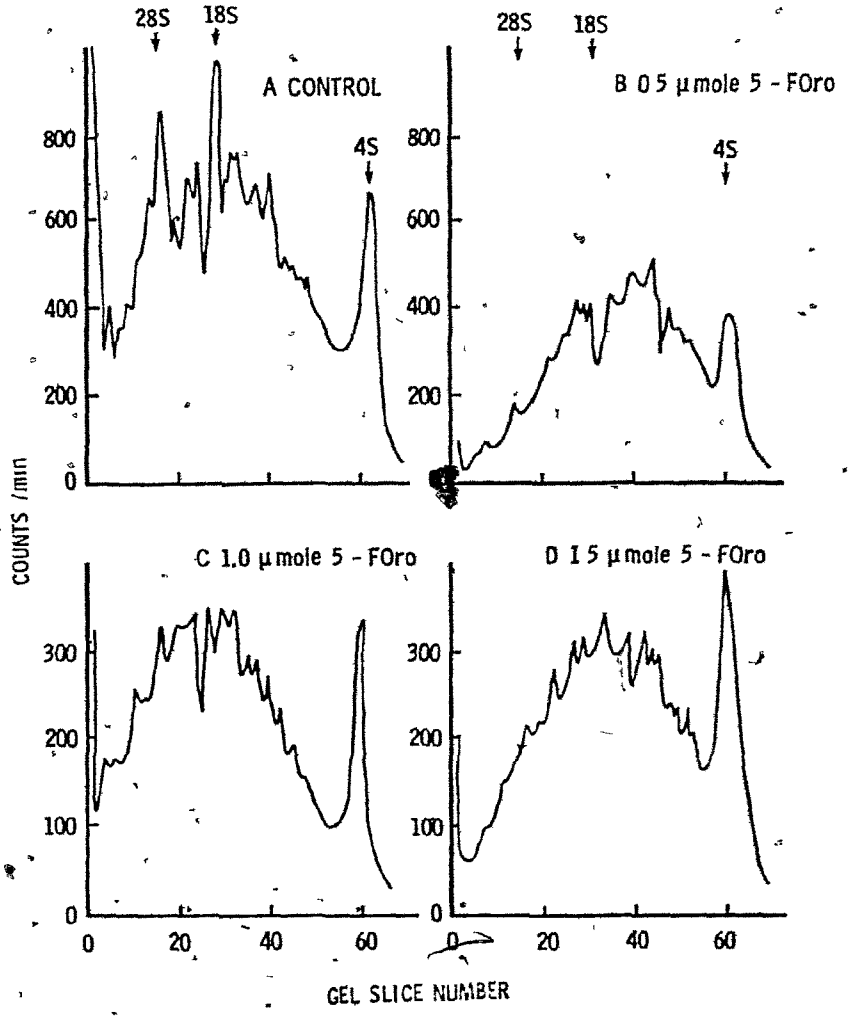


Fig. 27. Effect of actinomycin D on the labelling of total RNA from the sciatic nerve of 3-day chicks. Nerve tissue from 20 3-day chicks was preincubated for 15 min. in the presence or absence of the inhibitor and then incubated with 100 µCi [5-^{3}H]uridine/ml for 3 h. RNA was extracted from the tissue, fractionated on 8-cm long x 0.7-cm dia. gels of 2.6% (w/v) polyacrylamide for 120 min., and slices of the gel were counted as described in Section II. The arrows indicate the position and sedimentation values of the three major peaks of the bulk RNA, obtained after scanning the gels at 260 nm. (A) control nerve; 23 µg RNA applied, (B) actinomycin D treated nerve (0.1 µg/ml of incubation medium); 22 µg RNA applied.

Fig. 28. Effect of increasing doses of 5-Foro on the labelling of total RNA from the sciatic nerve of 3-day chicks. Nerve tissue (0.6 g wet weight) of 3-day chicks was incubated with 100 µCi [5-^{3}H]orotic acid/ml for 3 h. Conditions of incubation, extraction of RNA and fractionation were the same as described in the legend of Fig. 27 and "Methods". (A) control nerve; 28 µg RNA applied; specific activity 1407 cpm/µg RNA; (B) nerve incubated with 0.5 µmole/ml of 5-Foro; 30 µg RNA applied; specific activity 650 cpm/µg RNA; (C) nerve incubated with 1.0 µmole/ml of 5-Foro; 33 µg RNA applied; specific activity 465 cpm/µg RNA; (D) nerve incubated with 1.5 µmole/ml of 5-Foro; 33 µg RNA applied; specific activity 384 cpm/µg RNA.



in the specific activities of the nerve RNA.

3. Comparison of gel electrophoretic patterns of RNA labelled by [2-^{14}C]5-Foro and [5-^{3}H] orotic acid.

The results of Wilkinson et al. (1971) demonstrated that [2-^{14}C]5-Foro was selectively incorporated into non-ribosomal ribonucleoprotein particles. Therefore, the pattern of incorporation of labelled 5-Foro and labelled orotic acid into total nerve RNA was compared. Fig. 29A and B shows that after 1 h of incubation the radioelectropherograms of RNA labelled by either[2-^{14}C]5-Foro or [5-^{3}H] orotic acid were quite similar. Most of the radioactivity appeared between the 18S and 4S components, although the RNA labelled by the base analogue contained relatively more radioactivity in fractions heavier than 28S. After 3 h of incubation the 28S and 18S rRNA and 4S tRNA peaks showed incorporation of [5-^{3}H]orotic acid in the control nerve, whereas the eletropherogram of RNA labelled by [2-^{14}C] 5-Foro was not changed significantly from that seen after 1 h (Fig. 29C). This latter figure also demonstrates the good resolution of the gel electrophoretic technique for the mixed orotic acid and analogue-labelled RNA preparations.

4. Effect of unlabelled 5-Foro on the incorporation of [5-^{3}H]orotic acid into nerve RNA of 3-day chicks.

Time study.

The effect of unlabelled 5-Foro on the incorporation

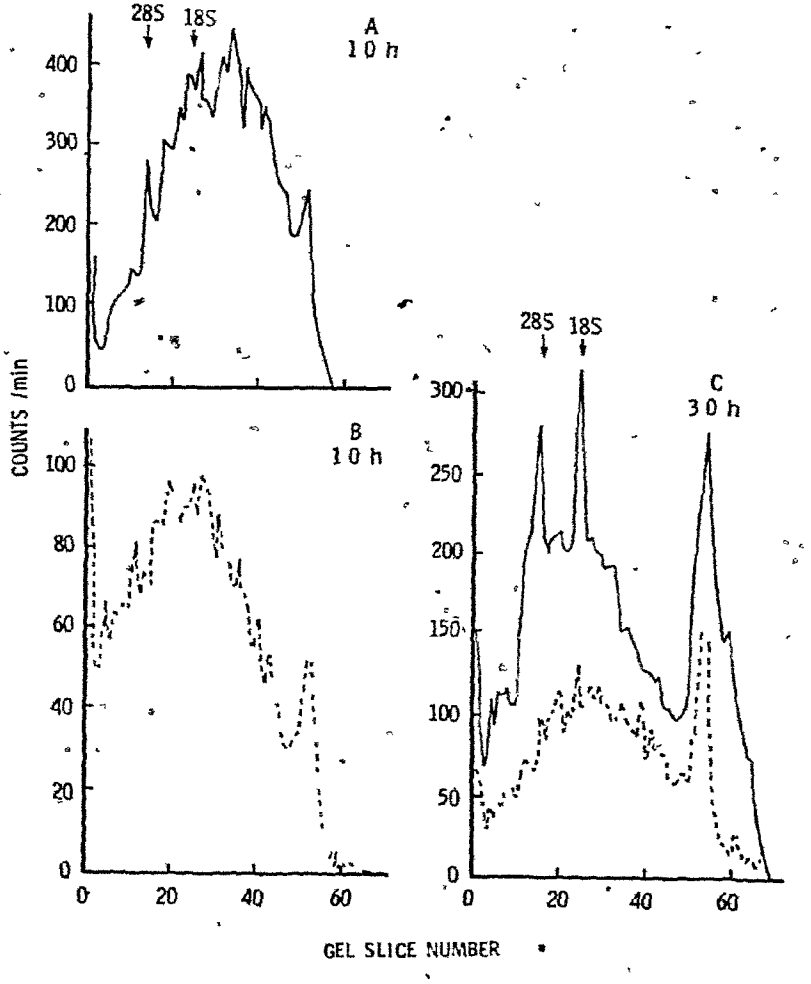


Fig. 29. Polyacrylamide gel electrophoresis of RNA from 3-day chick sciatic nerve incubated with either [5-^{3}H] orotic acid or [2-^{14}C]5-Foro for 1 h or 3 h. Nerve tissues of 15-20 3-day chicks were incubated with either 100 µCi of [5-^{3}H]orotic acid/ml or with 3.3 µCi of [2-^{14}C]5-Foro/ml. Conditions of incubation, extraction of RNA and fractionation were the same as described in the legend of Fig. 27, and "Methods". (A) Nerve tissue labelled with [5-^{3}H]orotic acid for 1 h; 39 µg RNA applied; (B) nerve tissue labelled with [2-^{14}C]5-Foro for 1 h; 90 µg RNA applied; (C) co-electrophoresis of mixed RNA samples from nerve tissues labelled for 3 h with either [5-^{3}H]orotic acid (6 µg RNA) (——) or with [2-^{14}C]5-Foro (56 µg RNA) (-----).

of radioactive orotic acid into total nerve RNA of 3-day chicks was followed as a function of time. Fig. 30A and B shows that after 0.5 h and 1.5 h incubation time a very diffuse patternwas obtained for the control preparations. Most of the radioactivity appeared between 28S and 4 S components of the bulk RNA. However, after 3 h, three distinct radioactive peaks, corresponding to the 28S, 18S and 4S components were evident in the control (Fig. 30C), as was shown previously for control preparations after this length of incubation time (Figs. 27A, 28A and 29 solid line). In contrast, the gel patterns of the 5-Foro-treated samples were similar at each incubation period, and even after 3 h resembled those produced after a short 0.5 h or 1.5 h "pulse" with [5-^{3}H]orotate alone (Fig. 30D, E and F). No radioactive peaks, corresponding to the 28S and 18S rRNA components were apparent in any of the 5-Foro treated RNAs. However, the presence of 5-Foro led to a considerable decrease in the overall specific activity of the RNA.

5. Effect of unlabelled 5-Foro on the incorporation of [5-^{3}H]orotic acid into embryonic nerve RNA.

The effects of 5-Foro on the pattern of RNA synthesis in embryonic nerves were also investigated. Fig. 31A and B shows that after 1.5 h incubation, the radioelectropherograms of RNA from 17-day embryonic nerve incubated in the presence of unlabelled 5-Foro and [5-^{3}H]orotate were quite

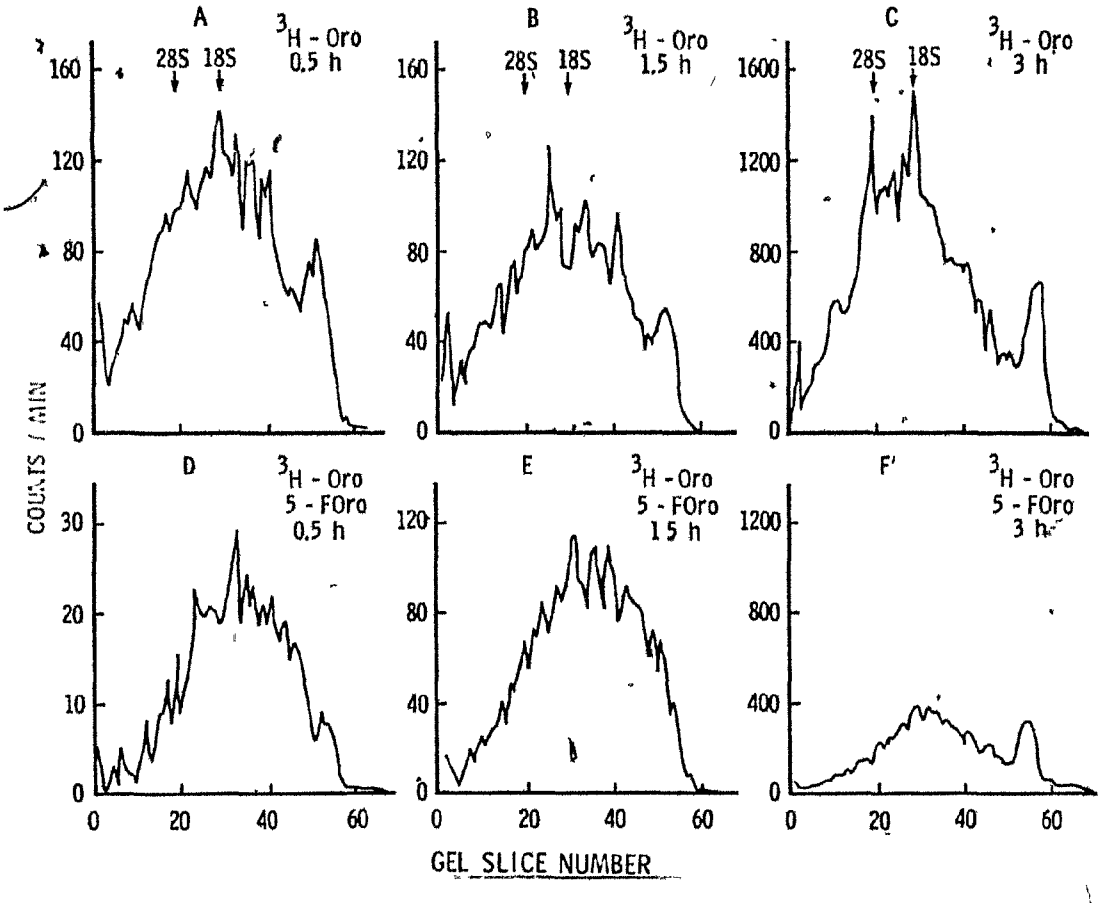


Fig. 30. Polyacrylamide gel electrophoresis of RNA from 3-day chick sciatic nerve isolated at various time intervals following incubation with either 100 µCi/ml of [5-^{3}H] orotic acid or with [5-^{3}H]orotic acid and unlabelled 5-Foro simulatneously. Conditions of incubation, extraction of RNA and fractionation were the same as described in the legend of Fig. 27 and "Methods." (A, B and C) nerve tissue labelled with [5-^{3}H]orotic acid for 0.5, 1.5 and 3 h; 32, 16 and 40 µg RNA applied respectively, (D, E and F) nerve tissue labelled with [5-^{3}H]orotic acid in the presence of 0.7 µmole 5-Foro/ml for 0.5, 1.5 and 3 h; 16, 30 and 30 µg RNA applied respectively.

similar to that of the control incubated with [5-^{3}H] orotate alone. However, the overall radioactive profile of the RNA was different from that of the 3-day chicks; relatively more radioactivity appeared in fractions heavier than 18S. After 3 h of incubation with [5-^{3}H] orotic acid alone, radioactivity appeared distinctly in the 28S and 18S rRNA components of the control preparation. (Fig. 31C). In contrast, the gel pattern of RNA labelled in the presence of 5-Foro was not changed significantly from that seen after 1.5 h of incubation (Fig. 31D). Furthermore, the radioactive profile of the RNA labelled for 3 h in the presence of the base analogue was very similar to that reported previously for the rapidly-labelled RNA from the 17-day embryonic nerve (Mezei & Hu, 1972). A considerable proportion of radioactivity was distributed in fractions heavier than 28S. The effect of the inhibitor on the 14-day embryonic nerve preparation was similar to that observed with the older embryos. After 3 h of incubation, the incorporation of radioactive orotic acid in the 28S and 18S rRNA peaks was inhibited, whereas no effect was evident in the tRNA region (Fig. 32A and B).

The differential effect of 5-Foro on the labelling (3 hr.) of RNA from different developmental stages can be more clearly demonstrated when the percentage of the total

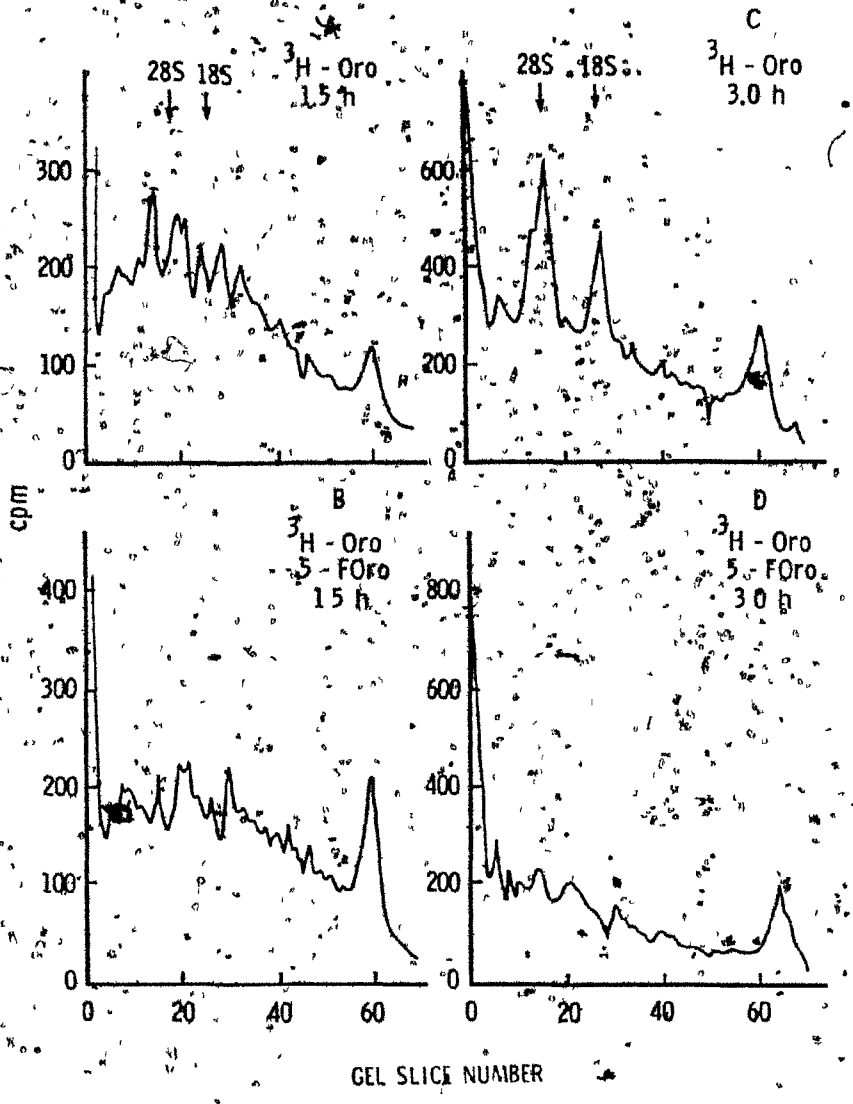


Fig. 31. Polyacrylamide gel electrophoresis of RNA from sciatic nerve of thirty 17-day embryos isolated at various time intervals following incubation with either 100 µCi/ml of [5-^{3}H]orotic acid or with [5-^{3}H]orotic acid and unlabelled 5-Foro simultaneously. Conditions of incubation, extraction of RNA and fractionation were the same as described in the legend of Fig. 27 and "Methods." (A and C) nerve tissue labelled with [5-^{3}H]orotic acid for 1.5 and 3 h; 70 and 48 µg RNA applied respectively, (B and D) nerve tissue labelled with [5-^{3}H]orotic acid in the presence of 0.7 µmole 5-Foro/ml for 1.5 and 3 h; 75 and 48 µg RNA applied respectively.

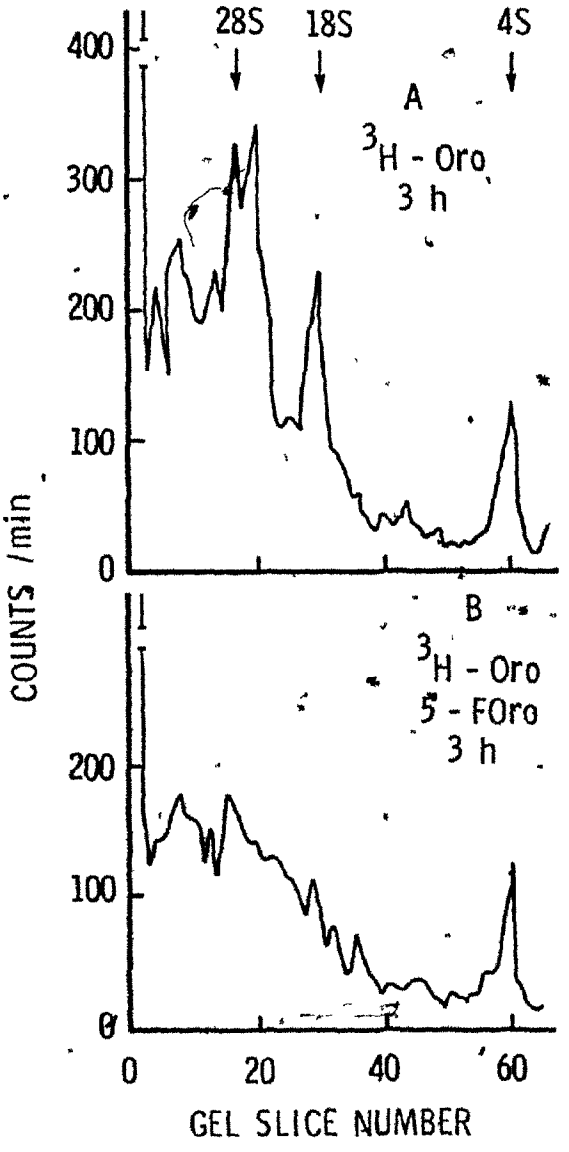


Fig. 32. Polyacrylamide gel electrophoresis of RNA from sciatic nerve of sixty 14-day embryos isolated following incubation with either 100 µCi/ml of [5-^{3}H] orotic acid or with [5-^{3}H] orotic acid and unlabelled 5-Foro simultaneously. Conditions of incubation, extraction of RNA and fractionation were the same as described in the legend of Fig. 27 and "Methods." (A) nerve tissue labelled with [5-^{3}H]orotic acid for 3 h; 33 µg RNA applied; (B) nerve tissue labelled with [5-^{3}H]orotic acid in the presence of 0.5 µmole 5-Foro/ml for 3 h; 36 µg RNA applied.

counts is plotted against the fraction number for each preparation (Fig. 33). This figure illustrates that the bulk of the radioactivity in the RNA fractions of treated 14-day embryonic and 17-day embryonic nerves appeared mainly in the high-molecular-weight region with diminished 28 S and 18S RNA peaks (Fig. 33 A, B). The high-molecular-weight RNA and heterodisperse low-molecular-weight RNA were less affected by 5-Foro. These results support the hypothesis that the high-molecular-weight RNA in younger animals is mainly heterogeneous nuclear RNA and large rRNA precursors. In contrast, the RNA in the high-molecular-weight region was more affected by 5-Foro in the 3-day nerve preparations, indicating that these species consist mainly of 28S rRNA and some of its precursor molecules. Actinomycin-D, at the specific dose used, does not seem to inhibit as extensively as 5-Foro the labelling of 28S and 18S rRNA and rRNA species larger than 18S.

Since these experiments indicated that the pattern of labelling of RNA molecules larger than 28S was relatively unaffected by the drug in the embryonic nerve, the radioactive RNA preparations were fractionated on 2.4% (w/v) gels to obtain better resolution of the very high-molecular-weight species. Fig. 34B and 35B show prominent radioactive peaks corresponding to molecular weights of 2.6×10^6 and 3.0×10^6 daltons, respectively, in the 5-Foro-treated

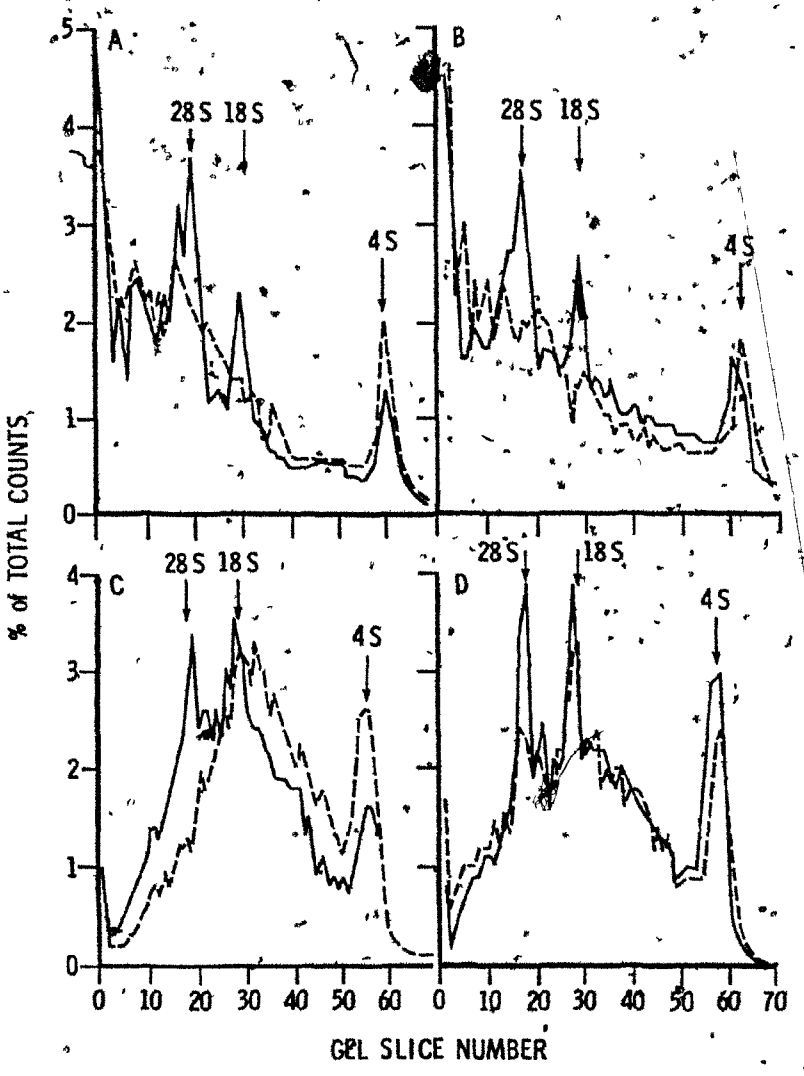


Fig. 33. Percentage distribution of total counts of RNA from 14-day embryonic (A), 17-day embryonic (B), and 3-day chick nerve (C,D) treated (------) with 5-Foro (A,B,C) or Act.-D (D) for 3 hr. and controls (——). The percentage distribution of total counts were calculated from the gel patterns of Fig. 30 C,F; Fig. 31 C,D; and Fig. 32 A,B.

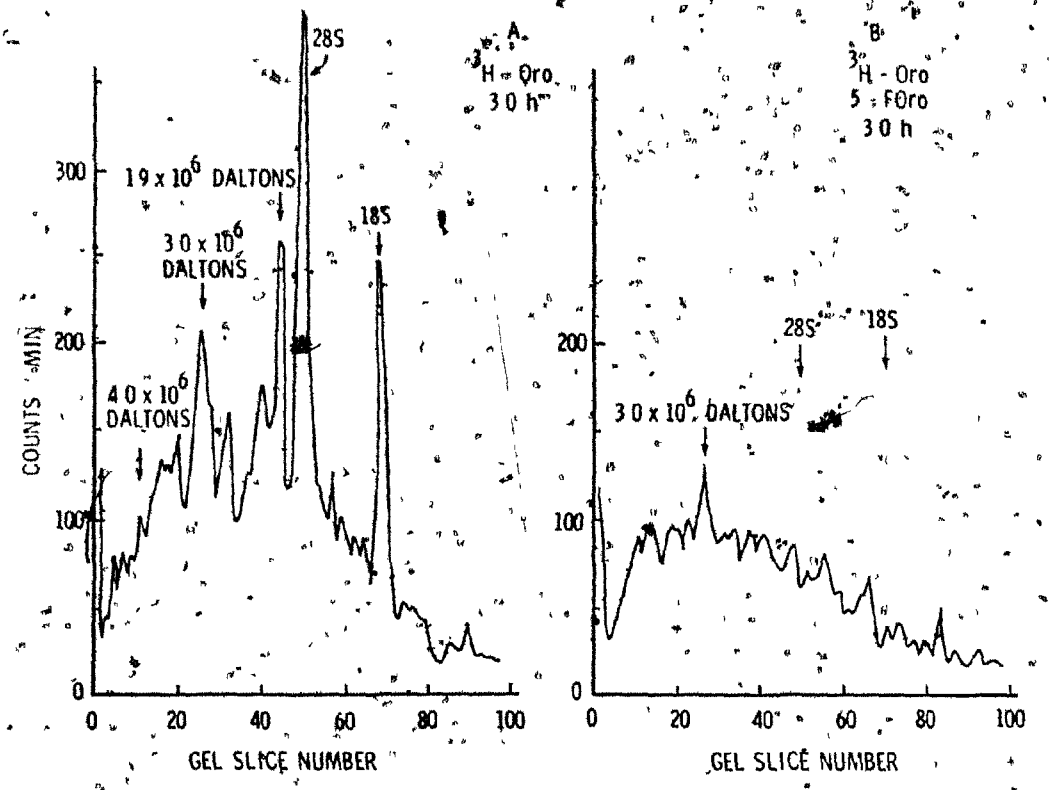


Fig. 34. Polyacrylamide gel electrophoresis on 2.4% (w/v) gels of RNA from sciatic nerve of 14-day embryos. RNA was isolated from nerve tissues of 60 embryos following incubation for 3 h with either [5-^{3}H]orotic acid alone (A), or with [5-^{3}H]orotic acid and unlabelled 5-Foro simultaneously (B), as described in the legend of Fig. 32 and "Methods." Control RNA (33 µg) and 5-Foro treated RNA (36 µg) were fractionated on 10-cm long x 0.7 cm dia. gels of 2.4% (w/v) polyacrylamide for 4.3 h as described in Section II.

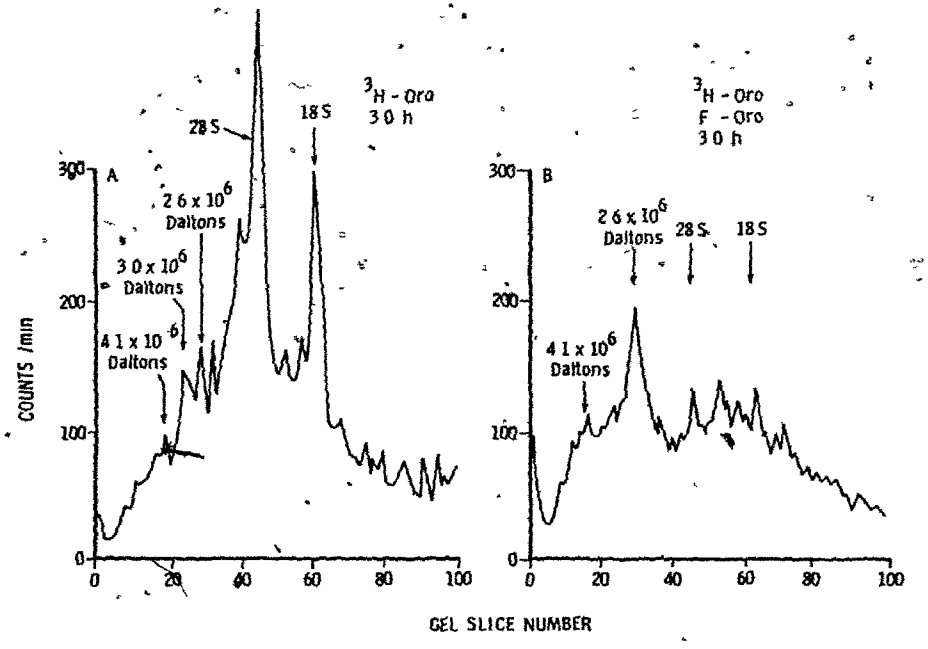


Fig. 35. Polyacrylamide gel electrophoresis of RNA from the sciatic nerve of 17-day embryos incubated for 3 hr. with either [5-^{3}H]orotic acid [^{3}H-Oro] (A); or with [5-^{3}H]orotic acid and unlabelled 5-fluoroorotic acid [F-Oro, 1.4 µmole] (B). Electrophoresis was carried out on 2.4% gels for 4 hr. 30 min. as described in the legend of Fig. 36. In each case 48 µg RNA was applied on the gels.

embryonic nerve RNA. These RNA species are also apparent in the control gels, which show several other radioactive peaks including the extensively-labelled 28S and 18S rRNA molecules.

6. Effect of 5-Foro on the incorporation of [5-^{3}H]orotic acid into polyribosomal RNA of sciatic nerve: a developmental study.

To investigate the effect of 5-Foro on synthesis of mature rRNA (cytoplasmic), the labelling of polyribosomal RNA was explored in the presence or absence of the inhibitor in embryonic and post-hatch animals. The solid line in Fig. 36A and B shows that the two rRNA species became distinctly labelled in the control polyribosomes after a 2 h incubation period with the labelled precursor in organ culture. In contrast, no significant radioactivity could be seen in the 28S and 18S polyribosomal RNA peaks in nerves incubated in the presence of 5-Foro. Furthermore, the dotted line indicates that the labelling of the heterodisperse, 5-Foro-resistant RNA of the 3-day chicks contained relatively more RNA species smaller than 18S than did that of embryonic nerve. An attempt was also made to ascertain whether the difference in the size of embryonic and post-hatch polysomal RNA was due to differential extraction or RNase degradation of these species during the cell fractionation and RNA extraction procedure. In these experiments

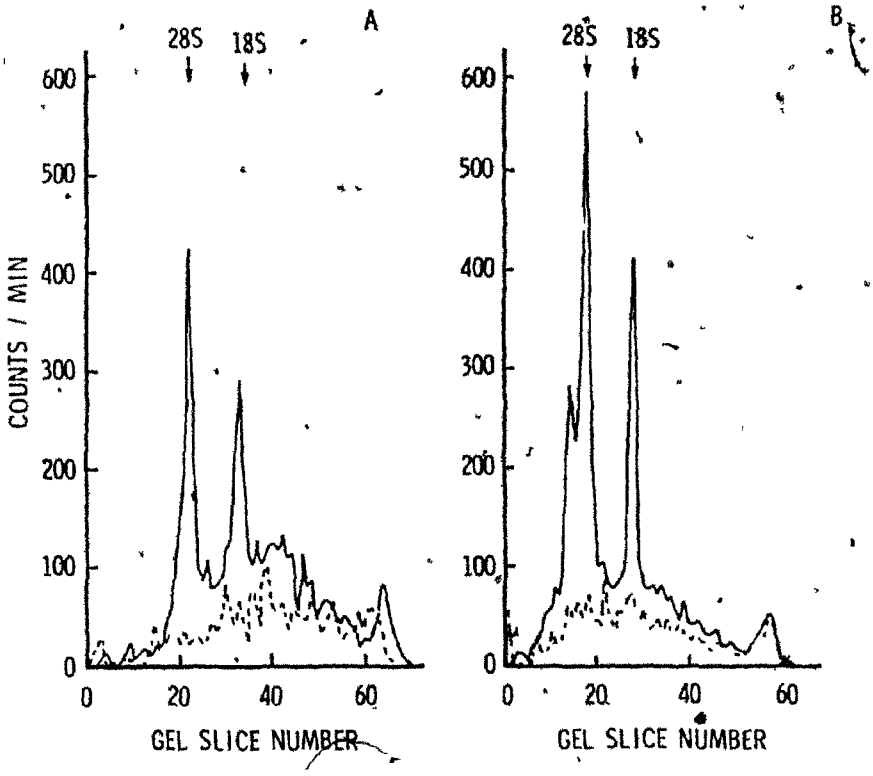


Fig. 36. Effect of 5-Foro on the labelling of polyribosomal RNA of the sciatic nerve from 3-day chicks and 17-day embryos. Nerve tissue from 3-day chicks (A) or 17-day embryos (B) was incubated for 2.0 h with [5-^{3}H]uridine (100 μCi/ml) in the absence (——), or in the presence (-----) of 0.5 μmole 5-Foro/ml. RNA was extracted from the polyribosomal pellet, purified and fractionated as described in the legend of Fig. 27 and "Methods." In each case approximately 31 μg of RNA was applied on the gels.

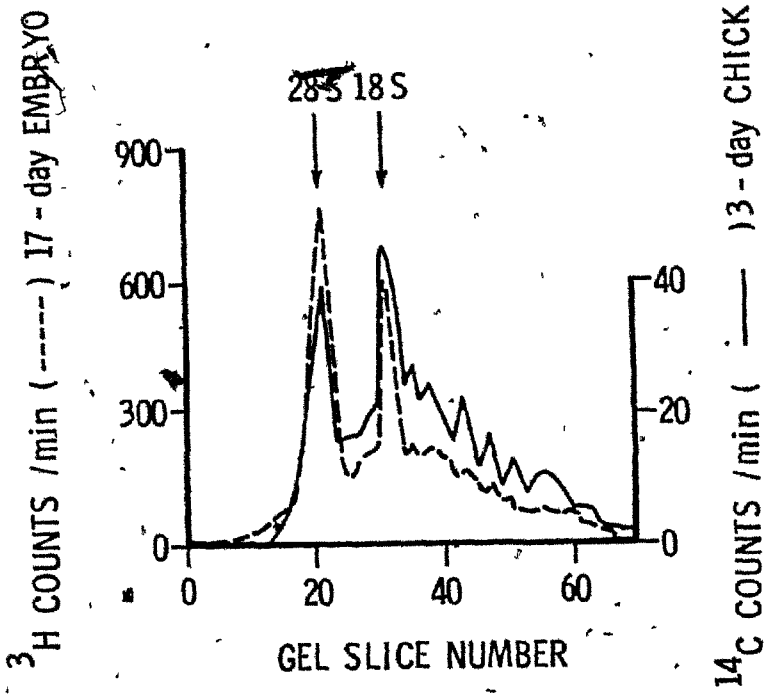


Fig. 37. Electrophoretic pattern of polysomal RNA of mixed sciatic nerve. Nerve from 3-day chick (——) and 17-day embryo (- - - -) were incubated in [^{14}C]uridine (12.5 µc/ml and [^{3}H]uridine (100 µc/ml) respectively. RNA extracted from the mixed polyribosomal pellet, purified and fractionated as described in the legend of Fig. 27 and "Methods."

nerve tissue from 17-day embryos and 3-day chicks was incubated with ^{3}H and ^{14}C-labelled uridine, respectively, and after 2.0 hr of incubation the nerve tissues were combined and polysomal RNA was isolated, purified, extracted, and fractionated as described in "Methods" of this section (Fig. 37). The gel patterns in Fig. 37 indicate that the electrophoretic profile of RNA from 17-day embryonic polysomes was somewhat similar to that shown in Fig. 36 (solid line). There was a higher proportion of 28S and 18S RNA in the 17-day embryonic nerve than in the 3-day chick nerve, in which more low-molecular weight RNA species were apparent (Fig. 37). This result demonstrates that the size difference of RNA species between 17-day embryonic and 3-day chick nerve RNA is not caused by preferential extraction or degradation of RNA in 3-day chick nerve. However, the proportion of the 28S rRNA peak in the 3-day chick preparation was smaller than that shown for the control (Fig. 36 solid line). These experiments, therefore, do not exclude the possiblity that during the incubation or cell fractionation procedure RNA is differentially degraded in the sciatic nerve of 3-day chicks.

D. Discussion

Two general techniques have been used to label mRNA in eucaryotes. One utilizes a "pulse label" brief enough

to allow a significant incorporation into cytoplasmic mRNA (but not rRNA or tRNA) (Spohr et al., 1970), whereas the other involves the administration of labelled precursor in the presence of appropriate doses of actinomycin-D to inhibit rRNA transcription selectively (Penman et al., 1968). Previously, using the first of these techniques it has been shown that the sedimentation and electrophoretic properties of the rapidly-labelled RNA of the sciatic nerve were different in the 14-day embryos, 17-day embryos and 3-day chicks (Hu & Mezei, 1971; Mezei & Hu, 1972). In the present investigation, inhibition of rRNA labelling was also attempted by using a relatively low dose of actinomycin-D (0.1 µg/ml of incubation medium), and it was found that the drug did indeed suppress the incorporation of radioactive precursor into the 28S and 18S rRNA components (Fig. 27). The pattern of labelling in the presence of actinomycin-D was similar to that observed previously for the "pulse-labelled" nerve RNA of that particular developmental stage. However, inhibition of rRNA labelling was not complete. Previous investigations from this laboratory (Mezei, 1970) and others (Kennel, 1964; Pastan & Friedman, 1968) have demonstrated that actinomycin-D has many toxic side effects unrelated to RNA metabolism. Furthermore, the short-pulse technique has the inherent disadvantage of focusing mainly on those

mRNAs with rapid synthetic rates, and therefore probably with rapid turnover rates as well. Recently Wilkinson et al. (1971) reported the selective inhibition of rRNA synthesis in rats by 5-Foro. On the basis of their relative toxicities 5-Foro appeared to be superior to actinomycin-D as a specific inhibitor of rRNA transcription (Wilkinson et al., 1971; Wilkinson & Pitot, 1973; Crhak et al., 1973). The results of the present investigation confirmed that 5-Foro was a selective inhibitor of rRNA labelling in the sciatic nerve of chicks for at least 3 h during incubation of tissues in organ culture.

A number of organisms incorporate fluorinated pyrimidines into their RNA (Heidelberger, 1965). Because experiments by Wilkinson et al. (1971) and Crhak et al. (1973) indicated that radioactive 5-Foro was selectively incorporated into nonribosomal RNA of rat liver in vivo, the electrophoretic properties of sciatic nerve RNA labelled by [2-^{14}C]5-Foro in organ culture were also investigated. In confirmation of the results of the above investigators, radioactive 5-Foro was incorporated into heterodisperse RNA species of 3-day chicks (Fig. 29). No discrete peaks of radioactive 5-Foro appeared in the 28S and 18S regions even after 3 h of incubation with the radioactive base analogue (Fig. 29C dotted line).

Recently a number of investigators demonstrated that

5-Foro blocks selectively the last steps of rRNA maturation in certain rodents (Wilkinson et al., 1971; Wilkinson & Pitot, 1973; Hadjiolova et al., 1973). According to Wilkinson and Pitot (1973), 5-fluorouridine only slightly inhibited the formation of the initial 45S rRNA precursor or the 38S rRNA intermediate in Novikoff rat hepatoma cells. Inhibition of 32S rRNA precursor production was greater than that of the larger precursor molecules, but less than that of the mature rRNA molecules. According to Hadjiolova et al. (1973) the labelling of nuclear and cytoplasmic 28S and 18S rRNA was almost completely inhibited by 5-Foro in mouse liver in vivo, while the transcription of 45S precursor rRNA and its early processing to 32S and 21S rRNA were not affected by the drug. Since in the present investigation, no detailed kinetic analysis was carried out of the various rRNA precursor species labelled in the presence or absence of the base analogue, definite conclusions could not be made about the mode of inhibition of rRNA maturation by 5-Foro in the sciatic nerve. However, results indicated that even after 3 h of incubation a significantly-labelled RNA species of molecular weight around 3 x 10^6 daltons persisted in the 5-Foro treated embryonic nerves, whereas the labelling of mature rRNA was significantly inhibited (Fig. 34B, 35B). Based on methylation and pulse-chase

experiments, it was previously concluded that the 3 x 10^6 dalton RNA species was one of the high-molecular-weight rRNA precursors of the sciatic nerve (Mezei & Hu, 1972). The results of the present investigation in embryonic nerve tissue are, therefore, at least consistent with the suggestion of Wilkinson and Pitot (1973) and Hadjiolova et al. (1973) that 5-Foro interferes with a definite step of rRNA maturation in eucaryotic cells. Since in 3-day nerve there was no accumulation of high-molecular weight (>28S) material in the drug-treated nerve, one may speculate that 5-Foro increased the degradation of 45S RNA precursors in this tissue. The findings of Hadjiolova et al. (1973), to the effect that this drug caused the appearance of rapidly-labelled degradation products in the cytoplasmic soluble RNA fraction also support the above speculation. Furthermore, according to Hadjiolova et al. (1973), the base analogue did not affect the pattern of synthesis of a number of other species, including heterogeneous nuclear RNA, messenger-like RNA of cytoplasmic polyribosomes, free post-microsomal ribonucleoproteins and tRNA. The results of the present investigation also indicate that the labelling of tRNA was not affected appreciably by the drug.

The radioelectropherograms of the 5-Foro-resistant RNA species of the three developmental stages were almost

identical to those observed previously in this laboratory after a relatively short "pulse" (30 to 60 min.) by the radioactive precursor alone (Mezei & Hu, 1972). There was a developmental-dependent change in the electrophoretic profiles of these preparations, and these changes were apparent even after 3 hr incubation with the radioactive precursor in the presence of the inhibitor.

The incorporation of both labelled 5-Foro, and radioactive orotic acid in the presence of unlabelled 5-Foro, into the nerve from 3-day chicks revealed the presence of a heterogeneous population of RNA, most of which migrate between the smaller rRNA and tRNA components of the bulk RNA. Previous studies demonstrated that in the post-hatch animals most of these heterodisperse species had a low methyl content and that their sedimentation and electrophoretic properties were very similar to those of the cytoplasmic RNA of other fully-differentiated vertebrate tissues (Hiatt, 1962; Peterson, 1970; Mezei & Hu, 1972). Therefore, these undermethylated, rapidly-labelled, 5-Foro and actinomycin-D-resistant RNA fractions may represent some of the functional mRNA of the cytoplasmic fractions of the sciatic nerve. However, the possibility cannot be excluded that the low-molecular-weight, 5-Foro-resistant-RNAs are degradation products of the high-molecular-weight rRNAs. Sciatic nerve RNA from different

developmental stages might have different sensitivities toward this drug. A base analysis experiment might provide an answer to this question. Recently Garrett et al. (1973) reported that the 5-Foro resistant-RNA of rats had a DNA-like base composition.

Previous results from this laboratory indicated that the "pulse-labelled" RNA in the embryonic nerves was a mixture of rRNA precursors and heterogeneous, mostly high-molecular-weight, messenger-like RNA (Mezei & Hu, 1972). If 5-Foro is a selective inhibitor of rRNA synthesis, the gel pattern of embryonic nerve RNA labelled in the presence of 5-Foro should contain a large proportion of high-molecular-weight rRNA precursor species even after long incubation periods. Results with unlabelled 5-Foro have borne out this expectation; relatively more radioactivity was distributed in fractions heavier than 18S in the embryonic 5-Foro-treated preparations. When these RNA preparations were examined further on more porous gels the presence of at least one prominent rRNA precursor species (Fig. 34, 35) could be discerned. These results are thus consistent with previous results and indicate that the large-molecular-weight, rapidly-labelled, 5-Foro-resistant fractions may represent a mixture of rRNA precursors and heterogeneous large-molecular-weight RNA of the nuclear fraction of the embryonic nerve.

Since the selective inhibition of rRNA maturation by the base analogue could be a useful tool in distinguishing between the different types of mRNAs present in the polyribosomes of the developing nerve, the effect of the inhibitor on polyribosomal RNA labelling was explored. As in the case of total RNA, 5-Foro completely abolished labelling of polyribosomal rRNA (Fig. 36A and B). Furthermore, the 5-Foro-resistant RNA of the 3-day chick nerve contained relatively more RNA smaller than 18S than did that of the embryonic nerves. Recent studies by Yamagami and Mori (1970) and Zomzely et al. (1970) revealed that the stability of certain large mRNA-ribosome complexes in rat cerebral cortex declined with age. In this connection, results of another study indicate that the adult brain may produce a higher proportion of relatively small mRNA molecules (Zomzely et al., 1970). Therefore, results on the 5-Foro-resistant total and polyribosomal RNA are consistent with the hypothesis that the embryonic nerve tissue contains a larger proportion of high-molecular-weight mRNA molecules than does that of young chicks or adults.

However, the experiments described in this section do not exclude the possiblity, that the small size of these molecules in the post-hatch animals is the result of differential degradation of the RNA occurring either

in vivo or *in vitro* (Fig. 37). A previous report from this laboratory (Mezei & Hu, 1973) indicated that the total and latent alkaline ribonuclease activities of the particulate fractions of the sciatic nerve increased significantly during development.

Section V

Conclusion and Bibliography

Conclusion

In multicellular organisms an ordered programme of changes occurs during the development of the mature organism from the fertilized egg to produce the final pattern characteristic of normal development. In general, two classes of processes can be recognized. The first is the emergence of functionally and morphologically-distinct cell types. The other is the association of cells in precise patterns to form tissues and organs. Although the exact mechanisms of these processes are unknown at present, there is evidence indicating that one of the regulatory modes in differentiation is at the level of transcription, to produce characteristic patterns of RNA molecules at particular developmental stages.

The purpose of the present dissertation was to investigate the nature of these characteristic patterns during myelination, a special case of cell differentiation which occurs during development of certain peripheral and central nerves. During the course of these and previous studies it was shown that the sciatic nerve of the chick indeed exhibits a pattern of RNA labelling which is characteristic of certain developmental stages of the animal. Some of the RNA molecules had properties similar

to those of mRNA or hnRNA of other fully-differentiated vertebrate tissues. These RNA species were rapidly-labelled, undermethylated, and had a relatively short half-life. The synthesis of these RNA species was resistant to the effects of low doses of actinomycin-D and 5-fluoroorotic acid, and a portion of the RNA population was enriched in poly(A) sequences. The electrophoretic and sedimentation properties of these molecules varied at different developmental stages. Sciatic nerve RNA from 14-day and 17-day embryos contain a larger proportion of high-molecular-weight RNA species (>18S) than did that of the post-hatch animals. Little of this RNA originated in the mitochondrial fraction of the cells, although mitochondria of brain and nerve tissue synthesized RNA both in vivo and in vitro.

The above results suggest that RNA metabolism is important and has a specific, probably regulatory function in the peripheral nerve. A great number of enzymes and other chemical constituents are synthesized in the nervous system just prior to or during the active phase of myelination. Nerve cells at a critical developmental stage may "turn on" the synthesis of specific messages which either separately or in a collaborative manner direct differentiation of these cells to carry out their specific functions, such as myelination of certain axons.

Although a very small amount of mitochondrial RNA was synthesized at different developmental stages, it is still possible that mitochondrial translational products could specifically interact with the nuclear gene products to give special signals for regulation of myelinogensis. However, the results of this thesis support the above hypothesis only in a very qualitative and indirect way. More conclusive interpretation of the data is complicated for the following reasons:

(1) Since RNA of sciatic nerves could not be labelled efficiently *in vivo*, all of the experiments with this tissue had to be carried out under *in vitro* conditions. It is possible that degradation of the rapidly-labelled RNA occurs during the isolation procedure. To circumvent this possibility adequate precautions could be made (see methods sections II-IV). However, breakdown of RNA may also occur during incubation, as the result of increased RNase activity due to tissue damage. In this respect the isolated and cultured peripheral nerve can be regarded as the distal portion of a nerve undergoing *in vitro* Wallerian degeneration (Muirhead & Mezei, 1973). It has been shown that the number of lysosomes increases in the degenerating nerve. Lysosomes are known to contain degradative enzymes and have been implicated as a source of degradative enzymes for the destruction of the myelin

sheath during the early stages of Wallerian degeneration (Porcellati, 1972).

(2) The results may reflect mere changes in the relative proportion of nuclei and cytoplasm in the peripheral nerve during myelination. It is known that during rapid myelination the cytoplasm of Schwann cells increases greatly (Friede & Samorajski, 1967).

(3) The relatively high proportion of low-molecular-weight (<18S), mRNA-like molecules in the myelinating, post-hatch animals may be the result of relatively faster processing of hnRNA at that stage of nerve development. It has been shown for example, that in sea urchin embryos during the early developmental stages a special class of low-molecular-weight mRNA appeared in the cytoplasm after a very short lag period (<5 min.) (Nemer et al., 1975). To determine if this is the case for sciatic nerve mRNA at a certain developmental stage, kinetic studies should be carried out in which the labelling characteristics of nuclear and cytoplasmic RNA are followed as a function of time.

(4) The changes in the electrophoretic and sedimentation patterns of RNA could be the result of activation of specific ribonucleases at different stages of development. In this context previous studies from this laboratory have indicated changes in the activity of the

alkaline ribonuclease system of the sciatic nerve during development (Mezei & Hu, 1973).

(5) The differential labelling of certain RNA species during development could reflect changes of the specific precursor pools for these molecules. For technical and practical reasons the size, specific activity and possible compartmentalization of the RNA precursor pool(s) of the sciatic nerve have not been determined in the present investigation. The work of Barbioli et al. (1973) established that the size of the total RNA precursor pool was decreasing, although the rate of total RNA synthesis of chick embryos was relatively constant during the 3rd to 12th day of embryonic development. Jude et al. (1973) showed that the specific activity of [^{3}H] uridine-containing material in the acid-soluble pool of chick embryo cerebral hemispheres did not change between 6 and 10 days but increased significantly (20%) at 14-days. The specific activity of UMP from total RNA relative to that of the precursor was constant between 6 and 10 days, and decreased by about 20% between 10 and 14 days. Judes and Jacob (1973) also indicated with [^{3}H]uridine as a precursor, that the rate of rRNA synthesis did not change significantly between 6 and 14 day, although the rate of tRNA synthesis increased significantly.

To determine if these changes occur in the developing

sciatic nerve, the sizes, specific activities, and possible compartmentalization of the RNA precursor pools should be determined for the 14-day and 17-day embryonic and 3-day post-hatch stages.

(6) Any interpretation of the data in this dissertation is further complicated by the fact that they have been derived from an organ containing a mixture of constituents such as Schwann cells, axons, myelin sheath and connective tissue such as peri - epi - and eudoneurium. These cells and their subcellular fractions may have quite different synthetic capacities for RNA and other materials. In order to arrive at a clear-cut interpretation of the data it would be necessary to carry out these experiments on separate axons or Schwann cells, or study cultured neurons and Schwann cells under conditions where myelinogenesis can be artificially induced, maintained and controlled. Using these well-established cell lines and/or mixed cell cultures the appearance of specific gene products such as mRNA and protein classes could be then followed quantitatively and characterized as a function of myelination. However, these experiments would not be feasible until such time as the necessary techniques become refined.

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