Physical, Chemical and Morphological Characteristics of Isolated Mammalian Brain Nuclei

by

GABY G. BADR

Göteborg 1973

Physical, Chemical and Morphological Characteristics of Isolated Mammalian Brain Nuclei

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av
medicinska fakulteten vid universitetet
i Göteborg för ernående av medicine doktorsgrad
offentligen försvaras på engelska språket
i stora föreläsningssalen, institutionen
för anatomi och histologi, lördagen den
15 december 1973 kl. 9 f.m.

av
GABY G. BADR
Doctor of Medicine

GÖTEBORG
GÖTEBORGS OFFSETTRYCKERI AB
1973

Institute of Neurobiology, Medical Faculty
University of Göteborg, Göteborg, Sweden

Physical, Chemical and Morphological Characteristics of Isolated Mammalian Brain Nuclei

by

GABY G. BADR

Printed in Sweden, Gotab, Kungälv 73.11867

Puisque, au fond même de notre conscience humaine, la face interne du Monde vient au jour et se réfléchit sur elle-même, il semblerait que nous n´ayons qu´à nous regarder nous-mêmes pour comprendre dans quelles relations dynamiques se trouvent, en un point quelconque de l´Univers, le Dehors et le Dedans des choses.

Le Phénomène Humain

P. Teilhard de Chardin

TO MY PARENTS

Contents

The present thesis is based upon the following publications:

I Electrophoretic studies of intact nuclei isolated from mammalian brain. I. Electrokinetic characteristics and effects of cations.

G.G. Badr and A.A. Waldman: Int.J.Neurosci. In press.

II Electrophoretic studies of intact nuclei isolated from mammalian brain. II. Electrokinetic characteristics following treatment and comparison of glial and neuronal nuclei.

G.G. Badr and A.A. Waldman: Int.J.Neurosci. In press.

III Microelectrode studies on intact nuclei isolated from mammalian brain.

G.G. Badr: Int.J.Neurosci. Accepted for publication.

IV Influence of pH, DNAase, RNAase and Heparin on the ultrastructure of isolated rat cerebrocortical nuclei.

G.G. Badr and S. Smeds (Submitted for publication).

V Inner structure of microdissected cerebrocortical nuclei as observed by whole mount electron microscopy.

G.G. Badr and S. Smeds (Submitted for publication).

VI In vitro response of nuclei isolated from rat brain to pH changes, enzymes and heparin treatment: cytochemical characteristics and incorporation of ^{3}H-uridine and ^{14}C-valine.

G.G. Badr: Cytobiologie. In press.

In the text, these papers will be referred to by Roman numerals I-VI.

The following abreviations are used:

AO	Acridine orange
DNA	Deoxyribonucleic acid
DNP	Deoxyribonucleoprotein
RNA	Ribonucleic acid
DNAase	Deoxyribonuclease
RNAase	Ribonuclease
^{3}H	Labelled with tritium
^{14}C	Labelled with Carbon 14

INTRODUCTION

Those properties unique to the brain are the processing, storage and recall of the information which impinge upon it as nerve impulses. To fulfil such a complexe function the brain is highly organized by means of specific interactions among the nerve cells and between the nerve cells and glia— forming a biochemical and functional unit (Hydén 1967). The intrinsic integrative properties of the brain cells allows them, moreover, to generate as well as to code and decode the nerve impulses.

The requirements for such a processing are met by the functioning of multiple and complex synaptic networks (Grundfest 1967, Mountcastle 1967, Eccles 1967) and by molecular mechanisms (Hydén 1967, 1972, Agranoff 1967).- RNA and protein synthesis are known to change during the processing of new information (Hydén 1967, Bocharova et al.1972). What are the mechanisms translating electrical activity into macromolecular patterns ?

The cell surface, acting as a transducer, transmits the information received from the environment into intracellular control sites which will regulate the specific cellular response. The instructions encoded in these control sites, within the genome, are translated into macromolecules that direct the synthesizing machinery of the cell. Hence, knowledge of the control of gene expression is required to understand the manner in which cells respond to functional, e.g. electrical, activity.

The primary substance of the genetic material is DNA present within the chromatin. The chromatin consists of fibres of various diameters containing DNA in supercoiled arrangement around which specific protein is bound (Zirkin and Kim 1972). Changes in DNA-protein interactions may play a role in the control of gene expression.

The chromatin exists in two forms: a condensed form which is functionally repressed and an active dispersed form engaged in RNA synthesis (Ringertz et al. 1970; Brasch et al. 1972; Leake et al. 1972). The state of the chromatin is highly dependent on the composition of the surrounding medium (Solari 1968). Thus, changes in physicochemical parameters, such as ions or pH, may be important factors in the activation of the chromatin (Ringertz 1967).

In all eukaryotic cells, the chromatin is contained in a well-defined nucleus, and it is often partly attached to the nuclear membrane (Comings and Okada 1970). Hence, intra and extracellular factors which regulate the release of information operate via the nucleus. The nature of the relationships between the nuclei and its surrounding is essential to an understanding of cell control through the process of gene expression. Nucleocytoplasmic exchange, activated after stimulation (Bocharova et al. 1972), is of great importance in the communication of the genetic instructions in the cell. In this, the nuclear surface, with the pore-complexes it contains, must play a major role (Wunderlich 1972; Scharrer and Wierzelmann 1969).

Alterations of the cytoplasm surrounding the nucleus are responsible for many of the nuclear responses. It is therefore of major importance to determine how nuclear responses are

related to changes in the surrounding secondary to the main manifestations of the nerve cell such as changes in the membrane potential.

In order to provide clues for the understanding of the nuclear behavior, investigations have been performed on nuclei in situ, using various methodological approaches, such as electrophysiology (Kanno et al. 1965; Loewenstein et al. 1966), electron microscopy (Wunderlich et al. 1972; Yasuzimi et al. 1966), biochemistry (Bocharova et al. 1972; Hogan and Gross 1972), and studies of cell hybrids (Bolund 1971; Harris 1967). The natural surrounding of the nucleus is however far too complex to allow accurate monitoring of its action on the nucleus. Therefore, studies have been carried out on isolated nuclei suspended in a well-defined milieu (Anderson 1956; Arnold et al. 1972; Brasch et al. 1971; Burdman and Journey 1969; Dravid et al. 1969; Haglid 1972; Haljamäe and Waldman 1973; Kato and Kurokawa 1967; Kraemer and Coffey 1970; Løvtrup-Rein 1970; Olins and Olins 1972; Ris and Mirsky 1948; Tres et al. 1972; Tosi et al. 1972; Waldman and Alm-Håkansson 1972).

However, to fully understand the in vitro behavior of nuclei, for example following alterations in their environment, many aspects of the nuclear response have to be considered simultaneously. Thus, the purpose of the present investigation was to compare the physical, chemical and morphological characteristics of isolated brain nuclei.

To achieve this aim, the work was carried out along the following methodological lines:

1) Electrophoresis: the aim of the electrophoresis experiments is to obtain information about the surface of the

particle under observation. Since the nuclear surface is the site of many important biological phenomena, knowledge of surface characteristics is of interest.

2) Microelectrode studies: microelectrode impalements of particles allow recording of possible internal potentials, the study of which provides clues to the ionic content as well as the structural organization of the internal elements.

3) Electron microscopy: Ultrastructure of isolated nuclei were observed. An ordered ultrastructure can be of importance for the physical characteristics of the nuclei.

4) Cytochemistry: Cytochemical methods are convenient to investigate the molecular interactions responsible for the ordered structure of the nuclei.

5) Biochemistry: That the observed ultrastructural and molecular alterations of the nuclei have a functional significance can be shown through biochemical methods such as the study of the incorporation of precursors into nuclear macromolecules.

The main purposes of the present investigation dealing with the *in vitro* behavior of isolated nuclei can then be summarized as attempts to:

- Determine the electrokinetic and electrostatic characteristics of isolated brain nuclei (I, II, III)
- To identify and characterize the ultrastructure of the nuclear population (IV, V)
- To study some cytochemical and biochemical parameters of isolated brain nuclei (VI)

- To analyze with the aid of these physicochemical parameters the nuclear response to alterations of the surrounding milieu
- To discuss some biological significance of the obtained results.

METHODOLOGY

The following is only a brief summary of the materials and methods used in this investigation. For further details the reader is referred to the individual publications.

Materials:

Experimental animals. Erythrocytes as well as brain and liver nuclei were usually isolated from Sprague-Dawley rats. Nuclei from neuronal- and glial-enriched fractions, however, were obtained from albino rabbits.

Human material. Normal grey and white matter, as well as tumor nuclei were prepared from samples removed at surgery (Department of Neurosurgery, Sahlgrenska, Göteborg).

Preparation of nuclear suspensions (I). Following removal and homogenization of the appropriate tissues nuclei were prepared by ultracentrifugation in a sucrose density gradient as previously described by Dravid et al.(1969). Rabbit neuronal- and glial-enriched fractions were prepared according to the procedure of Blomstrand and Hamberger (1969).

Preparation of rat erythrocytes (I). Blood was collected, heparinized and centrifuged. Packed erythrocytes were repeatedly washed and resuspended in a buffered medium.

Methods:

Treatment of nuclei. Freshly isolated nuclei were incubated in different solutions:Tris-HCl, potassium/sodium phosphate and NaHCO3 buffers were tested in a wide range of pHs as well as in different ionic strengths. Nuclei were treated with enzymes, such as DNAase, RNAase, Pronase, with monovalent and divalent cations, as well as with heparin and spermidine. The concentrations used, the time and the temperature of the incubation are presented in the appropriate publications.

Electrophoresis (I, II). The electrophoretic mobility of isolated nuclei suspended in a buffered milieu was studied using a rectangular electrophoresis chamber mounted on the stage of a phase contrast microscope. The chamber was calibrated and tested using rat erythrocytes. The surface charge was computed from the electrophoretic velocity (Brinton and Lauffer 1959; Tenforde 1970). The assumptions and limitations of the method are extensively described in publication I.

Microelectrode studies (III). Glass as well as gold-plated microelectrodes were used to impale, under microscopic observation, freshly isolated nuclei suspended in a buffered medium. The potentials were recorded and analyzed following the classical procedures used in electrophysiology (Burés et al. 1967).

Phase contrast observations (I, II, III, IV). The morphology of intact and treated nuclei in suspension was studied by phase contrast microscopy. The size of nuclei was measured on picture prints. Qualitative studies on the response of nuclei to an applied electric field were performed on nuclei suspended on a glass slide (Oil-chamber type) with electrodes mounted on each side of the slide (I).

Electron microscopy (IV, V). The ultrastructure of intact and treated nuclei was studied by electron microscopy, following fixation, embedding, sectioning and staining of the preparation (IV). Whole mount electron microscopy observations (V) were performed on isolated nuclei individually microdissected prior to fixation and staining.

Absorption spectra (VI). Freshly isolated nuclear preparations were suspended into quartz microcuvettes and their absorption

spectra measured in the far ultraviolet using a double beam spectrophotometer.

Microfluorimetry (VI). A microfluorimeter was used to quantify the fluorescence of individual nuclei stained with AO (Bolund 1971).

Microinterferometry(VI). The dry mass of nuclei was determined using a rapid scanning and integrating microinterferometer (Carlsson et al. 1970).

In vitro incorporation of ^{14}C-valine and ^{3}H-uridine into nuclear proteins and RNA (VI). The procedure of nuclear incubation was essentially that described by Ramirez et al. (1972). For the double labelling with ^{14}C-valine and ^{3}H-uridine a modification of the same procedure was used as presented in publication VI. All the necessary precautions were taken to avoid bacterial contamination. The radioactivity of the samples was determined in a scintillation counter.

Analytical procedure. Protein determinations were performed according to the folin phenol method (Lowry et al. 1958), DNA was determined by the diphenylamine method (Burton 1956) and RNA by the orcinol method (Mejbaum 1939).

RESULTS AND COMMENTS

During the isolation procedure of the nuclei, it is likely that some nuclear material is lost. Proteins may be solubilized (Laval and Bouteille 1973) or the nuclear envelope may also be altered at the molecular level. These changes can even be more pronounced after a longer preparation procedure such as the isolation of neuron and glia-enriched fractions. On the other hand, cytoplasmic remnants can contaminate the nuclear preparations. For these reasons the purity and integrity of the nucelar preparations were checked by microscopic observations.

A. Morphological features of isolated brain nuclei.(IV).

Observed with phase contrast microscopy, the preparations of isolated nuclei were essentially free of cytoplasmic contaminants. The nuclei appeared intact. In a population of mammalian brain nuclei suspended in a medium at neutral pH, at least two types of morphologically distinct nuclei are observed. One type of nuclei, accounting for approximately 25% of the total population appears as a relatively large, round structure with a light nucleoplasm and a centrally located nucleolus. Observed with electron microscopy, the nuclear envelope, as in other eukaryotic nuclei (Stevens and André 1969) consists of two membranes separated by a perinuclear space. The envelope is circular, although not regularly so. The nuclear pores, often surrounded by filamentous structures (50-100 Å) and electron opaque material, contain a central granule (150 Å). The nucleolar components are readily differentiated as fibrils (50-150 Å), granules (150-250 Å) and amorphous matrix. Chromatin bundles, either loose or densely packed, radiate from the nucleolus, extending as far as the nuclear membrane. In the nucleoplasm,

perichromatin granules (300-600 Å) as well as interchromatin granules (200-250 Å) are present.

The other type of nuclei encountered under the same conditions represents half of the total nuclear population. The nuclei of this group are smaller, some of them containing one or many eccentric circular or elongated nucleoli while no nucleolus can be observed in the others. Observed with electron microscopy the nuclear envelope appears circular. When present, the nucleoli have a poorly defined structure. The nucleoplasm is dense. Intranucleoplasmic structures, such as perichromatin and interchromatin granules are sparsely distributed. A thin and dense perinuclear layer is present.

A third group includes small and unidentifiable nuclei.

In accordance with previous descriptions (Kato and Kurokawa 1967; Lovtrup-Rein and McEwen 1966; Mori and Leblond 1970; Peters et al. 1970; Reger et al. 1972), the first type of nuclei is generally identified as being mainly of neuronal origin while the second type is mainly of glial origin. Nuclei isolated from cell debris of non-neuronal origin and from microglia as well as disrupted nuclei account for the unidentified nuclei. The observation of some nuclear preparations incompletely isolated from their surrounding cytoplasm confirmed the classification of the nuclei by the identification of the cytoplasmic components(Reger et al. 1972; Mori and Leblond 1970).

Ribosome-like particles are sometimes observed to be attached to the outer membrane of the nuclear envelope. In the envelope, the inner and outer membranes are held together by thread-like membrane-to-membrane intracisternal

linkers (Frank et al. 1973). Hence, treatment of either of the membranes could affect the entire envelope. Indeed, the various treatments described for the detachment of the ribosome-like particles or for the separation of the outer membrane affect the nuclear structure (Zbarsky 1972). Since the integrity of the nuclear envelope, which performs many specific functions (Zbarsky 1972), is necessary to maintain the integrity of the nuclei and the ultrastructure of the pores, no attempts were made to further purify the nuclear preparations.

B. Electrokinetic characteristics of isolated mammalian brain nuclei (I) (II)

1. Mobility of intact nuclei. Intact nuclei isolated from brain or liver cells migrate in an electric field, provided the suspending medium is of low ionic strength. Nuclei migrate slower than rat erythrocytes (taken as reference). The electrophoretic velocity is a function of the ionic strength of the buffer used, the velocity decreasing sharply as the molarity of the surrounding medium increases. The direction and velocity of migration of the nuclei are also pH-dependent. When observed at the stationary layer, the nuclei migrate to the positive electrode at all pH values above pH 4.0. The velocity increases progressively, peaking at a pH of about 6.8 with a value of 0.87 ± 0.01 $\mu m.sec^{-1}.V^{-1}.cm$. This peak is followed by a slight decrease; thereafter an increase in the velocity occurs. The same pH-characteristic curve is observed independently of the buffer used.

A heterogeneity in the distribution of the electrophoretic velocity of the nuclei is to be expected, since they are known to originate from different cell types. This heterogeneity is

depicted as a bimodal distribution in the histogram of the velocity. It is further indicated by the spreading of the velocity distribution observed when the nuclei are allowed to migrate for a longer time, when the ionic-strength of the suspending medium is increased, the current intensity lowered, or the pH changed. All of these factors decrease the electrophoretic velocity.

To ascertain the difference between the nuclear types, the electrokinetic properties of nuclei isolated from glial- and neuronal-enriched fractions were studied. The velocity of the glial nuclei is found to be significantly higher than the velocity of neuronal nuclei. Also, nuclei isolated from human tumors of presumably glial origin migrate faster than the nuclei from the normal grey matter. Further, a comparative analysis of the velocity values obtained for nuclei isolated from different organs, such as liver or brain, and from different species, such as rat, rabbit and human provides strong evidence for a specificity of the surface charge. This is in accordance with previous reports (Abramson and Mayer 1936) on erythrocytes. Differences in the surface charge values were observed for erythrocytes isolated from different species. This specificity in the surface charge makes the electrophoretic method suitable as a separation procedure of nuclear fractions.

2. Electrokinetic characteristics following treatment of the nuclei. In order to obtain information about the ionogenic groups giving rise to the surface charges, brain nuclei were submitted to various treatments following their isolation.

a) Cations. In vivo, the nuclear environment is rich in cations. Brain nuclei have been reported to differentiate between divalent and monovalent cations (Haljamäe and Waldman

1973). Some cations, such as Ca^{++} which are known to interact strongly with phospholipid membranes (Ohki 1972) also compete with proteins in binding to DNA (Chang and Carr 1968). Hence the presence of negative charges at the nuclear surface requires consideration of the interaction of cations, that can influence the ultrastructure as well as the enzyme functions of the nuclei (Meeker 1970, Stevens and André 1969).

The electrophoretic mobility of the nuclei do not change following incubation of the nuclei with a low concentration of cations, such as Li^{+}, Ca^{++}, Mg^{++}, Zn^{++}. However, a 4.0 mM concentration of cations leads to a drastic decrease in the mobility with all divalent cations used, whereas Li^{+} does not affect the mobility. Ca^{++} is the most active cation. The differences observed in the behaviour of nuclei following treatment with different cations can be due to a specificity in binding while different radii of the cations can affect the surface of shear differently. Furthermore, treatment of nuclei with cations often induces morphological alterations. Cations adsorbed at specific sites may be able to induce ultrastructural changes by reducing the repulsive electrostatic force between ionizable groups.

b) Enzymes. DNA, RNA and proteins constitute, together with phospholipids, the major molecular components present at the nuclear periphery (Zbarsky 1972). Furthermore, DNA is reported to be closely associated with the nuclear membrane (Ockey 1970), while ribosome-like particles are sometimes encountered attached at the outer membrane. This led to an investigation of the effect of DNAase, RNAase and pronase on the electrokinetic characteristics of isolated nuclei.

Treatment with DNAase results in a large, significant decrease of the electrophoretic velocity. From the brain nuclear population, two groups can be distinguished. One group, accounting for approximately 25% of the total population shows about 80% of a decrease in its velocity. RNAase-treatment does not affect the electrophoretic velocity provided the incubation time is less than an hour. Increasing the time of incubation with RNAase decreases the velocity. Treatment of nuclei with DNAase and RNAase results in a large decrease of the electrophoretic mobility together with a loss of the normal morphology of the nuclei. Treatment with pronase results in a large decrease of nuclear mobility associated with structural changes.

c) Polyions. Profound changes in the nuclear structure and the chromatin configuration are known to occur following the action of heparin on nuclei (Kraemer and Coffey 1970). The possibility that heparin, by formation of complexes with nuclear proteins might serve as a de-repressor of nucleic acid synthesis *in vivo* (Novello and Stirpe 1969; Kraemer and Coffey 1970) stimulated studies on the electrokinetic characteristics following treatment of nuclei with heparin.

Similarly, nuclei were treated with spermidine, which is known to form complexes with the nucleic acids and to affect the chromatin (Michelson 1963; Bachrach 1970).

Heparin-treated nuclei are found to increase their electrophoretic velocity. This effect is associated with a swelling of the organelles. Spermadine treatment results in a decrease in the nuclear mobility together with a shrinking of the nuclei.

These compounds can either adsorb at the nuclear surface and/or penetrate the nuclear envelope (Stevens and André 1969) with a direct action on the intranuclear structures.

3. Further characterization and origin of the surface charges. The electrophoretic mobility is a function of the net surface charge density. It reflects the composite effect of the surface components on the surface charge (Brinton and Lauffer 1959). The surface charges arise from the ionization of groups present at or near the nuclear surface and from adsorption of ions. Ionizable groups on the surface become charged by the loss or gain of ions, particularly hydrogen ions. The forces involved in adsorption of ions are essentially electrostatic in nature adsorption being limited by hydration. The ionogenic groups which contribute to the charge density are present on and below the slip plane. When the ionic strength of the suspending medium decreases, the coulombic screening of the charged groups decreases and thus ionic groups situated deeper may contribute increasingly to the surface charge density. At a very low ionic strength, the surface charges arise mainly from anion adsorption, while at higher ionic strength fixed anionic components of the nuclear surface become responsible for the surface charge. Further, since the nuclear envelope is permeable to water, small molecules, and some macromolecules (Stevens and André 1969), other structures situated deeper below the surface of shear can contribute to the total surface charge (I) (Haydon 1961). Moreover, any modification of the nuclear surface structures or morphological changes observed following treatment of the nuclei can alter the nuclear surface, so that components normally hidden deep in the surface may contribute to the negative charges

of the nucleus. Treatment with, for example, enzymes or polyions can induce many changes besides the specific action on the substrate. Changes in the effective thickness of the surface of shear with rearrangement of the molecular structures at the surface may follow adsorption of the active compound. Possible elution of components from the membrane, as well as adsorption of impurities from the autolytes may affect the electrokinetic characteristics of the surface, inducing changes in the viscosity and the dielectric constant of the envelope. Following adsorption, the compound can develop a "shadowing" effect masking some structural charges, or be active itself at the electrokinetic surface. The theory, limitations and assumptions of the electrophoretic method have been extensively discussed (I) (II) (Brinton and Lauffer 1959; Tenforde 1970).

Approximative values of the electrokinetic potential and the charge density of the nuclear surface under various conditions were derived and computed from the electrophoretic velocity. The number of charges per nucleus area is a function of the pH and ionic strength of the bathing medium. A decrease in the pH, which results in a decrease of the surface charge induces a shrinking of the nucleus, that is a decrease of the nuclear size, while heparin, which increases the surface charge, produces a swelling of the particles. Swelling must require stretching of the membrane; that may unmask ionizable structures which would thus prevent a decrease in the expected surface charge since the membrane would be thinner. Alternatively preassembled materials may be brought near the surface and account for the surface charges. However, although often associated with nuclear size variation, the charge density can vary, as a function of

the ionic surrounding, independently of any observable morphological alteration. This is observed for example between pH 6.5-7.5 when nuclei do not undergo visible volume changes although the surface charge is reaching a peak.

Distinct groups are responsible for the surface charges. Since pronase acting on the proteins known to account for a large part of the dry weight of the nuclear envelope (Zbarsky 1972) decreases the surface charge, it is conceivable that an important part of the charges on the nuclear surface arises from protein-associated compounds present at the nuclear envelope. Equally, the phospholipids present at the nuclear envelope (Stadler and Kleinig 1971) where they represent about 25% of the dry weight (Zbarsky 1972) also account for the surface charges.

On the other hand, DNAase treatment of the nuclei induces a decrease in the electrophoretic velocity. The nuclear shrinkage following DNAase treatment might contribute to a certain extent to the decrease in the total surface charge, since some structural charges may no longer be active at the surface of shear. However, most of the decrease follows a direct action of the enzyme on DNA-associated structures which carry the major part of the surface charges and are present near the nuclear surface. DNAase presumably acts on these surface structures prior to a further action on the more intranucleic structures.

It is noteworthy that, one group of nuclei, representing 25% of the total population, is very sensitive to DNAase-treatment. Hence, either most of its charges are carried by DNA-associated structures present at the surface, or it is more readily permeable to the enzyme. This fraction may well

represent the round-shaped type of intact nuclei identified previously as being of neuronal origin and which account for 25% of the total nuclear population.

Besides the ionic groups already described, other groups might account, to some extent, for the total surface charge, e.g. the carboxyl groups arising from the small amount of sialic acid found to be present at the membrane (Zbarsky 1972, Nicolson et al. 1971; Kashnig and Kasper 1969). Some glycolipid amines as well as some terminal groups of e.g. arginine provide positive charges (Mehrishi 1972).

Another point of interest is that the differences in surface charge density of nuclei isolated from different cell types, from different organs, and from different species, can reflect a fundamental difference in the molecular structure of the nuclei which can be related to nuclear activity. This is further suggested by the differences observed in the surface charge of nuclei isolated from tumoral cells as compared to normal cells.

C. Microelectrode studies.(III)

1. Intranuclear potential. In order to see whether the internal structures can develop a cationic distribution, microelectrode studies were performed on isolated nuclei in suspension. Following impalement, a negative internal potential is recorded at all pH values of the bathing medium above pH 4.0, provided the ionic strength is low, independently of the buffer used. The magnitude of this potential is 18 $\pm$ 1.6 mV in 10 mM phosphate buffer pH 6.8.

The intranuclear potential is found to be a function of the ionic strength. Increasing the ionic strength decreases the value of the recorded potentials. The potential appears also to be a function of the pH of the bathing medium. The largest values of the recorded potentials are observed between pH 6.7-7.0 in a 10 mM phosphate buffer. Further increases in the pH result in constant decreases in the potential.

A heterogeneity of the recorded potential is observed depending upon whether the nucleus has one central nucleolus (i.e. a nucleus of probably neuronal origin) or one or more noncentrally located nucleoli (nuclei of probably non-neuronal origin). The intranuclear potential is found to be larger for the single-nucleolated nuclei.

To ascertain the differences between the different types of nuclei, nuclei were isolated from glia- and neuron-enriched fractions and were impaled. The intranuclear potentials of nuclei isolated from neuronal-enriched fractions are found to be significantly higher than the potentials of nuclei from glial-enriched fractions.

Also, nuclei isolated from human tumors of presumably glial origin had a lower potential than nuclei from normal grey matter.

2. Origin of the intranuclear potential. This internal potential indicates an unequal distribution of ions. Although it is well established that cations accumulate within the nucleus (Haljamäe and Waldman 1973; Hanig et al. 1972; Tres et al. 1972) this recorded potential is not caused by an active transport of mobile ions. It is rather a Gibbs-Donnan potential based on fixed charges (Tupper and Tedeshi 1969). The nucleoproteins

present in the nucleoplasm can be anionic and capable of supporting a cationic distribution independently of any metabolism. Since neuron nuclei contain more macromolecules than glia nuclei (Poduslo and Norton 1972) a higher potential is expected for the former; this is indeed observed.

To further characterize the origin of this intranuclear potential intact nuclei were treated with various agents. DNAase induces an increase in the recorded internal negative potential of the nuclei, while RNAase has no major effect. Heparin decreases the potential. Treatment with divalent cations at a concentration of 4.0 mM results in a decrease in the value of the recorded potential.

DNAase, by acting primarily on the DNA-associated structures present at the nuclear membrane will enhance the intranuclear potential difference. On the other hand, the decrease of the potential following heparin treatment can be due to a neutralization of the fixed charges following adsorption of heparin. This may produce secondary molecular changes. However, it may also be that the structural changes result in a secondary decrease in the potential, since a reorganization of the macromolecules may be expected to occur. The differences in the potentials recorded following treatment of nuclei with various cations further suggest specific adsorption and/or penetration sites present at the nuclear surface. On the other hand, DNA present at the nuclear surface (Comings and Okada 1970a; Ockey 1970) could behave as a negative polyelectrolyte and could modify the ionic mobilities of small ions (Overbeek 1956) while strongly binding divalent cations (Katchalsky 1964) in competition with the cationic protein binding to DNA (Chang and Carr 1968).

D. Induced ultrastructural changes of isolated nuclei (IV)

Morphological changes of the nuclei are observed following alterations of the suspending medium such as pH changes (Ris and Mirsky 1949; Anderson 1956; Anderson and Wilbur 1952). Nuclei shrink in acid pH and swell in alkaline pH. The ordered structure of the nuclei appears thus to be much dependent on the ionic environment. Intranuclear elements, such as nucleoli are also sensitive to changes in the ionic environment of the nuclei, since morphological changes, such as displacement or deformation of the nucleoli are observed when the nuclei are subjected to the action of an electric field (I).

The visual observation of nuclear shrinkage is characterized ultrastructurally by a very prominent condensation of the nuclear chromatin as well as of the nucleolus, which appear very densely packed. A swelling state of the nuclei is characterized by an "unfolding" of the nucleolus while the chromatin is released from its dense granular packing and becomes filamentous. These changes are, to a certain extent, reversible. Hence, depending on the extranuclear milieu, the nucleolus can be present in the form of a condensed structure - droplets - or dispersed filaments. In a similar way, the intranucleoplasmic structures such as the perichromatin granules and interchromatin granules can condense or become filamentous. It is therefore suggested that many of the observed structures in the nucleoplasm can be the same elements with a different physical state.

DNAase treatment of the nuclei differentiates two types of nuclei from the total brain population. About 15% of the nuclear population have their nucleoplasm cleared, while opaque

material is observed at their periphery. This observation suggests either a different localization of the DNA-associated structures in the different types of nuclei or a different sensitivity towards DNAase digestion. A different nuclear permeability to the enzyme must also be considered. RNAase is effective only after a long period of incubation. It induces a condensation of the nuclear components. Heparin-treated nuclei are swollen and their ultrastructural changes are similar to the ones observed following alkalinisation of the nuclei environment.

E. Observations of nuclei by whole mount electron microscopy (V)

The general picture of the ultrastructure of normal nuclei suggests the existence of networks of dispersed or condensed filaments in the nucleoplasm, radiating from the nucleolus and occasionally observed to be attached to the nuclear membrane (IV) (Brasch et al. 1971; Comings and Okada 1970). In order to sustain this observation, isolated intact or treated nuclei were microdissected and mechanically stretched upon an electron microscope grid prior to observation. A dense network of compact filaments of various sizes is observed to radiate from the electron opaque central core of the nucleus and to branch into thinner filaments before ultimate attachment to the nuclear membrane. Both condensed and dispersed chromatin structures are observed in the nucleoplasm. Their respective presence is a direct function of the degree of stretching of the nuclear membrane.

Moreover, mild DNAase-treatment, while clearing the network, reveals a polygonal-patterned reticulum apparently

connected to the network of compact filaments. The fact that this reticulum is insensitive to DNAase,unless it is used at very high concentrations, suggests that it is a protein-rich structure.

F. Spectroscopy, cytochemistry of isolated nuclei. Incorporation of precursors into nuclear macromolecules (VI).

In order to determine whether the observed structural changes involve alterations in the interaction and the close packing of nucleoproteins, and are not secondary to fixation, the absorption spectra in the far ultraviolet range were recorded. A relation between the morphology of the nuclei and their absorption spectra is observed, which agrees with previous reports (Olins and Olins 1972; Kraemer and Coffey 1970). Swollen nuclei present well defined spectra, close to the spectra of purified nucleoproteins (Olins and Olins 1972), while the spectra of shrunken nuclei are flattened.

In order to further understand the mechanisms of nuclear volume changes, cytochemical analyses based on the binding properties of acridine orange (AO) were performed. The extent to which this basic dye binds to the nucleoproteins varies with the state of the nuclear chromatin (Ringertz et al. 1970) as well as with modifications of charge interactions. AO-binding is mainly electrostatic in nature, free phosphate groups in the nucleic acids providing the negative charges (Fredericq and Houssier 1972; Rigler 1966). AO-binding is known to be a function of the structural organization of the tightly packed DNP complexes (Ringertz and Bolund 1969). Following DNAase treatment, the binding capacity of AO decreases dramatically.

This is a strong indication that AO binds to DNP structures. Further, the binding of AO to DNP is observed to be pH-dependent and to peak at a neutral pH. It is worth noting that it is the same pH at which the nuclei present the peak of their charges (I) (II) (III).

The changes in AO-binding of nuclei subjected to pH alterations thus reflect changes in the physicochemical state of the nuclear DNP complexes. Since the dry mass remains constant, the possibility that AO changes are secondary to major loss or degradation of nuclear protein is excluded.

Changes in AO-binding to DNP were reported to parallel changes in RNA synthesizing cell nuclei (Ringertz et al. 1970). On the other hand, nuclear condensation is correlated with a decrease in RNA synthesis (Grasso et al. 1963, Grasso and Woodard 1966), while in vivo the condensation of chromatin may be associated with depression of RNA synthesis (Brasch et al. 1971). These findings stimulated the study of the in vitro metabolic response of nuclei, such as incorporation of precursors into RNA and proteins in order to see whether they are associated with the state of condensation of the chromatin. The function of the chromatin is thus measured as the amount of uridine incorporated. It is shown that the incorporation of both RNA and protein precursors are pH-dependent. However, while the incorporation of aminoacid decreases with an increase in pH and hence a swelling of the nuclei, uridine incorporation increases. Further, the maximum value for protein synthesis is found to occur at pH 6.8 which is the pH at which the nuclei present their most ordered ultrastructure.

GENERAL DISCUSSION

Intact nuclei, with their envelope organized as a mosaic of macromolecular complexes (Zbarsky 1972), possess electrical surface charges. These charges arise from selective adsorption and/or ionization of the proteophospholipids and DNA-associated structures present at/near the nuclear surface. They are specific for each type of nucleus. Electroneutrality dictates an elevated concentration of cations in the solution adjacent to the sites of fixed negative charges which may affect ion activities and diffusion rates across the nuclear membrane. Moreover, the nucleoplasm contains polyelectrolytes whose anionic charges are prevented from passing into and out the nucleus. A cationic distribution is expected to arise. Divalent cations such as Ca^{++} concentrate inside the nucleus (Haljamäe and Waldman 1973) and can be loosely bound to the intranuclear structures, while small monovalent cations with a smaller binding capacity will have a different distribution. This effect could account in part for the major role attributed to the divalent cations in the maintenance of the nuclear structure.

The ordered structure of the nucleus is determined by the interaction between the chromatin, the nucleolus and the nuclear membrane. It has been shown that the nucleolus is connected through a branched chromatin network to the inner leaflet of the nuclear envelope (V). A highly organized intranuclear structure is further suggested by a protein-rich reticulum observed on the nuclear membrane and possibly interacting with the chromatin network.

Another major factor accounting for the ordering of the nuclear structure is the packing of the chromatin which consists

of coiled and twisted fibrils (Comings and Okada 1970, Engelhardt and Pusa 1972) composed of DNA. Interactions between proteins and DNA in the chromatin may be responsible for the specific configuration of the chromatin. The DNA-containing structures may be held in a dispersed state by repulsion between negatively charged groups within them. Any modification of the charge interactions could induce ion-mediated configurational changes of the intranuclear elements.

The dramatic alterations of the chromatin morphology observed, including narrowing of chromatin fibres, a decrease in the number of granules and a general increase in electron translucency are all consistent with unfolding and extension of the chromatin fibres; this could be due to a loss of cross-linking substances from the chromatin fibres. A thinning in the chromatin fibre diameter may be a result of the marked extension of a supercoiled structure produced by nuclear membrane and chromatin stretching (V) (Comings and Okada 1970). Nuclear swelling is induced secondarily to these changes.

Thus, the volume changes are considered to be due primarily to ionic effects on the nuclear colloids rather than to osmotic behaviour. Any changes in the nuclear surface charges, secondary to a modification of the surrounding milieu, such as pH changes, will affect the ionic distribution. The configuration of the chromatin will be then modified with, as a result, a change in the osmotic pressure.Swelling or shrinking of the entire nucleus will then follow.

Does the dispersal of condensed chromatin have any significance in terms of activation of the genetic transcription? Probably, since the chromatin condensation is generally asso-

ciated with repression of the chromatin activity (Leake et al. 1972) while a dispersion of the chromatin is associated with an increase in RNA synthesis (Brash and Seligy 1972; Ringertz et al. 1970). On the other hand, changes in the ionic environment have been considered to be important factors in repression and derepression of the chromatin (Laval and Bouteille 1973). It could be expected that an induced dispersion of the chromatin in isolated nuclei, by the unfolding of the tightly packed DNP can make the structure suitable for its function as a template to the synthesis of RNA (Rigler 1966). Hence, the physical state of the chromatin which reflects the physicochemical properties of DNA is important in the expression of gene activity. The experimental results indeed strongly suggest an association between the organizational state of intranuclear DNP and the template activity of the nucleus. A state of condensed chromatin, which represents tightly packed DNP, parallels a minimum incorporation of precursor into RNA while an increase in the order of chain organization increases the synthesis of RNA (Wood and Berg 1964). When DNA reaches its maximum order of helix, the lability of the helix which follows facilitates the strand separation, and thus the template activity of DNA. A complete dispersal of the nuclear chromatin parallels a dramatic increase in RNA synthesis. The synthesis of protein within the nuclei may be connected with the RNA (Laval and Bouteille 1973). However, the problem of the nature and origin of the nuclear protein is still controversial.

In vitro, therefore, isolated brain nuclei respond specifically to alterations of their surrounding. They present their best characteristics at a pH of the bathing milieu of pH 6.8. Brain nuclei in situ, on excitation of neurons, are

exposed to alterations of the ionic composition of the surrounding cytoplasmic milieu due to generation of action potentials (Bocharova et al. 1972). The cytoplasmic pH - between pH 6.8-7.0 in nerve fibers (Bicher and Ohki 1972; Caldwell 1958; Chambers and Kao 1952; Spyropoulos 1960) - has been reported to change, following activity of nerve cells (Wasserman and Kallfelz 1970). Moreover, electrical stimulations of neurons induce ultrastructural changes in the neuronal nuclei together with changes in RNA synthesis (Bocharova et al. 1972).

Brain cells are required to process, store and recall vast amounts of information conveyed as sequences of nerve impulses. Such intense cellular activity necessarily results in changes in the cytoplasmic milieu surrounding the nuclei. Some of the physical, chemical and morphological characteristics observed in isolated nuclei following alterations in their surrounding milieu have been presented here. Such effects may be of biological significance in vivo as regards the response of nuclei to functional activity and hence the control of gene expression.

SUMMARY

An electrophoresis method was described and used for the determination of the electrical surface charge density of isolated mammalian brain nuclei. These charges were pH - and ionic strength - dependent. They were negative over a wide range of pH, presenting a maximal value at pH 6.8. A specificity of these electrical charges was strongly suggested. Treatment of isolated brain nuclei with cations, enzymes - DNAase, RNAase and pronase - heparin and spermidine affected the electrophoretic velocity. From these results attempts were made to determine the origin of the surface charge.

Intranuclear potentials were also recorded with micro-electrodes when isolated brain nuclei were suspended in a low ionic strength medium. These potentials were shown to be pH - and ionic strength - dependent. They were negative over a wide range of pH and peaked at pH about 6.8. The different potentials obtained with nuclei isolated from different sources suggested a specificity of these intranuclear potentials. Treatment of isolated brain nuclei with cations, enzymes and polyions affected the intranuclear potential. The results indicated that the potentials were of a Gibbs-Donnan nature.

These potentials were discussed in relation to the surface charge density and to the ultrastructural organization of the nuclei.

The ultrastructure of isolated brain nuclei was further studied by electron microscopy. Different types of nuclei were encountered, described and tentatively identified.

Following changes in the pH of the surrounding medium or treatment with DNAase, RNAase, pronase or heparin, marked alterations of the nuclear ultrastructure were observed. They consisted of changes in the condensation state of the nuclear chromatin and the nucleoli. A close association of the chromatin with the nuclear membrane and the nucleolus was suggested to account for these reversible alterations.

To confirm this view, investigations on isolated and microdissected nuclei were performed. Networks of chromatin fibrils were observed to radiate from the central mass of the nucleus, branching into thinner filaments before attachment to the nuclear membrane.

In an attempt to examine the molecular basis of the observed ultrastructural changes, the DNP complexes of nuclear chromatin were examined by cytochemical methods. Since a causal relationship exists between the structural and funtional state of the nuclei, the incorporation of precursors into nuclear RNA and proteins were also studied after alterations of the suspending medium.

The results were discussed in relation to a possible biological role of the physicochemical characteristics of brain nuclei studied in vitro.

ACKNOWLEDGEMENTS

Acknowledgement is made to the Lebanese National Scientific Research Council (CNRS) for a scientific fellowship. This investigation was also supported by grants from the Swedish Medical Council and from the Medical Faculty, University of Göteborg.

I wish to express my sincere gratitude to Professor Holger Hydén for his constant support and interest during these years at the Institute of Neurobiology. I also express my gratitude to Professor Ragnar Ekholm for kindly placing the facilities of his department at my disposal. My sincere thanks go to Professor Ingemar Engberg and Professor Nils Ringertz for offering the technical facilities of their respective laboratories and for their valuable advice.

I especially wish to thank Docent Hengo Haljamäe for his ready help and stimulating discussions in all respects. The cooperation I have had with Drs Alan Waldman, Galo Ramirez, Walter Mushinsky and Docent Staffan Smeds has been gratefully appreciated. Acknowledgement is to be given to Professor Anders Hamberger, Docent Kenneth Haglid, Docent Hans-Arne Hansson and Dr Ragnar Hultborn for their help and constructive criticism and to Dr Graham McLean for revision of the manuscript. Likewise my thanks go to all my other colleagues at the Institute for their interest and encouragement.

I am also grateful to Ing Lars-Ingemar Nilsson for his help with the electronic equipment used. The technical assistance of Mrs Birgitta Svensson-Karlsson, Gunnel Bokhed and Zaiga Purins was greatly appreciated.

I owe a special debt to Mrs Ingrid Lundberg and Mrs Hana Wilson for their untiring patience and care with the manuscripts. My thanks are also due to Mrs Irene Mogren and Mrs Elsa Hansson for secretarial help. Mrs Maria Ekmark, Miss Dala Sjögren, Mr Clarence Hall and Mr Bror Bergkvist are to be thanked for photographic assistance.

I would like to express a special and deep gratitude to my friends outside the department who encouraged me throughout these years simply by their sincere thoughts and by their presence.

REFERENCES

Abramson, H.A. and Mayer, L.S., J.Gen.Physiol.19 (1936) 601.

Agranoff, B.W., in: The Neurosciences, G.C. Quarton, Th.Melnechuk and F.O. Schmitt (eds) p 756. Rockefeller Univ.Press, New York 1967.

Anderson, N.G., The quartely review of biology, 31 (1956) 169.

Anderson, N.G. and Wilbur, K.M., J.Gen.Physiol. 35 (1952) 781.

Arnold, E.A., Yawn, D.H., Braun, D.G., Wyllie, R.C. and Coffey, D.S., J.Cell Biol. 53 (1972) 737.

Bachrach, U., Ann.Rev.Microbiol., 24 (1970) 1544.

Bicher, H.I. and Ohki, S., Biochim.Biophys.Acta, 255 (1972) 902.

Blomstrand, C. and Hamberger, A., J.Neurochem.,16 (1969) 1401.

Bocharova, L.S., Borovyagin, V.L., Dyakonowa, T.L., Warton, S.S. and Veprintsev, B.N., Brain Res., 36 (1972) 371.

Bolund, L., Thesis, Stockholm 1971.

Brasch, K., Seligy, V.L. and Setterfield, G., Exptl.Cell Res., 65 (1971) 61.

Brinton, C.C. Jr and Lauffer, M.A., In: Electrophoresis: Theory, methods and application, Bier, M. (ed) p 428, Academic Press, New York 1959.

Burdman, J.A. and Journey, L.J., J.Neurochem., 16 (1969) 493.

Burés, J., Petrán, M. and Zachar, J. Electrophysiological methods in biological research, Academic Press, New York, 1967.

Burton, K., Biochem.J., 62 (1956) 315.

Caldwell, P.C., J.Physiol., London, 142 (1958) 22.

Carlsson, L., Caspersson, T., Lomakka, G., and Silverbage, S. In: Introduction to quantitative cytochemistry, Vol. II, Wied, G. and Bahr, G. (eds) p 117, Academic Press, New York 1970.

Chambers, R. and Kao, C.Y., Exptl. Cell Res., 3 (1952) 564.

Chang, K.Y. and Carr, C.W.,Biochim.Biophys.Acta, 157 (1968) 127.

Comings, D.E. and Okada, T.A., Exptl.Cell Res., 62 (1970) 293.

Comings, D.E. and Okada, T.A., Exptl.Cell Res., 63 (1970) 62.

Dravid, A.R., Duffy, T.E. and Haglid, K., Brain Res., 12 (1969).

Eccles, J.C., In: The Neurosciences, G.C. Quarton, Th.Melnechuk and F.O. Schmitt (eds) p. 408 Rockefeller Univ. Press, New York 1967.

Engelhardt, P. and Pusa, K., Nature New Biology, 240 (1972) 163.

Franke, W.W., Zentgraf, H., Scheer, U.,Kartenbeck, J., Cytobiologie 7 (1972) 89.

Fredericq, E. and Houssier, C., Biopolymers, 11 (1972) 2281.

Grasso, J.A. and Woodard, J.W., J.Cell Biol., 31 (1966) 279.

Grasso, J.A., Woodard, J.W. and Swift, H., Proc.Natl.Acad.Sci. US, 50 (1963) 134.

Grundfest, H., In: The Neurosciences, G.C.Quarton, Th.Melnechuk and F.O. Schmitt (eds) p. 353 Rockefeller Univ.Press, New York 1967.

Haglid, K.G., J.Neurochem., 19 (1972) 19.

Haljamäe, H. and Waldman, A.A., J.Neurochem., 20 (1973) 1747.

Harig, R.C., Tachiki, K.H. and Aprison, M.H., J.Neurochem., 19 (1972) 1501.

Harris, H., J.Cell Sci., 2 (1967) 23.

Haydon, D.A., Biochim.Biophys.Acta, 50 (1961) 450.

Hogan, B. and Grass, P.R., Exptl.Cell Res., 72 (1972) 101.

Hydén, H. In: The Neurosciences G.C. Quarton, Th. Melnechuk and F.O. Schmitt (eds) p. 248 Rockefeller Univ. Press, New York 1967a.

Hydén, H., In: The Neurosciences, G.C. Quarton, Th.Melnechuk and F.O. Schmitt (eds) p. 765 Rockefeller Univ. Press, New York 1967b.

Hydén, H., In: Macromolecules and Behaviour, - Arthur Thomson Lectures - Ansell, G.B. and Bradley, P.B. (eds) MacMillan, London 1972.

Kanno, Y., Ashman, R.F. and Loewenstein, W.R., Exptl.Cell Res., 39 (1965) 184.

Kashnig, D.M. and Kasper, C.B., J.Biol.Chem., 244 (1969) 3786.

Katchalsky, A., In: Connective tissue: intercellular macromolecules, New York heart association (Eds), p. 9, Little, Brown, Boston, 1964.

Kato, T. and Kurokawa, M., J.Cell Biol., 32 (1967) 649.

Kraemer, R.J. and Coffey, D.S., Biochim.Biophys.Acta, 224 (1970) 553.

Kraemer, R.J. and Coffey, D.S., Biochim.Biophys.Acta, 224 (1970) 568.

Laval, M. and Bouteille, M., Exptl.Cell Res. 76 (1973) 337.

Leake, R.E., Trench, M.E. and Barry, J.M., Exptl. Cell Res., 71 (1972) 17.

Loewenstein, W., Kanno, Y. and Ito, S., Ann.N.Y. Acad.Sci., 137 (1966) 708.

Løvtrup-Rein, H., Brain Res., 19 (1970) 433.

Løvtrup-Rein, H. and McEwen, B.S., J.Cell Biol. 30 (1966) 405.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randaff, R.J. J.Biol.Chem., 193 (1951) 265.

Meeker, G.L., Exptl.Cell Res., 63 (1970) 165.

Mehrishi, J.N., Prog.Biophys.Mol.Biol., 25 (1972) 1.

Mejbaum, W., Z.Physiol.Chem., 258 (1939) 117.

Michelson, A.M., The chemistry of nucleosides and nucleotides, Academic Press, London, 1963.

Mori, S. and Leblond, C.P., J.Comp.Neur. 139 (1970) 1.

Mountcastle, V.B., In: The Neurosciences, G.C. Quarton, Th. Melnechuk and F.O. Schmitt (eds) p. 393 Rockefeller Univ. Press, New York 1967.

Nicolson, G., Lacorbiere, M. and Delmonte, P., Exptl.Cell Res., 71 (1972) 468.

Novello, F., and Stirpe, F., Biochem.J., 112 (1969) 721.

Ockey, C.H., Exptl.Cell Res., 70 (1972) 203.

Ohki, S., Biochim.Biophys.Acta, 282 (1972) 55.

Olins, D.E. and Olins, A.L., J.Cell Biol., 53 (1972) 715.

Overbeek, J.T., Progr.Biophys., London 6 (1956) 57.

Peters, A., Palay, S. and Webster, H., Harper and Row, New York 1970.

Poduslo, S.E. and Norton, W.T., J.Neurochem., 19 (1972) 727.

Ramirez, G., Levitan, I. and Mushynski, W., FEBS letters, 21 (1972) 17.

Reger, J.F., Holbrook, J.R. and Pozos, R.S., J.Submicr.cytol. 4 (1972) 135.

Rigler, R., Acta Physiol. Scand. 67 suppl. 267 (1966).

Ringertz, N.R., In: Handbook of molecular cytology, Lima-de-Faria, A. (ed.) p. 656, North-Holland Publ., Amsterdam 1967.

Ringertz, N.R. and Bolund, L., Exptl.Cell Res., 55 (1969) 205.

Ringertz, N.R., Bolund, L. and Darzinkiewicz, Z., Exptl.Cell Res., 63 (1970) 233.

Ris, H. and Mirsky, A.E., J.Gen.Physiol.32 (1949) 489.

Scharrer, B. and Wurzelmann, S., Z.Zellforsch., 101 (1969) 1.

Solari, A.J., Exptl.Cell Res., 53 (1968) 553.

Spyropoulos, C.S., J.Neurochem. 5 (1960) 185.

Stadler, J., and Kleinig, H., Biochim.Biophys.Acta, 233 (1971) 315.

Stevens, B.J. and André, J., In: Handbook of molecular cytology, Lima-de-Faria, A., (ed) p. 837, North-Holland Publishing, Amsterdam, 1969.

Tenforde, T., In: Advan.Biol.Med.Phys., Vol. 13, Laurence, J.H. and Gofman, J.W. (eds) p. 43, Academic Press, New York, 1970.

Tosi, L., Granieri, A. and Scarano, E., Exptl. Cell Res., 72 (1972) 257.

Tres, L.L., Kierszenbaum, A.L. and Tandler, C.J., J.Cell Biol., 53 (1972) 483.

Tupper, J.T. and Tedeshi, H., Science, 166 (1969) 1539.

Waldman, A.A. and Alm-Håkansson, G., Personal communication.

Wasserman, R.H. and Kallfelz, F.A., In: Biological calcification: cellular and molecular aspects, Schraer, M., (ed) p. 313, North Holland, Amsterdam, 1970.

Wood, W.B. and Berg, P.J., J.Mol.Biol., 9 (1964) 452.

Wunderlich, F., J.Membrane Biol. 7 (1972) 220.

Wunderlich, F. and Speth, V., J.Microscopie 13 (1972) 361.

Yasuzumi, G., Nakai, Y., Tsubo, J., Yasuda, M., Sugioka, T. Exptl.Cell Res., 45 (1967) 261.

Zbarsky, I.B., In: Methods in cell physiology, Vol. 5, Prescott, D.M., (ed) p. 167, Academic Press, New York 1972.

Zirkin, B.R. and Kim, S.R., Exptl.Cell Res., 75 (1972) 490.

GÖTEBORGS UNIVERSITET
GÖTEBORGS UNIVERSITETSBIBLIOTEK

Printed in Sweden, Gotab, Kungälv 73.11867